In vitro Control of *Chalara paradoxa* Isolated from *Raphia hookeri* (Mann and Wendel) Fruits using Diethyl ether, Acetone and Methanol Extracts of the Seeds of *Aframomum sceptrum*

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ABSTRACT

The study of *in vitro* biological control of *Chalara paradoxa* using the local spice *Aframomum sceptrum* was carried out by using different solvent extracts such as diethyl ether, acetone and methanol extracts of the seeds of *A. sceptrum* with the aim of assessing the antimicrobial potential of the seeds and the most active solvent extract on the pathogen. The results of the inhibitory activity of diethyl ether, acetone and methanol extracts on the *in vitro* mycelia growth of *C. paradoxa* showed that diethyl ether seed extract exerted the highest inhibitory effect compared to the other extracts and the fungicide Mancozeb used. The results of the phytochemical screening of the three extracts shows that methanolic extract to had the highest amount of phenols, reducing sugar and steroids. The acetone extract had the highest amount of alkaloids and oxalate while the diethyl ether seed extract had the least amount of oxalate. The ability of these extracts to have an inhibitory effect on the *in vitro* mycelia growth of *C. paradoxa* can be attributed to the presence of the phytochemical agents detected in them.

KEY WORDS: *Chalara paradoxa*, phytochemical agents and *Aframomum sceptrum*

INTRODUCTION:

Raphia hookeri (Raffia palm) is the largest palm in Africa and is restricted to the tropical rainforest, the ideal ecological condition for the Raffia palm (Ndon, 2003). In Nigeria, *Raphia* palms grow wild in the lowland forest region and swamps in the Southern parts of the country, Ndon, (2003). *Raphia* palms are peculiar for their hepaxanthic flowering and so a trunk usually flowers and fruits only once and dies after 3-35 years of vegetative growth (Otedoh, 1976).

Economic products of *Raphia* palm include building materials such as bamboos, *Raphia* fibre, thatch, fibrous piassava and palm wine. Most of the species are tapped for wine by tapping the young terminal inflorescence (Otedoh, 1976). The wine is now successfully bottled for commercial purposes at the Nigerian Institute for Oil Palm Research (NIFOR) Benin-City and other places in Nigeria such as Federal Institute of Industrial Research, Oshodi (FIIRO), and Lagos.

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The palm wine is also used in distilling local dry gin. In a recent study, *Raphia* was strongly recommended for its good quality pulp for paper making as well as for producing soft tissue paper (Otedoh, 1976).

One of the economic importance of *R. hookeri* fruits as reported by Ndon, (2003) is its oil which can be used for cooking and making of confectionery. The mature and ripe fruit serves as food for coastal people of Akwa Ibom state, Nigeria. Ndon , (2003) also reported that the fruits contain plant growth regulators such as auxins, cytokinins, ethylene, gibberellins and other chemicals which can used in tissue culture and also to stupefy fish.

The healthy ripe mesocarp of *R. hookeri* fruits have also been reported to posses some phytochemical agents such as phenols, flavonoid, alkaloids saponin oxalate, quinones and other nutritional components such as moisture, minerals, fat, protein, carbohydrates (Ekpa, 1996) and other mineral components which are beneficial for nutritional purposes to man (Esiegbuya, 2012 and 2013, Murray *et al.*, 2000). The ethanolic extract of the epicarp, mesocarp and seed of *R. hookeri* fruits have also been reported by Adaigbe *et al*., (2013) to posses some phytochemical agents which were toxic for the control of termites workers at 28^0C and 75%

Chalara paradoxa and *Xylaria feejensis* are the major fungi causing black rot black rot and dry rot diseases respectively of *R. hookeri* fruits. These fungi have the ability to affect the scale, mesocarp and endocarp of the *Raphia* fruits thus destroying the embryo and thereby making the seed unsuitable for planting (Oruade-Dimaro, 1989; Esiegbuya *et al*., 2013). Other fungi associated with the *Raphia* fruits as reported by these authors include *Aspergillus niger, Fusarium* sp and *Botryodiplodia theobromae*.

The disease incidence of black rot disease caused by *C. paradoxa* on *R. hookeri* fruits in storage as reported by Esiegbuya *et al.*, (2013), under favorable environmental conditions such as temperature, humidity and light condition was over 60%. The author also reported that the black rot disease caused by *C. paradoxa* can be controlled by storing the fruits under environmental conditions with low relative humidity and complete lighting system.

In order to preserve the economic importance of the *R. hookeri* fruits, it is imperative to develop control measures against the pathogen *C. paradoxa* causing fruit rot of the fruits so as to maximize its economic values.

Apart from the method of using environmental storage conditions to control *C. paradoxa* causing fruit rot of *R. hookeri*, proposed by Esiegbuya *et al*., (2013), no other method has been proposed for the control of the black rot disease of *R. hookeri* fruits caused by *C. paradoxa.*

The plant, *Aframomum sceptrum*, K. Schum is a tropical herbaceous crop of the genus *Aframomum* which belongs to the family, *Zingiberaceae* of the angiosperms in the kingdom, Plantae. It is widely spread across countries like Nigeria, Sierra Leone, Ghana, Togo and Cote D'ivore (George *et al.*, 2010). Biological investigations carried out on several species from the genus *Aframomum* revealed their antifungal, antiparasitic, antibacterial and antiviral properties (Cousins and Hoffman, 2002; Okwu *et al.*, 2003). The seeds of this indigenous spice have also been found to contain phytochemicals (Fasoyiro and Adegoke, 2007).

The objectives of this study was to assess the biofungicidal potentials of different extracts of the local spice *Aframomum sceptrum* on the *in vitro* control of mycelia growth of *Chalara paradoxa*

MATERIALS AND METHODS

Sample collection:

Fresh seeds of "Grains of paradise" (*Aframomum sceptrum*) were obtained from a market in Benin City, Edo State and identified by a Botanist in the Department of Botany, Delta State University, Abraka, Nigeria.

Culture Preparation

A stock culture of *C. paradoxa* (IMI no: 261637) previously isolated from *R. hookeri* fruit and identified at CABI, Surrey England was collected from the culture bank of Plant Pathology Division NIFOR. Fresh cultures from the stock was prepared by inoculation on potato dextrose agar (PDA, Oxoid, England) plates and incubated at room temperature (28-30^{o}C). After three (3) days of active mycelia growth, a subculture of the filamentous fungus was made unto Petri dishes containing PDA and into slants in sterile McCartney bottles. The plates and slants were incubated at room temperature of 28± 2^{o}C and allowed to grow over a period of seven (7) days in order for spores to be adequately produced. The slants were refrigerated to store for further use.

Sterilization of Materials

Autoclavable materials such as flasks, distilled water and medium were sterilized using a Gallenkamp autoclave at 121^{o}C at 15 psi (pounds per square inch) for 15 minutes. Petri plates and other metal apparatus such as spatula and forceps was sterilized using hot air oven at a temperature of 160^{o}C for 1hour. The wire loops were sterilized by heating in the blue flame of a Bunsen burner until red-hot and allowed to cool before using. The laminar flow chamber was swabbed with 70% (v/v) alcohol to prevent external contamination.

Extract preparation:

The seeds of healthy *A. sceptrum* were harvested from the pods by hand picking them.They were then washed thoroughly with sterile distilled water; oven dried at a temperature of 45^{o}C and pulverized using a clean Lexus mixer grinder with model No: MG 2053 to a fine powder form according to the method of Wokocha and Okereke, (2005).

Thirty grams (30 g) of the fine powder was weighed using a weighing balance with model No: ALC 201.3 15038730. Three different extracting solvents, diethyl ether, methanol and acetone (analytical grade) were used to prepare the seed extracts according to the methods of Bautista *et al*., (2003).

The comparative effect of the different solvents was evaluated using a single step, single solvent extraction procedure. Fifty mls (50mls) of each solvent was measured using a calibrated cylinder and poured carefully into sterilized 250ml Erlenmeyer flask respectively. The already weighed powder (30g) of the "grain of paradise" was then soaked in the respective solvents to give 60% w/v. The flasks were sealed with a sterile foil paper and held tightly with rubber bands. The labeled extracts were then placed in an orbit shaker with model No: 3521 for 24 hour duration to allow for uniform extraction of the active ingredients. The solvents were then recovered using a Buchii rotary evaporator (Manufactured by BibbySterlin LTD., England with model No: RE 100) at 40^{o}C under vacuum and the extracts reconstituted by mixing in appropriate amount of 15 % (v/v) Dimethyl sulphoxide (DMSO, a protic solvent) to obtain a homogenous mixture of the extract (Novak, 2002). A fungicide (mancozeb 80% wettable powder)

concentration of 2000ppm was equally dissolved in equal volume of the DMSO as used in the reconstitution of the extracts.

In vitro Antifungal activity assay

In vitro experiments were carried out to determine the effect of the three extracted solvents (Diethyl ether, Methanol and Acetone) of *A. sceptrum* against *C. paradoxa*. The diethyl ether, methanol and acetone extract were evaluated at 100% concentrations on *C. paradoxa* using the food poisoned technique (Nene *et al*., 2000). The desired concentrations were obtained by pipetting 1ml respectively of each extract into 10mls of PDA medium in sterile test tubes. The PDA medium modified with the 1ml of DMSO served as the negative control and the other, PDA amended medium with 1ml of the fungicide solution served as tshe positive control for the antifungal activity assessment. The amended PDA medium was poured gently into appropriately labeled Petri dishes and replicated four times for each treatment.

Each plate was inoculated with a mycelia disc of pathogen (5 mm) taken from the periphery of a 7 day old culture of the pathogen grown on PDA using a sterile cork borer. The inoculated plates were incubated at 28±1°C till the fungus growth covered the plate as in the case of the control. The average growth was determined for each isolates for the period of seven days.

Determination of mycelia radial growth

The radial growth of the mycelium was measured along the intersecting lines and the mean of the four measurements were recorded for each of five replicates by subtracting 0.5cm initial diameter of inoculum from the mean, the total growth of mycelium was thus determined using the formula:

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

Where dc = average increase in mycelia growth in control and dt = average increase in mycelia growth in treatment (Singh and Tripathi 1999).

The values of percentage inhibition of the test and controls were compared for each test sample and the difference in their percentage inhibition reflected the extent of inhibition by the extracts.

Phytochemical Screening:

Qualitative phytochemical screening of the extracts being investigated was carried out in the Biochemistry Laboratory of Nigerian Institute for Oil Palm Research (NIFOR) Benin City. The extracts were evaluated for the presence of Phenols, Reducing sugars, steroids, Oxalate and Alkaloids using standard procedures as described by Sofowora (1993), Trease and Evans, (1989), Harborne, (1998) and Ghani, (1998).

STATISTICAL ANALYSIS

Data obtained was subjected to statistical analysis using the one-way ANOVA, while significant differences among the means were determined by using Duncan's New Multiple Range (DMR) Test as outlined by Obi, 2002. The statistical analysis was conducted with SPSS software (SPSS 17, USA set at significant levels of 0.05).

RESULTS

The results in figure1below, shows the effects of diethyl ether, methanol and acetone extract of *A. sceptrum* seed on the mycelia growth of

C. paradoxa within a test period of seven days under temperature of 28± 2°C. The study revealed that the three different polar extracts of *A. sceptrum* and fungicide (Mancozeb) had significant (P<0.05) antifungal effect on the pathogen. Diethyl ether extract of *A. sceptrum* seed was observed to have the highest antifungal activity as it was able to inhibit the mycelia growth of *C. paradoxa* for the test period of seven days of observation. The extract with the closest inhibitory activity to diethyl ether extract was acetone, methanol extracts and mancozeb fungicide. The activities of these three extracts were compared individually to that of the fungicide, the inhibitory potentials of these extracts were observed to be significantly higher at p< 0.05 than the mancozeb fungicide used. Dimethyl sulphoxide used to reconstitute the extracts from the dried form did not affect the mycelia growth of the test microorganisms negatively as observed throughout this study.

The graphical representation of the antimicrobial activities of different seed extracts of *A.sceptrum* on the mycelia growth of *C. paradoxa* is shown below.

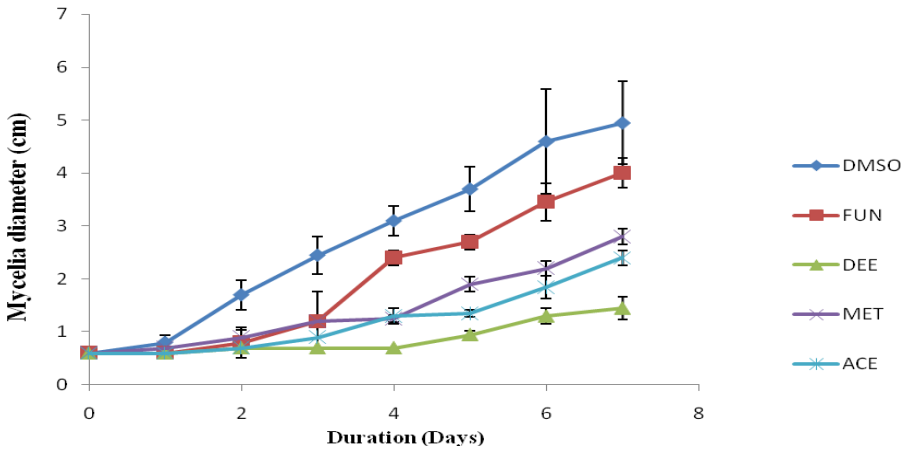

Figure 1: Graph showing the effect of three (3) different extracts of *A. sceptrum* on the mycelia growth rate of *Chalara paradoxa* (IMI 261637). DMSO- Dimethyl sulphoxide(Negative control), FUN--Fungicide (Mancozeb), DEE- Diethyl ether seed extract of A.sceptrum, MET-Methanol seed extract of A.sceptrum, ACE-Acetone seed sextract of A.sceptrum

The table below shows the results of quantitative phytochemical screening of the different extracts of *A. sceptrum*. The result showed that methanolic extract had the highest amount of phenols, reducing sugar and steroids. Acetone extract had the highest amount of alkaloids and oxalate while the diethyl ether seed extract has the least amount of oxalate.

Table 1. Quantitative phytochemical yields from seed extracts of *Aframomum sceptrum* expressed as PPM.

EXTRACTS	PHE	ALKA	RED. SUG.	STER.	OXA.
Methanolic seed extract	2790	158900	325	7.60	43200
Acetone seed extract	2610	88400	250	8.20	123900
Diethyl ether seed extract	2410	125900	320	0.20	49700

Key: PHE= Phenols, ALKA= Alkaloids, RED. SUD= Reducing sugars, STER.= Steroids, OXA= Oxalate

DISCUSSION

A*framomum* species have been reported to be fungitoxic against fungi such as *Aspergillus niger, Penicillium digitatum, Helminthosporium solani and Mucor Piriformis,* against *E.coli, Klebsiella spp and Salmonella spp.* (Chiejina and Ukeh, 2012; Doherty *et al*., 2010). The results from this study as shown on the graph in figure 1 revealed that the fungicide as well as the dimethyl sulphoxide used to reconstitute the extracts after extraction did not significantly (P>0.05) inhibit the growth of the pathogen when compared to the activity of the three extracts tested. The antimicrobial properties of *Aframomum species* reported by some authors are attributed to the phytochemical constituents such as flavonoids, phenolic compound tannins, saponin, terpernoids, cardiac glycosides and alkaloids present in the seeds.

The ability of the different extracting solvent of *A. sceptrum* to inhibit the mycelia growth of *C. paradoxa* in this study may be attributed to the presence of phytochemical agents such as phenols, reducing sugar, steroids, oxalate and alkaloids present in the different extracts. Alkaloids are heterocyclic nitrogen compounds and are commonly found to have antimicrobial properties (Oliver-Bever, 1986). These extracts have been reported by Matasyoh *et al*., (2007) to have the ability to diffuse through the cell membranous structures of fungal cells and cause damage to the cell thereby altering or lowering the physiological activities of the cell.

The presence of phenols compound in these extracts indicates that the seed extract of the plants can serve as antimicrobial agents. Phenols and phenolic compounds have been extensively used in disinfection and remain the standard with which other fungicides are compared (Doherty *et al.*, 2010).

According to Doherty *et al.*, (2010), alkaloids rank as the most efficient therapeutically significant plant substance. Pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects (Stary, 1998). They exhibit marked physiological activity when administered to animals.

The results of the *in vitro* screening of the different seed extracts used showed that diethyl ether extract was more effective in controlling *C. paradoxa* when compared with methanolic extract, acetone extract and the fungicide Mancozeb.

CONCLUSION:

The *in vitro* results of this study shows the potential of using seeds of *A. sceptrum* as one of the possible plant sources for controlling

Chalara paradoxa that affects *Raphia hookeri*. *In vivo* study is also required to confirm the resourcefulness of these results. Biofungicidal botanicals are environmentally safe and (Mushin *et al*., 2001; Okgbo and Emoghene, 2003) could successfully replace the toxic and hazardous synthetic fungicides for plant disease management.

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