Evaluation Of Azadirachta Indica extracts Invitro On The Growth And Development Of Phytophthora Megakarya Causal Organism Of Blackpod Disease Of Cocoa (Theobroma Cacao L.)

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Evaluation of *Azadirachta indica*extracts *invitro* on the growth and development of *Phytophthora megakarya* causal organism of blackpod disease of cocoa (*Theobroma cacao* L.)

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CERTIFICATION

This is to certify that this project work was carried out by **Adisa Habibat Ayomide** with the Matriculation number **14/06/3920** in the department of Science Laboratory Technology, School of Science, Abraham Adesanya Polytechnic, Ijebu-Igbo under my supervision.

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DATE

DEDICATION

This project is dedicated to the Almighty Allah the all satisfactory God of heaven and earth, for his magnificence throughout my programme and my parents Mr. and Mrs. **Adisa** for their continuous support from the beginning to the end of my National Diploma Programme.

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ABSTRACT

The efficacy of *Azadirachta indica* (Neem) leaf extracts were evaluated on the growth and development of *P. megakaryain vitro* using acetone, ethanol and methanol as extraction solvent.Fourier Tranform Infra-Red (FTIR) Spectrometer was used to determine the quantitative constituents of the plant extracts. Five levels of crude extracts concentrations were used viz; 20, 40, 60, 80 and 100% respectively to determine the best concentration at which the extract will be most effective against the pathogen.The fungus *Phytophthora megakarya* was isolated from infected cocoa pod. Food poisoning technique was used to determine the toxicity of the neem extracts against the growth of the pathogen.5 ml of *A. indica* extracts were mixed with 20 ml of Potato Dextrose Agar (PDA) to get 20, 40, 60, and 80 % concentration, while the undiluted extract was recorded as 100 %. The plates were inoculated with a 5mm assay disc cut from 7 day old culture fungus; data were taken starting from 1 day after inoculation and the experiment was terminated at 7th day after inoculation. A negative and positive control experiment was set up separately containing distilled water and reference synthetic fungicide (Kocide). Radial growth of *P. megakarya*was inhibited by the acetone, methanol and ethanol extracts. It was observed that ethanol extracts had the highest inhibiting activity on *P. megakarya* with an inhibition percentage of 78 at 100%

concentration compared to the synthetic fungicide (kocide) with an inhibition percentage of 53.70 at 100% concentration.

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CHAPTER ONE

INTRODUCTION

Cocoa (*Theobroma cacao)* is a native of Amazon of South America. *Theobroma cacao* belongs to the family *Malvaceae*. Cocoa trees attain a height of 8 to 10m; the shape of the pod is similar to that of cucumber. It is about 20cm long, 8 to 10cm thick and it weigh between 300 to 400g. The bulk of it is produced in the tropical areas of the African continent. Its seeds (beans) are used to make cocoa powder and chocolate.

There are two prominent competing hypotheses about the origin of the domestication of the wild *Theobroma cacao* tree. One is that wild examples were originally distributed from south eastern Mexico to the Amazon basin, with domestication taking place both in theLacando area of Mexico and in lowland South America. But recent studies on*Theobroma cacao* genetics seem to show that the plant originated from the Amazon and was distributed by human throughout Central America and Mesoamerica (Wales, 2010).

Cocoa (*Theobroma cacao*) is a native of Amazon basin of South America where it was harvested from the wild for religious ceremonies. The aztes of the Central America used the bean as currency and to prepare a drink known as chocolate (Lemin 2005). Cocoa is cultivated in central and south America, south-east Asia and in West Africa.

There are 20 species in the genus but the cocoa tree (*Theobroma cacao*) is the only one cultivated widely economically. *Theobroma cacao* L. is the most important species in the genus *Malvaceae* (Naturland, 2000). Naturland (2000) and Opeke (2005) further reported that, the three main subspecies; *Criollo, Forastero, and Trinitario*are in cultivation and the *forastero* subspecies is present on over 80% of all cocoa plantations. Cocoa generally originated from the Amazon forest (the subspecies *forastero*Amazonian) as well as the rainforest of Central America (the white seed subspecies *criollo*) the crop was already known to the Aztecs, who relished it as a "food of the gods".

Cocoa arrived in Europe through the former colonial powers, Portugal, and Spain and was later found in Africa. Today cocoa is cultivated in all of the humid tropical countries (Naturland 2000).

From the finding of Wood and Lass (1987), the principal cocoa-producing countries in West and Central Africa include (in order of importance) are Cote d'ivore, Ghana, Nigeria, Cameroon, and Togo. *Theobroma cacao* which is known as a tropical tree native to the main forest under story of South and Central America (Wood and Lass, 2001). Cocoa is an important crop in many countries including Brazil, Ghana, Ivory Coast, Cameroon, and Nigeria.

Global annual earnings from export of cocoa average are $2.9 billion (Gray, 2000). Between 1960 and 1969, Nigeria was ranked second world largest producer of cocoa in the world with an annual output of about 270,000 tonnes accounting for 18% of the world's total production (Opeke, 2003).

Nigeria's cocoa production output has declined from over 300,000 tonnes to 100,000 tonnes with an average annual rate of decline of 8.3% during 1992-96 to 1.8% during the 1997-2001 (ICCO, 2009). Currently, Nigeria ranks fourth in cocoa production in the world, after Cote d'Ivoire, Indonesia, and Ghana (FAO, 2009) and third largest producer in Africa (ICCO, 2009).

The downward trend in the production of cocoa has been attributed to a several factors including ageing of trees, poor agronomic practices, pests and diseases (Dongo and Orisajo, 2007). Of these, diseases are the most important factors contributing to decline in cocoa production in Nigeria, and the fluctuation of cocoa production worldwide with some estimates putting loses as high as 30 to 40% of global production (Woods and Lass, 1989; ICCO, 2009).

Over 200 different diseases are known to affect cocoa but about 10 of these are of economic importance (Woods and Lass, 1989). Diseases affect cocoa trees and fruits in a number of ways which may result in the death of parts of

the plant or the whole plant (Evans, 2001). Cocoa is affected by a number of virus and fungi diseases at different stages of growth and development.

The most prominent among cocoa-infecting fungi disease in cocoa-growing region is Blackpod disease of cocoa incited by soil-borne fungi pathogen, *Phytophthora* species (Oluyole and Lawal, 2008).

In economic terms, black pod disease is the most serious disease of cocoa worldwide and especially in Nigeria. It is prevalent only during the wet season. The disease is more devastating in areas of heavy rainfall. Major damage from the disease is the rotting of both small and large pods. Choupons, seedlings (in the nursery) and leaves of trees are attached and killed under specially severe disease conditions following long periods of cool and rainy weather. Losses due to black pod disease vary from place to place and from variety to variety. Adegbola (1972) reported about 40 % average loss over several parts of West Africa and up to 90 % in certain places in Nigeria.

Cocoa Research Institute of Nigeria (CRIN) noted in one of their data analysis that pod loss due to black pod disease infection varies with variety of cocoa. Babcock *et al* (1992) reported that those yield losses could be managed through the use of chemicals (synthetic pesticides), but various cases of

chemical residue, ill-health and poisoning associated with chemical use are of serious concern. Additionally, lack of technical know-how, high cost, scarcity, soil contamination and environmental pollution associated with these chemicals make them unpalatable in developing countries.

On the other hand, man has used plants for health care in many countries. Many molecules of medicinal uses were originally derived from plants. However, in the 50(s), with the advance in anti-biotic and particularly the enormous development of synthetic organic chemistry, the use of herbs and herbal products created considerably scientific interests. Responses from traditional society indicate the efficacy of leaf extract of commonly available local plants for combating fungal infections.

The antifungal and antiviral property of these plants has been reported by many authors. Consequence upon the above, and the need to source for alternative to chemical control, the role of higher plants as source of fungicides and their importance in controlling different plant pathogens are gaining prominence, in view of the ecological stability and cost effectiveness plant extracts with their biodegradable and environment friendly nature have shown some promise in recent years. The result could add to methods of control used by farmers, thereby reducing reliance on chemical fungicides that

are reported to predicate long term harmful consequences on environment, man and other wildlife.

1.1 Objectives of the study

- To analyse the antifungal properties of Neem leaf extract against *Phytophthora megakarya*.
- Comparative efficacy of Neem leaf extract using different solvents at different concentrations for in vitro control of *P. megakarya*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1History and cultivation of cocoa

Theobromacacao (L.), previously a member in the family *Sterculiaceae* (Coste, 1992), has recently been reclassified in the family *Malvaceae* through the use of molecular markers (Alvenson*et al.*, 1999).

Cacao is the only cultivated species of the entire *Theobroma* genus (Bartley, 1986). Recent studies on morphological diversity, along with extensive eco-geographical surveys carried out in major cocoa herbaria have pointed out that the upper Amazon of South America was the centre of origin of cacao (Opeke, 2003).

It is believed that cocoa reached Europe in 1585 (Coste, 1992).The crop was introduced to the Island of Fernando Po in Equatorial Guinea from Portugal in 18th century by a Sierra Leonean named William Pratt, a returnee from West Indies (Opeke, 2003) and introduced to Nigeria in 1874 by Chief SquissIbannigo (Ayorinde, 1966).

Later, cocoa cultivation spread to other parts of West Africa through trading agents, Ministries of Agriculture and research institutes (Opeke, 2003).

Cocoa is a popular tree crop grown under rain fed conditions of the tropics and its yields vary due to many agronomic factors. Cocoa production is carried out majorly by peasant farmers at subsistence level (< 3 hectares). Yield potentials in resource-poor farmers' fields average 300 – 400 kg per hectare.

In West Africa, the most common sources of cocoa planting materials are the government cocoa seed gardens and cocoa research institutes.

The importance of cocoa as a cash crop and foreign exchange earner led the then Government of Nigeria to set up field trials at Ibadan using cocoa seedlings from the old botanical garden at Ebute Meta in Lagos (Opeke, 2003). In 1912, total area planted to cocoa in Nigeria was estimated to be 4,000 hectare and this increased to over 12,000 hectare by 1930 (Olayemi, 1974).

Cultivation was intensified from 1930 to 1945 by which time nearly 400,000 ha had been planted (Adesimi and Ladipo, 1977), with an average contribution of 18 % to the total world production between 1960-1970 (CBN, 2004).

The Nigeria cocoa output, however, declined in 1980s due to the reduction in production capacity as a result of abandonment of many cocoa farms due to

pest and disease attack, and increasing dominance of oil exports in 1970s (FAO,1997). This changed the ranking of Nigeria as the fourth largest producer in the world (Thresh, 1989; ICCO, 2009). Table 2.1 shows the list of the major cocoa-producing countries in the world.

2.2Diseases of cocoa

Cocoa is affected by a variety of diseases and pests during growth and post-harvest and they constitute a major constraint to productivity leading to over 30 % production loss (Entwistle, 1972; Keane, 1995; Evans, 2001).

2.2.1Virus diseases of cocoa

Virus diseases affecting cocoa include, CSSVD caused by CSSV in Nigeria, Ghana, Côte d'Ivoire, Togo, Sri Lanka and Indonesia (Thresh, 1958); Cocoa necrosis virus disease (CNVD) in Nigeria and Ghana (Thresh, 1958; Owusu, 1971); Cocoa mottle leaf virus disease (CMLVD) in Nigeria (Thresh, 1958); Cocoa yellow mosaic virus disease(CYMVD) in Sierra Leone (Blencowe*et al.*, 1963; Brunt, 1970); Trinidad virus disease in Trinidad (Posnette, 1943); Cocoa yellowvein-banding disease (CYVBD) in Malaysia (Liu and Liew, 1975); watermark disease in Malaysia (Liu, 1979).

Cocoa virus diseases and their characteristics are summarized in Table 2.2. Of these, CSSV has been recognized as the most economically important virus.

CSSVD was first reported in Ghana in 1936 (Steven, 1936), in Nigeria in 1945 (Murray, 1945), Côte d'Ivoire (Mangenot*et al.*, 1946), Trinidad (Posnette, 1943) and Sri Lanka (Peiris, 1953). Presently, CSSV occurs in all cocoa-producing countries in West Africa (ICCO, 2009). CSSV particles are bacilliform and measure 121 - 130 nm × 28 nm (Frison and Feliu, 1989; Lockhart *et al.*, 2000).

Its genome is circular double stranded DNA (dsDNA) of 7.4 kb. CSSV is known to have latent period in its host, varying from a few weeks to more than 2 years (Posnette, 1947). Different CSSV strains are known to cause different symptoms (Lockhart *et al.*, 2000).

2.2.2 Fungal diseases of cocoa

Fungal diseases pose major constraint to cocoa production at global level. The greatest losses are as a result of black pod caused by *Phytophthora* species, witches' broom induced by *Crinipellisperniciosa* and frosty pod rot incited by *Moniliophytorararoreri.*

Low productivity accompanying infection represents a serious economic loss to cocoa-growing communities.Witches' broom caused by *Crinipellisperniciosa* is the second most important fungal diseases affecting cocoa production globally.

The disease is believed to have first occurred in South America. The pathogen infects all actively growing meristematic tissues including, shoots, flowers and pods, and causes hormonal imbalance which result in hypertrophy and hyperplasia of the cambial tissues distal to infection site (Evans, 2001).

The colonized tissues undergo several physiological and hormonal changes leading to swelling and formation of numerous succulent vegetative branches, known as brooms, within flower cushions and on vegetative apical or axillary buds (Orchard *et al*,.1994).

Black pod occurs throughout the tropics, and seven different pathogens have been identified to incite black pod disease all over the world. All of which are found in the *Phytophthora* genus (a plant damaging Oomycetes), the species includes; *P. capsici, P. citrophtora, P. megasperma, P. katsurae, P. palmivora* and *P. megakarya.* The most virulent and devastating among the species are *P. palmivora* and *P. megakarya. Phytophthora megakarya* is found only in West Africa (Guest, 2007).

The pathogen infects every part of the plant at all stages of development, and infection is more severe at the onset of the rainy season and few months prior to ripening of the fruit.

The infection process is initiated by motile zoospores, asexual spores released by *Phytophthora* under moist conditions. Zoospores are encased solely by a plasma membrane and produced in a vesicle or a sporangium, and when released they are responsible for the spread of the pathogen.

The most striking symptom caused by *Phytophthora* spp. is pod rot or black pod. Pod lesions begin as small, hard, dark spots on any part of the pod, at any stage of pod development.

Lesions grow rapidly covering the entire pod surface and internal tissues, including the beans, of susceptible genotypes within a few days. Colonized pods shrivel to form a mummified pod, which serves as reservoir of inoculum for at least 3 years (Dennis and Konam, 1994). Symptoms appear quicker and sporulation is usually more abundant (Gregory and Maddison, 1981, Opoku*et al.*, 2000).

Under humid conditions a single pod may produce up to 4 million sporangia (containing motile zoospores) that are disseminated by rain, ants, flying insects, rodents, bats, and flying foxes; on contaminated harvesting and pruning implements; and in contaminated soil (Gregory and Maddison, 1981). *P. megakarya* and *P. palmivora* infect bark, flower cushions, and chupons causing cankers. Cankers at the base of the trunk may extend to the main

roots. Canker lesions are hidden by the bark but often exude a reddish gum or infect flower cushions, killing the flowers. In humid conditions, the pathogenalso causes seedling and leaf blight. Infections of fine roots are also common; however these appear to be more important as a source of inoculum than as a cause of serious injury to the tree, particularly if a leaf mulch layer is present.

The pathogen commonly survives as mycelium and chlamydospores (thick-walled resistant spores) in infected plant material, usually roots, cankers or mummified pods (*P. palmivora*), or in the soil (*P. megakarya*) (Gregory and Maddison, 1981).

Although symptoms appear year-round, the most severe epidemics coincide with the proliferation of sporangia and insect vectors during the wet season. In the presence of moisture, sporangia release the infective propagules, zoospores.

Zoospores need 20 to 30 min in free water on plant surfaces before they encyst, germinate, and penetrate host tissues. Under favorable conditions, sporangia develop within 48h of infection. Rain splash, aerosols, contaminated equipment, rodents, and ants are potential mechanisms of inoculum movement into the canopy.

However, rain splash dispersal of *P. megakarya* from the soil surface or piles of pod cases is limited to 75cm, and there is little evidence to indicate that aerosols are created under the relatively protected canopy of cocoa trees (Gregory and Maddison, 1981).

Once in the canopy, reservoirs of inoculum are established in cankers, in infected flower cushions and mummified pods (Gregory and Maddison, 1981).

2.3 DISEASE MANAGEMENT

2.3.1 Quarantine

Despite vast distribution of the pathogen, pathogenic diversity within the species exists and the introduction of exotic isolates poses a significant threat to cocoa production (Appiah*et al.*, 2003, Drenth and Guest, 2004). Efforts should be made to prevent the movement of *Phytophthora* spp., particularly *P. megakarya* from West Africa to other cocoa-growing regions. The movement of soil between cocoa-growing areas must be avoided and cocoa germplasm must be exchanged thorough intermediate quarantine facilities (Kepe*et al.*, 2002, and Opoku*et al.*, 2000).

2.3.2 Resistance

Breeding for resistance offers the best long-term management strategy; however, progress incorporating durable resistance into cultivars with desirable agronomic and quality attributes has been slow.

As a genetically variable perennial tree, cocoa improvement presents significant challenges to breeders. Additionally, most breeding programs have focused on yield and quality under intense management regimes and correspondingly low rates of disease, thus neglecting the impact of disease on yields under smallholder farm conditions.

Amelonado-type Lower Amazon and Upper Amazon selections appear less susceptible to *Phytophthora* than Trinitario and Criollo types, and are widely used in breeding programs (Iwaro*et al.*, 2006). Reliable screening assays for resistance using detached leaves or pods have been developed and correlate well with field observations of pod rot incidence (Iwaro et al., 1997, Iwaro et al., 2005, and Tahi et al., 2007).

These assays are now used in breeding programs to identify and cull highly susceptible progenies. Resistance to *Phytophthora* has been identified as additive and polygenic (Despreaux*et al.*, 1989, and Flament*et al.*, 2001) and does not appear specific for at least the two most important species of

Phytophthora, *P. palmivora* and *P. megakarya* (Nyasse*et al.*, 2007). A number of different quantitative trait loci (QTLs) for resistance to *Phytophthora* have been identified in leaf disk, pod inoculation, and field studies, although so far none of these markers appear consistently (Figueira, 2004).

Nevertheless, with further development and improved precision, marker assisted selection for resistance should assist future cocoa breed programs.

2.3.3 Biological control.

There has been extensive research into the discovery and application of conventional inundative biological control agents against *Phytophthora* diseases of cocoa. Although there have been many reports of antagonistic and mycoparasitic fungi inhibiting the growth of *Phytophthora* in vitro, no commercial products have been released or widely adopted by cocoa farmers.

The short life cycle, phenomenal reproductive capacity, complex disease cycle, and zoospore motility of *Phytophthora* generates explosive epiphytotic in cocoa have so far rendered inundative biological control agents ineffective. The discovery and development of antagonistic endophytes offers more promise.

Endophytic fungi are naturally transmitted from mature trees to seedlings in the natural cocoa forest ecosystem, however they eventually disappear from plantations. Recent evidence suggests that antagonistic endophytes re-introduced into cocoa persist and protect the tree against *Phytophthora* (Arnold *et al.*, 2002). Endophytes could play an important role in integrated disease and pest management programs.

Biological suppression and antagonism following the use of mulches and composts improves soil health and microbial activity and suppresses *Phytophthora*, and these methods are also important components of integrated management programs (Aryanthae*t al.*, 2000, Konam and Guest, 2002).

2.3.4 Fungicides.

Chemicals are widely recommended for *Phytophthora* control, but their effectiveness is variable, particularly during high-disease pressure in the wet season. The implementation of recommendations is typically yield- and price-sensitive.

Protectant sprays of copper-based fungicides, together with the systemic fungicide metalaxyl, at 3- or 4-weekly intervals are frequently injections of the inexpensive inorganic salt potassium phosphonate are very effective against *P. megakarya*, particularly in reducing cankers, in very wet areas of

Papua New Guinea (PNG) (Guest *et al.*, 1994), and in Ghana against both *P. palmivora* and *P. megakarya* (Opokuet *al.*, 2007).

2.3.5 Botanicals.

Some plant contain compounds that are toxic to pathogens when extracted from the plant and applied on infested crops, these components are called botanical pesticides or botanicals. Commonly used botanicals are Neem (*Azadirachta indica* A. Juss), Garlic (*Allium sativum*, Linn), Eucalyptus (*Eucalyptus globulus*, labill.), Tumeric (*Curcuma longa*, Linn.), Tobacco (*Nicotianatobacum*, Linn.), Ginger (*Zingiberofficinale*, Rosc).

Essential Oils: Nettle oil (*Urtica*spp), Thyme oil (*Thymis vulgaris*, Linn) Eucalyptus oil (*Eucalyptus globulus*, Labill) Rue oil (*Rutagraveolens*, Linn), lemon grass oil (*Cymbopogonflexuosus*) and Tea Tree oil (*Malaleueaalternifolis*).Botanicals are sustainable solutions in agriculture, they reduce crop losses, are eco-friendly, easily bio-degradable, organic farming, cheaper, and integrated disease management.

2.3.6 Use of Plant Extracts to Control Blackpod of Cocoa

Plants have the ability to synthetize aromatic secondary metabolites like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. (Cowan, 1999).

The components with phenolic structures, like carvacrol, eugenol, and thymol, were highly active against the pathogen. These groups of compounds show antimicrobial effect and serves as plant defence mechanisms against pathogen micro-organisms (Das *et al.*, 2010).

Many plant and plant products have been reported to be antimicrobials against plant pathogenic fungi. (Bowers and Locke, 2000; Lawson and Dennedy, 1998; Grayer and Harbome, 1994; Shetty *et al.*, 1984).

Many of the earliest pesticides are extracts of plants, and several plants are exploited more widely as sources of insecticides.

But from 1940s, synthetic agrochemicals largely replaced plant-derived natural products for the use in agriculture went into decline for a number of years, but this trend is now reversed as it becomes evident that plant natural products still have enormous potential to inspire and influence modern agrochemical research.

It is estimated that there are at least 250,000 different species of plant in the world. But it was also estimated that only 10% of plant species have been examined chemically until 1993 (Benner, 1993), so there is enormous scope for further work.

2.4 HISTORY OF NEEM PLANT

Azadirachta indica(Meliaceae) commonly known as neem is a native of India and naturalized in most of The chemical constituents contain many biologically active compounds that can be extracted from neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones.

*Azadirachtin*is actually a mixture of seven isomeric compounds labeled as *azadirachtin* A-G and azadirachtin E is more effective (Verkerk*et al.*, 1993). Other compounds that have a biological activity are salannin, volatile oils, meliantriol, nimbin, nimbidin, gedunin and quercetin (Jacobson *et al.*, 1990; Ahana*et al.*, 2005).

Neem leaf is active in treating eczema, ringworm, acne, anti-inflammatory, antiheperglycemic properties and it is used to heal chronic wounds, diabetic food and gangrene developing conditions. It is believed to remove toxins from the body, neutralize free radicals and purify the blood. It is used as anticancer agent and it has hepato-renal protective activity and hypolipidemic effects (Fitoterapia part I and part II).

Medicinal plants have been found useful in the cure of a number of diseases including bacterial. Medicinal plants are a rich source of antimicrobial agents

(Mahesh and Satish*et al.*, 2008). Almost every part of the tree is bitter and finds application in indigenous medicine.

Neem extract has been reported to have antidiabetic, antibacterial and antiviral activity (Kirtikar and Basu, 1987). Almost every part of the tree has been in use since ancient times to treat a number of human ailments and also as a household pesticide.

The extract from bark, leaves, fruits and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children (Chattopadhyay et al., 1993). Flavonoids, flavonoglycosides, dihydrochalocones, tannins and others are also important constituents of bark, leaves, fruits and flowers of neem. The biological activities and medicinal properties of neem have recently been reported (Venugopal and Venugopal, 1994).

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids etc. which have been found *in-vitro* to have medicinal properties. Pharmacological studies have accepted the value of medicinal plants as potential source of bioactive compounds (Biswas and Chattopadhyay, 2002). Phytochemicals from medicinal plants serve as lead

compounds in antimicrobial discovery (Chakravarthy*et al.*, 1985; Ebi*et al.*, 2000; and Cohen *et al., 2002*).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Samples Collection

Leaves of *Azadirachta indica* were collected from AdekunleAjasin University campus. The collected samples were air-dried in the Laboratory of Plant Science and Biotechnology Department. Infected cocoa pod were collected from Federal University of Technology, Akure, Teaching and Research Farm.

3.2 Pathogen Isolation

The fungus *Phytophthora megakarya* was isolated from infected cocoa pod in the Department of Plant Science and Biotechnology laboratory. The pod was washed under gentle flow of tap water and surface sterilized with 5% Sodium hypochlorite (NaOCl), drained and rinsed three times with sterile distilled water. About 1mm portion was cut from the advancing edge of the diseased portion.

The samples were blotted on sterile paper towels in a laminar flow chamber for 10mins to dry, then four pieces were placed per Petri dish containing 20ml PDA (Merck KGaA, Darmstadt, Germany) acidified with 10% lactic acid. The plates were incubated at 27^0C for 72 hours.

Sub-culturing was carried out to get pure isolates. The isolate was prepared on a slide and stained with cotton blue in lactophenoland viewed under the compound microscope for the characteristic feature of *P. megakarya* with reference to Barnett and Barry (1972). The isolate was maintained on PDA slants and kept at 4^0C for further experiment.

3.3 Preparation of plant extracts

The dried plants were powdered using mortar and pestle. Methanol, Ethanol and Acetone were used for the extraction process. 20g of the powdered neem sample were loaded into catridges and placed in soxhlet extractor using 250ml each of the extraction solvent and then concentrated in vacuum at 40-50^0C using a rotary evaporator. Evaporation of the solvent in the rotary evaporator yielded a crude extract of the soluble components and these extracts were subjected to the Fourier Transform Infra-red Spectroscopy (FTIR).

3.4 Qualitative determination of *Azadirachta indica* crude extract using Fourier Transform Infra-Red (FTIR) Spectrometer.

FTIR analysis of *Azadirachta indica*crude extract was carried out at the Centre for Energy and Research for Development (CERD), ObafemiAwolowo University (OAU), through the potassium bromide (KBr) analytical grade (FTIR grade) method in 1:10 and the spectrum was recorded using thermoficial scientificNicolet-IS5 Fourier transform infrared spectrometer

mapped with Intron Infrared Microscope using transmittance mode operating at a resolution of $4cm^{-1}$(Nicolet IS5, USA).

3.5 Determination of antifungal properties of the extracts

Antifungal activities of the samples were carried out by poisoned food technique (Nene and Thapliyal, 1979). 5ml of each sample was mixed with 20ml of molten Potato Dextrose Agar (PDA) separately before pour plating and allowed to solidify at ambient temperature.

A 5mm disc cut from the periphery of the 7 day old culture of the test fungus was inoculated at the center of the PDA plates (mixture of PDA plus plant extracts). A negative and positive control experiment was set up separately containing distilled water and reference synthetic fungicide (Kocide). Plates were incubated aseptically at 25-27^{0}Cfor 72-96 hours.

The position of the disc was marked on the base of the dish with a marker pen and two orthogonal axes, passing through the centre of the disc, were marked to use as references for recording growth. Plates were incubated at 27^{0}C for 7 days.

Different concentrations of the extracts (20, 40, 60 and 80%) was equally prepared separately and set up as earlier explained. Mycelial growth of the test isolates (*P. megakarya*) were measured with the aid of digital verniercaliper

and recorded 24h intervals. However, the experiment was stopped when the Petri dishes were completely covered by the fungus. Each treatment was replicated three times and arranged in Completely Randomised Design (CRD). The percentage of mycelia growth inhibition was calculated and recorded appropriately using the following formula;

$$\frac{dc - dt}{dc} \times \frac{100}{1}$$

Where dc = diameter of fungi colony in negative control sets

dt= average diameter of fungicolony in treatment sets

The experiments were carried out in triplicate and the mean values were recorded.

3.6 Determination of minimum inhibitory concentration (MIC): To determine the MIC values, the method of Dulger and Aki (2009) was used. The MIC was considered as the lowest concentration of the sample that prevented visible growth.

CHAPTER FOUR

4.0 RESULTS

4.1 FTIR analysis result

The quantitative analysis of *Azadirachta indica* constituent were carried out using FTIR analysis in order to find out their roles in reducing and inhibiting the growth of the tested fungus.

The strongest band recorded from the fig. 4.1 below (3415.14 cm^{-1}) correspond to O-H groups, H bonded alcohols and phenols. A peak at 2359.51-2925.29 cm^{-1} denote –C-O-C-, ether linkages, -C-O-, germinal methyls, - C = - groups or from aromatic rings and alkyne bonds respectively which are responsible for the reduction and inhibition of the tested fungus.

The bands at peak 1635.21-1733.93 shows the presence of protein which helps in reducing the metallic ions or affinity for metal nanoparticles. Bands at peak 1405.54-1506.79 denotes stretching vibrational bands responsible for compounds like flavonoids and terpenoids (fig.4.1) which may be held responsible for efficient inhibition and stabilization of the antifungal properties of *A. indica* crude extract against *P.megakarya.*

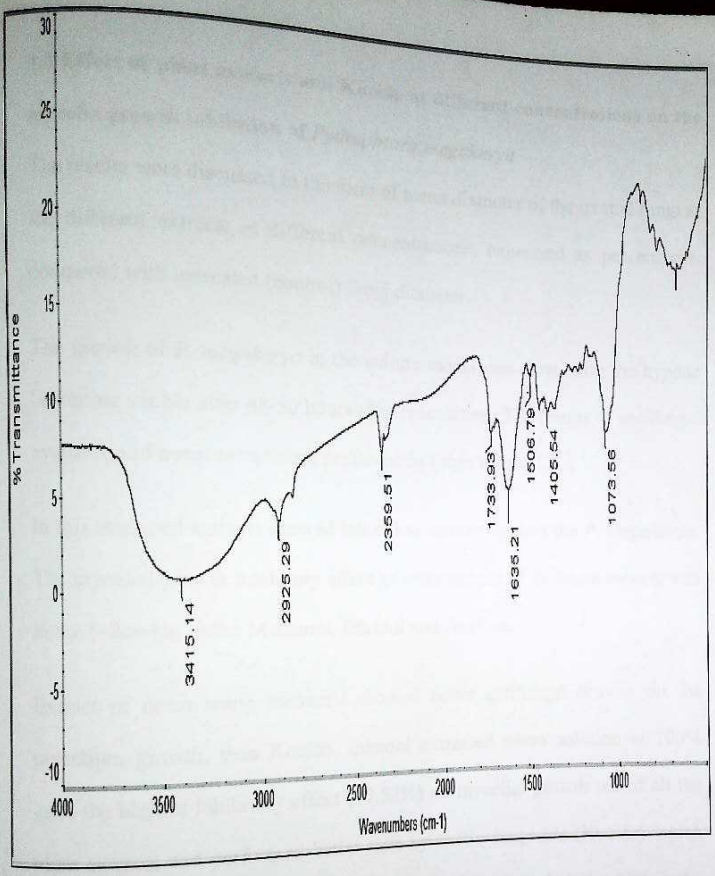

Fig.4.1 showing quantitative constituents of ethanol extract of *A. indica*

4.2 Effect of plant extracts and Kocide at different concentrations on the mycelia growth inhibition of *Pythophtora megakarya*

The results were discussed in the form of mean diameter of the treated fungi at the different extracts at different concentrations, expressed as percentages, compared with untreated (control) fungi diameter.

The growth of *P. megakarya* in the culture media was slow, with the hyphae becoming visible after 48-50 hours after inoculation. The results of antifungal evaluation of neem extracts are presented in Table below.

In this study, all extracts showed inhibition activity against the *P. megakarya*. The mycelial growth inhibitory effect of plant extract of different solvent was in the following order: Methanol, Ethanol and Acetone.

Extract of neem using methanol showed better antifungal activity on the mycelium growth, than Kocide. Ethanol extracted neem solution at 100% gave the highest inhibitory effect (77.52%) on mycelial growth out of all the plant extracts and performed better than synthetic fungicide (Kocide) which gave 53.70% inhibition. It was observed in Figure 2 that the higher the concentration the higher the inhibitory effect.

Table 4.1**Effect of plant extracts at different concentrations on mycelia growth of *P. megakarya***

Treatment	**Concentration (g/L)**	**Mycelia growth inhibition (%)**
Neem extract using Methanol	20	46.15
	40	56.04
	60	61.53
	80	65.93
	100	67.41
Neem extract using Ethanol	20	59.34
	40	61.53
	60	61.53
	80	63.73
	100	77.52
Neem extract using Acetone	20	45.05
	40	51.69
	60	53.84
	80	56.04
	100	52.80
Kocide	100	53.70

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Plants are a rich source of potentially useful antimicrobial products for the development of new chemotherapeutic agents (Mousavi*et al.*, 2009; Musyimi*et al.*, 2008; Ferreira *et al.*, 2009; Safary*et al.*, 2009).

Many reports are available on the antifungal, antibacterial, antiviral, antihelmintic, antimolluscal and anti-inflammatory properties of plants (Dey and De, 2010; Mahesh and Satish 2008; Samy and Ignacimuthu, 2000; Palombo and Semple, 2001).

Some of these observations have helped in identifying the active compounds responsible for such activities and in developing drugs for the therapeutic use in human beings. However not many reports are available on the exploitation of antifungal and antibacterial property of plants for developing commercial formulations for applications in crop protection.

In this study, the antifungal effect of ethanol, methanol and acetone extracts of neem (*Azadirachta indica)* were evaluated against *Phytophthora megakarya* at different concentrationsviz; 20, 40, 60, 80 and 100% respectively under in vitro condition. The results indicated that all tested extracts of *A. indica*

caused a significant reduction in the radial growth of *P. megakarya*. From the Table 4.1, it can be deduced that ethanol extract of neem gave the highest inhibition percentage of 77.52 at 100% concentration, followed by 80% with 63.73 inhibition percentage, while 20% concentration gave the lowest inhibition percentage value (59.34).

That is, the radial growth was gradually inhibited with increase in concentration of the extract.

Also, neem methanol extract at 100% concentration gave 67.41 inhibition percentage, then at 80% concentration 65.93 inhibition percentage, at 60% concentration (61.53), 40% (56.04) and at 20% (46.15). The higher the concentration, the higher the rate of inhibition.

Neem acetone extract gave the least inhibition percentage, at 100% concentration 52.80 inhibition percentage was recorded, at 80% (56.04), at 60% (53.84), at 40% (53.84), then at 20% concentration (45.05). Here, the highest inhibition percentage was recorded at 80%.

The results showed that *Azadirachta indica* caused a significant reduction in the radial growth of *P. megakarya*. Similar effects of various other plant products effective against *P. megakarya* were reported by several authors.Ambang*et al.* (2010) who obtained complete inhibition of the growth

of the *P. megakarya* strains with methanol extract of seeds of *T. peruviana.* These results are in agreement with Dube and Tripathi (1987) who showed that the aqueous extracts of *A. indica* obtained from bark and leaf, inhibited both spore germination and mycelial growth of *epidermophytonfloccosum, Microsporiumcanis*and *Trichophytonmentagrophytes.*

They also found that this antifungal toxic effect was also retained in organic extractsusing ethanol. These results were similar to previous work on the role of plant extracts in the fungal disease control.

Several authors including Curtis et al. (2004), Krebs et al. (2006), and Lathaet al. (2009) reported that plant extracts from 20 non-host plant species caused a reduction of the early blight disease and suppressed the mycelial growth of A. solani.

Upasana et al. (2002) found that neem seed extract in methanol was effective against Aspergillusniger, Fusariumoxysporum and Trichodermaresii and that both dried and fresh organic extracts from leaves were effective only against Trichodermaresii.

Results obtained from this study, indicate that the plant extract showed the strongest antifungal activity and more environmentally friendly than the

commercially available fungicides. For example, kocide showed maximum percentage of inhibition of 53.70 at 100% against P. megakarya.

5.2 CONCLUSION

It may be concluded from this study that *Azadirachta indica* leaf extracted with methanol, acetone and ethanol has antifungal activity and can be used as bio-fungicide against*P. megakarya*. From the result obtained above, the extracts proved to be more effective in controlling the black pod disease of cocoa especially the ethanol extract when compared to synthetic fungicide (kocide). Also, this method of control can contribute to minimizing the risks and hazards of toxic fungicides, especially on vegetables produced for fresh consumption.

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