ANTIFUNGAL ACTIVITIES OF THE LEAF EXTRACTS OF *HYPTIS SPICIGERA* LAM. ON SOME *ASPERGILLUS* AND *FUSARIUM* SPECIES

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DEPARTMENT OF BOTANY,

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AHMADU BELLO UNIVERSITY, ZARIA.

NIGERIA.

JANUARY, 2021

DEDICATION

This work is dedicated to my parents Alhaji Adamu Mohammad and Late Hajiya Fatima Abdulkadir and Professor M. Ilyas and Professor N. Ilyas

DECLARATION

I declare that the work in this thesis entitled 'Antifungal activities of the leaf extracts of *Hyptis spicigera* Lam. on Some *Aspergillus* and *Fusarium* species' has been performed by me in the Department of Botany under the supervisions of Dr. H. Musa and Dr. A. O. Musa. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

ADAMU, Kabiru ________________ ____________________

Signature Date

CERTIFICATION

This thesis titled 'ANTIFUNGAL ACTIVITIES OF THE LEAF EXTRACTS OF *HYPTIS SPICIGERA* LAM. ON SOME *ASPERGILLUS* AND *FUSARIUM* SPECIES' by ADAMU, Kabiru meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Several genera of fungi cause different important diseases of plants around the world. Plant diseases are mostly controlled by chemical pesticides and in some cases by cultural practices. The use of plant extract for the control of plant pathogenic fungi reduce the risk of food poisoning and increased the market value of crops. The antifungal activities of the leaf extract of *Hyptis spicigera* was screened against four species each of *Aspergillus* (*A. flavus, A. fumigatus, A. niger* and *A. parasiticus*) and *Fusarium* (*F. oxysporum, F. graminearum, F. proliferatum* and *F. verticilloides*). The powdered leaf was extracted with hexane, ethyl-acetate, methanol and water following an increase in polarity of these solvents. The qualitative and quantitative phytochemical screening was carried out on the extracts and fractions using standard procedures and the antifungal activities of the hexane, ethyl-acetate, methanol and aqueous extract with their terpenoids and flavonoids fractions was carried out to determine the zone of inhibition, minimum inhibitory concentration and minimum fungicidal concentration. The qualitative phytochemical screening indicates the presence of anthraquinones, unsaturated sterols and triterpenes, cardiac glycosides, saponins, flavonoids, tannins and alkaloids in all the extracts (n-hexane, ethyl-acetate, methanol and aqueous) except anthraquinones which was absent in the methanol and n-hexane extracts. The quantitative phytochemical screening indicates that saponins has the highest concentration of 920 mg/g/DE in the ethyl-acetate extract, phenolics was found to be highest in n-hexane extract with a quantity of 880 mg/g/GAE, flavonoid was higher in the aqueous extract with quantity of 220 mg/g/GAE. Alkaloids was found to be highest in n-hexane extract with a concentration of 170 mg/g/AE and tannins was having the highest concentration of 50 mg/g/QE as observed in the aqueous extract. The TLC chromatographic profile of terpenoids fraction indicates the presence of 7 terpenoids spots on the TLC plates while that of flavonoids fraction indicates 2 yellow and 2 red bands of flavonoids on the TLC plates when viewed under the UV light at 360nm. Ethyl-

acetate extract recorded wider diameter of inhibition against *A. parasiticus, A. fumigatus* and *A. flavus* with diameter of 24.67±0.88 mm, 21.33±0.67mm and 22.67±0.88 mm respectively. This inhibition zones were significantly higher than the control fungicide (mancozeb) and the other extracts. Methanol extract showed the highest zone of inhibition on *F. graminearum* (21.00±0.00 mm) which was also significantly higher than the control fungicide (18.33±1.45). The terpenoids fraction from ethyl-acetate extract showed the highest zone of inhibition on *F. oxysporum* with a diameter of 19.33±0.33mm while there was no inhibition zone against this fungus by the control fungicide (mancozeb) and the other extracts on this fungus. In the case of *F. proliferatum,* the control fungicide showed higher inhibition zone (21.00±0.00 mm) which was significantly higher than the extracts with their corresponding terpenoids and flavonoids fractions. The aqueous extract was the only extract that showed zone of inhibition on *A. niger* with a diameter of 16.00±0.58 mm. All the extracts, terpenoids and flavonoids fraction did not show any activity on *F. verticilloides* while the fungus was sensitive to the control fungicide (22.00±0.58) but *A. parasiticus* and *F. oxysporum* were resistance to the control fungicide. The MIC of the extracts and fractions on all the fungi species ranged from 3.13 to 12.5 mg/ml and their MFCs was between 6.25 to 25 mg/ml. The result from this research indicates that the leaf extract of *Hyptis spicigera* contains phytochemicals which exert a broad range of fungistatic and fungicidal activity on some species of *Aspergillus* and *Fusarium.*

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Fungi are ubiquitous in nature and vital for recycling of nutrients contained in organic matter. The vast majority of the known fungal species are strictly saprophytes, although there are a few capable of causing disease in humans (Bennett and Klich, 2003). However, there are several fungal genera containing species that cause diseases to plants and animals. These fungi can be categorized into two groups with regards to infection; saprophytic fungi which can be opportunistic pathogens that enter via wounds or due to a weakened state of the host and true pathogens that may depend on living plant or human tissues for nutrients but can also survive outside of the hosts (Anthony, 2007).

Several genera of fungi cause many important plant diseases around the world (Anderson *et al*., 2004; Strange and Scott, 2005). Plant diseases are of paramount importance to humans because they damage plants and plants products on which humans depend on for food, clothing, furniture and many other economical values. The kinds and amounts of losses caused by plant diseases on crop plants varies from species to species and also the type and nature of the pathogen, the locality, the environment, the control measures practiced and combination of these factors (Agrios, 2005).

Cereals and other agriculturally derived products represent an important nutrient source for mankind world-wide. In addition, they are the most important dietary food for most African populations (Riba *et al*., 2010). Unfortunately, agricultural products are naturally contaminated with fungi in the field, during drying, processing, transportation and subsequent storage and it may be difficult to completely prevent mycotoxins formation in contaminated commodities, particularly those that are produced in tropical and subtropical climates, in countries where high temperature and humidity promote the growth and proliferation of fungi (Kumar *et al*.,

2008). Thus, they are often colonized by fungi, including species from the genus *Aspergillus*, *Penicillium* and *Fusarium*, which cause significant reductions in crop yield, quality and safety due to their ability to produce mycotoxins (Alkenz *et al*., 2015).

Fungal infections which causes as great as 25-50% loss and still remain an important challenge in sustainable food production (Chuang *et al*., 2007; Zaker, 2014) with *Fusarium* being one of the most economically important genus of phyto-pathogenic fungi. Several *Fusarium* species can infect agricultural produce such as millets, guinea corn, maize, tomatoes and several other vegetables, the predominant species can vary according to crop species involved, geographic region and environmental conditions (Longrieco *et al*., 2002; Vander-lee *et al*., 2015).

Aspergillus mold fungus is a large genus consist of over 200 species to which humans are constantly exposed. Only few of these species are pathogenic among which more than 95% of the infections are caused by three species of Aspergillus including *A. fumigatus, A. flavus, A. niger* (Anaissie *et al*., 2009). *Aspergillus* spp. are among the pathogenic fungi causing infection through spores entering human body and these infections are invasive and very serious in individuals with deficient immune systems. Even in healthy people, Aspergillus may cause local infections in lungs, sinuses, and other organs of the body (Teles and Seixas, 2015). Aflatoxins are among the most important mycotoxins which are produced by species assigned to the Aspergillus genus. Among the numerous aflatoxins identified are; Aflatoxin B1 which is the most toxic aflatoxin being a potent genotoxic carcinogen in laboratory animals and there is strong evidence for its liver carcinogen in humans (Backhouse, 2014). The International Agency for Research on Cancer has classified aflatoxin B1 as a group I carcinogen. The most important producer of Aflatoxin B1 is *Aspergillus flavus,* it is also an important pathogen of various cultivated plants including maize, cotton, cowpea and peanut, and cause serious yield losses throughout the world. Since aflatoxin production is favoured by moisture and high

temperature, *A. flavus* is able to produce aflatoxins in warmer, tropical and subtropical climates (Varga *et al*., 2009).

Fusarium diseases that affect most crops are caused by several individual *Fusarium* or more commonly, co-occurring species. *Fusarium* spp. can cause indirect losses resulting from seedling blight or reduced seed germination, or direct losses such as seedling foot and stalk rots; however, the most important diseases in cereals due to severe reduction in yield and quality are head blight of small cereals such as wheat, barley, oat and ear rot of maize (Nganje *et al*., 2000; Munkvold, 2003). The coexistence of different *Fusarium* spp. in the field is a normal situation and although the number of detectable species can be high (Longrieco *et al*., 2007), only some of them are pathogenic, especially under suitable climatic conditions. The composition of species involved in Fusarium disease complex is dynamic (Kohl *et al*., 2007), the species comprises of a Fusarium community associate with each other and this cohabitation is particularly affected by climatic factors such as temperature and moisture.

Plant extracts act as contact fungicides by disrupting cell membrane integrity at different stages of fungal development, while others inactivate key enzymes and interfere with metabolic process (Rey *et al*., 2018). Crops treated with plant extract produce and accumulate elevated levels of specialized protein and other compound which inhibit the development of fungal diseases (Gebore *et al*., 2013).

Hyptis (*Hyptis spicigera*) is a genus of flowering plant in the Lamiaceae family. *Hyptis spicigera* is an erect, aromatic, annual or perenial herb growing up to 1meter tall, the plant is frequently grown as a food crop for its seeds in parts of tropical Africa (Ladan *et al*., 2014). The plant is commonly known as **bushmint** (due to the aromatic nature of their leaves), they are widespread in tropical North and South America, as well as parts of West Africa. There are 300 to 400 species, which may be annual or perennial, small or large shrub (Parak and Chanda, 2007). *Hyptis spicigera* is an important medicinal plant used in treatment of gastrointestinal

disturbances, wounds, skin infections and insect bites (Esquivel-Ferrino *et al*., 2014). The member of this genus are usually being used traditionally as mosquitoes and other insect repellent, leaves of this plants are normally kept at the edge of rooms to repel mosquitoes. However, studies have shown that the plant in this genus contain some major bioactive compounds among which includes carbohydrates, saponin glycosides, alkaloids and flavonoids (Ladan et *al*., 2011).

1.2 Statement of Research Problem

Fungi are predominant inducers of severe diseases causing huge economic loss. Most of these fungi are known to release mycotoxins which destroy the quality and nutritive value of food. In agricultural sectors, a crop is susceptible to fungal contamination (a stage of making the crop unhealthy or changes from natural state) at various stages, right from the sowing of seeds to post harvesting periods. Approximately 25- 40% cereals and other storage agricultural produce worldwide are contaminated with fungi (Singh *et al*., 2010). These fungi produce different types of mycotoxins that can be mutagenic, teratogenic or carcinogenic causing feed refusal and emesis in human and animals (Shukla *et al*., 2012). Hence to suppress these pathogenic fungi different synthetic fungicides are being commercialized. These synthetic chemicals are bound with various adverse effects (Wang and Jeffers, 2010). For many years' synthetic fungicides have been used for plant protection however their extensive use has led to resistance by most of the pathogenic fungi (Wang *et al*., 2014).

Death due to aflatoxins has been reported in humans, animals and birds (Gugnani, 2003; Agrios, 2005). Fumonisin produced by *Fusarium* species is also one of the toxin known globally to affect agricultural food and feed crops. Processing of infected grains and other agricultural produce from farms results in the release of airborne particulate matter that is contaminated with aflatoxins, thereby exposing the lungs of agricultural workers to these toxins

(Yiannikouris and Jouany, 2014). In humans, inhaled aflatoxin can cause inflammation and eventually irreversible pulmonary interstitial fibrosis which is the scarring of the lung tissues between the air sacs (Lougheed *et al.,* 2018)

1.3 Justification

The use of most synthetic fungicides have been restricted because of high acute toxicity, long degradation period, pathogen resistance, side effect on human, plants, animals and the ecology of most living organisms (Wuyep *et al*., 2017). Due to high usage of chemical fungicides on farm land near water bodies, there is an increase release of toxins in water bodies which will invariable enter into the food chains (Avasthi *et al.,* 2010)

The development of non-toxic, safe and effective biodegradable alternative source to synthetic fungicides has in recent years puzzled researchers to screening of plants for bioactivity on plant pathogenic microbes (Onifade, 2000). Plants generally contain a wide variety of free radical scavenging molecules including phenols, flavonoids, vitamins and terpenoids that are rich in antifungal activity (Cai *et al*., 2014). Furthermore, the use of fungicides generates health concerns due to their carcinogenic and teratogenic properties (Sharma *et al*., 2009).

Also, the use of plant extract for the control of plant pathogenic fungi will virtually reduce the risk of food poisoning as some species of fungi such as *Fusarium* and *Aspergillus* produces toxins on crop plants (Davide *et al*., 2016). Increasing pathogen resistance to key fungicides, lack of replacement of fungicides, consequent restrictions on fungicide use and high cost of chemicals requires alternative methods which are safer and eco-friendly. The activity of *Hyptis spicigera* as an antifungal agent on plant pathogenic fungi has not been scientifically proven as claimed by the locals and as such the need for the research.

1.4 Aim

This research is to evaluate the *in vitro* antifungal activities of *Hyptis spicigera* leaf extracts on *Aspergillus* and *Fusarium* species.

1.5 Objectives of the research

The objectives of the study are to:

1. Determine the comparative qualitative and quantitative phytochemical components of *Hyptis spicigera* leaf extracts (n-hexane, ethyle-acetate, methanol and aqueous)
2. Evaluate the antifungal activities of *Hyptis spicigera* leaf extracts (n-hexane, ethyl-acetate, methanol and aqueous) on some species of *Aspergillus* and *Fusarium*
3. Evaluate the antifungal activities of terpenoids and flavonoids fractions against the selected species of *Aspergillus* and *Fusarium*

1.6 Research Hypotheses

1. There are no significant phytochemicals in *Hyptis spicigera* leaf extracts (n-hexane, ethyle-acetate, methanol and aqueous) with antifungal activities
2. *Hyptis spicigera* leaf extracts (n-hexane, ethyle-acetate, methanol and aqueous) have no antifungal activity on *Aspergillus* and *Fusarium* species
3. Terpenoids and flavonoids fractions from *Hyptis spicigera* leaf extracts have no antifungal activity on *Aspergillus* and *Fusarium* species

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Fungi

Fungi are eukaryote that digest food externally and absorb nutrients directly through their cell walls. Most fungi reproduce by spores and have a body (thallus) composed of microscopic tubular cells called hyphae. Fungi are heterotrophs and like animals they obtain their carbon and energy from other organisms. Some fungi obtain their nutrients from a living host (plant or animal) and are called biotrophs, others obtain their nutrients from dead plants or animals and are called saprotrophs (Carratu *et al.,* 2012)

2.2 Pathogenic Fungi

The infection of plants by various fungi not only results in reduction in crop yield by causing a decrease in the available farm harvest but also there could be a chance of contamination of these farm produce with poisonous fungal secondary metabolites called mycotoxins which also causes drastic reduction in the quality of the farm produce. The ingestion of such mycotoxin contaminates by animals and human beings has enormous public health significance, because these toxins are capable of causing diseases in man and animals (Bhat and Vasanthi, 2003; Bhat *et al*., 2010). Although, the involvement of fungi and their toxins in causing diseases to man and animal had an historic evidence that dates back as the time of the dead sea scrolls that causes several deaths of humans and life-stocks, their chemical nature was not known until recently (Richard, 2007). It seems the evidence of their historic occurrence and impact were not obvious until the middle ages, when ergot alkaloids poisoning outbreaks in Europe were responsible for the death of thousands of people. Subsequently, between 1940s and 1950s a lethal human disease caused by *Fusarium* toxins also referred to as 'Alimentary Toxic Aleukia' was reported in Russia (Smith and Moss, 1985; Bankole *et al.*, 2005). Similarly, in 1938 in

Japan, *Penicillium* species were responsible for the colouring of rice that erratically led to the fatal human cardiac syndrome called 'yellow rice disease' (Kushiro, 2015). The livestock industry was also affected as seen by the devastation of the New Zealand sheep industry by facial eczema a fungal infection caused by *Pithomyces chartarum* in 1822. Other deadly animal syndromes arising from fungal infections and termed differently as equine leuko-ecephalomalacia (1930 to 1970 in USA), Satachybotryotoxicosis (1930 in USSR), red mould diseases (1945 to 1947 in japan), and red clover disease, vulvovaginitis and mouldy corn toxicosis (1920 to 1950 in USA) plagued the world (Gbodi and Nwude, 1998; Hussein and Brasel, 2001; Kishore *et al.*, 2007).

2.3 Types of plant pathogenic fungi

Most phytopathogenic fungi belong to the Ascomycetes and the Basidiomycetes. The fungi reproduce both sexually and asexually via the production of spores and other structures, spores may be spread long distances by air or water, or they may be soil borne (Adamu *et al.,* 2019). Fungal diseases may be controlled through the use of fungicides and other agriculture practices, in recent times new races of fungi often evolve that are resistant to various fungicides (Bennett, 2010). Biotrophic fungal colonize living plant tissue and obtain nutrients from living host cells, necrotrophic fungal pathogens infect and kill host tissue and extract nutrients from the dead host cells (Duan *et al.,* 2016). Significant fungal plant pathogens which include *Fusarium* species, *Aspergillus* species and *Sclerotinia sclerotiorum* all belonging to the ascomycetes, also a group of pathogenic fungi from the basidiomycetes are *Ustilago* species, *Rhizoctonia* species, *Puccinia* species and other classes of fungi had been known to affect plants at different stages of growth.

2.4 The Genus *Aspergillus*

The genus *Aspergillus* is one of the most important filamentous fungal genera, belonging to the phylum "Ascomycota" and family "Trichocomaceae". *Aspergillus* has about 250 species

but about 64% have no known sexual state. *Aspergillus* is present in the soil as conidia or sclerotia and as mycelia in plant tissues (Bennett, 2010). The conidia remain dormant in soil and only germinate when nutrient sources are present. The conidia can be black, green, brown, blue or yellow in colour, according to the species and the medium on which the fungus is growing.

On falling on a suitable substratum, each conidium germinates, by first producing a germ tube which grows into a new mycelium. The conidial stage is very prominent and commonly seen in nature and cultures. Fungi are opportunistic pathogens, most of them are encountered as storage molds on plant products. *Aspergillus* molds thrives best in oxygen-rich environments and on carbon-rich materials, hence they can be found on parts of living plants such as leaves, stems and fruits etc. During warm, dry periods, several Aspergilli increase rapidly in association with crop plants, although they are not considered to be major cause of plant diseases, *Aspergillus* species are responsible for several disorders in various plant and plant products. They are commonly found as a saprophyte, growing on dead leaves, stored grain, compost piles and other decaying vegetation (Bennett, 2010). They can contaminate agricultural products at different stages including pre-harvest, harvest, processing and handling. Changes due to spoilage by *Aspergillus* species can be of sensorial, nutritional and qualitative nature like: pigment discoloration, rotting, development of off-odors and off-flavors, but the most notable consequence of their presence is mycotoxins contamination of foods and feeds. The most common species are *A. niger* and *A. flavus*, followed by *A. parasiticus, A. fumigatus, A. ochraceus, A. carbonarius*, and *A. alliaceus* (Amaike and Keller, 2011).

2.4.1 Classification of *Aspergillus*

Aspergillus belong to the division ascomycota, class eurotiomycetes and family trichocomaceae. Aspergillus was first catalogued in 1729 by the Italian priest and biologist Pier

Antonio Micheli. Viewing the fungi under a microscope, Micheli was reminded of the shape of an Aspergillum (holy water sprinkler), from Latin *spargere* (to sprinkle), and he named the genus accordingly. (Bennett, 2010)

2.5 The Genus *Fusarium*

Fusarium is a large genus of filamentous fungi, part of a group often referred to as hyphomycetes, widely distributed in soil and associated with plants. Most species are harmless saprobes and are relatively abundant members of the soil microbial community. Some species produce mycotoxins in cereals and other food crops that can affect human and animal health if they enter the food chain. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes (Duan *et al.,* 2016). Despite most species apparently being harmless (some existing on the skin as commensal members of the skin flora), some *Fusarium* species and sub-specific groups are among the most important fungal pathogens of plants and animals (Covarelli *et al.,* 2012)

Fusarium diseases that affect most crops are caused by several individual Fusarium or more commonly, co-occurring species. *Fusarium* spp. can cause indirect losses resulting from seedling blight or reduced seed germination, or direct losses such as seedling foot and stalk rots; however, the most important diseases in cereals due to severe reduction in yield and quality are head blight of small cereals such as wheat, barley, oat and ear rot of maize (Nganje *et al*., 2000; Munkvold, 2003). The coexistence of different *Fusarium* spp. In the field is a normal situation and although the number of detectable species can be high (Longrieco *et al*., 2007), only some of them are pathogenic, especially under suitable climatic conditions.

The composition of species involved in *Fusarium* disease complex is dynamic (Kohl *et al*., 2007). The species comprises of a *Fusarium* community associate with each other and this cohabitation is particularly affected by climatic factors such as temperature and moisture.

2.5.1 Classification of *Fusarium*

The taxonomy of the genus is complex. A number of different schemes have been used, and up to 1,000 species have been identified at times, with approaches varying between wide and narrow concepts of speciation. The Genus Fusarium belong to the division ascomycota, class sordariomycetes and family nectriaceae.

2.6 Economic importance of *Aspergillus* and *Fusarium*

As agriculture struggles to support the rapidly growing global population, plant disease reduces the production and quality of food crops. Losses may be catastrophic or chronic, but on the average, 32% of the production of the six most important food crops are lost due to Aspergillus and Fusarium infection in Nigeria (NEARLS, 2017). Losses due to postharvest disease can be severe, especially when farms are a long way from markets and infrastructure and supply chain practices are poor. Many postharvest pathogens also produce toxins that create serious health problems to the farmers and consumers (Bennett, 2010). Farmers spend billions of dollars on disease management, often without adequate technical support, resulting in poor disease control, pollution and harmful results. In addition, plant disease can devastate natural ecosystems and compounding environmental problems caused by habitat loss and poor land management (Bau *et al.,* 2006)

Crop losses tend to be greatest in tropical countries where environmental conditions are particularly favourable for disease proliferation, incomes are low and the knowledge and investments in crop health management are minimal. In the case of severe disease incidence, the communities become dependent on imported foods, often replacing a balanced diet with processed foods that create further health problems (Serra *et al.,* 2005)

Aspergillus species are among the most widely plant pathogen known to affect a broad range of crop plants, contamination of crop plants by Aspergillus does not only have effect on the

plant alone but also create a serious contaminant which affect humans and animals. Many species of *Aspergillus* such as *A. glaucus A. flavus A. niger* are responsible for the spoilage of several crop plants such as tomatoes, grains, tubers onion and several other perishable vegetables. (Luongo *et al.,* 2008)

Fusarium species infect crop plants worldwide, Fusarium head blight, a well-known crop disease is caused by different *Fusarium* species and can result in yield loss and reduced grain quality. *Fusarium* species complex basically exert more harmful effect on cereal crops majorly, although they also affect other agricultural produce (Bennett, 2010) Fungal infection of cereals, such as barley, wheat, and maize, is often accompanied by mycotoxin contamination and hence causes serious effects on human and animal diets. Fusarium mycotoxins can be categorized in four major groups: Trichothecenes, zearalenone, fumonisins, and enniatins (Kouadio *et al.,* 2007)

Fusarium basal rot disease of onion is caused by the fungus *Fusarium oxysporum*. The symptoms in the field include yellowing of leaf tips that later become necrotic. This yellowing and/or necrosis may progress toward the base of infected plants, and sometimes infected leaves may exhibit curling or curving. Infected bulbs, when cut vertically, show a brown discoloration in the basal plate, which can move up into the bulb from the base. Losses to this disease can occur in the field and later when onions are in storage (Prakash *et al.,* 1989; Serra *et al.,* 2006)

Fusarium graminearum commonly infects barley if there is rain late in the season, this species is of economic impact to the malting and brewing industries as well as feed barley. Fusarium infection in barley can result in head blight and in extreme conditions the barley appears pink due the infection caused by the species (Kelly *et al.,* 2015). *F. graminearum* can also cause root rot and seedling blight, a total loss of barley and wheat crops between 1991 and 1996 in US was estimated to about $3 billion (Tian *et al.,* 2016)

Fusarium oxysporum f.sp. cubense is a fungal plant pathogen that causes Panama disease of banana (*Musa* spp.), also known as fusarium wilt of banana. Panama disease affects a wide range of banana cultivars, which are propagated asexually from offshoots and therefore have very little genetic diversity. Panama disease is one of the most destructive plant diseases of modern times, and caused the commercial disappearance of the once dominant Gros Michel cultivar in parts of tropical Africa (Yahaya *et al.,* 2018)

2.7 Control of *Aspergillus* and *Fusarium* species

Drying has been proven effective for preventing mycotoxin contamination of crops. However, drying equipment such as freeze drying machines may not be always available in the farm due to its cost and the lack of technical know-how on the farmers to operate such facilities. Besides, climatic conditions in the tropics are unpredictable and mostly harvesting seasons coincide with the onset of the wet season (Moss, 2002). The high moisture crops especially corn will be contaminated with aflatoxin within 48 hours. Therefore, chemical treatment is imperative.

It has been reported that 10% of the harvested foods are destroyed in storage by fungi thus, protecting our harvested crops from fungi can significantly increase availability of food resources (Fawzi *et al.,* 2009) Chemicals are applied as preventive and control measures to most fungal infection. However, thorough sanitation is the first and most effective step toward preventing infestation by fungal pathogens, hence storage areas should be clean to reduce infection of storage farm produce (Tournas and Katsoudas, 2005) The chemicals recommended for fungal protectants are chemicals belonging to the sulphur and copper group; copper sulphate and lime was effective in controlling downy mildew of grape vine caused by *Plasmopara viticola* and late blight of potatoe (Oliver and Hewitt, 2014)

The BP Chemicals of England has developed chemicals which when applied to moist grains prevent molds and bacterial activity. The name of the chemical is propcorn which is 100% active liquid preservative based on feed grade propionic acid. Propcorn prevents deterioration, loss of dry matter, nutrition loss and removes the associated health hazard to grain handlers, livestock and the end user. It has been found out that propcorn treated moist grain could be stored and transported with its condition retained for up to 12 months without the use of specialized storage (Page and Thomson, 2003; Burger *et al.,* 2010; Abhishek *et al.,* 2014; Oliver and Hewitt, 2014)

Kemin Industries in US has also produced a mould inhibitor called Myco Curb. It is a non-corrosive liquid mould inhibitor non-toxic and non-volatile, it contains propionic acid, acetic acid, ascorbic acid, benzoic acid, mono- and all-esters of 1,2 propanediol, hydrated ammonium phosphate, propyl benzoate, porpyl acetate, butylated hydroxyanisole (Amici *et al.,* 2013)

2.7.1 Mancozeb

Mancozeb is a dithiocarbamate non-systemic agricultural fungicide with multi-site, protective action on contact. It is a combination of two other dithiocarbamates: maneb and zineb (Shan *et al.,* 2006) The mixture controls many fungal diseases in a wide range of crops such as fruits, nuts, vegetables, and ornamentals. It is marketed as Penncozeb, Trimanoc, Vondozeb, Dithane, Manzeb, Nemispot, and Manzane, a mixture of zoxamide and mancozeb was registered for control of the mildew named Gavel as early as 2008 (FRAC, 2017)

Mancozeb reacts and inactivates the sulfhydryl groups of amino acids and enzymes within fungal cells, resulting in disruption of lipid metabolism, respiration, and production of adenosine triphosphate (Tomlin, 2003) Mancozeb is listed under FRAC code M:03 The "M:" refers to Chemicals with Multi-Site Activity. "M:" FRAC groups are defined as generally

considered as a low risk group without any signs of resistance developing to the fungicides (FRAC, 2017)

Major toxicological concern is ethylenethiourea (ETU), an industrial contaminant and a breakdown product of mancozeb and other synthetic pesticides. It has potential to cause goiter, a condition in which the thyroid gland is enlarged and has produced birth defects and cancer in experimental animals. ETU has been classified as a probable human carcinogen by the EPA (Shan *et al.,* 2006; Abhishek *et al.,* 2014)

2.8 *Hyptis spicigera* Lam.

Hyptis spicigera is a plant with a strong aromatic smell. It is erect, candelabra shaped. The stem is quadrangular and bears simple opposite and decussate leaves. The leaves are lance-shaped and are carried by a narrow stalk (Azevedo *et al.,* 2001)

2.8.1 Classification, Habitat and Distribution of *Hyptis spicigera* Lam.

Hyptis spicigera belong to the kingdom plantae, Phylum Tracheophyta, Class Magnoliopsida, Order Lamiales and Family Lamiaceae. *Hyptis spicigera* is widely spread at low altitudes, it occurs in moist soils in bush fallows and by the roadsides it is an invasive species and often found with alien species in the Guinea-savannah zone of Nigeria (Ladan *et al.,* 2014). It is indigenous in tropical America; widespread as a weed in tropical Africa from the Nile Land to Senegal and southwards to Mozambique and Madagascar

2.8.2 Uses of *Hyptis spicigera* Lam.

The seeds of *Hyptis spicigera* are used in sauce making/cooking as condiments, spices or flavourings. They have great medicinal use where the seeds, leaf and inflorescence in mixtures or singly are used to treat different ailments such as body pains (as pain killer), eye infection, naso-pharyngeal infection, pulmonary troubles, diarrhoea, dysentery, cutaneous and

subcutaneous parasitic infections. The genus *Hyptis* had long been used as insecticides by the local populace in several part of the world (Esquivel-Ferrino *et al.,* 2014)

2.8.3 Chemical constituent present in the genus *Hyptis*.

The essential oil of *Hyptis suaveolens* seed was characterized using GC-MS and UV-VIS spectrometry for its fatty acid profile, tocopherol and physicochemical properties. The oil content was 21% while unsaturated fatty acids were linoleic acid (71.85 %) and palmitic acid (16.06%) as the predominant fatty acids (Marinova *et al.,* 2005). Tocopherol content was 186.15mg/ml while Vitamin A was absent (Santos *et al.,* 2007). The potentials of *Hyptis spicigera* seed oil was analysed to have high oxidative stability which could be suitable for food and beverage as well as other industrial applications while the tocopherol content could improve human health (Sidibe *et al.,* 2001). Purification of the ethyl acetate fraction of *Hyptis suaveolens* by High Performance Liquid Chromatography (HPLC) yielded 3-buten-2-enol on the basis of GC-MS, the best separation using the analytical High Performance Liquid Chromatography (HPLC) was achieved at 0.8 ml/min (Din *et al.,* 1988)

2.9 Uses of Plants Secondary Metabolites as fungicides

There had been continuous use of natural product as fungicides in several countries, particularly in Nigeria where synthetic fungicides are readily not available, costly and the peasant farmers lack the technical know-how on the correct proportion and timing of such fungicides application.

Chemical control of most of fungal diseases of plants may be available and could extensively reduce the impact of plant diseases, but field application of synthetic fungicides may not always be desirable. During the last decades, there has been a global awareness that excessive and improper use of chemical fungicides is hazardous to the health of humans, animals and the environment, therefore an extensive research for environmentally safe and easily

biodegradable bio- fungicides is being carried out. Furthermore, these compounds are natural in origin, have minimum adverse effects on the physiological processes of plants and are easily convertible into common eco-friendly organic materials (Gnanamanickam, 2002). These products are generally assumed to be more acceptable and less hazardous for the ecosystems and could be used as alternative remedies for treatment of plant diseases (Chuang *et al*., 2007)

It is estimated that there are more than 250,000 higher plant species on the earth offering a vast virtually unlapped reservoir of bioactive chemical compounds with many potential uses, including their application as pharmaceuticals and agrochemicals (Cowan, 1999; Burt, 2004). As in pharmacology, bio-chemicals isolated from higher plants may contribute to the development of natural products for the agricultural industry in three different ways.

- ✓ By acting as natural fungicides in an unmodified state (crude extracts) (Cox, 1990; Bowers and Locke, 2004).
- ✓ By providing the chemical 'building blocks' necessary to synthesize more complex compounds (Cox, 1990; Bowers and Locke, 2004).
- ✓ By introducing new modes of fungicidal action that may allow the complete synthesis of novel products in order to counter the problem of resistance to currently used synthetic products by plant pathogenic fungi (Cox, 1990; Bowers and Locke, 2004).

Many reports approve the efficacy of natural products of plants in controlling fungal growth and mycotoxin production, eg. Cinnamon, clove, oregano, palmarosa and lemongrass leaf and aniseed oils (Cosic *et al*., 2010), sweet basil, neem, eucalyptus, datura, garlic and oleander extracts (Nashwa and Abo-Elyousr, 2012). Thymol and carvacrol were the most effective active constituents against the fungal species tested (Numpaque *et al*., 2011; Shin *et al*., 2014; Villanueva *et al*.,2015; Gavaric *et al*., 2015). The mechanism of action of these compounds against fungi is not completely understood but it is supposed to be in relation to their general ability to dissolve or otherwise disrupt the integrity of fungal cell walls and cell membranes

(Isman and Machial, 2006). Some more examples regarding antifungal potential of plants products are listed in (Appendix 1)

2.10 Chemical composition and mode of action of plant secondary metabolites

The most commonly plant products used for plant disease management are essential oils and extracts. These two types of plant-based products have many similarities but also differ for some characteristics. Essential oils are oily liquids obtained from plants through fermentation, effleurage and steam distillation of plant parts (Burt, 2004), whereas plant extracts, in contrast are obtained by filtration and evaporation using various solvents (Wang *et al*., 2004). The major compounds that have been investigated to date include phenols, flavonoids, quinones, terpenes, tannins, alkaloids, lectins, polypeptides, saponins and sterols (Halama and Van Haluwin, 2004). These products may have fungicidal or fungistatic effect on plant pathogens or they can cause conditions unfavourable for establishment and multiplication of pathogenic microorganisms on host plants (Scheuerell and Mahaffee, 2002).

Saponins from plant extracts had been associated with causing lysis of cells of living organism (Ibrahim *et al.,* 2017) Saponins isolated from essential oil of semicarpus plant had been associated with broad spectrum antifungal activity on *A. flavus* (Mohanta *et al.,* 2007). Similarly, Adamu *et al.* (2019) reported a wide spectrum fungicidal activity of Saponins obtained from the juice extract of *Gmelina arborea* on a wide range species of candida. Terpenoids bind to cell wall of fungi there by producing a hydro-oxyle complex which had been associated with membrane expansion and fluidity of cell leading to cell disruption and consequently lethality of cells (Rao *et al.,* 2010)

Simple phenols and phenolic acids are bioactive phytochemicals consisting a single substituted phenolic ring. Phenolic toxicity to microorganisms in higher concentration is due to the binding site (s) and number of hydroxyl groups present in the phenolic compound (Subhashini *et al.,*

2013). Quinones are characteristically highly reactive, coloured compounds with two ketone substitutions in aromatic ring. Flavones, flavonoids and flavonols are phenolic structures with one carbonyl group, these chemicals are synthesized by plants in response to microbial infection and are often found effective *in vitro* as antimicrobial substance against a wide array of microorganisms (Elegani *et al.,* 2012)

Tannins are polymeric phenolic substances possessing the astringent property, tannins had been known to bind to protein complex of cell wall of fungi species where is causes substrate deprivation and cellular destruction (Schiijlen *et al.,* 2004) These compounds are soluble in water, alcohol and acetone (Gurjar *et al*., 2012). The mechanisms of some important natural compounds on plant pathogenic fungi are given in Appendix 2.

2.11 Plant Secondary Metabolites

Phytochemicals literally means "plant chemicals" Scientist have identified thousands of different phytochemicals, found in various parts of plants such as vegetables, fruits, beans, whole grains, nuts and seeds. Eating lots of plants foods rich in phytochemicals may help to prevent at least one in every five cases of cancer, as well as other serious ailments such as heart diseases (Okigbo and Omodamiro, 2007). The phytochemicals investigation of plant may involve the extraction of the plant materials, separation and isolation of compounds or constituent of interest, characterization of the isolated compounds and investigation of the biosynthetic pathways of a particular compounds and quantitative evaluation (Abdul, 1990)

Phytochemical constituents of plant (secondary metabolites or natural products) are obtain from plants in nature in very small quantities, plants in general normally store or release these substances as waste products of metabolism i.e. they do not need it for their normal growth, reproduction or for any other function. Natural products have been very important to ensure the survival of man since ancient times, especially as remedies to treat different diseases of

both plants and animals. Today, despite the development of new fungicides; natural products continue to play a highly significant role in biological control of fungi through discoveries and developmental process (Nester *et al.,* 2007)

Phytochemicals of plant origin are currently in used in biological control of diseases of plants in the employment of natural enemies of pest or pathogen in the eradication or control of their population. It could also be in the form of induction of plants resistance using non-pathogenic or incompatible micro-organisms. Over the years, farmers in Nigeria has employed the use of composted organic materials such as plant debris and animal manure to add nutrient to the soil in other to increase its fertility. According to Muhammad *et al*. (2001) use of composted organic materials do not only improves plant growth but also makes them less prone to infections by soil inhabiting pathogens. Krause *et al*. (2003) reported that Rhizobacteria from composite suppress the severity of bacteria leaf spot of Radish and many fungal diseases.

2.11.1 Alkaloids

Alkaloids are basically nitrogen bases. The amino acids act as building blocks for the biosynthesis of alkaloids. Alkaloids are responsible for physiological effects in plants, man or in animal. Alkaloids have basic properties and are alkaline in nature, turning red litmus paper blue (Tyler *et al*., 2011). Alkaloids are basically compound of ammonia, where various radicals replace one or more atoms of hydrogen. Alkaloids combine with acid to form crystalline salts without the production of water. The majority of alkaloids exist in solid form like atropine and contain oxygen. Some alkaloids like lobe-line or nicotine occur in a liquid form and contain carbon, hydrogen, and nitrogen. Alkaloids have one peculiarity regarding solubility in organic solvents. They are readily soluble in organic solvents and sparingly soluble in water. The salts of alkaloids are usually soluble in water. In nature, the alkaloids exist in many plants; in larger proportion in the seeds and roots of plants and often in combination with vegetables acids. The solutions of alkaloids are intensely bitter (Hu *et al*., 2003).

Alkaloids such as Morphines, Berbins and Putrecines from plant extracts, essential oils, gums, resins etc. have been shown to exert biological activity against plant fungal pathogens *in vitro* and *in vivo* and can be used as bio-fungicidal products (Fawzi *et al*., 2009; Jalili-Marandi *et al*., 2010; Romanazzi *et al*., 2012). These products are generally assumed to be more acceptable and less hazardous for the ecosystems and could be used as alternative remedies for treatment of plant diseases (Chuang *et al*., 2007). Natural plants products have a narrow target range with specific mode of action, therefore are suitable for a specific target, mostly nontoxic for antagonistic microorganisms, show limited field persistence and have a shorter shelf life and no residual threats (Zaker, 2016).

2.11.2 Flavonoids

A flavonoid is identical to a bioflavonoid; furthermore, a flavonoid by definition is a polyphenol, since it contains more than one benzene ring in its structure. However, many regard polyphenols to be tannins. Green tea has an abundance of flavonoids and flavonals as well as tannin. These compounds have been well characterized in the herb and numerous papers support their use as antioxidants or free radical scavengers (Woo *et al.,* 1980; Trease and Evans, 2009)

Flavonoids are an important group of polyphenols, widely distributed in plant flora. Four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, Kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Flavonoids are derived from parent compounds known as flavans. They are yellow and are usually found in majority of plants. Leaves of *Calycopteris floribunda* contain flavanol calycopterin, which is considered to be anthelmintic. Flavanones, flavans and lipophilic flavones have been associated with antifungal activity (Grayer and Harbone, 1994; Mendoza *et al*., 2009). Sakuranetin, a flavonoid isolated from the surface of *Ribes nigrum*, inhibited germination of *B*. *cinerea* conidia (Grayer and Harbone, 1994; Mendoza *et al*., 2009). On the

other hand, three chalones from the wood of *Bauhinia manca* affected mycelial growth of *B. cinerea* (Grayer and Harbone, 1994; Mendoza *et al*., 2009). Resveratrol, a stilbene produced by grapes, inhibited the spread of *B. cinerea* infection (Langcake and McCarthy, 1979)

2.11.3 Glycosides

These are water-soluble constituents, found in a cell sap. They are colorless, crystalline substance containing carbon, hydrogen and oxygen. Some glycosides are peculiar in having nitrogen and sulfur. Glycosides are neutral in reaction. Chemically, glycosides contain a carbohydrate (glucose) and non-carbohydrate part (aglycone or genin). Alcohol, glycerol or phenol represents aglycones. A glycoside can be readily hydrolyzed into its components with ferments or mineral acids (Haorongbam *et al*., 2009)

The influence of the iridoid glycosides extracts from *Linaria genistifolia* (L.) Mill. has been investigated to be the cause of the resistance of the winter wheat plant to *Fusarium oxysporum* which causes root rot. It was also established, that the iridoid glycosides extract from this plant, containing four major known compunds:5-O- allosylantirrinoside, antirrinoside, linarioside and 6-β-hidroxiantirride, can be successfully employed in biological control of the mentioned wheat pathogen: it stimulates wheat grains germination and embryonic root growth in conditions of fungal infection (Yue *et al*., 2010)

2.11.4 Phenolic Compounds

Phenolic compounds are widely distributed in plant flora. They constitute an important part of glycosides (phenolic glycosides), flavonoids, and tannins. *Curcumins* are phenolics compounds from *Curcumalonga* (Velderrain-Rodríguez *et al*., 2014). Phenolic compounds such as querecetin, scopoletin and scoparone had been proved to be effective in reducing green mould severity and incidence in “Navelina” oranges (Feliziani *et al*., 2013). Some major class of glycoside had widely been used as fungicides. Other phenolic compounds of biological

importance include anthraquinones, unsaturated sterols and triterpenes which are basically used in mixtures with other secondary compounds in fungicides formulations (Gatto *et al*., 2013)

2.11.5 Saponins

Saponins are glycosides found in a number of plants. Saponins are regarded as high molecular weight compounds in which, a sugar molecule is combined with triterpine or steroid aglycone. Saponins have a characteristic feature of frothing. The term saponin is derived from *Saponaria vaccaria (Quillaja saponaria*) is known to contain toxic glycosides quillajic acid and *senegin* quillaji acid is strenutatory and senegin is toxic, Studies had also shown that saponin extracted from aerial parts of Alfalfa (*Medicago sativa*) against the causal agent of rice blast *Pyricularia oryzae* (Pamela *et al*., 2014)

2.11.6 Tannins

These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i.e. to convert things into leather. The tannins are acidic in nature and is attributed to the presence of phenolic or carboxylic group. Tannins form complex with proteins, carbohydrates, gelatin and alkaloids.

Tannins and tannic compounds are used in dietary and medicinal herbs with antioxidant and antimicrobial activity and have also been considered to prevent cancer (Field and Lettinga, 1992; Ahn et *al*., 2005; Huang *et al*., 2009). As early as in 1913, Knudson (1913) reported the tannic acid (TA; $C_{76}H_{52}O_{46}$) is even at low concentrations toxic to a large number of fungi. Antibiotic tannin compounds were found in many plants and play constitutively or induced by elicitors, a crucial role in defence of plant disease, examples of such induction of antibiotic tannins in wheat are seed treatments with Chitosan, a product based on chitin and silicon

(Bhaskara *et al*., 1999; Velosz-Garcia *et al*., 2010). Seed treatments with Chitosan reduced seed borne incidence by more than 50% whereas silicon sprayed on wheat plants induced the formation of phenolics which in turn reduced powdery mildew (*Erysiphe graminis*) incidence on wheat leaves.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in the Department of Pharmacognosy and Drugs Development, Ahmadu Bello University Zaria Located in Kaduna State between Latitude 11º 09' N and Longitude 7º 39' E at an altitude of 672 meters above sea level and Department of Botany, Ahmadu Bello University Zaria located between Latitude 11º 14' N and Longitude 7º 65' E and an altitude of 652 meters above sea level, all reading were taken with the aid of android version 10.11.1 global position system (GPS).

3.2 Source of Plant Materials

Fresh leaves samples of *Hyptis spicigera* Lam. (Plate 3.1) was collected in August, 2018 at Shika dam site located at Latitude 10º 01' N and Longitude 6º 84' E, the plant was taken to the herbarium unit of Department of Botany, Ahmadu Bello University Zaria for proper identification and documentation and the Voucher number of the plant was documented as ABU 2050

3.3 Preparation of Plant Materials

The fresh leaves were washed under running water to remove soil particles, the leaves were dried at room temperature (21-28 ^{0}C) for 21 days, the dried leaves were grinded into fine powder using the leaves grinding machine model number A006 Casella Germany. The powdered leaves were weighed and kept in an air tight jar and the extraction was carried out the following day (Ladan *et al*., 2014)

Plate 3.1: *Hyptis spicigera* Leaf and Infloresence

3.4 Extraction of Plant Materials

The powdered leaf was extracted using the method described by (Kokate *et al.,* 2002) with some modifications, 250g of the powdered leaf was extracted succesively with n-hexane, Ethyl-Acetate, methanol and distilled water using cold maceration at room temperature (25-36° C) as outline below (Figure 3.1) at each stage of extraction for every extraction solvent used the marc was dried at room temperature for 24 hours, the marc was re-weigh to account for the initial weight of the powdered leaf for each extraction solvent before further extraction with other solvents. The filtrate was further concentrated to dryness at room temperature without exposure to heat except for the Aqueous extract that was dried via water bath set at 40° C after which the extract was stored in the desiccator for further analyses

3.5 Determination of Percentage Yield of Extracts

Percentage Yield is the ratio of the actual yield to the theoretical yield of the extract. The percentage yield of the crude extract for each solvent was determined (Parak and Chanda, 2007; Mahmood and Ameh, 2007; Wuyep *et al.,* 2017). The percentage yield of the three extract as determined was calculated using the equation bellow.

$$\text{Percentage Yield} = \frac{\text{Weight of Extract after Extraction}}{\text{Weight of Extract before Extraction}} \times 100$$

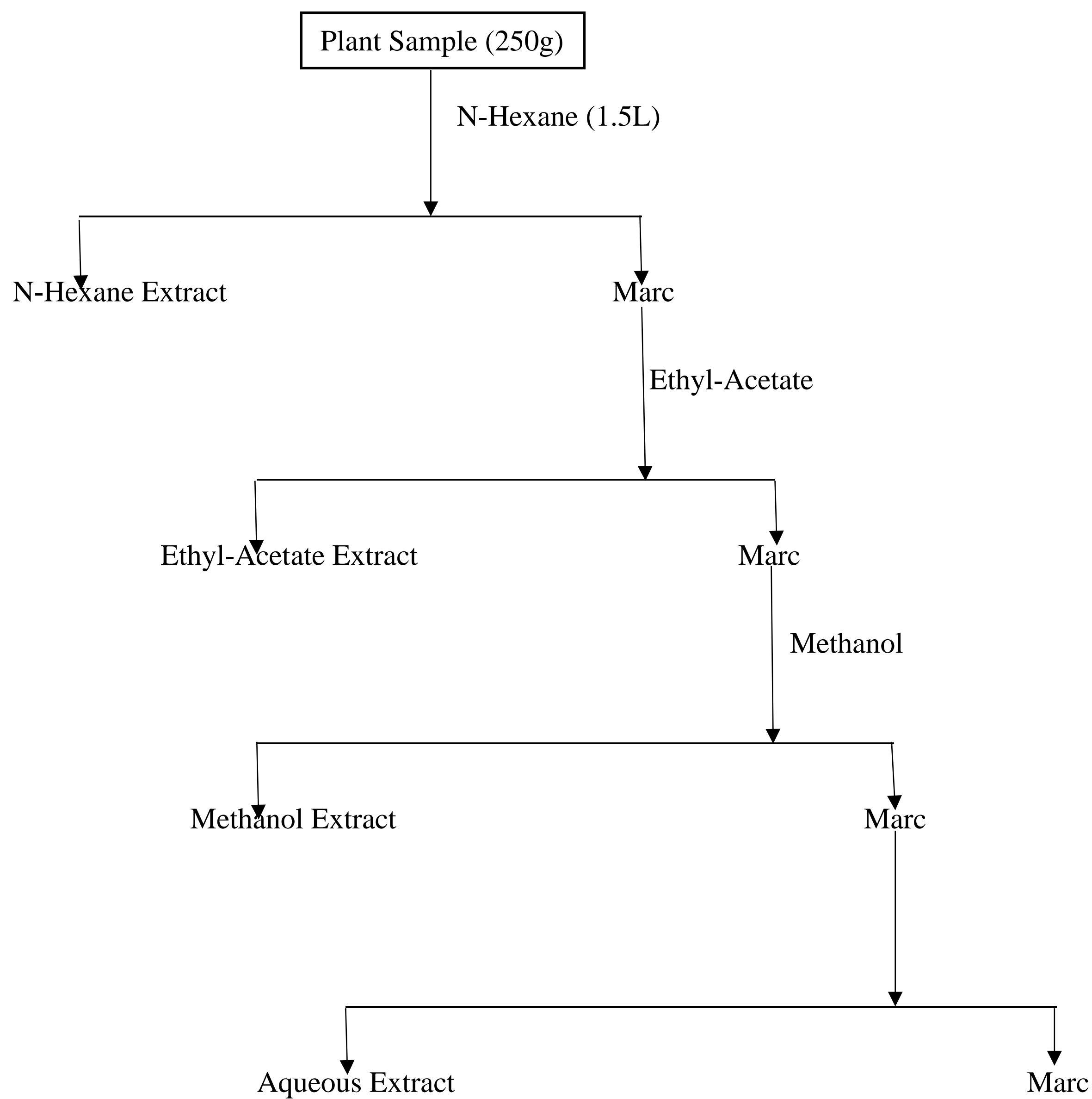


Figure 3.1: Schematic Diagram of Extraction Procedures with modification (Kokate *et al.,* 2002)

3.6 Isolation of Terpenoids from Ethyl-Acetate Leaves Extract of *Hyptis spicigera* Lam.

Terpenoids were extracted from the ethyl-acetate leaves extract by acid base extraction Assay, 5g of the extract was dissolved in 100 ml Ethyl-acetate and poured into a separation funnel and clamped on a retort stand, the extract was basified using 200 ml of 5% Potassium Hydroxide and the separation funnel was removed and tilted at an angle of 180^0 to the horizontal to allow a complete mixing of the base and the extract, it was then clamped back on the retort stand for clear separation (layer) between the aqueous and the ethyl-acetate phase, the aqueous phase which settled at the lower portion was collected and the ethyl-acetate phase was re-extracted with another 200 ml of 5% Potassium Hydroxide and this was done in triplicates. The ethyl-acetate phase was re-extracted with 5% Hydrochloric Acid by adding 200 ml of it to the ethyl-acetate phase in the separating funnel, the mixture was shaken for complete mixing, it was left to stand and was observed to form a layer i.e a clear separation between the aqueous (distilled water used in the preparation of 5% Hydrochloric Acid) and the ethyl-acetate phase and each layer was collected separately, this process was repeated thrice to obtained the organic fraction (ethyl-acetate phase/layer). The organic fraction (ethyl-acetate phase) which contained the neutral compound was washed with 50 ml of water and then concentrated in a rotary evaporator to 30 ml, it was then centrifuged for 10 min at 6000 rpm to remove the suspended particles. The solvent was evaporated to dryness, giving a residue which is the Terpenoid fraction. Figure 3.2 summarizes the procedure of the extraction procedure (Cordeiro *et al.,* 1999; Abubakar *et al.,* 2017)

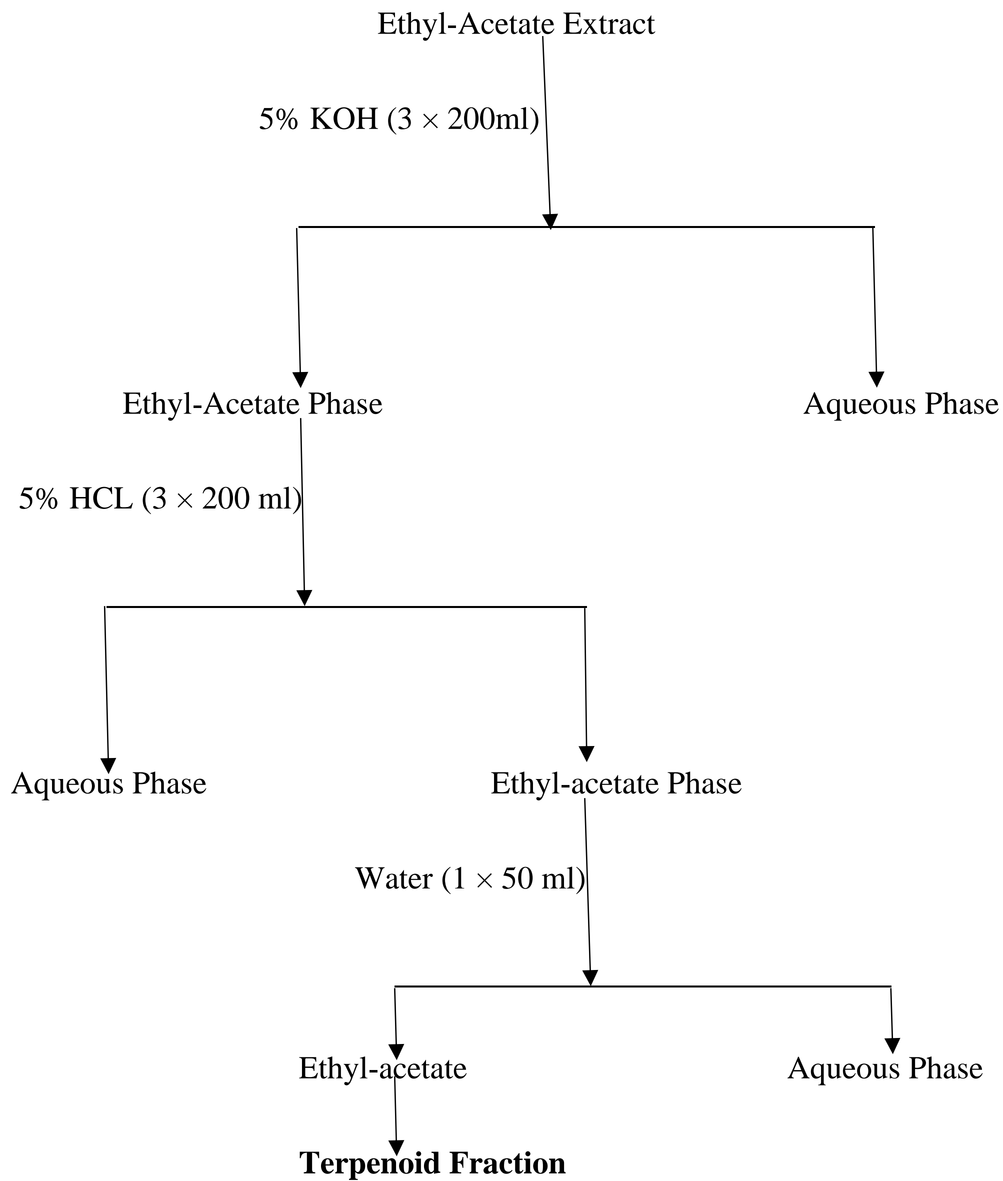


Figure 3.2: Terpenoid Extraction Procedure (Cordeiro *et al.,*1999)

3.6.1 Thin Layer Chromatographic Profile and chemical test for Terpenoids

The fraction was spotted on a silica gel coated plate and ran in a TLC tank using hexane ethyl-acetate 7:3 as the solvent system. Specific terpenoid test was done using Lieberman bucchard reagent for both the chemical test and spray. The terpenoid fraction was mixed with few drops of acetic anhydride, heat to boiling on water bath set at 60 ^{0}C. Concentrated sulphuric acid (5 ml of 98 %) was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. A Green coloration at the upper layer and the formation of deep red color in the lower layer indicate a positive test for steroids and triterpenoids. (Cordeiro *et al.,* 1999; Abubakar *et al.,* 2017)

3.7 Isolation of Flavonoid from Methanol Leaves Extract of *Hyptis spicigera* Lam.

Flavonoid and Saponins were extracted from the methanol leaf extract of *Hyptis spicigera* following standard method as described by Woo *et al.* (1980). Methanol extract (10 g) was dissolved in 100 ml of distilled water and passed over a filter paper, the filtrate was poured into a separating funnel and 250 ml of petroleum ether was added and shaken at an angel of 180 degrees and clamped back to the retort stand for separation between the aqueous phase and the pet-ether layer, after a separation was observed the tap below the separating funnel was released gradually and the aqueous portion was collected with the pet-ether layer separately. The aqueous phase was transferred into the separating funnel and continuous extraction was done with pet-ether until the pet-ether portion after successive extraction become colourless, the idea is basically to remove the pet-ether soluble portion which are mostly fat, terpenes and pigments.

Continuous extraction of the aqueous portion was done using ethyl-acetate to remove the ethyl-acetate soluble portion, in this 250 ml of ethyl-acetate was added to the aqueous portion in the separating funnel and tilted to allow even mixing, it was then clamped back for clear separation between the aqueous portion and the ethyl-acetate layer , the aqueous and ethyl-acetate layer was collected separately, continuous extraction of the aqueous layer was done until the ethyl-

acetate added unto the aqueous portion is colourless. The aqueous fraction was then treated with 250ml of N-Butanol in a separating funnel, the aqueous and N-Butanol soluble portion was collected separately, continuous extraction was done with the N-Butanol until the N-Butanol also become colourless, all the different N-Butanol soluble portion collected was then transferred into the separating funnel and was treated with equal volume of 2% KOH to obtain the Saponin fraction which is the first N-Butanol fraction, the aqueos layer was then acidified with equal volume of 2% Hydrochloric acid, 250ml of N-Butanol was added shaken and allowed to form a clear separation to obtain the second N-Butanol fraction containing the Flavonoids, all the fractions obtained were concentrated in the rotary evaporator and subsequently on a water bath, the concentrated fractions was then transferred into a universal bottle and kept in a desiccator prior to further analyses (Figure 3.3)

3.7.1 Thin Layer Chromatographic Profile and chemical test for Flavonoids

The fraction was spotted on a silica gel coated plate and ran in a TLC tank using Butanol Acetic-acid Water (10:1:1) as the solvent system. Specific flavonoid chemical test was done using aluminium chloride as the spray reagent for flavonoid detection on the TLC plates, the plate was sprayed with 1% ethanolic solution of aluminum chloride, the appearance of a yellow or red fluorescence in a long wavelength (360nm) of UV light confirms flavonoids. A series of chemical test was carried out for confirmatory test of flavonoids as follows:

3.7.1.1 *Shinoda test*

To a portion of the flavonoid fraction and the crude extracts (2 ml of 0.5 g dissolved in 10 ml of distilled water) 2 ml of methanol as well as 1 g of magnesium chips were added and heated on a water bath (60 ^{0} C) for 5 minutes (do not allow boiling), concentrated sulphuric acid was added down the side of the test-tube slowly, the presence of a red colour indicates the presence of flavonoids.

3.7.1.2 *Sodium hydroxide test*

About 2 drops of 10% NaOH (sodium hydroxide) was added to 2 ml (0.5 g dissolved in 10 ml of distilled water) of the terpenoid fraction and the crude extracts, the appearance of a yellow coloration indicated presence of flavonoids

3.8 Preliminary Qualitative Phytochemical Screening

Preliminary qualitative phytochemical screening is a test conducted using standard methods to test the presence and absence of the various secondary metabolites (phytochemicals) that might be presence in the extract which makes it responsible for it activities. A stock of the extract was prepared by dissolving 1 g of the extract in 20 ml of distilled water and this was used for the chemical analyses

3.8.1 Test for Carbohydrates

3.8.1.1 *Molisch's test*

A 2 ml of the stock extract was transferred into the test-tube, 3 drops of Molisch's reagent was added followed by concentrated sulphuric acid (5 ml) down the side of the test-tube slowly to form a lower layer of reddish colored ring at the interphase, these indicates the presence of carbohydrates (Trease and Evans, 2009)

3.8.1.2 *Fehling's test*

The extract (2 ml) was mixed with 2 ml of distilled water in a test-tube, 5ml of an equal mixture of Fehling's A and B solution was added and boiled in a water bath, a brick red precipitate indicates the presence of carbohydrate (Trease and Evans, 2009)

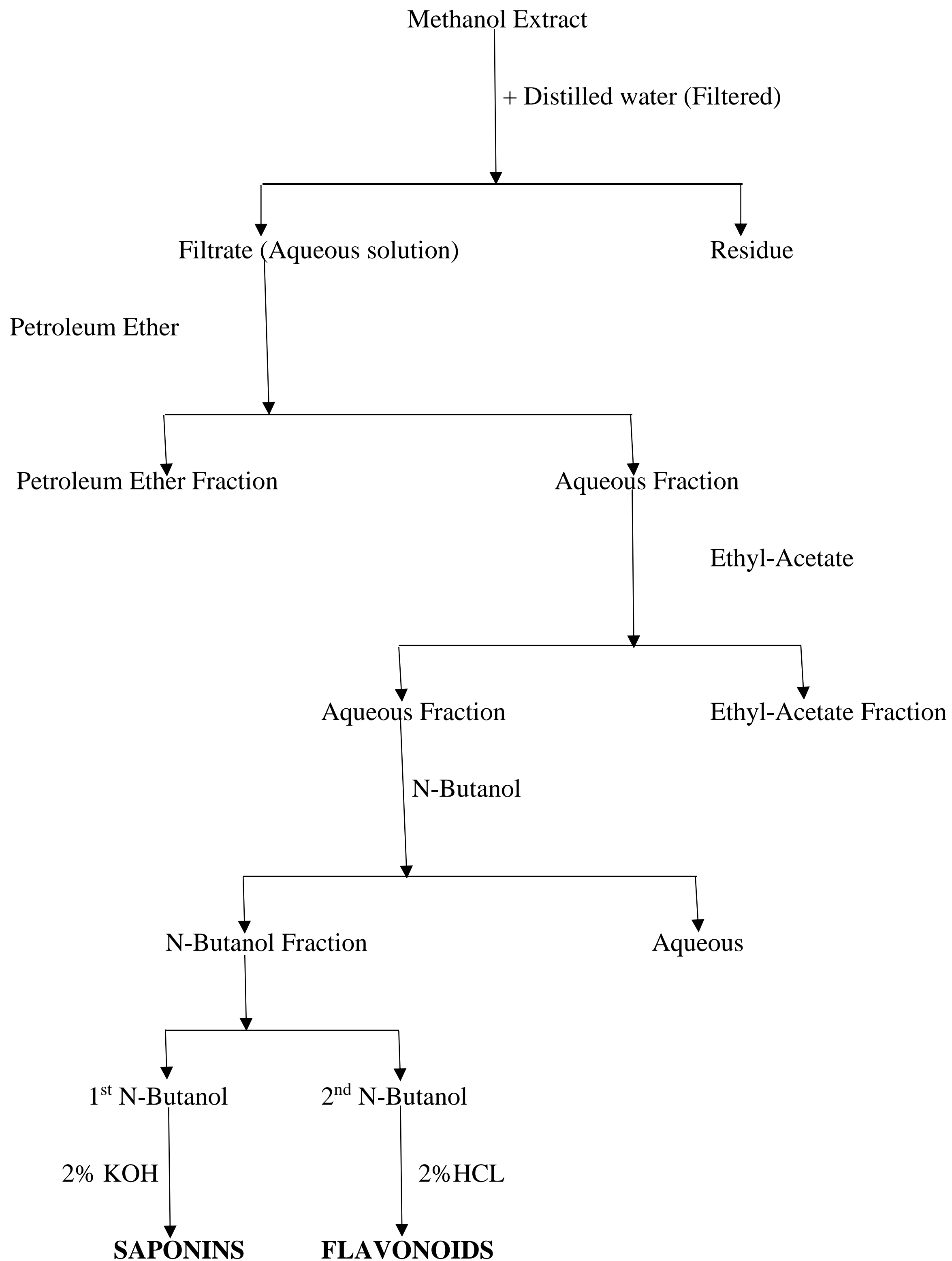


Figure 3.3: Flavonoid Extraction Procedure (Woo *et al.,* 1980)

3.8.2 Test for Glycosides

Fehling's solution test

A 2 ml of the stock extract was transferred into a test tube, 5ml of 50 % dilute sulphuric acid was added and boiled on water bath for about 10-15minutes. It was then allowed to cool (35 °C) at room temperature and neutralized with 20% KOH (potassium hydroxide). The fraction was now divided into two portions in separate clean test-tubes.

- To the first portion, 5ml of equal mixture of Fehling's A and B solution was added and boiled in a water bath, a brick red precipitate shows the release of reducing sugar as a result of hydrolysis of Glycoside.
- To the second portion, 3 drops of ferric chloride solution was added, a dark green to blue colour indicates the presence of phenolic aglycones released due to the hydrolysis of the glycosides (Trease and Evans, 2009)

3.8.3 Test for Anthraquinones Derivatives

3.8.3.1 *Test for anthraquinones (Borntrager's test*)

A test tube containing 2 ml of the stock extract was mixed with equal volume (2 ml) of chloroform, shaken for 5 minutes. This was pipetted to collect the chloroform layer as it absorbs certain component of the extract and transferred into a test tube. Equal volume of 10% NH_30H (ammonium hydroxide) was added to the filtrate (chloroform), a bright pink colour in the upper layer indicates the presence of free anthraquinones (Trease and Evans, 2009)

3.8.3.2 *Test for combined anthraquinones (modified Borntrager's test)*

The stock extract (2 ml) was transferred in a test-tube, 5ml of 10% HCl (hydrochloric acid) was added and boiled in a water bath for 3 minutes. These hydrolyzes the glycoside to yield aglycones which are soluble in hot water. This was then filtered and the filtrate was cooled to 50 °C, 5 drops of chloroform was added. Equal volume of 10% NH_3OH was then added, a

rose pink or cherry red colour indicates that the plant contains anthraquinones derivatives (free or in combined state) (Trease and Evans, 2009)

3.8.4 Test for Unsaturated Sterols and Triterpenes

3.8.4.1 *Liebermann-Bucchard Test*

The stock extract (2 ml) was transferred in a test tube, equal volume (2 ml) of acetic acid anhydride was added and mixed gently, 1 ml of 98% concentrated sulphuric acid was added gradually down the side of the test-tube to form a lower layer. Colour changes were observed immediately which stayed over a period of one hour. Blue to blue-green colour in the upper layer and a reddish, pink or purple colour indicate the presence of triterpenes.

3.8.4.2 *Salkowski Test for Unsaturated Sterols*

The stock extract (2ml) was transferred in a test tube, 3 drops of 98% concentrated sulphuric acid was added at the side of the test-tube. A cherry red colour at the interphase of the sulphuric acid usually indicates the presence of unsaturated sterols (Trease and Evans, 2009)

3.8.5 Test for Cardiac Glycoside

3.8.5.1 *Kella-killian test*

The stock extract (2 ml) was transferred in a test-tube, 1ml of glacial acetic acid containing traces of ferric chloride solution was added, concentrated sulphuric acid (1 ml) was added gradually down the side of the test tube to form a lower layer. A purple brown ring at the interphase indicates the presence of a deoxy sugar and a pale green colour in the upper acetic layer indicates the presence of cardiac glycoside (Trease and Evans, 2009)

3.8.5.2 Kedde's *Test*

The stock extract (1 ml) was transferred in a test tube, 1ml of 3, 5-Dinitrobenzoic acid in 95% alcohol was added. The solution was made alkaline with 1ml 5% sodium hydroxide and appearance of a purple-blue colour indicates the presence of cardenolides which is the basic compound in cardiac glycoside (Trease and Evans, 2009)

3.8.6 Test for Saponins

3.8.6.1 *Frothing test*

The stock extract (1 ml) was transferred into a test-tube, 10ml of distilled water was added and shaken vigorously for about 30 seconds. The test-tube was allowed to stand in a vertical position; a honeycomb that persisted for 10-15 minutes indicated the presence of Saponins (Trease and Evans, 2009)

3.8.6.2 *Heamolysis test*

Aqueous sodium chloride (1.8%) solution was prepared, 2 ml of it was transferred in a test-tube, 2ml of the stock extract was added, 5 ml of animal blood was added in drops, the test-tubes was observed for 10-15 minutes for haemolysis (settling down of the red blood cells) which indicated the presence of saponins (Trease and Evans, 2009)

3.8.7 Test for Tannins

3.8.7.1 *Lead sub acetate test*

Lead sub-acetate solution (2 drops) was added to 3 ml of the stock extract, a brown or reddish coloured precipitate indicates the presence of hydrolysed and un-hydrolysed tannins respectively (Trease and Evans, 2009)

3.8.7.2 *Ferric chloride test*

To the dissolved stock extract in a test tube (2 ml), 3 drops of ferric chloride solution was added A greenish-black precipitate indicated the presence of condensed tannins while hydrolyzed tannin gives a blue or brownish-blue precipitate (Trease and Evans, 2009)

3.8.8.3 *Bromine water Test*

Bromine water (2 ml) was added to 2 ml of the extract in a test-tube. A buff coloured precipitate indicates condensed tannins while hydrolyzed tannins give none at all (Trease and Evans, 2009)

3.8.9 Test for Alkaloids

3.8.9.1 *Mayer's test*

A drop of Mayer's reagent was added to 3 ml of the extract, a creamy precipitate indicated the presence of alkaloids (Trease and Evans, 2009)

3.8.9.2 *Dragendoff"s test*

A drop of Dragendoff"s reagent was added to 2 ml of the extract in a test tube, a reddish brown precipitate indicated the presence of alkaloids (Trease and Evans, 2009)

3.9 Quantitative Phytochemical Screening

Quantitative phytochemical screening is a test conducted using various standard methods and procedures to ascertain the concentration of various secondary metabolites present in the plant extract equivalent to the standard

3.9.1 Determination of Total Phenolic Compound (TPC)

Estimation of Total Phenolic compound in the leaf extract (N-Hexane, Ethyl-Acetate, Methanol and Aqueous Extract) of *Hyptis spicigera* Lam was measured spectroscopically by Folin Ciocalteu Colometric method, using Gallic acid as standard and expressing the result as Gallic

acid equivalent (GAE) per gram of sample. Different concentration of (0.01-0.1mg/ml) Gallic acid was prepared. Aliquot of 0.5ml of the test samples and the standard solution were taken in a separate test tubes, mixed with 2ml of Folin Ciocalteu reagent (1:10 in deionized water) and 4ml of standard solution of Sodium carbonate (7.5% w/v) was added to each of the test tubes. The tubes were covered with silver foil and incubated at room temperature for 30 min with intermitted shaking. The absorbance was taken at 765nm using the dissolving solvent for each extract as blank. All samples (N-Hexane, Ethyl-acetate, Methanol and Aqueous extracts) were analysed in three replicates. The total phenolic compound was determined with the help of a standard curve prepared from pure phenolic standard (Gallic acid), the concentration of TPC was expressed in mg/g per standard (Alhakmani *et al.,* 2013)

3.9.2 Determination of Total Flavonoid Content (TFC)

The TFC of *Hyptis spicigera* leaf extract was determined by Aluminium chloride colometric assay (Zhishen *et al.,* 1999). Aliquot of 0.5 ml of each sample (n-hexane, ethyl-acetate, methanol and aqueous extracts) a standard solution (0.01-1.0 mg/ml) of quercetin was taken in separate test tubes, 2ml of distilled water was added and subsequently, 0.5 ml of sodium nitrites (5% NaOH w/v) solution was added. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added. The solution was allowed to stand for 6 min and after that, 2 ml of sodium hydroxide (4% NaOH w/v) solution was added to the mixture. The final volume was adjusted to 5ml with immediate addition of distilled water, mixed thoroughly and allowed to stand for another 15min. The absorbance of each mixture was determine at 510nm. The TFC was determined as mg/g quercetin equivalent with the help of calibration curve of quercetin. Each determination was done in triplicates.

3.9.3 Determination of Total Alkaloids Content (TAC)

TAC was determined by spectroscopic method, this was based on reaction between alkaloid and bromocresol green (BCG). In this method, 0.5 g of each plant extract (n-hexane, ethyl-

acetate, methanol and aqueous) was dissolved in 5 ml of distilled water, 1 ml each of the stock was transferred into a test tube. The standard atropine in a test-tube each (having a set of 5 test tubes) were set (0.05 g, 0.1g, 0.15g, 0.2 g and 0.25g) and dissolved in 2 ml HCl and then filtered. The pH of phosphate buffer was adjusted to neutral with 0.1 ml NaOH, 1 ml of this solution was transferred to a separating funnel and then 5 ml of BCG solution along with 3 ml of phosphate buffer added as well. The mixture was taken and the complex formed was extracted with chloroform by vigorously shaking. The extract was collected in a 10 ml volumetric flask and diluted to the volume of the flask with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The whole experiment was conducted in three replicates and the concentration of TAC was expressed in mg/g (Shamsa *et al.,* 2008; Sharief *et al.,* 2014). TAC was determined in mg/ml Atropine equivalent.

3.9.4 Determination of Total Tannin Content (TTC)

TTC was determine spectroscopically by Folin Ciocalteu method. About 0.1 ml of the extract (n-hexane, ethyl-acetate, methanol and aqueous) was added to a 10 ml volumetric flask containing 7.5 ml of distilled water and 0.5 ml Folin Ciocalteu reagent, 1 ml of 35% Na_2CO_3 solution was then added and adjusted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A reference standard solution of Gallic Acid set (20, 40, 60, 80 and 100 mg/ml) was prepared in the same manner as described earlier. Absorbance for the test extract and standard solution was measured against the blank for each extract at 750 nm. The tannin content was expressed in terms of mg/g/GAE of each extract (Marinova *et al.,* 2005; Rajeev *et al.,* 2012; AfifyAel-M *et al.,* 2012)

3.9.5 Determination of Total Saponin Content (TSC)

TSC was determined according to the method described by Makkar *et al.* (2007). A known quantity (0.5 g) of the extract (n-hexane, ethyl-acetate, methanol and aqueous) was dissolved in a suitable dissolving solvent (n-hexane, ethl-acetate, methanol and aqueous) for each extract

(An aliquots of 5 ml/ml) was taken. Vanillin reagent (0.25 ml of 8%) was added followed by sulphuric acid (2.5ml of 72% v/v). The reaction mixture was mixed well and incubated at 60 ^{0}C in a water bath for 10 min after incubation the reaction mixture was cooled and its absorbance was taken at 544 nm against the blank for each extract. The standard calibration curve was obtained from suitable aliquots of Diosgenin (0.5 mg/ml). The total saponin concentration was expressed as mg/g/ Diosgenin equivalent.

3.10 Source of Fungal Isolates

Four different species each of *Aspergillus* and *Fusarium* known to affect cereals and other farm produce which had been identified by Innovative Medicine Initiative (IMI) was used for the study. Already cultured isolates was collected from the Department of Crop Protection, Ahmadu Bello University, Zaria-Kaduna, Nigeria and was taken to the Department of Botany, Ahmadu Bello University, Zaria-Kaduna

The four species of *Aspergillus* used were:

1. *Aspergillus flavus* IMI 506180
2. *Aspergillus niger* IMI 305683
3. *Aspergillus parasiticus* IMI 507181
4. *Aspergillus fumigatus* IMI 506179

The four species of *Fusarium* used were:

1. *Fusarium verticilloides* IMI 617849
2. *Fusarium graminerum* IMI 702314
3. *Fusarium oxysporum* IMI 631004
4. *Fusarium proliferatum* IMI 419160

3.11 Media Preparation

Potato Dextrose Agar (CRITERION™) was prepared according to the manufacturer's instructions, by dissolving 39g in a liter of distilled water and heated until there was complete dissolution of the mixture, it was autoclaved at 121°C for 15 mins. and 20 ml each of the prepared medium was dispensed in each petri dish and allowed to solidify before usage

3.12 Culturing of Fungal Isolates

The petri dish with solidified PDA were inoculated with a fragment of the colony using a sterilized wire loop and incubated for 7-10 days at 30°C.

3.13 Preparation of Fungal Inoculum

The spores from the surface of the plates was collected with inoculating needle and suspended in 10ml of normal saline solution, 2 ml of 10% tween 20 was added, the mixture was homogenized and left to stand for 6 hours, the spore settle below and was collected in a sterilised test tube. The suspension was adjusted to 0.5 McFarland standard equivalent to the turbidity of the suspension by a spectrophotometer at a wavelength of 530nm to obtain a final concentration that will match 0.5 McFarland standard for mould base on the optical density of the solution and the optical density of one is regarded as 0.5 McFarland which corresponds to the spore count between (4×10^5-5×10^6) CFU/ml (CLSI, 2014)

3.14 Sensitivity Test

Agar well diffusion method was used to screen the extract against the test organisms. The already prepared PDA was re-heated and poured into sterile petri-dish and allowed to solidify. The medium was seeded with 0.1ml standard inoculum of the test microbe each in three replicates, by the use of a standard cork borer of 6mm in diameter a well was made at the middle of the petri dish, 0.5g of the extract was dissolved in 10 ml of 20% Dimethyl sulfoxide (DMSO) at this stage the concentration of the extract obtain was 50 mg/ml, then 0.1ml of the

prepared extract was introduced into the well and incubated at 30°C for 7 days after which the plates was observed for zone of inhibition, the inhibition zone was measured with a meter rule and recorded excluding the diameter of the well (CLSI, 2014) A positive control Mancozeb (Fungicide) and a negative control which are Normal Saline and 20% dimethyl sulphur oxide (DMSO) was set to account for their inhibitory action.

3.15 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) is the least concentration of the extract that did not show any sign of fungal growth in a test tube. The MIC was determined using the broth dilution method, Potato Dextrose (PD) Broth was prepared as prescribed by the manufacturer, two-fold serial dilution of the extract was done in the sterile broth to obtain a concentration of 50, 25, 12.5, 6.25, 3.13 and 1.57 mg/ml. The already prepared standard inoculum 0.5 ml was introduced into each of the test tube containing varied concentration of the extracts dissolved in the PD broth, incubation was done for 48 hours at a temperature of 30 °C in an incubator. The MIC was read as the test tube having the least concentration of the extract with no sign of fungal growth, this could easily be seen from the surface of each tube as the spores if present will show visible sign of coloured spores depending on the species (CLSI, 2014)

3.16 Minimum Fungicidal Concentration (MFC)

Minimum fungicidal concentration (MFC) is the least concentration of the extract obtained by sub-culturing the contents of the MIC that did not show any trace of growth of microorganism on a petri dish which indicated that 99% of the test organisms are not viable. Potato Dextrose Agar was prepared, 20 ml each was dispensed into sterile petri dish and allowed to solidify, the content of the MIC starting from the test tube which the MIC was recorded and those with increase concentration of the extract in the sterile dilution were sub-cultured onto prepared medium, incubation was made at 30 °C for 48 hours in an incubator after which the plates of

the medium was observed for colony growth. The least concentration of the extract without colony growth is referred to as the minimum fungicidal concentration. (CLSI, 2014)

3.17 Data Analysis

The quantity of the various secondary metabolites was tested in each of the extract using one way Analyses of variance (ANOVA). The data collected on zone of inhibition was subjected to Two Way Analyses of Variance (ANOVA) where significant, Duncans Multiple Range Test was used to separate the means.

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Yield of the Leaf Extracts of *Hyptis spicigera* Lam. using different Extraction Solvent

The percentage yield of the leaves extract of *Hyptis spicigera* showed that the aqueous extract had the highest percentage yield of 9.09% followed by methanol with 6.12%, ethyl-acetate had 3.59% while n-hexane had the least yield of 2.71%. (Table 4.1)

4.2 Qualitative Phytochemicals contents

The qualitative phytochemical screening of the leaf extract (n-hexane, ethyl-acetate, methanol and aqueous) of *Hyptis spicigera,* indicates the presence of Carbohydrates, Unsaturated sterols and triterpenes, Cardiac glycosides, Flavonoids, Tannins and Alkaloids in all the extracts. Glycosides was absent in all the extracts except in the aqueous. However, anthraquinones was present in the ethyl-acetate and aqueous extracts but absent in n-hexane and methanol extracts. Saponins was also present in all the extracts except in n-hexane (Table 4.2).

4.3 Quantitative phytochemicals constituents

Ethyl-acetate extract had the highest concentration of Saponins (920±0.33 mg/g/DE) while the lowest was in aqueous (490±0.04 mg/g/DE), n-hexane had the highest concentration of phenolic (880±0.01 mg/g/GAE) while the least concentration was in ethyl-acetate (200±0.00 mg/g/GAE). Alkaloids was higher in the n-hexane extract (170±0.12 mg/g/AE) and the least was in the ethyl-acetate extract (70±0.13 mg/g/AE). The highest concentration of flavonoids was detected in the aqueous extract (220±0.00 mg/g/QE) and it least concentration was observed in n-hexane (40±0.07 mg/g/QE). Tannins had the highest concentration in the aqueous extract (50±0.33 mg/g/GAE) and it least concentration was in n-hexane extract (20±0.07 mg/g/GAE). There was a significant difference in the concentrations of all the

phytochemical in all the extract except for the tannins where the difference was not significant in n-hexane (20±0.07 mg/g/GAE) and methanol (20±0.17 mg/g/GAE) extarcts (Table 4.3).

Table 4.1: Percentage yield of the leaf extracts of *Hyptis spicigera* using different extraction solvents

Extract/solvents	Initial weight (g)	Recovery rate (g)	Percentage yield
N-Hexane	250	6.78	2.71
Ethyl-Acetate	243	8.72	3.59
Methanol	234	14.32	6.12
Aqueous	219	19.91	9.09

Table 4.2: Qualitative phytochemical contents of *Hyptis spicigera* leaf extracts

Phytochemicals	Test	N-Hexane	Ethyl-Acetate	Methanol	Aqueous	Fraction A	Fraction B
Carbohydrates	Molish	+	+	+	+		
	Fehlings	+	+	+	+		
Glycosides	Fehling	-	-	-	+		
Anthraquinone	Bontragers	-	+	-	+		
Unsaturated Sterols and Triterpenes	Lieberman-Bucchard	+	+	+	+		+
	Salkwoski	+	+	+	+		
Cardiac Glycoside	Kella- killiani	+	+	+	+		
	Keddes	+	+	+	+		
Saponins	Frothing	-	+	+	+		
	Haemolysis	+	+	+	+		
Flavonoids	Shinoda	+	+	+	+	+	
	NaOH	+	+	+	+	+	
	Ferric Chloride	+	+	+	+		
Tannins	Lead-Acetate	+	+	+	+		
	Ferric-Chloride	+	+	+	+		
	Bromine-water	+	+	+	+		
Alkaloids	Mayers	+	+	+	+		
	Dragendoff	+	+	+	+		
	Wagners	+	+	+	+		

Key: + = Positive; - = Negative; Fraction A= Flavonoid; Fraction B= Terpenoids

Table 4.3: Quantitative Phytochemical constituents of *Hyptis spicigera* Leaf Extracts

	Solvents of extraction			
Phytochemicals	N-hexane	Ethyl-acetate	Methanol	Aqueous
Phenolic	$880±0.01^a$	$200±0.00^d$	$220±0.13^c$	$280±0.71^b$
Tannins	$20±0.07^c$	$30±0.03^b$	$20±0.17^c$	$50±0.33^a$
Flavonoids	$40±0.07^d$	$80±0.33^c$	$140±0.33^b$	$220±0.00^a$
Alkaloids	$170±0.12^a$	$70±0.13^d$	$90±0.13^c$	$120±0.00^b$
Saponins	$890±0.06^b$	$920±0.33^a$	$690±0.70^c$	$490±0.05^d$

Key: Mean with the same superscript along each row are not significantly different at $P \geq 0.05$

4.4 Chemical confirmation and TLC chromatographic Profile of Terpenoids fraction from Ethyl-acetate leaf extract of *Hyptis spicigera*

Green coloration was observed at the upper layer and the formation of deep red color in the lower layer which indicate a positive test for steroids and triterpenoids respectively. About 7 different spots of different classes of terpenoids were noticed and their retention factors were measured with a meter rule (Plate 4.1)

4.5 Chemical confirmation and TLC chromatographic Profile of Flavonoid from Methanol leaf extract of *Hyptis spicigera* Lam.

A red color and yellow was observed which indicates the presence of flavonoids, a yellow fluorescence was observed in long wavelength UV light (360nm) on the TLC plates which confirms flavonoids (Plate 4.2)

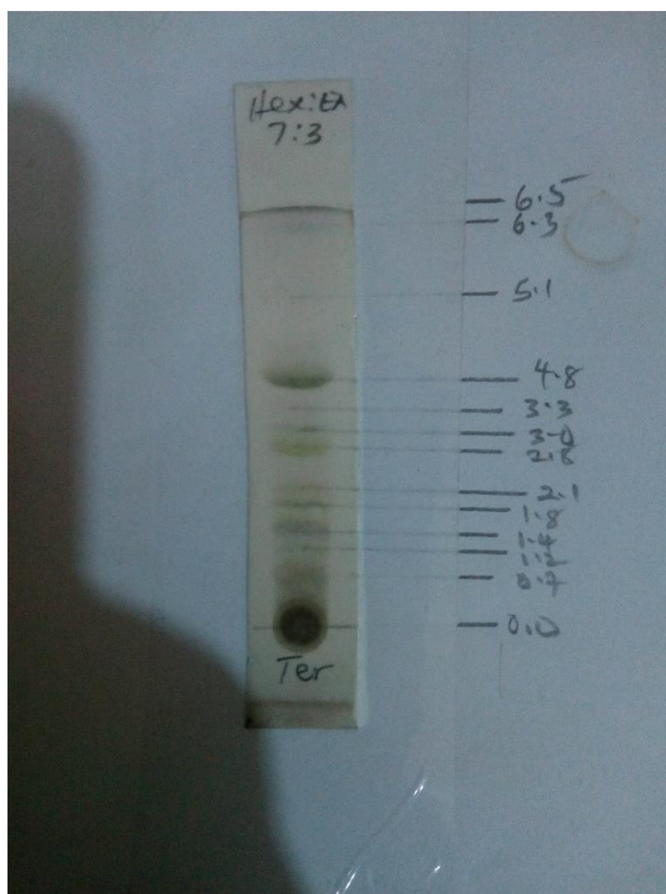


Plate 4.1: Thin layer chromatographic profile for terpenoids from ethylacetate leaf extracts of *Hyptis spicigera* showing the calibrations (cm) of spots

Plate 4.2: Thin layer chromatographic profile for Flavonoids for methanol leaf extracts of *Hyptis spicigera* under UV light

4.6 Zones of Inhibition of Extracts, Terpenoids and Flavonoids from *Hyptis spicigera* on *Aspergillus* and *Fusarium* species

All the extracts and fractions showed zone of inhibition on *A. fumigatus* (15.67 - 21.33 mm) and *F. oxysporum* (14.00 - 19.33 mm) (Table 4.4). There was no significant difference between the inhibition zone of ethyl acetate (21.33±0.67 mm) and the control fungicides (22.67±0.88 mm) on *A. fumigatus* while there was a significant difference between the inhibition zone of the ethyl-acetate and the control fungicides with respect to all the other extracts, terpenoids and flavonoid fractions on this fungus. A highly significant difference was observed between the terpenoid fraction (19.33±0.33 mm) and the control fungicide (0.00±0.00 mm) on *F. oxysporum.* Similarly, a significant difference was also observed between the terpenoid fraction and all the other extract including the flavonoid fraction.

Inhibition zone observed on *A. flavus* (16.67 - 22.67 mm) and *A. parasiticus* (18.33 - 24.67 mm) for all the extracts and fractions were significantly different except for n-hexane extract. Inhibition zone of ethyl-acetate extract (22.67±0.88 mm) and the control fungicide (20.00±1.16 mm). However, the inhibition zone of the control was not significant with that of the terpenoid fraction (21.00±0.58 mm). Inhibition zone of *F. proliferatum* was also observed on aqueous (18.33 mm) and terpenoids fraction (19.00 mm) which were not significantly different, but the inhibition zone of the control (21.00±0.57 mm) was significantly different from that of the aqueous extract and the terpenoid fraction on *F. proliferatum*

All the extracts and flavonoids fraction showed zone of inhibition against *F. graminearum* (13.00 -21.00 mm) except for the aqueous extract, significant difference was observed between the inhibition zone of the methanol extract (21.00±0.00 mm) and the control fungicides (18.33±1.45 mm). However, there was no significant difference between the inhibition zone of the ethyl-acetate (20.23±0.02 mm) and the methanol extract.

A. niger was found to be resistant to all the extracts and fractions except for the aqueous extract (16.00 mm) and mancozeb (19.33 mm), although the inhibition zone of the control fungicide was significant from that of the aqueous extract. *A. parasiticus* and *F. oxysporum* was found to be resistance to the fungicide

(Mancozeb). *F. verticilloides* was resistance to all the extracts although the control fungicide was sensitive to this fungus species with inhibition zone of 22.00 mm (Table 4.4)

4.7 Minimum Inhibitory Concentration (MIC) of the leaf extracts and fractions from *Hyptis spicigera* on *Aspergillus* and *Fusarium* species

The organisms sensitive to the n-hexane extract were *A. fumigatus, F. graminearum* and *F. oxysporum* and they all showed MIC values of 12.5mg/ml; ethyl-acetate extract had an MIC of 6.25 mg/ml for *A. flavus, A. fumigatus* and *A. parasiticus* and 12.5 mg/ml for *F. graminearum* and *F. oxysporum.* For methanol extract, the MIC on *A. flavus, A. fumigatus A. parasiticus, F. graminearum* and *F. oxysporum* was 12.5 mg/ml. The MIC of aqueous extract on *A. fumigatus* and *F. proliferatum* was 6.25mg/ml while on *A. niger* and *F. oxysporum* it was 12.5mg/ml, for *A. parasiticus* the MIC was 3.13mg/ml.

The flavonoids fraction had MIC of 3.13 mg/ml on *A. flavus* and *F. graminearum;* for *A. fumigatus* and *A. parasiticus* the MIC was 6.25mg/ml and 12.5 mg/ml for *F. oxysporum*. The MIC of terpenoids fraction on *A. flavus* and *A. fumigatus* was 6.25 mg/ml and 12.5 mg/ml for *A. parasiticus, F. proliferatum* and *F. oxysporum* (Table 4.5).

Table 4.4: Inhibition Zones (mm) of the Leaf Extracts and fractions from *Hyptis spicigera* Lam on *Aspergillus* and *Fusarium* species

	Extracts and Fractions							
Fungal Species	N-hexane	Ethyl-acetate	Methanol	Aqueous	Fraction A	Fraction B	+Control	- Control
A. flavus	$0.00±0.00^{e}$	$22.67±0.88^{a}$	$18.33±0.33^{c}$	$0.00±0.00^{e}$	$16.67±0.33^{d}$	$21.00±0.58^{ab}$	$20.00±1.16^{b}$	0.00
A. fumigatus	$15.67±0.88^{cd}$	$21.33±0.67^{a}$	$18.33±0.88^{b}$	$17.67±0.33^{bc}$	$14.33±0.33^{d}$	$19.00±0.58^{b}$	$22.67±0.88^{a}$	0.00
A. parasiticus	$0.00±0.00^{d}$	$24.67±0.88^{a}$	$18.33±0.88^{c}$	$21.33±0.33^{b}$	$18.33±0.33^{c}$	$21.33±0.33^{b}$	$0.00±0.00^{d}$	0.00
A. niger	$0.00±0.00^{c}$	$0.00±0.00^{c}$	$0.00±0.00^{c}$	$16.00±0.58^{b}$	$0.00±0.00^{c}$	$0.00±0.00^{c}$	$19.33±0.88^{a}$	0.00
F. graminearum	$13.00±0.58^{c}$	$20.23±0.02^{a}$	$21.00±0.00^{a}$	$0.00±0.00^{d}$	$17.67±0.33^{b}$	$0.00±0.00^{d}$	$18.33±1.45^{b}$	0.00
F. proliferatum	$0.00±0.00^{c}$	$0.00±0.00^{c}$	$0.00±0.00^{c}$	$18.33±0.33^{b}$	$0.00±0.00^{c}$	$19.00±0.00^{b}$	$21.00±0.57^{a}$	0.00
F. oxysporum	$14.00±0.00^{c}$	$18.00±0.58^{b}$	$18.00±0.58^{b}$	$18.00±0.58^{b}$	$17.00±0.09^{b}$	$19.33±0.33^{a}$	$0.00±0.00^{d}$	0.00
F. verticilloides	$0.00±0.00^{b}$	$0.00±0.00^{b}$	$0.00±0.00^{b}$	$0.00±0.00^{b}$	$0.00±0.00^{b}$	$0.00±0.00^{b}$	$22.00±0.58^{a}$	0.00

KEY: A= Flavonoids B= Terpenoids +Control= Mancozeb -Control= 20% DMSO

NOTES: Values are expressed as means ± SEM (standard error of mean) of three replicates
Means with the same superscript along each row are not significantly different at P≥0.05

Table 4.5: Minimum Inhibitory Concentration (MIC) of extracts and fraction from *Hyptis spicigera* on *Aspergillus* and *Fusarium* species

	Concentration (mg/ml) of extracts and fractions					
Test organism	hexane	ethyl-acetate	methanol	aqueous	flavonoids	terpenoids
A. flavus	-	6.25	12.5	-	12.5	25
A. fumigatus	12.5	6.25	12.5	6.25	12.5	12.5
A. parasiticus	-	6.25	12.5	3.13	12.5	12.5
A. niger	-	-	-	12.5	-	-
F. graminearum	12.5	12.5	12.5	-	6.25	-
F. oxysporum	12.5	12.5	12.5	12.5	12.5	25
F. proliferatum	-	-	-	6.25	-	25
F. verticilloides	-	-	-	-	-	-

KEY: - = fungus was resistance to the extract

4.8 Minimum Fungicidal Concentration (MFC) of the leaf extracts and fractions from *Hyptis spicigera* on *Aspergillus* and *Fusarium* species

The MFC of n-hexane on *A. fumigatus, F. graminearum* and *F. oxysporum* was 25 mg/ml., for ethyl-acetate extract on *A. flavus, A. fumigatus* and *A. parasiticus* it was 6.25 mg/ml while on *F. graminearum* and *F. oxysporum* it was 25 mg/ml and 12.5 mg/ml respectively. The MFC of Methanol extract on *A. flavus, A. fumigatus A. parasiticus, F. graminearum* and *F. oxysporum* was 25 mg/ml. The MFC of aqueous extract on *A. fumigatus, F. oxysporum and F. proliferatum* was 12.5mg/ml, on *A. niger* and *A. parasiticus* it was 25 mg/ml and 6.25 mg/ml respectively. The MFC of flavonoids fraction on *A. flavus, A. fumigatus, A. parasiticus* and *F. oxysporum* was 12.5 mg/ml, on *F. graminearum* it was 6.25 mg/ml. The MFC of terpenoids on *A. flavus, F. proliferatum* and *F. oxysporum* 25 mg/ml and 12.5 mg/ml on *A. fumigatus* and *A. parasiticus* (Table 4.6).

Table 4.6**:** Minimum Fungicidal Concentration (MFC) of extracts and fraction from *Hyptis spicigera* on *Aspergillus* and *Fusarium* species

	Concentration (mg/ml) of extracts and fractions					
Test organism	hexane	ethyl-acetate	methanol	aqueous	flavonoids	terpenoids
A. flavus	-	6.25	25	-	3.13	6.25
A. fumigatus	25	6.25	25	12.5	6.25	6.25
A. parasiticus	-	6.25	25	6.25	6.25	12.5
A. niger	-	-	-	25	-	-
F. graminearum	25	25	25	-	3.13	-
F. oxysporum	25	12.5	25	12.5	12.5	12.5
F. proliferatum	-	-	-	12.5	-	12.5
F. verticilloides	-	-	-	-	-	-

KEY: - = fungus was resistance to the extract

CHAPTER FIVE

5.0 DISCUSSION

The percentage yield of extract from *Hyptis spicigera* was increasing with increasing polarity of extraction solvents. As polarity of solvent increased the percentage or number of phytochemicals extracted also increased. Also the solubility of plant extract was determined by the polarity of the extracting solvent in which more polar chemical constituents dissolved more in highly polar solvent, this trend is similar to the findings of Ojo *et al.* (2006), Sultana *et al.* (2009) and Ibrahim *et al.* (2017) who reported an increased concentration in the leaf extract of their plant by increasing polarity of extraction solvent (chloroform, ethyl-acetate and methanol)

The qualitative phytochemical constituents of leaf extracts of *Hyptis spicigera* using varied solvents indicates the presence of Carbohydrates, Unsaturated sterols and triterpenes, Cardiac glycosides, Flavonoids, Tannins and Alkaloids in all the extracts. The presence of carbohydrates in sufficient quantity had been attributed to the fact that they act as bacteriostatic or fungistatic and also serves as preservatives in some certain biological systems of food items (Tyler *et al.,* 2011)

Glycosides, anthraquinones and tannins are also among the phytochemicals detected; tannins were present in all the extracts while glycoside was absent in all the extract except the aqueous extract, this could be as a result of the hydrolyses of the glycosides during extraction with organic solvent. The presence of glycosidic compound such as tannin would likely result in enzyme inhibition and substrate deprivation to microorganism (Cowan, 1999).

Also flavonoids, alkaloids and saponins were present in all the extracts, the presence of chemical components such as flavonoids in an extract could results in the formation of complexes around the cell wall components of some microorganism which could lead to the

disruption of ezymes and inactivation of metabolic processes (Zaker, 2016). Similar trends of phytochemicals were also detected in other *Hyptis* species (Santos *et al.,* 2007)

The high concentration of phenolic compound detected in the n-hexane (880±0.01 mg/g/Gallic acid equivalent) could be attributed to the fact that the leaves of this plant contains high concentration of volatile aromatic phenolic compounds (Marinova *et al.,* 2005). This phenol present in higher concentration in the n-hexane could also reflect the fact that this solvent generally extracts the less polar or volatile compounds or constituents of phytochemical which phenol is among. The mode of action and inhibition of phenolic compound either simple phenols or phenolic acids to microorganism includes the formation of complex with the cell wall of fungi spores and also the disruption of membrane and substrate deprivation (Cowan, 1999). Similarly, Subhashini *et al.* (2013) reported higher concentration of phenolic compound in the n-hexane extract of *Ecbolium viride* (720 mg/g/GAE) while the least concentration of phenolic compound was observed in the ethyl-acetate with a concentration of 200±0.00 mg/g/ per Gallic acid equivalent.

Gavaric *et al.* (2015) reported that phenolic compound basically forms a shield adhesion complex of poly-hydroxyle furanyle complex around the conidium of fungi leading to substrate deprivation, in such situation when nutrient medium is not accessible to conidia of the fungi all metabolic activities is halted which will invariably lead to the death of the mycelia. In this research it was observed that the n-hexane leaf extract despite having the highest concentration of phenolic compound also showed lesser activity as compared to the other extract, this could be as a result of the presence of pigment molecules in the hexane extract. Sharief *et al.* (2014) reported that pigments molecules were present in less polar solar solvent (n-hexane) and tends to mask the activities of noble phytochemicals that are present in the extract.

Tannins were also present in high concentration in the aqueous extract with a concentration of 50±0.33 mg/g/GAE while the least concentration was observed in n-hexane and methanol extract with a concentration of 20±0.07 mg/g/GAE and 20±0.17 mg/g/GAE, the presence of these phytochemical in varied concentration in the various extracts could results in enzyme inhibition and substrate deprivation when in contact with any microorganism (Schijlen *et al.,* 2004).

The flavonoids were highest in the aqueous extract with a concentration of 220 mg/g per quercetin equivalent (QE), while the least concentration was observed in n-hexane extract with a concentration of 40 mg/g/QE. Moreover, the concentration gotten from this work is in accordance with the work of Elegani *et al.* (2012) who reported a close range concentration of 180 mg/g/QE of flavonoids in the methanol extract of *Hyptis suaveolens*. Flavonoids play an important role in biological processes, beside their function as pigments in flowers and fruits, to attract pollinators and seed dispersers, flavonoids are involved in UV-scavenging, fertility and disease resistance especially to fungal infection (Schijlen *et al.,* 2004).

The highest concentration of alkaloid was seen in the n-hexane extract with a concentration of 170±0.12 mg/g per atropine equivalent (AE) while the least concentration noticed in ethyl-acetate with a concentration of 70 mg/g/AE. Alkaloids basically act as intercalating enzymes on the cells of most microorganisms there by causing disruption of the cell wall leading to the death of the cells or spores as in fungi (Jalili-Marandi *et al.,* 2010). Cowan, 1999 also reports that alkaloids present in plant extract had greatly been associated to intercalate into the cell wall component of cells and cause spore disruption in fungi.

Saponins present in the plant indicates a high concentration in the ethyl-acetate extract having a concentration of 890±0.06 mg/g per diosgenin equivalent (DE) while the least concentration was observed in aqueous extract with a concentration of 490±0.05 mg/g/DE. Masih *et al.*

(2014) reports that saponin present in the leaves extract could also be responsible for causing lyses of the spores of fungal species serving as a toxic chemical to the spore there by leading to the death of the cell. Similar activity of toxic effect of Saponins had been reported on *Aspergillus flavus* from essential oils of *Semicarpus anarcadium* (Mohanta *et al.,* 2007) Saponins from plants serve as chemotheraphy of mycotic infections on plant pathogenic microbes (Esquivel-Ferrino *et al.,* 2014). Similar potentials of saponins was reported on the juice extract of *Gmelina arborea* on *Candida* species where it was observed to cause cell lysing and membrane disruption (Adamu *et al.,* 2019)

The chemical test confirmation for terpenoids showed green coloration at the upper layer and there was formation of deep red color in the lower layer which indicate a positive test for steroids and triterpenoids respectively (Satish *et al.,* 2012). The chemical test for flavonoids also indicates a red and yellow coloration which confirms a positive test for flavonoids (Woo *et al.,* 1980; Trease and Evans, 2009)

The TLC chromatogram of the terpenoid fraction from the ethyl-acetate leaves extract showed 7 different spots. The retention factors for each of the spots were 0.97, 0.78, 0.74, 0.55, 0.46, 0.43, 0.32, 0.28, 0.22, 0.18 and 0.11. The mechanism of action of terpenoids on fungi is related to the hydrophobic nature of terpenoids which makes it bind to the Ca^{2+} ion on the cell wall of fungi there by producing a hydro-oxyle complex which had been associated with membrane expansion and fluidity of cell leading to cell disruption and consequently lethality of mycelium (Rao *et al.,* 2010). The TLC chromatogram of the flavonoids isolated from the methanol leaves extract showed 2 yellow and 2 red fluorescence which confirms flavonoids.

The inhibitory activity of the leaves extracts, terpenoids and flavonoids fraction from *Hyptis spicigera* using varied extracting solvent revealed the various zones of inhibitions on *Aspergillus* and *Fusarium* species. All the extracts, terpenoids and flavonoids tested shown

significant zones of inhibition on *A. fumigatus* and *F. oxysporum* having mean diameter of inhibition ranging from 14.33±0.33 mm to 21.33±0.67 mm and 14.00±0.00 to 19.33±0.33 mm respectively. The widest diameter of inhibition on *A. fumigatus* was exerted by the ethyl-acetate extract with a mean diameter of inhibition of 21.33±0.67 mm which was not significantly different from the control fungicides (mancozeb).

There was a significant difference between the zones of inhibition of the terpenoids fraction gotten from the ethyl-acetate extract on *A. fumigatus,* as the ethyl-acetate showed wider zone of inhibition than the terpenoids fraction isolated from this extract. These might be as a result of the synergistic effect of other metabolites along with the terpenoids present in the ethyl-acetate extract. Flavonoids and n-hexane leaf extract are the least having a mean inhibition zone of 14.33±0.33 mm and 15.67±0.88 mm respectively on *A. fumigatus* which were no significantly different from each another.

All the extracts and fractions showed zone of inhibition against *F. oxysporum* while the control fungicides showed no inhibitory zone on this fungus, the widest zone of inhibition was showed by the terpenoids fraction (19.33±0.33) and the least was observed on n-hexane (14.00±0.00) The control fungicides does not show inhibition zone on *F. oxysporum,* the resistance of these fungi species to the fungicide could be that the fungicide (mancozeb) as reported by Abhishek *et al.* (2014) is a broad spectrum (wide range) fungicides and might not show activity against some fungi, so therefore the fungus *F. oxysporum* used in this research is resistance to mancozeb. as a result of the broad spectrum activity of the fungicide and it's been used for a wide range of fungi species which makes it less specific to certain species of fungi

There was inhibitory activity on *A. flavus* by all the extract and fractions except the n-hexane and aqueous extract where they do not show inhibition on the fungus, the widest diameter of inhibition on this fungus was exerted by the ethyl-acetate extract with zone of inhibition of

22.67±0.88 mm which was significantly higher than the control fungicide and the least diameter of inhibition on this fungus was observed on the flavonoid fraction (16.67±0.33 mm)

Similarly, all the extracts and fractions showed zones of inhibition on *A. parasiticus* except the n-hexane extract and the control fungicides, the widest diameter of inhibition on this fungus was observed on ethyl-acetate extract with diameter of 24.67±0.88 which was significantly different than the control fungicides (mancozeb) and the terpenoids fraction isolated from the ethyl-acetate extract, this could be as a result of synergetic activity of other metabolites and the terpenoid in the ethyl-acetate extract.

Inhibition zone was also observed on *F. graminearum* on n-hexane, ethyl-acetae, Methanol extract and flavonoids having inhibition zone of 13.00±0.58 mm, 20.23±0.02 mm, 21.00±0.00 mm and 17.67±0.33 mm respectively. Methanol was the extract that exerts a higher diameter of inhibition on *F. graminearum* with mean inhibition of 21.00±0.00 mm which was significantly higher than the control fungicides used for this study. Inhibition zone of *F. proliferatum* was only observed on aqueous and terpenoid having diameter of inhibition zone of 18.33±0.33 mm and 21.00±0.00 mm respectively. Aqueous extract was the only treatment that shows activity on *A. niger* having a mean zone inhibition of 16.00±0.58 mm, this exceptional activity observed on the aqueous extract could be as a result of higher profile concentration of flavonoids and alkaloids as compared to the other extracts, flavonoids and alkaloids play an important role in biological processes, flavonoids binds to cell wall of fungi spores leading to the formations of a complex which form serves as substrate deprivation, with the combine effect of alkaloid which intercalates into the spores of the fungi lead to destructions of fungal spores and inhibition of key enzyme activity (Makkar *et al.,* 2007). Although there was no activity seen on all the extract and fraction on *F. verticilloides* while the control fungicide shows activity on it.

The MIC of all the extract's and fraction on the fungi species sensitive to the extract's and the fractions ranges 3.13 mg/ml to ≤ 12.5 mg/ml while there MFCs ranges from 6.25 mg/ml to ≤ 25 mg/ml. this indicates that at the MIC concentration the fungi are still viable but could not reproduce due to the fungistatic nature of the extracts and fractions while at the MFC stage the fungi lost it viability due to the fungicidal activity of the extracts and fractions

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. The ethyl-acetate leaf extract of *Hyptis spicigera* contains phytochemicals of higher fungicidal activity than the other extract (n-hexane, methanol and aqueous extract), the qualitative analyses of the varied leaf extracts indicates the presence of flavonoids, tannins, alkaloids, unsaturated sterols and triterpenes. The quantitative analyses indicate that Saponins was the phytochemical having the highest concentration of 920±0.70 mg/g/DE in the ethyl-acetate extract while tannins had the least concentration of 20±0.07 mg/g/GAE and 20±0.17 mg/g/GAE as seen in n-hexane and methanol extracts respectively.
2. The varied leaf extracts of *Hyptis spicigera* proved effective in inhibiting mycelia growth of all the fungi species tested except for *F. verticilloides* that showed resistance to all the extracts. Also the ethyl-acetate extract showed highly significant inhibition zones on *A. flavus* when compared to the control fungicide used (Mancozeb). The control fungicides (mancozeb) could not inhibit the growth of *A. parasiticus* and *F. oxysporum* while the extracts inhibited their mycelia growth. The broad spectrum activity observed by these extracts indicates that they could be utilized as bio-fungicidal products against the species of fungi tested and could be used as alternative fungicides.
3. Terpenoids and flavonoids fractions from the leaf extract of *Hyptis spicigera* significantly inhibited mycelia growth of *A. flavus, A. fumigatus, A. parasiticus* and *F. oxysporum.* Inhibitory activity of terpenoid and flavonoids fraction were observed only on *F. graminearum* and *F. proliferatum* respectively.

6.2 Recommendations

It is recommended that;

1. The chemical analyses of *Hyptis spicigera* revealed the presence of phenols, tannins, Saponins, flavonoids and alkaloids, comparative quantitative screening should be carried out using varied solvent system for each plant extract before subjecting the extract to any biological activity such as antifungal activity to have idea of the phytochemicals responsible for growth inhibitions on the fungi species

2. Biological control of plant pathogenic fungi proves to be an alternative method to the use of synthetic fungicides, the antifungal activity of the leaf extract of *Hyptis spicigera* showed a broad spectrum of growth inhibition on *Aspergillus* and *Fusarium* species. Antifungal activity of *Hyptis spicigera* leaf extract should be carried out on other fungal pathogens to evaluate its inhibitory activity, the extracts could also be tested on bacteria towards the development of an antibacterial agent. *In-vivo* antifungal activities should be carried out at both seeding level and maturity on crops to determine the mechanism of action of the plant extract so as to developed a bio-fungicidal product

3. Terpenoids and Flavonoids extracted from the leaf extract of *Hyptis spicigera* also showed a wide range of growth inhibition on *Aspergillus* and *Fusarium* species, these fractions obtained gave an idea of their capability to inhibit the growth of these fungal species individually and in there combine form. Fractionation of phytochemicals should be carried out in the most active extract to give a baseline on the group of chemicals causing fungal inhibition. There should be isolation of the active compound present in the fractions for antifungal activities

REFERENCES

Abdul, G. (1990). Introduction to Pharmacognosy, University Press Ibadan. First Edition. 298 p.

Abhishek, W., Preeti, M., Shiwani, G., Anjali, C. and Shirkot, C. K. (2014). Impact of fungicides Mancozeb at different application rates on soil microbial population, soil biological process and enzyme activities in soil, *The Scientific World Journal*., 10: 702909-702918

Abubakar, H. M. G., Karumi, Y. and Usman, H. (2017). Assessment of microbially active phytochemicals from the partitioned portions of the stem bark extract of *Diospyros mespiliformis* Hochst. ex. A. DC. *Nigerian Journal of Pharmaceutical Sciences,* 10 (2): 0189-0197

Adamu, K., Musa, H., Adelanwa, M. A., Ibrahim, R. and Rabilu, S. A. (2019). Effect of the crude juice extract of *Gmelina arborea* on four *Candida* species. *Nigerian Journal Mycology* 11: 204-209

Afify-Ael, M., El-Beltagi, H. S. and El-Salam, S. M. (2012). A biochemical change in phenols, flavonoids, tannins, vitamin E, β-carotene and antioxidant activity during soaking of three white sorghum varieties, *Asian Pacific Journal of Tropical Biomedicine,* 2(3):203–209

Agrios, G. N. (2005). *Significance of plant disease in plant pathology*, Academic Press, London; 205 p.

Ahn, Y. J., Lee, H. S., Oh, H. S., Kim, H. T. and Lee, Y. H. (2005): Antifungal activity and mode of action of Gallarhois-derived phenolics against phytopathogenic fungi. *Journal of Biochemical Physiology*, 81: 105–112

Akerele, O. (1991). Natures Medicinal Bounty: Don't Throw It Away, *World Health Forum,* 14(4): 390- 395

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F. A., Oswald, I. P. and Puel, O. (2013). New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. *Toxicology of Applied Pharmacology,* 272:191–198.

Alhakmani, F., Kumar, S. and Khan, S. A. (2013). Estimation of total phenolic content, *invitro* antioxidant and anti-inflammatory activity of flower of *Moringa oleifera. Asian Pacific Journal of Tropical Biomedicine.* 3 (8): 623-627

Alkenz, S., Sassi, A. D., Abugnah, Y. S. and Alryani, M. B. (2015). Isolation and Identification of Fungi Associated with Some Libyan Food, *African Journal of Food Science.* 9 (7): 406-416

Amaike, S., and Keller, N.P. (2011). *Aspergillus flavus*. *Annual review of Phytopathology,* 49(1): 107-133

Amici, E. S., Bozat, G. and Akbulut, I. (2013). Investigation of potential biological control of Fu*sarium oxysporum* f. sp. *lyscopersici* and *F. oxysporum* f. sp. *cubense* by essential

oils, plant extracts and chemical elicitors *In vitro*, *Pakistan Journal of Botany*, 45: 2119-2124

Anaissie E. J, Kuchar, R. T., Rex, J. H., Francesconi, A, Kasai, M., Müller, F. M., Lozano-Chiu, M., Summerbell, R. C., Dignani, M. C., Chanock, S. J. and Walsh, T. J. (2009). Fusariosis associated with pathogenic *Fusarium* species colonization of a hospital water system: a new paradigm for the epidemiology of opportunistic mold infections. *Clinical Infectious Diseases,* 33: 1871-1878

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R. and Daszak, P. (2004). Emerging Infectious Diseases of Plant: Pathogen Pollution, Climate Change and Agro-technology Drivers. *Trend in Ecology and Evolution.* 19 (10): 535-544

Anonymous, (2010). FAOSTAT, Food and Agricultural Organization of the United Nations, Annual Reports. http://faostat.fao.org

Anthony, J. D. (2007). Harmful fungi in both agricultural and medicine, *Review of Annual Mycology Spain,* 24:3-13

Aoki, T., Ward, T. J., Kistler, H. C. and O'donnell, K. (2012). Systematics, phylogeny and trichothecene mycotoxin potential of *Fusarium* head blight cereal pathogen. *Journal of South Mexico Mycotoxin.* 62, 91-102

Avasthi, S., Gautam, A. K. and Bhadauria, R. (2010). Antifungal activity of plant products against *Aspergillus niger*: A potential application in the control of a spoilage fungus. *Biological Forum- An International Journal,* 2(1): 53-55

Azevedo, N. R., Campos, I. F. P., Ferreira, H. D., Portes, T. A., Santos, S. C., Seraphin, J. C., Paula, J. R. and Ferri, P. H. (2001). Chemical variability in the essential oil of *Hyptis suaveolens, Phytochemistry,* 57: 733-736

Backhouse, D. (2014). Global Distribution of *Fusarium gramenearum, Fusarium asiaticum,* and *Fusarium boothii* from Wheat in Relation to Climate. *European Journal of Plant Pathology,* 139: 161-173

Bankole, S. A., Mabekoje, O. O. and Enikuomehin, O. A. (2005). *Fusarium moniliforme* and FBs B1 in stored maize from Ogun State, *Nigerian Journal of Tropical Sciences,* 43:76-79

Bau M., Castella, G., Bragulat, M. R. and Cabanes, F. J. (2006). RFLP characterization of *Aspergillus niger* aggregate species from grapes from Europe and Israel, *International Journal of Food Microbiology*, 111: 18-21

Bennett, J.W. (2010). An overview of the genus Aspergillus. *Molecular Biology and Genomics.* Caister Academic Press Limited, UK, 198 p.

Bennett, J.W. and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Review*, 16: 497-516

Bhaskara, R. M. V., Arul, J., Angers, P. and Couture, L. (1999). Chitosan treatment of wheat seeds induces resistance to *Fusarium graminearum* and improves seed quality. *Journal of Agriculture and Food Chemistry,* 47: 1208–1216

Bhat, R. V. and Vasanthi, S. (2003). Mycotoxin food safety risks in developing countries. Food Safety in Food Security and Food Trade. Vision 2020 for Food, *Agriculture and Environment Focus,* 10(3): 17–19

Bhat, R., Rai, R. V. and Karim, A. A. (2010). Mycotoxins in food and feed: Present status and future concerns, *Comprehensive Review of Food Science and Food Safety,* 9: 57-81

Bowers, J. H. and Locke, J. C. (2004). Effect of formulated plant extracts and oils on population density of *Phytophthora nicotianae* in soil and control of Phytophthora blight in the greenhouse. *Plant Disease*, 8(8): 11–16

Burger, Y., Jonas-Levi, A., Gurski, E., Horev, C. and Saar, U. (2010). Variation in antifungal activity in extracts from Momordica plants, *Israel Journal of Plant Sciences,* 58: 1-7

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods, a review. *International Journal of Food Microbiology*, 94: 223-253

Cai, Y. Z., Sun, M. and Zhang, Y. (2014). Antioxidant activities of betalins from plants of the Amaranthaceae*, Journal of Agriculture and Food Chemistry,* 51:2288-2294

Campbell, C. K., Johnson, E. M., Philpot, C. M. and Warnock, D. W. (1996). *Identification of Pathogenic Fungi,* London: Public Health Laboratory Service, 198P

Carratu, M. R., Cassano, T., Coluccia, A., Borracci, P. and Cuomo, V. (2012). Antinutritional effects of fumonisin B1 and pathophysiological consequences, *Toxicology Letter,* 141: 459-463

Chuang, P. H., Lee, C. W., Chou, J. Y., Murugan, M., Sheik, B. J. and Chen, H. M. (2007). Antifungal Activity of the Crude Extract and Essential oil of *Moringa oliefera* Lam. *Bioresources Technology.* 98: 232-236

Clinical and Laboratory Standard Institutes (CLSI) (2014): Document M39-A3. Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data. Approved Guideline, Third Edition. CLSI, 940 West Valley Road, Suite 1400; Wayne, Pennsylvania. USA.2588 p.

Cordeiro P. J, Vilegusj, H. and Lancasj, F. M. (1999): Chemical and biological investigations of *Dillenia indica* Linn. *Journal of Brazilian Chemical Society,* 10: 523-526.

Cosic, J., Vrandecic, K., Postic, J., Jurkovic, D. and Ravlic, M. (2010). In vitro antifungal activity of essential oils on growth of phytopathogenic fungi, *Europen Journal of Plant Pathology*, 16(2): 25-28

Covarelli, L., Stifano, S., Beccari, G., Raggi, L., Lattanzio, V. M. T., Albertini, E. (2012). Characterization of *Fusarium verticillioides* strains isolated from maize in Italy: Fumonisin production, pathogenicity and genetic variability. *Journal of Food Microbiology*, 31: 17–24

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12: 564-582

Cox, P. A. (1990). Ethnopharmacology and the search for new drugs. **In**: *Bioactive compounds from plants*. Chadwick D. J. and Marsh, J. (eds.), John Willey, Chichester, UK, 198 p.

Davide, F., Alessandro, R. and Robert, C. (2016). *Fusarium* toxins in cereals: Occurance, legislation, factors promoting the appearance and their management. *Molecules Review,* 4: 2-5

Din, L. B., Zakaria, Z., Samsudin, M. W., Brophy, M. and Toia, R. F. (1988). Composition of the steam volatile oil from *Hyptis suaveolens* Poit, *Pertanika*, 11: 239-242

Elegani, A. A., El-nima, M. S. and Muddathir, A. K. (2012). Antimicrobial activities of some species of the family Combretaceae, *Phytochemistry Research Journal,* 16: 551-561

Esquivel-Ferrino, P. C., Clemente-Soto, A. F., Ramirez-Cabriales, M. Y., Garza-Ganzalez, E., Alvares, L. and Camacho-Corona, M. R. (2014). Volatile constituents identified in hexane extract of *Citrus sinensis* pell and anti-mycobacterium tuberculosis activity of some of its constituents. *Journal of Mexican Chemical Society,* 5 (4), 431-434

Fawzi, E. M., Khalil, A. A. and Afifi, A. F. (2009). Antifungal effect of some plant extracts on *Alternaria alternata* and *Fusarium oxysporum*. *African Journal of Biotechnology,* 8(11): 2590–2597

Feliziani, E., Santini, M., Landi, L. and Romanazzi, G. (2013). Pre-and postharvest treatment with alternatives to synthetic fungicides to control postharvest decay of sweet cherry. *Postharvest Biological Technology*, 7(8): 133–138

Field, J. A. and Lettinga, G. (1992). *Toxicity of tannic compounds to microorganisms*. **In**: Hemingway RW, Lanks E (eds) Plant Polyphenols: synthesis, properties, significance. Plenum, New York, pp 673–692

Fog, N. K. (2000). Mould growth on building materials, secondary metabolites and biomarkers, PhD Thesis Bio centrum DTU Technicals, 51 p.

FRAC (2017). Fungicides resistance action committee, Cornell London, 85 p.

Fredlund, E., Gidlund, A., Sulyok, M., Borjesson, T., Kriska, R., Olsen, M. and Lindblad, M. (2013). Deos-nivalenol and other selected Fusarium toxins in Swedish oats occurance and correlation to specific *Fusarium* species. *International Journal of Food Microbiology.* 167: 276-283

Gato P., Vrhovsek U., Muth J., Segala C., Romualdi C., FontanaP., Pruefer D., Stefanini M., Moser C., Mattivi, F. and Velasco R., (2013). Ripening and geotype control stilbene accumulation in healthy grapes. *Journal Agriculture and Food Chemistry*, 56: 11773-11785

Gavaric, N., Mozina, S. S., Kladar, N. and Bozin, B. (2015). Chemical profile, antioxidant and antibacterial activity of thyme and oregano essential oils, thymol and carvacrol and their possible synergism. *Journal of Essential Oil Bearing Plants*, 18(4): 1013-1021.

Gbodi, T. A. and Nwude, N. (1998). Mycotoxicosis in domestic animals. *A Review of Veterinary Human Toxicology,* 30(3): 235-245

Gebore, J., Benhamou, N., Vallance, J., Le-floch, G., Grizard, D., Regnault-roger, C. and Rey, P. (2013). Biological control of plant pathogen advantage and limitation seen through

the case study of *Pythium oligandrum. Enviromental Science and Pollution Research,* 21(7): 1-14

Gnanamanickam, S. S. (2002). *Biological control of crop diseases*. Marcel Dekker Inc., New York, USA, 468 P.

Grayer, R. J. and Harborne, J. J. (1994). A Survey of antifungal compounds from higher plants, 1982-1993, *Phytochemistry,* 37: 19-42

Gugnani, H. C. (2003). Ecology and taxonomy of pathogenic aspergilli, *Frontiers of Biosciences*, 8: 346-357

Gurjar, M. S., Ali, S., Akhtar, M. and Singh, K. S. (2012). Efficacy of plant extracts in plant disease management. *Agricultural Sciences*, 3(3): 425-433

Halama, P. and Van Haluwin, C. (2004). Antifungal activity of lichen extracts and lichenic acids. *Biological Control,* 49: 95-107

Haorongbam, S., Elangbam, D. and Nirmala, C. (2009). Cyanogenic glucosides in juvenile edible shoots of some Indian bamboos. Paper presented at: 8th World Bamboo Conference, November, Bangkok, Thailand, pp: 12-18

Hu, Y., Liu, L., and Kmiec, E. B. (2003). Reduction of HTT inclusion formation in strains of *Saccharomyces cerevisiae* deficient in certain DNA repair functions: a statistical analysis of phenotype. *Experimental Cell Research.* 291(1):46-55

Huang, W., Li, Z., Niu, H., Li, L., Lin, W. and Yang, J. (2009): Utilization of acorn fringe for ellagic acid production by *Aspergillus oryzae* and *Endomyces fibuliger*. *Bioresources and Technology*, 8:100-101

Hussein, H. S. and Brasel, J. M. (2001): Review: Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology*, 167(2): 101–134.

Ibrahim, R., Abubakar, E. M. Modibbo, S. M. and Lamaran, B. G. (2017). Percentage yield and acute toxicity of plant extracts of *Ceiba pentandra* grown in Bauchi state, North Eastern part of Nigeria. *Journal of Pharmacognosy and Phytochemistry,* 6(5): 1777-1779

Isman, M. B. and Machial, C. M. (2006). Pesticides based on plant essential oils: from traditional practice to commercialization. **In:** Naturally occurring bioactive compounds. Rai, M. and Carpinella, M. C. (eds.). *Elsevier, Amsterdam, Netherlands,* Pp: 29-44

Jalili-Marandi, R., Hassani, A., Ghosta, Y., Abdollahi, A., Pirzad, A. and Sefidkon, F. (2010). *Thymus kotschyanus* and *Carum copticum* essential oils as botanical preservatives for table grape. *Journal of Medicinal Plants Research*, 4(22): 2424-2430.

Kelly, A. C., Clear, R. M., O'Donnell, K., McCormick, S., Turkington, T. K., Tekauz, A., Gilbert, J., Kistler, H. C. Busman, M., Ward, T.J. (2015): Diversity of Fusarium head blight populations and trichothecene toxin types reveals regional differences in pathogen composition and temporal dynamics, *Fungal Genetic and Biology Journal*, 82: 22–31

Kishore, K. G., Pande, S. and Harish, S. 2007. Evaluation of essential oils and their components for broad-spectrum antifungal activity and control of late leaf spot and crown rot diseases in peanut, *Plant Disease*, 91(4): 375–379.

Knudson, L. (1913). Tannic acid fermentation. *International Journal of Biological Chemistry.*, 14: 159–184

Kohl, J., De-Hass, B. H., Kastelein, P., Burgers, S. and Waawijk, C. (2007). Population dynamics of *Fusarium* spp. and *Microdochium nivale* in crops and crop residues of winter wheat. *Phytopathology,* 97: 971-978

Kokate, C. K., Purohit, A. P. and Gokhale, S. B. (2002). Textbook of pharmacognosy, *Nirali Prakasan*: Pune, 205 p.

Kouadio, J. H., Dano, S. D., Moukha, S., Mobio, T. A. and Creppy, E. E. (2007). Effects of combinations of *Fusarium* mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, {DNA} methylation and fragmentation, and viability in Caco-2 cells. *Toxicon,* 49:306–317

Krause, M. S., DeCeuster, T. J. J., Tiquia, S. M., Jr Michel, F. C., Madden, L.V. and Hoitin, H. A. J. (2003). Isolation and characterization of Rhizobacteria from compost that suppress the severity of Bacterial leaf spot of Radish. *Phytopathology*, 93: 1292-1300.

Kumar, K. V., Basu, S. and Rajendran, T. P. (2008): Mycotoxin Research and Mycoflora in Some Commercially Important Agricultural Commodities. *Crop Protection,* 27: 891-905

Kushiro, M. (2015). Historical review of researches on yellow rice and mycotoxigenic fungi adherent to rice in Japan, *Japanese Society of Mycotoxicology,* 65(1):19-23

Ladan, Z., Amupitan, J. O., Oyewale, A. O., Ayo, R. G., Temple, E. and Ladan, E. O. (2014). Phytochemical screening of the leaf extract of *Hyptis spicigera* plant. *African Journal of Pure and Applied Chemistry,* 8(5): 83-88

Ladan, Z., Anupitan, J. O., Oyele, A. O., Okonkwo, E. M., Ladan, E. O., Odjobo, B. and Habila, N. (2011): Chemical composition and biological activity of the volatile oil of *Hyptis spicigera* against *Trypanosome brucei brucei* found in Northern-Nigeria. *African Journal of Pure and Applied Chemistry,* 5 (4): 53-58

Langcake, P. and McCarthy, W. (1979). The relationship of resveratrol production to infection of grapevine leaves by *Botrytis cinerea* Vitis, *Molecules,* 18: 244–253.

Longrieco, A., Moretti, A., Perrone, G. and Mule, G. (2007). Biodiversity Complexes of mycotoxigenic fungal species associated with Fusarium Ear Rot of Maize and Aspergillu*s* Rot of Grape. *International Journal of Food Microbiology,* 119: 11-16

Longrieco, A., Mule, G., Moretti, A. and Bottalico, A. (2002). Taxogenic *Fusarium* species and mycotoxin associated with maize ear rot in Europe, *Journal of Plant Pathology,* 108: 597-609

Lougheed, M. D., Roos, J. O., Waddell, W. R. and Munt, P. W. (2018). Desquamative interstitial pneumonitis and diffuse alveolar damage in textile workers, *Chemistry,* 108: 1196-1200

Luongo, D., De Luna, R., Russo, R. and Severino L. (2008). Effects of four *Fusarium* toxins (fumonisin B_1, α-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation. *Toxicon,* 52:156–162

Mahmood, A. M. and Ameh, J. M. (2007*). In-vitro* antibacterial activity of *Parkia biglobosa* (Jacq) root, bark extract against some microorganisms associated with urinary tract infections. *African Journal of Biotechnology,* 6 (11): 195-200

Makkar, H. P., Siddhuraju, P. and Becker, K. (2007). *Methods of molecular biology:* Plant secondary metabolites, Totowa, Human Press, 310 p.

Makun, H. A., Gbodi, T. A., Akanya, H. O., Salako, E. A. and Ogbadu, G. H (2012): Health implications of toxigenic fungi found in two Nigerian staples: Sorghum and rice. *African Journal of Food Sciences,* 3 (9): 250 – 256

Marinova, D., Ribarova, F. and Atanassova, M. (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *Journal of University Chemistry and Technical Metallurgy*, 40 (3): 255–260

Masih, H., Peter, J. K. and Tripathi, P. A. (2014). Comparative evaluation of antifungal activity of medicinal plant extracts and chemical fungicides against four plant pathogens. *International Journal of Current Microbiology and Applied Sciences*, 3(5): 97-109

Mendoza, L., Espinoza, P., Urzúa, A., Vivanco, M. and Cotoras, M. (2009). *In vitro* antifungal activity of the diterpenoid 7α-Hidroxy-8(17)-labden-15-oic acid and its derivates against *Botrytis cinerea. Molecules*, 1(4): 1966–1979

Miller, J. D. (2001): Fungi and mycotoxins in grain: Implications for stored product research, *Journal of Stored Product Research*, 3(1): 1–16

Mohanta, T. K., Patra, J. K., Rath, S. K., Pal, D. K. and Thatoi, H. N. (2007). Evaluation of antimicrobial activities and phytochemical screening of oil and nuts of *Semicarpus anacardium* L., *Science Research Essentials,* 2 (11): 486-490.

Moss, M. O. (2002). Mycotoxin review-1. *Aspergillus* and *Penicillium, Mycologist*, 1(6): 116-119

Muhammad, S., Amusa, N.A., Suberu, H. A., Abubakar, A. and Magaji, M. D. (2001). The effects of soil amendment with sawdust and rice husks on the incidence of seedling blight caused by *Fusarium solani* and *Rhizoctonia solani* and the growth of *Parkia biglobosa* Moor., *Journal of Agricultural research*, 2: 40-47

Munkvold, G. P. (2003). Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. *European Journal of Plant Pathology,* 109 (7): 705-713

Naqvi, S. A. (2004) *Disease of Fruit and Vegetables.* Diagnosis and Management 1, Kluwer Academic Publishers. Neitherlands, 104 p.

Nashwa, S. M. A. and Abo-Elyousr, K. A. M. (2012). Evaluation of various plant extracts against the early blight disease of tomato plants under greenhouse and field conditions. *Plant Protection Science,* 48 (2): 74–79

NEARLS (2017). Prospect and Problem of the 1996 Cropping Season. A report of a Study Conducted by the National Agricultural Extension Research and Liaison Services (NEARLS) and Agricultural Planning Monitoring and Evaluation Unit (APMEU), 2-3 Oct. 2017. (NEARLS), Ahmadu Bello University, Zaria pp: 62.

Nester, E.W., Anderson, D.G., Robert, C.E., Persal, N. N., Nester, N. N. and Hurley, D. (2007). *Microbiology. A human perspective.* (Fourth Edition). Mc. Graw Hill Consp. Inc. 786 p.

Nganje, W. E., Bangsund, D. A., Leistritz, F. L., Wilson, W. W. and Tiapo, N. M. (2000). Regional Economic Impacts of *Fusarium* Head Blight in Wheat and Barley. *Applied Economic Perspective Policy,* 26(3): 332-347

Numpaque, M. A., Oviedo, L. A., Gil, J. H., García, C. M. and Durango, D. L. (2011). Thymol and carvacrol: biotransformation and antifungal activity against the plant pathogenic fungi *Colletotrichum acutatum* and *Botryodiplodia theobromae, Tropical Plant Pathology*, 36 (1): 3-13

Ojo, O. O., Nadro, M. S. and Tella, I. O (2006). Production of rats by extracts of some common Nigerian Trees Against Acetaminophen-Induced hepatotoxicity, *African Journal of Biotechnology,* 5(9):755-760

Okigbo, R. N. and Omodamiro, O. D. (2007). Antimicrobial effect of leaf extracts of Pigeon Pea (*Cajanus cajan* (L.) Millsp.) on some human pathogens. *Journal of Herbs Spices and Medicinal Plants,* 12(1-2): 117-127

Oliver, R. P. and Hewitt, H. G. (2014): Fungicides in crop protection. 2nd edition, CABI Publishers, 182 P.

Onifade, A. K. (2000). Antifungal effect of *Azadrachta indica* Juss extract on *Collectrichum linderthiamum. Global Journal of Pure and Applied Sciences,* 4: 32-34

Page, B. G. and Thomson, W. T. (2003): The 2003 newly revised insecticides, herbicides, fungicides quick guide. Kovak Books. Bakersfield,CA. 45 P.

Pamela, P., Mawejje, D. and Ugen, M. (2014). Severity of angular leaf spot and rust diseases on common beans in central Uganda. *Uganda Journal of Agricultural Sciences,* 15 (1): 63-72.

Parak, J. and Chanda, S. V. (2007*). In vitro* antimicrobial activity and phytochemical analyses of some Indian medicinal plants. *Turkish Journal of Biology,* 31: 53-58

Placinta, C., D'mello, J. P. and Macdonald, A. M. (1999). A Review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxin. *Animal Feed Science and Technology,* 78: 21-37

Prakash, O. Pune, L. and Raoof, M. A. (1989) Control of mango fruit decay with post-harvest application of various chemicals against black rot, stem end rot and anthracnose disease. *International Journal of Tropical Plant Diseases,* 6: 99-106

Rajeev, S., Pawan, K. V. and Gagandeep, S. (2012). Total phenolic, flavonoids and tannin contents in different extracts of *Artemisia absinthium. Journal of Complement and Medicinal Research,* 1(2):101–104

Rao, A., Zhang, Y., Muend, S. and Rao, R. (2010). Mechanism of antifungal acitivites of terpenoids phenols resembles calcium stress and inhibition of the TOR pathway. *American Society of Microbiology,* 10(4):50-60

Rey, P., Henhamou, E. and Tirilly, Y. (2018). *Phythium oligandrum* biological control. *Pakistan Journal of Science,* 10: 22-29

Riba, A., Bouras, N., Mokranea, S., Mathhieub, F., Lebrihi, A. and Sabaou, N. (2010). *Aspergillus* section flavi and aflatoxin in algeria wheat and derived products. *Food Chemical Toxicology,* 48: 2772-2777

Richard, J. (2007). *Effect of mycotoxin on immunity of animals, plants and microbial*, Rosemberg, ed. Peragmon press, New York, USA. 1012 p.

Romanazzi, G., Lichter, A., Gabbler, F. M. and Smilanick, J. L. (2012). Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. *Postharvest Biology and Technology,* 63:141–147

Santos, T. S., Marques, M. S., Menezes, I. A. C., Dias, K. S., Silva, A. B. L., Mello, I. C. M., Carvalho, A. C. S., Cavalcanti, S. C. H., Antoniolli, A. R. and Marçal, R. M. (2007). Antinociceptive effect and acute toxicity of the *Hyptis suaveolens* leaves aqueous extract on mice. *Fitoterapia*, 78: 333336-333343

Satish, S., Mohana, D. C., Raghavendra, M. P. and Raveesha, K. A. (2007). Antifungal activity of some plant extracts against important seed borne pathogens of Aspergillus spp., *An Journal of Agricultural Technology,* 3(1): 109-119

Scheuerell, S. and Mahaffee, W. (2002). Compost tea: Principles and prospects for plant disease control. *Compost Science and Utilization*, 10: 313-338

Schijlen, E. G., Ric, D., Vos, C. H., Tunen, A. J., and Bovy, A. G. (2004). Modification of flavonoid biosynthesis in crop plants, *Phytochemistry*, 6(5): 2631-2648

Serra, R., Braga, A. and Venancio, A. (2005). Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A, *Research in Microbiology,* 5(6): 515-521

Serra, R., Cabanes, J., Perrone, G., Kozakiewicz, Z., Castella, G., Venancio, A. and Mule, G. (2006). *Aspergillus ibericus:* a new species of the Section *Nigri* isolated from grapes, *Mycologia*, 8 (2): 295-306

Shamsa, F., Hamidreza, M., Rouhollah, G. and Mohammadreza, V. (2008). Spectrophotometric determination of total alkaloids in some Iranian medicinal plants, *Thailand journal of pharmaceutical sciences,* 32: 17-20

Shan, M., Fang, H., Wang, X., Feng, B. X., Chu, Q. and Yu, Y. L. (2006). Effect of chlorpyrifos on soil microbial populations and enzyme activities. *Journal of Environmental Sciences,* 8(1): 4–15

Sharief, M. D., Srinvasulu, A. and Uma, M. R. (2014). Estimation of alkaloids and total phenol in roots of *Derris trifoliate* L. and Evaluation for antibacterial and antioxidant activity. *Indian Journal of Applied Research,* 4 (5): 1-11

Sharma, R. R., Singh, D. and Singh, R. (2009): Biological control of fungi. *Molecules,* 50 (3): 205-221

Shin, M. H., Kim, J. H., Choi, H. W., Keum, Y. S. and Chun, S. C. 2014. Effect of Thymol and Linalool fumigation on postharvest diseases of table grapes. *Mycobiology*, 42(3): 262–268

Shukla, A. M., Yadav, R. S., Shashi, S. K. and Dikshit, A. (2012): Use of plant metabolites as an effective source for the management of post-harvest fungal pest: A review. *International Journal of Current Discoveries Innovation.* 1(1): 33-45

Sidibe, L., Chalchat, J. C., Garry, R. P. and Harama, M. (2001). Aromatic plants of Mali (III): Chemical composition of essential oils of two *Hyptis* species: *H. suaveolens* (L.) Poit. and *H. spicigera* Lam. *Journal of Essential Oil Research*, 13: 55-57

Singh, H., Fairs, G. and Syarhabil, M. (2011). Anti-fungal activity of *Capsicum frutescence* and *Zingiber officinale* against key post-harvest pathogens in citrus. *In International Conference on Biomedical Engineering and Technology*, 11: 1-7

Singh, V. K., Naresh, P., Biswas, S. K. and Singh, G. P. (2010): Efficacy of fungicides for management of wilt disease of lentils caused by *Fusarium oxysporum* f. sp. *lentis. Annals of Plant Protection Sciences,* 8(2):411-414

Smith J. E. and Moss, M. O. (1985). *Mycotoxins: formation, analysis and significance*. Wiley, Chichester, 348 p.

Strange, R. N. and Scott, P. R. (2005). Plant Diseases: A threat to global food security. *Annual Review of phytopathology,* 43 (1):83-116

Subhashini, S., Poonguzhali, T. V. and Madha, V. S. (2013). Quantitative phytochemical analyses of *Ecolium viride* (Forks) Merrill and *Justicia gandarussa* Burn. *International Journal of Current Research,* 2(1):34-37

Sultana, L., Anwar, R. and Ashraf, M. (2009). Effects of extraction solvent technique on the antioxidant of selected medicinal plants extracts, *Molecules,* 14: 2167-2180

Teles, F. and Seixas, J. (2015). The future of novel diagnostics in medical mycology. *Journal of Medical Microbiology*, 64(4): 315-22.

Tian, Y., Tan, Y. L., Liu, N., Liao, Y. C., Sun, C. P., Wang, S. X. and Wu, A. B. (2016). Functional agents to biologically control deoxynivalenol contamination in cereal grains, *Frontiers of Microbiology*, 7: 12-17

Tomlin, C. D. S. (2003). *The fungicide manual a world compendium*, 13ed. British Crop Protection Council, 135 P.

Tournas, V. H. and Katsoudas, E. (2005). Mould and yeast flora in fresh berries, grapes and citrus fruits. *International Journal of Food Microbiology,* 105: 11-17

Trease G. E. and Evans, W. C. (2009). *A textbook of Pharmacognosy*, 13, London Bacilliere Tinall Limited, pp. 234

Tyler, E. V., Brady, L. R. and Robbers, E. J. (2011). *Pharmacognosy* 9th edition. Published by Lippincott Williams and Wilkins, New York, London. 512 p.

Vander-lee, T., Zhang, H., Diepeningen, V. and Waalwijik, A. C. (2015). Biogeography of *Fusarium graminearum* species complex and chemotypes: A Review. *Food Additive Contamination,* 3(2): 453-460

Varga, J., Frisvad, J. C. and Samson, R. A. (2009): A reappraisal of fungi production aflatoxin. *World mycotoxin Journal,* 2: 263-277

Velderrain-Rodríguez, G. R., Palafox-Carlos, H., Wall-Medrano, A., AyalaZavala, J. F., Chen, C. Y. O., Robles-Sanchez, M., Astiazaran-García, H., Alvarez-Parrilla, E. and González-Aguilar, G. (2014): A Phenolic compounds: Their journey after intake. *Food and Function,* 5: 189–197

Veloz-Garcia, R., Marín-Martínez, R., Veloz-Rodríguez, R., Rodríguez-Guerra, R., Torres-Pacheco, I., Gonzalez-Chavira, M. M., Anaya-Lopez, J. L., Guevara-Olvera, L., Feregrino-Perez, A. A. and Loarca-Pina, G. (2010). Antimicrobial activities of cascalote (*Caesalpinia cacalaco*) phenolics-containing extract against fungus *Colletotrichum lindemuthianum. Industrial Crops and Production,* 31: 134–138

Villanueva B. D., Angelov, I., Vicente, G., Stateva, R. P., Rodriguez-GarciaRisco, M., Reglero, G., Ibañez, E. and Fornari, T. (2015). Extraction of thymol from different varieties of thyme plants using green solvents. *Journal of Science Food and Agriculture*, 95 (14): 2901-2907

Wang, B. and Jeffers, S. N. (2010). *Fusarium* root and crown rot: a disease of container-grown hostas, *Plant Disease,* 84: 980-988

Wang, B., Brubaker, C. L., Burdon, J. J. (2014). Fusarium species and Fusarium wilt pathogens associated with native *Gossypium* populations in Australia. *Mycological Research,* 108: 35-44.

Wang, H., Hawang, S. F., Chang, K. F., Turnbull, G. D. and Howard, R. J. (2004). Suppression of important pea diseases by bacterial antagonists. *Biological Control*, 48: 447-460

Woo, S. W., Shin, H. K. and Kang, S. S. (1980). Chemistry and pharmacognosy of flavone-C-glycosides from Ziziphus Seeds. *The Korean Journal of Pharmacognosy*, 11(3-4): 141-148

Woo, S. W., Shin, H. Y. and Kang, K. S. (1990). Chemical profile, isolation of phenols and flavonoids from *Ficus polita*. *The Korean Journal of Pharmacognosy*, 5(4): 231-244

Wuyep, P. A., Musa, H. D., Ezemokwe, G. C., Nyam, D. D. and Silagtang, M. D. (2017): Phytochemicals from *Agerantum conyzoids* L. extracts and their antifungal activities against virulent *Aspergillus* Spp. *Journal of Academia and Industrial Research,* 6: 32-39

Xu, X. M., Nicholson, P., Thomsett, M. A., Simpson, D., Cooke, B. M., Doohan, F. M., Brennan, J., Monaghan, S., Moretti, A. and Mule, G. (2008). Relationship between the fungal complex causing *Fusarium* head blight of wheat and environmental condition. *Phytopathology,* 98: 69-78

Yahaya, S. M., Abubakar, Y., Ali, M. U., Lawan, M. and Abba, H. (2018): Fungal infection of banana (*Musa sapientum*) sold at wudil and yanlemo market of Kano State, *Dutse Journal of Pure and Applied Sciences,* 4(1):254-262

Yiannikouris, A. and Jouany, J. P. (2014). Mycotoxins in feeds and their fate in animals: *A Review in Animal Research,* 51: 81-99

Yue, X., Abdallah, A. M. and Xu, Z. (2010). Distribution of isoflavones and antioxidant activities of soybean cotyledon, coat and germ. *Journal of Food Processing and Preservation*, 34: 795–806

Zaker, M. (2014). Antifungal evaluation of some plant extracts in controlling *Fusarium solani,* the causal agent of potato dry rot in vitro and in vivo. *International Journal of Agriculture and Biosciences,* 3(4): 190-195

Zaker, M. (2016). Natural products as eco-friendly fungicides for plant diseases control. A Review: *The Agriculturist,* 14(1): 134-141

Zhisen, J., Mengcheng, T. and Jiaming, Wu. (1999): The determination of flavonoids content in mulberry and their scavenging effect on superoxide radical, *Chemistry,* 64: 555-559

APPENDICES

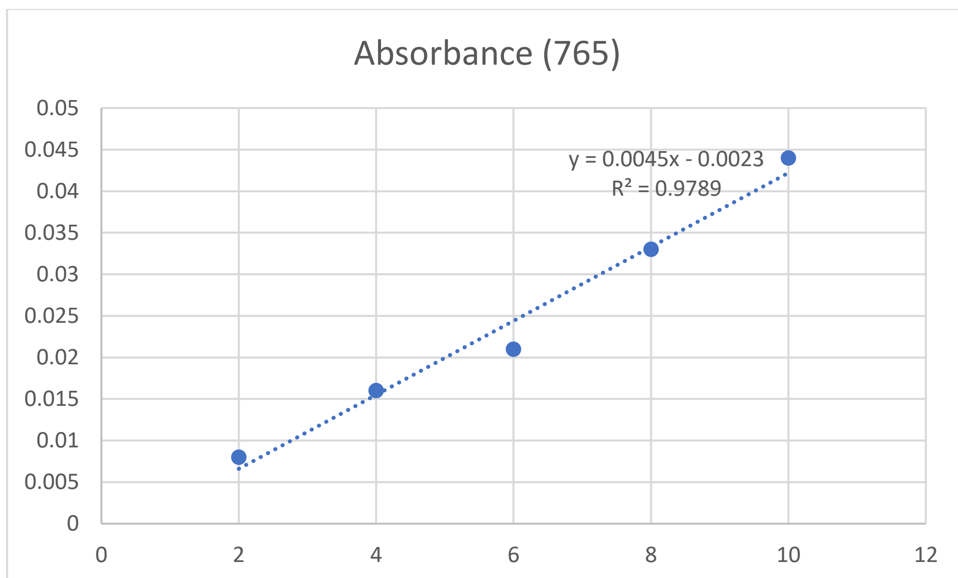
Appendix I: Fungicidal properties of some plant products

Name of plant products/Compounds	controlled pathogen	Reference
Acacia, Sapodilla, Datura *Eucalyptus*, pomegranate and Black plum Extracts	*Aspergillus candidus* *A. flavus, A. fumigatus, A. niger, A. ochraceus*	Satish *et al.* (2007)
Eucalyptus and *Lavender* extracts	*Alternaria alternate*	Zaker and Mosallaneja (2010)
Clove bud, cinnamon, Ginger Extracts	*Aspergillus niger* (2010)	Avasthi *et al.*
Neem, china berry, garlic and turmeric Extracts	*Fusarium oxysporum* *Rhizoctonia solani*	Hadian. (2012)
Kokum, wild turmeric and jasmine Extracts	*Rhizopus stolonifer*, *Collectotrichum coccodes*	Bhagwat and Datar (2014)
Artemisia, Thyme and *Eucalyptus* extracts	*Fusarium solani*	Zaker (2014)
Indian beech, milk weed And Oleander	Aspergillus *fumigatus*	Masih *et* al. (2014)

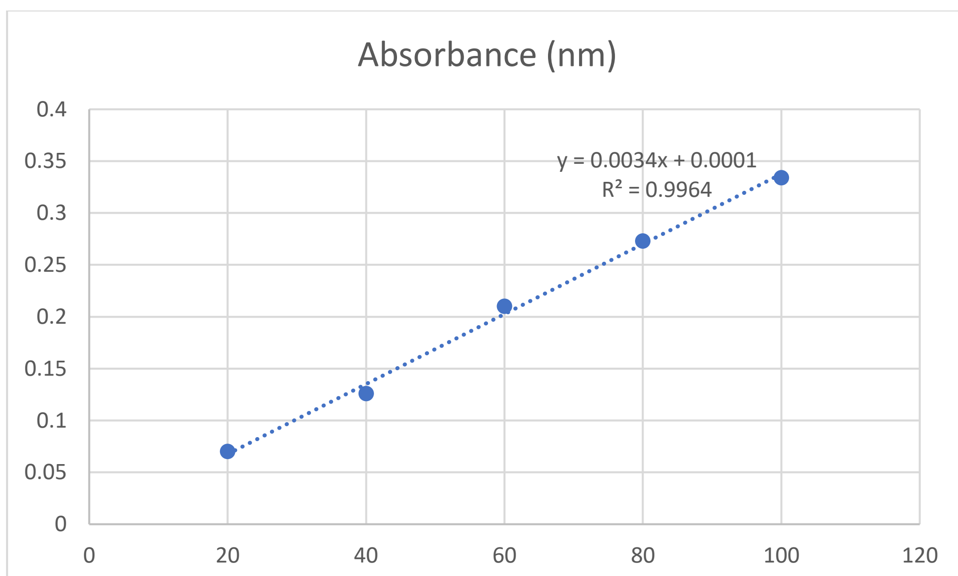
Appendix II: Mechanism of Action of phytochemicals/secondary metabolites from plants (Cowan, 1999)

Name of compounds	Mode of action
Simple phenols	Membrane disruption and substrate deprivation
Phenolic Acids	Bind to adhesions, form complex with cell wall and Inactive enzymes
Terpenoids	Membrane disruption
Essential oils	Membrane disruption
Alkaloids	Intercalate into the cell wall and cause disruption
Tannins	Bind to protein, enzyme inhibition and substrate deprivation
Flavonoids	Bind to adhesion, form complex with cell wall and inactivate enzymes
Coumarins	Interact with eukaryotic D.N.A
Lectins and polypeptides	Form Disulphide Bridges

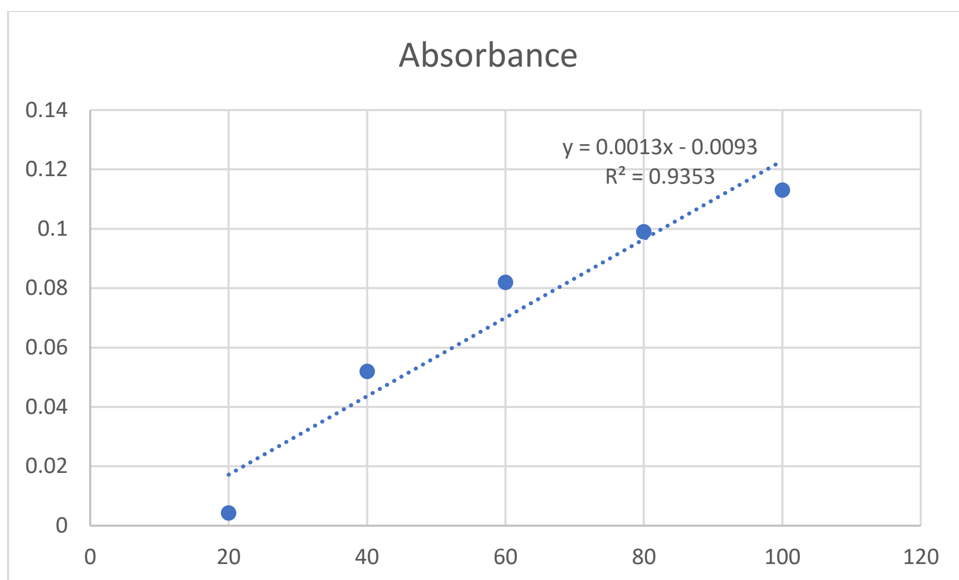
Appendix III: Standard curves used for Quantitative Analyses of Phytochemicals



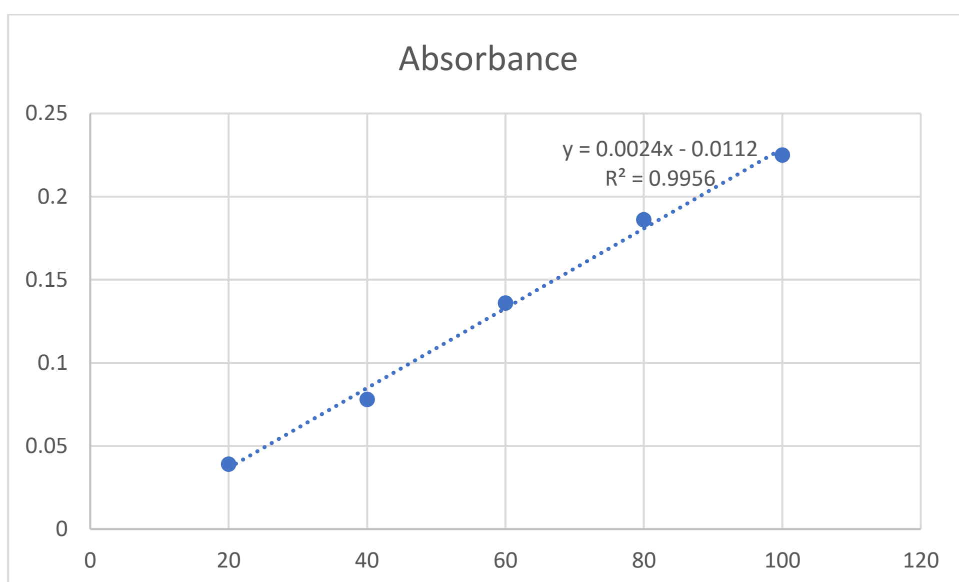
Appendix III a: Gallic acid standard curve



Appendix III b: Quercetin standard curve

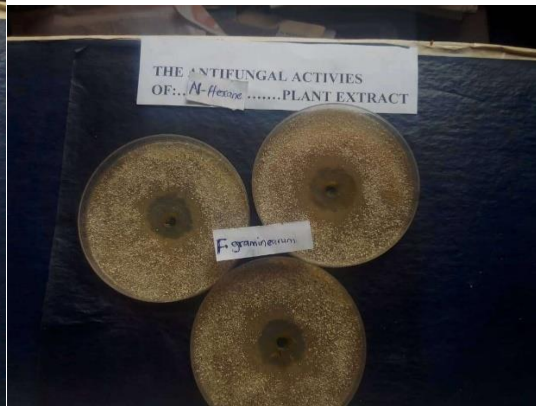


Appendix III c: Diosgenin standard curve

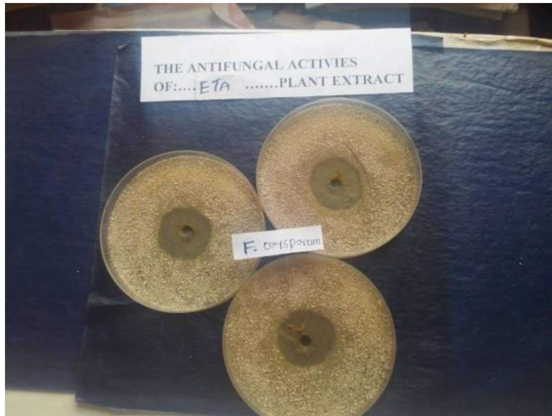


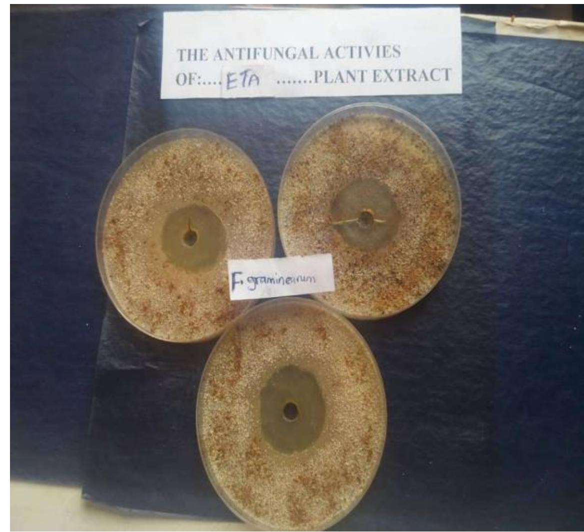
Appendix III d: Atropine standard curve

Appendix IV: Plates showing zones of inhibition of n-hexane leaf Extract on *Aspergillus* and *Fusarium* species

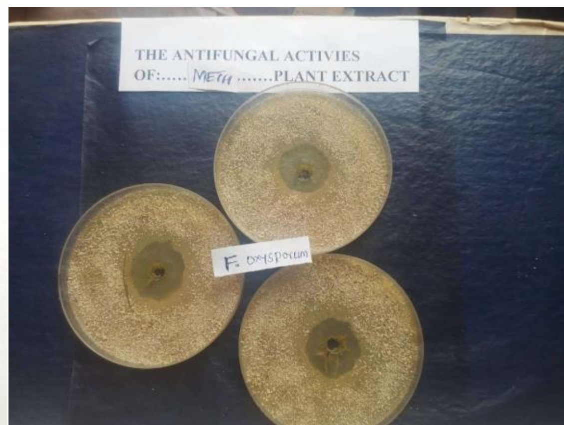


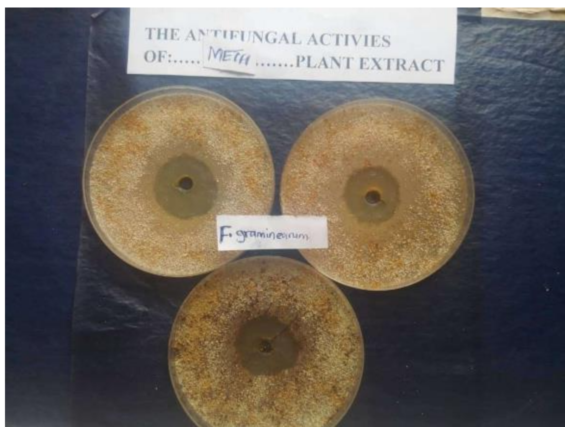
Appendix V: Plates showing zones of inhibition of Ethyl-acetate leaf Extract on *Aspergillus* and *Fusarium* species

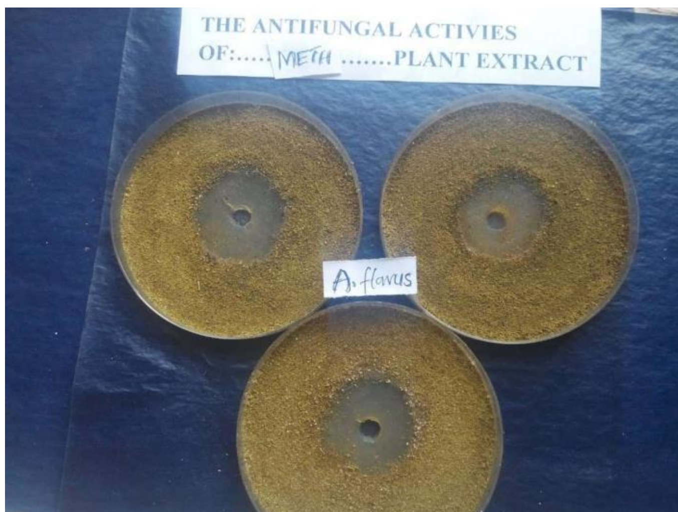




Appendix VI: Plates showing zones of inhibition of Methanol Leaf Extract on *Aspergillus* and *Fusarium* species







Appendix VII: Plates showing zones of inhibition of Aqueous Leaf Extract on *Aspergillus* and *Fusarium* species

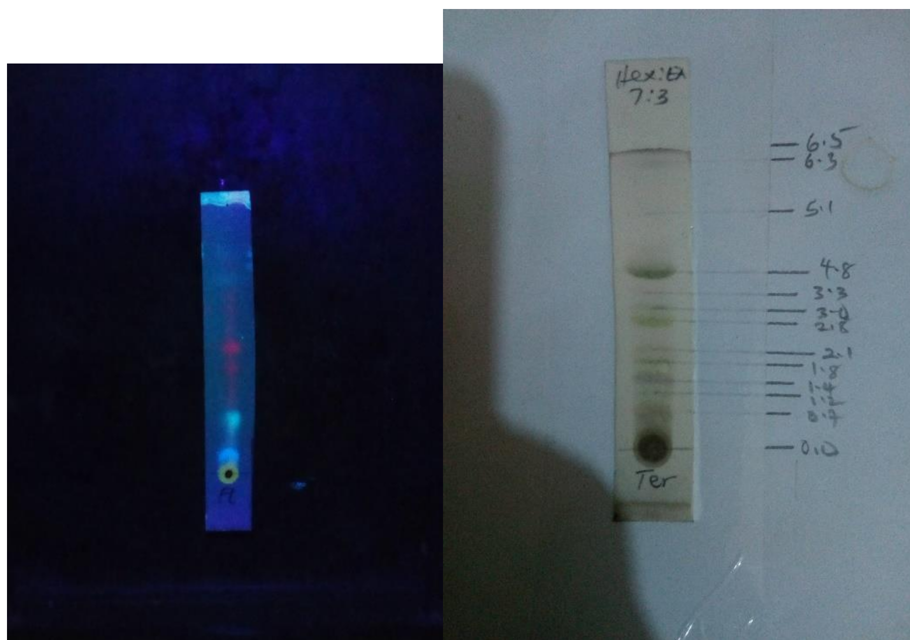
THE ANTIFUNGAL ACTIVIES
OF:.... X ......PLANT EXTRACT
1
2
3

THE ANTIFUNGAL ACTIVIES
OF:.... X ....PLANT EXTRACT
1
2
3
A. nigre

THE ANTIFUNGAL ACTIVIES
OF:..... X ....PLANT EXTRACT
1
2
3

THE ANTIFUNGAL ACTIVIES
OF:..... X ....PLANT EXTRACT
1
2
3

Appendix VIII: Plates showing the TLC chromatographic profile of flavonoids and terpenoids extracted from the methanol and ethyl-acetate leaves extract of *Hyptis spicigera* Lam.



Appendix IX: Isolation procedure for flavonoids and terpenoids from methanol and ethyl-acetate leaf extract of *Hyptis spicigera* Lam.