FUNGICIDAL EVALUATION OF *Rhizophora racemosa* AND *Ocimum gratissimum* EXTRACTS AND GREEN SYNTHESIZED SILVER NANOPARTICLES AGAINST MYCOTOXIGENIC FUNGI

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FEDERAL UNIVERSITY OF TECHNOLOGY,

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ABSTRACT

The presence of fungi in feed grains can produce secondary metabolites, including mycotoxins in the mold of target feedstuffs, which is known to pose the most serious threats to human and animal health and also cause significant economic losses. Due to the phytochemicals in plants, Botanicals present the prospect of substituting natural for chemical synthetic preservatives/pesticides which is more eco-friendly and furthermore silver nanoparticles (AgNPs) has the potential to provide solution to the problem of antibiotic resistance due to its mode of actions against microorganism. In the present study, the antifungal activity of hexane, ethyl acetate and methanol leaf extracts of *Ocimum gratisimmum* and *Racemosa rhyzophora* were tested against three important mycotoxin producing fungi; *Aspergillus niger, Aspergillus flavus* and *Fusarium verticilloides* isolated from contaminated groundnut seeds, maize-cob and maize seeds and morphologically identified. Concentration of extracts used were 3mg/ml, 6mg/ml and 10mg/ml, and the culture media without treatment served as negative control while the media treated with fluconazole served as the positive control. The poisoned food technique was used to determine antifungal activity and the percentage growth inhibition was measured. The three fractions of *R. rhyzophora* extracts had no significant ($p<0.05$) antifungal activity against *A. niger A. flavus* and *F. verticcilloides*. In the case of *O. gratissimum*, the ethyl acetate and methanol extracts showed significant ($p<0.05$) level of fungicidal effect against the three fungi and the antifungal activity occurred in a dose dependent manner, while n-hexane fraction of *O. gratissimum* showed no significant ($p<0.05$) antifungal activity. The AgNP was green synthesized using methanol and ethyl acetate extract as reducing and capping agent (average diameter/polydispersity index of 68.88nm/0.398 and 84.67nm/0.203 respectively) with peak wavelength of 405.5nm and 370nm respectively. The AgNP showed a significantly ($p<0.05$) higher antifungal activity compared to the plant extracts. Therefore methanol and ethyl acetate extract of *O.gratissimum* and even more potently the green AgNPs can be applicable in the development of more eco-friendly fungicidal formulations.

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2.0 INTRODUCTION

1.1 Background to the Study

In recent times, nutrition and food security have become two of the primary worries of human society, with an emphasis being placed on the quality of food. This is either because of the need for locally-produced items or due to the presence of artificial contaminants like pesticides, hormones, additives, and mycotoxins. Fungi within feed grains can bring about the formation of secondary metabolites, such as mycotoxins, which can contaminate food and feed. Even though the crops may seem alright, they might have a high amount of various types of fungi and their derivatives. The contamination of food and feed by mycotoxins is becoming a more pressing issue each day, leading to major health issues for both humans and animals, along with financial damages to countries (Cinar and Onbaşı, 2019; Balqees *et al*., 2020). The gravity of this problem is highlighted by the fact around 25% of the world's crops are affected by mycotoxin most of which is aflatoxin (Kumar *et al*., 2017)

Fungi secrete secondary metabolites known as mycotoxins, which are mainly produced by five genera: Aspergillus, Penicillium, Fusarium, Altenaria and Claviceps. Although there are many types of fungal toxins, a few of them are particularly pertinent to food safety. Mycotoxins have been found to negatively impact human and animal health (Puri *et al*., 2019). According to a study conducted by the World Health Organization, it was estimated that one-fourth of the world's grains and crops are contaminated by mycotoxins, which have been reported to be toxic to humans and animals (Galvano *et al*., 2001; Kumar *et al*., 2017). The most notorious mycotoxins are aflatoxins, fuminosins, trichothecenes, ocharatoxin A, cyclopiazonic acid, zearalenone, deoxyvalenol, citrinin, gliotoxin and

sterigmatocystin, all of which have been linked to cancer and other severe health risks (Reddy *et al*., 2010). These toxins can cause genotoxicity, teratogenicity and nephrotoxicity hepatotoxicity, reproductive disorders and immune suppression (Mohsenzadeh *et al.,* 2016; Desjardins *et al.,* 2000). On a cellular level, mycotoxins interact with nucleic acids and prevent the production of macromolecules such as DNA, RNA, and proteins, as well as the structural and functional components of biological membranes, leading to impaired energy metabolism (Diaz, 2005).

Over the last few years, the antimicrobial properties of plant extracts have been reported with increased regularity from different areas of the world (Khameneh *et al*, 2019). Several studies on medicinal plant extracts have shown resistance against phytopathogenic fungi in laboratory conditions (Oyedeji *et al*., 2011). Plants contain a range of phytochemicals including vitamins, alkaloids, flavonoids, terpenoids, carotenoids and coumarins, which are known to be helpful in defending themselves against bacteria, fungi, herbivores, insects and viruses (Saravanan, 2022). Because of their biodegradability and low toxicity, these phytochemicals are highly valued in food industry, and they offer the opportunity to replace natural preservatives and other products with synthetic ones (Hochma *et al*., 2021). Possible mechanisms that are thought to be responsible for the toxicity against fungi could involve different targets: interfering with the production of cell walls, changing cell permeability, interference with the transport of electron, the nutrient absorption, the adenosine triphosphatase and other metabolic processes of the cell, deactivation of various cellular enzymes and denaturation of cellular proteins (Al-Amiery *et al.,* 2012).

The tremendous growth in nanotechnology has opened a new-fangled window in biomedical science, physical science, biological science, and engineering. Metallic nanoparticles have received considerable attention because of their unique size and shape-

dependent properties that are of interest for applications such as catalysis, sensing, optics, and antibacterial materials. (Shahabadi *et al*., 2021) Among the large variety of metallic nanoparticles, silver nanoparticles (AgNPs) are one of the noble nanomaterials which have been known for their antimicrobial activity as reported in many studies. Study by Kaplan *et al*., (2021) showed AgNPs synthesized using *Coriolus versicolor* and *Boletus edulis* extract exhibited significant inhibitory effects against fungal strains of *Candida utilis*. In another study it was reported that rice leaf extract was utilized for the green synthesis of AgNPs and the antifungal activity of the synthesized NPs was tested against mycelium and sclerotia of *R. solani*, a fungus that causes sheath blight disease in rice and found that it inhibits the growth of the fungus and the growth inhibition is dependent on the concentration of the AgNPs (Kora *et al*., 2020). Other plant extract used in the biosynthesis of AgNP exhibiting antimicrobial activity include aqueous extract of *Azadirachta indica* leaves (Chinnasamy *et al.*, 2021), leaf extract of *Parkia biglandulosa* (Pb-AgNP) (John *et al*., 2021), *Echinophora platyloba* DC extract (Shahabadi *et al*., 2021).

Rhizophora racemosa belongs to the species of mangrove tree in the family Rhizophoraceae. It has a patchy distribution on the Pacific coast of Central and South America, occurs in places on the Atlantic coast of that continent, and has a more widespread range on the Atlantic coast of West Africa and also in the Niger Delta Region of Nigeria. Members of the genus *Rhizophora* are very similar to each other in morphology. *Rhizophora racemosa* is known by various names in different part of Nigeria some of the local names for it are as follows: Yoruba: Ogba; Edo; Odonowe; Itsekiri; Odo; Urhobo: Urhe Nwerim; Ijaw: Agala; Igbo: ngala (Arbonnier, 2004; Udeozo *et al.*, 2011).

Traditionally, different parts of *Ryzophora racemosa* has been used for therapeutic purposes for years. The bark is used to treat boils and fungal infections, an infusion of its leaf and bark is used to treat human microbial infections, diarrhoea, dysentery, fever, malaria, diabetes, snake bites, asthma, skin diseases, throat pains, intestinal worms, among others (Chiavaroli *et al*., 2020). However most of these traditional therapeutic applications have not been verified yet.

Even though Literature reports on this medicinal plant are currently very few, the antimicrobial potential of its fungal endophytes has been confirmed and the lethal dosage (LD50) of the methanolic leaf extract has been reported to be 1583.33 mg/kg which is considered safe for consumption. (Angalabiri-Owei & Isirima, 2014; Ariole & Akinduyite, 2016).

Ita and Eduok, (2022) reported that the ethylacetate and ethanol stem bark extracts of *Rhizophora racemosa* showed significant antioxidants and antifungal activity against some selected fungi. It has also been reported that the species *R. racemosa* from Australia contains primary and secondary metabolites, such as sugars and polysaccharides, amino acids as well as polyphenols, triterpenes and tannins. The phytochemical profile of water extract from the bark of *R. racemosa* includes saponins and terpenes and possesses anti-microbial activity against the phytopathogen fungus *Lasiodiplodia theobromae* (Ukoima *et al*., 2013). The presence of multiple classes of secondary metabolites was qualitatively confirmed (Udeozo *et al*., 2018)

Ocimum gratissimum is an herbaceous plant which belongs to the Labiatae family. The plant is indigenous to tropical areas such as West Africa, India and South America (Akara *et al*., 2021). In Nigeria it is cultivated in different parts of the country (Prabhu *et al.*, 2009). It is commonly known as scent leaf and also known by various names across the country such as "effinrin-nla" by the Yoruba speaking tribe in the southern part of

Nigeria, it is called “nchuawu” by the Igbos, while in the Northern part of Nigeria, the Hausas call it “Daidoya”.

Scientific reports have shown that *O. gratissimum* has a wide range of bioactive compounds such as flavonoids and polyphenols (Venuprasad *et al*., 2014; Irondi *et al*., 2016) and essential oils with beneficial effects (Melo *et al*., 2019). Furthermore, several studies have shown this plant possesses numerous pharmacological properties such as anti-hyperglycaemic (Casanova *et al*., 2014), hypoglycaemic (Shittu *et al*., 2019), anti-inflammatory (Ajayi *et al*., 2019), anti-anaemic, hepatoprotective (Akara *et al*., 2021), anti-hypertensive (Shaw *et al.*, 2017), antibacterial (Melo *et al*., 2019), antifungal (Mohr *et al*., 2017), and anti-oxidative properties (Mahapatra and Roy, 2014) as well as exhibits many other pharmacological activities.

1.2 Statement of the Research Problem

Contamination of food and feed by mycotoxin is a global concern and is generally considered to play important roles in food safety. Mycotoxins are well known for their health-hazardous effects in humans and animals; the main toxic effects are terratogenicity, nephrotoxicity, genotoxicity, hepatotoxicity, nephrotoxicity, reproductive disorders and immune suppression (Desjardins *et al.*, 2000) Mycotoxins can also be passed along in the food chain and contaminate milk, meat and eggs, posing a greater danger to the quality regulations of animal products (Bryden, 2007). As well as the health effect, it has also been a major cause of serious losses in nation’s economy (Cinar and Onbaşı, 2019). It is estimated that 25% of world’s food crop is contaminated by fungi-producing mycotoxins, causing huge losses in billions of dollars in domestic and international trade involving agricultural products (Kumar *et al*., 2017; Pankaj *et al*., 2018; Cinar and Onbaşı, 2019).

Chemical control of fungi and mycotoxins result in environmental pollution, health hazard and also affects the natural ecological balance (Yassin *et al.*, 2011; Mohammed *et al.,* 2012). Therefore Use of plant products in form of plant extracts in the control of fungi and its mycotoxin provides an opportunity to avoid synthetic chemical fungicide and its risks.

1.3 Aim and Objectives

1.3.1 Aim:

The aim of this study is to evaluate the inhibitory effects of different fractions of *Ocimum gratissimum* (scent leaf) and *Rizophora racemosa* (odo nowe) leaf extracts and plant-derived silver nanoparticle on growth of three mycotoxin producing fungi (*Aspergillus flavus, Fusarium verticilloides* and *Aspergillus niger*).

1.3.2 Objectives:

The objectives were to;

i. Isolate and Identify *Aspergillus flavus, Fusarium verticilloides* and *Aspergillus niger*

ii. Evaluate the fungicidal activity of *Ocimum gratissimum* and *Rhizophora racemosa* leaf extracts

iii. Bio-synthesize Silver nanoparticles using the extract fractions with significant anti-fungal activity.

iv. Characterize the Silver nano-particles synthesized.

v. Evaluate the fungicidal effect of the synthesized silver nanoparticles.

1.4 Justification for the Study

The development of safer antifungal agents, from plant products are recognized as one of the most promising alternative strategy in ensuring food safety (Varma and Dubey, 2001). Providing food security to devastatingly increasing population with limited natural resources along with destruction caused by pre- and post-harvest pathogens are the foremost concerns for developing countries like Nigeria. Numerous pesticides, herbicides and chemical fertilizers are being applied by the farmers to deal with the existing situation but leave very disastrous and undesirable after effects on ecosystem as non-degradable molecules. Botanicals can be utilized as an ecofriendly and effective alternative against chemical as they are of natural origin and acclaim less or non-toxic effects (Ikpeazu *et al*., 2018; Ijioma *et al*., 2021) and have also been safely used by the public for thousands of years as part of food.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mycotoxin, Food Safety and Plant

Mycotoxin-producing fungi are major contaminants and destroyers of agricultural products and seeds in the field, in storage, in processing and in markets, reducing their nutritional value (Jimoh and Kolapo, 2008). Mycotoxin contamination in food and feed poses a serious threat to animal and human health (Mokhles *et al.,* 2007; Iheshiulor *et al.,* 2011). Mycotoxins are commonly produced by species of *Aspergillus, Fusarium* and *Penicillium* (Maheshwar *et al.,* 2009). Various strategies are currently being employed to control fungal growth and mycotoxin biosynthesis in seeds, grains and feedstuffs through chemical treatments as well as physical and biological methods. These methods often require sophisticated equipment and expensive chemicals or reagents. The chemical control of fungi and mycotoxins also leads to environmental pollution, health hazards and affects the natural ecological balance (Yassin *et al.,* 2011). The use of herbal products in the form of plant extracts and essential oils offers the opportunity to avoid synthetic chemical preservatives and fungicide risks (Mohammed *et al.,* 2012).

Phytochemicals, a term for naturally occurring, non-nutritive, biologically active chemical compounds of plant origin, have some protective or disease-preventing properties. Some phytochemicals are harmful to fungi and could be used to protect crops, animals, humans, food and feed from toxigenic fungi and mycotoxins (Deresa and Diriba, 2023). Phytofungicides could be prepared or formulated from leaves, seeds, stem bark or roots of plants with pesticidal activity and could be applied in the form of extracts, powders and cakes or as plant exudates (Owino and Wando, 1992; Anjorin and Salako, 2009). Phytochemicals vary in plants depending on growing conditions, cultivar

differences, harvest age, extraction methods, storage conditions, and age of the sample. The use of plant derivatives for fungal control was common in developing countries before the advent of synthetic fungicides and because of the relative cost implications of imported fungicides (Galvano *et al.,* 2001). Efforts have been made over the years to search for new antifungal materials from natural sources for use in food preservation. (Juglal *et al.,* 2002; Onyeagba *et al.,* 2004; Boyraz and Ozcan, 2005). Several edible botanical extracts have been reported to have antifungal activity (Ferhout *et al.,* 1999; Pradeep *et al.,* 2003), for example *Allium sativum* has a broad antifungal spectrum and achieves approximately 60-82% inhibition of growth of seed-borne *Aspergillus* and *Penicillium* fungi. This has been attributed to the phytochemical compound of the garlic plant, namely allicin, which can break down into several potent antimicrobial compounds such as diallyl sulphide, diallyl disulphide, diallyl trisulphide, allyl methyl trisulphide, dithiins and ajoene (Salim 2011; Tagoe *et al*., 2011).

2.2 Green Synthesized Nanopartcles as Antimicrobial Agent

The usage of nanomaterials, synthesized via nanotechnology, has become part of the routine life of humans in the 21st century. Since the advent of nanotechnology in the late 1950s, the realization of its enormous potential has been actualized through a rapid increase in its applications in almost all domains of life, and in particular, health-related and biomedical fields (Kolahalam *et al*., 2019) Therefore, the green synthesis of various nanoparticles has been comprehensively deliberate to provide a more natural, safe and eco-friendly nanoparticles especially in biomedical and agricultural application. In the green bioprocess, plant, algal, fungal and cyanobacterial extract solutions have been utilized as nucleation/capping agents to develop effective nanomaterials for advanced medical applications. Metal oxides generally used as nanoparticles such as silver, iron

oxides, zinc oxides, manganese oxides, and copper oxides have been reported to have very potent antimicrobial effects. (Verma *et al*., 2023).

AgNPs synthesized with *Coriolus versicolor* (CV-AgNPs) and *Boletus edulis* (BE-AgNPs) synthesized by coupling AgNPs with extracts from *C. versicolor* and *B. edulis* mushrooms exhibited significant inhibitory effects in fungal strains of *Candida utilis* (Kaplan *et al*., 2021). Aqueous extract of *Azadirachta indica* (*AI*) leaves used as reducing and capping agent in the formation of nanoparticles from $AgNO_3$ (AI-AgNPs) demonstrated antibacterial activity against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* (Chinnasamy *et al.*, 2021). AgNPs green synthesized with leaf extract of *Parkia biglandulosa* (Pb-AgNP) serving as reducing and capping agent for Ag^+ reduction compared with AgNPs chemically synthesized (cAgNPs) with a reducing agent (sodium borohydride) showed that the green synthesized AgNPs (Pb-AgNPs) had significant increased antimicrobial activity than CAgNPs (John *et al*., 2021). In another study by Shahabadi *et al*., (2021), *Echinophora platyloba* DC extract used to green synthesize AgNPs displayed a remarkable antibacterial and antifungal activity against various microorganisms and was further used in conjugation with an antibiotic (Chloroxine) which displayed elevated efficacy. In another work, rice leaf extract was utilized for the biosynthesis of AgNPs and the antifungal activity of the synthesized NPs was tested against mycelium and sclerotia of *R. solani*, a fungus that causes sheath blight disease in rice and found that it inhibits the growth of the fungus and the growth inhibition is dependent on the concentration of the AgNPs (Kora *et al*., 2020)

2.3 *Ocimum gratissimum*

Ocimum gratissimum is a herbaceous plant which belongs to the Labiatae family. The plant is indigenous to tropical areas especially West Africa and also India. In Nigeria, it

is found in the Savannah and coastal areas. It is known by various names in different parts of the world. In the southern part of Nigeria, the plant is called “effinrin-nla” by the Yoruba speaking tribe. It is called “nchuawu” by the Igbos, while in the Northern part of Nigeria, the Hausas call it “Daidoya” (Effraim *et al.,* 2003)

2.3.1 Morphology

Ocimum gratissimum is a shrub up to 1.9m in height with branched stems. The leaves measure up to 10 x 5 cm, and are ovate to ovate-lanceolate, sub-acuminate to acuminate at apex, cuneate and decurrent at base with a coarsely crenate, serrate margin, pubscent and dotted on both the sides. The leaves show the presence of covering and glandular trichomes. Stomata are rare or absent on the upper surface while they are present on the lower surface. Ordinary trichomes are few, while the long ones up to 6-celled are present on the margins mostly; the short ones which are 2 celled, are mostly found on the lamina. Petioles are up to 6 cm long and racemes up to 18 cm long. The peduncles are densely pubescent. Calyx is up to 5mm long, campanulate and 5-7 mm long, greenish-white to greenish-yellow in colour. Nutlets are mucilaginous when they are wet (Prabhu *et al.,* 2009).

Plate I: *Ocimum gratissimum*

2.3.2 Traditional Uses

Ocimum gratissimum has been used extensively in the traditional system of medicine in many countries. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion (Rabelo *et al.,* 2003). In Africa, the plant has traditional importance ascondiments and for treating various diseases such as pyorrhea, bronchitis and dysentery and dried leaves are used in treating headache and fever. Igala community of Kogi State, Nigeria uses leaves and root in treating diabetes, gastro intestinal problems and gonorrhea and in Abia State leaf juice are used to treat malaria fever (Prabhu *et al.,* 2009). In South- In the coastal areas of Nigeria, the plant is used in the treatment of epilepsy, high fever and diarrhoea (Effraim *et al.,* 2003). In the Savannah areas decoctions of the leaves are used to treat mental illness (Akinmoladun *et al.,* 2007). *Ocimum gratissimum* is used by the Igbos of Southeastern Nigeria in the management of the baby's cord, to keep the wound surfaces sterile. It is also used in the treatment of fungal infections, fever, cold and catarrh (Ijeh *et al.,* 2005). Brazilian tropical forest inhabitants use a decoction of *Ocimum gratissimum* roots as a sedative for children (Cristiana *et al.,* 2006). People of Kenyan and sub Saharan African communities' use this plant for various purposes like viz., the leaves are rubbed between the palms and sniffed as a treatment for blocked nostrils, they are also used for abdominal pains, sore eyes, ear infections, coughs, barrenness, fever, convulsions, and tooth gargle, regulation of menstruation and as a cure for prolapse of the rectum (Matasyoh *et al.,* 2007). In India, the whole plant has been used for the treatment of sunstroke, headache, influenza, as a diaphoretic, antipyretic and for its anti-inflammatory activity (Taˆnia *et al.,* 2006).

2.4 *Rhizophora racemosa*

Rhizophora racemosa is a mangrove plants that belongs to the family Rhizophoraceae (mangrove family). It occurs naturally and dominates tropical tidal areas along both sides

of the Atlantic. In West Africa, estuaries, bays and lagoons are fringed by tidal mangrove forests, dominated by *Rhizophora* and *Avicennia*. When new mudflats are formed, seagrasses are the first plants that grow on the mud, with *Rhizophora racemosa*, a pioneering species, being the first mangrove to appear. With time, the mud solidifies and more tree and plant species arrive. On the seaward side the trees are short but get steadily taller further inland. (Hughes and Hughes, 1992). At one time considered to be a subspecies of *Rhizophora mangle*, *R. racemosa* is now accepted as a full species, most easily distinguished by the fact that the stem of the axillary flowers branches up to six times, making a maximum cluster size of 128. However, the inflorescences more usually contain 32 to 64 flowers. Other distinguishing characteristics include thick, short rounded bracteoles and rounded flower buds (Ellison *et al.*, 2010)

2.4.1 Morphology

Rhizophora racemosa are medium to tall trees. They may reach 30–50 m (100–160 ft) in height, often with aerial stilt roots, but in more marginal habitats are shorter, around 5–8 m (16–26 ft) more branched and scrubby. Stem diameters are about 15–35 cm (6–14 in) taken just above the highest prop root. This method of measurement differs fundamentally from the standard diameter at breast height (dbh) used for most forest surveys, as diameter height above the substrate varies from 0.5–7 m (1.5–23 ft) (Ellison *et al.*, 2010)

Leaves are opposite, simple, bright green, obovate, leathery, margins revolute, generally curved surface, obtuse blunt apex with a minute lip folded under. Cork wart spots occur on leaf undersurfaces, scattered evenly, not raised, reddish brown (may be distinguished from infections and wounds by their uniform cover). Note that non-spotted leaves are found on an unusual form of *R. racemosa* in northern Brazil. Leaf emergence occurs chiefly during November–February in the southern hemisphere and May–August in the

north. Leaf fall occurs chiefly over the wet summer period, October–February in the southern hemi-sphere and April–August in the northern hemisphere

Bark is smooth and red-brown in seaward and exposed locations (rocky and sandy substrates), to gray-fissured with smooth, red-brown prop roots in sheltered locations (mud substrates). There is total coverage of gray-fissured bark in some localities.

Fruits, when mature, are pear-like, elongate, waist constriction, smooth brown surface, calyx lobes elongate spreading (when the hypocotyl is ready to emerge). Mature fruit located in leaf

Plate II: ***Rhizophora racemosa***

2.4.2 Traditional uses

In West Africa, *Rhizophora racemosa* is used for construction poles and firewood on a limited scale. The smoke has antimicrobial properties and is also used for smoking meat. In the Americas it is less likely to be harvested as it is scarce and not considered to be of

much value (Ellison *et al.,* 2010). Extracts from the bark of *Rhizophora racemosa* has been proven for its potential as an indicator in titration. (Korfii *et al.,* 2021)

2.5 Fungicidal Plant

An emerging alternative to random chemical synthesis is the study and exploitation of naturally occurring products with fungicidal properties. Plants produce an enormous array of secondary metabolites, and it is commonly reasoned that a significant part of this chemical diversity serves to protect plants against plant pathogens. A problem with plant-produced compounds as potential fungicides is that in the natural state, they are generally only weakly active compared to synthetic fungicides. The antimicrobial and antitoxin properties of some plants, herbs, and their components have been documented since the late 19th century (Saadabi, 2006, Fawzi *et al*., 2009; Zaker and Mosallanejad 2010, Abdulghaffar *et al*., 2010; Abdel Ghany and Hakamy, 2014). Some of these natural plants involve garlic, lemon grass, *Datura, acacia*, a triplex, ginger, black seed, neem, basil, eucalyptus, *Juniperus procera*, alfalfa and basil (Omar and Abd-El-Halim, 1992; Aly *et al.,* 2000; Abdel Ghany, 2014; Noor, 2016).

Development of safer anti-fungal agents such as plant extracts to control phytopathogens in agriculture was reported in recent years (Tumen *et al.,* 2013). Extracts from plants such as garlic (*Allium sativum*) (Obagwu and Korsten, 2003), *Azadirachta indica*, *Moringa oleifera* (Adandonon *et al.*, 2006), *Ferula communis* and *Dittrichia viscose*, *Juniperus communis* (Menghani and Sharma, 2012) have been tested on many soil borne fungi. Alkhail, (2005) showed that extracts of *Allium sativum, Azadirachta indica* and *Eugenia caryophyllus* presented remarkable biological activity when tested against fungi viz., fusarium oxysporum.

2.6 Mycotoxins

The term mycotoxin was first used in the 1960s to describe the toxin associated with contaminated peanuts in animal feed and the loss of turkeys in England (Turkey-X-disease). This mycotoxin was later identified as the *Aspergillus flavus* toxin aflatoxin B1 (Ismaiel and Papenbrock, 2015).

mycotoxins are toxic secondary metabolites produced by fungi when they colonize foodstuffs (Pandey *et al*., 2023). Mycotoxins defined by Bennett and Bentley (Bennett and Bentley, 1989) as "metabolic intermediates or products, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism". The term was later applied to other toxic fungal natural products (Bennett, and Klich 2003).

Traditionally, toxigenic fungi contaminating agricultural grains have been conventionally divided into two groups those invade seed crops have been described as "field" fungi (e.g., *Cladosporium, Fusarium, Alternaria* spp.), which reputedly gain access to seeds during plant development, and "storage" fungi, (e.g., *Aspergillus; Penicillium* species), which proliferate during storage (Ismaiel and Papenbrock, 2015). Currently, this division is not so strict because according to Miller (Miller, 1995) four types of toxigenic fungi can be distinguished: (1) Plant pathogens as *Fusarium graminearum* and *Alternaria alternata*; (2) Fungi that grow and produce mycotoxins on senescent or stressed plants, e.g., *F. moniliforme* and *Aspergillus flavus*; (3) Fungi that initially colonize the plant and increase the feedstock's susceptibility to contamination after harvesting, e.g., *A. flavus*; (4) Fungi that are found on the soil or decaying plant material that occur on the developing kernels in the field and later proliferate in storage if conditions permit, e.g., *P. verrucosum* and *A. ochraceus*.

In terms of abundance and toxicology, the most important mycotoxins are produced by the *Penicillium, Aspergillus, Fusarium* as well as *Alternaria*. They grow normally at a degree of temperature 10 or 40 °C, in a pH range from 4 to 8, although these conditions can vary between fungi species (Bhat *et al.,* 2010). It is very important to note that one mycotoxin can be produced by different fungal species or one fungal species can produce several mycotoxins (Agriopoulou *et al*., 2020).

The most important mycotoxins produced by *Aspergillus* as well as *Penicillium* are ochratoxin A (OTA) and Aflatoxins (AFs), of which aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are the most prevalent. Otherwise, the most common Fusarium mycotoxins are fumonisins (FBs) – of which fumonisin B1 (FB1) and fumonisin B2 (FB2) are predominant – zearalenone (ZEA) and trichothecenes, of which the best known are deoxynivalenol (DON) and HT-2 and T-2 toxins (Habschied *et al*., 2021). These kinds of fungi are generally associated with the climate and crop stages of different geographical regions. The genera *Fusarium* and *Alternaria* are common in field contaminations, and *Penicillium* and *Aspergillus* are common to crop storage (Filtenborg *et al.,* 1996). Consequently, mycotoxins such as FBs and DON are mainly produced before harvest, and AFs and OTA are mainly produced during post-harvest stages (pre-harvest mycotoxins and post-harvest mycotoxins). However, depending on the producer fungus, they can appear in any crop and harvest stage and, depending on when they are produced, they can be reduced in various ways. Pre-harvest mycotoxins can be reduced by applying good agricultural practices (GAP), using control methods, developing resistant varieties of crops, using crop protection chemicals (Agriopoulou *et al*., 2020).

Contamination of food and feed by mycotoxin has been reported to be both direct and indirect (Tola and Kebede, 2016). Direct contamination explains a situation when food

and feed commodities are tainted with mycotoxin-producing fungus. A condition whereby toxigenic fungus is no longer in the contaminated food/ingredient used in the production of food but the toxin released are present due to their resistant to processing procedures is an indirect contamination (Alshannaq and Yu, 2017; Sokefun *et al*., 2018). This has led to a situation where the quality of such commodities were compromised causing negative effects on international trade (Dikhoba *et al*., 2019). This also poses a huge threat to human and animal health as the food crops are rejected for not meeting international trade standards are left for the average or low-income earners to consume. This is a common occurrence in developing countries like Nigeria, Ghana, Republic of Benin, Lesotho, Ethiopia, Kenya and many others. (Imade *et al*., 2021)

2.7 Fungi and type of Mycotoxin Produced

2.7.1. *Aspergillus* and *Penicillium* Mycotoxins

Aspergillus and *Penicillium* are capable of producing such mycotoxins as ochratoxins (OTA) and Aflatoxins (AFs), which have hazardous effects in humans as well as animals. OTA and AFs are considered to be the most toxic mycotoxins because they have carcinogenic and teratogenic effects (Omotayo *et al*., 2019)

2.7.1.1 Ochratoxin A

Ochratoxins A, B and C are secondary metabolites produced by filamentous species belonging to the genera *Penicillium* and *Aspergillus*. Ochratoxins A (OTA) is the most hazardous (Kőszegi and Poór, 2016). It was first isolated from A. ochraceus and chemically characterised in 1965 in corn meal samples (Van der Merwe *et al.,* 1965A; Van der Merwe *et al.,* 1965B). To date a wide range of *Aspergillus* species have been shown to produce OTA in foodstuffs, among others *A. niger* and *A. carbonarius* (Malir *et al.,* 2016 and El Khoury and Atoui, 2010). These toxigenic filamentous fungi

preferentially grow in hot and wet climatic conditions like South Asia, South America and Africa. Even so, in North Europe and North America two *Penicillium* species grow at low temperatures producing the OTA *P. verrucosum* and *P. nordicum* (El Khoury and Atoui, 2010; Denli and Perez, 2010). Hence, this mycotoxin can be found nearly all over the world and in a wide variety of matrices, principally in insufficiently dried cereals and cereal products, but also in wines, musts and grape juices (Belli *et al.*, 2002). They are also present in other commodities such as beer, pork coffee, peanuts, spices, cocoa, pulses, cow milk and cheeses Malir *et al.*, 2016; European Food Safety Authority (EFSA), 2006; Benkerroum, 2016).

OTA is a cyclic pentaketide and it is regarded as the second most important mycotoxin (Bhat *et al.*, 2016). One of the most important characteristics of this mycotoxin is that it is extremely stable to high temperatures and acidity. For this reason, cooking processes are not enough to completely remove OTA contamination from foodstuffs. This conclusion was drawn from previous research that observed that OTA did not fully degrade: heating wheat to 250 °C was not enough (Boudra *et al.*, 1995), roasting coffee reduced it only by 69% (Van der Stegen *et al.*, 2001) and baking at 200 °C under acidic conditions degraded it slowly but not fully (Trivedi *et al.*, 1992). Moreover, when OTA is consumed by pigs and other animals in contaminated feeds, it is rapidly absorbed and enters the systemic circulation, where it largely binds to plasma proteins, especially to albumin (Malir *et al.*, 2016). This is why OTA can be found in internal organs, predominantly in blood, kidneys and liver (European Food Safety Authority (EFSA), 2004), so it may be present in edible tissues and meat products. It is not excreted easily because of its solubility to fat. In the human body, OTA has been reported to have a long half-life of 35 days after ingestion (Studer-Rohr *et al.*, 2000), and *in vivo* experiments reveal that OTA accumulates in the kidneys, which is the cause of its related nephrotoxic

properties (EFSA, 2006), its main toxic effect. For this reason, kidneys are the main target organ of OTA (Pfohl-Leszkowicz and Manderville 2007). Apart from nephrotoxicity, exposure to OTA has also been related to carcinogenicity, teratogenicity, immunotoxicity and possibly neurotoxic properties (EFSA, 2006). The International Agency for Research on Cancer (IARC) has classified some compounds, such as mycotoxins, according to their carcinogenicity in humans and experimental animals (IARC, 1993).

2.7.1.2 Aflatoxins

Another important Aspergillus group of mycotoxins are (Aflatoxins) AFs. AFs are produced by the two species *A. parasiticus* and *A. flavus*. The word aflatoxin comes from the first letter "a" for Aspergillus, the following letters "fla" for the species flavus and the ending "toxin" (Ellis *et al.*, 1991). AFs were discovered at the end of the 1950s (Bennett and Klich, 2003) and are currently the most studied group of mycotoxins, with AFB1, AFB2, AFG1 and AFG2 being the most important of the approximately 20 AFs that have been chemically characterised (Loi *et al*., 2023). AFs have a difuranocoumarin chemical structure. They are distinguished and designated according to their fluorescence under ultraviolet light. Thus, AFB1 and AFB2 provide blue fluorescence and AFG1 and AFG2 provide green fluorescence (Schincaglia *et al*., 2023).

Aspergillus can grow on a wide variety of commodities and under several climatic conditions. Consequently, AFs are as widespread as OTA. Moreover, food can be contaminated by *A. parasiticus* and *A. flavus* during growth, harvest or storage, so it appears extremely unexpectedly. Furthermore, when the aflatoxigenic moulds have disappeared from the substrate, AFs may persist extensively. AFs are considered by IARC to be genotoxic and carcinogenic to humans (IARC, 1993). Of all AFs, AFB1 is the most common and also the most toxic, in both acute and chronic terms. The carcinogenicity of AFB1 has been well-established in several animal species, the liver being the primary

target organ (Kensler *et al.,* 2016). As a consequence, AFB1 is related to a high incidence of hepatocellular carcinoma and in regions with a greater exposure to AFs, the disease occurs more frequently (Bennett and Klich, 2003 and Kensler *et al.,* 2011). Some clinical manifestations such as vomiting, anorexia, gastrointestinal affections, pulmonary edema, depression, weight loss, haemorrhages and liver necrosis are related to AF exposure (EFSA, 2004 and Kensler *et al.,* 2011). Because of these toxic characteristics, there is no threshold dose below which consumption is safe. Consequently, TDI or NOAEL values cannot be suggested without risk.

2.7.2 *Fusarium* mycotoxins

The genus *Fusarium* is an ascomycete fungus, one of the most important genera of fungi and the most predominant toxin producer in cereals from the temperate regions of America, Europe and Asia and the development of toxicogenic *Fusarium* species can depend, to a large extent, on the geographical location of the fields, and particularly on the temperature and humidity typical to the region. (Bryła *et al*., 2022). Some species of *Fusarium* are widespread plant pathogens with toxic characteristics and feedgrain contamination is commonplace. Additionally, Fusarium mycotoxins usually appear together in contaminated matrices to produce cocontaminations. These toxigenic species produce cereal crop diseases that are complicated to control (D'Mello, 1999) and create serious problems for crops and the economy. The *F. graminearum* species has been the most widely studied since the Fusarium secondary metabolites responsible for mycotoxicoses were first identified and characterised at the beginning of the 1960s (Summerell and Leslie, 2011). Other common mycotoxin producing species are *F. verticillioides, F. culmorum* and *F. cerealis*. As has been mentioned above, Fusarium mycotoxins (fuminosin) are commonly present in crop fields.

2.7.2.1 Fumonisins

Fuminosin B1 (FB1) and Fuminosin B2 (FB2) are the most common FBs. One of the most representative characteristics of FB1 and FB2 is their long hydrocarbon chain which contributes to their toxicity (Hussein and Brasel, 2001). This chemical structure enables FBs to interact with molecular membranes and interfere with the sphingolipid metabolism (Dutton, 1996), because they have hydrophilic characteristics unlike most other known mycotoxins. This makes them more difficult to extract and detect. The main difference between FB1 and FB2 is the existence of a hydroxyl group in FB1. This makes FB1 the most toxic Fumonisin and it is classified as possibly carcinogenic to humans (group 2B) by the IARC. The effects of FBs ingestion on animals can range from brain lesions in horses to lung edema in swine (Summerell and Leslie, 2011). The animal species more sensitive to FB1 consumption are pigs and horses (EFSA, 2014). In humans, FBs are related to oesophageal cancer, especially in the case of FB1, although these connections have never been completely verified (Bennett and Klich 2003). For this reason, FBs are considered to be cancer promoters, but not mutagenic. Contaminations by FBs commonly come about during pre-harvest or at the beginning of storage (Marin *et al.,* 2013), and maize is the matrix in which almost all FB contaminations are produced (EFSA, 2014). Contamination levels can vary drastically between maize samples, especially between maize fractions intended for animal feed and raw maize (EFSA, 2014). Despite this high presence of FBs in maize samples, concentration levels do not increase during storage (Marin *et al.,* 2013), which makes it easier to control them.

2.7.2.2 Zearalenone

ZEA It is another mycotoxin produced by Fusarium fungi species and commonly found in maize samples. Its name is a collection of letters from different origins: “Zea-” comes from Gibberella zeae, which is the name of a producing organism that was the first to be

studied; resorcylic acid lactone ("-ral-") is the generic name for these natural products; and finally, "-ene-" and "-one" are the suffixes which indicate the existence of the C-1' to C-2' double bond and the C-6' ketone, respectively (Krska and Josephs, 2001). This structure is highly stable, so generally ZEA is not affected by cooking conditions (Marin *et al.,* 2013). But the most important characteristic of ZEA is that its chemical structure is similar to that of oestrogens (Gzyl-Malcher *et al.,* 2017). Thus, ZEA can interact and bind with plant cytoplasmic receptors for oestrogens and act as a plant hormone (Krska and Josephs, 2001; GzylMalcher *et al.,* 2017), or otherwise bind to the receptors from the membranes of animal cells and cause hyperestrogenism, which leads to reproductive and infertility problems (GzylMalcher *et al.,* 2017). For instance, swine are especially sensitive to ZEA and can become sterile if concentration levels are sufficiently high (Summerell and Leslie, 2011). Other clinical symptoms resulting from oestrogen alterations are retention or absence of milk and rectal prolapse in females, and lower testosterone levels and spermatogenesis in males (Marin *et al.,* 2013). Despite this, ZEA is not acutely toxic, and because of its lack of teratogenic and mutagenic activity it is not considered to be a human carcinogen by the IARC.

2.7.2.3 Trichothecenes

The compounds in group 3, which are not classified as carcinogenic to humans, also include trichothecenes. Native trichothecenes are classified as type A or type B according to their functional groups. Group A is characterised by a functional group other than a carbonyl in C-8 position, and group B is characterised by a carbonyl group in C-8 position. Thus, type A trichothecenes are less polar than type B trichothecenes. Hence, HT-2 and T2 toxins belong to the type A group, and DON, DON acetylated forms and nivalenol (NIV) to type B. Although the mycotoxins 3-acetyl-deoxynivalenol (3AcDON) and 15-acetyldeoxynivalenol (15AcDON) are modified forms, they are produced by fungi

and are also considered to be native trichothecenes (Birr *et al.,* 2021). Other DON secondary metabolites are not considered to be native and are discussed in the following section. Trichothecenes are sesquiterpenoids that can inhibit the synthesis of protein, RNA and DNA, what becomes cytotoxic (Hussein and Brasel, 2001). Of all the known trichothecenes, DON is the most common and is present in most of the cereal crops (wheat, maize, barley, oats and rye) and processed grains (malt, beer and bread) that have been studied (Birr *et al.,* 2021). DON is also known as vomitoxin since acute doses can cause vomiting. The chemical structure of DON, has a 12, 13 epoxide group which is largely responsible for its high toxicity. DON is soluble in water and in polar organic solvents and it is highly stable, which means that it can be stored long term and is stable to heat and UV light. Its stability also means that it can withstand several food processing methods, such as milling and heating (up to 350 °C), and enables it to stay in the food chain (Maresca, 2013). Some researchers have been studying the stability of DON and its derivatives through such cooking processes as baking, boiling, frying, steaming and extrusion (De Nijs *et al.,* 2016; Wu *et al*., 2017). The reduction in DON depended on pH, the length of cooking and temperature (higher temperatures do not involve greater reductions). Even though boiling provided the most effective degradation, it cannot be considered as a detoxification process. For this reason, DON needs to be stopped from emerging and controlled with regulation limits.

2.8 Mycotoxins Contamination of Food and Feeds in Nigeria

In sub-Saharan Africa (SSA), the prevalent mycotoxins of concerned affecting the health of human, animals and economy are aflatoxins (43.75%) followed by fumonisins (FUM, 21.87%), ochratoxin (12.5%), zearalenone (ZEN, 9.38%), deoxynivalenol (DON, 6.25%), beauvericin (BEA, 6.25%) (Darwish *et al*. 2014), while others constitute 3.13% (Figure 1). Globally in 2019, data from January to December showed the most prevalent

mycotoxins were FUM (70%) and DON (68%) (Biomin, 2020). A 10- year survey by Gruber-Dorninger *et al*. 2019 in SSA, reviewed an increased shift in prevalence in Fumonisins (72.6%); ZEN (52.2%) and DON (49.5%) amidst other emerging mycotoxins (Ladeira *et al*. 2017; Chilaka *et al*. 2018b; Ojochenemi *et al*. 2019; Ikeagwulonu *et al*. 2020; Kebede *et al*. 2020).

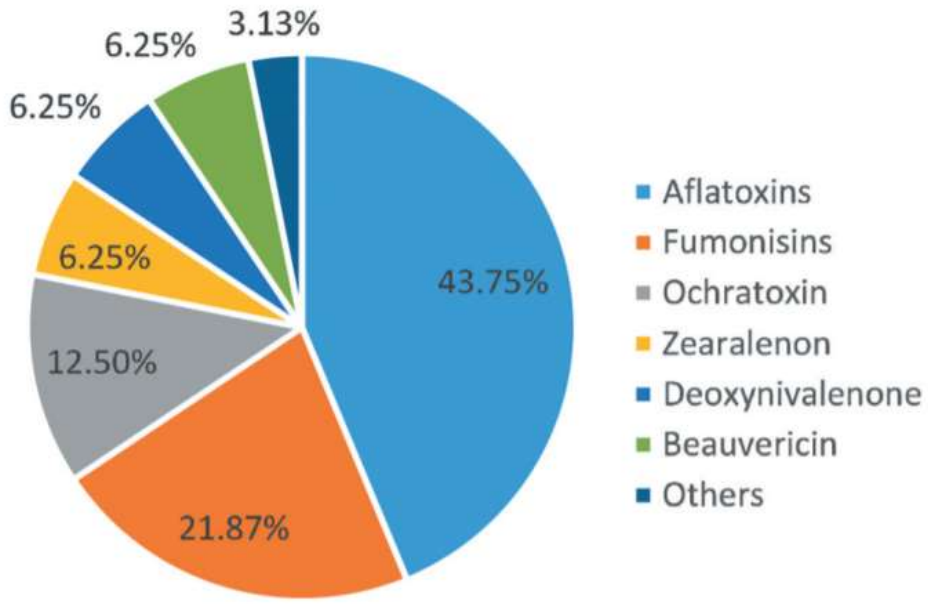


Figure 2.1 Percentage of contamination rate of concern mycotoxin in sub-Saharan Africa. (Imade *et al.*, 2021)

2.9 Nanoparticles and Green Synthesis

Nanotechnology is the science that deals with matter at the scale of 1 billionth of a meter (i.e., 10^{-9} m = 1 nm), and is also the study of manipulating matter at the atomic and molecular scale. A nanoparticle is the most fundamental component in the fabrication of a nanostructure, and is far smaller than the world of everyday objects that are described by Newton ' s laws of motion, but bigger than an atom or a simple molecule that are governed by quantum mechanics (Vert *et al.*, 2012)

Nanomaterials can be obtained either naturally or incidentally or can be manufactured. The particles with one or more peripheral dimensions in the size ranging from 1 to 100 nm are referred to as nanoparticles, which exist in unbound state or as an aggregate or agglomerate. Nanoparticles have various applications in the different fields of science and technology, including electronics to structural engineering and agriculture to medicine. Materials at the nanoscale do not exhibit the properties of the bulk material. The qualitative differences in material behavior at the nanoscale are attributable to the quantum mechanical effects that bring about new physical and chemical characteristics and large surface-to-volume ratio of the nanoparticles (Tadeusz *et al.,* 2014).

High demands have led to the large-scale production of nanoparticles. Therefore, a broad range of industrial methods have been developed to synthesize metal nanoparticles. However, some of these methods use toxic solvents or high energy, which has led to a growing awareness regarding the necessity of using clean, nontoxic and environmental-friendly methods to synthesize nanoparticles. Currently, the top-down and bottom-up approaches are the two major approaches used for synthesizing nanoparticles. In the top-down approach, the nanoparticles are produced by breaking down its bulk material. However, in the bottom-up approach, the nanoparticles are synthesized through chemical reactions among atoms and/or molecules (Fendler 1998). In general, the nanoparticles can be synthesized primarily by using physical and chemical methods. In addition, the biosynthesis of nanoparticles, which is also known as green synthesis, is also used because of its advantages of reduced toxicity and costs over the other two methods. Often, chemical synthesis of nanoparticles may lead to the existence of some toxic chemical traces adsorbed onto the surface of nanoparticles that may have adverse effects when applied in the biomedical field (Parashar *et al.,* 2009); however, this can be avoided by using biosynthesis of nanoparticles.

The various methods for nanoparticle synthesis are categorized mainly under physical, chemical, and biological synthesis. Each of these methods has its advantages and disadvantages in terms of yield potential, scalability, particle size and distribution, shape uniformity, and costs involved.

In the recent years controllable synthesis of noble metal nanoparticles has attracted much attention due to their potential applications in many areas (Yang and Cui, 2008). They have been extensively exploited for use in biomedical areas, such as targeted drug delivery (Jayanth and Vinod, 2003), imaging (Ji-Ho *et al.,* 2009), sensing (Yıldız and Ibtisam, 2010) and antimicrobial activity (Pallab *et al.,* 2008). Among these metal nanoparticles, silver nanoparticles have attracted intensive research interest because of their important applications as antimicrobial, catalytic, and antifungal activity (Jayanth and Vinod, 2003; Yang and Cui, 2008; Ji-Ho *et al.*, 2009). Silver has been used as an antimicrobial agent for centuries, the recent resurgence in interest for this element particularly focuses on the increasing threat of antibiotic resistance, caused by the abuse of antibiotics (Pallab *et al.*, 2008; Yıldız and Ibtisam, 2010). It is generally recognized that silver nanoparticles may attach to the cell wall, thus disturbing cell-wall permeability and cellular respiration. The nanoparticles may also penetrate inside the cell causing damage by interacting with phosphorus- and sulfur containing compounds such as DNA and protein (Yogeswari *et al.,* 2011). Another possible contribution to the bactericidal properties of silver nanoparticles is the release of silver ions from particles (Pallab *et al.,* 2008). Ag nanoparticles can be successfully synthesized traditionally by chemical and physical methods. However, these methods strongly depend on severe reaction conditions, for example, aggressive agents (sodium borohydride, hydrazinium hydroxide, cetyltriethylammnonium bromide), harmful solvent system to environment and ecology, higher temperature and higher pressure, and so on. To pursue a healthy life

and space, it is imperative to develop a clean synthetic approach ("green chemistry") to obtain nanomaterials targeted on different applications, especially in biomedical fields.

2.9.1 Green synthesis of nanoparticles

In the natural environment, various processes are available for the synthesis of nano- and micro-scaled materials that have contributed to the development of this relatively new and largely unexplored area of research on the biosynthesis of nanomaterials (Mohanpuri *et al.*, 2008; Ying *et al*., 2022). Owing to the high costs and toxicity of physical and chemical methods, the demand for green synthesis of nanoparticles has risen dramatically. Therefore, in search of cheaper alternatives, researchers have started using biological components/molecules that act as reducing agents, including microorganisms, biomolecules, and extending to plants and plant extracts. The biosynthesis method follows the bottom-up approach and involves either reduction or oxidation reactions. The reducing or antioxidant properties of microbial enzymes (Ahmad *et al.,* 2002; Jang *et al.,* 2012) or the plant phytochemicals (Shukla *et al.,* 2008; Lee *et al.*, 2014) are typically responsible for the reduction of metals into their respective nanoparticles. The success of green synthesis depends on the choice of the solvent medium, eco-friendly reducing agent and nontoxic stabilization material used. The majority of the synthetic methods (physical and chemical) depend highly on organic solvents due to the hydrophobicity of capping agents (Shukla *et al.,* 2003). However, in green synthesis, reducing and stabilization agents are generally present within the bioextracts (Inbakandan *et al.*, 2010; Nellorea *et al.,* 2012). In the case of silver nanoparticles, the components present in the bio-extract act as reducing agents to reduce silver ions formed from precursors to silver nanoparticles (Figure 2.2).

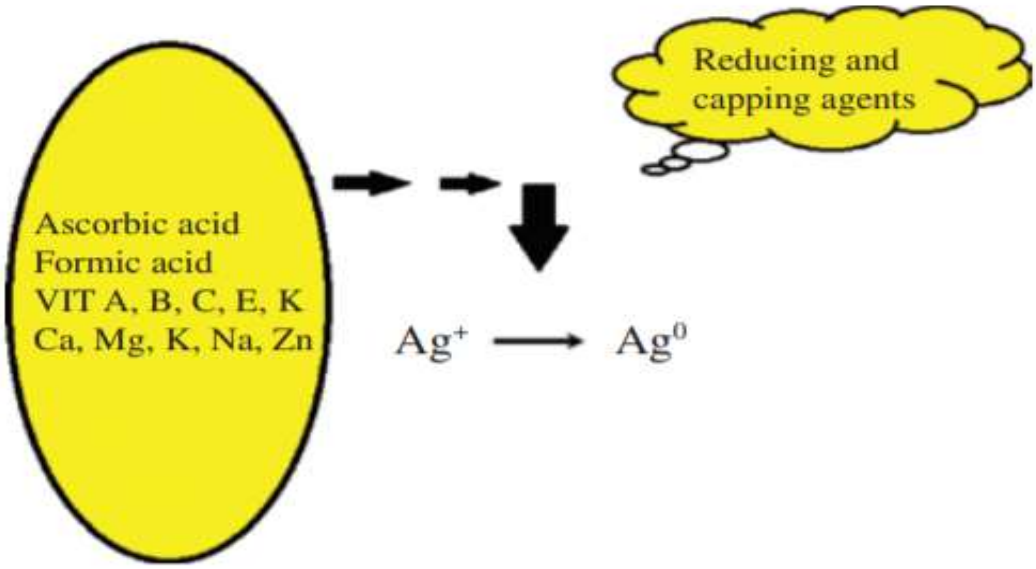


Figure 2.2 Mechanism for the formation of silver nanoparticles (Tejaswi *et al.*, 2013)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Seed samples of maize, groundnut and maize cob were obtained from local farmers in Minna and brought to the laboratory to monitor and harvest fungi isolates from them. Potato Dextrose Agar (PDA) was used as culture medium for the entire study. Solvents used for plant extraction were n-hexane, ethylacetate, and methanol. Filter paper, petridishes, conical flask, culture tubes, measuring cylinder, glass rod, beaker, broken glass beads, Dettol antiseptic, alcohol sanitizer, parafilm. Auto clave, Microscope, benchtop freeze dryer, spectrophotometer, laminar airflow, incubator, digital balance were all used at different stages in the study.

3.2 Preparation of Plant Extracts

3.2.1 Plant Collection

The two plants used were *Ocimum gratissimum* and *Rhizophora racemosa*. The former was collected in Minna, Niger state, while the latter was collected in Lagos state, Nigeria. The leaves collected were healthy, uninfected and washed under running tap water to remove any traces of soil particles and other dirt, cut into small pieces, dried for 15 days under shade in the laboratory. The leaves were then pulverized and sieved to get fine powder. Cold, Exhaustive extraction was done by multiple maceration using three solvents: n-Hexane, Ethyl acetate and Methanol successively to obtain 3 fractions of the extract.

3.2.2 Extraction of Plant Leaf

One hundred and fifty grams of the powdered leaves was added to 750 ml (1:5) of 100% N-hexane in a 1000 ml conical flask, sealed foil paper and thoroughly stirred at intervals, filtered after 24 hours with sterile 150 mm diameter whatman filter paper and the marc was used to repeat the process with fresh n-hexane 3 times and the filtrate were collected while the marc was allowed to dry under room temperature. The entire procedure was repeated for ethyl acetate and methanol solvents respectively. The filtrate for each solvent was allowed to dry up in fume cupboard under room temperature to obtain the extract. The procedure was applied for the extraction of *Ocimmum gratissimum* and *Rhyzophora racemosa* used for this study.

3.3 Isolation and Identification of Fungi

The fungi were isolated from groundnut, and maize. Visibly contaminated stored maize and groundnuts were collected and used for isolation.

Identification of fungi was based on the growth patterns, colour of mycelia and microscopic examinations of vegetative and reproductive morphological characteristics of conidia and mycelia of the fungi that were isolated compared with the standard contained in literature (Bhadauria and Guatam, 2009).

The standard protocol (Hocking *et al.,* 2006) recommended by the International Commission on Food Mycology (ICFM), was used for isolation and purification of fungal species. This technique involves several steps:

3.3.1 Surface Disinfection: Surface disinfection removes the inevitable surface contamination arising from dust and other sources and permits recovery of the fungi

actually growing in the grains. This process provides an effective measure of inherent mycological quality.

Whole grains were completely immersed in 0.4% excess sodium hypochlorite (NaOCl) solution for about two minutes, with occasional stirring. The grains were then drained and rinsed with sterile distilled water. This procedure was repeated thrice, each time with fresh NaOCl solution.

3.3.2 Plating: Potato Dextrose Agar (PDA) enriched with Chloramphenicol was used. Chloramphenicol, being an antibiotic, will inhibit the growth of bacteria in the medium. 10 disinfected surface sterilized grains were placed spaciously on Petri dishes containing solidified medium.

3.3.3 Incubation: Petri plates were incubated upright at 28±2°C for 5-10 days. To reduce evaporation, the dishes were sealed with parafilm. Distinct colonies representing different species were sampled for sub-culturing and eventual purification.

The microscopic examination was done by wet mount using 1% lactophenol blue stain, then viewed under x10 and x40 objective lens which was then compared with the vegetative and reproductive Morphological characteristics of conidia and mycelia of existing taxon for identification.

3.4 Maintenance of Pure Culture on PDA Media

The isolated and identified fungi were maintained using methods adopted by Mukul and Nilesh (2020). The fungi were sub-cultured differently on PDA slants, incubated at 28± 2^{o}C and allowed to grow for 15days. Such slants were preserved in a refrigerator at 5-10^{o}C and renewed once in a month. This pure culture was used for subsequent studies.

3.5 Organisms/inocula and Preparation of Fungal Spores

The fungi strains used in this study were *Aspergillus flavus, Fusarium verticilloides* and *Aspergillus niger,* All cultures were maintained on potato dextrose agar (PDA) slants, as previously reported (Mukul and Nilesh, 2020). The strains were cultivated on PDA slants and incubated at room temperature (28 ± 2^0C) until they sporulate in 7 days. Spores were harvested by adding 10 ml of sterile distilled water containing 0.05% Tween 20 and scraping the surface of the culture using glass bits to dislodge the spores and a suspension with 106 spores/ml was obtained.

3.6 Synthesis and Characterization of Silver Nanoparticles.

Method used by Rautela *et al*. (2019) was adopted. A 2mM silver nitrate solution was prepared by dissolving 1.70g of $AgNO_3$ in 500 mL of distilled water. Silver nitrate and extract solution were mixed together in a ratio of 1:9 in a beaker, with beaker, then covered with an aluminum foil. Sunlight was used to photo catalyze the reducing action of biological agent and accelerate the synthesis of the silver nanoparticle (AgNP) as reported by Srikar *et al.*, (2016) and changes in the solution color were observed after 2 hours . After 24h, the solution was centrifuged at 15000 rpm for 15 min, the supernatant was transferred to a clean dry beaker for further settlement of particles and repeated centrifugation was carried to purify AgNPs. The sample obtained was lyophilized and stored at 4°C.

Silver ion reduction in colloidal solution was checked on UV-Visible spectroscopy to confirm the synthesis of silver nanoparticles. For this purpose, small sample amounts were taken and analyzed between wavelengths of 300 and 700 nm after 2hours in a UV-Visible Spectrometer (Shimadzu UV 1800-series). Then, particle size analyzer (Zetasize

ver.7.01, Malvern Instruments Ltd.) laser was used to analyze the particle size of silver nanoparticles.

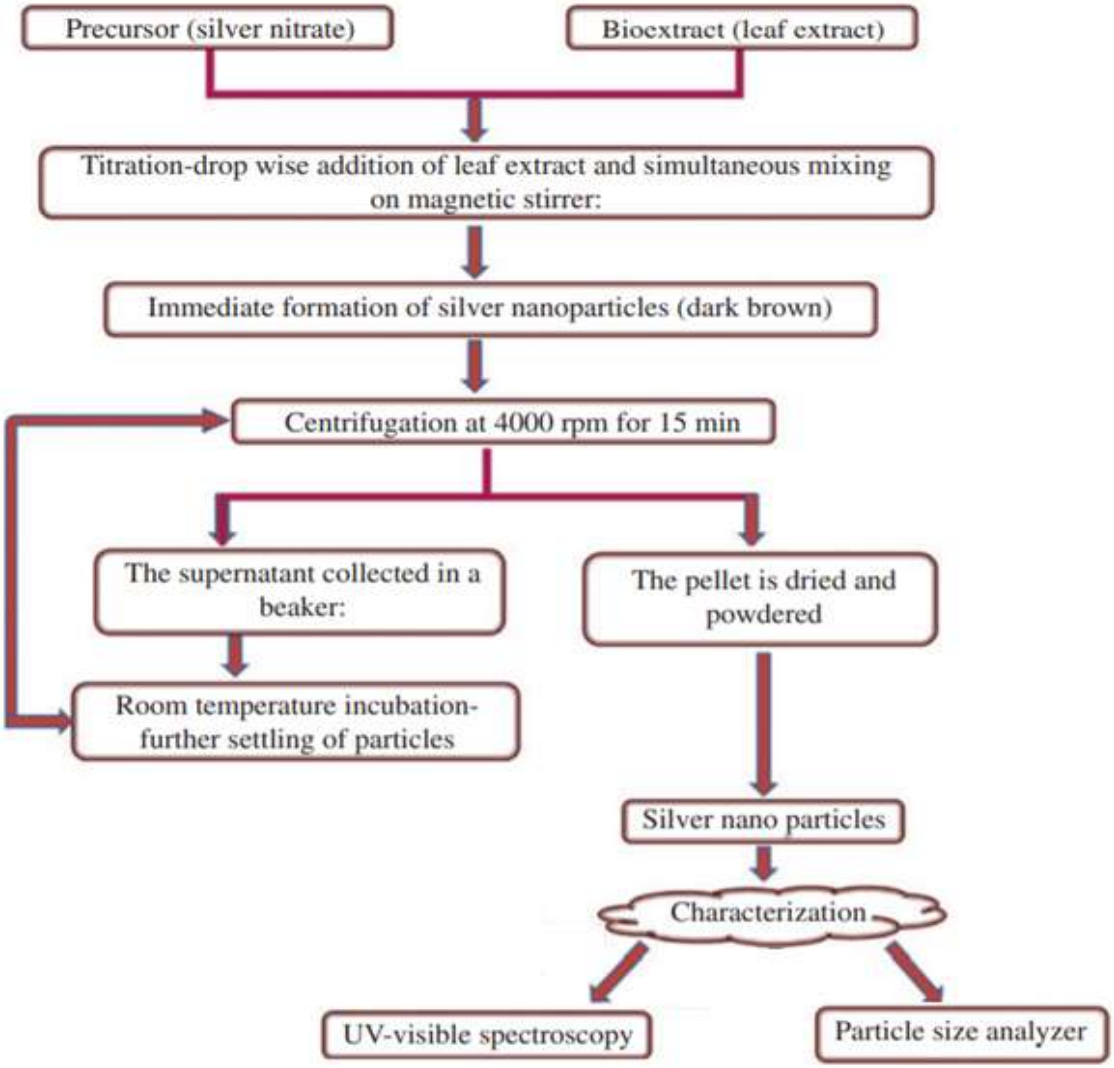


Figure 3.1 Methodology Flowchart of AgNP Biosynthesis

3.7 Antifungal Activity

A pilot study was carried out with lower concentrations (500 µg/ml - 3000 µg/ml) of plants samples against the three fungi to determine the concentration range of the plant samples to use for the study.

The antifungal activity was measured using the methods adopted by Vijayalakshmi *et al.*, (2014). The different fractions of Plant extract was thoroughly mixed with the medium

(PDA) after autoclaving. Prepared medium (19 ml) transferred into 180ml flat glass bottles and 1ml of various concentration of the extracts (3000, 6000, 10000µl/ml) was added while the media is in its molten state, each in triplicate and then cooled. The media without extracts samples served as negative control while the media containing Minimum inhibitory Concentration (MIC) of fluconazole for the fungus served as positive control. After complete solidification of the medium in the bottles, diluted spore suspension of *Aspergillus flavus, Fusarium verticilloides* and *Aspergillus niger* were added, spread, sealed with parafilms and incubated for 5 days at 28°C for the control reach of full growth. Antifungal properties of the samples was judged by counting the colonies after 5 days. Fungal growth was measured for each colony and percent inhibition (I %) of the fungal growth was calculated according to the following formula: Percent inhibition = (Growth in Control-Growth in treatment/Growth in control) x 100

3.8 Data Analysis

Colony fungal growth and antifungal activity data will be evaluated using analysis of Variance ANOVA. The mean values will be tested for all significance difference by Duncan's multiple range test (DMRT) as the mean ± S.E and p values < 0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Plants Extracts Yield

Plant samples were extracted using hexane, ethyl acetate, and methanol successively in order of least polarity index to high and the results are presented in table 4.1 and 4.2 below.

Table 4.1 Percentage yield of *Rhizophora racemosa* leaf extract

Fraction	Percentage yield (%)
Hexane	3.4
Ethyl acetate	4.0
Methanol	9.4

Table 4.2 Percentage yield of *Ocimum gratisimum* leaf extract

Fraction	Percentage yield (%)
Hexane	2.3
Ethyl acetate	6.8
Methanol	12.8

4.1.2 Fungal Morphological Identification

The microscopic examination was done by wet mount using 1% lactophenol blue stain, then viewed under x10 and x40 objective lens which was then compared with the vegetative and reproductive Morphological characteristics of conidia and mycelia of existing taxon for identification.

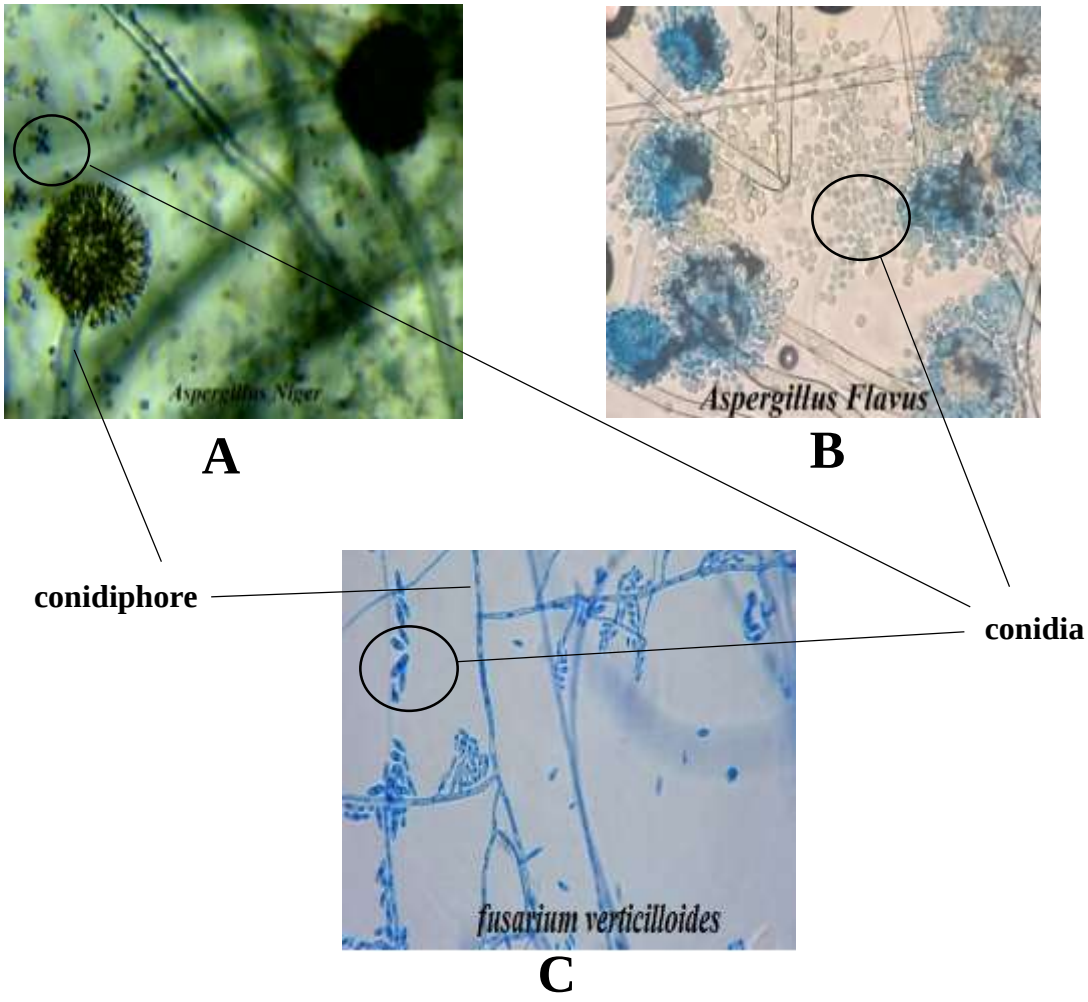


Plate III: Microscopic view of Fungi isolate.

Each fungus showing the structures of the conidia, conidiphore, and the hyphae

A – ***Aspergillus niger****:* Black colony, conidia terminated in vesicles, Septate hyphae

B – ***Aspergillus flavus****:* Yellowish green colony, globose conidia, long and large conidiphore, and septate hyhae

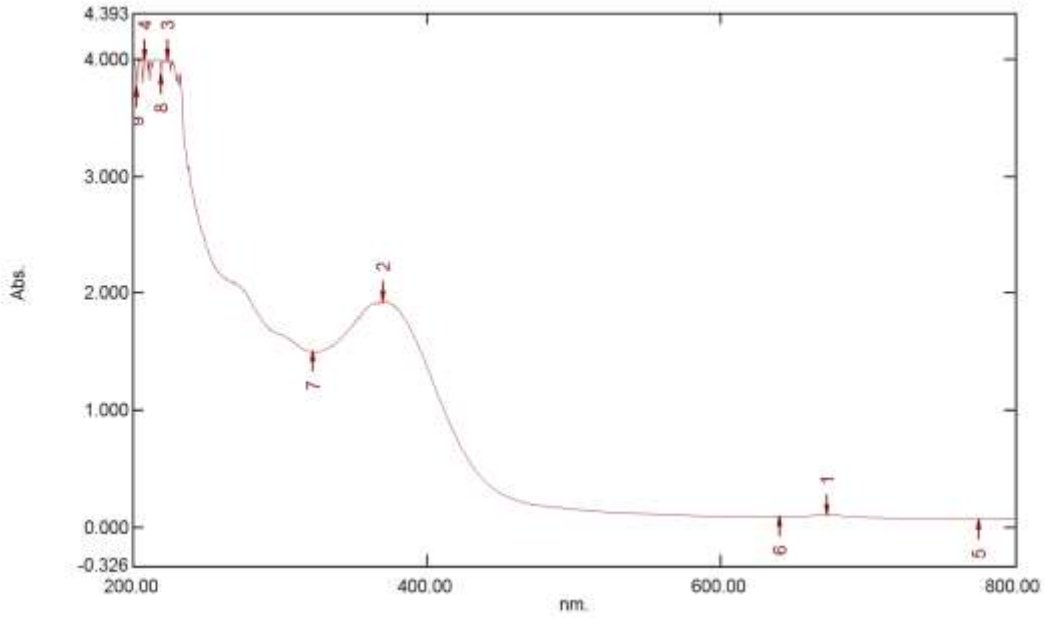
C – ***Fusarium verticilloides:*** pink to violet colour and aerial mycelium, microconidia are oval-shaped and arranged in chains and clusters

4.1.3 Green Synthesized Silver Nanoparticle

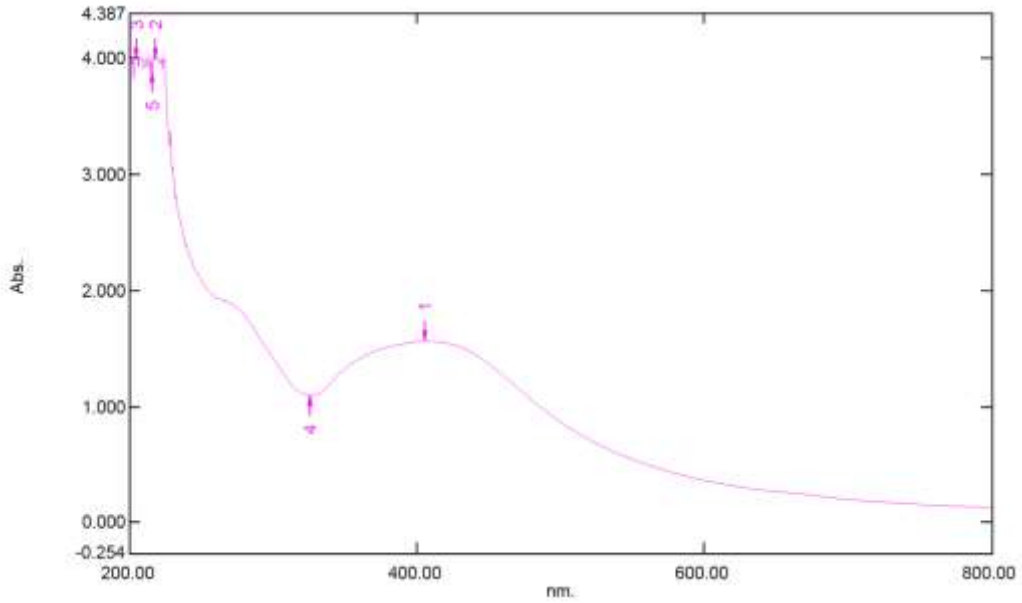
The reduction of pure Ag+ ions was monitored by first observing the colour change from faint green to dark and cloudy green when the Silver nitrate solution was mixed with the extracts solution, then measuring the UV-Vis spectrum of the reaction medium by diluting a small aliquot of the sample in distilled water.

UV-Visible Spectroscopy

The absorption peaks of the AgNPs were identified, for ethyl acetate extract synthesized AgNP (E-AgNP) and methanol extract synthesized AgNP(M-AgNP) to be 370.0nm and 405nm respectively (figure 4.1).



A



B

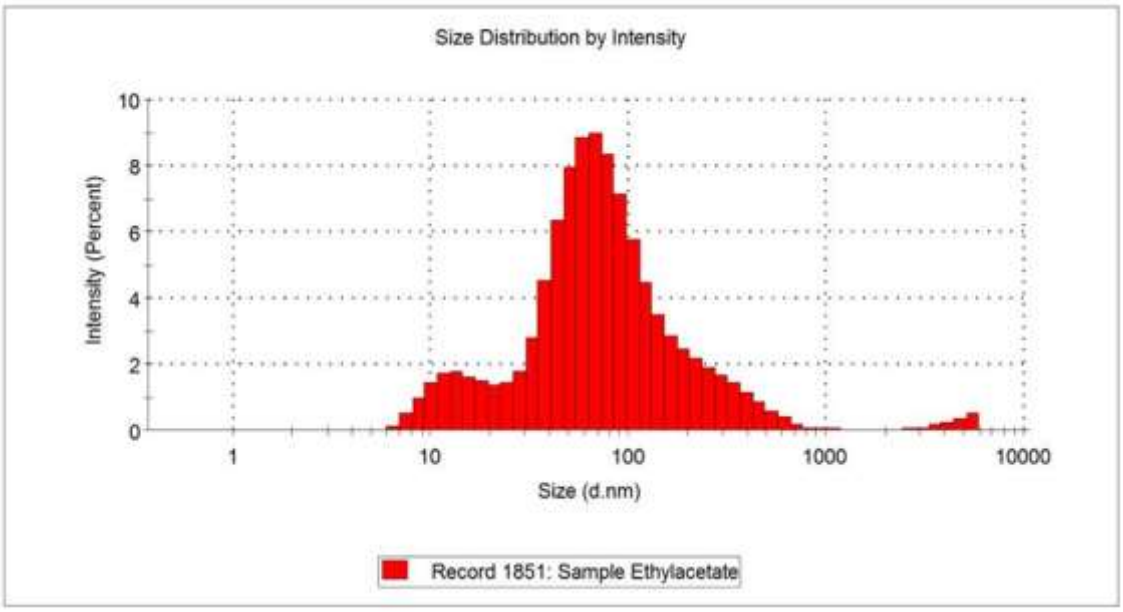
Figure 4.1. UV-Visible analysis of *Ocimum gratissimum* silver nanoparticles

A = E-AgNP showing absorption peak at 370nm
B = M-AgNP showing absorption peak at 405.5nm

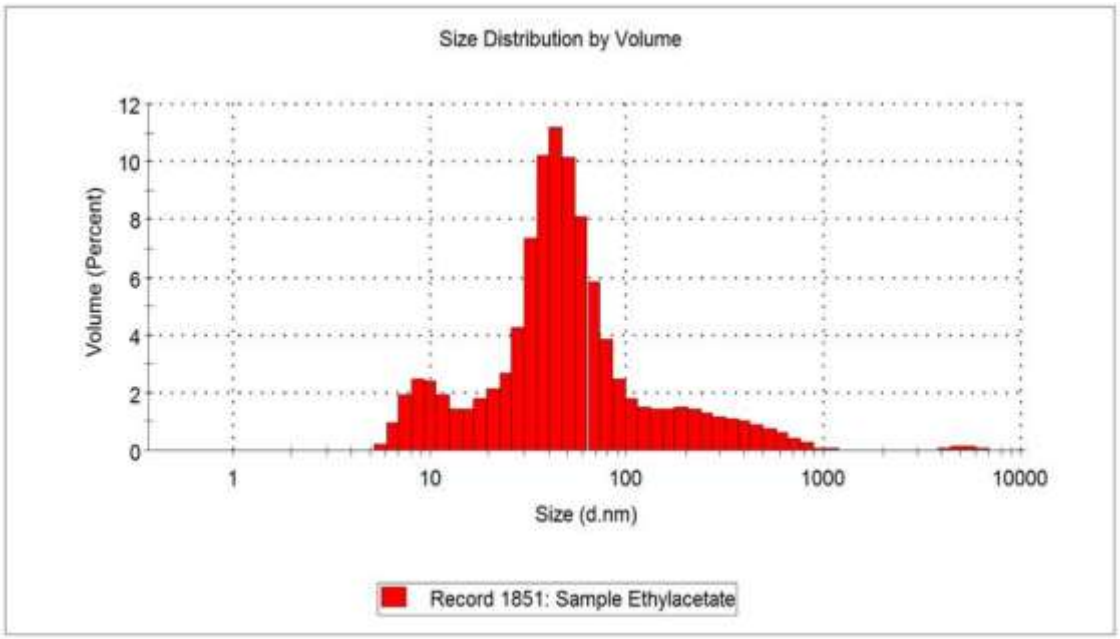
Characterization of AgNP

The hydrodynamic diameter of AgNPs was measured by using a dynamic light scattering technique. It collectively represents the size of a metallic silver core and biochemical attire of AgNPs that is designed by functional groups originally belong to plant secondary metabolites.

The volume distribution demonstrates the total volume of particles in various size bins (figure 4.2b and 4.3b) while The intensity distribution gives the amount of light scattered by the particles in the different size bins (figure 4.2a and 4.3a)



A

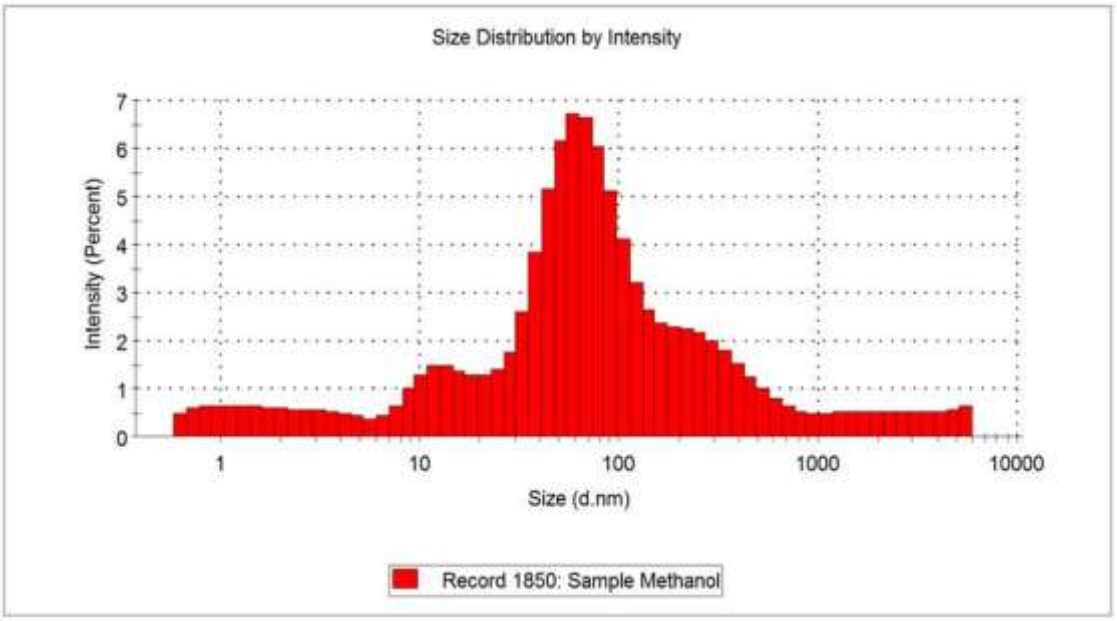


B

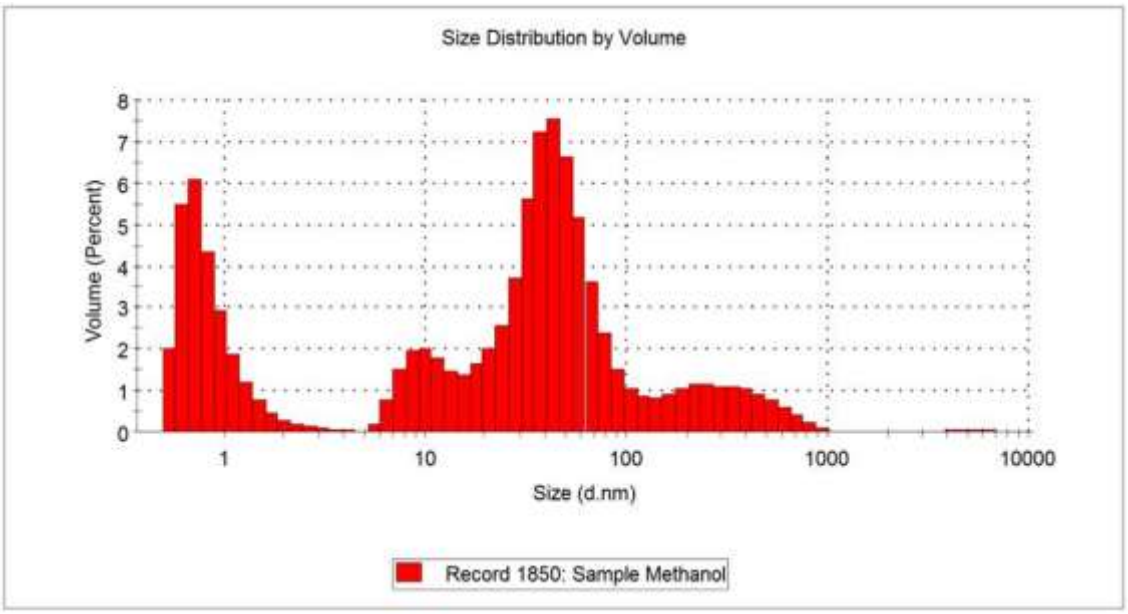
Figure 4.2 Size distribution of E-AgNP (average diameter = 84.67nm)

A= Size distribution by intensity

B= size distribution by volume



A



B.

Figure 4.3 Size distribution of M-AgNP (average diameter = 68.88nm)

A= Size distribution by intensity

B= size distribution by volume

4.1.4 Antifungal Activity of *Rhizophora racemosa* and *Ocimmum gratissimmum* Leaf Extracts

4.1.4.1 Percentage Inhibition (I %) of the fungal growth

Percentage inhibition (I %) of the fungal growth calculated using the formula: Percent inhibition = (Growth in Control - Growth in treatment/Growth in control) x 100.

The tables (4.3 and 4.4) below indicating percentage inhibition (I %) range of the highest and lowest percentage exhibited from the triplicate treatment of each fungus and concentration while table 4.5and 4.5 shows the statistical analysis of the data collected.

Table 4.3. Percentage Inhibition range of fungi growth on different concentration of *Ocimum gratissimum* extracts

Conc.	**Hexane extract**			**Ethyl acetate extract**			**Methanol extract**		
(µg/ml)	**(I %)**			**(I %)**			**(I %)**		
	A. niger	*A. flavus*	*F .verticilloides*	*A. niger*	*A. flavus*	*F. verticilloides*	*A. niger*	*A. flavus*	*F. verticilloides*
3000	0-6	0-3	0-0	29-38	0-7	50-70	12-20	0-6	0-17
6000	0-6	0-3	0-11	63-67	4-7	80-100	20-28	45-52	42-50
10000	0-12	0-6	0-11	92-100	52-63	90-100	96-100	94-97	92-100

Table 4.4. Percentage Inhibition of fungi growth on different concentration of *Rizophora racemosa* extracts

Conc. (µg/ml)	**Hexane extract (I %)**			**Ethyl acetate extract (I %)**			**Methanol extract (I %)**		
	A. Niger	*A. flavus*	*F. verticilloides*	*A. niger*	*A. flavus*	*F. verticilloides*	*A .niger*	*A. flavus*	*F. verticilloides*
3000	0-4	0-5	0-0	0-5	0-6	0-0	0-0	0-6	0-8
6000	0-4	0-9	0-9	0-5	0-0	0-15	0-0	0-6	0-0
10000	0-4	0-9	0-9	0-5	0-17	0-8	0-9	0-12	0-17

Table 4.5. Antifungal activities of *Ocimum gratissimum* leaf extracts

Extract	Concentration (µg/ml)								
	3000			6000			10000		
	A. niger	*A. flavus*	*F. verticciloides*	*A. niger*	*A. flavus*	*F. verticciloides*	*A.niger*	*A. flavus*	*F. verticciloides*
E	15.67 ± 0.67^{b}	26.00 ± 0.58^{b}	4.00 ± 0.58^{b}	8.33 ± 0.33^{b}	25.67 ± 0.33^{c}	0.33 ± 0.33^{a}	0.00 ± 0.00^{a}	18.33 ± 0.88^{b}	0.00 ± 0.00^{a}
H	16.67 ± 0.33^{b}	30.00 ± 0.58^{c}	8.33 ± 0.67^{c}	17.00 ± 0.58^{c}	30.00 ± 0.00^{d}	9.00 ± 0.58^{c}	16.33 ± 0.33^{b}	30.00 ± 0.58^{c}	8.67 ± 0.33^{b}
M	21.33 ± 0.67^{c}	30.00 ± 0.58^{c}	10.67 ± 0.67^{d}	18.67 ± 0.67^{cd}	16.33 ± 0.67^{b}	5.67 ± 0.33^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.67 ± 0.33^{a}
NC	22.00 ± 2.52^{c}	29.33 ± 1.20^{c}	10.33 ± 0.88^{d}	22.00 ± 2.52^{d}	29.33 ± 1.20^{d}	10.33 ± 0.88^{c}	22.00 ± 2.52^{c}	29.33 ± 1.20^{c}	10.33 ± 0.88^{c}
PC	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are presented as mean±standard error of mean (SEM) of three replicates.
Values with different superscripts in a column are significantly different at $p < 0.05$
Key: E=Ethylacetate; H=Hexane; M=Methanol; NC=Negative Control; PC=Positive Control.

Table 4.5 above indicates the organisms are more susceptible to ethyl acetate and methanol leaf extracts of *Ocimum gratissimum* in the higher doses and the fungicidal effect of the extracts on the organisms is generally dose dependent.

Table 4.6. Antifungal activities of *Rhizophora racemosa* leaf extracts

Extract	Concentration (µg/ml)								
	3000			6000			10000		
	A.niger	***A. flavus***	***F. verticciloides***	***A.niger***	***A. flavus***	***F. verticciloides***	***A.niger***	***A. flavus***	***F. verticciloides***
E	20.67 ± 0.88^{b}	17.67 ± 0.88^{bc}	12.33 ± 1.33^{b}	20.33 ± 0.33^{bc}	17.00 ± 1.00^{b}	12.33 ± 0.67^{b}	20.00 ± 0.58^{b}	17.67 ± 0.67^{b}	11.00 ± 0.58^{b}
H	24.00 ± 0.58^{c}	20.00 ± 0.58^{c}	11.33 ± 0.33^{b}	22.67 ± 0.88^{c}	20.00 ± 0.00^{c}	9.67 ± 0.88^{b}	24.33 ± 0.67^{c}	20.00 ± 0.58^{b}	10.00 ± 1.00^{b}
M	19.67 ± 0.33^{b}	16.67 ± 1.20^{b}	10.67 ± 9.67^{b}	19.33 ± 0.33^{b}	17.33 ± 0.88^{bc}	11.33 ± 0.33^{b}	19.67 ± 0.88^{b}	17.67 ± 0.88^{b}	11.67 ± 1.20^{b}
NC	21.33 ± 1.45^{b}	18.33 ± 0.88^{bc}	12.00 ± 0.58^{b}	21.33 ± 1.45^{bc}	18.33 ± 0.88^{bc}	12.00 ± 0.58^{b}	21.33 ± 1.45^{b}	18.33 ± 0.88^{b}	12.00 ± 0.58^{b}
PC	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Key: E=Ethylacetate; H=Hexane; M=Methanol; NC=Negative Control; PC=Positive Control

Values are presented as mean ± standard error of mean (SEM) of three replicates.
Values with different superscripts in a column are significantly different at $p < 0.05$

4.1.5 Antifungal Activity of E-AgNP and M-AgNP

Table 4.7 Percentage Inhibition (%I) of E-AgNP and M-AgNP against *A. niger, A. flavus and F. verticilloides*

Conc. (µg/ml)	Ethyl acetate extract (% I)			Methanol extract (% I)		
	A.niger	*A.flavus*	*F.verticilloides*	*A.niger*	*A.flavus*	*F.verticilloides*
3000	42-53	0-9	0-0	0-13	0-0	0-13
6000	58-79	27-36	67-67	48-61	38-50	50-75
10000	100	91-100	89-100	91-100	92-100	88-100

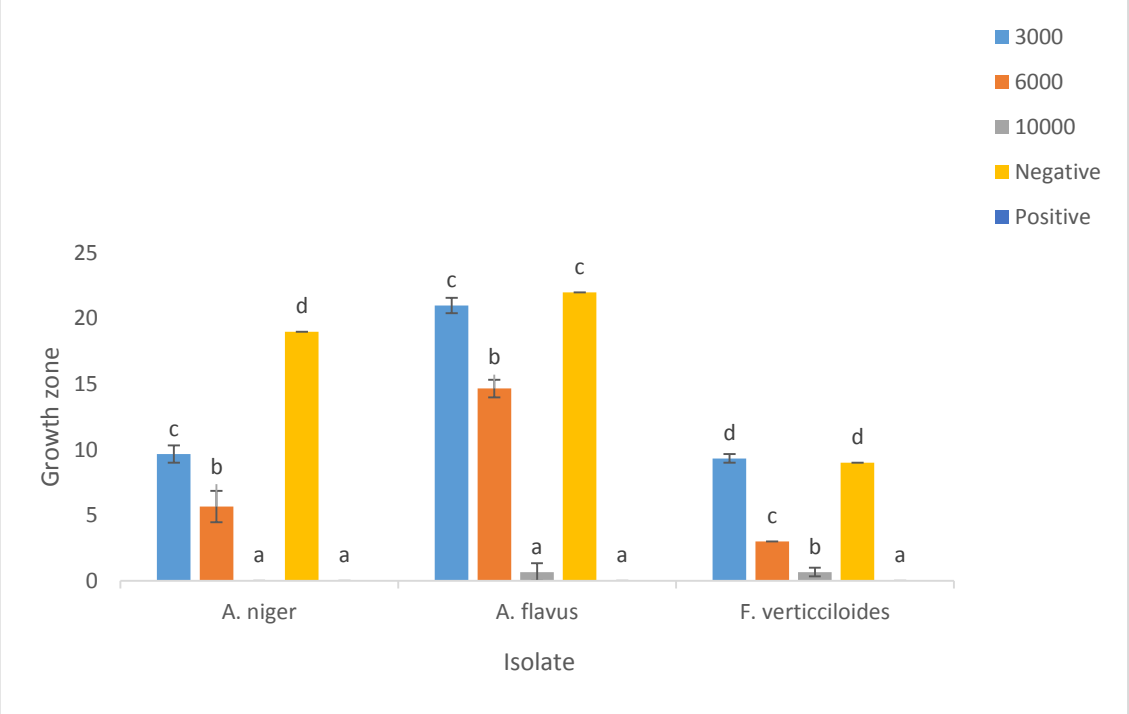


Figure 4.4. Antifungal activity of E-AgNP against *A.niger, A.flavus* and *F. verticilloides*

The AgNP treatments showed significant ($p<0.05$) antifungal activity against *Aspergillus niger* and *Aspergillus flavus Fusarium verticilloides* from the the chart above (figure 4.4)

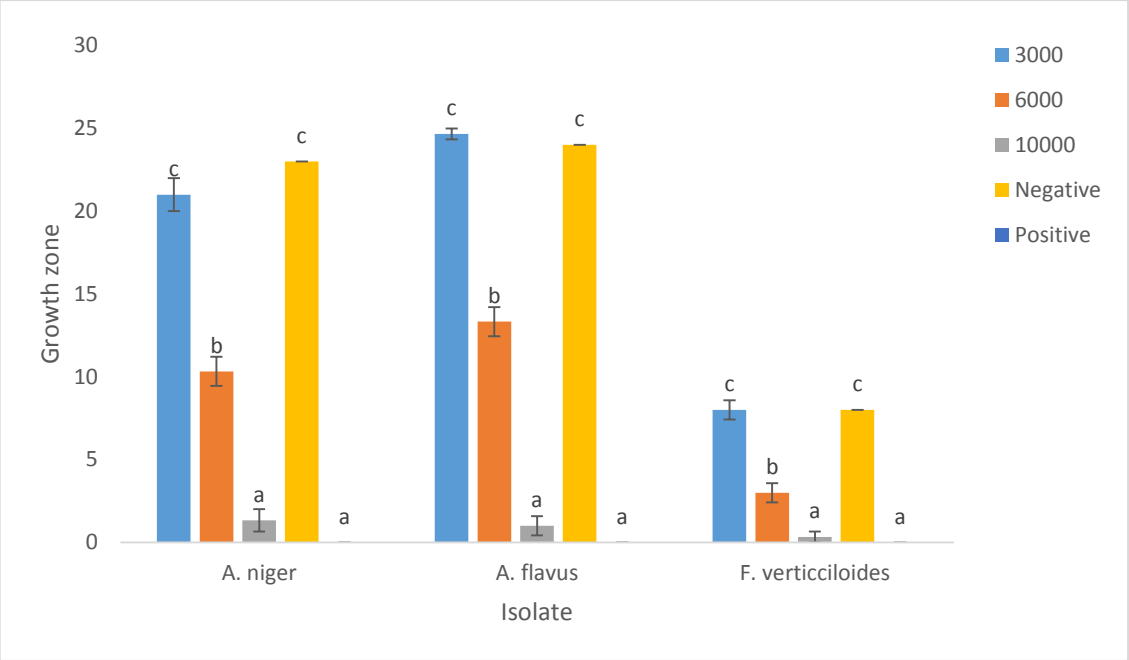


Figure 4.5. Antifungal activity of M-AgNP against *A.niger, A.flavus and F. verticilloides*

The AgNP treatments showed significant ($p<0.05$) antifungal activity against *Aspergillus niger* and *Aspergillus flavus Fusarium verticilloides* from the the chart above (figure 4.5)

4.1.6 Antifungal activity of AgNP Compared with *Ocimmum Gratissimum* Extracts

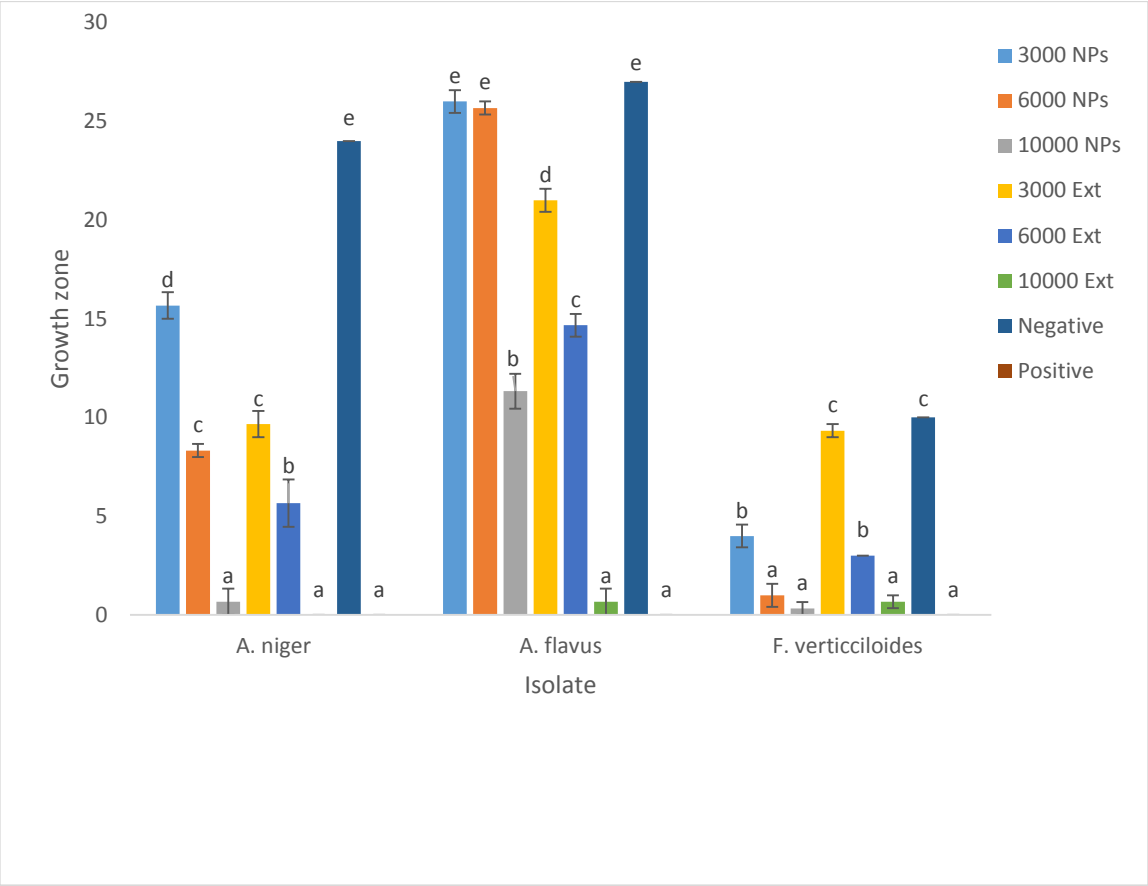
4.1.6.1 Antifungal activity of Ethyl acetate extract compared with E-AgNP

The antifungal activity of biosynthesized AgNP produced using the ethyl acetate extract compared to the ethyl acetate extract against *A.niger, A.flavus and F. verticilloides* is shown in table 4.8 and further illustrated in the chart below (figure 4.6) where Values with different superscripts in a column are significantly different at $p < 0.05$.

Table 4.8. Antifungal activity of E-AgNPs compared with *Ocimmum gratissimum* ethyl acetate Extracts

Sample	Conc. (µg/mL)	*A. Niger*	*A. flavus*	*F. verticciloides*
AgNPs	3000	15.67 ± 0.67^{d}	26.00 ± 0.58^{e}	4.00 ± 0.58^{b}
	6000	8.33 ± 0.33^{c}	25.67 ± 0.33^{e}	1.00 ± 0.58^{a}
	10000	0.67 ± 0.67^{a}	11.33 ± 0.88^{b}	0.33 ± 0.33^{a}
Extract	3000	9.67 ± 0.67^{c}	21.00 ± 0.58^{d}	9.33 ± 0.33^{c}
	6000	5.67 ± 1.20^{b}	14.67 ± 0.67^{c}	3.00 ± 0.00^{b}
	10000	0.00 ± 0.00^{a}	0.67 ± 0.67^{a}	0.67 ± 0.33^{a}
Negative control	0	24.00 ± 0.00^{e}	27.00 ± 0.00^{e}	10.00 ± 0.00^{c}
Positive control	800	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are presented as mean ± standard error of mean (SEM) of triplicates.
Values with different superscripts in a column are significantly different at $p < 0.05$.



Ethyl acetate, NPs= Nanoparticles & Ext= extract

Figure 4.6 Antifungal activity of E-AgNP *versus* Ethyl acetate extract against *A.niger, A.flavus and F. verticilloides.*

E-AgNP showing significant ($p<0.05$) increase in antifungal activity compared with the antifungal activity of ethyl acetate extract against *A.niger, A.flavus and F. verticilloides.*

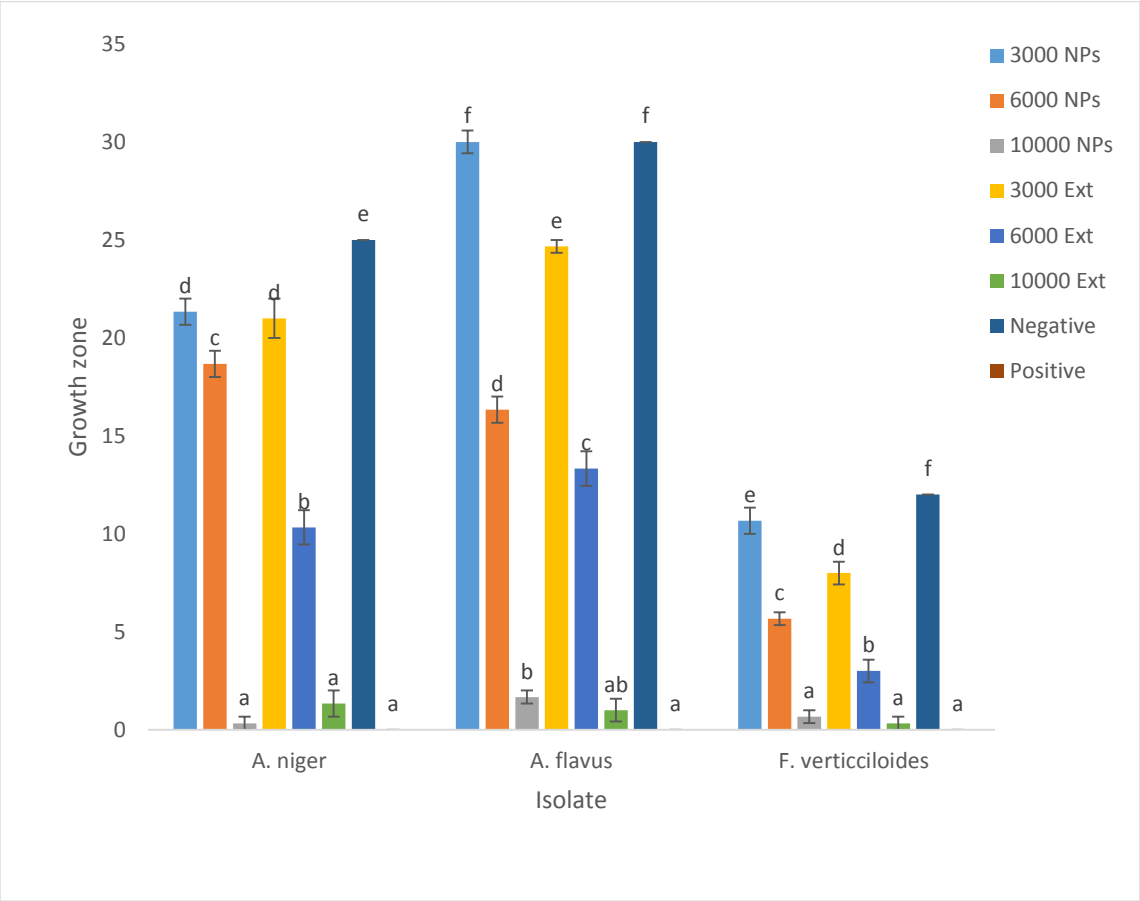
4.1.6.2 Antifungal Activity of Methanol extract compared with M-AgNP

The antifungal activity of green synthesized AgNP produced using the methanol extract (M-AgNP) compared with the methanol extract antifungal activity against *A.niger*, *A.flavus and F. verticilloides* is shown in table 4.9 and further illustrated in the chart below (figure 4.7) where Values with different superscripts in a column are significantly different at $p < 0.05$.

Table 4.9: Antifungal activity of AgNPs compared with *Ocimmum Gratissimum* methanol Extracts

Sample	Conc. (µg/mL)	*A.niger*	*A.flavus*	*F. verticciloides*
AgNPs	3000	21.33 ± 0.67^{d}	30.00 ± 0.58^{f}	10.67 ± 0.67^{e}
	6000	18.67 ± 0.67^{c}	16.33 ± 0.67^{d}	5.67 ± 0.33^{c}
	10000	0.33 ± 0.33^{a}	1.67 ± 0.33^{b}	0.67 ± 0.33^{a}
Extract	3000	21.00 ± 1.00^{d}	24.67 ± 0.33^{e}	8.00 ± 0.58^{d}
	6000	10.33 ± 0.88^{b}	13.33 ± 0.88^{c}	3.00 ± 0.58^{b}
	10000	1.33 ± 0.67^{a}	1.00 ± 0.58^{ab}	0.33 ± 0.33^{a}
Negative ctr	0	25.00 ± 0.00^{e}	30.00 ± 0.00^{f}	12.00 ± 0.00^{f}
Positive ctr	800	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Ctr = control
Values are presented as mean ± standard error of mean (SEM) of triplicates.
Values with different superscripts in a column are significantly different at $p < 0.05$.



Methanol extract, NPs= Nanoparticles & Ext= extract

Figure 4.7 Antifungal activity of (M-AgNP) *versus* the antifungal activity methanol extract against *A.niger, A.flavus and F. verticilloides.*

M-AgNP showing significant ($p<0.05$) increase in antifungal activity compared with the antifungal activity of methanol extract against *A.niger, A.flavus and F. verticilloides.*

4.2 Discussion

4.2.1 Plant extracts antifungal activity

Secondary metabolites such as flavonoids, alkaloids, tannins and sterols are responsible for plants antimicrobial potential. These secondary metabolites are responsible for the alteration of biochemistry and cytology of microbes. Saponins have been found to be one of the important bioactive compounds responsible for inhibitory activity on fungi growth (Ahmed *et al.,* 2014)

Poisoned food technique was used to determine the percentage inhibition by counting the number of colony growth on the media treated with hexane, ethyl acetate and methanol extracts of *Ocimum gratissimum* and also *Rhyzophorah racemosa* each as shown in table 4.3 to 4.4. The extract fractions of *Rhyzophorah racemosa* generally showed minimal percentage inhibition with an average of 2% (hexane extract), 4% (ethylacetate) and 2% (methanol extract) for doses up to 10000µg/ml against the three fungi which was not dose dependent and had no significant ($p<0.05$) inhibitory activity, this could be as a result of some physical factors such as duration of plant storage, temperature, pH, and oxygen known to affect the amount and stability of active compounds in plants samples. (Bhanu *et al.,* 2022). The ethyl acetate and methanol extract fractions of *Ocimum gratissimum* on the other hand exhibited significant antifungal activity against *A. niger, A. flavus and F. verticilloides* at $p<0.05$ with average percentage inhibition of 83% at higher dose (10000µg/ml) and 32% at lower dose (3000µg/ml) for ethylacetate extract while for methanol extract it was 97% at higher dose (10000µg/ml) and 9% at lower dose (3000µg/ml). The percentage inhibition occurred in a dose dependent manner while the Hexane fraction of *Ocimum gratissimum* showed no significant ($p<0.05$) antifungal potential. The result suggest that methanolic and ethyl acetate extract of *Ocimum*

gratissimum possesses some bioactive compounds with antifungal activities which may be phenolic and/or saponins compounds (Ahmad & Akram, 2019) present in these fractions.

4.2.2 Characterization of synthesized of AgNPs

From UV results obtained, it is evident that the silver nanoparticles were formed & this was confirmed by the peak absorption in the UV spectrum in the wavelength range of 340 - 620nm which falls within reported range. (Mano *et al.,* 2011; Fu *et al*., 2021).

The absorption peaks of the AgNPs were identified, for ethyl acetate extract synthesized AgNP (E-AgNP) and methanol extract synthesized AgNP(M-AgNP) to be 370.0nm and 405nm respectively (figure 4.1). Silver nitrate used as the silver precursor in producing AgNPs with different capping and reducing agents have generally shown absorption peaks in the wavelength range of approximately 400nm (Ashraf *et al*., 2016; Bastús *et al*., 2014). The dominant wavelength range varies with the use of different capping and reducing agents for AgNP generation (Fu *et al*., 2021)

According to Mie's theory, small particles absorb light at a smaller wavelength while large particles absorb light at a longer wavelength (Sharma *et al.,* 2006) which suggest the M-AgNPs formed were relatively smaller than E-AgNPs as further evident by the average diameter which was 84.67nm for ethyl acetate and 68.8nm for methanol. Theoretically and experimentally, it is found that when size decreases, the SPR peak shifts towards shorter wavelength side. It is also found that with the decrease in size, absorption spectra becomes weak and broad (Drachev *et al.,* 2004).

According to Njagi *et al.* (2011), this bands corresponds to the absorption by colloidal silver nanoparticles in the region due to the excitation of surface plasmon vibration. Surface Plasmon Resonance band formed is a response to the interaction of free electrons

of silver in oscillation with the electromagnetic waves of the light (Nindawat and Agrawal 2019). The flat curves of the graph represent the poly-dispersed particles in the solution. Sedimentation was not observed after a week of storage which suggests that the synthesized silver nanoparticles were stabilized by the presence of polyphenols in the two extracts. (Njagi *et al*. 2011).

Polydispersity index of AgNPs was recorded at 0.203 for E-AgNP and 0.398 for M-AgNP which indicates the relatively narrow particle size distribution and express their suitability in biomedical application. (Javed *et al.,* 2020). The average diameter of E-AgNPs and M-AgNPs were 68.88nm and 84.67nm respectively. These NPs have negative zeta potential on their surfaces, which improves their stability (Nandhini *et al*., 2023).

The double major peak in the size distribution by volume of ethyl acetate silver nanoparticles indicate agglomeration due to higher surface area to volume ratio of the particles causing weak bond interaction between particles (Rucha *et al*., 2012**).**

4.2.3 AgNP antifungal activity

The AgNP treatment showed an increase in the percentage fungi inhibition compared to the methanol and ethyl acetate extract of the plant and it was evident in the test for significant difference which showed the AgNP treatment against the fungi has significantly ($p<0.05$) higher inhibitory activity than the treatment with the corresponding concentrations of the methanol and ethyl acetate extracts of the plant at 3000µg/ml, and 6000µg/ml on *Aspergillus niger* and *Fusarium verticilloides* while not significantly different on the 10000µg/ml concentration because there was equally a 100% inhibition in both the AgNP and also the extract treatment at 10000µg/ml. For *Aspergillus flavus* On the other hand, there was a significantly higher inhibitory effect when treated with AgNP compared to the Extract treatment in all the concentration.

Generally, the AgNP synthesized using the methanol, and ethyl acetate extract of *Ocimum gratissimum* tested against the three fungi, *Aspergillus flavus*, *Aspergillus niger* and *Fusarium verticilloides* showed a dose-dependent antifungal activity and there was significant increase in inhibitory action in the activity of the AgNP treatment compared to the extracts fractions and this could be attributed to the fact AgNPs has multiple and potent mode of action, one of which is its attachment to the cell wall of the microorganism thus disturbing cell-wall permeability and cellular respiration and also its higher surface area to mass ratio could also be responsible of the higher inhibitory activity (medda *et al*., 2015). However, in the case of managing food products against fusarium specie, a combination of AgNPs with (Deoxynivalenol) DON-reducing mechanisms might also be a choice for the management of Fusarium head blight as recent studies carried out by Yunqing *et al.*, (2022) showed the in-vitro antifungal activity of AgNP against some fusarium species is accompanied with the production of the mycotoxin deoxynivalenol.

According to Imade *et al.* (2021), aflatoxins (43.75%) followed by fumonisins (FUM, 21.87%), ochratoxin (12.5%), are the top three prevalent mycotoxins in Sub Sahara Africa posing great threat to human and animal health and also the economy at large. This problems can therefore be potentially controlled and managed by formulations containing methanol, ethyl acetate extract and even more potently bio-synthesized AgNP which will generally be more eco-friendly, safer and reduce economic loses.

LD_{50} of ethyl acetate fraction extracted from methanol leaf extract of *Ocimum gratissimum* has been reported to be 2154.1mg/kg (Anoka *et al.*, 2019), which makes the tested doses in this study, safe for application in food products against the mycotoxin producing fungi.

CHAPTER FIVE

5.0 CONCLUSION, RECOMMENDATION AND CONTRIBUTION OF THE RESEARCH TO KNOWLEDGE

5.1 Conclusion

The fungi (*A. niger, A. flavus,* and *F. verticilloides*) were isolated from contaminated food samples (groundnut seeds and maize seeds) and morphologically identified while plants samples used were extracted using three solvents; hexane ethyl acetate and methanol. Silver nanoparticles was further produced using the most potent extract as reducing and capping agent for the studies which revealed that methanol and ethyl acetate leaf extract of *Ocimum gratissimum* and also the green synthesized AgNP exhibited anti-fungal activity against the three fungi producing three of the most prevalent mycotoxins. However, the green synthesized AgNPs were more potent so therefore methanol and ethyl acetate extract of *Ocimum gratissimum* can be used as treatment in food safety management and even more potently, green AgNPs can be applicable in developing fungicidal formulations.

5.2 Recommendation

Future advance studies are needed to develop products under agricultural conditions for field application in the control of mycotoxins contamination of crops.

Synergistic combination of AgNP with another active compound/antibiotic treatment should also be further studied against resistant strains of mycotoxin producing fungi as AgNP-fluconazole combination has been reported be effective against the resistant *Candida albicans*.

5.3 Contribution to Knowledge

Hexane, methanol, and ethyl acetate extract of *Ocimum gratissimum* and *Rizophora racemosa* leaf extracts tested against the mycotoxigenic fungi (*A. niger, A. flavus and F. verticilloides*) in this study, showed that at $p<0.05$ the methanol, ethylacetate and hexane extract of *Rhyzophora racemosa* generally has no significant antifungal activity against *A. niger, A. flavus and F. verticilloides* with an average percentage inhibition of 2% (hexane extract), 4% (ethyl acetate extract) and 2% (methanol extract) in doses between 3000 µg/ml to 10000µg/ml while only methanol and ethylacetate extract of *Ocimum gratissimum* exhibited significant ($p<0.05$) antifungal activity against *A. niger, A. flavus* and *F. verticilloides* with average percentage inhibition of 83% at higher dose (10000µg/ml) and 32% at lower dose (3000µg/ml) for ethylacetate extract while for methanol extract it was 97% at higher dose (10000µg/ml) and 9% at lower dose (3000µg/ml). The AgNPs synthesized using each of methanol and ethyl acetate extract of *Ocimum gratissimum* as reducing and capping agent showed significant increase in activity than the extracts against the three fungi at $P<0.05$.

It can then be deduced from this study that the significant health (animals and humans) concerns and economic losses caused by these fungi and the mycotoxins they produce can effectively be managed using formulations containing the appropriate dose of ethyl acetate and methanol extract of *Ocimum gratissimum* and more potently the Green synthesized AgNP for safer use and improved ecological impact due of their biodegradability and low toxicity

LD_{50} of ethyl acetate fraction extracted from methanol leaf extract of *Ocimum gratissimum* has been reported to be 2154.1mg/kg, which makes the highest dose

(10000µg/ml) tested in this study with an average of 90% inhibition rate an effective and safe dose for application in food products against the mycotoxin producing fungi: *A. niger, A. flavus and F. verticilloides*.

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APPENDICES

APPENDIX A

ANTIFUNGAL ACTIVITIES OF DIFFERENT CONCENTRATION OF *Ocimum gratissimum* FRACTIONS

Colony growth of the three fungi; *Aspergillus niger*, *Aspergillus flavus* and *Fusarium verticilloides* treated with 3000µg/ml, 6000µg/ml, and 10000µg/ml of the Ethyl acetate extract (Appendix A), Hexane(Appendix B), and methanol (Appendix C) of *Ocimum gratissimum* compared to the negative and positive (fluconazole) control, each in triplicates and percentage inhibition determined.

APPENDIX A: Antifungal activities of different concentration of *Ocimum gratissimum* ethyl acetate extract.

Ethyl Acetate Extract/Drug(µg/ml)	***A. niger***			***A. flavus***			***F. verticciloides***		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
3000	15	17	15	26	25	27	5	3	4
6000	8	8	9	25	26	26	1	0	2
10000	0	0	2	13	11	10	1	0	0
Control (negative)	24	24	23	28	27	26	10	10	10
Fluconazole (positive)	0	0	0	0	0	0	0	0	0

*A, B, C represents the sample of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml

APPENDIX B

Antifungal activities of different concentration of *Ocimum gratissimum* hexane extract

Hexane Extract (µg/ml)	***A.niger***			***A. flavus***			***F. verticciloides***		
	A	B	C	A	B	C	A	B	C
3000	16	18	17	31	31	30	9	10	9
6000	16	17	17	31	32	30	10	8	9
10000	16	15	17	30	29	31	8	9	9
Control(negative)	17	16	18	31	31	31	9	10	9
Fluconazole (positive control)	0	0	0	0	0	0	0	0	0

* A, B, C represents the concentrations of each study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml.

APPENDIX E

Antifungal activities of different concentration of *Ocimum gratissimum* methanol extract

Methanol Extract(µg/ml)	***A.niger***			***A flavus***			***F. verticciloides***		
	A	B	C	A	B	C	A	B	C
3000	22	22	20	29	31	30	10	10	12
6000	20	18	18	17	15	17	6	5	6
10000	0	1	0	2	2	1	0	1	1
Control (negative)	25	26	24	30	32	31	12	12	11
Fluconazole (positive)	0	0	0	0	0	0	0	0	0

*A, B, C represents the sample of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml.

ANTIFUNGAL ACTIVITIES OF DIFFERENT CONCENTRATIONS AND FRACTIONS OF *Rhizophora racemosa* EXTRACT

Colony growth of the three fungi; *Aspergillus niger*, *Aspergillus flavus* and *Fusarium verticilloides* treated with 3000µg/ml, 6000µg/ml, and 10000µg/ml of the Ethyl acetate (Appendix F), Hexane (Appendix G), and methanol (Appendix H) extracts of *Rhizophora racemosa* compared to the negative and positive (fluconazole) control, each in triplicates and percentage inhibition determined.

APPENDIX F

Antifungal activities of different concentrations of *Rhizophora racemosa* ethyl acetate extract

Ethyl Acetate Extract (µg/ml)	**A.Niger**			**A Flavus**			**F. Verticciloides**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
3000	20	22	21	17	20	18	15	11	13
6000	21	20	21	19	18	18	11	14	13
10000	20	20	21	17	15	20	12	12	13
Control (negative)	21	21	20	18	19	17	13	14	12
Fluconazole (positive)	0	0	0	0	0	0	0	0	0

*A, B, C represents the sample of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml.

APPENDIX G

Antifungal activities of different concentration of *Rhizophora racemosa* extract

Hexane Extract (µg/ml)	***A.Niger***			***A Flavus***			***F. Verticcilloides***		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
3000	25	24	23	19	21	20	12	11	11
6000	23	25	25	20	20	20	11	10	13
10000	24	23	25	20	19	21	12	10	10
Control(negative)	24	25	23	20	20	21	11	11	11
fluconazole (positive)	0	0	0	0	0	0	0	0	0

*A, B, C represents the sample of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml.

APPENDIX H

Antifungal activities of different concentration of *Rhyzophora racemosa* methanol extract

Methanol Extract(µg/ml)	***A.niger***			***A flavus***			***F. verticciloides***		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
3000	19	20	20	19	18	16	13	11	12
6000	20	19	19	19	16	17	12	12	12
10000	18	18	20	16	15	18	13	10	10
Control (negative)	19	18	20	17	16	18	12	13	11
Fluconazole(positive)	0	0	0	0	0	0	0	0	0

*A, B, C represents the sample of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml.

APPENDIX I

Antifungal activity of different concentration AgNP of ethyl acetate extract.

Ethyl Acetate Extract (µg/ml)	***A. niger***			***A. flavus***			***F. verticciloides***		
	A	B	C	A	B	C	A	B	C
3000	9	11	9	20	22	21	9	9	10
6000	8	5	4	14	14	16	3	3	3
10000	0	0	0	0	0	2	0	1	1
Negative Control	19	18	20	22	23	21	9	9	10
Fluconazole (positive)	0	0	0	0	0	0	0	0	0

A, B, C represents the samples of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800µg/ml.

APPENDIX J

Colony growth of fungi on media treated with different concentration AgNP of the methanol extract.

Methanol Extract (**µg/ml**)	*A. niger*			*A. flavus*			*F. verticciloides*		
	A	B	C	A	B	C	A	B	C
3000	23	20	20	24	25	25	8	9	7
6000	12	10	9	15	13	12	3	4	2
10000	2	0	2	0	2	1	1	0	0
Negative Control	23	23	24	24	23	25	8	8	7
Fluconazole	0	0	0	0	0	0	0	0	0

*A, B, C represents the sample of each of the study organisms showing the number of colony growth at 3000µg/ml, 6000µg/ml, 10000µg/ml, negative control and positive control (fluconazole) treatment which was 800 µg/ml.