ANTIMICROBIAL ACTIVITIES OF OIL EXTRACTS OF *PIPER GUINEENSE* AND *XYLOPIA AETHIOPICA* ON SOME MICROBIAL PATHOGENS OF FOOD.

BY

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CERTIFICATION

This is to certify that this research project entitled '**Antimicrobial activities of oil extracts of *Piper guineense* and *Xylopia aethiopica* on some microbial pathogens of food'** was carried out by Azeke, Ehijie Augusta with Reg No: 20154942695 in partial fulfillment of the requirements for the award of Post Graduate Diploma (PGD) in Food Science and Technology Owerri.

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DEDICATION

This project work is dedicated to Almighty God for his grace upon my life and for His wisdom, strength and understanding to carry out this work.

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ABSTRACT

Oils extracted from the fruits of two Africa spices- *Piper guineense* and *Xylopia aethiopica* were evaluated for their antimicrobial activities using *Escherichia coli, Salmonella sp, Aspergillus sp, Bacillus cereus, Penicillum commune* and *Rhizopus stolonifer* as test isolates. Well-in-agar, disc diffusion and dilution susceptibility testing methods were used to assay for their antimicrobial properties. For the bacterial isolates the antimicrobial activity of the oils increased with increase in concentration. *B. cereus* was more susceptible to the oils than other isolates with zones of growth inhibition measuring 19.5 mm and 18.0 mm for *P. guineense* and *X. aethiopica* respectively. However, the disc diffusion method produced lower inhibitory effect on the isolates. The MIC values ranged from 8.0 mg/ml – 16.0 mg/ml while MBC values ranged from 16-32mg/ml. Percentage inhibition against concentration of the oils revealed that percentage inhibition increased with increase in concentration ranging from 91.28% as observed on *B. cereus* produced by *P. guineense* to 96.86% on *Salmonella sp*. produced by *X. aethiopica*. The antifungal activities of the oils showed moderate to high activities on the isolates. This shows that essential oils of *Piper guineense* and *Xylopia aethiopica* has both bactericidal and fungicide effects which can be exploit in disease prevention and control as well as food preservation.

Key words: Antimicrobial activities, Oil extract, *Piper guineense*, *Xylopia aethiopica, Escherichia coli, Salmonella sp, Aspergillus sp.*

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Spices are plant substances either from indigenous or exotic source, with aromatic smell or strong taste (Olorunjuwon and Adeleke, 2012). Most chemicals responsible for those distinctive taste and smell are compounds known as essential oils or volatile oils (diffusing readily into the air). Spices give aroma and flavour to food and at the same time, they can serve as food preservatives because they possess active ingredients which are either microbistatic or microbicidal (Ogbonna *et al*, 2013). Spices are also used in perfumes and cosmetics purposes. Many have been used in medicine as well. Spices contain poly- and mono-unsaturated oils which are essential for normal growth and development. These oils also play important roles in the prevention and treatment of coronary artery disease, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders (Wang, 2004). Spices are different from other plant products used for similar purposes, such as herbs which are green leafy parts of plants aromatic vegetables and dried fruits (Olorunjuwon and Adeleke, 2012). Pungent spices can cause sweating, which may give a cooling sensation in tropical climates, on the other hand they can give inner warmth when

present in food in cold climates (Olorunjuwon and Adeleke, 2012). Spices also fit into philosophic concepts of improving health; they can affect the four humours (blood, phlegm, yellow blue and black bile) and influence moods sanguine, phlegmatic, choleric and melancholic (Jennifer and Paul, 1998).

Food safety depends on their adequate manipulation, transportation and storage. Foods are not sterile, in the sense that they normally contain some bacteria, viruses, yeasts and molds, some of which can lead to food intoxication and infections when present above the acceptable levels (Agatemor, 2008).

1.2 STATEMENT OF PROBLEMS

Of the world's health problems, food poisoning and food borne infections are becoming increasingly prevalent, especially in tropical countries with elevated temperature and humidity which favours microbial growth (Ogbonna *et al.,* 2013). There is growing interest in exploiting plants for food preservative and medicinal purposes especially in Africa (Nwinyi *et al.,* 2009). This is because food microorganisms are becoming resistant to many chemical preservatives and as such has created the need to seek alternative less expensive natural antimicrobial agents.

1.4 OBJECTIVES OF THE STUDY

The main objective of this work is to determine the antimicrobial activities of *Piper guineense* and *Xylopia aethiopica* on *Escherichia coli, Salmonella sp, Bacillus cereus, Penicillium commune, Rhizopus stolonifer and Aspergillus sp.*

The specific objectives are:

i) To extract essential oils from *Piper guineense* and *Xylopia aethiopica* using cold extraction method, using n-hexane solvent.

ii) To determine the antimicrobial activities of *Escherichia coli, Salmonella sp, Bacillus cereus* on oil extracts using well in agar and disc diffusion methods.

iii) To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the different plant extracts on each microorganism.

1.4 JUSTIFICATION

In the global food industry today, natural is a powerful force as there is increasing resistance at regulatory and consumer levels against chemical food preservatives (Agatemor, 2008). With the increasing rate of food poisoning and the need to reduce intake of chemical preservatives, there is a high demand for cheap and natural

alternatives. The use of plant materials traditionally used as food spices, condiments and or as medicine would be more beneficial to human health than the use of synthetic chemical food preservatives (Ogbonna et al., 2013). Spices contain phytochemicals (peptides, alkalonoids, essential oils, phenols and flavonols) (Okigbo and Igwe, 2007) which have been implicated in disease prevention as well as having antimicrobial effects on microbes (Abolaji *et al*., 2007; Nwinyi *et al*., 2009). *Piper guineense and Xylopia aethiopica* have been asserted to provide various culinary and medicinal properties. These medicinal properties have bacteriostatic and bacteriocidal effects (Ogbonna et al., 2013) on some bacteria which can be exploited in food preservation, diseases control and treatment.

1.5 SCOPE

This research work focus only on the the antimicrobial activities of *Piper guineense* (Uziza) and *Xylopia aethiopica* (Uda) on common bacterial (*Escherichia coli, Salmonella sp and Bacillus cereus)* and fungi (*Penicillium commune, Rhizopus stolonifer and Aspergillus sp*) using well in agar and disc diffusion methods.

CHAPTER TWO

LITERATURE REVIEW

2.1 ESSENTIAL OILS

Essential oils are derived from a variety of natural sources including plants or components of plants such as flowers, leaves, bark, roots, berries, seeds and/or fruit. These oils are complex mixtures of chemicals, and include various alcohols, aldehydes, terpenes, ethers, ketones, phenols, and oxides. Many essential oils have limited solubility in aqueous solutions and form emulsions with non-ionic surfactants. Previous investigators have reviewed the effect of essential oils, their components and antimicrobial activity (Burt, 2004; Baser and Demirci, 2011). However, few studies have determined the antimicrobial-specific mechanism (s) of action of various essential oils or their components (Cox *et al*., 2000; Fisher and Philips, 2006). Since essential oils are complex mixtures of compounds, it is likely the observed antimicrobial activity is due to inhibition or interaction with multiple targets in the cell (Skandamis and Nychas, 2001; Carson *et al*., 2002). However, many essential oils exert non-specific antimicrobial effects due to the hydrophobic properties of the mixtures and components. For instance, the hydrophobic character of many essential oils facilitates entry into cell membranes leading to alteration in

architecture, leakage of cell contents, and eventually death (Prabuseenivasan *et al*., 2006).

The essential oils of *Piper guineense* and *Xylopia aethiopica* demonstrated antibacterial and antifungal activities. These oils are edible and are therefore prospects for traditional preservation of foods in Nigeria. Their fragrance and aroma makes it possible also for application as food as flavourings, perfumes, fragrances, after shaves, and pharmaceuticals for their functional properties.

2.2 INDIGENOUS SPICES

Some world spices include Peppers (*Capsicum* spp), ginger (*Zingiber* spp), thyme (*Thymus* spp), nutmeg (*Myristica* spp), Garlic (*Allium sativum*), *Ocimum gratissimum, Piper guineense* (Uziza), Xylopia aethiopica (Uda) etc.

Piper guineense commonly called African black pepper or Ashanti pepper is a plant that grows in the tropical and sub-tropical regions of the world. It belongs to the family Piperaceae with more than 700 species (Nwinyi *et al*., 2009). *Piper guineense* is a gnarled vine native to West Africa, which is used as a substitute for black pepper. Their leaves are heart shaped, oval, petiolate, and alternate and 12 cm long. It is a perennial plant. The inflorescence is a pedicel led flower spike between 3cm and 6cm long. The peduncle and flowers are greenish yellow and arranged in spirally along the spine (Opara, 2014). It is a climbing plant, grows to about 20m in length

and bears pepperish berry fruits (Ogbonna *et al.*, 2015). It is known as Uziza in Igbo; *iyere* in Yoruba and *odusa* in Ibibio (Nwinyi *et al.*, 2009).

Figure 1(A): Fresh *Piper guineense* Seeds

Figure 1(B): Dried *Piper guineense* Seeds

Xylopia aethiopica is a slim, tall tree of about 60 - 70 cm in diameter and can reach up to 15-30cm tall (Acquaye *et al.*, 2011) with small aromatic fruit hull that looks like twisted bean-pods (Acquaye *et al.*, 2011). It belongs to the family Annonaceae, genus *Xylopia* and has many species. It is commonly known as West African pepper. It is called *ata* in Ibibio, *eeru* in Yoruba and Uda in Igbo (Abolaji *et al.*, 2007).

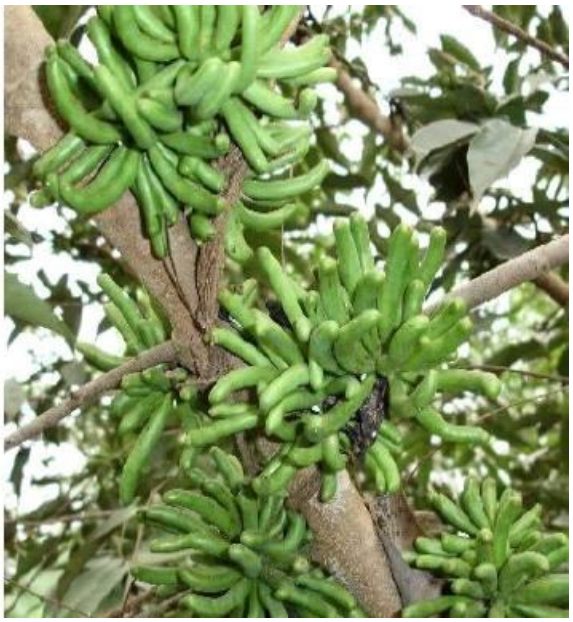

Figure 2 (A): Fresh *Xylopia aethiopica* Fruit Figure **2** (B): Dried *Xylopia aethiopica* Fruit

Galic (*Allium sativum*) has been used in all part of the world not only as spice or food but also for treatment of diseases. It is a vegetable member of the onion family which consist of a member of segment of cloves, each surrounded by a paper skin, joined together to form a spherical bulb (Olorunjuwon and Adeleke, 2012). Garlic contains several active substances, including sulphur compounds which are responsible for the pungent, aromatic odour.

Nutmeg (*Myristica fragrams*) seed is enclosed in a mottled yellow, edible fruit, the approximate size and shape of a small peach. Nutmeg is usually associated with sweet, spicy dishes-pies, puddings, custards, cookies and spice cakes. It

compliments egg dishes and vegetables like cabbage, spinach, broccoli, beans onions and eggplant.

Ginger (*Zingiber offinale)* is a strong antioxidant with antimicrobial properties used to treat sores and wounds. It was one of the earlier spices known in Western Europe. It is used in cooking either as silvers of fresh root or dried and ground as a reddish-coloured spices. It contains several active substances and volatile aromatic oil (Olorunjuwon and Adeleke, 2012).

Scent leaves *Ocimum gratissimum*is an herbaceous plant which belongs to the family *leguminocaeae*. *Ocimum gratissimum*is found in the tropical and warm temperature regions such as India and Nigeria (Okigbo and Ogbonnaya, 2006). The plant is indigenous to tropical areas especially India and also West Africa. In

Nigeria, it is found in the Savannah and coarse areas. It is commonly grown in Ceylon, South Sea Islands, Nepal, Bengal, Chittagong and Decan. It is known by various names in different parts of the world. In Nigeria for instance, the plant is called Effinrin-nla by the Yorubas, Ahuji or Nchuanwu by the Ibos and Diadoya in Hausa. *Ocimum gratissimum* has been described to have other species in the flora of tropical West Africa. These include: *Ocimum viride*Linn, *Ocimum suave* Linn, *Ocimum basilicum* Linn and *Ocimum canum*Sims.

2.3 MEDICINAL USES OF INDIGENOUS SPICES

Piper guineense has culinary, medicinal, cosmetic and insecticidal uses. *Piper guineense* has insecticidal effect against *Zonocerus variegatus a characteristic* attributed to its piperine amide content. The leaves are appetitive, carminative and eupeptic. They are used for the treatment of cough, bronchitis, intestinal diseases and rheumatism (Sumathykutty et al., 1999). *Xylopia aethiopica* (Uda) seeds have an aromatic pungent taste which is used as flavourings to prepare local dishes in West Africa. The fruit has medicinal uses which include treatment of cough, stomachache, dizziness, amenorrhea, bronchitis and dysentery. Concoction made from the mixture of the fruits and roots is used in the treatment of rheumatism.

Garlic (Allium sativum) has traditional and medicinal applications as anti-infective agent (Olorunjuwon and Adeleke, 2012). The therapeutic effects of garlic as hypolipidemic, antithrombotic, anti-hypertensive, anti-hyperglycemic, antihypercholesterolemic and immuno-modulatory have been reported (Mann, 2011). The bioactive components responsible for the health benefits of garlic are assumed to be allylic sulfur compounds. Garlic is commonly used for the reduction of cholesterol and cardio vascular risk. Consumption of garlic or garlic oil has been associated with a reduction in total cholesterol, low density lipoprotein (LDL) cholesterol and triglyceride levels. An intake of between one-half and one clove of

garlic per day may reduce the total cholesterol by 9%. Garlic extracts have been shown to have anti-clotting properties (Mann, 2011), and to cause modest reductions in blood pressure (approximately 5.5%decrease in systolic pressure) (Balentine et al., 1999). The effectiveness is associated with the active substances in garlic such as Allicin and other breakdown products. It has antiviral and antibacterial properties and recent congestion, bronchitis and cold symptoms and protect against re-infection.

Ginger (*Zingiber offinale)* is used as a spice as well as an important medicinal product. It has many therapeutic attributes such as antimicrobial, antithrombotic, anti-inflammatory and anticancer activity. Ginger has also demonstrated to be antimutagenic, inducers of detoxification, and preventers of DNA damage *in vitro* (Ohaeri and Adoga, 2006). Ginger has been shown to reduce nausea and vomiting during pregnancy. Ginger owes its characteristic organoleptic properties to two classes of constituents. The aroma of ginger is due to the constituents of its steamvolatile oil which are mainly sesquiterpene hydrocarbons, monoterpene hydrocarbons and oxygenated monoterpenes while its pungency is due to the nonsteam volatile components also known as the gingerols. Ginger is a major tranquilizer, carminative, and an antihypertensive agent due to its gingerol. Ginger

has been suggested for potential utility in treating peptic ulceration due to its action as a thromboxane synthetic inhibitor.

Nutmeg (Myristica fragans) is used to treat complaints of the digestive tract, such as stomach cramps and diarrhea, as well as catarrh of the respiratory tract. Nutmeg oil possesses strong antibacterial, antifungal, anti-inflammatory and insecticidal properties due to the presence of sabinen, β- and α-pinenes, eugenol, isoeugenol, methyl engenol, safrol, neolignan, myristicin, elemicin, and linalool. The lignin constituents are anticarcinogenic (Narasimhan and Dhake, 2006).

Scent leave (*Ocimum gratissimum*) has extensively been employed in the traditional medicine. In some parts of Brazil, it is used both as medicine and for culinary purposes. It is naturally used in the treatment of different diseases which include: upper respiratory tract infections, diarrhea, headache, conjunctivitis, skin disease, pneumonia, tooth and gum disorder, fever and as mosquito repellants. The flowers and leaves of this plant are rich in essential oils, so it is used in preparation of teas and infusion. In the southern parts of Nigeria, *Ocimum gratissimum* is used in the treatment of epilepsy, febrile conditions. Decoctions of the leaves of *O. gratissimum* have been used to treat mental illness. The Ibos of southeastern Nigeria use *Ocimum gratissimum* in sterilizing wound surfaces of their babies cord. It is also used in treatment of fungal infections, fever, cold and catarrhs.

2.4 ANTIMICROBIAL EFFECTS OF EXTRACTS FROM INDIGENOUS SPICES ON COMMON BACTERIAL AND FUNGI.

The *Ocimum* oil has been described to be active against several species of bacteria and fungi. These include *Listeria monocytogenes, Shigella, Salmonella* and *Proteus,* for fungi *Trichophytonrubrum, Trichophytonmentagrophytes, Cryptococcus neoformans, Penicillum islandi cum,* and *Candida albicans* (Akinyemi et al., 2004). Ajibola *et al.*, (2013) observed the antimicrobial activities of ethanol and water extracts (dissolving 25g spice in 50 ml distilled water) of *Xylopia aethiopica* (Uda), *Syzgium aromaticum* (Kanafuru), *Monodora myristica* (Ariwo), and *Piper guineense* (Uziza) against eight microorganisms (*Acinetobacter* spp., *Bacillus cereus*, *Escherichia coli*, *Salmonella* spp., *Shigella dysenteriae*, *Staphylococcus aureus*, *Aspergillus flavus* and *Aspergillus niger*). Their finding which shows more antibacterial activity for ethanol extract than water extracts as shown in tables 2.1and 2.2; antifungal activity on table 2.3.

Table 2.1 Antibacterial activity of the water extracts from the spices, mm

Bacteria tested	*Piper guineense*	*Xylopia aethiopica*	*Monodora myristica*	*Syzygium aromaticum*
Bacillus cereus	-	10	-	8
Shigelladysenteriae	-	8	-	8
Escherichia coli	-	-	-	-
Salmonella spp.	-	10	-	10
*Acinetobacter*spp.	-	10	-	10
Staphylococcus aureus	-	-	-	-

– No inhibition

Table 2.2 Antibacterial activity of ethanol extracts from the spices, mm

Bacteria tested	*Piper guineense*	*Xylopia aethiopica*	*Monodora Myristica*	*Syzygium aromaticum*
Bacillus cereus	10	27	15	10
Shigelladysenteriae	12	27	25	12
Escherichia coli	15	20	12	15
Salmonella spp.	15	20	16	20
*Acinetobacter*spp.	20	20	-	10
Staphylococcus aureus	18	-	-	20

– No inhibition

Table 2. 3. Antifungal activity of ethanolic extracts from the spices, mm

Bacteria tested	*Aspergillus flavus*	*Aspergillus niger*
Piper guineense	25	10
Xylopia aethiopica	11	15
Monodora myristica	-	20
Syzygiuma romaticum	25	20

– No inhibition

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

The following equipment were used for the project work; A Forced Convection Oven, An Autoclave, Incubator, soxhlet extraction unit, and glass wares used for the projects work include petri dishes, universal bottles, conical flasks, beakers, pipette, test tubes, measuring cylinder and amber coloured bottles.

Two African spices, *Piper guineense* and *Xylopia aethiopica* were purchased from Relief Market located in Owerri municipal in Imo state, Nigeria. The spices were transported to the laboratory in polythene bags for preparation and oil extraction. Pure cultures of *Escherichia coli, Salmonella sp, Bacillus cereus, Penicillium commune, Rhizopus stolonifer and Aspergillus sp* were obtained from the Anthony van Leeuwenhoek Research centre, Umuoma, Nekede. They were re-identified in the laboratory before use.

3.2 METHODS

3.2.1 Sample preparation

The spices were washed in sterile water, then sun dried for 5 days and sorted to remove every form of impurities. The dried spices were ground in a sterile blender and filtered to obtain fine particles with a large surface area exposed for extraction. The powdered samples were then packaged in polythene bags and used for extraction.

3.2.2 Extraction of oils from the samples

Cold extraction, using n-hexane as solvent, was adopted in this study. The powdered samples were soaked in the solvent and kept for 24 hours. The extract was filtered using a sterile white handkerchief and the solvent was recovered by distillation leaving behind the viscous oils as residue.

3.2.3 Media preparation

All media used were prepared according to the manufacturer's directives. Nutrient Agar, Eosin methylene Blue Agar, Mueller Hinton Agar and Potato dextrose agar were used in this study. The Nutrient Agar was used for plating out the organisms as well as storing them in slants while the Muller- Hinton Agar was used for sensitivity test.

3.2.3.1 Preparation of Nutrient Agar

Twenty-eight grams (28 g) of the powdered Nutrient Agar was dissolved in 1000ml of distilled water, according to the manufacturer's specifications. The medium was swirled to dissolve properly and sterilized by autoclaving at 121^{o}C and 15psi for 15 minutes; it was allowed to cool to 45^{o}C before plating it out into sterile petri-dishes. The media was allowed to solidify. Then it was surface dried in the oven at 40^{o}C to remove the moisture that settled at the surface to avoid contamination.

3.3.2 Preparation of Eosin methylene blue Agar

36 g of the Eosin methylene blue Agar (EMB) was suspended in 1000ml of distilled water. The mixture was heated to boiling with frequent stirring to dissolve the medium completely. The mixture was sterilized in an autoclave at 121°C (15 psi.) for 15 minutes and; then cooled to 45°C. The resulting mixture was swirled gently to avoid the formation of bubbles and poured into petri dishes. It was then surface dried in the oven at 40^{o}C to remove the moisture present at the surface to avoid contamination.

3.3.3 Preparation of *salmonella shigella Agar*

66g of *salmonella shigella Agar* was suspended in 1000ml distilled water and heated with frequent agitation until boiling. The mixture was cooled quickly and mixed before pouring into the sterile petri dishes.

3.3.4 Preparation Mueller Hinton Agar

38 grams Mueller Hinton Agar (MHA) was suspended in 1000 ml distilled water. The mixture was heated to boiling to dissolve the medium completely. It was then Sterilized by autoclaving at 15 psi pressure (121°C) for 15 minutes then allowed to cool to 45°C. The resulting mixture was swirled gently, avoiding the formation of bubbles and poured into petri dishes. It was surface dried in the oven at 40^{o}C to remove the moisture at the surface to avoid contamination.

3.3.5 Preparation of Potato Dextrose Agar

39 grams was Suspended in 1000 ml distilled water. The mixture was heated to boiling to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The resulting mixture was swirled gently, avoiding the formation of bubbles and poured into petri dishes. Then, surface dried in the oven at 40^{o}C to remove the moisture that can settle at the surface to avoid contamination.

3.3.6 Characterization of bacterial isolates

Distinct colonies sub-cultured on Nutrient agar at 37°C for 24 hours can be identified through microscopy (gram stain reaction, motility, sporulation and capsules were determined) and biochemical tests; catalase, oxidase, coagulase, methyl red, indole, Vogues Proskauer, citrate and carbohydrate fermentation tests; glucose, fructose, maltose, lactose and sucrose (Cheesbrough, 2000). The identities of the microbial isolates can be determined using standard manuals (Buchanan and Gibbon, 1984)

2.5.1 Microscopic Identification

i) Gram Staining

This is done for selected colonies on a particular culture medium (Cheesbrough, 2000). Using a wire loop, 24hours culture colony is emulsified in a drop of distilled water on a grease free slide and then heat fixed appropriately. The fixed smear is flooded with crystal violet for 60 seconds. The stain washed with clean water and the smear flooded with Lugol's iodine for 60seconds and then washed with clean water. Decolorizing with acetone for 15 seconds should be done and washed immediately with clean water. The smear can then be covered with safranin red dye for 60seconds and excess stain washed off accordingly. The slide should be placed on a draining rack to dry. Dropping a

drop of oil immersion on the smear and viewing using 100x objectives lens under a microscope gives the shapes and arrangements of cells as well as the gram stain reactions. **ii) Motility Test**

This is usually done by a loopful of the 24-hours-old broth culture of the test organism at the centre of Vaseline made into a circle on a clean grease free slide. A coverslip put in place and inverted. The slide can then be observed with a low power magnification (10x and 40x). The appearance and absence of darting in the broth showed a positive and negative motility respectively.

iii) Endospore Staining Test

This involves heat fixing air dried smeared organisms on a slide, then adding few drops of malachite green to the smear. The slides placed directly on a pre-warmed hot plate set on low heat (50^0C) for 3 minutes. Then removed from the hot plate and allow them to cool. This is followed by gentle rinsed with water after which it is flooded with safranin red dye and allowed to stand for 30 seconds, then rinsed with water to remove excess stain and dried. When examined under oil immersion, the spore forming bacteria appeared pink while its spores appeared green.

iv) **Capsule Test**

This involves placing a loopful of Indian ink slide. The Indian ink mixed with a little suspension of 24-hours-old bacteria on the slide is covered with a coverslip in such a way that there are no air bubbles and a blotting paper used to form a thin film will be formed on the slide. Observing the slide under oil immersion lens, capsules appeared as a clear area around the organisms.

2.5.2 Biochemical Tests

The biochemical tests are conducted in order to obtain characteristics which will be helpful in identification of the microorganisms. To aid in the more definitive identification of microorganisms, microbiologist have developed a series of biochemical tests that can be used to differentiate even closely related organisms. These various tests were designed to identify various metabolic properties of different microbial species (Buchannan and Gibbon, 1974). The following biochemical tests were carried out: catalase test, oxidase test, coagulase test, urease test, nitrate test, IMViC test and sugar fermentation. These tests were carried out in order to confirm the presences of certain microorganisms in the biofilms on the cathodes and anodes of the MFCs.

v) Catalase Test

This is done by placing a 24-hours-old organism suspension on a grease-free slide with 2 drops of 3% hydrogen peroxide added to the suspension. The occurrence of bubbling on the preparation indicates a positive test (Cheesbrough, 2000). **vi)**

Coagulase Test

A drop of distilled water was placed on two clean slides. The colony of the test organism was emulsified in the distilled water. A loopful of plasma was then added to one of the suspensions on the slides and mixed gently. It was then observed for clumping within 10 seconds and compared with control. Clumping is a positive test while no clumping is a negative test (Cheesbrough, 2000). **vii)**

Oxidase Test

This involves observation of a blue-purple colour after ten seconds indicating a positive result while its, a negative result (Cheesbrough, 2000) when a colony of the test organism is transferred and smeared on drops of freshly prepared oxidase reagent placed on a clean filter paper.

viii) Indole Test

When a loopful of the 24-hours-old test organism is inoculated in 3ml of sterile tryptone broth in a bijou bottle and the preparation incubated at 37°C for 48 hours. 0.5ml of Kovac's reagent added to the bijou bottle using a micropipette shook gently gives a red colour at the surface layer within 10 minutes. A positive indole test is characterized with a red ring while a negative test result gives no red ring (Cheesbrough, 2000).

ix) Methyl Red (MR) Test

This involves adding five drops of Methyl Red indicator on 5 ml of the broth cultured from a 24-hours-old test organism inoculated in a MR-VP broth and incubated for 48 hours at 37°C. A red colouration indicated a Methyl Red positive organism.

x) Vogues Proskauer (VP) Test

24-hours-old test organism inoculated in a MR-VP broth and incubated for 48 hours at 37°C. 1ml of the broth culture, 1ml of 6% alcoholic solution of alpha-naphtol and 1ml of 16% Potassium hydroxide mixed and allowed to stand for 15-20 minutes which gives a red colouration indicates a VP positive organism.

xi) Citrate Utilization Test

This involve preparing and autoclaving Simmon citrate medium in a bijou bottle according to manufacturer's instruction, then allowed to solidify in a slanting position. Streaking the test organism on the surface of the media using a sterile wire-loop and incubated at 37°C for 48 hours gives a deep blue colour as a positive test while a negative result gives a dirty green colour (Cheesbrough, 2000).

xii) Carbohydrate Fermentation Tests

Carbohydrate medium for glucose, fructose, maltose, xylose and lactose are all prepared according to manufacturer's instruction into 100 ml volumetric flasks. Medium is dispensed into test tubes and sterilized by autoclaving and sterile durham tubes are filled then gently inverted into the test tubes. Inoculating the test organisms into the test tubes and incubated for 24 hours at 37°C gives a colour change from red to yellow and an air space in the durham tubes indicating acid and gas production respectively.

3.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING

The antimicrobial susceptibility testing was determined using the modified method of Kirby-Bauer (1966). Cultures of *Salmonella sp, E. coli, B. cereus*, *Penicillium sp, Rhizopus stolonifer and Aspergillus sp* were subjected to susceptibility testing using

the well-in-agar and disc diffusion methods. The turbidity of the inocula were equivalent to 0.5 McFarland's standard (1.5×10^8 cfu/ml). Sterile cork borers were used to produce wells of about 5 mm in Mueller Hinton agar.

The oils were diluted using 5% dimethylsulphoxide (DMSO) solution to obtain the desired concentrations. These were stirred thoroughly for complete homogeneity. Samples were plated out on Nutrient agar and Potato Dextrose agar plates, and incubated at 37ºC and 30ºC respectively for 24h and 72h to ensure sterility of the solutions. Different concentrations of the oils prepared ranging from 20% to 100% for the bacteria and 1, 1:1, 1:2, 1:3 and 1:4 (volume of agar/volume of oil) for the moulds.

3.4.1 Well in Agar Method

0.1ml of the different concentrations of the oils was introduced into the wells made on Mueller - Hinton agar using sterile Pasteur pipette. A control was set up with 0.1 ml 5% dimethylsulphoxide. The dishes were allowed to stand for 45 min at room temperature to allow proper diffusion of the extracts to occur, and then incubated at 37^0C for 24 hours. The zones of growth inhibition were measured using a meter rule to the nearest millimeter (mm) after incubation.

3.4.2 Disc Diffusion Method

A filter paper was perforated to get paper discs of 5 mm diameter. The filter paper disc was sterilized at 110^0C for 30 minutes and then impregnated with each concentration of the extracts and placed on the surface of Mueller-Hinton agar inoculated with the test isolates. A control was set up with 0.1 ml 5% dimethylsulphoxide. The resulting plates were incubated at 37^0C for 24 hours. The zones of inhibition were measured using a meter rule to the nearest millimeter (mm) after incubation.

3.5 DILUTION SUSCEPTIBILITY TESTING

3.5.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Oils from Spices.

The MIC and MBC of the extracts for each test organism were determined by a modification of the broth macro tube dilution method described by Okeke *et al*. (2001) and Okoli et al. (2002). The MIC and MBC of the extracts for each susceptible test organism were determined by making double fold serial dilutions of the reconstituted extract 400 mg/ml in Nutrient broth. A 0.1 ml suspension of the test organism was inoculated into 1 ml of each concentration of the extract in duplicates. A tube containing Nutrient broth only and seeded with the test organism serve as control. All culture tubes were incubated at 37ºC for 24 h. Growth was

scored visually by the turbidity of the culture. The least concentration showing no growth was taken to be the MIC.

To determine the MBC, 0.1 ml inoculum was taken from the tubes in which there was no growth and sub cultured on Muller-Hinton Agar plates. After incubation at 37ºC for 24 hours, the plates were observed for bacterial growth. The least concentration showing no growth was taken as the MBC.

3.6 ANTIFUNGAL ASSAY

The biological activity of the extracts was evaluated according to a modification of the method described by Tatiana et al., (2015). The biological activity was evaluated on mycelial growth of fungal isolates. The media were prepared by making 20ml of different concentration of Potato Dextrose Agar (PDA) and the oil extracts (1:1=10ml of extract and 10ml of agar, 1:2=6.6ml of extract and 13.34ml of agar, 1:3=5ml of extract and 15ml of agar and 1:4=4ml of extract and 16ml of agar.). Mycelia of fungi removed from a 7 days old culture were transferred to

Potato Dextrose Agar (PDA) media containing the extracts of different concentrations. Three replicates were used per treatment. For each extract and concentration, inhibition of radial growth compared with the untreated control was calculated after 7 days of incubation at 24ºC, in the dark.

3.7 DATA ANALYSIS

All the data collected were analyzed using Microsoft excel 2010 computer software.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. ANTIMICROBIAL SUSCEPTIBILITY TESTING

Figures 4.1 and 4.2 show the antimicrobial susceptibility testing of *Piper guineense* and *Xylopia aethiopica* against selected test isolates using the well-in-agar diffusion method and the disc diffusion method respectively. The results from the well in agar diffusion test (Figure 4.1) revealed that *B. cereus* was more susceptible to the activity of both spices with a zone of inhibition (diameter) of 19.5 mm and 18.0 mm for *Piper guineense* and *Xylopia aethiopica* respectively at 100% concentration. The activity of the spices increased with increase in concentration. On the other hand, the spices had lower activities on *E. coli* both having a zone of inhibition of 9mm at 100% concentration. *E. coli* was resistant at concentration lower than or equal to 33.33% of the spices. Similar trend was recorded using the disc diffusion method (Figure 4.2), although, results obtained showed lower sensitivity compared to the well in agar diffusion method. Resistance to the activities of the extracts by *E. coli* and *Salmonella sp* was observed at

concentrations similar to the well in agar diffusion results.

The oils extracted from these spices exhibited broad spectrum antimicrobial activity which is consistent with previously published work (Schelz *et al*., 2006;

Hammer *et al*., 1999, Lopez *et al*., 2005; Ilori *et al*., 1996; Agatemor, 2009). The rate of diffusion and thickness of oils play a major role in the use of this protocol in assessing the activities of the oils. Amadioha and Obi (1999), Okigbo and Ajalie (2005) and Okigbo *et al.* (2005) reported that inactivity of plant extracts may be due to age of plant, extracting solvent, method of extraction and time of harvesting of plant materials. Insolubility of active compounds in water or the presence of inhibitors to the antimicrobial components also plays major roles in the activity of essential oils (Okigbo and Ogbonnanya, 2006). Previously published works have shown that the essential oils as well as the crude extracts (both alcoholic and aqueous) of the plants possess antimicrobial property against a wide range of Gram positive and Gram-negative bacteria, and Candida albicans (Boakye-Yiadom *et al*., 1977; Thomas, 1989; Tatsadjieu *et al*., 2003; Asekun and Adeniyi, 2004; Okigbo *et al*., 2005).

Essential oils or their constituents are odoriferous substances from plants and are extensively used as medicinal products, in the food industry as flavours and in the cosmetic industry as fragrances (Evans, 2003). It has also been reported that almost every morphological part of these plants is used in traditional medicine for managing various ailments including skin infections, candidiasis, dyspepsia, cough and fever

(Burkhill, 1985; Irvine, 1961; Mshana *et al.*, 2000; Ghana Herbal Pharmacopoeia, 1992). The antimicrobial activity of the oils therefore justifies these applications.

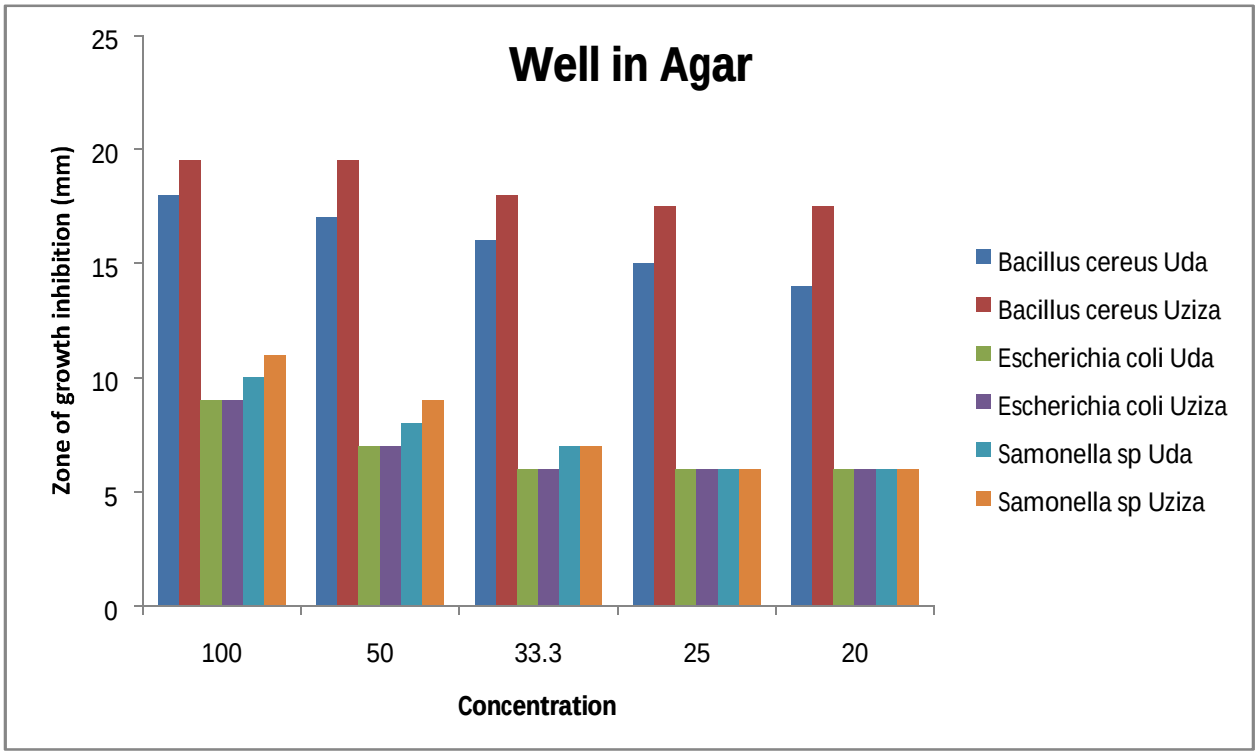

Figure 4.1: Antimicrobial Susceptibility Testing of Isolates Using Well in Agar Diffusion Method.

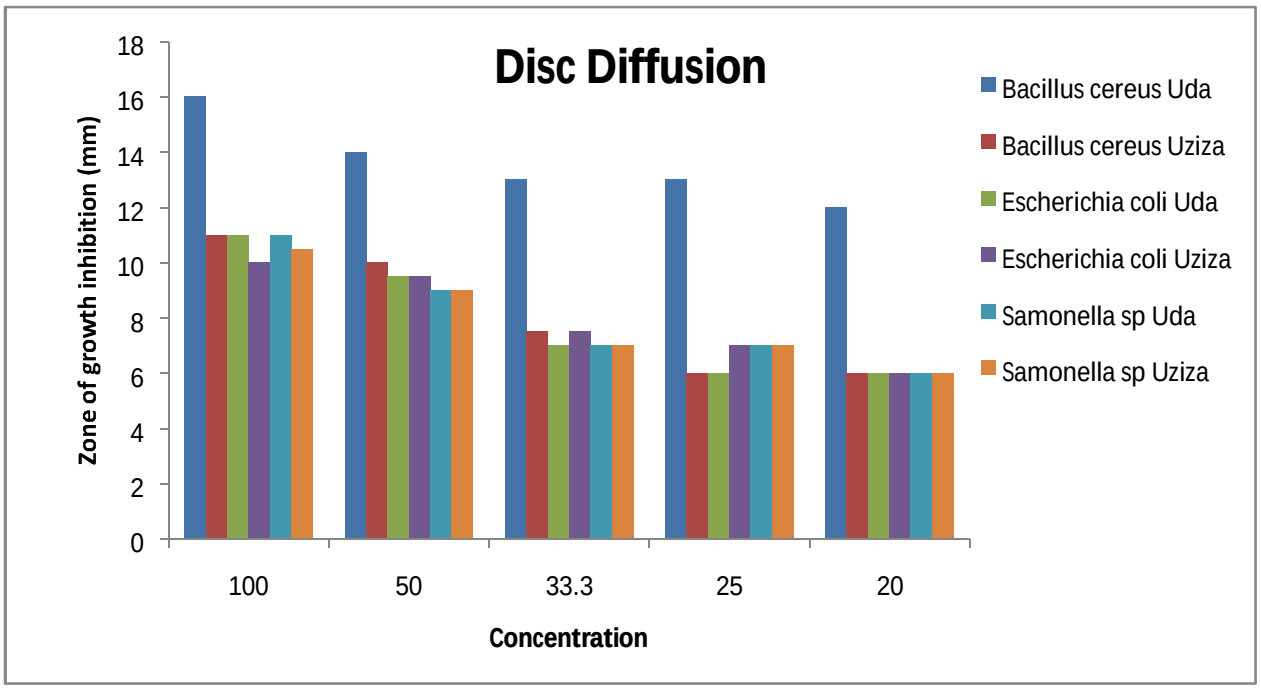

Figure 4.2: Antimicrobial Susceptibility Testing of Isolates using the Disc Diffusion Method

4.2. DILUTION SUSCEPTIBILITY TESTING (DST)

Dilution susceptibility testing (DST) has proven to be better protocol for working with essential oils than the disc diffusion or the well in agar diffusion method (Table 4.1). Results from the DST shows Minimum inhibitory concentration (MIC) values of 8 mg/ml for the essential oils on most tested isolates except *E. coli*, upon which the activity of *Piper guineense* produced 16mg/ml. MBC value range of 1632mg/ml was recorded for the essential oils on the tested isolates. As stated earlier, insolubility of active compounds in water or the presence of inhibitors to the antimicrobial components also play major roles in the activity of essential oils (Okigbo and Ogbonnanya, 2006).

Figures 4.3-4.5 show the graphs of % inhibition and linear % inhibition against time. The results show that the % inhibition increased with increase in concentration of the extracts. This further justifies the results obtained from the DST.

Table 4.1. MINIMUM INHIBITORY CONCENTRATION OF *PIPER GUINEENSE* AND *XYLOPIA AETHIOPICA* AGAINST SELECTED ISOLATES

Extract codes	MIC(mg/ml)	MBC(mg/ml)
BUZ(OD)	8	16
EUZ(OD)	16	32
SUZ(OD)	8	32
EUD(OD)	8	32
SUD(OD)	8	16
BUD(OD)	8	32

From table 4.1 above, it shows that the MIC value of 8mg/ml was common in BUZ, SUZ, EUD, SUD and BUD except for EUZ which has MIC of 16mg/ml. The table also shows that the MBC value of 32 mg/ml was similarly common on EUZ, SUZ, and BUD except for BUZ and SUD which has MBC values of 16mg/ml.

KEY:

BUZ: *Bacillus cereus Piper guineense*

EUZ: *Escherichia coli Piper guineense*

SUZ*: Salmonella spp Piper guineense*

EUD: *Escherichia coli Xylopia aethiopica*

SUD: *Salmonella spp Xylopia aethiopica*

BUD: *Bacillus cereus Xylopia aethiopica*

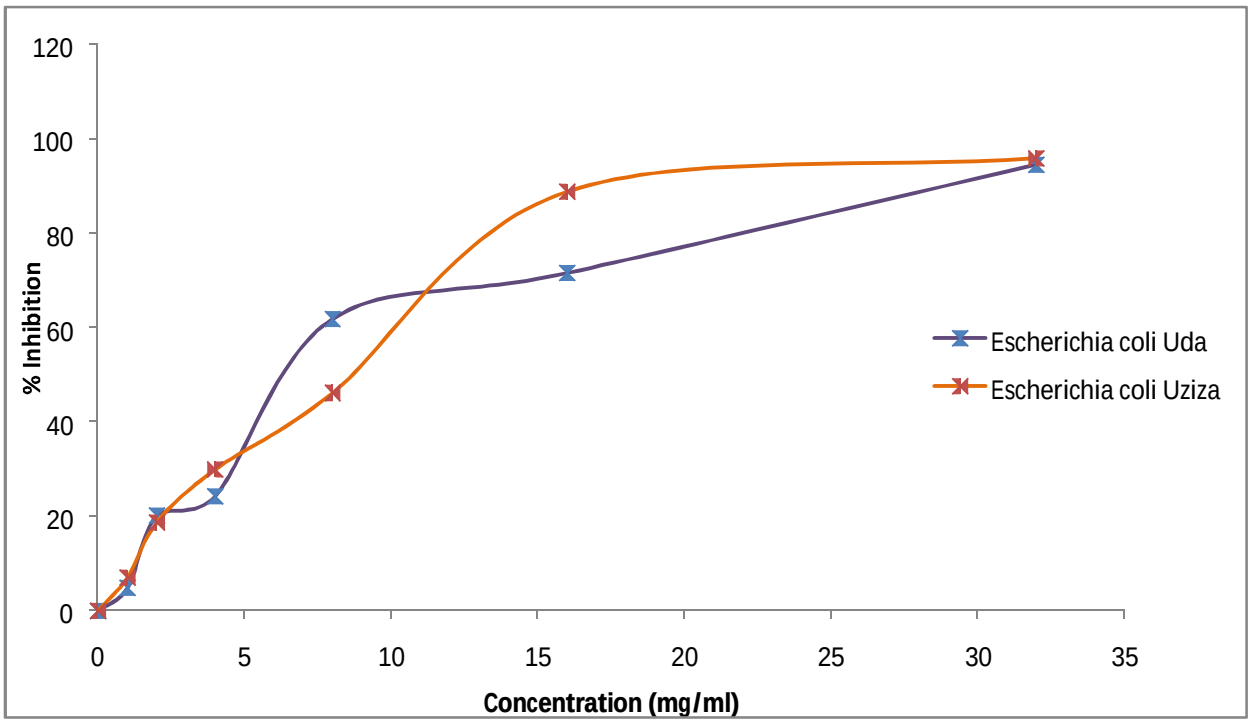

Figure 4.3: Graph of % Inhibition against Concentration Showing Activity of *Piper guineense* and *Xylopia aethiopica* against *Escherichia Coli.*

From the graph 4.3 above, it shows that *Piper guineense* and *Xylopia aethiopica* has a positive effect on *E.coli*. The graph shows that as the concentration of the extracts increases, the % inhibition increases. The maximum % inhibition was achieved at 94% at concentration of 32mg/ml for *Xylopia aethiopica and* 96% at a concentration of 32mg/ml for *Piper guineense* respectively.

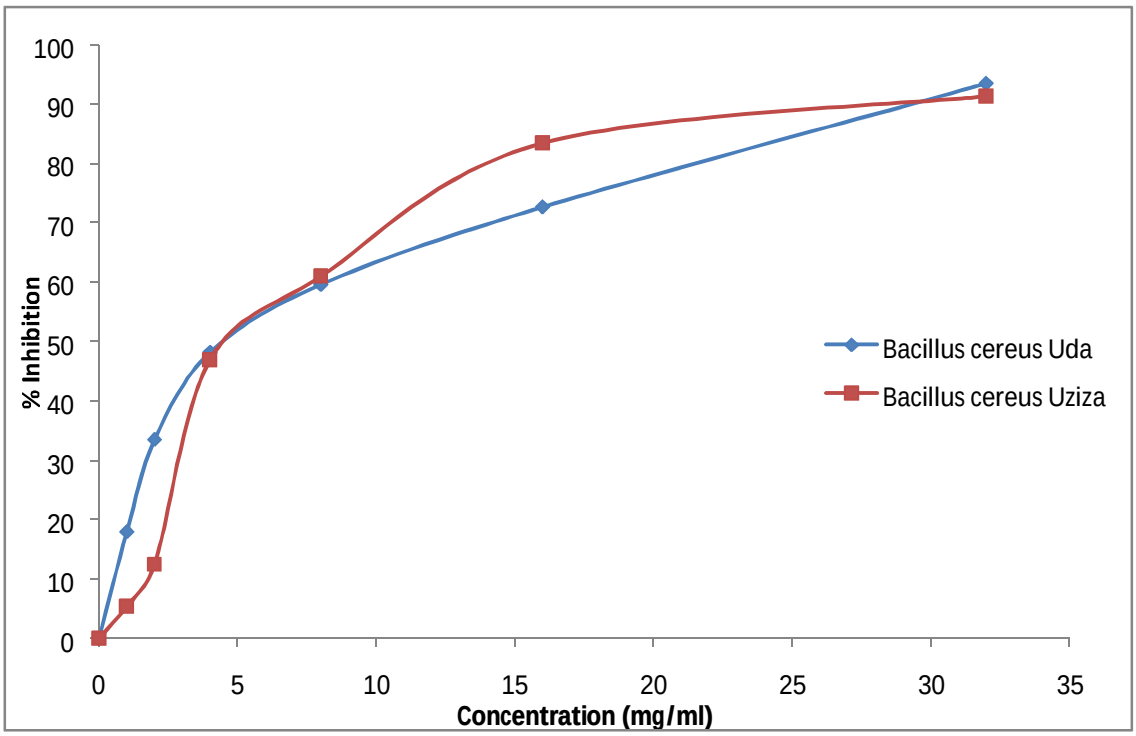

Figure 4.4: Graph of % Inhibition against Concentration Showing Activity of *Piper guineense* and *Xylopia aethiopica* against *Bacillus cereus.*

From the graph 4.4 above, *Piper guineense* and *Xylopia aethiopica* also exhibited antimicrobial property on *B. cereus.* The inhibition increases as the concentration increases up to a maximum of 32mg/ml thereafter.

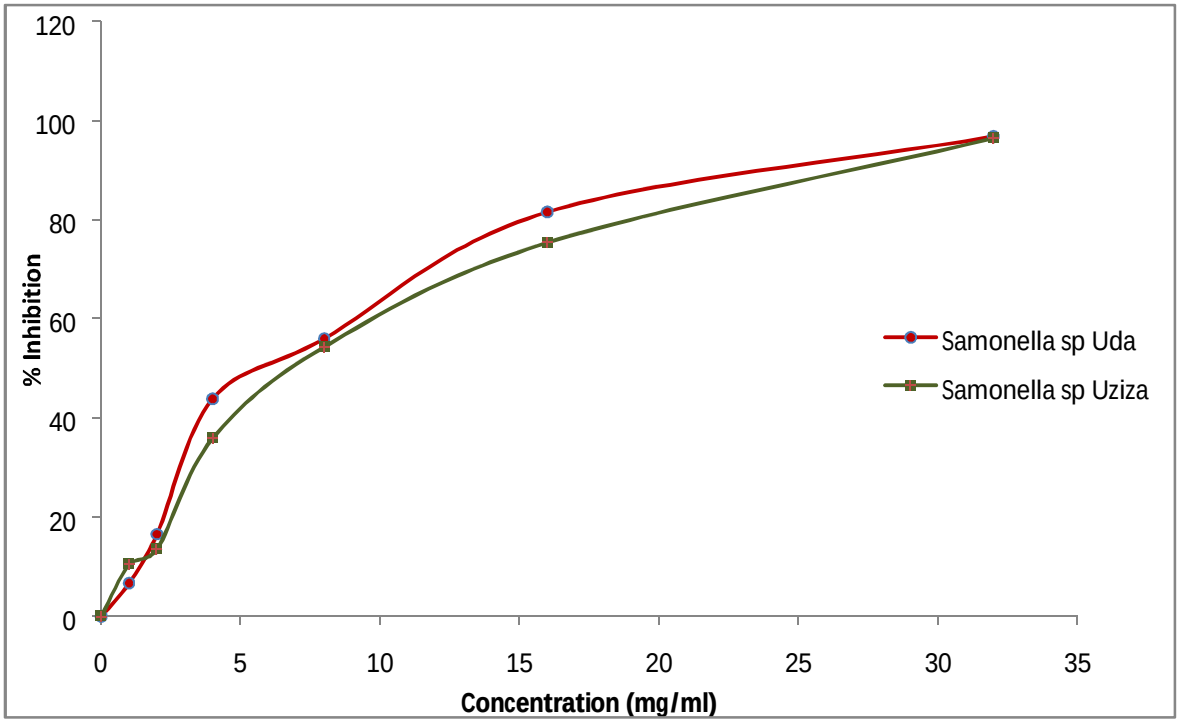

Figure 4.5: Graph of % Inhibition against Concentration Showing Activity of *Piper guineense* and *Xylopia aethiopica* against *Salmonella spp.*

Graph 4.5 above shows that *Piper guineense* and *Xylopia aethiopica* both had antimicrobial effect on *Salmonella spp*. The inhibition increased with increase in concentration. As the concentration increases, the activity of the organism reduced.

4.3. ANTIFUNGAL ACTIVITY OF *PIPER GUINEENSE* AND *XYLOPIA AETHIOPICA* AGAINST SELECTED MOLDS

Table 4.2 below shows the result of the antifungal activities. The results that the extract of *Piper guineense* and *Xylopia aethiopica* inhibited the growth of the molds. The activity of *Piper guineense* was higher than that of *Xylopia aethiopica.* The activity of the molds showed proportionality with the concentration of the spices. Previous research has tested the plant material against *Candida albicans* and has shown to have inhibitory activities (Ngane *et al*., 2003; Tatsadjieu *et al*., 2003). The activity of *Xylopia aethiopica* may be affected by viscosity and diffusibility (Tatsadjieu *et al*., 2003).

Piper guineense extract at ration 1.1 exhibited total inhibition on the growth of all tested molds. At ratio 1.2, *Piper guineense* extract similarly inhibited the growth of the molds for *Penicillium spp* and *Rhizopus stolonifer* but inhibited fairly lower % of growth of *Aspergillus spp* (70-90). At ratio 1.3, % inhibition of growth for the molds was between (70-90). At ratio 1.4, similar level of inhibition of growth was observed for *Aspergillus spp Penicillium spp Rhizopus stolonifer.*

Table 4.2 also shows the antifungal activity of *Xylopia aethiopica* extract. At ratio 1.1, *Xylopia aethiopica* extract inhibited between 70-90 % of *Aspergillus spp* and *Rhizopus stolonifer* and 30-60% for *Penicillium spp*. At ratio 1.2, the inhibition of growth followed similar trend. At ratio 1.3, Inhibition of growth reduced to 3060% in *Aspergillus spp* and *Rhizopus stolonifer* and lowest 10-20% for *Penicillium spp*. Similar observation was also made at ratio 1.4. The result showed that *Piper guineense* extract was more effective for controlling the growth of these molds at higher concentration.

Table 4.2: ANTIFUNGAL ACTIVITY OF *PIPER GUINEENSE AND XYLOPIA AETHIOPICA* AGAINST SELECTED MOLDS

s/n	Mold	Plant extract	Antifungal activity			
			1:1	1:2	1:3	1:4
1	*Aspergillus spp*	*PIPER GUINEENSE*	100	90	80	80
		XYLOPIA AETHIOPICA	80	80	60	40
2	*Penicillium spp*	*PIPER GUINEENSE*	100	100	90	80
		XYLOPIA AETHIOPICA	40	30	20	20
3	*Rhizopus stolonifer*	*PIPER GUINEENSE*	100	100	90	80
		XYLOPIA AETHIOPICA	90	80	60	40

KEY:

100% = No growth or formation of hyphae after 7 days

70-90% = early formation of hyphae, no conidiophores or basidiospores formation Inhibited

30-60% = the appearance of the spores and late formation of spores.

10-30% = Partial deviation to normal growth observed in pigmentation of the spores on the growth media.

0% = growth was normal in the presence of the plant material.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

From the data obtained from this research work, the following conclusions are made.

- The antimicrobial activity of the spices increased with increase in concentration.
- The spices had lower activities on *E. coli* both having a zone of inhibition of 9 mm at 100% concentration. *E. coli* was not sensitive to the activities of the extracts at concentrations lower than or equal to 33.33% of the spice.
- *B. cereus* was more susceptible to the activity of both spices with a zone of inhibition (diameter) of 19.5 mm and 18.0 mm for *Piper guineense* and *Xylopia aethiopica* respectively at 100% concentration.
- Similar activity with well in agar was recorded using the disc diffusion method. Although, results obtained showed lower sensitivity compared to the well-in-agar diffusion method.
- Results from the DST shows Minimum inhibitory concentration (MIC) value of 8mg/ml for the essential oils on most tested isolates except *E. coli*,

upon which the activity of *Piper guineense* had 16mg/ml MIC. MBC value range of 16-32mg/ml was recorded for the essential oils on the tested isolates.

- The oils had microbial activities against the molds with the activity of *Piper guineense* higher than that of *Xylopia aethiopica*.

5.2 RECOMMENDATIONS

Upon proper evaluation of their organoleptic properties of the essential oils of *Piper guineense* and *Xylopia aethiopica* the following recommendations are made.

- The information obtained from this work may be useful in food preservation. Further studies should be done on application of theses extracts in certain food processes like preservation of ogi, meat and fruit juices.

- The antibacterial properties of essential oils and their components should be exploited in such diverse commercial products as dental root canal sealers, antiseptics and feed supplements for lactating sows and weaned piglets.

  More work is therefore needed to explore these possibilities.

5.3 CONTRIBUTION TO KNOWLEDGE

The study has generated useful information on the use of these extracts (*Piper guineense* and *Xylopia aethiopica*) in controlling the growth of moulds. The information will also be useful in essential oil value chain activities.

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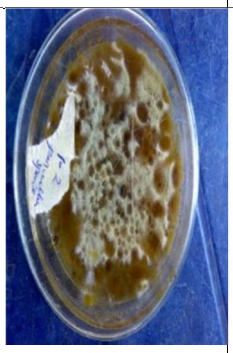
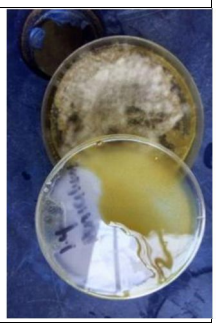
APPENDIX 1

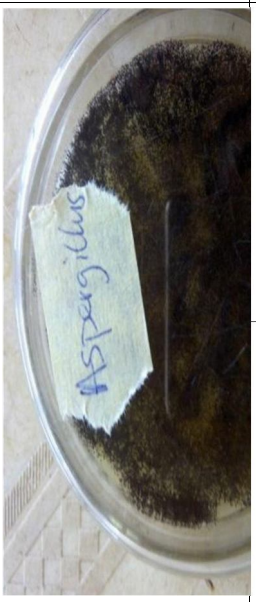
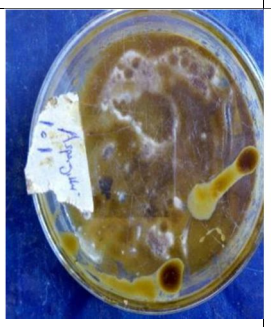
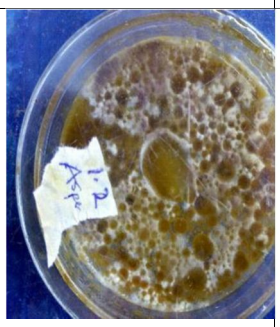
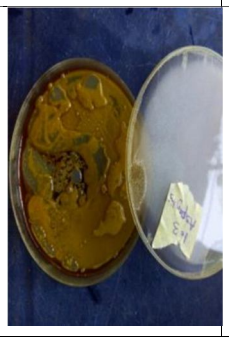
CONCENTRATIONS							
Concentration	0	0.1	0.2	0.4	0.8	1.6	3.2
BUZ(OD)	0.241	0.228	0.211	0.128	0.094	0.04	0.021
% Inhibition	0	5.394191	12.44813	46.88797	60.99585	83.40249	91.28631
EUZ(OD)	0.284	0.264	0.231	0.199	0.153	0.032	0.012
% Inhibition	0	7.042254	18.66197	29.92958	46.12676	88.73239	95.77465
SUZ(OD)	0.228	0.204	0.197	0.146	0.104	0.056	0.008
% Inhibition	0	10.52632	13.59649	35.96491	54.38596	75.4386	96.49123
EUD(OD)	0.288	0.274	0.23	0.218	0.11	0.082	0.016
% Inhibition	0	4.861111	20.13889	24.30556	61.80556	71.52778	94.44444
SUD(OD)	0.223	0.208	0.186	0.125	0.098	0.041	0.007
% Inhibition	0	6.726457	16.59193	43.94619	56.05381	81.61435	96.86099
BUD(OD)	0.245	0.201	0.163	0.127	0.099	0.067	0.016
% Inhibition	0	17.95918	33.46939	48.16327	59.59184	72.65306	93.46939

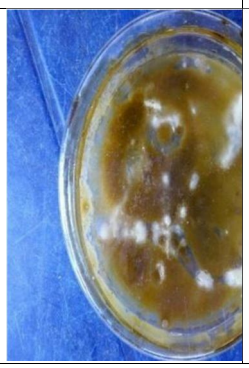
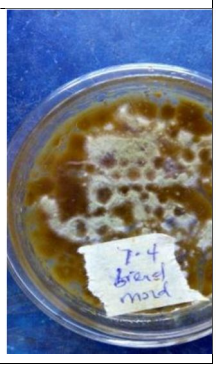
The percentage inhibition was calculated using

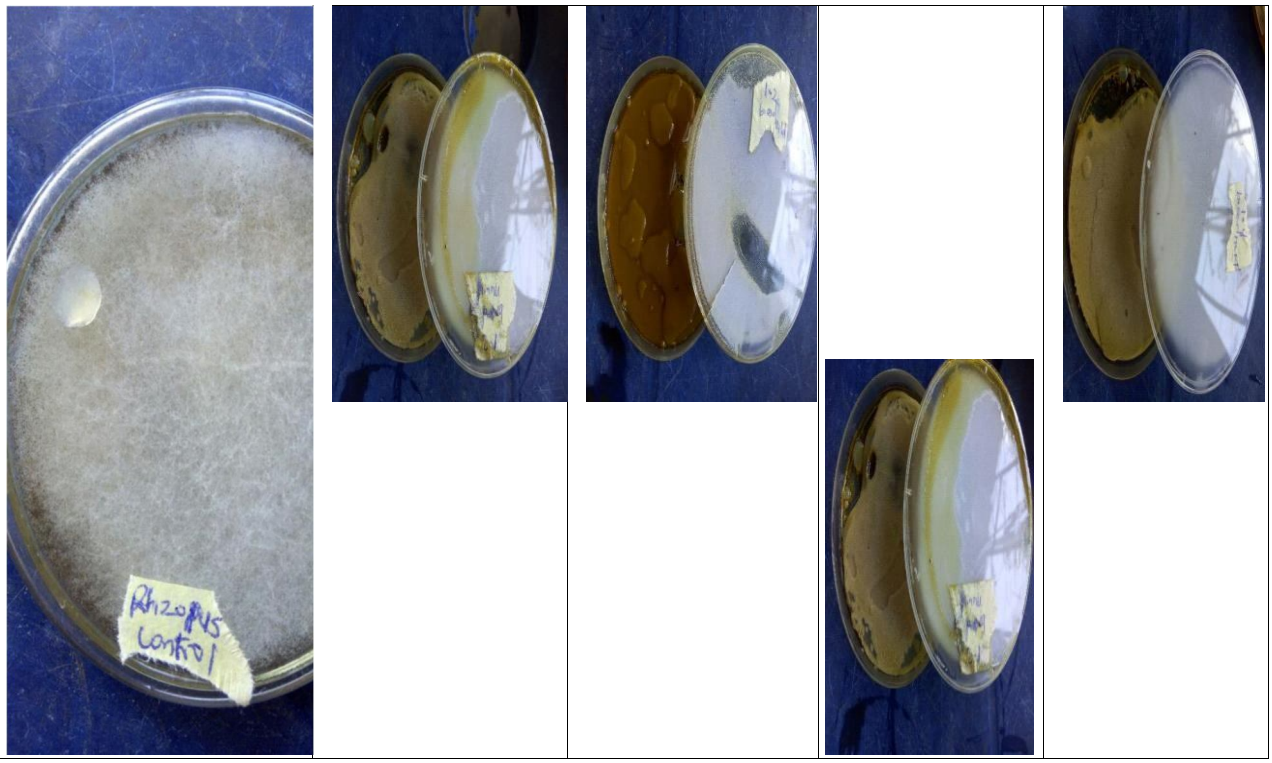
$$\% \text{ inhibition} = \frac{OD_{control} - OD_{test} \times 100}{OD_{control}}$$

APPENDIX 2

CONTROL	1:1	1:2	1:3	1:4
				
				

CONTROL	1:1	1:2	1:3	1:4
	 	 	 	 

CONTROL	1:1	1:2	1:3	1:4
				



APPENDIX 3

Disc diffusion

Zone of growth inhibition (mm)	concentration				
	100	50	33.3	25	20
Bacillus cereus Xylopia aethiopica	16	14	13	13	12
Bacillus cereus Piper guineense	11	10	7.5	6	6
Escherichia coli Xylopia aethiopica	11	9.5	7	6	6
Escherichia coli Piper guineense	10	9.5	7.5	7	6
Samonella sp Xylopia aethiopica	11	9	7	7	6
Samonella sp Piper guineense	10.5	9	7	7	6

APPENDIX 4

Well in agar method

Zone of growth inhibition (mm)	concentration				
	100	50	33.3	25	20
Bacillus cereus Xylopia aethiopica	18	17	16	15	14
Bacillus cereus Piper guineense	19.5	19.5	18	17.5	17.5
Escherichia coli Xylopia aethiopica	9	7	6	6	6
Escherichia coli Piper guineense	9	7	6	6	6
Samonella sp Xylopia aethiopica	10	8	7	6	6
Samonella sp Piper guineense	11	9	7	6	6