EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF *ALLIUM SATIVUM* LINN. (GARLIC) AND *ZINGIBER OFFICINALE* ROSCOE (GINGER) AGAINST SOME BETA-LACTAMASE PRODUCING RESPIRATORY TRACT PATHOGENS

BY

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BEING A DISSERTATIONSUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, BAYERO UNIVERSITYKANO, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (MSc.) DEGREE IN MICROBIOLOGY(MEDICAL)

SUPERVISOR
DR. NASIR TUKUR DABO

DECLARATION

I hereby declare that this work is the product of my own research efforts, undertaken under the supervision of Dr. Nasir Tukur Dabo and has not been presented anywhere for the award of a degree or certificate. All sources have been duly acknowledged.

_____

Signature & Date

Aisha Abdulhameed Saeed

SPS/11/MBI/00020

CERTIFICATION

This is to certify that the research project titled **EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF *ALLIUM SATIVUM* LINN. (GARLIC) AND *ZINGIBER OFFICINALE* ROSCOE (GINGER) AGAINST SOME BETA-LACTAMASE PRODUCING RESPIRATORY TRACT PATHOGENS** by Aisha Abdulhameed Saeed (SPS/11/MBI/00020) meets the regulations governing the award of the degree of MSc. Microbiology of Bayero University Kano, and is approved for its contribution to knowledge and literary presentation.

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APPROVAL

This dissertation has been read, examined and approved by the undersigned as meeting the requirement for the award of Master of Science Degree in Microbiology (Medical) of Bayero University, Kano, Nigeria.

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DEDICATION

I dedicate this research project to My Husband.

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Abstract

Dried rhizomes of *Zingiber officinale*(ginger) and *Allium sativum*(garlic)bulbs were extracted in three different solvents (Ethanol, Chloroform and Water) using soxhlet and percolation methods. Only the aqeous fraction was extracted using percolation. The extracts were screened for the presence of biologically active phytochemicals using standard procedures. Antibacterial activities of the crude extracts against some β-lactamase producing respiratory tract bacteria were tested using agar disc diffussion, agar well diffussion and bioautography methods. The organisms used were *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *proteus spp.* All fractions of the two plants tested positive for alkaloids. Saponins were present in all ethanolic and chloroform extracts of the plants, but absent in the aqeous extract. Tannins were present in only the aqeous fractions of both plants. Flavonoids were detected in only the ethanolic and chloroform extracts of garlic and all of the the extracted plant material tested positive for reducing sugars. Results of the bioassay (agar disc and agar well diffussion) using the crude extracts indicated that all the organisms were resistant to all the plant extracts at concentrations of 25µg, 50 µg, 100µg and 200µg/disc. Same concentrations were used for the well diffussion method. Bioautography studies revealed that some of the compounds in each of the six plant extracts separated using thin layer chromatography (TLC) had inhibitory activities against the test organisms. Therefore, results of the bioautography suggests that garlic and ginger have compounds that have the potential for antibacterial activity if used in higher concentrations.

CHAPTER ONE

1.0 INTRODUCTION

1.1 The Use of Plants in Traditional Medicine

Medicinal plants are natural resources for valuable products that can be used in the treatment of various ailments or may provide leads for the development of new antimicrobials.Many medicinal plants produce antimicrobial principles against pathogens, therefore highlighting the importance of search for natural antimicrobial drugs (Mothana and Lindequist, 2005, Bajpai *et al*., 2005; Wojdylo *et al*., 2007). The Use of Medicinal Plants to treat ailment associated with pains is well known throughout history (Ernst and Pittler, 2000).Historians from all around the world have produced evidence to show that apparently all primitive people used plants often in a sophisticated way. Quinine fromCinchona bark was used to manage the symptoms of malaria long before the disease was identified (Raphael, 2011).

By the middle of the 19th century at least 80 of all the medicines were derived from plants. Moreover, today many pharmaceutical classes of drugs include a natural product prototype (Gilani *et al.,* 2000). Traditional medical practice has been knownfor centuries in many parts of the world. It is, however, observed that these practicesvary from one country to another. Numerous plantsand herbs are used all over Nigeria by traditionalmedicine practitioners. The use of herbs is the mostancient approach to healing known. The herbalmedicines may be in form of powders, liquids, ormixtures, which may be raw, or boiled. Roots, barks,and leaves of various plants are employed inethno-medicine. Plant extracts are given singly or asconcoctions for various ailments.

According to Gregory (2004), Traditional medicine has given us very useful synthetic clues of modern drugs in the past (Table 1). Most of these plant derived drugs were originally discovered through the study of herbal cures and folk knowledge of traditional people and some of these could not be substituted despite the enormous advancement in synthetic chemistry (Gilani *et al*., 2005).

Table 1: Few examples of plants derived modern drugs

Active ingredients	Botanical source
Aspirin	Willow bark
Atropine	Belladonna
Capsaicin	Pepper plant
Colchicine	Autumn crocus
Digitalis	Fox glove
Morphine	Opium poppy
Pilocarpine	Jaborandi tree
Podophyllin	May Apple
Quinine	Chinchona bark
Reserpine	Indian snake root
Taxol	Pacific yew tree bark

1.2 Justification of the study

Infections with multi-drug resistant Gram negative pathogens impose a significant and increasing burden on both patients and healthcare providers. Infection with an extended spectrum β-lactamase producing pathogen particularly *Escherichia coli* or *Klebsiella pneumoniae*, is associated with greater mortality and delay in treatment

compared to infection due to organisms that are not β-lactamase producing (Braykov *et al.,* 2013). Ho *et al.* (2002) noted that resistance to β-lactam antibiotics among clinical isolates of Gram negative bacteria is on the rise worldwide.Increase in multi-drug resistant strains of bacterial pathogens necessitates the need for the scientific search to find alternative compounds with minimal side effects for treatment of infectious diseases. Thus *Allium sativum* Linn(Garlic)and *Zingiber officinale* Roscoe(Ginger) reported as medicinal plants were selected for their reported antimicrobial activity.

1.3Aim and objectivesof the study

The aim of this study was to evaluate the antibacterial activity of *A. sativum* Linn (Garlic) and *Z. officinale* Roscoe (Ginger) against some β-lactamase producing respiratory tract bacterial isolates. The following were the objectives;

1. To extract the plant materials using three solvents-Ethanol, Chloroform and Water.
2. To determine the Phytochemical properties of the extracts.
3. To assess the antibacterial activity of the plants extracts against β-lactamase producing respiratory tract bacterial isolates using:
    i. Agar disc diffussion
    ii. Agar well diffussion
    iii. Bioautography (TLC).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Literature Review

Herbs and spices are very important and useful as therapeutic agents against many pathological infections (Gull *et al*., 2012). Today, a majority of the world's population resort to one form of phytomedicine or the other as their first line of treatment for various diseases (Pandy *et al*., 2011). Various parts of plants have been shown to have remarkable lethal effect on both prokaryotic and eukaryotic organisms and therefore they have been employed in the treatment of diseases caused by such organisms (Joshi *et al*., 2011; Shobana *et al*., 2009).Several reports had been published that describe the antibacterial and antifungal properties of different herbs and spices. However, still there is little information about the exact mechanism of their antimicrobial action (Gur *et al*., 2006; Yusha'u *et al*., 2008; Belguith *et al.,* 2010; Yin *et al*., 2002; Oskay *et al*., 2009 and Poeloengan, 2011)

Allium sativum Linn.(Garlic) is one of those plants that was seriously investigated over the years. It has been used for centuries to fight infections (Onyeagba *et al*., 2006). The early Egyptians used it to treat diarrhoea, the ancient Greeks used it to treat intestinal and extraintestinal diseases, while the ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever. In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhoea, otitis media and respiratory tract infections (Ankri and Mirelman, 1999; Jaber and Al-Mossawi, 2007). The phytochemical constituents of garlic have been established in previous studies (Farbman *et al*., 1993; Cavallito and Bailey, 1994; Ankri and Mirelman, 1999). The

antimicrobial properties of garlic were first described by Louis Pasteur (Adetumbi and Lau., 1983) and since then, research had demonstrated its effectiveness against bacteria, protozoa, fungi and some viruses (Jaber and Al-Mossawi, 2007). The ability of garlic to inhibit the growth of both Gram-positive and Gram negative bacteria shows that it has a broad spectrum of activity and can be used for formulation of newer broad spectrum antibacterial substances (El-Mahmood, 2009). The antimicrobial activity of spices is due to specific phytochemicals or essential oils (Avato *et al*.,2000). The phytoconstituents of garlic have long been known and its antimicrobial properties have been widely reported (Roy *et al*., 2006).

A.sativum contains at least 33 sulfur compounds, several enzymes and the minerals germanium, calcium, copper, iron, potassium, magnesium, selenium and zinc; vitamins A, B1 and C, fiber and water. It also contains 17 amino acids: lysine, histidine, arginine, aspartic acid threonine, swine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tryptophan and phenylalanine (Josling, 2005). It has a higher concentration of sulfur compounds than any other Allium species which are responsible both for garlic's pungent odor and many of its medicinal effects. One of the most biologically active compounds in garlic is allicin (diallyl thiosulfinate or diallyldisulfide). Its antibacterial activity is mainly due to the presence of allicin produced by the enzymatic activity of allinase on alliin..Allicin has been found to be the active ingredient in garlic and it works as an antimicrobial agent by inhibiting DNA and protein synthesis moderately and inhibiting RNA synthesis completely as a primary target (Shobana *et al*., 2009; Rahman *et al*., 2011). Garlic is also rich in anionic components such as nitrates, chlorides and sulfates and other water soluble components found in plants and these components may have antimicrobial

properties (Shobana *et al*., 2009).Allicin is considered to be the most potent antibacterial agent in crushed garlic extracts, but it can be unstable, breaking down within 16 h at 23°C (Hahn, 1996), Typical garlic food preparation such as chopping, mincing and crushing disturbs S-allyl cysteine sulfoxide and exposes it to the allinase enzymes, then quickly converts it to diallyl thiosulfinate, which give off garlic's characteristic aroma. The allinase enzyme responsible for diallyl thiosulfanate conversion becomes inactivated below a pH of 3.5 or with heating (Pedrazza-Chaverri *et al*., 2006). Although allicin is considered the major antioxidant and free radical scavenging compounds, recent studies showed that other compounds may play stronger roles; such as polar compounds of phenolic and steroidal origin, which offer various pharmacological properties without odor and are also heat stable (Lanzotti, 2006).

Garlic extract inhibits the growth of Gram positive and Gram negative bacteria, such as *Staphylococcus, Streptococcus, Micrococcus*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pseudomonas*, *Shigella, Salmonella, Proteus*, and *Helicobacter pylori* (Tsao and Yin, 2001). Previous authors have described the antibacterial activity of garlic extract against microorganisms. Bulbs belonging to the Allium genus had the most antibacterial activity against *Streptococcus mutans* (Ohara *et al*., 2008) and against *Streptococcus agalactiae* (Alsaid *et al*., 2010). In addition, garlic was shown to have antimicrobial activity against *Streptococcus olaris, Streptococcus mitis, Staphylococcus aureus* (Silva and Fernandes, 2010; Daka, 2011); *Escherichia coli, Salmonella typhi, Shigella flexineri, Proteus mirabilis* (Shobana *et al*., 2009); and *Vibrio parahaemolyticus, Escherichia coli* and *Staphylococcus aureus* (Venugopal*et al*, 2009). Few studies have shown some bacteria to be resistant towards

garlic extract and these include *Escherichia coli* and *Staphylococcus aureus* (Esimone *et al*., 2010).

Fresh Rhizomes of *Z.officinale* has been used for the treatment of cold-induced diseases, nausea, asthma, cough, colic, heart palpitation, swelling, dyspepsia, less of appetite, and rheumatism, in ancient china (Foster, 2000). In the nineteenth centuryginger served as a popular remedy for cough and asthma when the juice of fresh ginger was mixed with a little juice of fresh garlic and honey (Foster, 2000). A paste of powdered dried ginger was applied to the temples to relieve headache and fresh ginger was mixed with a little honey, tapped off with a pinch of burnt peacock feathers to alley nausea. One modern government health guide suggests one to two tea spoons of ginger juice with honey as a cough suppressant (Tyler, 2002).*Z.officinale* inhibits the growth of *Escherichia coli*, *Proteus sp*, *Staphylococci*, *Streptococci* and *Salmonella*. Ginger has been traditionally exploited for having broad range of antimicrobial activity against both Gram positive and Gram negative bacteria and fungi. *In vitro* studies have shown that active constituents of ginger inhibit multiplication of colon bacteria. These bacteria ferment undigested carbohydrates causing flatulence, this can be counteracted with ginger (Gupta and Ravishankar, 2005). White (2007) also stated that ginger inhibits the growth of *Escherichia coli, Proteus sp, Staphylococci, Streptococci* and *Salmonella*. Ginger has strong antibacterial activity and to some extent antifungal properties (Nielsen and Rios, 2000). Ginger inhibits *Aspergillus sp*, a fungus known for the production of aflatoxin, a carcinogen. Fresh ginger juice showed inhibitory action against *Aspergillus niger, Sacharomyces cerevisiae, Mycoderma sp.* and *Lactobacillus acidophilus* (Nanir and Kadu, 1987). Ginger which is a normal ingredient of our routine food preparations can

provide protection against our natural enemies like bacterial and fungal pathogens.In ginger, the gingerol related components have been found to have antimicrobial activities (Rahman *et al*., 2011). There are several reports of the inhibitory effect of ginger in the form of extract against several bacteria (Nanasombat *et al*., 2005; Joe *et al*., 2009; Patel *et al*., 2011). Moderate to good antimicrobial properties of ginger were shown in previous studies (Ibrahim *et al*., 2003; Singh *et al*., 2008).

However, some studies have reported ginger as having weak antimicrobial effects (Indu *et al*., 2006; Eruteya and Odunfa, 2009; Esimone *et al*., 2010; Silva and Fernandes, 2010). Similar results were reported where ginger did not show any antibacterial activity against the multi-drug resistant bacteria viz: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella Typhi* (Adeshina *et al*., 2011). Study by Venugopal *et al*. (2009) also showed no antibacterial activity of ginger to *Vibrio parahaemolyticus, Escherichia coli* and *Staphylococcus aureus*.

2.2 Biology, classification and uses of the plants studied

2.2.1 *Allium sativum*Linn (Garlic)

*Allium sativum*Linn (Garlic) in the family Liliaceae is a perennial bulb-forming plant. It is known world-wide, andfor several centuries, it has been used for dietary andmedicinal purposes (Ross *et al*., 2001). The leaves of the plant are usedas spice and as medicine in West Africa.Several studies have confirmed that garlic hasantimicrobial properties (Eja *et al.*, 2007).*A.sativum*is a condiment, which for several years has been in India, Egypt and China for its medicinal purposes. It has been used for conditions like fever, cough, digestive disorders and respiratory diseases like tuberculosis. Garlic has also been implicated to alleviate some of the risk factors

associated with nicotine induced hyperglycemia and hypercholesteremia (Effraim *et al.*, 2000). Chemical analysis of garlic cloves have revealed an unusual concentration of sulfur-containing compounds (1–3%). The phytochemical constituents of garlic have been established inprevious studies. The antimicrobial activity of spices is due to specific phytochemicals or essential oils. The main factors that determine the antimicrobial activity are the type and composition of the spice, amount used, type of microorganism, composition of the food, pH value and temperature of the environment (Avato *et al*., 2000).

Botanical classification of garlic according to Rajsekhar *et al*. (2012).

Kingdom - Plantae

Class – Angiosperms

Order – Asparagales

Family – Amaryllidaceae

Sub-family - Allioideae

Genus – *Allium*

Species – *A. sativum*

Local name- Tafarnuwa(Hausa), Aayu (Yoruba) andAyo-ishi (Igbo)

*Allium sativum*bulbs

2.2.2*Zingiber officinale*Roscoe (Ginger)

Ginger is a member of the family Zingiberaceae; a family with more than 45 genera, and 800 species (Newall *et al,* 1996). Its scientific name is *Zingiber officinale* named by an English botanist William Roscoe (1753 – 1831) in 1807 (Foster, 2000). Ginger has a wide range of action on the human body and has been found effective in the treatment of cataract, heart disease, migraines, stroke, amenorrhea, athlete's foot, bursitis, chronic fatigue, cold, flu, coughs, depression, dizziness, fever, erectile difficulties, kidney stones, Reynard's disease and viral infection. Ginger has also been historically used to treat inflammation which several scientific studies support (Auta *et al*., 2011). In Venezuela, ginger is pounded into a paste and applied to the abdomen for difficult menstruation. In Costa Rica, it is used in a decoction to relieve throat inflammation and asthma with the addition of honey,it is a valued remedy for coughs and bronchitis and also serves as a sudorific in fever. Its natural diuretic stimulatesthe kidney to flush out toxins faster. In Panama, it is used to relieve rheumatism. In Guatemala and Trinidad, it is the best remedy for stomach ache, malaria and indigestion, the fumes from an infusion in urine are inhaled to relieve head colds(Auta *et al*., 2011). Indian traditional medicinal remedies especially for cough and asthma consists of juice of fresh ginger with a little juice of fresh garlic mixed with honey. Besides, ginger is very often used to cure many illness such as indigestion, tastelessness, loss of apetite, flatulence, nausea, vomiting, allergic reactions, acute and chronic cough, common cold, fever, sinusitis, acute chronic bronchitis e.t.c. Other medicinal uses include gastrointestinal relief, anti inflammatory effects, reduction of blood pressure, hypoglycemic and hyperglycemic activity, cancer prevention and larvicidal activity.

Botanical classification of ginger according to Rajsekhar *et al*., 2012.

Kingdom – Plantae

Class – Angiosperms

Order – Zingiberales

Family – Zingiberaceae

Genus – *Zingiber*

Species – *Z. Officinale*

Local name- Citta (Hausa), Ata ile (Yoruba) and Jinja (Igbo).

*Zingiber officinale*rhizomes

2.3The β-lactamase producing Bacteria

Drug resistance to pathogenic microorganisms has been commonly reported worldwide. Antibiotic resistance refers to the ability of a microorganism to withstand the effects of an antibiotic. The increasing frequency of microorganisms that are resistant to common and generally accepted antibiotics is on the increase. Furthermore, the rate of resistance to these drugs is higher in developing countries as compared to developed countries because of extensive and indiscriminate use of

antibiotics over the last few decades (Akram *et al*., 2007) and people's ability to self-medicate without a prescription from a physician. Among the wide array of antibiotics, β-lactams are the most varied and widely used (Bronson and Barrett, 2001). The most widespread cause of bacterial resistance to β-lactam antibiotics like penicillin is the production of enzymes called β-lactamases.β-lactamases are a family of enzymes produced by many Gram positive and Gram negative bacteria that inactivate β-lactam antibiotics by opening the β-lactam ring.The β-lactam ring is part of the core structure of several antibiotic families, the principal ones being the penicillins, cephalosporins, carbapenems and monobactams which are also called β-lactam antibiotics. Nearly all these antibiotics work by inhibiting bacterial cell wall biosynthesis. This has a lethal effect on bacteria. Bacteria can exhibit resistance by expressing one of many β-lactamase genes. More than 1,000 different β-lactamase enzymes have been documented in various species of bacteria (Ehmann, 2012).

Classification of β-lactamases

There are two globally accepted classification schemes for β-lactamases. The first one is based on amino acid sequence classification and the second is based on functionality. Ambler divided β-lactamases into four classes (Class A-D)based on their sequence similarity in 1980. Classes A,C and D function by the serine ester hydrolysis mechanism, whereas Class B β-lactamases known as Metalloβ-lactamases have a zinc ion participating in catalysis (Ambler, 1980). The Classification scheme based on functionality gave rise to three major groups: Group 1 cephalosphorinases (Class C), Group 2 serine β-lactamases (Class A and Class D) and Group 3 metalloβ-lactamases (Class B), each of which is divided into several subgroups (Bush *et al*., 1995; Bush and Jacoby, 2010). The functionality based classes of the β-lactamases were determined according to their hydrolysis rates of some pre-defined drugs such

as EDTA and benzylpenicillin. Over 890 unique protein sequences of β-lactamases were reported by Bush and Jacoby by the end of 2009 (Bush and Jacoby, 2010). The table below shows a summarized classification of β-lactamases (Rubtsova et al., 2010).

Summarized classification of β-lactamases

Functional group	Subgroup	Molecular class	Main substrate	Pecularities of β-lactamase members
1	1	C	All groups of β-lactam antibiotics except carbapenems	Chromosome encoded AmpC β-lactamases, some plasmid encoded AmpC β-lactamases-not inhibited by clavulanic acid
2	2a	A	Penicillins	Penicillases of Gram positive bacteria- inhibited by clavulanic acid
	2b	A	Penicillins, cephalosporins	Broad spectrum β-lactamases (TEM-1, TEM-2, SHV-1)-inhibited by clavulanic acid
	2be	A	Penicillins, cephalosporins, monobactams	Extended spectrum β-lactamases (ESBL)- inhibited by clavulanic acid
	2bf	A	Penicillins	Inhibitor resistant β-lactamases of TEM and SHV types
	2c	A	Penicillins, carbenicillin	Carbenicillin hydrolyzing PSE type β-lactamases
	2e	A	Cephalosporins	Inducible cephalosporins from *Proteus spp.*- inhibited by clavulanic acid
	2f	A	Penicillins, cephalosporins, carbapenems	Serine carbapenamases- inhibited by clavulanic acid
	2d	D	Penicillins, oxacillin	OXA type β-lactamases hydrolyzing oxacillin- mainly inhibited by clavulanic acid
3	3a, 3b, 3c	B	Most β-lactams including carbapenems	Metallo β-lactamases- not inhibited by clavulanic acid but are inhibited by EDTA
4	Not determined		Penicillins	Penicillinases not belonging to other groups

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection, Handling and Extraction of the Plant Materials

The two test plants evaluated in this work were *Allium sativum* (Garlic) and *Zingiber officinale* (Ginger). These were purchased from Abubakar Rimi local market within Kano Municipal Local Government Area. The fresh samples of the plants were obtained, washed, cut into pieces, air-dried and made into powdered form by grinding (Fatope *et al*., 1993). This powdered form was used for extraction. Extraction was carried out using both soxhlet and percolation methods(Fatope *et al*., 1993). The solvents used include 100%Ethanol, 99 Chloroform and Distilled Water. Only the aqueos fraction was subjected to percolation.

3.2.1 Test for alkaloids

One (1) ml of each extract was placed in two separate test tubes. To the first test tube, 2 drops of Dragendoff's reagent was added while 2 drops of Meyer's reagent was added to the second test tube. The formation of an orange-red precipitate in the test tube in which Dragendoff's reagent was added or a white precipitate in the case of Meyer's reagent indicated the presence of alkaloids (Cuilei,1994).

3.2.2 Test for reducing sugars

This was carried out as described by Brain and Turner (1975). Fehling's solution was added to 1ml of the extract which was diluted with 2mls of distilled water and warmed. Development of a brick-red precipitate at the bottom of the test tube was indicative of the presence of reducing sugars.

3.2.3 Test for tannins

Two (2) mls of the extract was diluted in a test tube and 2 drops of Ferric Chloride ($FeCl_3$) solution was added. A change in colour to dark green or blue-black indicated the presence of tannins (Cuilei, 1994).

3.2.4 Test for flavonoids

Ten (10) mls of dimethyl sulphoxide (DMSO) was added to two (2) mlsof the extract. The mixture was heated followed by the addition of Magnesium metal and a few drops of concentrated Hydrochloric Acid. The presence of red colour was indicative of the presence of flavonoids (Sofowora, 1993).

3.2.5 Test for saponins

Two (2) mls of the extract contained in a test tube was vigorouly shaken. The presence of froth indicated the presence of saponins (Brain and Turner, 1975).

3.2.6 Test for amino acids

One (1) ml of the extract was treated with 2 drops of Ninhydrin reagent. Appearance of purple colour showed the presence of amino acids.

3.3Collection and Confirmation of the Bacterial Isolates

Four clinical bacterial isolates (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus sp.* and *Klebsiella pneumoniae*) were obtained from the Microbiology Department of Murtala Mohammed Specialist Hospital in Kano. The isolates were confirmed usingthe following biochemical testsas described by Cheesbrough(2004).

3.3.1 Indole test

The suspected test organisms were inoculated into bijou bottles containing 3 mls of sterile peptone water, and incubated at 37^oC for 48 hours. After incubation, 0.5 ml of Kovac's reagent was added and shaken gently. Appearance of a red colour in the surface layer within 10 minutes indicated a positive result.

3.3.2 Oxidase test

A filter paper was placed inside a petri dish, 2-3 drops of oxidase reagent was added. A colony of the test organism was smeared on the filter paper. Development of a blue-purple colour within 10 seconds indicates a positive oxidase test.

3.3.3 Citrate utilization test

The suspected organism was suspended in normal saline, streaked onto Simmon's citrate agar slope and then stabbed at the bottom. This was incubated at 35^{o}C for 48 hours, a change in colour to bright blue indicated a positive result.

3.3.4 Urease test

The test organisms were inoculated into bijou bottles containing 3 ml of sterile urea broth and incubated at 37^{o}C for 24 hours. After incubation, the bottles were observed for a pink colour which indicates a positive urease test.

3.3.5 Kligler Iron Agar (KIA) test

The test organism was streaked and stabbed on the KIA in a test tube. This was incubated at 37^{o}C for 24 hours. Change in the colour of the butt, slope, gas production ahd hydrogen sulphide production was observed.

3.3.6 Nitrocefin test

The β-lactamase producers were determined using the nitrocefin test,specifically with the Oxoid β-lactamase (nitrocefin) Touch Stick. Nitrocefin is a chromogenic cephalosporin which changes from yellow to red when its β-lactam ring is hydrolyzed by β-lactamase. The test was performed by touching a colony of the test organism with a stick and allowing it for a few minutes. A colour change from yellow to pink-red indicatedβ-lactamase production.

3.4 Bioassay Procedures

3.4.1 Agar disc diffussion

3.4.1.1 Preparation of sensitivity discs

The procedure of Kirby-Bauer (1966) was adopted. Discs of 6mm diameter were punched using Whatman No. 1 filter paper with the aid of a paper puncher and placed in Bijou bottles. The discs were thensterilized by autoclaving. Stock solutions of theplant extracts were prepared by dissolving 0.02g of each of the twoin 1ml of Dimethyl sulphoxide (DMSO). Each stock solution had a concentration of20,000µg/ml. This was prepared in duplicate.The first stock solution which was 20,000µg/ml was introduced into 100 sterile discs in bijou bottles to give a concentration of 200µg/disc.Each paper disc was capable of absorbing 0.01ml (Kirby-Bauer, 1966). Using the remaining stock, other concentrations were prepared using serial doubling dilution. Half (0.5ml) of the stock was added to 0.5ml of DMSO, this yielded 10,000µg/ml. This was introduced into 100 sterile discs in bijou bottles, which yielded disc potency of 100µg/disc. The same procedure was used to arrive at disc potencies of 50µg/disc and25µg/disc.

3.4.1.2Standardization of inoculum

This was carried out as described by Cheesbrough (2004), whereby 0.5 McFarland standard (barium sulphate suspension) was prepared by mixing 0.6ml of 1% w/v barium chloride with 9.94 ml of 1% v/v sulphuric acid. For the Standardization, a loopful of the colony from a 24hr culture of the test organism was inoculated into a test tube containing normal saline using a sterile wire loop until the turbidity of the suspension matches the turbidity of the 0.5 mcfarland standard as described by the National Committee for Clinical Laboratory Standards (2008).

3.4.1.3 Agar disc diffussion

The bioassay was carried out according to the procedure described by Cheesbrough (2004). Using a sterile swab stick, the test organism that was standardized was inoculated onto the surface of sterile Mueller Hinton agar which was allowed to

solidify. Using sterile forceps, prepared discs of different concentrations (i.e. 25µg/disc, 50µg/disc, 100µg/disc and 200µg/disc) for each of the Ginger and garlic extracts were then placed on the inoculated agar media. Each plant extract had a separate petri dish. The plates were incubated aerobically at 37^{o}C for 24hrs. Meropenem was used as control. The plates were observed for zones of inhibitions after overnight incubation.

3.4.2 Agar well diffussion

Stock solutions of the extracts were prepared by dissolving 0.002g of the extract in 1ml of DMSO. This yielded a concentration of 2000µg/ml. Different concentrations were prepared using the stock, 0.1ml of the stock was dissolved in 0.9ml DMSO. This gave a concentration of 200µg/ml. Half (0.5ml) of this was added to 0.5ml DMSO to give a 100µg/ml concentration. Same method was used to get concentrations of 50µg/ml and 25µg/ml.Thesusceptibilities of the test organisms to the plantextract were assesed as described by Kirby Bauer (1966).Six millimetre diameter wells were punched using corkborer on the agar and filled with the desiredconcentrations (200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml) of each of the plantextracts. A standardized inoculum wasinoculated onto the surfaces of Mueller Hinton agarplates using sterile swab sticks. Using a syringe, 100µl(0.1 ml) solution of ginger and garlic were added to different wells in the plates. Meropenem (10µg) was used as reference standardto determine the sensitivity of the isolates. Discs were directly placed onto thebacterial culture. The plateswere allowed to stand for some time at roomtemperature for the extracts to diffuse into the agar andthen incubated at 37^{o}C overnight. The diameters of inhibition zone (in mm) were measured.Antibacterial

activities wereevaluated by measuring inhibition zone diametersasrecommended by National Committee for ClinicalStandard (2008).

3.4.3 Bioautography (Agar Overlay)

Thin Layer Chromatography (TLC) was carried out on the plant extracts as described by Thomas and Veda (2007). Twenty (20) grams of Silica gel and 40 ml of distilled water were mixed to form slurry. The slurry was poured on to a clean grease free glass slide and spread evenly. This was activated in a hot air oven at 100 ºC. The plates were taken out. A pencil line was drawn at a distance of 1cm from the bottom of the glass plate. The extracts were applied on to the plate as small spots using a syringe. The plates were developed using ethyl acetate/methanol/water (76.5:13.5:10) as the solvent system and examined under UV light after drying, spots indicating compounds were marked and their retardation factor (R_f)values recorded. These chromatograms were used for the bioautography as described by Marston (2011), the chromatogram was covered with a molten, seeded agar medium. After solidifying, the plates were incubated at 37^{o}C for 24 hours. One (1%) percent aqeous solution of triphenyl tetrazolium chloride was used to stain the plates so as to help with the visualization of inhibition zones, tetrazolium salts were converted by the dehydrogenases of living microorganisms to intensely colored formazen. These salts were sprayed onto the bioautogram and re-incubated at 37°C for 3 - 4 h (Runyoro *et al.*, 2006).

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction Yield

Yield obtained from the extraction of the two plants (*A.sativum*and *Z.officinale*) using three different solvents (Ethanol, Chloroform and Water) are presented in table 1. The highest percentage (%) yield for *A. sativum* was obtained using water 65.65g (65.65 %), chloroform gave the least yield 1.91g (1.91%) and the ethanol fraction produced 3.78g (3.78%). On the other hand the highest percentage yield for *Z. officinale* was 8.94g (11.92%) from the ethanol fraction, 5.61g (7.48%) was obtained from the chloroform fraction and the least yield for ginger extract was from the aqeous fraction. All fractions of the extract have a brown colour except the chloroform fraction of garlic which is light brown in colour. The smell of all the ginger extract was pungent whereas the garlic extract have allicin odour. All the extracts had a gummy texture except the garlic fraction extracted with chloroform which is soft.

Table 1: Physical properties of *A.sativum* and *Z.officinale* extracts

	A.sativum			*Z.officinale*		
Properties	Ethanol	Chloroform	Water	Ethanol	Chloroform	Water

Weight of plant material used for extraction(grm)	100	100	100	75	75	75
Weight of extract(grm)	3.78	1.91	65.65	8.94	5.61	5.25
Percentage yield(□)	3.78	1.91	65.65	11.92	7.48	7.0
Colour	Brown	Light brown	Brown	Brown	Brown	Brown
Smell	Allicin odour	Allicin odour	Allicin odour	Pungent	Pungent	Pungent
Texture	Gummy	Soft	Gummy	Gummy	Gummy	Gummy

4.2 Phytochemical properties of the plant extracts

Phytochemical screening of the plant extracts indicated that alkaloids are present in all the extracted plant material. Amino acids were absent in all the fractions of both garlic

and ginger. The ethanol and chloroform fractions of both garlic and ginger contained saponins, which were absent in the aqeous fraction of both plants. On the contrary, tannins were present in only the aqeous fractions of garlic and ginger, and were absent in all other fractions. Flavonoids were present in the ethanol and chloroform fractions of garlic but absent in the aqeous fraction and the three fractions of ginger. Screening test indicated the oresence of reducing sugars in all six fractions (Table 2).

Table 2:Phytochemical properties of the *A.sativum* and *Z.officinale* extracts

Phytochemical	*A.sativum*	*Z.officinale*

component	Ethanol	Chloroform	Water	Ethanol	Chloroform	Water
Alkaloids (dragendoff's)	+	+	+	+	+	+
Alkaloids (Meyer's)	+	-	-	+	-	-
Amino Acids	-	-	-	-	-	-
Saponins	+	+	-	+	+	-
Tannins	-	-	+	-	-	+
Flavonoids	+	+	-	-	-	-
Reducing Sugars	+	+	+	+	+	+

KEY: + - Present - - Absent

4.3 Bioassay Results

4.3.1 Antibacterial properties of the *A.sativum* and *Z.officinale* extractsusing Agar diffussion technique

Results of the antibacterial activity of the crude extracts of the plants against all the four test organisms are shown in Tables 3(a and b) and 4(a and b). All the bacterial isolates were resistant to all the test plants but they showed sensitivity towards the commercial antibiotic meropenem. The highest zone of inhibition (46mm) was recorded for *Klebsiella pneumoniae*, followed by *Proteus spp*. and *Escherichia coli*. The least zone of inhibition was 24mm for *Pseudomonas aeruginosa*.

Table 3a: Antibacterial activity of *Allium sativum* extracts against β –lactamase producers using agar disc diffussion

Bacterial isolates	Ethanol Fraction				Chloroform fraction				Water fraction				MER
	(µg/disc)												
	25	50	100	200	25	50	100	200	25	50	100	200	10
	Zone of inhibition in mm												
Escherichia coli	00	00	00	00	00	00	00	00	00	00	00	00	28
Klebsiella pneumoniae	00	00	00	00	00	00	00	00	00	00	00	00	46
Proteus spp.	00	00	00	00	00	00	00	00	00	00	00	00	30
Pseudomonas aeruginosa	00	00	00	00	00	00	00	00	00	00	00	00	26

KEY:MER-Meropenem

Bacterial isolates	Ethanol fraction	Chloroform fraction	Water fraction	MER

	Concentration (µg/ml)												(µg/disc)
	25	50	100	200	25	50	100	200	25	50	100	200	10
	Zone of inhibition in mm												
Escherichia coli	00	00	00	00	00	00	00	00	00	00	00	00	29
Klebsiella pneumoniae	00	00	00	00	00	00	00	00	00	00	00	00	44
Proteus spp.	00	00	00	00	00	00	00	00	00	00	00	00	30
Pseudomonas aeruginosa	00	00	00	00	00	00	00	00	00	00	00	00	24

Table 3b: Antibacterial activity of *Allium sativum* extracts against β –lactamase producers using agar well diffussion

KEY: MER-Meropenem

Table 4a: Antibacterial activityof *Zingiber officinale* extracts against β–lactamase producers using agar disc diffussion

Bacterial isolates	Ethanol fraction				Chloroform fraction				Water fraction				MER
	Concentrations (µg/disc)												
	25	50	100	200	25	50	100	200	25	50	100	200	10
	Zone of inhibition in mm												
Escherichia coli	00	00	00	00	00	00	00	00	00	00	00	00	28
Klebsiella pneumoniae	00	00	00	00	00	00	00	00	00	00	00	00	46
Proteus spp.	00	00	00	00	00	00	00	00	00	00	00	00	30
Pseudomonas aeruginosa	00	00	00	00	00	00	00	00	00	00	00	00	26

KEY: MER-Meropenem

Table 4b: Antubacterial activity of *Zingiber officinale* extracts againstβ**–lactamase producers using agar well diffussion**

Bacterial isolates	Ethanol fraction				Chloroform fraction				Water fraction				MER/mm
	Concentrations(µg/ml)												(µg/disc)
	25	50	100	200	25	50	100	200	25	50	100	200	10
	Zone of inhibition in mm												
Escherichia coli	00	00	00	00	00	00	00	00	00	00	00	00	29
Klebsiella pneumoniae	00	00	00	00	00	00	00	00	00	00	00	00	44
Proteus spp.	00	00	00	00	00	00	00	00	00	00	00	00	30
Pseudomonas aeruginosa	00	00	00	00	00	00	00	00	00	00	00	00	24

KEY: MER-Meropenem

4.3.2 Bioautographic studies of *A. sativum* and *Z. officinale* extracts

The thin layer chromatograms of the ethanol, chloroform and water fractions of both garlic and ginger revealed the presence of a number of compounds with different R_f values (Table 5). The results of the bioautography tests revealed that some of the compounds in the plant extracts separated by TLC had good activities against the organisms as shown in Table 6. The ethanol and chloroform fractions of both plants studied indicated some activity against *Escherichia coli* and *Proteus spp*. Only the ethanolic and chlororm extracts of ginger inhibited *Klebsiella pneumoniae*. The aqeous fractions showed no activity against all organisms. *Pseudomonas aeruginosa* was resistant to all fractions of both plants.

Table 5:R_f values for different compounds present in the plant extracts.

FRACTION OF PLANT EXTRACT	COMPOUNDS SEPARATED					
	A	B	C	D	E	F
GLE	0.12	0.32	0.50	0.61	0.79	0.85
GLC	0.72	0.86	0.96	-	-	-
GLW	0.62	0.88		-	-	-
GRE	0.20	0.56	0.71	-	-	-
GRC	0.28	0.44	0.75	0.88	0.92	-
GRW	0.76	0.86	-	-	-	-

KEY:GLE-Garlic Ethanolic extract

GLC-Garlic Chloroform extract

GLW-Garlic Water extract

GRE-Ginger Ethanolic extract

GRC-Ginger Chloroform extract

GRW-Ginger Water extract

Table 6: Inhibitory activities of *A.sativum*and *Z.officinale* using bioautography

	A.sativum			*Z.officinale*		
Organisms	Ethanol	Chloroform	Water	Ethanol	Chloroform	Water
Escherichia coli	+	+	-	-	-	-
Klebsiella pneumoniae	-	-	-	+	+	-
Proteus spp.	+	+	-	+	+	-
Pseudomonas aeruginosa	-	-	-	-	-	-

KEY: + activity - no activity

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Results of the phytochemical tests carried out on both plant extracts revealed the presence of alkaloids, tannins, flavonoids, saponins and reducing sugars.This is in conformity with the work of previous studies by some other authors. The antimicrobial activities of plant extracts including garlic and ginger have been linked to the presence of some bioactive compounds. These bioactive compounds are known to work synergistically to produce various effects on the human and animal subjects (Amagace, 2006).

Results of the agar diffussion methods used in the present study showed that all the four test organisms(*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus sp.*) were resistant to both garlic and ginger extracts. This contradicts a study by Melvin*et al*. (2009), whichfound out that ginger extractexhibited maximum inhibitory effect against *P.aeruginosa* while the antimicrobial activity against *E.coli* was found to be moderate.Similar results were reported where ginger did not show any antibacterial activity against some multidrug resistant bacteria including *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi* (Adeshina *et al*., 2011). A study by Venugopal*et al*.(2009) also showed no antibacterial activity of ginger to *Vibrio parahaemolyticus, Escherichia coli* and *Staphylococcus aureus*. Akintobi *et al*. (2013) also had a similar result with the present one but with a slight difference whereby the ethanolic extract of ginger had inhibitory activity against *pseudomonas aeruginosa.*A study by Yusha'u *et al*.(2008) showed that *Escherichia coli, Pseudomonas*

aeruginosa, Klebsiella pneumoniae and *Proteus sp.* were inhibited by ethanolic extract of both plants tested, which is not the case in the present study. Few studies have shown some bacteria to be resistant towards garlic extract and these include *Escherichia coli* and *Staphylococcus aureus* (Esimone *et al*., 2010). The resistance exhibited by the test organisms in the present study may be due to elaboration of enzymes that possibly destroy or inactivate some of the bioactive phytoconstituents in plant extracts (Ahmadu *et al*., 2006). Resistance may also be asa result of transfer of resistance plasmids or indiscriminate and sub-therapeutic use of the extractsas stated by Bibitha *et al*., (2002). El-Mahmood and Amey (2007) stated that extracts of herbalmedicines are subject to degradation and decompositionon storage. This might also be a contributing factor to the resistance exhibited. The standard antibiotic, meropenem,displayed superior potencycompared to the crude extracts of the test plants. This may be attributedto the fact that meropenem, as a conventional antibiotic,is prepared by means of manufacturingprocesses and procedures(El-Mahmood and Amey,2007).

Bioautographic studyin the present study revealed that some of the compounds separated using Thin Layer Chromatography inhibited the growth of the test organisms. It can also be noted that all the test organisms were resistant to compounds of the aqeous fraction of both plant extracts. This could be due to the presence of the dissolved phytochemicals at low concentrations in the aqueous extracts.Of particular recognition in this study is the non susceptibility of *Pseudomonas aeruginosa*to all the extracts tested. This may partly be explained by some earlier reports that *Pseudomonas aeruginosa* exhibited strong resistance against a host of antibiotics including plant extracts (Bibitha *et al*., 2002). Intrinsic and acquired antibiotic

resistance makes *P.aeruginosa*one of the most difficult organisms to treat. The high intrinsic antibiotic resistance of *P.aeruginosa*is due to several mechanisms: a low outer membrane permeability, production of an AmpC β-lactamase and the presence of numerous genes coding for different multidrug resistance efflux pumps (Livermore, 2002).

5.2 Conclusion

It can be concluded from the present study thatboth *A.sativum* and *Z.officinale*tested revealed the presence of pharmaceutically important phytochemicals- alkaloids, tannins, flavonoids , saponins and reducing sugars. The Bioautographic studies showed that these compounds in the plant extracts separated using TLC have activity against the organisms to some extent. Therefore, it can also be concluded that garlic and ginger have potentials to yield biologically active compounds which could have antibacterial activities.

5.3Recommendations

From the results obtained in the present study, it is therefore recommended that further research be carried out to isolate the active constituents and determine their toxicity and side effects. Further research can also be carried out to find out the synergistic activity of *A.sativum* and *Z.officinale* against the test organisms used in this study.

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