THE ANTI-ANTHRACNOSE ACTIVITIES OF POLAR AND NON-POLAR COMPOUNDS EXTRACTED FROM MEDICINAL PLANTS IN THE NIGER DELTA REGION OF NIGERIA ON SPORE GERMINATION OF COLLECTOTRICHUM GLOEOSPORIOIDES (PENZ.) PENZ. & SACH.

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ABSTRACT: Polar and non-polar compounds extracted from twenty-one medicinal plants used in folklore medicine for treating infectious diseases were screened for anti-anthracnose activity with Colletotrichum gloeosporioides isolated from Carica papaya L. The polar compounds were extracted with ethanol, methanol and water while the non-polar compounds were extracted with n-hexane and chloroform. Screening of the various extracts for anti-anthracnose activity was done using the paper disc diffusion method with chlorothalonil as control. The extracts from test medicinal plants were active against the anthracnose fungus with inhibition zones ranging from 5.67-18.00mm. The polar compounds were more active than the nonpolar compounds and the methanol extracts were the most active among the polar compounds. The aqueous extracts were the least active. The most active plants were Azadirachtha indica A. Juss. and Etlingera elatior (Jack) R.M. Sm. with the methanol extract of these plants recording inhibition zones of 18 and 14.67mm respectively. The anti-anthracnose agent chlorothalonil recorded an inhibition zone of 9mm against C. gloeosporioides. The most active aqueous extract was that of the plant Cymbopogon citratus (DC.) Stapf. The degree of inhibition of the aqueous extract of this plant was lower than that recorded by the n-hexane and methanol extracts of the plant. The minimum inhibitory concentrations of

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methanol extracts of significantly active plants varied between 10-100mg/ml. The plants with significant activity are potential sources of anti-anthracnose agents.

**Key words:** Anthracnose; medicinal plants; Inhibition zone; folklore medicine; fungicide

### 1. Introduction

*Carica papaya* L. commonly called pawpaw or papaya in Nigeria is a common and economically significant fruit tree in tropical and subtropical countries of the world. Nigeria is one of the foremost global producers of papaya among countries like Brazil, Thailand, Ethiopia, Democratic Republic of Congo, Indonesia, Mexico and Colombia. The fruit is rich in vitamins A, C and calcium [1]. The ripened fruit is predisposed to a lot of diseases with anthracnose been one of the most common and devastating. The disease is a post-harvest one occurring during transit, storage and at the market or grocery shops. The post harvest loss from this pathogen could be as much as 40-100% in developing countries where proper storage conditions are a problem. *C. papaya* is susceptible to a lot of fungal pathogens such as the Phytophthora root and fruit rot (*Phytophthora palmivora* (E. J. Butler) E. J. Butler), anthracnose (*Colletotrichum gloeosporioides* (Penz) Penz & Sacc.), powdery mildew (*Oidium caricae* F. Noack) and black spot (*Asperisporium caricae* (Speg.) Maubl.) [2]. The causative agent of anthracnose of most plants is *C. gloeosporioides*, which belongs to the family Phyllachoraceae of the division Ascomycota. The fungus *C. gloeosporioides* actually initiates infection as soon as the papaya plant starts flowering and stays dormant until the postharvest environmental conditions favour colonization of the fruit tissue [3].

Treatment options for anthracnose are reliant on fungicides as the primary means of control [4] (Zhang, 2007). Some of the highly effective fungicides used for the control of the anthracnose disease are azoxystrobin, chlorothalonil, mancozeb and copper oxychloride [5]. Pathogens have however over the years developed resistance to these fungicides. Pathogen resistance and concern for public safety because of the adverse effects of these fungicides has lead to deregistration of some effective fungicides, making the search for alternative methods of control like bio-control and use of biocides for controlling fruit diseases imperative [6].
The cost implication of developing new effective chemicals is high and not an option for developing countries. The application of higher concentrations of currently used chemicals in an attempt to overcome the anthracnose disease is also not acceptable. This could potentially increase the risk of high levels of toxic residues accumulating in the plant and fruit [7]. High levels of these toxic residues when found in the fruit, could be dangerous as they are consumed immediately after harvest. Toxic levels of these compounds can therefore be bio-accumulated in humans and other non-target animals [7].

Plants have quite a number of bioactive compounds, which have found application as medicines and also antimicrobials for both plant and animal diseases. These bioactive products are less persistent in the environment and are safe for mammals and other non-target organisms.

Some plant species have been reported to possess natural compounds that are toxic to plant pathogenic fungi. Aqueous extracts of plants have been reported to inhibit the growth of pathogenic fungi like *Alternaria carthami* S. and *Fusarium oxysporum* f. sp. cubense [8, 9]. Some of the biocides are essential oils, which have been extracted from medicinal plants with solvents such as n-hexane and ethyl acetate. The non-polar compounds are lipophilic or hydrophobic like essential oils and can be extracted with non-polar solvents like chloroform. Active Compounds extracted from the plant *Etlingera elatior* with n-hexane were identified as lauric acid, β-sitosterol and stigmasterol, which were active against *C. gloeosporiodes* [10]. Polar compounds extracted from plants with ethanol and methanol, are mostly hydrophilic compounds [11]. These polar compounds are secondary metabolites of plants and are involved in plant defense against ultraviolet radiation or attack by pathogens [12]. They are naturally synthesized by plants to fight against invading pathogens and makes perfect sense to investigate their potentials as source of antimicrobials.

This study is necessitated by the fact that Papaya anthracnose is a major post harvest disease in Nigeria and it’s incidence is very high with the farmers been at the mercy of the disease. The study seeks to look for local alternative cheaper and safer means of treating the infection. The need to also look for new phytochemicals is also a consideration in embarking on this research. The aim is to explore plants used by the locals to effectively treat infectious diseases with the aim of finding new active compounds among these plants.
The type of compound extracted is also important and solvent used for extraction; hence different solvents are used for extracting polar and non-polar compounds from the test plants.

2. Materials and Methods

2.1. Isolation of Colletotrichum gloeosporioides

The test pathogen –C. gloeosporioides was isolated from papaya fruits showing anthracnose lesions on malt extract agar medium. Modification of the methods used by [13] Appiah-Kubi et al., (2016) was adopted for the isolation of the pathogen from diseased tissues. Malt extract agar was used for isolation instead of PDA. Spore suspensions were also got adopting the aforementioned methods [13]. A spore suspension of $1 \times 10^{-5}$ was used for spore germination inhibition studies. The spores were harvested with sterile water. The pure culture isolate of the pathogen was maintained in MEA culture tubes at $4^\circ$C and used as stock culture throughout the experiment. This pathogen is defined as “test pathogen” and will be abbreviated henceforth as TPh in the text.

2.2. Collection of plant specimens

The plants used for this study were collected from the wild in Ughelli Local Government Area of Delta State, Nigeria after consultation with the local herbal practitioners. The identity of the plants was confirmed at the University of Lagos Herbarium where voucher specimens were deposited. Fresh and healthy leaves of 21 medicinal plant species across 15 families collected viz. *Senna alata* (L.) Roxb. (Caesalpinaceae), *Carica papaya* Linn. (Caricaceae), *Etlingera elatior* (Jack) R. M. Sm, *Canna indica* Linn. (Cannaceae), *Vernonia amygdalina* Del. (Compositae), *Cymbopogon citratus* (DC.) (Poaceae), *Ocimum gratissimum* Linn. (Labiatae), *Aspilia africana* (Pers.) C.D. Adams (Compositae), *Momordica charantia* Linn. (Cucurbitaceae), *Gossypium barbadense* L. (Malvaceae), *Blighia sapida* König. (Sapindaceae), *Carpologia lutea* G. Don. (Polygalaceae), *Luffa cylindrica* (Linn.) M. Roem (Cucurbitaceae), *Phyllanthus amarus* Schum & Thonn. (Euphorbiaceae), *Alstonia congensis* Engl. (Apocynaceae), *Nauclea latifolia* Sm. (Rubiaceae), *Diodia scandens* Sw. (Rubiaceae), *Sphenocentrum
jollyanum Pierre (Minispermaceae), Azadirachtha indica A. Juss (Meliaceae), Acalypha wilkesiana Müll.-Arg (Euphorbiaceae) and Acalypha godseffiana Mast. (Euphorbiaceae). The specimens of the 21 TP were dried in a dehydrator (Stockli Dorrex Dehydrator, Switzerland) at 40°C until constant weight is attained. The specimens were then milled to fine powder with a Kenwood Multi-Mill (Kenwood Ltd., UK). The ground samples were dried to constant weight in the dehydrator at 25°C. These plants are herein defined as “test plants” and abbreviated as TP henceforth in the text.

2.3. Extraction of Polar Compounds

Fifty grams of powdered samples were extracted with 250ml of ethanol and methanol. The extraction was repeated three times and extracts combined and concentrated using a rotary evaporator following a modification of the method used by [14] Ademe et al., (2013). The extracts were dried and weight of extracts got. One hundred milligrams of extracts were weighed and dissolved in 1ml of 70% ethanol and methanol respectively. This concentration was tested for antifungal activity. The 100mg/ml concentration was serially diluted and the following concentrations; 100, 75, 50, 25 and 10mg/ml were used to get the minimum inhibitory concentrations for the methanol extract only.

2.4. Extraction of water-soluble fraction of plant materials (Aqueous extracts)

Boiling was used to get the aqueous extracts, 50g of powdered plant material was boiled in 250ml of distilled water (80°C) in a water bath with a shaker (Shaking Bath 5B-16; Techne Ltd, UK) for 1hour and filtered after cooling [14, 15]. The residues were boiled again and the process repeated three times and the extracts were pooled together and concentrated using a rotary evaporator. The filtrates are combined and centrifuged at 5000rpm for 10mins and filtered with a Millipore filter syringe with a 0.45μM nylon membrane. The crude aqueous extracts were freeze-dried and then weighed.

2.5. Extraction of Non-Polar Compounds

Non-polar compounds were extracted from 50g of powdered ground plant materials with 250ml of chloroform and n-hexane. The extraction was done for 2hours in a magnetic stirrer and process repeated three times. The extracts were combined and concentrated
using a rotary evaporator. After which they were dried and the weight of the extracts recorded [14, 15].

2.6. Inhibitory Effects of TP Extracts on spore germination using the Paper Disc Diffusion Assay

Filter paper discs (6mm) were sterilized in an autoclave after pouring into bottles that were covered with foil. Conidia of *C. gloeosporioides* were harvested from a 7days growth plate and the concentration of the conidia adjusted to a concentration of 100 conidia/ml following a modification of the method used by [14] Ademe *et al.*, (2013). Culture media containing spore suspension of *C. gloeosporioides* was poured into 15cm Petri dishes and allowed to solidify. Paper discs were placed on the plates and impregnated with 30μl of different plant extracts. After the carrier solvent has evaporated from the paper discs they were incubated for 4 days. The diameter of inhibition zone was measured in mm and the degree of inhibition of spore/conidia germination was measured and scored according the method used by [14, 15].

2.7. Data management and statistically analysis

Inhibition zones for the 21 TP used were recorded for polar, aqueous and non-polar compounds extracts with ethanol; methanol, water, and n-hexane; chloroform respectively in triplicates. Extracts that recorded inhibition zones equal to or higher than 8mm were considered significantly active. Data were expressed as mean ±SD at *P*<0.05.

3. Results

3.1. Effect of non-polar compounds extracted from medicinal plants on the growth of *C. gloeoeporiodes*

The non-polar compounds extracted from twelve out of the 21 TP used were significantly active against the TPh (Figure 1). Non-polar compounds from *Senna alata* (L.) Roxb., *Etlingera elatior*, *Momordica charantia* Linn., *Luffa cylindrical* (L.) M. Roem., *Phyllanthus amarus* Schum. & Thonn., *Nauclea latifolia* Sm., *Azadirachtha indica*, *Acalypha wilkesiana* Müll.-Arg. all showed significant inhibition with both
chloroform and n-hexane (Figure 1). The n-hexane extracts were more active than the chloroform extracts with most plants that showed activity with both solvents. Twelve out of the thirteen plants that were active with the n-hexane extracts showed significant activity (Figure 1). On the other hand, only eight out of the fourteen plants that showed activity (inhibition) with the chloroform extracts were significantly active.

![Non-polar Extracts](image)

Figure 1: The effect of Non-polar compounds extracted from TP on the growth of *C. gloeosporioides*

In plants like *Cymbopogon citratus*, *Gossypium barbadense* L., *Alstonia congensis* Engl., only the n-hexane and not the chloroform extracted non-polar compounds showed significant activity. The plants *Ocimum gratissimum* Linn., *Sphenocentrum jollyanum* Pierre, *Acalypha godseffiana* Mast. and *Aspilia Africana* (Pers.) C. D. Adams were active only with the chloroform extract but not significant (inhibition zones less than 8mm). The non-polar compounds from plants like *Vernonia maygdalina* Del., *Blighia sapida* König, *Diodia scandens* Sw. did not show activity (Fig. 1).

Plants that showed significant activity higher than the standard anti-anthracnose agent (chlorothalonil) used were *Senna alata* (n-hexane extracts), *Etlingera elatior*, *Cymbopogon citratus* (n-hexane extracts), *Momordica charantia* (n-hexane extracts), *Gossypium barbadense* (n-hexane extracts), *Phyllanthus amarus*, *Azadirachtha indica* (n-hexane extracts), *Acalypha wilkesiana* (n-hexane extracts) (Fig. 1).
The n-hexane extracts of plants like *E. elatior, Cymbopogon citratus, Momordica charantia, Azadirachtha indica* and *Acalypha wilkesiana* with inhibition zones between 12-13.67mm are potential sources of antimicrobials particularly of a plant pathogen like TPh (Figure 1). These were comparable to the test fungicide and recorded relatively higher inhibition zones (Fig. 1).

### 3.2. Inhibitory Activity of Polar Compounds Extracted from TP

Polar compounds (ethanol and methanol extracts) of nineteen out the twenty-one TPs used inhibited the growth of the TPh (that is 90.48% of the plants tested). The extracting solvent affected the activity of the extracts and perhaps the composition of the polar compounds extracted. Sixteen out of the nineteen active plants with the methanol extracts were significantly active that is recorded inhibition zones >8.00mm. On the other hand six out of the thirteen active ethanol extracts (i.e. extracts that elicited inhibition against TPh) were significantly active (Fig. 2).

![Figure 2: The effect of polar compounds on the growth of Colletotrichum gloeoporioides](image)

Generally the polar compounds extracted with methanol recorded inhibition zones higher than those extracted with ethanol. Plants like *Senna alata, Etinglera elatior, Canna indica*...
*indica, Cymbopogon citratus, Ocimum gratissimum, Momordica charantia, Luffa cylindrica, Nauclea latifolia, Azadirachta indica, Acalypha wilkesiana* with the exception of *Gossypium barbadense* recorded higher inhibition zones with methanol than ethanol-extracted compounds (Fig. 2). The polar compounds extracted from plants like *Carpologia lutea, Alstonia congensis, Diodia scandens* and *Sphenocentrum jollyann* only showed activity with the methanol extract. The only exception was found in the plant *Phyllanthus amarus* where only the ethanol extract showed activity (Fig. 2). The most active plants extracts (that is plants whose extracts recorded the highest inhibition zones) were methanol extracts of *E. elatior, Acalypha godseffiana* and *A. indica* with very high inhibition zones-14.67 and 18.00mm respectively (Fig. 2). The best extracting solvent for polar compounds from the TP was methanol.

3.3. **Aqueous Compounds (Polar)**

The aqueous compounds extracted from the TP showed different degrees of inhibition against the *C. gloeosporioides*. Eight out of the twenty-one plants tested showed activity against the test plant pathogen (that is 42.86% of the plants tested). The plants that showed significant activity with the aqueous extracts from the TP were *E. elatior, C. citratus, G. barbadense, P. amarus* (Fig. 3).

The aqueous compounds extracted from plants like *S. alata, Carica papaya, Canna indica, Ocimum gratissimum, Aspilia Africana, Momordica charantia, Blighia sapida, Carpologia lutea, Luffa cylindrica, Acalypha wilkesiana* did not show any activity against *C. gloeosporioides* (Fig. 3).
Figure 3: The effect of aqueous extracts from TP on the spore germination of *Colletotrichum gloeosporioides*

The polar compounds from the plant *Cymbopogon citratus* one of the most active plants are potential sources of antimicrobials for plant pathogens like *C. gloeosporioides*. The plant recorded inhibition zone higher than that of the standard anti-anthracnose agent used in this study (chlorothalonil) (Fig. 3).

### 3.4. The minimum inhibitory concentrations of methanol extract of TP

The minimum inhibitory concentration for methanol extracts from plants like *Senna alata, Cymbopogon citratus, Momordica charantia, Azadirachtha indica*, and *Acalypha wilkesiana* was 50mg/ml against *C. gloeosporioides* (Fig. 4). The minimum inhibitory concentration of one of the most active plants *Etlingera elatior* was 10mg/ml with an inhibition zone of 6mm (Fig. 4). The plant *Canna indica* was only active at high concentrations with the minimum inhibitory concentration been 75mg/ml. The plants *Ocimum gratissimum, Gossypium barbadense, Luffa cylindrica, Phyllanthus amarus, Nauclea latifolia* were only active at a high concentration of 100mg/ml with an inhibition zones of 6, 10, 6, 7 and 6mm respectively (Fig. 4). The plants - *S. alata, G. barbadense, A. indica, M. charantia* only showed significant activity at 100mg/ml. Other plants like *E. elatior* and *C. citratus* showed significant activity at 50mg/ml (Fig. 4).
The use of medicinal plants for the treatment of human diseases is an ancient practice. The discovery of a lot of medicines is plant based. The inhibitory effects of the medicinal plants used in folklore medicine in the Niger Delta region of Nigeria for treating infectious diseases also showed a lot of promise as anti-anthracnose agents. Most of the plants tested (TP) were active against the anthracnose pathogen *C. gloeosporioides* but not all of them showed significant activity. The inhibitory activity of the TP against the TPh shows that there are biocides in the plants and justifies their use for treatment of diseases caused by pathogenic organisms in folklore medicine. The inference here is that there are compounds in these plants that can act against or stop the growth of pathogen causing diseases. The aim of this research was also to investigate which groups of compounds in the plants are most effective for treating anthracnose of papaya. The research also seeks to find out the best extracting solvent between ethanol and methanol, which are most often used in literature for the extraction of polar compounds. The efficacy of some of the medicinal plants used in this study had been recorded in previous researches like those of Bazie *et al.*, (2014) [15]. Bazie et al., (2014) [15] recorded inhibition zones of 9.7mm for *Vernonia amygdalina* and 7.3mm for *Azadirachtha indica* against Anthracnose of banana. In this

**Figure 4**: Minimum inhibitory concentrations of methanol extracted polar compounds of test plants with high degree of activity

**4. Discussion**

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study, the plant *Vernonia amygdalina* did not show any activity with the polar and non-polar compounds against anthracnose of papaya. The aqueous extracts of this plant (*V. amygdalina*) showed activity against anthracnose but not significant activity (inhibition zone-6.67mm) in this study (Fig. 3).

This study also showed that the methanol extracts were more active than the aqueous extract (Fig. 2 and 3); a conclusion reached by Bazie *et al.*, (2014) [15] and Ekpo and Etim, (2009) [16]. This study agrees with other researchers like [17] Chigayo *et al.*, (2016), that concluded that 80% methanol is one of the best extracting solvents for extraction of biocides. Other researchers like Zlotek *et al.* (2016) [18] concluded that acetone mixtures are more effective than methanol mixtures for the extraction of bioactive compounds from medicinal plants like *Ocimum basilicum* (basil). According to Dawa *et al.*, (2016) [19] the yield and activity of bioactive compounds extracted with methanol was higher than that with chloroform and n-hexane. Inhibitory activity was also higher with the methanol extract as recorded here in this research. The plants used in this investigation have also been shown to be active against human pathogens like *Candida albicans* indicating that plants used in folklore medicine for treating infectious diseases are potential sources of new antimicrobials [20]. Records also show that essential oils from medicinal plants have inhibitory effects on human pathogens like *Staphylococcus aureus* Rosenbach. and *Candida albicans* (Robin) Berkhout [21] indicating that non-polar compounds also show a lot promise as new antimicrobial agents.

Various extracts from the same plant varied in their inhibitory activity against the plant pathogen *C. gloeosporioides* indicating that extraction method affects the efficacy of the antimicrobial agent extracted from plants or other natural sources. The different extracting solvents also elute different compounds that vary in their chemical structure and activity. Sales *et al.*, (2016) [22] demonstrated that different plant extracts have varying degrees of inhibition against plant pathogens like *Fusarium guttiforme* and *Chalara paradoxa*. The aqueous extracts *Cymbopogon citratus* and *Azadirachta indica* showed significant activity against the test organism *C. gloeosporioides* (Fig. 3). In other investigations for instance in Silva *et al.*, (2014) [23], the aqueous extracts of *C. citratus* and *A. indica* did not show any activity against the test organism contrary to the results recorded here. This indicates that the method extraction affects or determines the components of the
antimicrobials extracted and the efficacy of the extract. The plant *Ocimum gratissimum* recorded significant inhibition against TPh with the methanol extracts as recorded by Silva et al., (2008) [24] against *C. gloeosporioides*. Some of the medicinal plants tested showed high degree of inhibition and therefore emphasizes the need to look into traditional medicines for new sources of antimicrobials. The plants used for treating infectious diseases could be very potent as they target microorganism that are pathogenic. A lot of work has been done and is on-going for the discovery of drugs used in treating human pathogens. This work concentrated on the effects of medicinal plant extracts on a plant pathogen and the effects recorded were promising as a number of the extracts recorded significant inhibition.

The plants *Etingerla elatior*, *Cymbopogen citratus* *Acalypha godseffiana* and *Azadirachtha indica* are potential sources of anti-microbial agents against plant pathogens like *C. gloeosporioides*. The extracting solvents affect the activity and degree of inhibition of the compounds extracted and definitely the composition. Work is still on going to identify the active compounds in these various extracts.

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