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ANTIFUNGAL PROPERTIES OF LEAF EXTRACT OF *ANTHOCLESITA DJALONENSIS* A. CHEV. ON THREE FUNGAL PATHOGENS OF POSTHARVEST FRUIT ROT OF BANANA (*MUSA ACUMINATE* COLLA) IN CALABAR, NIGERIA.

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Abstract

The antifungal properties of leaf extract of *Anthocleista djalonensis* was examined in the control of three fungal pathogens of postharvest rot of banana (*Musa acuminate* colla) in vitro and in vivo. The concentrations of the plant extract used for the test were 10, 20, 40, 60, 80, 100 mg/ml on potato dextrose leaf extract agar (PDLA). Ethanolic leaf extract of *Anthocleista*, *djalonensis* significantly inhibited the mycelial growth of *Verticillium theobromac, Botryodiplodia theobromae* and *Collectotrichum musae* over those treated with hot and cold water extract in both in vitro and in vivo. At 100 mg/ml. the ethanolic extract concentration significantly inhibited the mycelial growth of *Colletotrichum musae* each by 99.5%. The inhibition levels of hot and cold water extract at same concentrations were lower with *Verticillium theobromae* (40.5% and 11.5%), *Colletotrichum musae* (45.0% and 30.5%) and *Botryodiplodia* theorbo (47.6% and 31.0%) respectively. Also, the ethanolic extract on in vivo inhibition at 100mg/ml also recorded the highest inhibition of the fungal growth with *Collectotrichum musae* (100%) while *Verticillium theobromae* and *Botryodiplodia theobromae* recorded 98.5% inhibition each. Again, the results of hot and cold water extract at same concentration (100mg/ml) were significantly lower when compared with ethanolic extract with *Colletotrichum musae* (50.5% and 31.0%), *Ventricillium theobromae* (52.5% and 38.5%) while *Botryodiplodia theobromae* (40.5% and 33.5%) respectively. Generally, media plates without extract inoculation showed maximum growth of mycelia with no inhibition.

Keywords: Antifungal properties, leaf extract, *Anthocleista djalonensis*, fungal pathogens, postharvest, fruit rot banana

1.0 Introduction

Banana is an edible, succulent, sweet fruit with an appetizing aroma when fully ripen (Salmon *et al.*,1996). The fruit is of great nutritional significance because it is an excellent source of vitamin B, thiamine, riboflavin niacin, vitamin C; and minerals like Iron, Magnessium, Manganese, Potassium and Phosphorous. Its dietary fibre serves as rouphage for the promotion of

digestion (Nutrition data. Com. 2015). Unripe bananas are also a staple starch for many tropical populations; they are eaten all over the world in their raw or cooked state (Gilbert *et al.,* 2009); and can therefore be used in a variety of ways such as banana jams, pancakes, fried chips and banana flower (Mui *et al.,* 2002).

Freshly harvested bananas like most vegetable crops are often faced with the problem of ripening and rapid deterioration before reaching commercial centres and the final consumers. This is because the physiological processes of a mature fruit like banana, harvested from the farm under goes rapid metabolic activities leading to ripening (Wills *et al.,* 1998); and deterioration with increased development of postharvest rot organisms in the fruit (Meredith, 1971). Post-harvest fungal diseases of banana (*Musu acuminate* cola) have been reported by several researchers and include crown rot anthracnose, cigar end rot *Botryodiplodia* rot, fluffy white rot etc. (Dadzie and Ochard, 1997; Dionisio, 2012; Ogawa, 1990; Ploetz, 2000 and Onyeke*et al.,* 2003). In south eastern Nigeria, the mean percentage incidence of these diseases were reported to be between 5.6-26.0% (Onyeke, *et al.,* 2003).The fungal pathogens associated with these diseases were also reported, and include *Fusarium oxysporum* (Schlecht) Arx; Botryodiplodia theobromae Pat.; *Aspergillus niger* Van Tiegh; *Verticillium theobromae* (Jurc.) Mason and Hurghes; and *Penicillium* sp Onyeke, *et al.,* 2003 and Meredith, 1971).

In Cross River State of Nigeria, Ikom and Biase Local Government Areas (LGAs) produces large quantities of banana and plantain, often shifted in commercial quantities to consumer centres in Calabar, Uyo and PortHarcount, the headquarters of Cross River, AkwaIbom and Rivers States of Nigeria respectively. This same postharvest diseases of banana reported in the south eastern Nigeria (Onyeke and Maduewesi, 2006) have been observed to be endemic in these banana producing areas of Ikom and Biase LGAs; and have adversely affected retailers who are deprived of large percentage, of their produce which get deteriorated before reaching consumer centres. However, no research has been carried out on the postharvest diseases of bananas in Calabar, one of the banana consuming town and headquarters of Cross River State, Nigeria.

Several workers have reported on the use of synthetic fungicides in disease control in plants and plant products but there is the fear of possible human toxicity (Olufolaji, 1999). Also, due to the phytotoxicity and non-biodegadable nature of conventional fungicides there is need therefore, for safer and environmentally friendly alternatives (Owolade *et al.,* 2000 and Thiangavelu *et al.,* 2004). Research on plant extracts have shown significant activities against rot causing organisms and as such, control rotting in fruit crops and other plant products have become very necessary with the use of botanicals. (Bankole and Adebanjo, 1995 and Okoi *et al.,* 2013).This research therefore, is aimed at evaluating the antifungal properties of leaf-extract of cabbage tree, *Anthocleista djalonensis* A. Chev. on three fungal pathogenic isolates of banana (*Musa accuminata* colla) in Calabar, Nigeria.

2.0 Materials and methods

2.1 Plant collection

Partially decayed banana fruits (*M. acuninata* colla) used as experimental material were obtained from Marian Market in Calabar Metropolis, Cross River State, Nigeria. The leaves of cabbage tree (*A. djalonensis*) used as plant extract were obtained from the bushes, at the out skirts of the Cross River University of Technology, Calabar campus. They were identified at the Herbarium unit of the Department of Biological Sciences, Cross River University of Technology, Calabar. The research work was carried out in the Biology Laboratory of the Department of Biological Sciences, Cross River University of Technology, Calabar.

2.2 Isolation of test fungi from banana

Partially rotted fruits of ripe banana (*M. acuminate* colla) were washed in sterile distilled water in the laboratory. They were peeled and cut into smaller sizes (2x2cm) with a sterile surgical blade, and about ten (10) pieces of the slized banana were soaked in 1000ml sterile beaker containing sterile distilled water for 10 minutes. The contents of the beaker was stirred with a sterile glass rod to dislodge morepropaqules into the water.The banana pieces were later removed from the beaker and the suspension filtered through sterile cheese cloth. Exactly 1.0ml aliquot of the filtrate was aseptically inoculated into each of the five petri-dishes containing fresh potato dextrose agar (PDA) cultures. The inoculated media plates were left for observation of fungal growth for 7days at room temperatures. 27±1^{o}C. Observed fungal colonies were identified on the basis of their growth habits and characteristic. These were further confirmed by examining slide preparations of the spores/mycelia using binocular light microscope with the aid of "Illustruted Genera of Imperfect Fungi" (Barneth and Hunter, 1972). Mycelia from banana seeds were picked with a sterile inoculating loop, sub-cultured on sterile PDA medium until pure cultures were obtained. The pure cultures were stored in the refrigerator at 9^{o}C until needed.

2.3 Preparation of test plant and extraction of active ingredients

Fresh leaves of cabbage tree (A. djalonensis) were washed with sterile distilled water and dried indoors by spreading on the laboratory benches for 2 weeks. When dried, the leaves were ground to fine powder using a house hole blender (Model 830 L. Hong Kong). The crude extract of the plant was prepared using standard procedures (Fatope *et al.,* 1999). This involved soaking 50g of the powdered leaves separately in 500ml of 95% ethanol. Hot and cold water used as solvents, in the ratio of 1:10 were left to stand for 48 hours at room temperature (25-28^{o}C) to allow for maximum extraction of the components. The solutions were later filtered through a single layer of Whatmanfilter paper through a sterile glass funnel. The filtrates were poured into sterile petri-dishes and placed before a standing electric fan (STUARC-SCIENTIFIC) which evaporated the solvents leaving behind the solidified plant extracts. The solidified extracts were then put into sterile reagent bottles, labelled and stored in the refridgerator at 12^{o}C until needed.

2.4 Preparation of extract concentration

The undiluted extract in the reagent bottles were each dissolved in a solvent, dimethyl sulphoxide (DMSO) in the ratio of 1g of extract to 10ml of DMSO (1:10) to give a concentration of 100mg/ml. Serial dilutions

of 80mg/ml, 60 mg/ml, 40mg/ml, 20mg/ml and 10mg/ml were made from the stock concentration.

2.5 Effect of plant extract on in-vitro inhibition of mycelial growth of fungal isolates.

Two mililitres (2ml) from each of the concentration of the extract (10, 20, 40, 60, 80 and 100 mg/ml was added serially into 9cm diameter sterile petri-dish and agitated thoroughly with 18ml of melted PDA forming potato dextrose leaf extract agar (PDLA). The agar-extract mixture was allowed to cool and solidify, and then inoculated centrally with a 3mm diameter mycelia disc obtained from the periphery of the actively growing culture of each of the test fungi using a sterile inoculating loop. Five treatments corresponding to the five concentrations of each solvent-extract were made.The PDA plates with the test fungi but without extract served as control. All the inoculated plates were incubated at 27±2^{o}C.Daily, measurement of the zones of inhibition on colony diameter (mm) of mycelial growth was measured for 7 days.Percentage inhibition of the plant extract was calculated and analysed statistically according to Pandy *et al.,* 1982. Benlate, a standard fungicide with a concentration of 8.5mg/ml was used to determine the efficacy of the plant extract on the fungal pathogens according to the method of Owolade and Osikanlu (1999). The experiment were repeated five times in a randomized complete block design with 6 treatments and 3 replications.

2.6 Effect of plant extract on in-vivo inhibition of disease development by fungal isolates.

Five fingers per hand of ripe and intact banana of *Musa accuminata*colla. of the green colour variety were used for inoculation experiment of the plant extract. The banana fruits were surface sterilized in 95% ethanol and rinsed in sterilized water in the biology laboratory. A modified method of Uzuegbu and Okoro (1999) and Adeniji (1970) was used. The fruits were bored with a sterile 3mm cork-borer and the core removed from the fruit with sterile forceps.Exactly 0.5ml of *A. djalonensis* leaf extract of known concentration was injected into the holes and allowed to percolate for 5 minutes. Discs of each fungal mycelia measuring 3mm in diameter, were cut from the periphery of an actively growing culture of the fungus on PDA in Petri dishes. The fungal culture-disc was placed inside the hole where the plant extract was injected. The core of the tissue was divided into two and each half used to plug the hole from both sides. The inoculated site was sealed with masking tape to prevent contamination. Discs of 3mm diameter sterile PDA on banana fruit/sites but without extract served as control. All the banana fruits were inoculated at room temperature (27±2^{o}C) and left in the incubator for 7days. The diameter of the rotted area was measured with a transparent ruler, and percentage inhibition calculated according to Pandey *et al.,* 1982. Benlate solution at 8.5mg/ml was again used to ascertain the efficacy of the plant extract.

3.0 Statistical analysis

Results of the fungitoxic effect of the treatments of extract concentrations on the test fungi was determined as a percentage inhibition of fungal growth and calculated statistically, using the formula of Pandey *et al.,* 1982 as shown below.

$$FP = \frac{F_1 - F_2}{F_1} \times 100$$

Where FP = Percentage inhibition of fungal growth

F_1 = Fungal growth in control petri-dish

F_2 = Fungal growth in treatment pertri-dish

4.0 Results

The results of the efficacy of plant extract of A. djalonensis isolated from 95% ethanol, hot and cold water media on the in-vitro inhibition of mycelial growth of three fungal isolates are shown in Table 1. The results showed that extract of A. djalonensis used exhibited some level of inhibition on the mycelial growth of the fungal isolates. The level of inhibition generally increased with increasing concentration of the extract. The ethanolic extract of the test plant at a concentration of 100mg/ml significantly inhibited the mycelialgrowth of *Verticillium theobromae* and *Colletotrichum musae* each by 99.5% while that of *Botryodiplodis theobromae* was 98.5%, Table 1. However, the inhibition levels of the extract from hot and cold water at same concentration showed *V. theobromae* (40.5%, and 11.5%); *C. musae* (45.0% and 30.5%) while *B. theobromae (*47.6% and 31.0%) respectively.

Also, at 80mg/ml concentration, the ethanolic extract of the test plant inhibited the mycelial growth of *C. musae* by 98.5% and *V. theobromae* (95.0%) while *B. theobromae* was 68.5%. These results closely resembled that of Benlate, a systemic fungicide often used in the control of several fruit rots at a concentration of 8.5mg/ml. At 60mg/ml concentration, the ethanolic concentration of the extract also recorded the highest.Inhibitory effect on *C. musae* (85.6%) while that of hot and cold water on same fungus caused 12.5% and 7.2% inhibition respectively Table 1. The results also showed that the inhibitory levels of the test plant on the three fungal isolates were significantly low when compared with that of ethanolic extract. At 10mg/ml concentration, the highest inhibition of the test plant was observed at *C. musae* (12.5%) treated with 95.5% ethanol while the lowest (0%) was recorded in *V. theobromae* and *B. theobromae* treated with hot and cold water respectively. Generally, the control plates without inoculation of the plant extract showed maximum growth of the mycelia with no trace of inhibition.

Table 1: In vitro effects of different concentrations of plant extract on mycelial growth of fungal isolates 7 days after inoculation

Fungal isolate	Mycelial growth inhibition (%)								
	Verticullium theobromae			***Colletotrichum musae***			***Botrydiplodia theobromae***		
Extract conc. 100mg/ml	**95% ethanol**	**Hot water**	**Cold water**	**95% ethanol**	**Hot water**	**Cold water**	**95% ethanol**	**Hot water**	**Cold water**
Control (0)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	12.5	1.3	1.5	0.0	0.0	0.0
20	0.0	0.0	0.0	30.6	2.5	2.3	10.0	5.0	0.0
40	34.5	2.1	1.5	55.6	6.4	5.2	20.5	10.0	0.0
60	50.0	17.3	3.1	85.6	12.5	7.2	35.2	18.4	1.2
80	95.0	20.4	4.1	98.5	34.5	16.3	68.5	30.0	3.1
100	99.5	40.5	11.5	99.5	45.0	30.5	98.5	47.6	31.0
Benlate (8.5mg/ml)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

The results of the effect of *A. djalonensis* extract on in vivo inhibition of disease development by the fungal isolates are shown in Table 2. The ethanolic extract at 100mg/ml recorded the highest inhibition of the fungal growth with *C. musae* (100%) while *V. theobromae* and *B. theobromae* recorded at 98.5% inhibition each. Also, the hot and cold water extracts at same concentration showed very low inhibition levels with *C. musae*(50.5% and 31.0%), *V.theobromae* (52.5% and 38.5%) while *B. theobromae* (40.5% and 33.5%) respectively. Also, at 80mg/ml of the extract highest inhibition of disease development was observed in ethanolic extract of *C. musae*(92/5%) closely followed by *V. theobromae* (90.5%) and *B. theobromae* (90.0%). However, at same concentration of 80mg/ml, hot and cold water extract of the plant reduced the mycelial growth of *V. theobromae* by 27.5% and 30.5% respectively; and in*C. musae* by 32.6% in hot water and 15.4% in cold water extract. Also, the vegetative growth of B. *theobromae* was reduced by 16.0% in hot water and 2.5% in cold water extract (Table 2). The control media plates without inoculation of plant extract also showed maximum growth of the mycelia with no inhibition.

Table 2: In vivo effects of different concentrations of plant extract on mycelial growth of fungal isolates 7days after inoculation

	Mycelial growth inhibition (%)								
Fungal isolate	***V. theobromae***			***C. musae***			***Botrydiplodia theobromae***		
Extract conc. 100mg/ml	**95% ethanol**	**Hot water**	**Cold water**	**95% ethanol**	**Hot water**	**Cold water**	**95% ethanol**	**Hot water**	**Cold water**
Control (0)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20	6.0	0.0	0.0	20.0	1.0	3.0	2.5	1.5	1.0
40	18.4	15.0	0.0	50.5	4.8	4.5	33.5	6.5	1.5
60	40.0	20.0	4.5	80.5	15.5	8.2	43.0	12.5	2.0
80	90.5	27.5	30.5	92.5	32.6	15.4	90.0	16.0	2.5
100	98.5	52.5	38.5	100.0	50.5	31.0	98.5	40.5	33.5
Benlate (8.5mg/ml)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

5.0 Discussion

This study showed that ethanolic leaf extract of *Anthocleista djalonensis* significantly inhibited mycelial growth of *Verticillium theobromae, Botryodiplodia theobromae* and *Colletotichum musae,* the fungal isolates of postharvest rot of banana over hot and cold water treatment in both in-vitro and in-vivo. It was observed that fungi toxicity of the extract was higher at increased concentrations and extracts by hot water were more effective than those extracted by cold water. This is similar to the observation of Ohazurike*et al.,* (2003), who reported that steam and chemical extraction methods of *Jatrophacurcas* seed and leaf extract yielded more effective control of maize weevil *(Sitophilus zeamais).*

The ethanol extract of *A. djalonensis* was most effective than hot and cold water extract in inhibiting growth and disease development in the infected banana fruits. This also agreed with the results of Olukemi *et al.,* (1997) who reported that ethanoic extracts of *Parkiafilicoidea* (L.) had more antibacterial activity than that of aqueous extract. This may be due to the ability of ethanol to extract both polar and non-polar antimicrobial compounds from infected plant parts than water (Heath and Reineccius, 1986).

The study also revealed that the antifungal activities of the plant extract did not stop in vitro but continues in vivo as well. These results also corroborates earlier reports by Bankole and Adebanjo (1995) on the ability of plant extracts to inhibit growth and development of plant pathogenic fungi in-vitro and in-vivo. Similar results were also reported by Thaigavelu *et al.,* (2004), who achieved 100% of plant growth of *Colletotrichum musae* both in-vitro and in-vivo, using plant extracts from *Solanum torvum, J. Curcus* and *Embicaofficinalis* in the control of anthracnose disease of banana.

Earlier researchers have reported on the antimicrobial potentials of extract of *A. djalonensis.* A comparison of ethanolic and cold water extracts from the root of *A. djalonensis* were reported to possess some

significant antimicrobial potency against *Staphylococcus aureus*and *Echerichie coli* (Okoli and Iroegbu, 2004). Also, studies on the phytochemical properties of different parts of *A. djalonensis* showed that the plant contains high doses of alkaloids like moneterpenes and loganines (Akubue *et al.,* 1983). Others are iribacholine, triterpene, djalonenol and lichexanthrone, indicating medical potency in ethnomedicine. The presence of these bioactive substances confirms its use in the treatment of human diseases like constipation, regulate menstrual disorders and as abortifacient (Newinger 2000). Other diseases treated by extracts of *A. djalonensis* are leprosy, veneral diseases, oedema, scrotal elephantiasis, abdominal pain and hernia (Togola *et al.,* 2005); as well as intestinal disorders like diarrhoea and dysentery, skin inflamations, boils and liver cirrhosis (Onocha *et al.,* 2003).

Based on the results of this study on the efficacy of *A. djalonensis* extract on the three fungal isolates a positive indication has been made on the fungicidal potentials of this plant. Future research efforts could therefore, be directed towards producing organic fungicides from this plant, as it is safer and readily available in our locality. Also, it is a known fact that the use of conventional chemicals are very scarce, environmentally hazardous and very expensive when seen (Owolade and Osikanlu, 1999; Thangavelu *et al.,* 2004).

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