ANTI-BACTERIAL PROPERTIES AND GC-MS ANALYSIS OF EXTRACTS AND ESSENTIAL OILS OF SELECTED PLANT PRODUCTS

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A Thesis submitted in partial fulfillment of the requirements for the award of Master of Science Degree in Applied Analytical Chemistry in the School of Pure and Applied Sciences of Kenyatta University

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DECLARATION

This thesis is my original work and has not been presented for an award of a degree in any other university or any other award.

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DEDICATION

To my late mother Margaret Ombuna, my husband Elijah and our children Brenda, Linda and Lincoln. For you I will undertake all that is desirable within my reach.

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Special thanks to the Almighty God for giving me knowledge, patience and strength to accomplish this work. This study comes to fruition trailing clouds of kindness of many institutions and people who deserve formal recognition for their love, help and support throughout the period of this research.

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ABBREVIATIONS AND ACRONYMS

AHMD	The American Heritage Medical Dictionary
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CSF	Cerebral Spinal Fluid
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
GC	Gas Chromatography
GC-MS	Gas Chromatography - mass spectrometer
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome
ICIPE	International Centre for Insect Physiology and Ecology
KEMRI	Kenya Medical Research Institute
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistant
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
NCCLS	National Committee for Clinical Laboratory Standards
pH	Potential of Hydrogen
SE	Standard Error
SNK	Student Newman Keuls
TMP-SMX	Trimethoprim/Sulfamethoxazole
TSS	Toxic Shock Syndrome
U.S.A	United States of America
UTI	Urinary Tract Infections
WHO	World Health Organization

ABSTRACT

Bacteria, as common microorganisms found in air, food, water and soil remain a major problem in developing countries where disease outbreaks frequently occur in congested areas. Antibiotics are used to treat common bacterial diseases such as respiratory, ear, gastrointestinal and skin infections but bacteria develop resistance to most of the drugs. The drugs also have serious side effects and the cost of medication is high. Plants are traditionally used for treatment of bacterial infections though they are not clinically regulated due to lack of awareness and enough data to support the reported therapeutic claims. Some plants used as food and vegetables are hardly considered in such studies. The aim of this study was to investigate the antibacterial properties associated with garlic, ginger, lemon, turmeric and onion. The bioactivities of juices, methanol extracts and essential oils of these materials were tested, individually and as blends, against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*. Bioassay data obtained from the active extracts and oils were subjected to analysis of variance (ANOVA). Treatment means showing significant difference ($p \leq 0.05$) were separated using Student-Newman-Keuls test (SNK). Identification of suspected antibacterial compounds was done by comparison of retention indices and the mass spectra with those in National Institute of Standards and Technology (NIST) libraries using GC-MS analysis. Garlic juice was bactericidal against *Salmonella typhi* (17.7±2.5), *Staphylococcus aureus* (14.7±2.5), *Pseudomonas aeruginosa* (10.0±0.0) and *Escherichia coli* (11.7±0.6). Lemon/garlic juice exhibited significantly higher activity against *Escherichia coli* (15.0±0.0) and *Salmonella typhi* (12.0±0.0). Turmeric/lemon/garlic methanol extracts blend was most active against *S. aureus* (12.0±1.0). Preliminary screening of the essential oils indicated significant antibacterial activity of lemon/garlic essential oil blend (10.0±0.0) against *Pseudomonas aeruginosa*. Garlic recorded time-course increasing activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* from day 1 to day 5. GC-MS analysis of the active samples confirmed the presence of compounds containing -OOH, -OH, -N, -Cl, -F, $-NH_2$ and –S groups which are associated with bacterial inhibition in conventional antibiotics. The 10 major constituents obtained from samples suspected to contain antibacterial activity include limonene (85.08%); 3-vinyl-1,2-dithiacyclohex-4-ene (21.43%); α-zingiberene (33.75%); diallyl disulphide (10.84%); 2-butanone,4-(-hydroxy-3-methoxyphenyl)- (14.14%); 3-chlorothiophene (8.93%); methanehydrazonic acid,N-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethyl ester (8.87%); *n*-hexadecanoic acid (8.01%); γ-sitosterol (8.00) and propanamide,2-amino-3-phenyl (6.71%). Since juices of garlic, lemon and lemon/garlic blend were found to be active against one or more of the bacteria tested unlike methanol extracts and essential oils, they should be used in raw form as heating and drying is likely to render them inactive. Further studies on methanol extract and fresh juice of lemon/garlic blend need to be undertaken in order to elucidate the active principles in these extracts. These may provide novel antimicrobial agents and/or models for new generation of synthetic antibiotics.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

The continuous spread of multidrug-resistant pathogens has become a threat to public health and a major concern for infection control practitioners worldwide (Borowitz and Naser, 2011). In addition to increasing the cost of drug regimens, this scenario has paved way for re-emergence of previously controlled diseases and has contributed substantially to high frequency of opportunistic and chronic infection cases in developing countries (Collins *et al*., 1999). Some of the pathogens include bacteria, viruses, fungi and prion (Borowitz and Naser, 2011).

Bacteria cause a wide range of infections, resulting in mild to life-threatening illnesses that require immediate interventions (Martin and Edzard, 2003). Common bacterial infections include respiratory infections, ear infections, gastrointestinal infections and skin disorders (Mandal *et al*., 2005; Arthur, 2006).

In developing countries, outbreaks of bacterial infections occur most often in congested areas such as refugee camps, slums and in areas with high population density. Food vendors, slum dwellers, riparian communities, fishermen and school children are among the risk groups (Brooks *et al*., 2005; Change, 2009). In Kenya, studies conducted by Center for Microbiology Research in KEMRI, Nairobi, shows that 41% of about 33 million people contracted the typhoid fever in 2008 (Kariuki, 2008). The research further reveals that 52% of the cases affected children younger than 10 years and 40% people aged 15 and 45 years and more than halve of these cases were from the informal settlements (slum areas) surrounding the capital city

(Kariuki, 2008). There have been reports of sporadic outbreaks of bacterial infections in many areas including three districts in Central Kenya, Malindi and Kwale in the Coast Province and some parts of Nyanza Province (Onyango, 2005). However, not all outbreaks were confirmed, leading to lack of reliable data on the prevalence of diseases caused by bacteria (WHO, 2009).

Conventional antibiotics usually provide effective therapy for bacterial infections (Martin and Edzard, 2003). However, these bacteria have become resistant to one or more antibiotics and the population of Multidrug Resistant (MDR) bacteria is increasing (Stewart and Costerton, 2001). Mechanisms which microorganisms have developed to resist antibiotics include inactivation of antibiotics by enzymes, alteration of drug target sites, blockage of drugs from entering into the cell membrane, chromosomal and plasmid mediated resistance.

Closely related to bacterial infections is malaria which is caused by *Plasmodium* parasite (Ndyomugyenyi *et al*., 2007; Charles, 2010*)*. Malaria Symptoms include fever, shaking chills, headache, muscle aches, tiredness, nausea, vomiting, and diarrhea (Ali *et al*., 2007; Mohanna *et al*., 2007). Some of the bacterial infections (especially typhoid) have similar signs and symptoms to that of malaria. This makes patients focus attention on treating malaria instead of the bacterial infections (Balentine, 2009). The symptoms are more dramatic in children and if untreated they may kill fast (Onyango, 2009).

Antibiotics such as ampicillin, chloramphenicol, Trimethoprim/Sulfamethoxazole (TMP-SMX), amoxicillin and ciprofloxacin have been commonly used to treat bacterial infections (Wain and Kidqell, 2004). The bioactive parts against bacteria in these conventional antibiotics include structural moieties that include -Cl, -F, -N, -NH_2, -S, -COOH and –OH, which are also found in many herbs used traditionally against bacterial infections. Studies have shown that sulphur containing compounds have strong inhibitory antibacterial activities (Julia and Ann 1947; Kyung and Fleming, 1996; Yanyali *et al*., 2001). Nitrite has toxic properties while nitrous acid is bactericidal, chlorine releasing compounds such as chlorine dioxide (ClO_2), acidic and alcoholic compounds act as antibacterial agents (Gerald and Russell, 1999).

Some vegetable trials have been reported to be as effective as conventional treatments and provide therapy for bacterial infections (Martin and Edzard, 2003). Herbs, foods and spices contain many compounds, and it is often unclear which one is associated with beneficial effects. The compounds in drugs vary in different species. However, even within a single species the phytochemical composition may be affected by the plant's growing conditions and different parts of a herb can have different chemical compositions (Linda *et al*., 2008). Most herbs, foods and spices contain antibacterial properties, for example, allicin a compound released from garlic was found to be active against bacteria and fungi (Serge, 2001; Lian-fang *et al*., 2009; Onyeagba *et al*., 2007); Pandey *et al*., 2011).

1.2 Problem statement and justification

Bacterial infections have been a major health challenge to most health care systems in Kenya. Their effective treatment options are expensive not only for the patients but also for the organizations they may be working for. Treatment, hospitalization and loss of wages are some of the costs the employee and employer may have to grapple with (Hatil, 2009). For the employer, workers' absenteeism, disorganization and loss of productivity may be the price to pay when an employee suffers from such infections.

Several antibiotics are used for treatment of bacteria though most of which have developed resistance. Resistance to antibiotics is the acquired ability of a microorganism to resist the effects of an antibiotic to which it is normally susceptible (Baliga, 2005). Emergence of resistance and multi-drug resistant bacteria has become a worldwide problem (Meng and Ramamoorthy, 1998; Aarestrup and Wegener, 1999). This is attributed to overuse and misuse of antibiotics (Usera *et al*., 2002). Resistance is also due to heavy metal pollution of water from industrial effluents, automobiles and agricultural practices. A long exposure of heavy metals to bacteria makes them resistant and clinically untreatable (Ndungi, 2007).

Use of vegetables in controlling bacterial infections has continued despite advances in modern pharmaceutical products and dominance of synthetic drugs all over the world (Deresse, 2010). Garlic (*Allium Sativum* L.), onion (*Allium cepa* L.), lemon (*Citrus lemon*), turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) and their products are some of the vegetables used in controlling bacterial infections though not clinically regulated due to lack of awareness and data to support the reported therapeutic claims (Wilde-Duyfjes, 1973; Rehman *et al*., 2011). There is need to develop new antibacterial agents from known potential vegetables and fruits to keep in check the growing resistance of bacteria to common conventional drugs (Wangari, 2008). In Kenya herbal medicine is gaining popularity because of its availability and affordability (Hatil, 2009). In spite of the presence of locally available foods that can be used to prevent or treat the bacterial infections, minimum research has been done on them, leaving most people to rely on antibiotics that are relatively expensive and have enormous side effects (Linda *et al*., 2008).

Essential oils, juices and methanol extracts of lemon, garlic, turmeric, ginger and onion have been previously investigated on their antibacterial properties against *Escherichia coli* (*E. coli*), *Salmonella typhi* (S. *typhi*), *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Seenivasan *et al*., 2006; Theresa, 2008). They have revealed structurally unique biologically active compounds. However, less attention has been given to the activities of their blended components (Viuda-Martos *et al*., 2008). Therefore, this study was designed to assess the antibacterial properties of the selected vegetables and lemon individually and their blends.

1.3 Hypothesis

The antibacterial properties of juices, methanol extracts and essential oils of different vegetables and their blends are significantly different and contain antibacterial constituents.

1.4 Objectives

1.4.1 General objective

To determine the antibacterial properties of juices, methanol extracts, essential oils and blends of selected vegetables and lemon.

1.4.2 Specific objectives

i. To determine *in vitro* antibacterial activities of juices, methanol extracts and essential oils of garlic (*A. Sativum*), ginger *(Z. officinale*), onion (*A. cepa*), turmeric (*C. longa*) and lemon (*C. lemon*) individually and as blends.

ii. To determine the time-course antibacterial activities of garlic (*A. Sativum*), ginger *(Z. officinale*), onion (*A. cepa*), turmeric (*C. longa*) and lemon (*C. lemon*) juices individually and as blends

iii. To identify suspected antibacterial constituents of the active samples and blended essential oils by GC-MS.

1.5 Significance of the study

This research was done to assess therapeutic antibacterial agents from garlic, lemon, onion, turmeric and ginger mixtures. It was envisaged to lay down the groundwork for enhancing traditional knowledge and practices through modern approaches of drug development. The study is also aimed at contributing to scientific knowledge locally, nationally and internationally in the area of bacterial infections.

1.6 Scope and limitations of the study

Bioassays of garlic, ginger, onion, turmeric and lemon juices, methanol extracts and essential oils were done against three gram-negative bacteria and one gram-positive bacterium. Bioassays using juices were carried out over five-day periods. Only three available conventional standards (+ve controls) and two solvents (-ve controls) were included. Climatic conditions, soil conditions, method of growth, mode of transportation, harvesting time, use of pesticides and inorganic fertilizers on the samples were not considered.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bacterial infections

Bacteria are microscopic organisms whose single cells have neither a membrane-bounded nucleus nor other membrane-bounded organelles like mitochondria and chloroplasts (Figure 2.1) (Todar, 2009; Paul *et al*., 2011). The term bacterium was introduced in the 19th century by the German botanist, Ferdinand Cohn (1828-98). It is derived from the Greek word 'bakterion' meaning a small rod or staff. Cohn categorized bacteria as one of the three types of microorganisms which are bacteria (short rods), bacilli (longer rods), and spirilla (spiral forms) (Guerrero *et al*., 1999; Martes, 2010). Bacteria are found in air, water, soil and food. They live on plants, insects, animals, pets, human digestive system and in the upper respiratory tract. There are several kinds of bacteria, but only a few cause diseases in human beings.

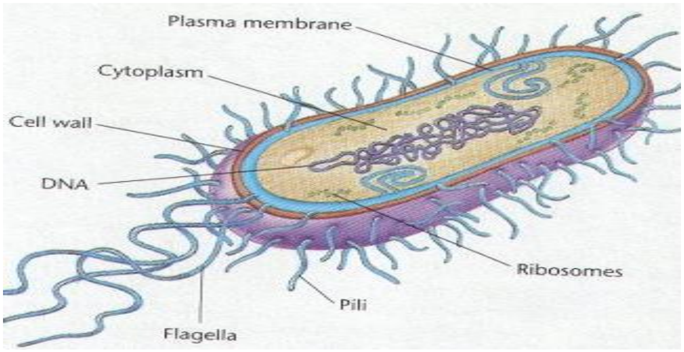


Figure 2.1 Typical magnified bacterium (Todar, 2009).

Bacteria are grouped as 'gram-positive' and 'gram-negative', based on the results of gram staining method. Gram-negative bacteria are those that do not retain crystal violet dye in the gram staining protocol (Figure 2.2(a)) while gram-positive bacteria retain the crystal violet dye when washed in a decolorizing solution (Figure 2.2(b)) (Crowley, 2009).

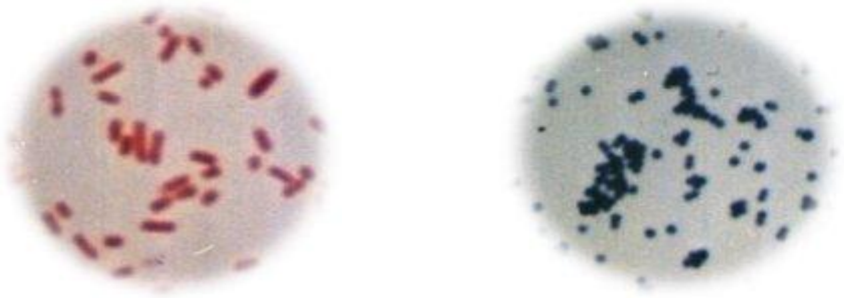


Figure 2.2 (a) *Escherichia coli* (b) *Staphylococcus epidermidis* (Crowley, 2009).

Salmonella typhi (*S. typhi*) is a gram-negative bacterium which belongs to the species *S. enterica* originally isolated in 1880 by Karl J. Erberth (John et al., 1997; Doughari et al., 2007; Doughari and Okaator, 2008). It is acquired through ingestion of water, milk, or fruits and vegetables contaminated by urine or feaces of infected carriers (Duguid *et al*., 1983; Doughari and Okaator, 2008). It grows in either the presence or absence of oxygen, but under the latter (under nitrogen) growth is only slightly less. *S. typhi* is a multi-organ pathogen that inhabits the lymphatic tissues of the small intestine, liver, spleen, and bloodstream of infected people (Mandal *et al*., 2005).

The multiplied bacteria retained in the bloodstream causes typhoid (Bavdekar, 1996; Dutta and Ghotekar, 2001; Prescott *et al*., 2005; Doughari *et al*., 2007). Typhoid fever is a common illness in areas where sanitation is poor. Nowadays, outbreaks occur most often in developing countries, in refugee camps and in overwhelmed areas with high population density. At least 17 million new cases and up to 600,000 deaths are reported annually worldwide (Steel, 2008).

Symptoms of *S. typhi* infections include minor abdominal disturbances, fatigue, slight headaches, rose spots, body ache and fever as high as 39 °C – 40 °C (Zavala *et al*., 2005; Onyango, 2005). Typhoid fever in most cases is not fatal. With appropriate treatment, its mortality rate is generally under 1% (Arthur, 2006). However, mortality rate depends on many factors, including the strain of the bacteria and its sensitivity to specific antibiotics (Arthur, 2006). Antibiotics such as ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, amoxillin and ciprofloxacin have been commonly used to treat typhoid fever (Wain and Kidqell, 2004).

Escherichia coli (*E. coli*) (Figure 2.1(a)) is a gram-negative bacterium which belongs to the genus *Escherichia* named after Theodor Escherich who was the first to isolate the species from the genus (WHO, 2010). It is usually transferred to humans by ingesting contaminated water, or contaminated food such as meat, which has not been properly cooked. *E. coli* is bacterium that can survive in environments with or without air (facultative anaerobes). Depending on the environment, it may or may not produce thin hair-like structures (flagella or pili) that allow the bacteria to move and to attach to human cells. These bacteria commonly live in the intestines of people and animals (Erica, 2011; Aarestrup and Wegner, 1999).

There are many strains (over 700 serotypes) of *E. coli* and most are normal inhabitants of the small intestine and colon which are non-pathogenic (Barton, 2008). They suppress the growth of pathogenic bacteria species and synthesize appreciable amounts of vitamin K and vitamin B complex (Erica, 2011). They can cause disease if they spread outside the intestines into the urinary tract where they cause bladder or kidney infections, urinary tract infections (UTI) or into the blood stream (sepsis)

(Arimi *et al*., 2000). Other *E. coli* strains cause poisoning or diarrhea, meningitis, cholecystitis, bacteremia, cholangitis and pneumonia (Tarun and Chi Hiong, 2009; WHO, 2010). Accumulated research data recognizes this organism as an important food borne pathogens and a zoonosis (Arimi *et al*., 2000). *E. coli* possess unusual acid resistant properties in that the microorganism has the ability to survive and grow in both acidified media and food containing added or biologically produced organic acids (Greg *et al*., 2007).

Although reported outbreaks of *E. coli* in Africa have been few to date, available information indicate that the pathogen has wide geographic distribution. Since 1992, culture-proven *E. coli* diarrheal illness has been reported from multiple locations, including Kenya, Nigeria, Côte d'Ivoire, and Central Africa Republic (Sang *et al*., 1996; Germani *et al*., 1998; Ahmed, 2009). In Egypt, 6 (5%) of 125 meat specimens obtained from slaughterhouses contained *E. coli* (Abdul-Raouf *et al*., 1996). Because *E. coli* is not detected by the usual methods used to isolate and identify traditional enteric bacterial pathogens and microbiology laboratories many countries in Africa do not routinely test for this pathogen, thus *E. coli* infections may go unrecognized (Erica, 2011).

Escherichia coli symptoms can range from mild diarrhea to haemorrhagic colitis - a combination of severe abdominal cramps and blood in the stools. Various antibiotics are used for treatment including ticarcillin, piperacillin, cephalosporins, aminoglycosides, trimethoprim/sulfamethoxazole (TMP-SMX), and fluoroquinolones. Studies show that urinary-tract infections (UTIs) are resistant to TMP-SMX, the first line of therapy, and other antibiotics (Shari, 2010).

Pseudomonas aeruginosa is a gram-negative organism that belongs to the family *Pseudomonaceae* which is commonly found in soil, water, plants and animals (Conan *et al*., 2005). It is an opportunistic pathogen of human beings which is able to infect hosts with defective immune system function (Rogers and Barbara 2003). It causes (UTIs), pneumonia, septicemia, gastrointestinal infections and a variety of systematic infections, particularly in patients with severe burns, cystic fibroids and in cancer and HIV/AIDS patients who are immune-suppressed (Azu *et al*., 2007; Nadia, 2009). It is the most common pathogen isolated from patients who have been hospitalized longer than one week (Christensona *et al*., 2000). Pseudomonal infections are complicated and life threatening.

Staphylococcus aureus is a common facultative anaerobic gram-positive bacterium. The Center for Disease Control and Prevention (CDC) estimates that 25–30 % of the population is “colonized” in the nose with *S. aureus,* meaning that the bacteria are present but cause no symptoms (CDC, 2005). The word comes from two Greek words; staphyle, meaning a bunch of grapes and kokkos, meaning berry (Mellisa, 2011). In the hospital setting, *S. aureus* transmission is through incomplete or ineffective hygienic practices and a high prevalence of *S. aureus* (Miller *et al.*, 2009). *S. aureus* causes a variety of suppurative (pus forming) infections and toxinoses in human beings. It causes minor skin infections, such as pimples, impetigo, boils (furuncles), folliculitis, cellulitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), chest pain, bacteremia and sepsis (Rybak and Laplante, 2005; Turnidge *et al*., 2008). It causes soft tissue, respiratory, bone, joint, endovascular to wound infections. *S. aureus* causes food poison by releasing enterotoxins into food and toxic shock syndrome by the release of super antigens into the blood stream (Diana, 2010). Staphylococcal sepsis is a leading cause of shock and circulatory collapse, leading to death, in people with severe burns over large areas of the body. When untreated, Sau sepsis carries a mortality (death) rate of over 80% (Turnidge *et al*., 2008).

Some strains of *S. aureus* are resistant to a variety of different antibiotics including methicillin and other antibiotics (penicillin, oxacillin, amoxacillin). Hospital acquired infection is often caused by antibiotic resistant strains and can only be treated with vancomycin or combination therapy using sulfa drugs and minocycline or rifampin (Bancroft, 2007). Until recently, infections acquired outside hospitals have been treated with penicillinase-resistant ß-lactams (Siegel *et al*., 2006). However, many of the community associated staphylococcal infections are now methicillin resistant. The ability of *S.aureus* bacteria to evade standard antibiotic treatment has been growing steadily over the past three decades. In 1972, only 2 percent of *staph* infections were drug resistant; that figure stood at 20–25 percent in the early 1990s and at 63 percent by 2004 (Hazariwala *et al*., 2002: CDC, 2006: Borowitz and Naser, 2011).

2.2 Treatment of bacterial infections

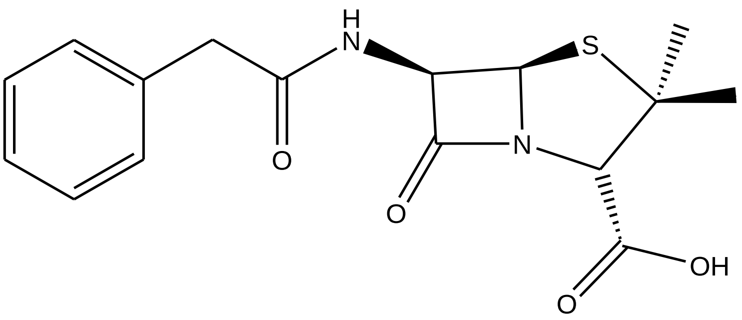
2.2.1 Conventional treatments

Bacterial infections are treated using antibiotics. Antibiotics are those substances which are produced by microorganism that kills or prevents the growth of another micro-organism. Antibiotics are generally used against bacteria. Several microorganisms' derived antibiotics are currently in use to treat a variety of human disease, therefore the action must be taken to control the use of antibiotics, develop new drugs either synthetic or natural, for a long period of time (James and Ruth, 2010). Plants are a valuable source of natural products for maintaining human health (Pandey *et al*., 2011).

There are different classes of antibiotics some of which include macrolides (erythromycin and its relatives), sulphurs (sulphunilides or sulphonamides), penicillins (ampicillin, amoxicillin, piperacillin), aminoglycosides, quinolones (ciprofloxacin, levofloxacin), tetracyclines and chloramphenicol (Salim *et al*., 1996: Ayogu and Amadi, 2009: Durante-Mangoni *et al*., 2009). Bacteria may be naturally resistant to different classes of antibiotics or may acquire resistance from other bacteria through exchange of resistant genes. Indiscriminate, inappropriate, and prolonged use of antibiotics also leads to resistance (Gallagher and Hanman, 1989).

Besides increased drug resistance, high-dose and prolonged antimicrobial therapy can eliminate helpful bacterial flora and predispose people to infection (Meng and Ramamoorthy, 1998). Some adverse effect of antibiotics include diarrhea, which can lead to loss of essential vitamins and minerals, especially vitamin K, magnesium, and zinc vitamin deficiencies, seizures, allergic shock, autoimmune disease, decreased platelets, kidney injury, drug/drug interaction, and death (Bavdekar, 1996; Correne *et al*., 2003).

Penicillin (**1**) is a group of antibiotics derived from *Penicillium* fungi. The discovery of penicillin is attributed to Scottish scientist and Nobel laureate Alexander Fleming in 1928 (Brown, 2004). The active ingredient in a mold, *Penicillium notatum,* which Fleming named penicillin, turned out to be an infection fighting agent of enormous potency. The penicillin antibiotics include ampicillin, amoxicillin, carbecillin and piperacillin. They were the first drugs that were effective against many previously serious diseases such as syphilis and infections caused by *staphylococci* and *streptococci* (Barbosa *et al*., 2003). Penicillins are still widely used today, though many strains of bacteria are now resistant (James *et al*., 2001). All penicillins are ß-lactam antibiotics and are used in treatment of bacterial infections, usually gram-positive organisms (Kasten and Reski, 1997; Brakhage, 1998).



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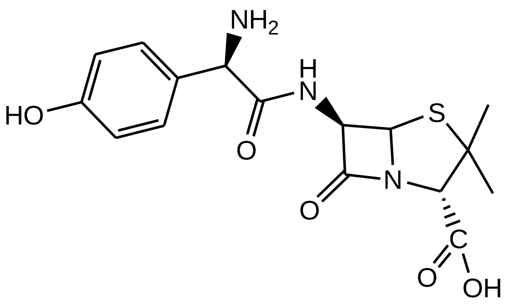
Amoxicillin (**2**) is a moderate spectrum ß- lactam antibiotics used to treat bacterial infections caused by susceptible microorganisms (Brakhage, 1998). It is usually the drug of choice within the class since it is better absorbed, following oral administration as compared to other ß-lactam antibiotics (Zimmerman, 1990). It is one of the most common antibiotics prescribed for children (Correne *et al*., 2003). Amoxicillin is always combined with clavulanic acid (**3**) and marketed under one name augmentin (Kerstin, 2010). The combination means that it remains effective against amoxicillin resistant bacteria (Nathan *et al*., 1998).



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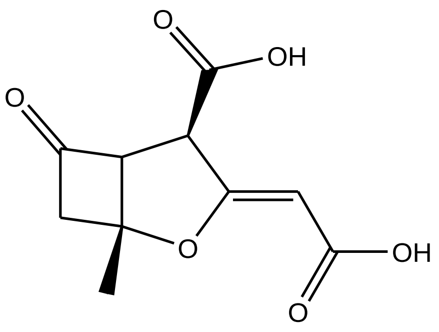

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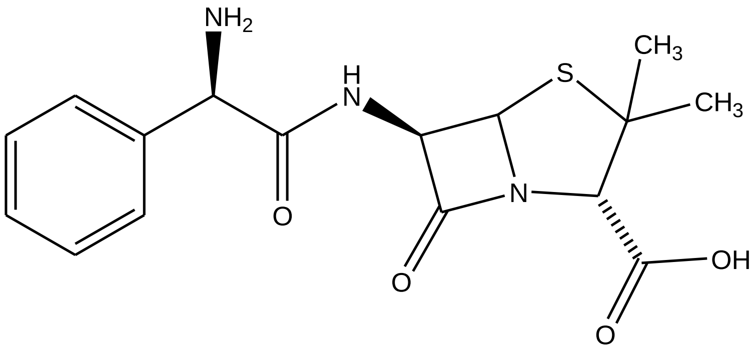
Amoxicillin is used in the treatment of a number of infections including: pneumonia, skin infections, urinary tract infections, *Salmonella, Chlamydia* and cystic acne. (Zimmerman, 1990). It is used to prevent *Streptomycin pneomococus* infections in those without spleen and prevention and treatment of Anthrax (Pawel *et al*., 2007). The adverse effects of the drug include: nausea, vomiting, rashes, colitis and diarrhea (Turck *et al*., 2003). Rare side effects include mental changes, light headedness, insomnia, confusion, anxiety, sensitivity to light and sound and unclear thinking (Pawel *et al*., 2007).

Ampicillin (6[D (-) a- aminophenylacetamido] penicillanic acid) (**4**) is synthetic penicillin having a broader antibacterial spectrum of action (Rolinson and Stevens, 1961). It is effective against gram- negative and gram-positive bacteria (Stewart and Costerton, 2011; Witman and Rogers, 2003) It has been used extensively to treat bacterial infections since 1961 and is considered part of the amino penicillin family, which is roughly equivalent to amoxicillin in terms of spectrum, and level of activity.



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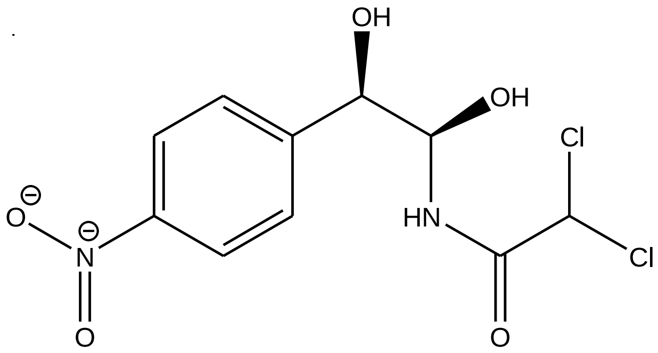
The antibacterial spectrum of ampicillin includes the *Pyogenic cocci, Enterococci, Neisseria* species including the *Gonococcus*, *Haemophilus influenza, Salmonella* species, and strains of *Escherichia coli, Shigella* and *Proteus*. Not all strains of these organisms are uniformly sensitive to ampicillin, however, and conflicting statements regarding the activity of this penicillin have appeared in the literature, particularly against coliforms and Proteus species (Sutherland and Rolinson, 1964). It can result in non-allergic reactions that range in severity from a rash to potentially lethal anaphylaxis. Other adverse effects include; gastro-intestinal disturbances including diarrhea, nausea and vomiting, skin rashes are frequently observed.

Chloramphenicol (**5**) is a bacteriostatic antimicrobial originally derived from the bacterium *Streptomyces venezuelae* isolated by David Gottlieb and introduced into clinical practice in 1949 (Gottlieb *et al*., 1962). It was the first antibiotic to be manufactured synthetically on a large scale (Feder, 1986). Gottlieb used the following compounds to make chloramphenicol: p-nitrophenylserinol (best in stimulating antibiotic production), dichloroacetic acid, norvaline, threonine, leucine, isoleucine, methionine, tryptophan, glutamic acid and phenylalanine (Gottlieb *et al*., 1962).



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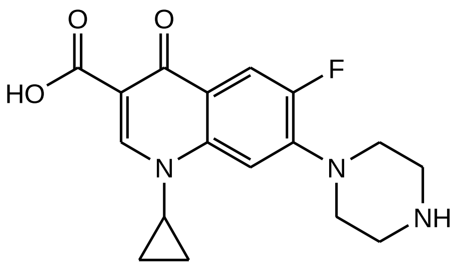
The original indication of chloramphenicol was in the treatment of typhoid, but the now almost universal presence of multidrug resistant to *S. typhi* has meant that it is seldom used for this indication except where the organism is known to be sensitive (Shu *et al*., 1987) Chloramphenicol is effective against a wide range of microorganisms. It is still very widely used in low income countries because it is exceedingly inexpensive, but has fallen out of favour in the west due to a very rare but very serious side effect: aplastic anaemia (Bisma *et al*., 2011). There is no treatment and there is no way of predicting who may or may not get aplastic anaemia and it occurs weeks or months after chloramphenicol treatment has been stopped (McLeman, 1962: Feder, 1986).

It is common for chloramphenicol to cause bone marrow suppression drug treatment- this is a direct toxic effect of the drug on human mitochondria and an increased risk of childhood leukemia and the risk increases with length of treatment (Shu *et al*., 1987). The use of intravenous chloramphenicol has been associated with the so called gray baby syndrome. This problem occurs in newborn infants because they do not yet have fully functional liver enzymes and so chloramphenicol remains unmetabolised in the body. This causes severe adverse effects including hypo tension and cyanosis.

Quinolones (**I-IV**) are synthetic, broad spectrum anti-microbial agents effective in the treatment of selected community- acquired and nosocomial infections (Nelson *et al*., 2007). Quinolones are bactericidal and exhibit concentration-dependent killing (Sarkozy, 2001). Earlier fluoroquinolone agents (ciprofloxacin (**I**), ofloxacin (**II**), norfloxacin (**III**), were more readily absorbed and displayed increased activity against gram-negative bacteria. Newer fluoroquinolones (levofloxacin (**IV**) trovafloxacin and grepafloxacin) are broad spectrum agents with enhanced activity against many gram-negative and positive bacteria (Mohamoud *et al*., 2007). Resistant to quinolones has been reported in a variety of bacterial pathogens including *E.coli, klebsiella pneomiae, p. aeruginosa, S.aureus* and *streptococcus pneumoniae* (John *et al*., 1997: Fu *et al*., 1992; Ramasamy *et al*., 2011).



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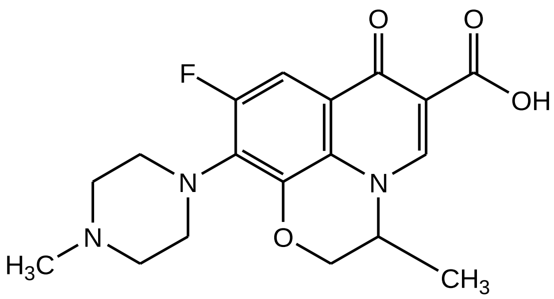

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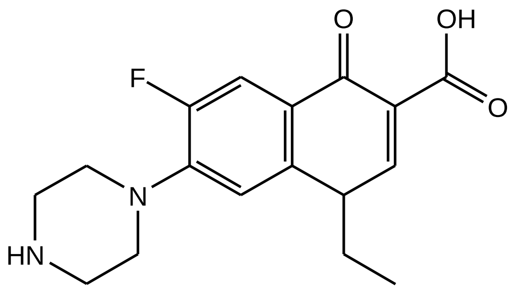

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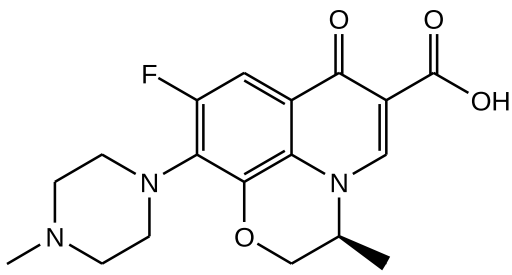


IV

Ciprofloxacin (**I**) is a synthetic chemotherapeutic agent used to treat severe and life threatening bacterial infections (Dutta and Ghotekar, 2001). It is commonly refered to as a fluoroquinolone (or quinolone) drug and is a member of the fluoroquinolone class of antibacterials. Ciprofloxacin was first patented in 1983 by Bayer and approved by United States Food and Drug administration for use in the U.S.A in 1987 (Sharma *et al*., 2010) After the emergence of chloramphenicol resistant *S. typhi* strain, ciprofloxacin has become the drug of choice for the treatment of typhoid fever even in paediatric age group (Dutta and Ghotekar, 2001).

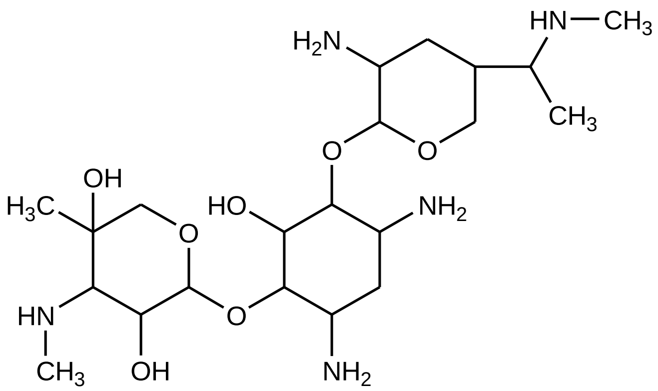
Its adverse effects include: psychosis and chorea (involuntary muscle movements) phototoxicity, neurological symptoms, impaired colour vision, exanthemal abdominal pain, malaise, drug fever, peripheral neuropathy, dysaesthesia and eosinophilia, bone marrow depression, interstitial nephritis and hemolytic anaemia (Smith, 1987).

Levofloxacin (**IV**) is a synthetic chemotherapeutic antibiotic used to treat severe or life-threatening bacterial infections that have failed to respond to other antibiotic classes. It is used for treatment of urinary tract infections and acute pyelonephritis (Jessina *et al*., 2008). Levofloxacin is a chiral fluorinated carboxyquinolone which is associated with a number of series and life threatening side effects. It can cause spontaneous tendon rapture irreversible peripheral neurapathy and hepatoxicity (Ziganshina and Squire, 2008)

Aminoglycoside is a molecule composed of amino-modified sugars. Aminoglycosides are a group of antibiotics derived from various species of streptomyces or produced synthetically (Levison, 2009). They were discovered in 1943 by Waksman and are still commonly used worldwide (Forge and Schacht, 2000). Depending on their concentration they act either as bacteriostatic or bactericidal (Shakil *et al*., 2007)

They are used to treat infections caused by gram-negative and gram-positive bacteria including Pseudomonas, Enterobacter, Mycobacteria, respiratory and urinary tract infections (Nicole *et al*, 1995). The major irreversible toxicity of aminoglycosides is ototoxicity and kidney damage. The aminoglycocides group includes gentamycin, streptomycin, amikacin and neomycin (Crofton, 2006).

Gentamycin (**6**) is an aminoglycoside antibiotic used to treat infections caused by gram-negative bacteria. It is active against *pseudomonas*, *proteus*, *serratia* and *staphylococcus* (Mathew *et al.*, 2005). Gentamycin is toxic to sensory cells of the ear and sometimes complete hearing loss. They cause acute tubular necrosis which can lead to acute renal failure, balance difficulty, unsteady vision and difficulty in multi-tasking, particularly when standing (Buabeng *et al*., 1999)



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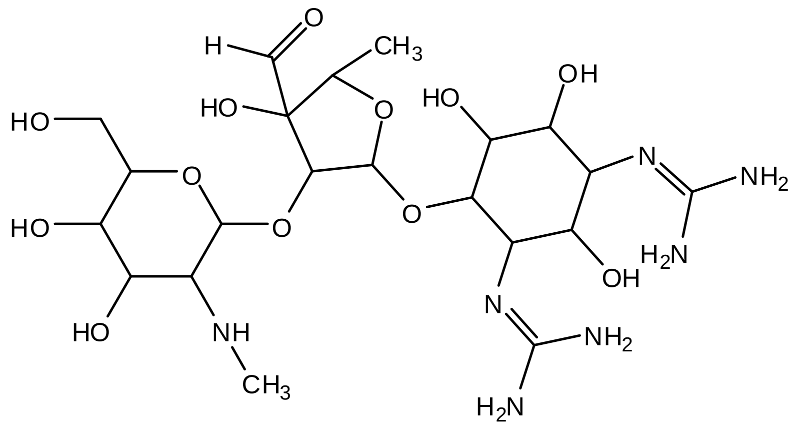
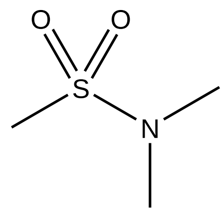
Streptomycin **(7)** is effective and safe in treating tuberculosis and active against 70 different types of bacteria which do not respond to penicillin including infections of the abdomen, urinary tract, pelvis, and meninges. Toxic effects of streptomycin can be severe. By far the most important toxic effect is damage to the inner ear, causing giddiness, nausea and vomiting which develops in a number of patients after about six weeks of treatment due to a hypersensitivity reaction to streptomycin. Both vestibular and auditory dysfunction can follow the administration of streptomycin. The degree of impairment is directly proportional to the dose and duration of Streptomycin administration, the age of the patient, the level of renal function and the amount of underlying existing auditory dysfunction (Alvarez-Elcoro and Enzler, 1999).



(**7**)

Sulphonamides are synthetic antimicrobial agents that contain the sulphonamide group (**8**) and are used to prevent growth of bacteria in the body (Salaheldin and Swapna, 2008). They are used to treat many kinds of infections caused by bacteria and certain other microorganisms. These drugs may treat urinary tract infections, ear infections, bronchitis, bacterial meningitis, eye infections, pneumonia and diarrhea (American Academy of Pediatrics, 2000; Witman and Rogers, 2003). Sulphonamides have the potential to cause a variety of untoward reactions, including urinary tract disorders, haemopoietic disorders, porphyria, hypersensitivity reactions, dizziness and increased sensitivity to sunlight leading to sun burns (Witman and Rogers, 2003)



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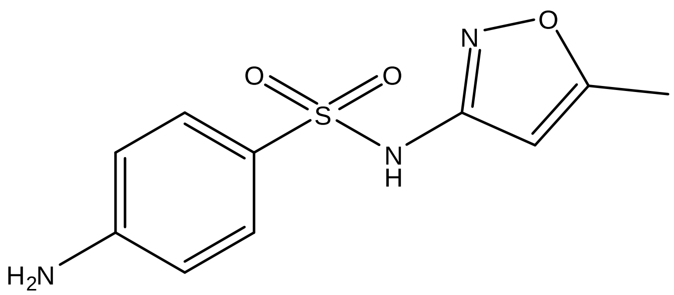
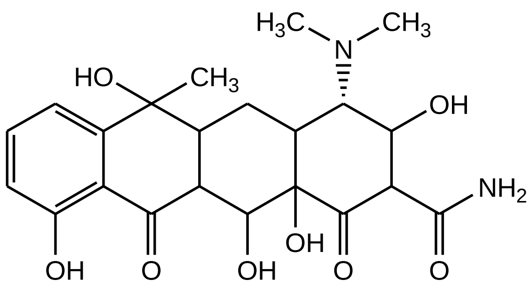
Sulphamethoxale (SMX) (**9**) is a sulphonamide bacteriostatic antibiotic used in combination with trimethoprim in a ratio of 5:2. It is also known as septrin or bactrime (Garg *et al*., 1986) it is commonly used to treat urinary tract infections, streptococcus, staphyloccus aureas, *E. coli* and oral anaerobes. It is also an alternative to amoxicillin-based antibiotics to treat sinusitis and a drug of choice for pneumocystis pneumonia in HIV patients (Halasa, 2004). The side effects are gastrointestinal upset, skin rashes, hives, unexpected increase in blood clotting time and uncontrolled bleeding, nausea, severe abdomen or stomach pains, muscle pain and headaches (Salaheldin and Swapna, 2008)



(**9**)

Tetracyclines (**10**) are broad-spectrum bacteriostatic agents derived from a species of *Streptomyces* bacteria. They got their name because they share a chemical structure that has four rings. They are derived from a species of *Streptomyces* bacteria. Tetracyclines may be effective against a wide variety of microorganisms, including rickettsia and amebic parasites. They are used in the treatment of infections of the respiratory tract, sinuses, middle ear, urinary tract, skin, intestines. Tetracyclines also are used to treat gonorrhea, Rocky Mountain spotted fever, Lyme disease, typhus. Their most common current use is in the treatment of moderately severe acne and rosacea (Jenner *et al*., 2013).



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Tetracycline antibiotics are: tetracycline, doxycycline, minocycline and oxytetracycline. Drugs in the tetracycline class become toxic over time. Expired drugs can cause a dangerous syndrome resulting in damage to the kidneys. Common side effects associated with tetracyclines include cramps or burning of the stomach, diarrhea, sore mouth or tongue. Tetracyclines can cause skin photosensitivity, which increases the risk of sunburn under exposure to UV light. Rarely, tetracyclines may cause allergic reactions. Very rarely severe headache and vision problems may be signs of dangerous secondary intracranial hypertension (Jenner *et al*., 2013).

2.2.2 Herbal treatment

Herbal medicines are drugs of plant origin used to treat diseases and to attain or maintain a condition of improved health (Vivek *et al*., 2007). Herbs with medicinal properties are useful and effective source of treatment for various diseases. Many drugs used in medicinal science have their origin in medicinal plants (Chatuverdi, 2009). Plants have been used as herbal remedies in the treatment of many infectious diseases throughout the history of humankind (Jones, 1996: Nenad *et al*., 2007)

The global perspective in treatment and control of diseases is slowly changing from the use of conventional drugs to herbal applications due to prevalence of organic products (Gower *et al*., 2009). They are also preferred due to easier accessibility, affordability and rarity of complications (Wangari, 2008; Lian-Fang *et al*., 2009). This is particularly so in the sub-Saharan Africa where the WHO estimates that 85 % of the population relies on herbs for their primary health care needs (Chooto, 2004).

Herbalists prefer using crude extracts rather than extracting single components from them. The majority of herbs are used in their dried form. There are those, however, which must be used in their fresh form to be useful as medicinals, since they lose their healing properties when dried (Angela, 2009). Whole plant extracts have many components. These components work together to produce therapeutic effects and also to lessen the chances of side effects from any one component. Several herbs are often used together to enhance effectiveness and synergistic actions, and to reduce toxicity (Junior *et al*., 2005).

Essential oils are volatile natural plant products that have been used for many thousands of years in pharmaceutical alternative medicine and natural therapies (Matos *et al*., 1999). They can be distinguished from the fatty vegetable oils such as canola and sunflower by the fact that they evaporate or volatilize with the air and they usually possess a strong aroma (Vivek *et al*., 2007). These products are complex mixtures of organic chemicals, the nature and relative proportions of which are primarily determined by the genetics of the plant species. Variables that may affect the chemical constituents of the plant essential oils include climate, altitude, soil conditions, and nutrition, time, and harvest conditions, methods of post harvest handling and process of extraction. The essential oils are composed of terpenoids and aromatic polypropanoids

Essential oils are potential sources of novel antimicrobial compounds especially against bacteria pathogens. It is therefore necessary to scientifically investigate those plants which have been used in traditional medicine to improve the quality of health care (Seenivasan, 2006). For an extract to be classified as an essential oil, only heat and water may be used in its extraction from the plant (Lucchesi *et al*., 2004).

The antimicrobial properties of essential oils, organic and aqueous extracts have been recognized for many years, and their preparations have found applications as naturally occurring antimicrobial agents in the field of pharmacology, pharmaceutical botany, phytopathology, medical and clinical microbiology and food preservation among others (Ayogu and Amadi, 2009).

Many plant essential oils, fresh and solvent extracts have been found to exhibit antibacterial properties (Atalay *et al*., 2003). They include garlic, ginger, lemon and *Aloe vera* methanol extracts and their juices (Yona *et al*., 2004; Onyeagba *et al*., 2007; Lee *et al*., 2008; Shabnam *et al*., 2011), turmeric essential oil (Jayaprakasha *et al*., 2002), onion (Ewa *et al*., 2004) and eucalyptus essential oil (Lu *et al*., 2004; Salari *et al*., 2006).

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional healers use water as the solvent but studies done show that plant extracts in organic solvent (methanol) provide more consistent antimicrobial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay (Parekh *et al*., 2005)

Herbal preparations are available in varieties, either singly, or in mixtures formulated for specific conditions. They play a role in relieving and treating serious life threatening diseases such as AIDS, as well as in immune- enhancement (Barrett, 2003). The consistent presence of flavonoids, tannins, glycosides, and quinones in herbal products in previous studies may be taken to indicate that the products are effective as prescribed by the herbalists (Ajibola and Motoyoshi, 1992). These phytochemicals occur in varied amounts in various herbal preparations indicating that, their therapeutic effect(s) are not the direct effect of a single group or compound, but rather that the compounds possibly act in combination to bring about an effect (Abba *et al*., 2009).

Garlic (*A. Sativum* L.) (Figure 2.3) is a species in the onion family *Alliaceae.* Its close relatives include; the onion, shallot, leek and chive. It has been used throughout recorded history for culinary and medical purposes (Langer and Hill, 1991). It has a characteristic pungent, spicy flavour that mellows and sweetens considerably with cooking. It develops a group of small bulbs enclosed in a membrane like skin

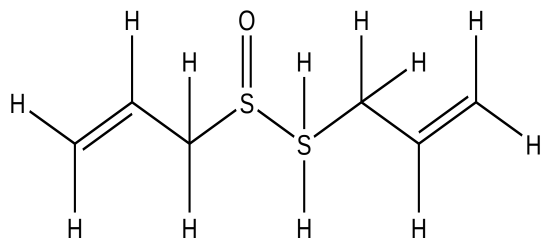
(Edmond *et al*., 1990). Garlic bulbs are formed underground (Larkcom, 1976). It is native to Central Asia, and has long been a staple in the Mediterranean region, as wellas a frequent seasoning in Asia, Africa and Europe (Peggy, 2009; Edmond *et al*., 1990).

Figure 2.3 Garlic bulbs

Garlic is claimed to help prevent heart disease, including atherosclerosis, high cholesterol and high blood pressure (Shanmugavelo, 1989). *A. sativum* may have beneficial properties, such as preventing and fighting the common cold. This assertion has the backing of long tradition in herbal medicine, which has used it as an expectorant for coughs and croup (Sharma *et al*., 1977: Yona *et al*., 2004). It is also alleged to reduce platelet aggregation and hyperlipidemia, blood sugar levels and thus prevent some complications of diabetes mellitus (Fawzy *et al*., 2012).

Garlic is the only natural antibiotic that actually kills infecting bacteria and at the same time protects the body from the poisons that cause infection, thus making it nature's ultimate antibiotic (Bergner, 2009). One very significant advantage the nutrient has over modern medications is that the body does not seem to build up a resistance to it as it does to many modern antibiotics (Larkcom, 1976). This also makes it a potentially effective nutrient for people who work with the public, like hospitals, day care facilities and malls where superbugs tend to be prevalent. Research shows that blood of garlic eaters can kill bacteria and is also reported that the vapor from freshly cut garlic can kill bacteria at a distance of 20 centimeters (Rayc, 2010). Traditional treatment of typhoid by herbs includes the use of allicin of garlic (Shanmugavelo, 1989). Garlic directly attacks bacteria and viruses, stimulates the body's natural defenses; enhancing the immune system against foreign invaders and also increases the activity of white blood cells that are central to the activity of the entire immune systems (Didill, 2008).

The major medicinal compound obtained from garlic is alliin (**11**) a powerful anti-biotic and anti-fungal agent. When garlic is crushed or damaged the enzyme allinase reacts with alliin to form allicin (**12**) (diallyl thiosulphinate).



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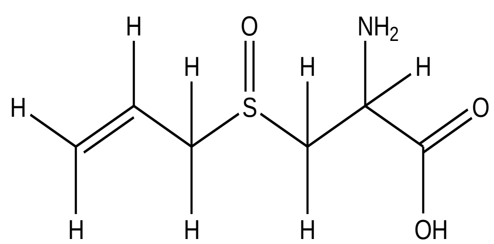

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In Traditional Chinese Medicine, garlic was considered a warm, bitter herb with particular effects on the large intestine, spleen and stomach meridians. It is used to lower blood pressure, to treat parasitic infections, food poisoning and tumors, and as a mild anticoagulant (Minyi, 1992; Bensky *et al.*, 1993; Huang, 1999).

Potentially active chemical constituents of China garlic include sulphur compounds, such as alliin, allicin, ajoene, allylpropyl disulfide, diallyl trisulfide, sallylcysteine, vinyldithiines, S-allylmercaptocystein (Bensky *et al*., 1993). Enzymes such as allinase, peroxidases, myrosinase, amino acids and their glycosides such as arginine, and trace metals such as selenium, germanium and tellurium are present in garlic (Kathi, 2000).

Garlic contains at least 33 sulfur compounds, several enzymes, 17 amino acids, and minerals such as selenium (Newall *et al*., 1996). It contains a higher concentration of sulfur compounds than any other *Allium* species. The sulfur compounds are responsible both for garlic's pungent odor and many of its medicinal effects. Allicin, which was first chemically isolated in the 1940's, has antimicrobial effects against many viruses, bacteria, fungi and parasites (Bradley, 1992). Garlic oil, aged garlic and steam-distilled garlic do not contain significant amounts of alliin or allicin, but instead contain various products of allicin transformation; none appears to have as much physiologic activity as fresh garlic or garlic powder (Block, 1985; Miething, 1988; Lawson, 1991).

Crude garlic extracts exhibited activity against both gram negative *(E. coli, Salmonella, Serratia, Citrobacter, Enterobacter, Pseudomonas, Klebsiella)* and gram positive *(Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus sanguis*) bacteria at room temperature, but there were no significant effects when the garlic had been boiled for five minutes before testing (Sharma *et al*., 1977; Elnima *et al*., 1983; Caceres *et al*., 1987; Hughes and Lawson, 1991; Farbman *et al*., 1983; Yongabi *et al*., 2009). Ajoene, a garlic-derived sulfur-containing compound, demonstrated antimicrobial activity against gram-positive bacteria, such as *Bacillus cereus, Bacillus subtilis, Mycobacterium smegmatis, Streptomyces griseus, Staphylococcus aureus* and *Lactobacillus plantarum* and against gram-negative bacteria, such as *E. coli, Klebsiella pneumoniae,* and *Xanthomonas maltophilia*; ajoene also inhibited yeast growth at concentrations below 20 micrograms/mL. Allicin exerted antibacterial activity against *Salmonella typhirium*, primarily by interfering with RNA synthesis (Feldberg *et al*., 1988; Mirelman, 1989).

Onion (*A. cepa*) (Figure 2.4) is probably a native to southern Asia and it consists of its herbaceous plant part. The leaves are bluish-green and hollow. The bulbs are large, fleshy and firm. There are three main varieties white, red and purple skinned. The relative pungency of onion has both genetic and environmental components (Azu *et al*., 2007).

Figure 2.4: Onion

Onions are a wonderful source of allicin. Allicin is a mineral recognized as an effective antimicrobial responsible for this vegetables' ability to fight infection in the body (Gareth *et al*., 2002). Epidemiological studies have shown a correlation between diets rich in onions and reduced risk of stomach cancer in human subjects (Zhou *et al*., 2011). Compounds that have been implicated in providing a number of health-promoting attributes of onion include flavonoids, particularly the flavonol quercetin and the organosulphur compounds such as cystein sulfoxides (WHO, 1999: Ewa *et al*., 2004).

Sulphur compounds in onions have shown to be ant-inflamatory both by inhibiting formation of thromboxanes and inhibiting the action of platelet-activating factor (Azu *et al*., 2007). Onion contains numerous sulfur compounds, including thiosulfinates and thiosulfonates; cepaenes; S-oxides; S,S-dioxides; mono-, di-, and tri-sulfides; and sulfoxides. Mincing or crushing the bulb releases cysteine sulfoxide from cellular compartments, making contact with the enzyme allinase from the adjacent vacuoles. Hydrolysis results with the release of reactive intermediate sulfenic acid compounds and then to the various sulfur compounds (Graefe *et al*., 2001; Rose *et al*., 2005; Arnault and Auger, 2006; Lanzotti, 2006; Nemeth and Piskula, 2007; Wiczkowski *et al*., 2008)

Two flavonoid subgroups are found in onion anthocyanins, which impart a red/purple colour to some varieties and flavanols such as quercetin and its derivatives responsible for the yellow and brown skins of many other varieties (Gareth *et al*., 2002). Flavonoids are chemical compounds active against microorganisms. However, the relative instability of the organosulphur compounds and the strong odor seem to limit the use of practical food preservatives. Onions have functional properties with the ability to modify lipid metabolism and stimulate the immune system (Ewa *et al*., 2004). Crude extracts of onion exert potent antifungal and antibacterial properties. Onion oils and aqueous extracts have been reported to be active against several gram-positive bacteria including *S. aureus* but ineffective against gram-negative bacteria such as *P. aeruginosa*, *E. coli* and *S. typhi* (Boulden, 2010).

In vitro studies have shown onion to possess antibacterial (including *H. pylori)*, antiparasitic, and antifungal activity (Elnima *et al*., 1983; Zohri, 1995; Rose *et al*., 2005). Clinical applications for this activity have not been determined, and use as a food preservative is limited by the strong odor and instability of sulfur compounds (Griffiths et al., 2002).

Lemon (*C. lemon)* (Figure 2.5) is used for culinary and nonculinary purposes throughout the world. All parts of lemon are used, that is, juice, rind and pulp (Ayalla *et al*., 2005; Annie, 2005). Citric acid from lemon is used for fermentation processes, insecticide and as antibacterial because it has a low pH (Seenivasan *et al*., 2006; Viuda – Martos, 2008). The high vitamin C content of the lemon is used to ward off scurvy. The fruit is good as flavouring for those on low salt diet (Del *et al*., 2004; Qian *et al*., 2012).

Figure 2.5: Lemon

Lemon essential oil has a bright, citrus smell. It has antiseptic properties and contains compounds which improve skin problems, kill bacteria, repels insects and boosts immune system (Theresa, 2008; Sharon, 2009). Studies done by Fisher and Phillips

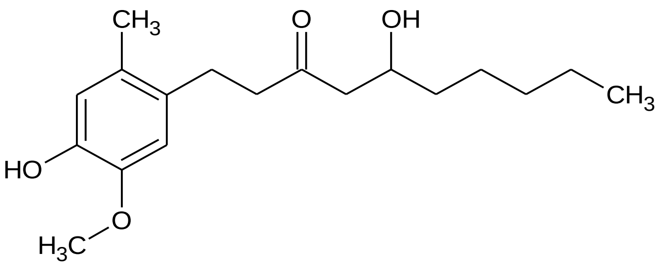
(2006) show that *C. lemon* is an effective anti-bacterial component. Gram-positive bacteria (especially *S. aureus*) are more susceptible than gram-negative bacteria *in vitro* (Fisher and Phillips, 2006).

Studies done on lemon essential oil showed maximum activity against *P. aeruginosa* (21.4 mm), *B. subtilis* (16.9 mm), *P. vulgaris* (22.5 mm), *K. pneumonia* (16.5 mm), *S. aureus* (17.5 mm) and E. coli (21.6 mm) (Seenivasan *et al*., 2006). Further studies show that the peel of lemon is not only an astringent but also is a good antimicrobial agent. This is an important finding as certain skin flora like *Pseudomonas* can grow in presence of sebum, especially when it is secreted in excess (in certain people), and cause purulent skin infections. Some time it can serve as a predisposing factor for other types of skin infections like acne. Simple use of lemon juice can prevent such types of infections and could help in keeping a good and healthy skin (Maruti *et al*., 2011).

Citrus fruit in general contain sugar, polysaccharide, organic-acid, lipids, carotenoids, vitamins, minerals, flavonoids, bitter lemonoids and volatile compounds. Lemon is a good source of potassium, calcium and vitamin C. Lemon juice has been reported to exhibit antimicrobial activity against *Vibrio cholera* (Hiroyuki *et al*., 2006).

Ginger (*Z. officinale*) (Figure 2.6) is used as delicacy or medicine (Sheri, 2007; Foster, 2009; Malu *et al*., 2009). *Zingiber* is native to tropical Southeast Asia and cultivated in the West Indies, Africa and India. A mixture of zingerone (**13**)**,** shogaols and gingerols causes the characteristic odour and flavour of ginger root (White, 2007).

Figure 2.6: A thick tuberous rhizome of ginger root



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Ginger compounds are active against diarrhea and treatment of nausea caused by seasickness, morning sickness and chemotherapy (Langner *et al*., 1998; Apariman *et al*., 2006; Chan *et al*., 2009). Studies conducted show that crude extracts of ginger in combination with lime inhibited *Staphylococcus spp* (Onyeagba *et al*., 2007; Riazur *et al*., 2011).

Turmeric *(C. longa)* (Figure 2.7), is an herbaceous perennial plant of the ginger family *Zingiberaceae* with orange, oblong tubers 2 or 3 inches in length and 1 inch in diameter, pointing or tapering at one end (Chaturvedi, 2009). It has lily-like leaves and yellow to yellowish white flowers (Chan *et al*., 2009). It has a strong taste and when dried it is made into yellow powder with a bitter, slightly acrid (unpleasantly pungent**)**, yet sweet taste (Chaturvedi, 2009). It is similar to ginger.

Figure 2.7: Photo of a turmeric tuber

Turmeric is a native of southern Asia, India and Central America (John and Timothy, 1997; Chan *et al*., 2009). Turmeric is well suited for growing in Kenyan “shambas” therefore readily available in local supermarkets and open air markets. It has been used for many years as a dye, flavouring and a medicinal herb (Jayaprakasha *et al*., 2002). Turmeric has antimicrobial, antioxidant, astringent properties; it is also used in dentistry (Gopalan *et al*., 2000). Turmeric powder is used in some communities as a contraceptive and induces sterility in women (Ghulam *et al*., 2009).

An analysis of turmeric shows that it consists of 13.1 % moisture, 6.3 % protein 5.15 fat 3.1 % minerals, 2.6 % fiber and 69.4 % carbohydrates; its mineral and vitamin contents are calcium, phosphorus, iron, carotene, thiamine and niacin (John and Timothy, 1997). Turmeric is a very useful intestinal antiseptic (Diran *et al*., 2008). Dry powder or juice from the rhizome mixed in butter milk or plain water is beneficial in intestinal problems especially chronic diarrhea. The juice of raw turmeric mixed with a pinch of salt is considered an effective remedy for expelling worms. Turmeric being rich in iron is valuable in anaemia; a teaspoon of raw turmeric juice mixed with honey is taken every day in the treatment of this condition. This herb is beneficial in the treatment of measles. Turmeric roots are dried in the sun and ground into powder, an effective household remedy for bronchial asthma, a teaspoon of turmeric powder in a glass of milk twice or thrice daily is effective (John and Timothy, 1997).

Turmeric volatile oil contains aromatic turmerone (20-30 %), which is a mosquito repellent and may be an effective drug for the treatment of respiratory disease and dermatophytosis (Govindarajan, 1980). It acts as anticarcinogenic, antivemon, antifungal and antibacterial. Turmeric is used traditionally to treat inflammation, skin wounds and tumors (Gopalan *et al*., 2000). It consists of two major parts: curcuminoids and turmeric oil (Jayaprakasha *et al*., 2002).

Laboratory tests have found that turmeric is antimutagenic, as it potentially helps prevent new cancers that are caused by chemotherapy or radiation used to treat existing cancers. It effectively inhibits metastasis (uncontrolled spread) of melanoma (skin cancer) cells (Chaturvedi, 2009).

2.3 Antibacterial compounds in herbal plants

The use and search for drugs and dietary supplements derived from plants have accelerated in recent years. Research done by Pavithra show that presence of phytochemicals such as alkaloids, tannins, triterpenoids, steroids and glycosides in the extracts of *Delonix elata*, *Enicostemma axillare, Merremia tridentata, Mollugo cerviana* and *Solanum incanum* supports their traditional uses as medicinal plants for the treatment of various ailments in India (Pavithra *et al*., 2010). While 25 to 50% of current pharmaceuticals are derived from plants, none are used as antimicrobials (Marjorie, 1999).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963; Narayana *et al*., 2000). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (for example, the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Schultes, 1978; Martini and Eloff, 1998).

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. Caffeic acid is effective against viruses (Wild, 1994), bacteria (Brantner *et al*., 1996), and fungi (Duke, 1985). Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well.

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988: Furniss *et al*., 1989). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al*.,1996), often leading to inactivation of the protein and loss of function. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. (Mohamoud *et al*., 2007).

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996).

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Narayana *et al*., 2000).

Terpenes or terpenoids are active against bacteria, fungi, viruses and protozoa (Tassou *et al*., 1995; Sun *et al*., 1996; Taylor *et al*., 1996; Xu, 1996). The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV.

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden and Fessenden *et al*., 1982).

Among the potential sources of new agents, medicinal plants have long been investigated. In rational drug therapy, the concurrent administration of two or more drugs is often essential and sometimes mandatory in order to achieve the desired therapeutic goal or to treat co-existing diseases. However, the drug interaction may have different effects on the host as well as the infecting microorganism. The potential benefits of using combined antimicrobial therapy can be treatment of mixed infections, therapy of severe infections in which a specific causative organism is known, enhancement of antibacterial activity, reducing the needed time for long-term antimicrobial therapy and prevention of the emergence of resistant microorganisms (Muhammad and Ahsan, 2005). Antibacterial synergism between bioactive plant extracts is a novel concept and has been recently reported (Nascimento *et al*., 2000; Junior *et al*., 2005; Chang *et al*., 2007; Horiuchi *et al*., 2007).

2.4 Methods of determining antibacterial properties

All of the susceptibility test methods commonly performed by clinical microbiology laboratories (disk diffusion, broth dilution, and agar dilution) measure the inhibitory activity of an antimicrobial agent. Antimicrobial assays can provide valuable information on the pharmacokinetics of the agent(s) being used and, when combined with Minimum inhibitory concentration (MICs) and Minimum bactericidal concentration (MBCs), may allow bacterial eradication to be predicted. In the present research disk diffusion, MIC and MBC tests are used to predict the activity of plant materials (Baron and Yolken, 1999).

2.4.1 Disk diffusion susceptibility test

In this method a filter paper disc impregnated with a sample is placed on agar, the chemical(s) from the sample diffuses from the disc into the agar. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a “zone of inhibition”. Experimental conditions which must be kept constant from test to test include agar, amount of organism , concentration of chemical, and incubation conditions (time, temperature, and atmosphere) (Rehman *et al*., 2011).

The amount of organism used is standardized using a turbidity standard. This may be a visual approximation using a McFarland standard 0.5 or turbidity may be determined by using a spectrophotometer (optical density of 1.0 at 600 nm). For antibiotic susceptibility testing the antibiotic concentrations are predetermined and commercially available. Each test method has a prescribed media to be used and incubation is to be at 35-37 °C in ambient air for 18-24 hours (Wayne, 1999).

Mueller-Hinton (MH) agar is prepared by using manufacturer’s instructions, autoclaved, cooled and poured into flat-bottom glass or plastic petri dishes on a level pouring surface to a uniform depth of 4 mm. McFarland 0.5 turbidity standards and physiological saline may be prepared or bought. Each culture to be tested should be streaked onto a non-inhibitory agar medium to obtain isolated colonies. After incubation at 35 °C overnight, 4 or 5 well-isolated colonies are selected with an inoculating needle or loop to a tube of sterile saline and mixed using a vortex. Inoculation is then done within 15 minutes after adjusting the turbidity of the inoculum (Yongabi *et al*., 2009).

After incubation at 35°C for 18 to 24 hours, the diameter of the zones of inhibition is measured (including the diameter of the disk) and recorded it in millimeters The measurements can be made with a ruler on the undersurface of the plate without opening the lid and inhibition zone ≥ 9.0 mm shows that the bacteria is susceptible to the antibacteria (Jorgensen *et al*., 2009; NCCLS, 2002).

2.4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Mann and Markham 1998). Agar dilution and broth dilution methods can be used to determine MIC. Agar dilution involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium followed by the application of a standardized number of cells to the surface of the agar plate. For broth dilution, often determined in 96-well microtiter plate format (Figure 2.8), bacteria are inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent (Wiegand, 2008).

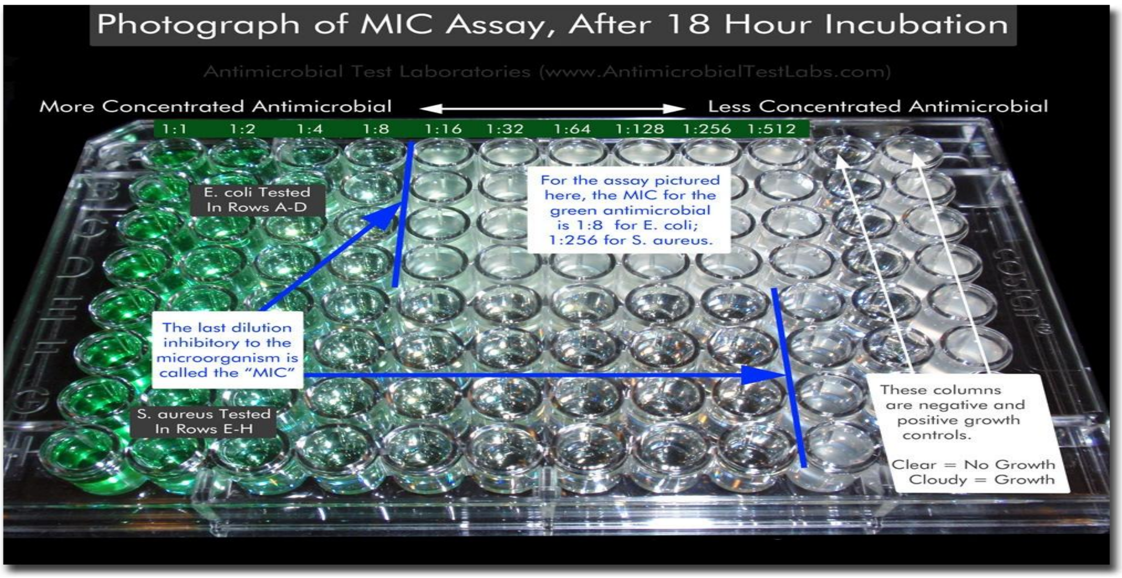


Figure 2.8: A 96-well microtiter plate

After incubation, turbidity is recorded either visually or with an automated reader, and the breakpoint concentration established. A lower MIC is an indication of a better antimicrobial agent (Donna *et al*., 1999). MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints (Andrews, 2001).

Minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (NCCLS, 1997). Dilutions and inoculations are prepared in the same manner as for determination of MIC and incubated overnight at 37 ^{0}C.

2.5 GC-MS analyses

Gas chromatography mass spectrometry (GC-MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals (Douglas, 2012).

This technique is used for the analysis of the hundreds of relatively low molecular weight compounds found in environmental materials. In order for a compound to be analyzed by GC/MS it must be sufficiently volatile and thermally stable. In addition, functionalized compounds may require chemical modification (derivatization) prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. Samples are usually analyzed as organic solutions consequently materials of interest need to be solvent extracted and the extract subjected to various 'wet chemical' techniques before GC/MS analysis is possible.

The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions (Skoog *et al*., 2007).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Experimental procedures

This study involved bioassay of garlic, ginger, turmeric onion and lemon juices, methanol extracts and essential oils over time. Identification of active compounds was performed using GC-MS. The experimental procedures are summarized using a flow diagram (Figure3.1).

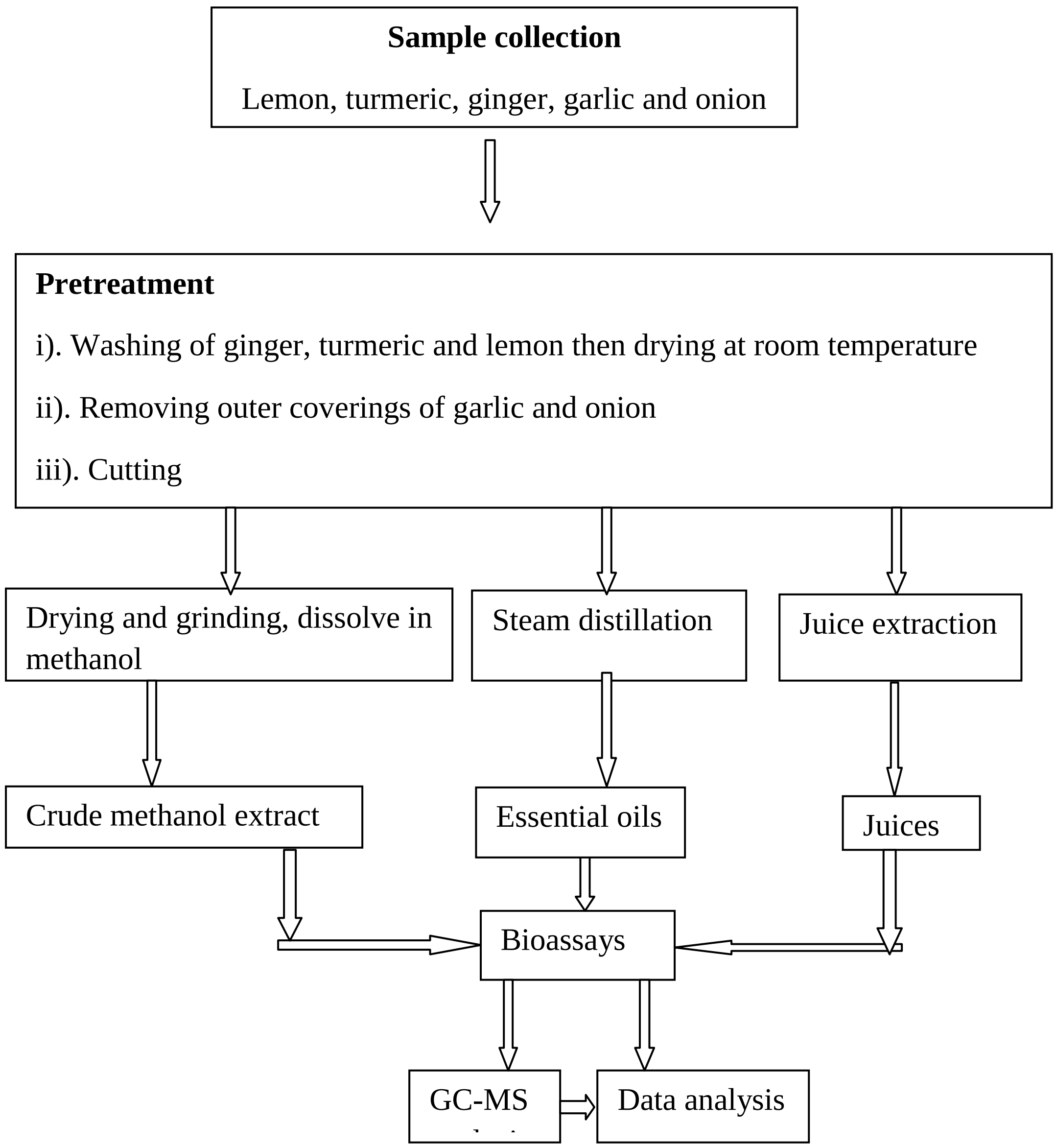


Figure 3.1 Flow chart showing the experimental procedures

3.2 Sample collection and pretreatment

The vegetable materials (garlic, ginger, onion and turmeric) and lemon were purchased from Githurai market in Nairobi. The rhizomes of ginger and turmeric, lemon fruit and onion were washed using tap water to remove dirt then dried. The outer coverings of garlic and onion were removed using a sharp knife disinfected with methanol. The materials were left to air dry at room temperature in a well-ventilated room away from sunlight for six hours. They were then stored in a dry cabin at room temperature awaiting extraction.

3.3 Apparatus and reagents

The glass apparatus were washed with hot water and detergent, rinsed three times with tap water, dried in an oven at 105^0 C for one hour and then cooled to room temperature. Sample bottles and equipments were rinsed with acetone followed by dimethylsulphoxide (DMSO) before use. The chemicals and reagents used in this study including acetone, methanol, anhydrous sodium sulphate, pentane, DMSO, barium chloride, dichloromethane (DCM) and sulphuric acid were of analytical grade from Sigma-Aldrich.

3.4 Instrumentation

The HP 5890 series II Gas Chromatograph interfaced to a 5973 Mass Selective Detector (MSD) and controlled by HP Chemstation software (version b.02.05, 1989-1997) was used. The chromatographic separation was achieved using a HP5-MS capillary column (30.0 m x 250 m x 0.25 m). The column stationary phase comprised of 5:95% diphenyl:dimethylpolysiloxane blend. The operating GC condition was an initial oven temperature of 35 °C for 3 min, then programmed to 280^0 C at the rate of

10^0 C/min, and then kept constant at 280^0 C (23 min). The injector and detector temperatures were set at 270^0 C and the carrier gas was nitrogen flowing at a rate of 1.2 ml/min. The mass spectrometer was operated in the electron impact mode at 70 eV. Ion source and transfer line temperature was kept at 280^0 C. The mass spectra were obtained by centroid scan of the mass range from 40 to 800 amu. Identification of the constituents was done on the basis of retention index, library mass search database (NIST & WILEY) and by comparing with the mass spectral data.

3.5 Isolation and extractions

3.5.1 Methanol extractions

The vegetable materials and lemon were cut into small pieces using a sharp knife and dried at room temperature for three weeks. They were ground into powder using a blender and soaked in methanol for 72 hours with occasional stirring. The extracts were filtered into clean dry glass containers using Whatman's No. 1 filter paper (9 cm). The extracts were concentrated using rotatory evaporator and dried to a paste in a hood. The crude extract was then used for bioassay.

3.5.2 Steam distillation

Essential oils were isolated from all the materials except onion (which did not produce significant amount of hydrodistillate). The materials were chopped into small pieces. Using a round-bottomed flask 1 kg of each material was mixed with 1 liter of water and then steam distilled using Clevenger-type apparatus (Figure 3.2). A flask containing the homogenate was heated for three to four hours and the oil was separated from water using a Pasteur pipette. The essential oils were put in amber colored vials, labeled and stored at −4° C before bioassay (Tassou *et al*., 1995)

Figure 3.2 Clevenger-type apparatus used for extraction of essential oils

The oil isolated from garlic was miscible with water and it was extracted using DCM. The mixture of oil and DCM was treated with anhydrous sodium sulphate to remove any dissolved water and evaporated using rotatory evaporator. The oil was labeled, put in amber colored vial then stored at −4° C before bioassay.

3.5.3 Juice extractions

The bulbs of garlic and onion, rhizomes of ginger and turmeric, and lemon were cut into small pieces and crushed using a juice extractor. The juice was sieved, put in amber colored vials and concentrated by freeze drying. The extractions were done two hours before commencement of sensitivity test. The sensitivity test was done within five days of preparation.

3.6 Bioassays

3.6.1 Preparation of McFarland standard

McFarland equivalent turbidity standard (0.5 McFarland) was prepared by adding 0.6 ml of 1 % barium chloride solution ($BaCl_2$. $2H_2O$) to 99.4 ml of 1 % sulphuric acid solution (H_2SO_4) and mixed. About 5 ml of the turbid solution was transferred to stopped test tube of the same type that was used to prepare the test and control inoculums. This was then stored in the dark room at a temperature of 25^0 C. Exactly 0.5 McFarland gives an equivalent approximate density of bacteria $1x10^{-8}$ Colony Forming Units (CFU) (Baron and Yolken, 1999).

3.6.2 Preparation of inoculums by direct colony suspension method

Microorganisms obtained from KEMRI included one gram positive bacteria; *S. aureus* (ATCC 25923) and three gram negative bacteria; *E. coli*, (ATCC 25922), *S. typhi* (ATCC 20613) and *P. aeruginosa* (ATCC 27853). The test strains were tested biochemically for viability and purity before use (Elgayyer *et al*., 2000). A small volume of sterile water was poured inside a test tube to which general colonies of the test organisms, taken directly from the plate were emulsified and the suspension was adjusted to match the 0.5 McFarland's standard (10^8 CFU/ ml) which has a similar appearance of an overnight broth culture by adding distilled water (Azu *et al.*, 2007).

3.6.3 Screening for antibacterial activity

3.6.3.1 Disc diffusion test

Antibacterial efficacy was tested using the filter paper disc diffusion method (Elgayyer *et al*., 2000). Using analytical balance, 3.0 g of each extract was dissolved in DMSO and 10 µl (100 mg/ml) loaded onto 6 mm (Whatman's No. 3) filter paper discs and air dried. The vegetable and lemon blends were made in the ratio of 1:1. The nutrient agar (NA) was used in culturing the bacteria. The media (NA) was prepared using manufacturer's instructions, sterilized using an autoclave set at 121^0 C. Plates were prepared by pouring 20 ml Mueller-Hinton (MH) agar into sterile petri dishes and allowed to set then allowed to cool in a sterile environment.

Each plate was inoculated with 0.1 ml of bacteria culture directly from the 24 hour broth culture (Figure 3.3) and diluted to match 0.5 McFarland standard. The discs loaded with the extracts were placed onto the seeded plates. The bacterial cultures were incubated at 37^0 C for 24 hours after which zones of inhibition were measured and recorded in mm. Negative control plates had discs with DMSO and water; positive control had standard antibiotic discs of chloramphenicol, ciprofloxacin and ampicillin. An inhibition zone of 9.0 mm was taken as the base and any sample that recorded less value was treated as inactive against the test organism.

Figure 3.3 Preparation of plates for bioassays

3.6.3.2 Minimum inhibitory concentration (MIC) Test

The active samples (with inhibition zone of ≥9) from the antibacterial screening were tested for minimum inhibitory concentration (MIC). To determine the MIC, different concentrations of essential oils, juices and methanol extracts were prepared by dissolving 3.0 g of the crude samples in 2.0 ml of DMSO. The resultant mixture was mixed using a vortex. The blends were made by mixing the resultant mixtures in the ratio of 1:1 and 100 µl of the samples were drawn into a 96-well micro titer plate. Concentrations of 750 mg/ml, 375 mg/ml, 188.5 mg/ml, 93.8 mg/ml, 46.9 mg/ml, 23.4 mg/ml, 11.7 mg/ml, 5.9 mg/ml, 2.9 mg/ml and 1.5 mg/ml were made using serial dilution method (Elgayyer *et al*., 2000; Kariba, 2001).

The test organism adjusted to 0.5 McFarland standard was drawn into wells. Blends of active essential oils and methanol extracts were made in the ratio of 1:1. The MIC for bacteria was determined using a broth dilution method of the active extracts. Tubes containing nutrient broth only were seeded with the test organism, as described above, to serve as control. The cultures were incubated at 37 °C for 24 hours. After incubation, they were examined for bacterial growth by observing turbidity. The first tube showing no growth (lowest concentration that inhibited growth) was the MIC (Kariba, 2001; Michael *et al*., 2003).

3.6.3.3 Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) of the active extracts was done by sub-culturing 0.1 ml (100µl) of all the tubes showing no growth on nutrient agar. After 24 hour incubation at 37 °C the first plate showing no growth was the MBC (Michael *et al*., 2003).

3.7 GC-MS analyses

Samples of 3.0 g garlic, ginger, lemon and turmeric were crushed and dissolved separately in 5 ml of DCM. They were shaken and mixed using the ultra sound path for 3 min, then filtered using glass wool. The sample was drawn into small vials and then 1 µl was injected into the GC-MS. China garlic was also prepared in similar manner and analyzed for comparison with garlic (used for bioassays). The active methanol extracts were blended in the ratio of 1:1 and 2 ml of pentane was added to each blend. The resultant mixture was left overnight, filtered using glass wool and 5 µl of the filtrate was dissolved in 1 ml of pentane. The sample (1 µl) was injected into the GC-MS for analysis. The active essential oil blends (in the ratio of 1:1) were also drawn into small vials and then 1 µl was analyzed.

3.8 Data analyses

The inhibition zone data obtained from juice and methanol extracts was subjected to analysis of variance (ANOVA). Individual essential oils recorded activities which were less than 9 mm and their results were not subjected to ANOVA. The mean inhibition zones of active individual juices and methanol extracts against *S. typhi*, *P. aeruginosa*, *S. aureus* and *E. coli* were compared to their blends. Treatment means showing significant difference ($p \leq 0.05$) were separated using Student-Newman-Keuls (SNK) at 5% significance level. The GC-MS chromatograms obtained from each active sample was subjected to HP Chemstation software; each peak was analyzed for the most abundant compound that contains active constituents -OH, -COOH, - Cl, -S, N, -F and $–NH_2$. The compounds were identified by direct comparison of their mass spectra to the Wiley NBS and MIST database library of mass spectra.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 Introduction

This chapter covers the results of antibacterial activities, MBC's and MIC's of juices, methanol extracts and essential oils individually and as blends. The results of time course antibacterial efficacies and GC-MS analyses of active juices, methanol extracts and essential oils are also discussed.

4.2 Antibacterial activities

4.2.1 Vegetable and lemon juices

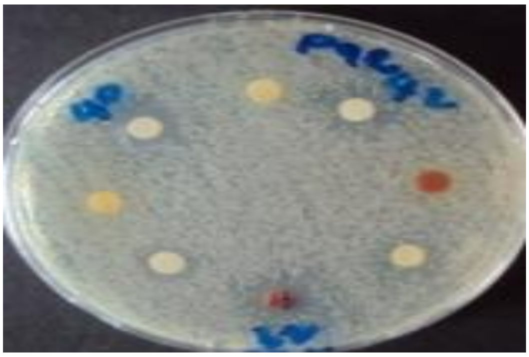
The inhibition zones of juices on gram positive and gram negative bacteria was determined using filter paper disc diffusion method (Elgayyer *et al*., 2000) and the results are indicated in Table 4.1

Table 4.1: Antibacterial activity exhibited by various juices against *P. aeruginosa, E .coli, S. aureus* and *S. typhi*

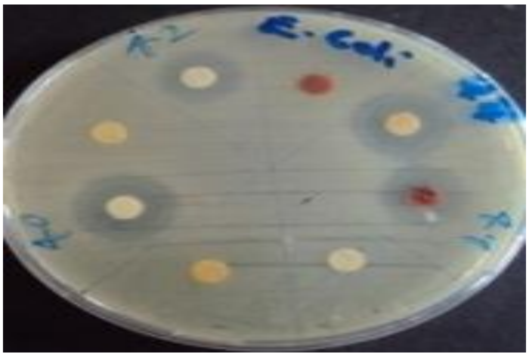
Juice/ antibiotic	Inhibition zone in mm[a]			
	P. aeruginosa	*E.coli*	*S.aureus*	*S.typhi*
Turmeric	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon	6.0±0.0	6.0±0.0	6.0±0.0	11.0±1.0
Ginger	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Garlic	10.0±0.0	11.7±0.3	14.7±2.5	17.7±2.5
Onion	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
DMSO (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Water (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Ampicillin (+ve)	6.0±0.0	11.7±0.3	6.0±0.0	18.7±0.6
Ciprofloxacin (+ve)	41.7±0.6	30.7±0.3	17.3±2.1	34.7±0.6
Chloramphenicol (+ve)	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.3

[a] - includes the diameter (6 mm) of the disk used
+ve - positive control
-ve - negative control

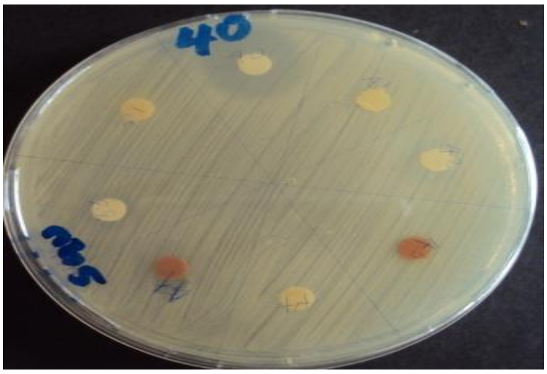
Garlic juice (Figure 4.1, (sample 40)) inhibited the growth of all bacteria tested to variable levels (10.0 mm for *P. aeruginosa*, Figure 4.1a; 11.7 mm for *E. coli,* Figure 4.1b; 14.7 mm for *S. aureus* Figure 4.1c*;* and 17.7 mm for *S. typhi,* Figure 4.1d.). Lemon juice inhibited only the growth of *S. typhi* with a zone of 11.0 mm. Turmeric, lemon and ginger juices had no activity against *P. aeruginosa*, *E. coli* and *S. aureus*, this can be attributed to the low concentration (10 µl) of samples which were used for bioassay. Earlier studies on their activity show that they had antifungal and antibacterial agents at concentrations of 50 µl and 100 µl (Gopalan *et al.*, 2000; Jayaprakasha *et al.*, 2002; Fisher and Phillips, 2006)

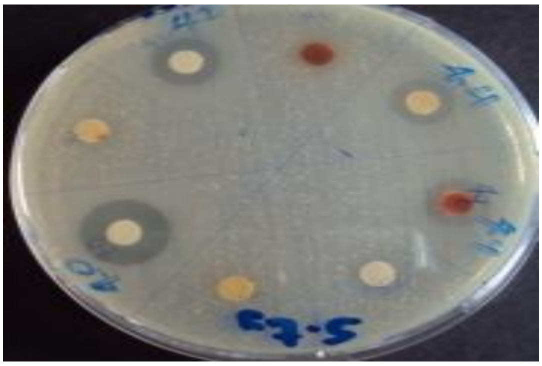


(a) *P. Aeruginosa*



(b) *E. coli*





(c) *S. aureus* **(d) *S. typhi***

40 – Garlic

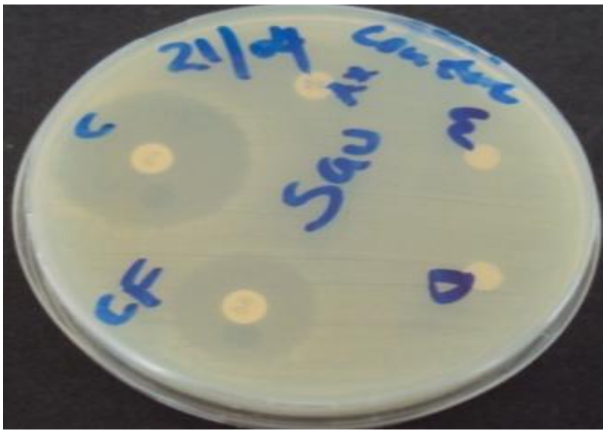
Figure 4.1(a, b, c, d): Plates showing inhibition zones of garlic against *P. aeruginosa*, *E. coli, S. aureus* and *S. typhi*

The high antibacterial activity exhibited by garlic as compared to lemon may be attributed to the presence of sulphur-based compounds such as alliin (**9**), which possess strong antibacterial activities (Larkcom, 1976: Bocchini *et al*., 2001). These compounds are present in the intact bulbs, flavorants formed on cutting or crushing the bulbs, substances derived from further reactions of these flavorants or metabolic degradation of these three types of compounds (John and Timothy, 1997). The results agree with earlier reports where garlic was effective against a plethora of gram positive and gram negative bacteria such as *S. aureus*, *Proteus*, *Pseudomonas*, *E. coli*, *Salmonella* and *Klebsiella* (O'Gara and Hill, 2000). The activity of lemon on the other hand can be attributed to the presence of –COOH and -OH group which acts against the bacteria (Angel, 2006). However *E. coli* was not susceptible to lemon juice containing due to its unusual acid resistant properties. The microorganism has the ability to survive and grow in acidified media (Greg and Ann, 2007).

Research has found aqueous extract of garlic to be more potent than organic extracts (Roy *et al.*, 2006; Jaber and Al-Mossawi, 2007). This could be as a result of the fact that when plant materials are ground in water, a number of phenolases and hydrolases are released and these enzymes might serve to modulate the activity of the active compounds in the extract (De and Ifeoma, 2002). Since the herbalist usually uses water to prepare infusions and decoctions, and since most constituents of garlic are soluble in water, there is likelihood that the herbalist is able to extract all the bioactive drug components in garlic making it a good home remedy against some infections.

Positive controls had varying activities depending on the type of the sample used. The activity of ampicillin on *E. coli* and *S. typhi* was 11.7 mm and 18.7 mm respectively

(Table 4.1). Ciprofloxacin had an activity of 41.7 mm against *P. aeruginosa*, 30.7 mm against *E. coli*, and 17.3 mm against *S. aureus* and 34.67 mm against *S. typhi*. Chloramphenicol had the highest activity against *E. coli* (35.0 mm) and *S. aureus* (34.0 mm) (Figure 4.2).



Key

C- Chloramphenicol

CF- Ciprofloxacin

Figure 4.2: Plate showing inhibition zones of chloramphenicol and ciprofloxacin, on *S. aureus*

The activity of standards (+ve controls) is considerably high compared to the samples used. This can be accredited to the pure form of the standards and therefore no interferences from other compounds. The natural juices contain mixtures of compounds including non-active constituents which may result in dilution of the active constituents (Narayana *et al.*, 2000).

Juices of turmeric, ginger and onion did not significantly inhibit growth of any microorganism tested; their inhibition zones were 6.0±0.0 mm each. The sulphur based compounds which are accredited to bacterial activities might have been destroyed during cutting and crushing of onion, the bacteria may have developed resistance to onion, ginger and turmeric or the relative percentage of the active compounds in the samples was low (Griffiths *et al*., 2002). Onion has also been reported to be ineffective against gram-negative bacteria such as *S*. *aureus*, *E*. *coli* and *S*. *typhi* due to less amounts of allicin (Farbman *et al*., 1983).

Although ginger, turmeric and onion juices tested individually showed no significant inhibition, some blends of these vegetables (1:1, v/v) were active (Table 4.2). The highest activity was exhibited by lemon/garlic (15.0 mm) and lemon/garlic/turmeric (14.7 mm) against *E. coli*. The blends of lemon/garlic, ginger/garlic, lemon/garlic/ginger, turmeric/ginger/garlic and lemon/garlic/turmeric had appreciable activities against *E. coli* and *S. typhi*. Ginger/lemon, lemon/turmeric, ginger/turmeric and lemon/ginger/turmeric blends had no significant activity against all the four bacteria tested. *S. typhi* was susceptible to turmeric/garlic and lemon/garlic/turmeric/ginger at a zone of 12.0 mm and 9.7 mm respectively. The test bacteria, *P. aeruginosa* and *S. aureus* did not record any activity when the juice blends were used.

Table 4.2: Antibacterial activity exhibited by various juice blends against *P. aeruginosa, E .coli, S. aureus* and *S. typhi*

Sample juice blends	Mean inhibition zone in mm [a]			
	P. aeruginosa	*E .coli*	*S. aureus*	*S. typhi*
Lemon/garlic	7.7±0.6	15.0±0.0	6.0±0.0	12.0±0.0
Ginger/garlic	6.0±0.0	12.0±0.0	6.0±0.0	11.0±0.0
Turmeric/garlic	6.0±0.0	6.0±0.0	6.0±0.0	12.0±0.0
Ginger/turmeric	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Ginger/lemon,	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon/turmeric,	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon/garlic/ginger	6.0±0.0	13.7±0.6	6.0±0.0	9.7±0.6
Turmeric/ginger/garlic	6.0±0.0	11.0±0.0	6.0±0.0	10.0±0.0
Lemon/garlic/turmeric	8.7±0.6	14.7±0.6	6.0±0.0	11.0±0.0
Lemon/ginger/turmeric	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Lemon/garlic/turmeric/ginger	6.0±0.0	6.0±0.0	6.0±0.0	9.7±0.6
DMSO (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Water (-ve)	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Ampicillin (+ve)	6.0±0.0	11.7±0.3	6.0±0.0	18.7±0.6
Ciprofloxacin (+ve)	41.7±0.6	30.7±0.3	17.3±2.1	34.7±0.6
Chloramphenicol (+ve)	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.3

[a] - includes the diameter (6 mm) of the disk used

Table 4.3 gives the results of Student-Newman-Keuls (SNK) test on the mean inhibition zones of individual juices and their blends against *S. typhi*, *P. aeruginosa*, *S. aureus* and *E. coli* bacteria. The mean inhibition zone of garlic juice against *S. typhi* was significantly different ($p < 0.05$) compared to other tested materials and not significantly different to that of ampicillin. Lemon/garlic/turmeric blend gave inhibition zones against *E. coli* and *S. typhi* that are significantly different to those of pure garlic ($p < 0.05$).

Table 4.3: The mean (±SD) inhibition zones exhibited by individual juices and their blends against *P. aeruginosa*, *E. coli*, *S. aureus* and *S. typhi*

Sample juice / Antibiotic	Inhibition zone (mm) (±SD)			
	P. aeruginosa	***E. coli***	***S. aureus***	***S. typhi***
Lemon	N.A	N.A	N.A	11.0±1.0[a]
Garlic	10.0±0.0[a]	11.7±0.6[ab]	14.7±2.5[a]	17.7±2.5[b]
Lemon/garlic	N.A	15.0±0.0[d]	N.A	12.0±0.0[a]
Ginger/garlic	N.A	12.0±0.0[b]	N.A	11.0±0.0[a]
Turmeric/garlic	N.A	N.A	N.A	12.0±0.0[a]
Lemon/garlic/ginger	N.A	13.7±0.6[c]	N.A	9.7±0.6[a]
Turmeric/garlic/ginger	N.A	11.0±0.0[a]	N.A	10.0±0.0[a]
Lemon/garlic/turmeric	N.A	14.7±0.6[d]	N.A	11.0±0.0[a]
Lemon/garlic/turmeric/ginger	N.A	N.A	N.A	9.7±0.6[a]
Ampicillin	N.A	11.7±0.3[ab]	N.A	18.7±0.6[b]
Ciprofloxacin	41.7±0.6[c]	30.7±0.6[e]	17.3±2.1[a]	34.7±0.6[d]
Chloramphenicol	20.0±0.0[b]	35.0±0.0[f]	34.0±0.0[b]	30.7±0.6[c]

Mean (±SD) followed by the same small letters within the same column are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)
N.A- not active

From the mean inhibition zones it can be noted that ginger and turmeric lowers the activity of blends and lemon/garlic blend has lower activity on *S. typhi* (12.0±0.0) compared to pure garlic (17.7±2.5). This may be due to the deactivating effect of citric acid on the allinase, an enzyme that converts alliin to allicin (Bocchini *et al*., 2001). The transformation of alliin to allicin is extremely rapid, taking mere seconds. Even more intriguing is the instability of allicin (Blania and Spangenberg, 1991). The most crucial and reactive part of the allicin molecule is the sulfur-sulfur bond coupled to an atom of oxygen (Mohammad *et al*., 2007). It remains active only for a short period before degrading. When allicin degrades, as many as 200 other sulfur compounds are formed (Bocchini *et al*., 2001).

Blends that comprised garlic had antibacterial activity against one or more microorganisms tested. Studies done on rats infected with *Klebsiella pneumoniae* using plant extracts (ginger and garlic) for seven days show that garlic treated group recovered fully on day four, but all the animals in ginger treated group died. No death was, however, recorded in rats treated with mixture of garlic and ginger (Olatunde *et al.*, 2009). All tests performed against *P. aeruginosa* showed inactivity except garlic. This might be as a result of the bacteria developing resistance against individual juices and blends (Baliga, 2005).

4.1.2 Methanol extracts

All individual methanol extracts except lemon showed no activity against the tested microorganism (Table 4.4). The lemon extract had activity of 11.0±0.0 mm against *P. aeruginosa* and 10.0±0.0 mm against *S. aureus* respectively.

Table 4.4: Antibacterial activity exhibited by individual methanol extracts against *P. aeruginosa*, *E. coli*, *S. aureus* and *S. typhi*

	Inhibition zone in mm[a]			
Sample methanol extract	*P. aeruginosa*	*E. coli*	*S. aureus*	*S. typhi*
Turmeric	6.0±0.0	6. 0±0.0	8.7±0.6	6. 0±0.0
Onion	7.7±0.6	7.3±0.6	6. 0±0.0	7.3±0.6
Lemon	11.0±0.0	7.3±0.6	10.0±0.0	6.3±0.6
Ginger	6. 0±0.0	6.0±0.0.	7.3±0.6	6. 0±0.0
Garlic	6. 0±0.0	6. 0±0.0	6. 0±0.0	6. 0±0.0
Ampicillin	6. 0±0.0	11.7±0.3	6. 0±0.0	18.7±0.6
Ciprofloxacin	41.7±0.6	30.7±0.6	17.3±2.1	34.7±0.6
Chloramphenicol	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.6

[a] includes the diameter (6mm) of the disk used

The methanol blends, also made in the ratio of 1:1 (v/v) had sensitivities against the bacteria as shown in Table 4.5. Turmeric/lemon extract blend had activity of 11.0±0.0mm against *S. aureus*. The increase in activity can be attributed to positive interactions between the natural compounds present in the blend leading to synergism (Bocchini *et al*., 2001). Addition of garlic to the blend of turmeric/lemon methanol extract increases the activity to 12.0±1.0 mm. The activity of turmeric/ginger/lemon extract on *S. aureus* is 10.0±0.0 mm, but on addition of garlic the activity reduces to 9.3±0.6 mm. The blend of turmeric/garlic/ginger/lemon/onion extracts had an activity of 9.0±0.0 mm against *S. aureus*. The methanol blends recorded an inhibition zone of less than 9.0±0.0 mm against *E. coli* and *S. typhi*, thus inactive. The factors associated with the reduced activities of the blends are not clear and therefore require further studies to be undertaken on the blends.

Table 4.5: Antibacterial activity exhibited by various methanol extract blends against *P. aeruginosa E. coli*, *S. aureus* and *S. typhi*

Sample methanol extract	Mean Inhibition Zone in mm [a]			
	P. aeruginosa	*E. coli*	*S. aureus*	*S. typhi*
Turmeric/lemon	8. 0±0.0	6.3±0.6	11.0±0.0	8.3±0.6
Lemon /ginger	9.67±0.6	6.3±0.6	8.0±0.0	8.0±0.0
Ginger/garlic	6. 0±0.0	6.0±0.0	7.0±0.0	6.0±0.0
Turmeric/ginger/lemon	6· 0±0.0	6.0±0.0	10.0±0.0	6.0±0.0
Turmeric/lemon/garlic	6. 0±0.0	6.0±0.0	12.0±1.0	6.0±0.0
Turmeric/garlic/ginger/lemon	6. 0±0.0	7.0±1.0	9.3±0.6	8.0±0.0
Turmeric/garlic/ginger/lemon/onion	6· 0±0.0	6.7±0.6	9.0±0.0	8.0±0.0
Ampicillin	6. 0±0.0	11.7±0.3	6. 0±0.0	18.7±0.6
Ciprofloxacin	41.7±0.6	30.7±0.6	17.3±2.1	34.7±0.6
Chloramphenicol	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.6

[a] includes the diameter (6mm) of the disk used

Table 4.6 gives a summary of the overall mean inhibition zones of individual methanol extract and blends against *S. aureus*. The overall mean inhibition zone of turmeric/lemon/garlic methanol blend against *S. aureus* is significantly different ($p < 0.05$) to the other test materials. The activities of individual lemon, and turmeric/ginger/lemon, turmeric/garlic/ginger/lemon/onion and turmeric/garlic/ginger/lemon blends are not significantly different. The data obtained from the susceptibility tests on *P. aeruginosa, E. coli* and *S. typhi* was not subjected to ANOVA as only lemon/ginger was active against *P. aeruginosa*.

Table 4.6: The mean (±SD) inhibition zones exhibited by individual methanol extracts and blends against *S. aureus*

Sample/Antibiotic	**Mean inhibition zone(mm) of *S. aureus*(±SD)**
Lemon	10.0±0.0[a]
Turmeric/lemon/garlic	12.0±1.0[b]
Turmeric/ginger/lemon	10.0±0.0[a]
Turmeric/garlic/ginger/lemon	9.3±0.6[a]
Turmeric/garlic/ginger/lemon/onion	9.0±0.0[a]

Mean (±SD) followed by the same small letters within the same column are not significantly different at $\alpha = 0.05$ (Student-Newman-Keuls test)

The result of antibacterial susceptibility assay showed promising evidence for the antibacterial effects of lemon methanol extract against *S. aureus* (10.0±0.0 mm) and *P. aeruginosa* (11.0±0.0 mm). This is in agreement with a study conducted by Pandey *et al*., (2011) which showed methanol extract of lemon to be effective against *P.*

aeruginosa with inhibition zone of 23 mm. The results of antibacterial testing revealed that methanol extract of lemon had inhibitory effect on *P. aeruginosa* (11.0 mm) and *S. aureus* (10.0 mm) due to better solubility in the organic solvent as compared to the juice (Malu, 2009: Mohamma *et al*., 2009: Pandey *et al*., 2011).

4.1.3 Essential oils

Essential oils of garlic, lemon, turmeric and ginger were obtained through steam distillation using Clevenger-type apparatus. Onion did not yield sufficient oil with steam distillation using Clevenger-type apparatus. Bioassay of all the essential oils gave an inhibition zone of less than 9.0 mm and thus inactive against the test gram positive and gram negative bacteria (Kariba *et al.*, 2001). The inactivity of garlic may be attributed to the relative instability of the organosulphur compounds which might have been destroyed during hydro- distillation and drying (Ewa *et al*., 2002). Steam-distilled garlic does not contain significant amounts of alliin or allicin, but instead contains various products of allicin transformation; none appears to have as much physiological activity as fresh garlic (Mohammad et al., 2009: Salem *et al*., 2010).

Bioassay results obtained from blends of essential oils are summarized in Table 4.7. The lemon/garlic blend gave an inhibition zone of 10.0 mm with *P. aeruginosa*. Lemon/ginger essential oil blend was active against *E. coli* and *S. typhi* with inhibition zone of 9.0 mm and 9.7 mm, respectively. Lemon/garlic/turmeric blend had an inhibition zone of 9.3 mm against *S. aureus*.

Table 4.7: Antibacterial activity exhibited by various essential oil blends against *P. aeruginosa E. coli, S. aureus* and *S. typhi*

Sample	Inhibition zone in mm [a]			
	P. aeruginosa	*E. coli*	*S. aureus*	*S. typhi*
Lemon	6.3±0.6	6 .0±0.0	6.3±0.6	6.3±0.6
Garlic	6.3±0.6	6 .0±0.0	7 .0±0.0	6 .7±0.6
Turmeric	6 .0±0.0	6 .0±0.0	6 .0±0.0	6 .0±0.0
Ginger	6 .0±0.0	6 .0±0.0	7 .0±0.0	6 .0±0.0
Lemon/garlic	10.0±0.0	6.3 ±2.1	6.7±0.6	6.3±0.6
Lemon/ginger	7.7±0.6	9.0±1.0	7.0±1.0	9.7±0.6
Lemon/garlic/turmeric	7.0±1.0	6 .0±0.0	9.3±0.6	6 .0±0.0
Ampicillin	6. 0±0.0	11.7±0.3	6. 0±0.0	18.7±0.6
Ciprofloxacin	41.7±0.6	30.7±0.6	17.3±2.1	34.7±0.6
Chloramphenicol	20.0±0.0	35.0±0.0	34.0±0.0	30.7±0.6

[a] - includes the diameter (6 mm) of the disk used

The results indicate that lemon/garlic essential oil blend showed an increase in the antibacterial activity against *P. aeruginosa* (10.0±0.0 mm) as compared to their individual essential oils. The increase may be due to synergistic interaction of essential oil constituents of lemon and garlic (Esimone *et al*., 2006). Lemon/ginger also showed an increase in the antibacterial activity against *E. coli* (9.0±1.0)and *S. typhi*(9.7±0.6). These results are consistent with previous reports which showed that some blends of plant essential oils can have higher *in vitro* activity against bacteria (Junior *et al*., 2005; Betoni *et al*., 2006; Horiuchi *et al*., 2007). Interestingly, although neither lemon nor garlic essential oil exhibited activity; the blend of the two was active, suggesting that the volatile constituents of lemon interact synergistically with the transformed products of garlic (Ewa *et al*., 2002).

4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The fresh juice of garlic inhibited the growth of *S. aureus*, *E. coli*, *S. typhi* and *P. aeruginosa* at a concentration of 375 mg/ml, 187.5 mg/ml, 93.8 mg/ml and 46.9 mg/ml respectively (Table 4.9).

Table 4.8: MIC and MBC results for active samples

Sample		MIC's mg/ml				MBC's mg/ml			
		P. aeruginosa	*E. coli*	*S. aureus*	*S. typhi*	*P. aeruginosa*	*E. coli*	*S. aureus*	*S. typhi*
Fresh extracts	Garlic	46.9	187.5	375	93.8	46.9	187.5	375	93.8
Methanol extracts	Lemon	2.9	ND	187.5	ND	2.9	ND	187.5	ND
	turmeric/lemon	ND	ND	23.4	ND	ND	ND	23.4	ND
	lemon/ginger	5.9	ND	ND	ND	5.9	ND	ND	ND
	turmeric/lemon/ginger	ND	ND	46.9	ND	ND	ND	46.9	ND
	turmeric/lemon/garlic	ND	ND	93.8	ND	ND	ND	93.8	ND
	turmeric/ginger/garlic	ND	ND	187.5	ND	ND	ND	187.5	ND
	turmeric/lemon/ginger/garlic	ND	ND	187.5	ND	ND	ND	187.5	ND
	turmeric/lemon/ginger/garlic/onion	ND	ND	23.4	ND	ND	ND	23.4	ND
Essential oils	lemon/garlic	187.5	ND	ND	ND	187.5	ND	ND	ND
	lemon/ginger	ND	750	ND	187.5	ND	750(static)	ND	187.5
	lemon/garlic/turmeric	ND	ND	375	ND	ND	ND	375	ND

ND- Test not done, static- bacteriostatic

Methanol extract of lemon and lemon/ginger inhibited the growth of *P. aeruginosa* at a concentration of 2.9 mg/ml and 5.9 mg/ml respectively. Lemon, turmeric/lemon, turmeric/lemon/ginger, turmeric/lemon/garlic, turmeric/ginger/garlic, turmeric/lemon/ginger/garlic, and turmeric/lemon/ginger/garlic/onion methanol extracts exhibited an MIC of 187.5 mg/ml, 23.4 mg/ml, 46.9 mg/ml, 93.8 mg/ml, 187.5 mg/ml, 187.5 mg/ml and 23.4 mg/ml against *S. aureus* respectively. All the methanol extracts had no activity against *S. typhi*. Essential oils of lemon/garlic inhibited growth of *P. aeruginosa* at a concentration of 187.5 mg/ml, and lemon/ginger inhibited growth of *E. coli* and *S. typhi* at 750 mg/ml and 187.5 mg/ml respectively. Lemon/garlic/turmeric essential oil had an MIC of 375.0 mg/ml against *S. aureus*.

The plates showing no growth on nutrient agar were sub-cultured and incubated for 24 hours at 37 ^{o} C. The MBC results obtained are shown in Table 4.8. Garlic juice, methanol extracts and essential oils were bactericidal on all the bacteria tested at concentrations similar to their MIC's except essential oil blend of lemon/ginger which was bacteriostatic against *E. coli* bacteria at 750 mg/ml.

Methanol extracts inhibited the growth of bacteria at lower concentrations (2.9 mg/ml, 23.4 mg/ml and 5.9 mg/ml) as compared to juices and essential oils. The bactericidal properties of the essential oils might have been evaporated, destroyed or transformed to other forms during hydro distillation and drying while methanol extracted most of the components from the samples (Ewa *et al*., 2002). Interestingly, ginger and turmeric are basic culinary spices and condiments consumed for relief during abdominal discomforts (Jayaprakasha *et al*., 2002; Apariman *et al*., 2006), but did not show notable bactericidal activity on the tested microorganisms.

4.3 Time-course antibacterial efficacy

Juices which had recorded activity against any one or more bacteria of ≥9.0 mm were tested for efficacy within five days and their results summarized in Table 4.9.

Table 4.9: Antibacterial activity exhibited by juices and blends against *P. aeruginosa*, *E. coli*, *S. aureus* and *S. typhi* for a period of 5 days

Antibiotic/ Sample	Bacteria	Mean (±SD) inhibition zone(mm) [a]				
		Day 1	Day 2	Day 3	Day 4	Day 5
Lemon	*S. aureus*	6.7±1.2[b]	7.0±1.0[b]	6.7±0.6[b]	6.0±0.0[b]	11.0±0.0[a]
	S. typhi	11.3±0.6[a]	7.0±1.0[b]	6.7±1.2[b]	6.3±0.6[b]	6.3±0.6[b]
Turmeric	*S. aureus*	6.0±0.0[a]	6.0±0.0[a]	6.0±0.0[a]	6.0±0.0[a]	6.0±0.0[a]
Ginger	*S. typhi*	6.0±0.0[a]	6.0±0.0[a]	6.0±0.0[a]	6.0±0.0[a]	6.0±0.0[a]
Garlic	*P. aeruginosa*	10.0±0.0[b]	6.0±0.0[c]	10.3±3.8[b]	9.7±3.2[b]	15.0±0.5[a]
	E. coli	11.7±0.6[ab]	19.7±3.8[a]	17.0±3.6[a]	10.3±3.8[b]	6.0±0.0[b]
	S. aureus	14.7±2.5[b]	26.0±2.6[a]	27.0±6.1[a]	11.0±2.9[b]	6.0±0.0[b]
	S. typhi	17.7±2.5[a]	13.7±1.2[ab]	11.7±2.8[b]	10.7±0.6[b]	11.0±1.0[b]
Lemon /garlic	*E. coli*	15.0±0.0[a]	10.3±0.6[c]	6.0±0.0[d]	12.3±0.6[b]	14.3±0.6[a]
	S. typhi	12.3±0.6[b]	11.3±0.6[b]	7.0±1.0[c]	11.7±2.1[b]	18.7±1.2[a]
Ginger/garlic	*E. coli*	12.3±0.6[b]	9.3±0.6[a]	7.0±1.0[a]	7.3±1.5[b]	7.7±1.5[a]
	S. typhi	12.0±1.0[a]	12.3±1.2[a]	7.0±1.0[b]	7.3±1.5[b]	7.3±1.5[b]
Turmeric/garlic	*S. typhi*	13.0±1.0[a]	8.0±1.0[b]	6.3±0.6[b]	6.7±1.2[b]	6.7±1.2[b]
Lemon/garlic/ginger	*E. coli*	13.7±0.6[a]	13.3±1.5[a]	6.7±1.2[b]	9.0±1.0[b]	6.7±1.2[b]
	S. typhi	10.3±0.6[ab]	9.3±0.6[b]	6.3±0.6[c]	6.3±0.6[c]	12.0±2.0[a]
Turmeric/garlic/ginger	*E. coli*	12.3±1.5[a]	7.7±1.5[b]	6.3±0.6[b]	7.0±1.0[b]	7.0±1.7[b]
	S. typhi	11.3±1.5[a]	8.0±1.0[b]	7.0±1.0[b]	7.0±1.7[b]	7.0±1.0[b]
Lemon/garlic/turmeric	*E. coli*	14.7±0.6[a]	13.3±1.5[ab]	6.7±0.6[c]	11.7±2.1[b]	7.0±1.0[c]
	S. typhi	11.7±1.2[a]	10.3±0.6[a]	6.7±1.2[b]	7.3±1.5[b]	6.7±1.2[b]
Lemon/garlic/turmeric/ Ginger	*E. coli*	6.3±0.6[b]	12.0±1.0[a]	7.5±1.4[b]	6.7±1.2[b]	7.3±1.5[b]
	S. typhi	10.0±1.0[a]	6.7±0.6[a]	7.7±1.5[a]	7.0±1.7[a]	7.3±1.5[a]

[a] includes the diameter (6mm) of the disk used

Garlic showed inhibitory activity against all the microorganisms used for the five days (Figure 4.3). Turmeric and ginger individual juices recorded an inhibition zone of <9 and thus did not show activity against any of the tested bacteria.

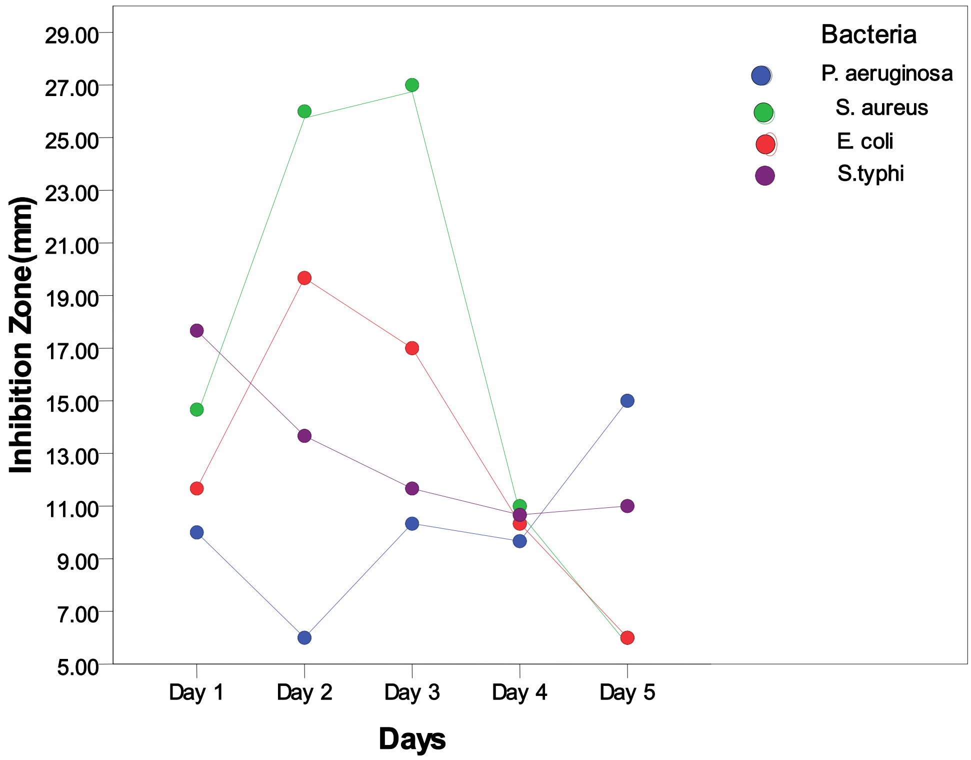


Figure 4.3: Comparison of mean (±SE) inhibition zones of garlic juice against *S. typhi, E. coli, P. aeruginosa* and *S. aureus*

Lemon and garlic individual juices showed decreasing activities against *S. typh*i from day 1 to day 5. The individual juice of lemon and turmeric/garlic blend did not show any changes in activity against *E. coli*. Ginger/garlic, lemon/garlic/ginger and turmeric/garlic/ginger blends show decreasing activities against *E. coli* with time. The activity of lemon/garlic against *E. coli* dropped from day 1 to 3 then increased again up to day 5 (Figure 4.4). The activity of lemon/garlic/turmeric against E. coli dropped from day 1 to 3 then increased again up to day 4 (Figure 4.4). Individual lemon juice and the other blended test materials did not record any activity against *P. aeruginosa* for the 5 days.

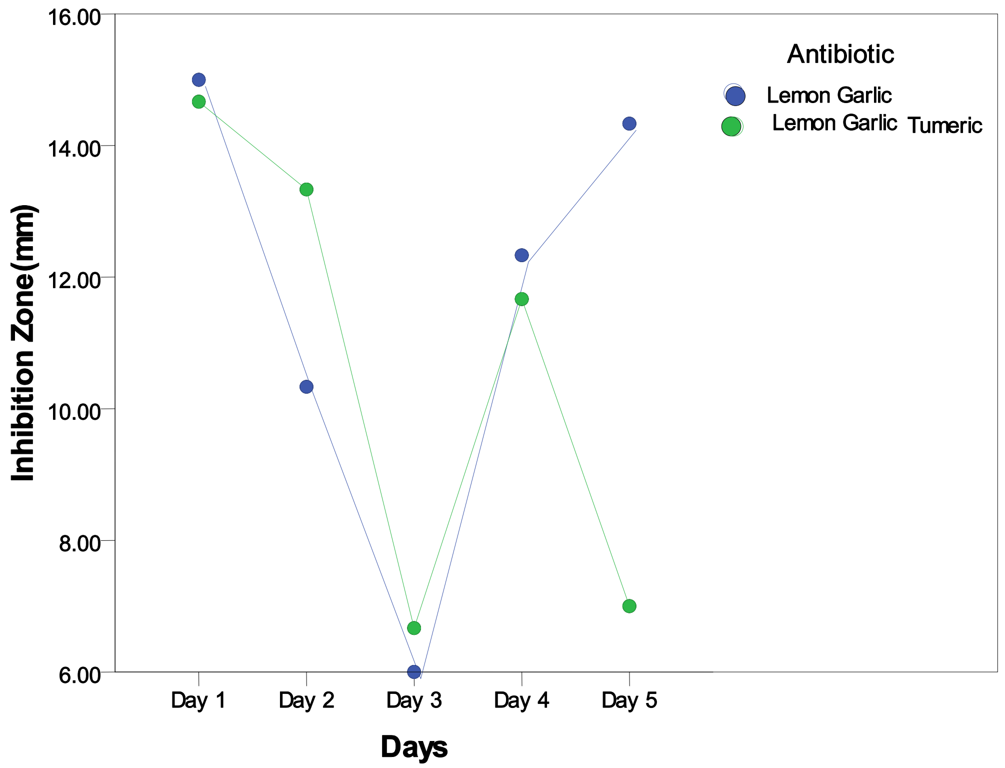


Figure 4.4: Comparison of mean (±SE) inhibition zones of two juice blends against *E. coli.*

Lemon/garlic blend showed a very interesting pattern against *S. typhi*: the activity dropped from 12.3±0.6 to 7.0±1.0 by day 3, but increased to 18.7±1.2 on day 5 (Fig 4.5). A similar pattern of activity was shown by lemon/garlic/ginger blend against the same bacterium (Fig 4.5). This pattern suggests that the intermediate products formed are inactive, but that their further transformation leads to products that are inhibitory to *S. typhi* (Farbman *et al*., 1983). Monitoring (by GC-MS or LC-MS) of the specific transformations of the constituents that take place can shed light on these interesting findings.

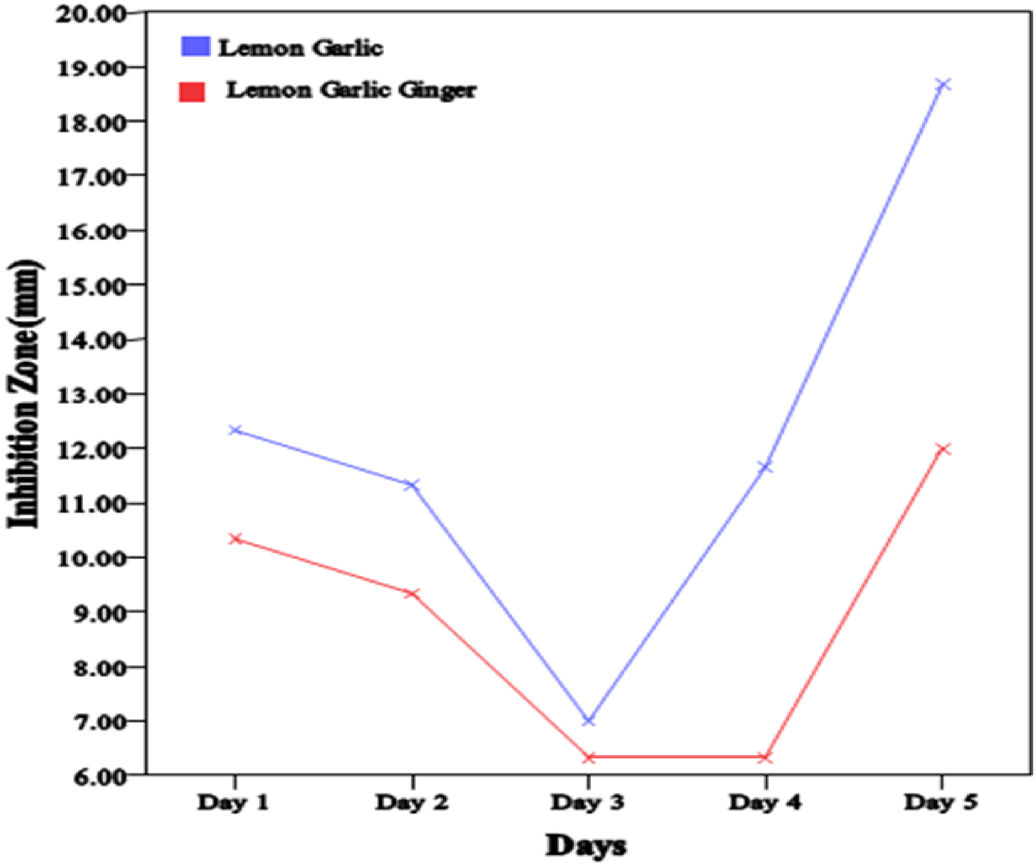


Figure 4.5: Comparison of mean (±SE) inhibition zones of two juice blends against *S. typhi*

4.6 GC-MS analyses

4.6.1 Fresh juices

Juices of lemon, local garlic, ginger, and turmeric were analyzed by GC-MS and each sample gave a chromatogram having several peaks. The suspected antibacterial compounds with their molecular formula and weight are listed in Table 4.10. The chromatograms obtained from garlic lemon, ginger and turmeric juices with peaks representing suspected antibacterial compounds are shown in Appendix I

Table 4.10: The GC-MS profile of compounds suspected to contain antibacterial properties identified in lemon, China garlic, local garlic, ginger and turmeric juices

					Relative %				
No	Compound	Molecular formula	M+(g/mol)	Retention time (min)	Lemon	China garlic	Local garlic	Ginger	Turmeric
1.	α –Terpineol	$C_{10}H_{18}O$	154	14.562	0.41	-	-	0.61	-
2.	Limonene	$C_{10}H_{16}$	136	11.906	85.08	-	-	-	-
3.	3-Hexen-1-ol	$C_6H_{12}O$	100	8.154	0.16	-	-	-	-
4.	Mentha-2,8-dien-1-ol	$C_{10}H_{16}O$	152	13.428	0.18	-	-	-	-
5.	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	23.749	0.46	-	-	-	-
6.	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280	25.434	0.14	-	-	-	-
7.	2-Ethoxycarbonyl-3-methyl-7-nitro-4-azafluorenone,phenylimine	$C_{22}H_{17}N_3O_4$	387	40.316	0.72	-	-	-	-
8.	Pyrrolo[2,3-b] indole	$C_{14}H_{16}N_2O_4$	218	20.502	-	-	-	-	0.73
9.	Methanehydrazonic acid, *N*-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester	$C_6H_9N_4OS_2$	218	21.331	-	-	-	-	8.87
10.	Selenourea, phenyl-	$C_7H_8N_2Se$	200	21.531	-	-	-	-	0.17
11.	Imidazole, 4-methyl-5-[3,3,3-trifluoropropionylpropyl]-	$C_{10}H_{13}\ F_3N_2O$	234	22.248	-	-	-	-	0.47
12.	1,6,10-Dodecatriene-3 ol,3,7,11-trimethyl-	$C_{15}H_{26}O$	222	22.358	-	-	-	-	0.48
13.	2-Butenoic acid, 3-methyl-, methylester	$C_6H_{10}O_2$	114	22.43	-	-	-	-	0.60
14.	2-Azabicyclo[3.2.1]octan-3-one	$C_7H_{11}NO$	125	22.58	-		-	-	0.12
15.	2,4-Quinolnediol	$C_9H_7NO_2$	161	22.724	-	-	-	-	0.44
16.	3-[4-Hydroxybenzoylhydrazono]-*N*-mesitylbutyramide	$C_{20}H_{23}N_3O_3$	353	22.974	-	-	-	-	0.29
17.	Phthalic acid, cyclohexylmethyl-3-phenylpropylester	$C_{24}H_{28}O$	380	23.14	0.40	-	-	-	0.53
18.	Linalool	$C_{10}H_{18}O$	154	13.066	-	-	-	0.50	0.05
19.	Terpinen-4-ol	$C_{10}H_{18}O_4$	154	14.563	-	-	-	-	0.05
20.	Bicyclo[3.2.2]non-8-en-6-ol, (1R,5-cis,6-cis)-	$C_9H_{14}O$	138	16.105	-	-	-	-	0.03
21.	Guaiacol<para-vinyl->	$C_9H_{10}O_2$	150	16.377	-	-	-	-	0.07
22.	*N*-(2-Phenylethenyl)acetamide	$C_{10}H_{11}NO$	161	17.266	-	-	-	-	0.03
23.	Ethanone,1-cyclopropyl-2-[3-pyridinyl]-	$C_{10}H_{11}NO$	161	19.5	-	-	-	-	0.73

Table 4.10: Cont'd

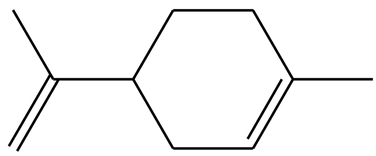
					Relative %				
No	Compound	Molecular formula	M+(g/mol)	Retention time (min)	Lemon	China garlic	Local garlic	Ginger	Turmeric
24.	1,5-Dimethyl-2-pyrrolecarbonitrile	$C_7H_8N_2$	120	20.104	-	-	-	-	0.61
25.	6-Octen-1-yn-3-ol, 3,7-dimethyl-	$C_{10}H_{16}O$	152	20.207	-	-	-	-	1.11
26.	Ethyl homovanillate	$C_{11}H_{14}O_4$	210	23.353	-	-	-	-	0.47
27.	Ezlopitant , dehydro-	$C_{32}H_{24}N_2O$	452	32.758	-	-	-	-	0.14
28.	Phenol, 4-pentyl-	$C_{11}H_{16}O$	164	33.341	-	-	-	-	0.76
29.	[1,3,5]Triazine-2,4-diamine,6-	$C_9H_{13}N_7$	219	34.608	-	-	-	-	0.21
30.	*O*-methoxy- α,-methylbenzyl alcohol	$C_9H_{12}O_2$	152	36.307	-	-	-	-	0.22
31.	Methyl-4-deoxy-2-0-methyl.beta.1-threo-hex-4-enopyrid urinate	$C_8H_{12}O_4$	204	5.878	-	3.77	-	-	-
32.	Benzenethiol	C_6H_6S	110	8.952	-	1.18	-	-	-
33.	3,4-Dimethylthiophene	C_6H_8S	112	9.268	-	1.21	-	-	-
34.	Ethylthiazole	C_5H_7NS	113	13.64	-	0.29	-		-
35.	Thiophene, 3-methyl	C_5H_6S	98	16.804	-	1.18	-	-	-
36.	Disulphide, methyl-2-propenyl	$C_4H_8S_2$	120	5.878	-	3.38	-	-	-
37.	1-propene-3, 3-thiobis	$C_6H_{10}S$	114	8.170	-	1.33	2.90	-	-
38.	Thiourea,*N*-*N*'-dimethyl	$C_3H_8N_2S$	104	9.869	-	0.48	0.84	-	-
39.	Diallyl disulphide	$C_6H_{10}S_2$	146	12.734	-	3.62	10.84	-	-
40.	3-Chlorothiophene	C_4H_3ClS	118	13.069	-	7.24	6.49	-	-
41.	3-Vinyl-1,2-dithiacyclohex-4-ene	$C_6H_9S_2$	144	14.541	-	18.25	21.43	-	-
42.	3-Vinyl-1,2-dithiacyclohex-5-ene	$C_6H_9S_2$	144	14.723	-	4.02	3.09	-	-
43.	Cyclohexen-1-ol, 3-methyl	$C_7H_{12}O$	108	15.500	-	-	0.62	-	-
44.	Ethyl trifluoromethyl trisulphide	$C_3H_5F_3S_3$	194	17.163	-	-	1.67	-	-
45.	1,3-Dioxolane-2-[dichloromethyl]-	$C_4H_6Cl_2O_2$	157	17.584	-	-	0.36	-	-
46.	Acetic acid, chloro-2-butoxyethyl ester	$C_6H_{15}ClO_3$	196	17.852	-	-	2.73	-	-

Table 4.10: Cont'd

					Relative %				
No	Compound	Molecular formula	M+(g/mol)	Retention time (min)	Lemon	China garlic	Local garlic	Ginger	Turmeric
47.	Acetamide,n-tetrahydrofurfuryl-2-methoxy	$C_8H_{15}NO_3$	173	14.250	-	1.44	1.35	-	-
48.	Octadecanoic acid,3-hydroxy, methyl ester	$C_{19}H_{38}O_3$	314	20.178	-	-	0.66	-	-
49.	1,2,3-Thiadiazole,5-methyl-	$C_3H_4N_2S$	100	14.727	-	2.59	-	-	-
50.	1,4-benzenediol-2-chloro	$C_6H_5ClO_2$	144	16.656	-	1.61	-	-	-
51.	Propanoic acid,2-chloro	$C_6H_5ClO_2$	108	17.163	-	1.86	-	-	-
52.	3,4-Dimethylthiophene	C_6H_8S	112	9.268	-	1.46	-	-	-
53.	Disulphide,methyl-2-propenyl	$C_4H_8S_2$	120	9.528		4.07	-	-	-
54.	1,2-dithiolane	$C_3H_6S_2$	106	10.885	-	0.32	-	-	-
55.	2-ethylthiacyclohexane	$C_7H_{14}S$	130	12.192	-	0.67	-	-	-
56.	(methylthio)-acetonitrile	C_3H_5NS	87	13.738	-	0.88	-	-	-
57.	3-Vinyl-1,3-dithiane	$C_6H_{10}S_2$	146	15.009	-	1.22	-	-	-
58.	1,4-Diathiane	$C_4H_8S_2$	120	9.527	-	1.65-	3.176	-	-
59.	Octadecanoic acid,3-hydroxy, methyl ester	$C_{19}H_{38}O_3$	314	20.179	-	1.24	-	-	-
60.	N-Methoxy-N-methyl	$C_2H_6NF_2OP$	129	21.927	-	0.44	-	-	-
61.	Amidinothiourea	$C_2H_6N_4S$	118	12.341	-	0.94	0.671	-	-
62.	2-Heptanol	$C_7H_{16}O$	58	9.222	-	-	-	0.24	-
63.	Borneol	$C_{10}H_{18}O$	154	14.49	-	-	-	0.81	-
64.	Citronellol	$C_{10}H_{20}O$	156	15.067	-	-	-	0.50	-
65.	Geraniol	$C_{10}H_{18}O$	154	15.458	-	-	-	1.05	-
66.	Geranic acid	$C_{10}H_{16}O_2$	168	16.825	-	-	-	0.15	-
67.	Elemol	$C_{10}H_{16}O_2$	222	19.447	-	-	-	0.73	-
68.	E –Nerolidol	$C_6H_{26}O$	222	19.537	-	-	-	0.43	-
69.	2-Butanone,4-(-hydroxy-3-methoxyphenyl	$C_{11}H_{14}O_3$	194	20.628	-	-	-	14.14	-
70.	Ketone,1-cyclohexen-1-yl methyl,semicarbazone	$C_9H_{15}N_3O$	181	28.736	-	-	-	0.51	-
71.	α-Zingiberene	$C_{15}H_{24}$	204	18.769	-	-	-	25.08	-

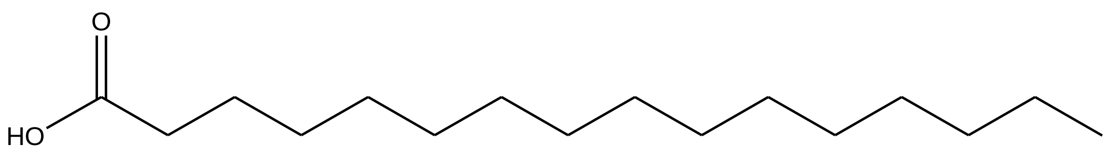
Garlic originating from China was also analyzed by GC-MS for comparison with garlic used (local garlic) in the bioassays. The candidate antibacterial compounds are listed with their molecular formula, percentage abundance and weight in Table 4.10. The chromatograms representing peaks suspected to have candidate antibacterial compounds for China garlic and local garlic are shown in appendix 1.

The GC-MS analyses showed that lemon juice contained limonene (**14**) (85.08%), an antibacterial agent (Hiroyuk *et al.*, 2006) ; 3-hexen-1-ol (0.16%); mentha-2,8-dien-1-ol (0.18%); hexadecanoic acid (**15**) (0.46%); 9,12-octadecadienoic acid (0.14%); 2-ethoxycarbonyl-3-methyl-7-nitro-4-azafluorenone,phenylimine (0.72%); phthalic acid, cyclohexylmethyl-3-phenylpropylester (0.40%) and α-terpineol (0.14), which may have confered bacterial inhibition property to this terpene (Angel, 2006; Fisher and Phillips, 2006).



limonene

14

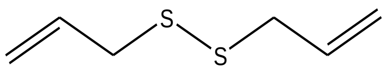


hexadecanoic acid

15

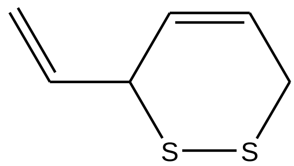
GC-MS analysis of local garlic juice showed the presence of: diallyl disulphide (**16**) (10.84%); 3-chlorothiophene (6.49%); 3-vinyl-1,2-dithiacyclohex-4-ene (21.4%); 3-

vinyl-1,2-dithiacyclohex-5-ene (**17**) (3.09%); acetic acid, chloro-2-butoxyethyl ester(2.73%); ethyl trifluoromethyl trisulphide (1.67%); acetamide, *n*-tetrahydrofurfuryl-2-methoxy (1.35%); 1-propene, 3,3'thiobis (2.90%); 1,4-diathiane (3.18%); thiourea, *N*,*N*'-dimethyl- (0.84%); octadecanoic acid,3-hydroxy, methyl ester (0.66%); Cyclohexen-1-ol, 3-methyl (0.62%); 1,3-Dioxolane-2-[dichloromethyl]-(0.36%) and amidinothiourea (0.67). All these compounds except acetamide n-tetrahydrofurfuryl-2-methoxy are sulphur-containing compounds, which might be responsible for antibacterial activity of garlic juice (Kathi, 2000; O'Gara *et al*., 2000). China garlic gives additional sulphur compounds compared with local garlic (appendix 1). This may reflect some genetic or chemotypic differences between the two.



diallyl disulphide

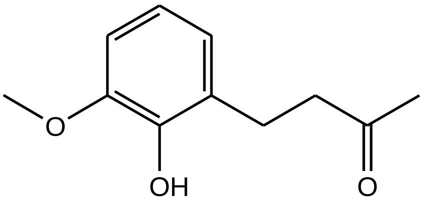
16



3-Vinyl-1,2-dithiacyclohex-4-ene

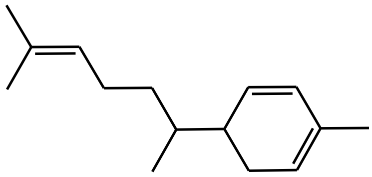
17

Ginger juice reaveled the presence of α –Terpineol (0.61%); 2-heptanol (0.24%); linalool (0.50%); borneol (0.81%); citronellol (0.50%); geraniol (0.05%); geranic acid (0.15%); elemol (0.73%); *E*-nerolidol (0.43%); 2-butanone,4-(-hydroxy-3-methoxyphenyl)- (**18**) (14.14%); ketone,1-cyclohexen-1-ylmethyl,semicarbazone (0.51%) and α-zingiberene (**19**) (25.08%). These compounds are mainly terpenoids, some of which have shown strong inhibitory activity against pathogenic bacteria (Malu *et al*., 2009).



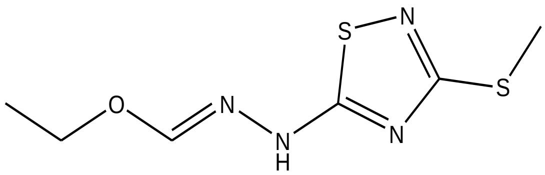
2-butanone,4-(-hydroxy-3-methoxyphenyl)-

18



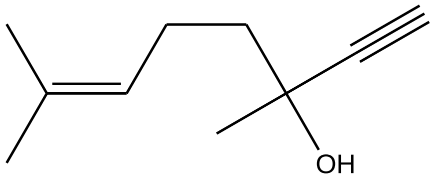
19 (α-zingiberene)

Turmeric had a wide range of suspected antibacterial components including; pyrrolo [2,3-b] indole (0.73%); Methanehydrazonic acid, *N*-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester (**20**) (8.87%) ; Selenourea, phenyl- (0.17%);Imidazole, 4-methyl-5-[3,3,3-trifluoropropionylpropyl]- (0.47%); 1,6,10-Dodecatriene-3 ol,3,7,11-trimethyl- (0.48%); 2-Butenoic acid, 3-methyl-, methylester (0.60%); 2-Azabicyclo[3.2.1]octan-3-one (0.12%); 2,4-Quinolnediol(0.44%); 3-[4-Hydroxybenzoylhydrazono]-*N*-mesitylbutyramide (0.29%) ; Phthalic acid, cyclohexylmethyl-3-phenylpropylester (0.53%); Linalool (0.05%); Terpinen-4-ol (0.05%); Bicyclo[3.2.2]non-8-en-6-ol, (1R,5-cis,6-cis)- (0.03%); Guaiacol<para-vinyl->(0.07%); *N*-(2-Phenylethenyl) acetamide (0.03%); Ethanone,1-cyclopropyl-2-[3-pyridinyl]- (0.73%); 1,5-Dimethyl-2-pyrrolecarbonitrile(0.61%) ; 6-Octen-1-yn-3-ol, 3,7-dimethyl- (**21**) (1.11%); Ethyl homovanillate (0.47%); Ezlopitant , dehydro- (0.14%) ; Phenol, 4-pentyl- (0.76%); [1,3,5]Triazine-2,4-diamine,6- (0.21%) and *O*-methoxy-α,-methylbenzyl alcohol (0.22%) but they exhibited low or no anti-bacterial activity. This may be attributed to their low concentrations (Gopalan *et al*., 2000; Ghulam *et al*., 2009)



Methanehydrazonic acid, N-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester

20



6-Octen-1-yn-3-ol, 3,7-dimethyl-

21

4.6.2 Methanol extracts

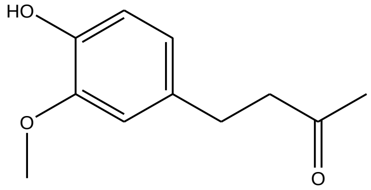
The suspected antibacterial compounds identified from active methanol extracts by GC-MS are listed in Table 4.11 with their percentage relative abundance, molecular formula and weight. Chromatograms representing some peaks suspected to be of antibacterial compounds from methanol extracts are shown in Appendix 2.

Table 4.11: The GC-MS profile of compounds suspected to contain antibacterial properties identified in methanol crude extract and blends

					Relative %					
No.	Compound	Molecular formula	M^+(g/mol)	Retention time (min)	Lemon	LG	GiLT	LGT	GGiT	LGTGi
1.	Cyclohexanol,2-methylene-5-(1-methylene-5-[1-methylethenyl]-	$C_{10}H_{16}O$	152	14.517	4.41					
2.	Carveol	$C_{10}H_{16}O$	152	14.987	1.49					
3.	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	23.767	8.01	0.81		0.41	0.37	0.69
4.	Linoleic acid	$C_{18}H_{32}O_2$	280	25.460	5.86					
5.	Heptadecanoic acid	$C_{17}H_{34}O_2$	312	25.933	2.37					
6.	γ-Sitosterol	$C_{29}H_{50}OH$	414	39.136	8.00					
7.	Borneol	$C_{10}H_{18}O$	154	14.197		0.59				
8.	Citronellol	$C_{10}H_{20}O$	156	15.076		0.64				
9.	2-Butanone,4-[4-hydroxy-3-methoxyphenyl]-	$C_{11}H_{14}O_3$	194	20.585		5.15	4.27	0.63	2.54	5.50
10.	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	284	24.082		1.28				
11.	2-[3-Hydroxy-2-nitrocyclohexyl]-1-phenylethanone	$C_{14}H_{17}NO_4$	263	20.856	-	-	2.53	-	-	-
12.	Propanamide,2-amino-3-phenyl	$C_9H_{12}N_2O$	164	21.216	-	-	6.71	-	-	-
13.	5,6,7,8-Tetrahydroindolizine	$C_8H_{11}N$	121	21.089	-	-	-	1.03	-	-
14.	*E*-Nerolidol	$C_6H_{26}O$	222	19.540	-	-	-	-	-	0.59
15.	1,5-Dimethyl-2-pyrrolecarbonitrile	$C_7H_8N_2$	120	20.722	-	-	-	-	-	1.10
16.	Beta-cadren-9-alpha-ol	$C_{15}H_{24}O$	220	22.407	-	-	-	-	-	0.69
17.	Phenol, 4-ethyl-2-methoxy-	$C_9H_{12}O_2$	152	26.900	-	-	-	-	-	0.94
18.	α-Zingiberene	$C_{15}H_{24}$	204	18.769		33.75				

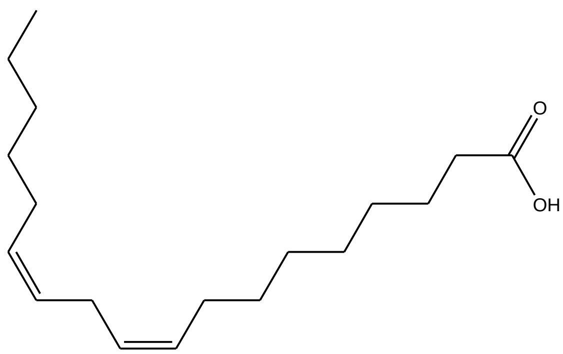
LG -Lemon/ginger. **GiLT** -Ginger/lemon/turmeric. **LGT** -Lemon/garlic/turmeric. **GGT** -Garlic/ginger/turmeric. **LGTG** -Lemon/garlic/ginger/turmeric.

The candidate antibacterial constituents obtained from methanol extracts include cyclohexanol,2-methylene-5-(1-methylene-5-[1-methylethenyl]- (4.41%); trans-carveol (1.49%); *n*-hexadecanoic acid (8.01%%); heptadecanoic acid (2.37%); γ-sitosterol (8.00%); borneol (0.59%); citronellol (0.64%); 2-butanone,4-[4-hydroxy-3-methoxyphenyl]- (**22**) (5.15%); linoleic acid (**23)** (5.86%); ethyl hexadecanoate (1.28%); 2-[3-hydroxy-2-nitrocyclohexyl]-1-phenylethanone (2.53%); propanamide,2-amino-3-phenyl (6.71%); 5,6,7,8-tetrahydroindolizine (1.03%); *E*-nerolidol (0.59%); 1,5-dimethyl-2-pyrrolecarbonitrile (1.10%); β-cadren-9-alpha-ol (0.69%); α-zingiberene (33.75%) and phenol,4-ethyl-2-methoxy- (0.94%). Methanol extracts contain aromatic hydrocarbons, ketones, phenols, organic acids and terpenes which have good inhibitory effect against gram positive and gram negative bacteria. Their varied occurrences in various blends may indicate that, their therapeutic effect(s) are not the direct effect of a single group or compound, but rather that the compounds possibly act in combination to bring about antibacterial effect (Abba *et al*., 2009).



2-Butanone,4-[4-hydroxy-3-methoxyphenyl]-

22



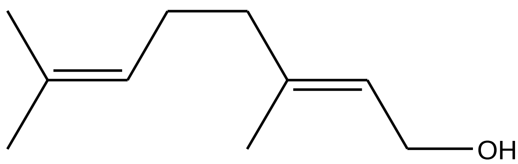
Linoleic acid

23

4.6.3 Essential oils

Three essential oil blends (lemon/garlic, lemon/ginger and lemon/garlic/turmeric) that were active against *S. typhi*, *P. aeruginosa*, *E. coli* and *S. aureus* were analyzed by GC-MS. The compounds suspected to have antibacterial properties with their molecular formula, mass and their relative proportions in the essential oils are given in Table 4.12 in relation to the sample of origin. The chromatograms showing suspected antibacterial compounds are shown in Appendix 3.

The compounds suspected to have antibacterial properties are fewer in the essential oils as compared to juices and methanol extracts. The compounds which were present include: diallyl disulphide; [4-Aminophenyl]2- methylpiperidin-1-yl) methanone; limonene; terpinen-4-ol; α-terpineol; borneol; geraniol (**24**) and elemol. Limonene and α-terpineol are present in all the analyzed essential oils. Lemon/garlic essential oil does not show any sulphur derived compound in the GC-MS analysis due to the fact that during cutting and heating of garlic to obtain the oil, the compounds might have escaped (Lawson, 1991; Yongabi *et al*., 2009; (Ahmet *et al*., 2006; Hérent *et al*., 2007; Ahmed *et al*., 2009; Mohamed *et al*., 2010).



geraniol

24

Table 4.12: The GC-MS constituents identified from three essential oil blends with antibacterial properties against *S. typhi*, *P. aeruginosa*, *E. coli* and *S. aureus*

					Relative %		
No.	Compound	Molecular Formula	M^+ (g/mol)	Retention time (min)	GL	LGi	GLT
1.	Diallyl disulphide	$C_6H_{10}S_2$	146	12.771	1.87	-	0.66
2.	Limonene	$C_{10}H_{16}$	136	11.921	84.27	49.78	36.16
3.	Linalool	$C_{10}H_{18}O$	154	14.612	0.91	1.13	-
4.	Terpinen-4-ol	$C_{10}H_{18}O$	154	13.113	4.46		1.72
5.	α-Terpineol	$C_{10}H_{18}O$	154	14.415	1.74	1.65	0.82
6.	[4-Aminophenyl]2-methylpiperidin-1-yl) methanone	$C_{13}H_{18}N_2O$	436	21.275	-	-	8.56
7.	Borneol	$C_{10}H_{18}O$	154	14.411	-	2.95	-
8.	Geraniol	$C_{10}H_{18}O$	154	15.510	-	0.09	-
9.	Elemol	$C_{10}H_{16}O_2$	222	19.490	-	0.67	-

GL-Lemon/garlic LGi-Lemon/ginger GLT-Lemon/garlic/turmeric

Citrus essential oils contain large amounts of terpenes, oxygenated derivatives and aromatic hydrocarbons (Ahmet *et al*., 2006; Hérent *et al*., 2007; Ahmed *et al*., 2009; Mohamed *et al*., 2010). Among the components (limonene and linalool) limonene was more abundant than linalool. Limonene shows the lowest effect against microorganisms. (Hérent *et al*., 2007; Tao *et al*., 2009; Palakawong *et al*., 2010). The inhibitory effect against microorganisms resulted from linalool rather than limonene (Fisher and Phillips, 2006). Results of the previous studies showed that greater antimicrobial potential could be ascribed to the oxygenated terpenes, including phenols (Maruti *et al*., 2011).

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The most potent sample among the juices was garlic juice which inhibited the growth of all bacteria tested with inhibition zones of 10.0±0.0 mm (*P. aeruginosa*), 11.7±0.3 mm (*E. coli*), 14.7±2.5 mm (*S. aureus*) and 17.7±2.5 (*S. typhi*). Garlic was also bactericidal on *S. aureus*, *E. coli*, *S. typhi* and *P. aeruginosa* at concentrations of 375 mg/ml, 187.5 mg/ml, 93.8 mg/ml and 46.9 mg/ml respectively. Lemon juice inhibited only the growth of *S. typhi* with a zone of 11.0±1.0 mm. The mean inhibition zones of Lemon/garlic juice against *E. coli* (15.0±0.0) and *S. typhi* (12.0±0.0) were significantly higher among the juice blends. Lemon/garlic/ginger, Lemon/garlic/turmeric juice blends also recorded high activity 13.7±0.6 and 14.7±0.6 respectively against *E. coli*.

Among methanol extract samples, lemon had the highest activity against *P. aeruginosa* (11.0±0.0) and *E. coli* (10.0±0.0). The activity of turmeric/lemon/garlic against *S. aureus* (12.0±1.0) was significantly higher compared to other methanol extracts. Methanol extract of lemon and lemon/ginger was bactericidal on *P. aeruginosa* at a concentration of 2.9 mg/ml and 5.9 mg/ml respectively. Lemon/garlic essential oil blend recorded the highest inhibition zone against *P. aeruginosa* (10.0±0.0) among the essential oils. Methanol extracts were bactericidal on bacteria at lower concentrations (2.9 mg/ml, 5.9 mg/ml against *P. aeruginosa* and 23.4 mg/ml against *S. aureus*) as compared to juices and essential oils. Time-course antibacterial tests of lemon/garlic and lemon/garlic/ginger juice blends showed an interesting pattern against *S. typhi*: the activity dropped by day 3 but increased up to day 5

In the bioassay tests, lemon and garlic juices and lemon methanol extract inhibited the growth of both gram positive and gram negative bacteria. The results of antibacterial testing revealed that the juices of garlic and lemon had higher inhibitory effects as compared to methanol extracts and essential oils. The results of this study support the traditional usage of the studied vegetables and lemon, and suggest that some of the extracts possess compounds suspected to have antimicrobial properties that can be used as agents in new drugs for therapy of infectious diseases caused by pathogens.

GC-MS analysis of lemon juice revealed the presence of α-terpineol; *n*-hexadecanoic acid; limonene; linalool; 3-hexen-1-ol; mentha-2,8-dien-1-ol; 9,12-octadecadienoic acid; 2-ethoxycarbonyl-3-methyl-7-nitro-4-azafluorenone,phenylimine while lemon methanol extract had cyclohexanol, 2-methylene-5-(1-methylene-5-[1-methylethenyl]-; trans-carveol; *n*-hexadecanoic acid; linoleic acid; heptadecanoic acid and γ-sitosterol suspected to contain antibacterial properties. Garlic juice contained thiourea,*N*-*N*'-dimethyl; diallyl disulphide; 3-chlorothiophene; 3-vinyl-1,2-dithiacyclohex-4-ene; 3-vinyl-1,2-dithiacyclohex-5-ene; 1,4-diathiane; acetic acid, chloro-2-butoxyethyl ester; ethyl trifluoromethyl trisulphide ; acetamide, *n*-tetrahydrofurfuryl-2-methoxy; 1-propene, 3,3'thiobis; 1,4-diathiane; thiourea, *N*,*N*'-dimethyl-; octadecanoic acid,3-hydroxy, methyl ester; Cyclohexen-1-ol, 3-methyl; 1,3-Dioxolane-2-[dichloromethyl]- and amidinothiourea.

Most abundant compounds that were suspected to have antibacterial properties in blends of essential oils were diallyl disulphide ; limonene ; linalol ; terpinen-4-ol ; α-terpineol ; [4-aminophenyl]2- methylpiperidin-1-yl) methanone; borneol; geraniol and elemol. The compounds which were present in all the samples contain one or more of the following functional groups: -COOH, -OH, -N, -Cl, -F, $-NH_2$ and –S groups which may be associated with bacterial inhibition and found in conventional antibiotics.

Individual juices and methanol extracts contained more compounds that were suspected to have antibacterial properties as compared to the blends. For example lemon and garlic individual juices had a total of 22 compounds that were suspected to have antibacterial properties, lemon methanol extract contained 6, while lemon/garlic blend contained only 6 compounds.

Compounds which were present in more than one extract include: α-terpineol in lemon juice, ginger juice and all the essential oil blends tested; linalool in ginger juce, turmeric juice, garlic/lemon essential oil and lemon/ginger essential oil; 2-Butanone,4-[4-hydroxy-3-methoxyphenyl]- in all methanol blends and limonene in lemon juice and all oil blends tested. None of the compounds suspected to contain antibacterial properties from the chromatograms was found in all the samples analyzed.

5.2 Recommendations

The synergism between garlic and lemon in the lemon/garlic essential oil shows the need for more studies concerning the molecular basis of these interactions to understand the synergistic mechanism. This may be fundamental to development of pharmacological agents to treat bacterial infections using medicinal plants. In addition, the way in which different constituents interact may be helpful in elucidating the blend effects of these products. The time course anti-bacterial activities of lemon/garlic and lemon/garlic/ginger blends that initially lose activity and then have relatively high activity suggests the formation of intermediate compounds which are not active but show inhibitory activity on further transformation.

With the evidence of antibacterial activities of the juices and methanol extracts of the herbal preparations under study, it can be rationally suggested that further work needs to be done to identify the chemical nature of the active principles as well as their modes of actions on bacterial cells and their role in diseases curing. It is also important that more species of pathogenic bacteria be tested in order to ascertain the spectra of activities of the antibacterial substances present in the herbal preparations.

5.2.1 Recommendations from the study

i. Lemon/garlic juice blend is suspected to possess compounds which have exhibited antibacterial properties that can be used as antimicrobial agents.

ii. Garlic and lemon should be used in their raw form as heating and drying may make the active compounds to be evaporated, destroyed or transformed to less active forms.

5.2.2 Recommendations for further studies

From the findings of this research, it is recommended that;

i. *In vitro* antibacterial activities of garlic from different ecological zones against *E. coli* and *S. typhi* should be carried out as GC-MS analysis of China garlic and local garlic gave varying compounds.

ii. A blend of lemon/garlic juice is a viable combination for further *in vitro* and *in vivo* studies.

iii. Phytochemical analysis of lemon/garlic methanol and fresh juice blend should be carried out in order to elucidate active antimicrobial principles.

iv. Time-course bioassays, GC-MS and LC-MS analyses of intermediate products suspected to be formed by lemon/garlic and lemon/garlic/ginger blends need to be done in order to explain the fluctuating activity.

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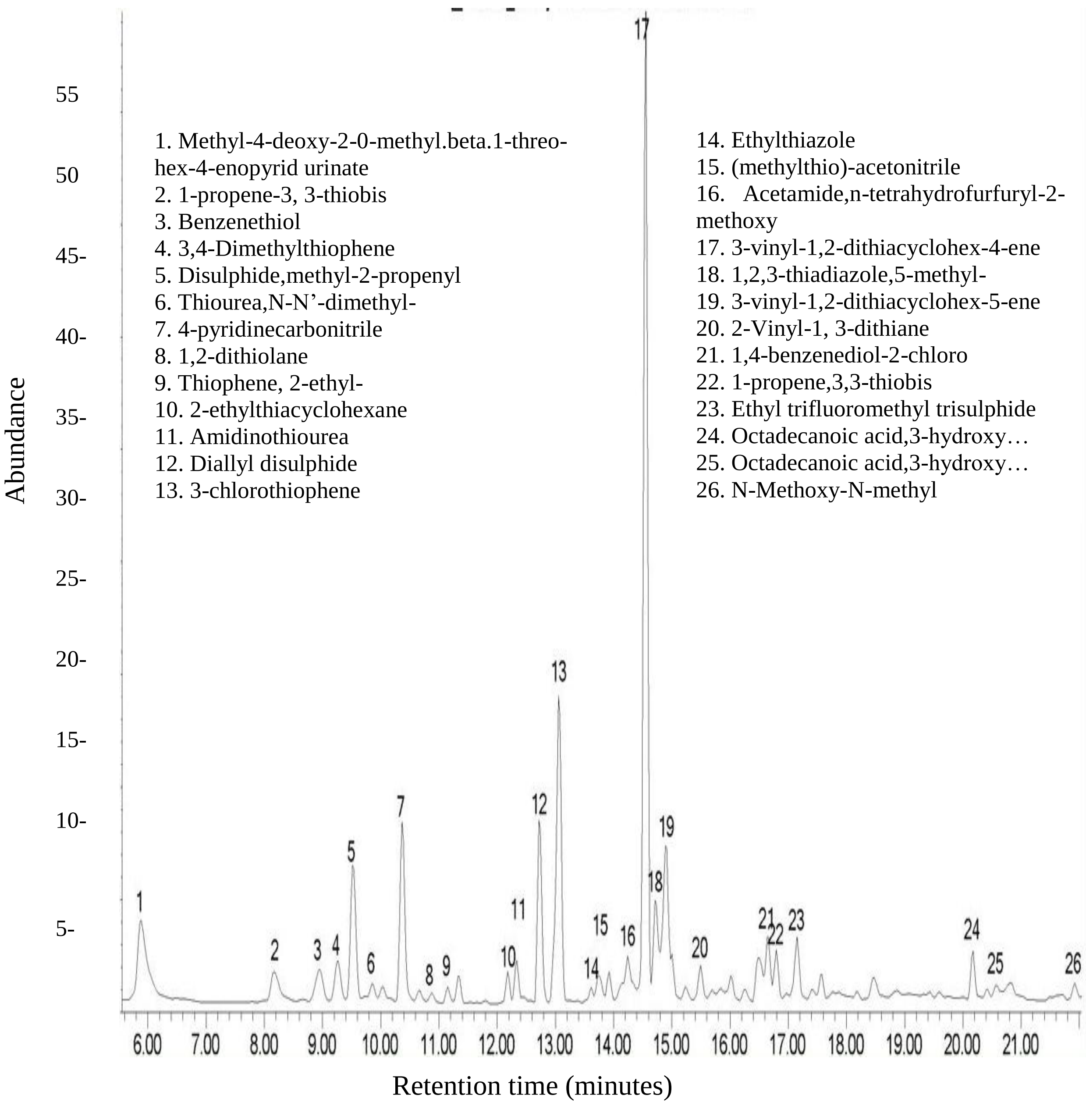
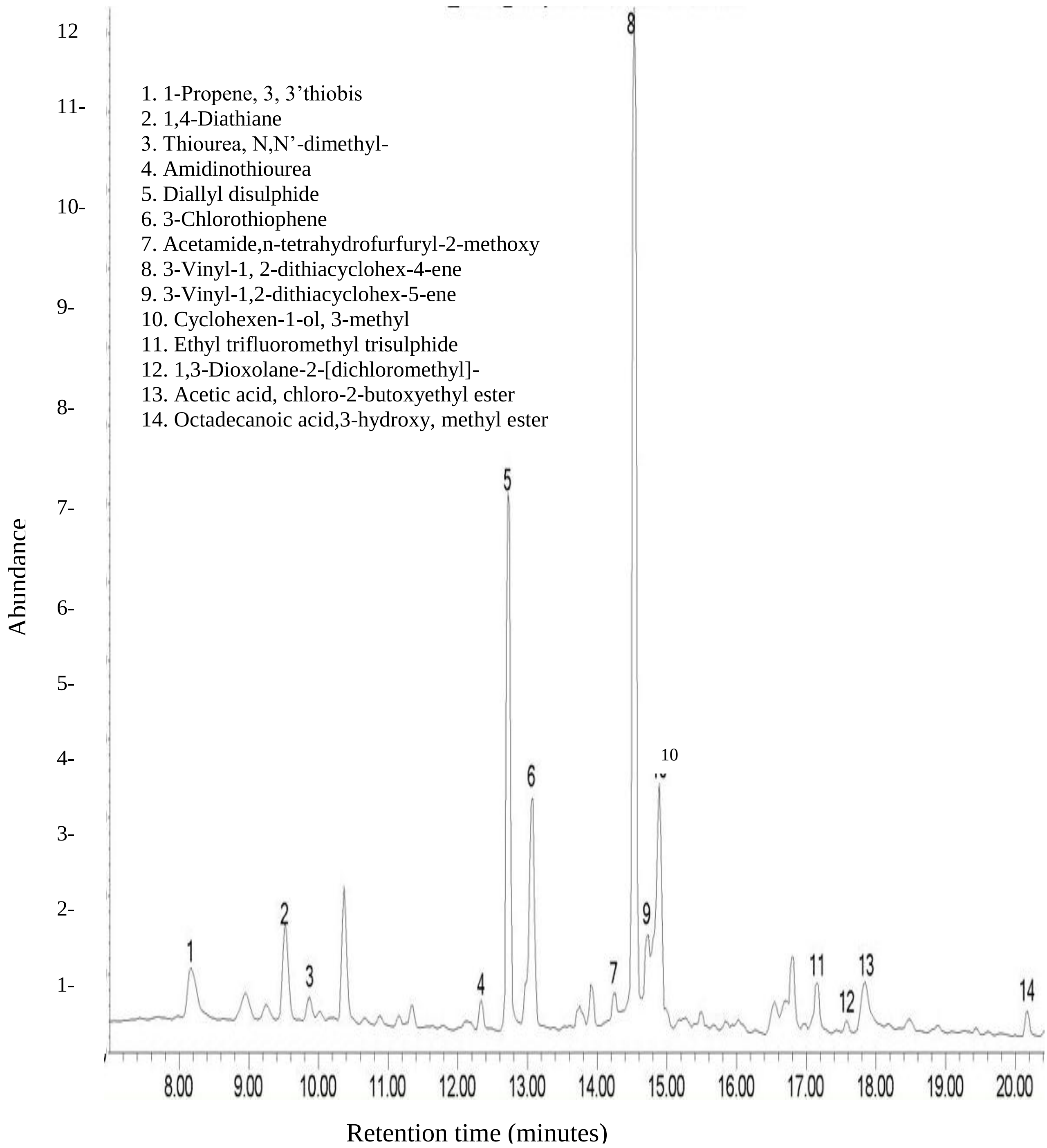
APPENDICES

Appendix 1: GC-MS Chromatogram obtained from garlic, lemon, ginger and turmeric juices



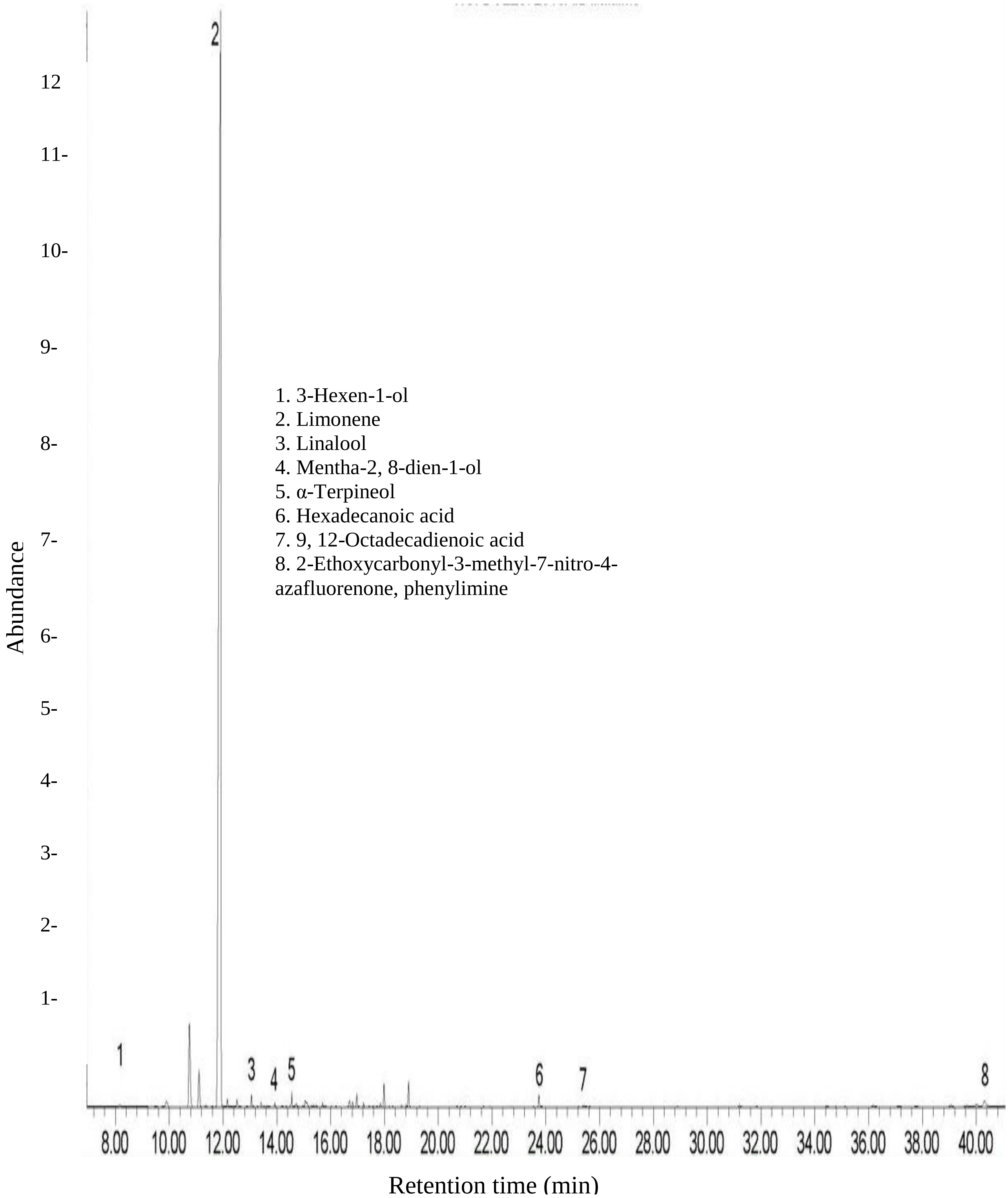
GC-MS Chromatogram obtained from China garlic juice

Appendix 1: Cont'd



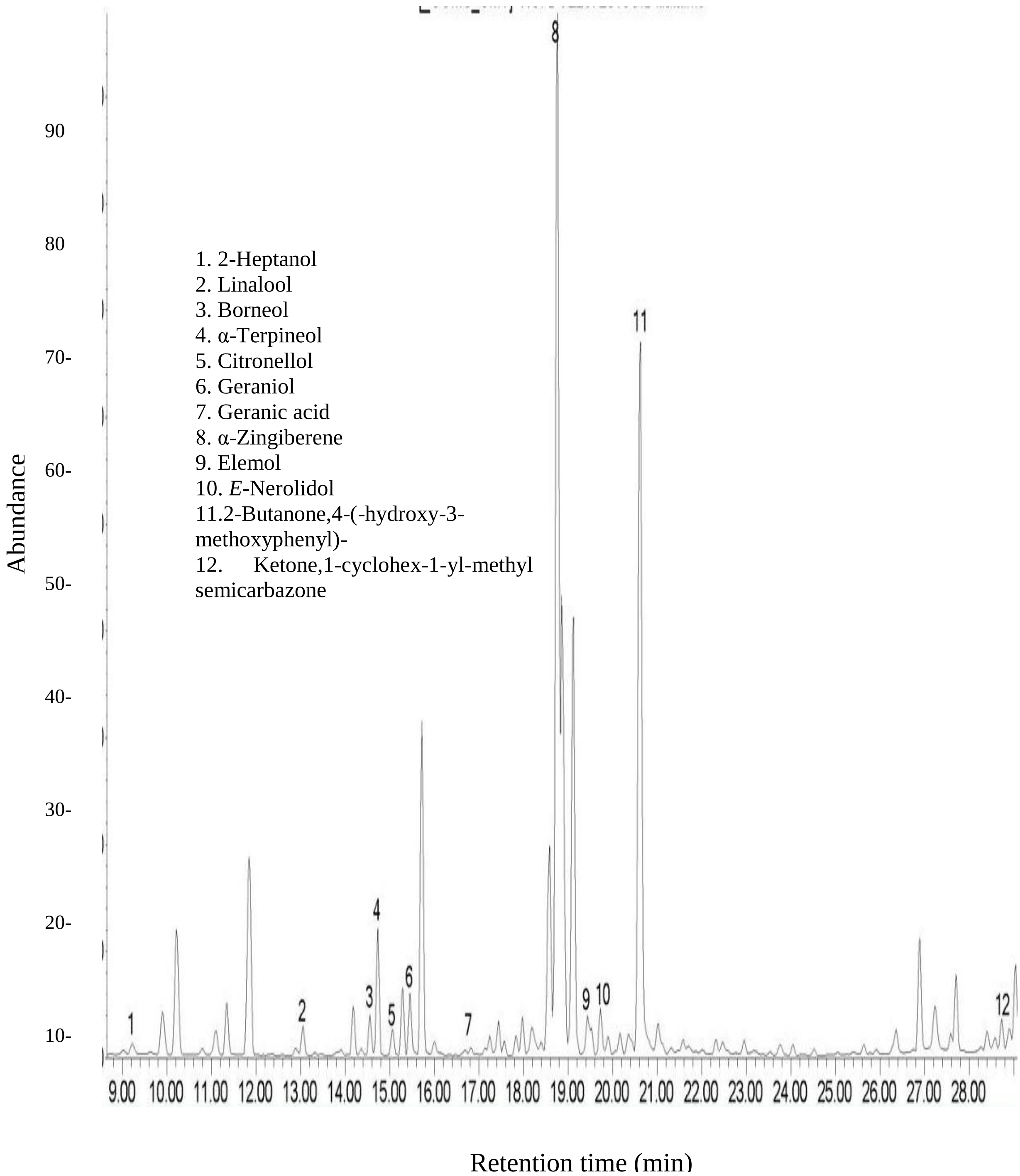
GC-MS Chromatogram obtained from local garlic juice

Appendix 1: Cont'd



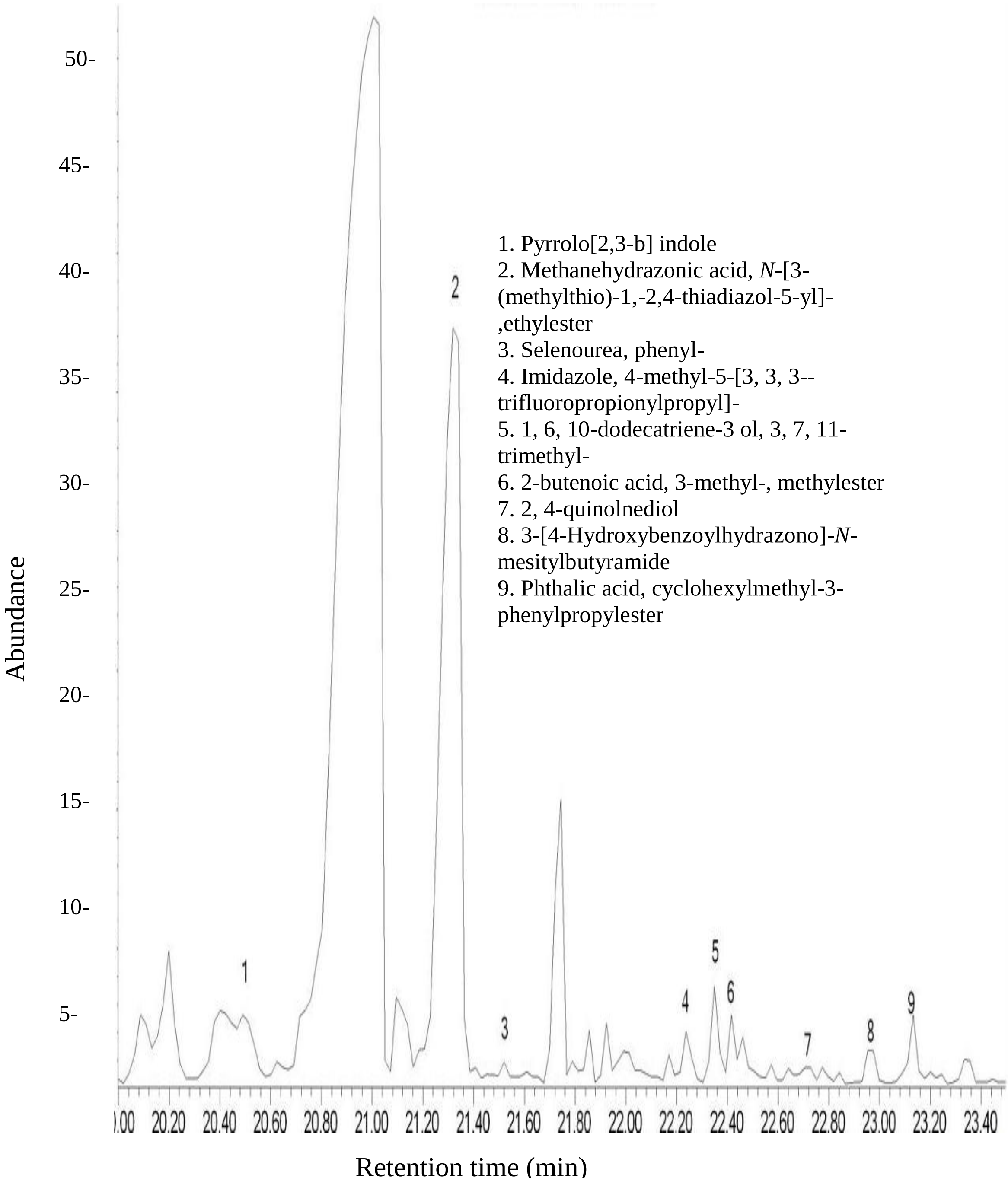
GC-MS chromatogram obtained from lemon juice

Appendix 1: Cont'd



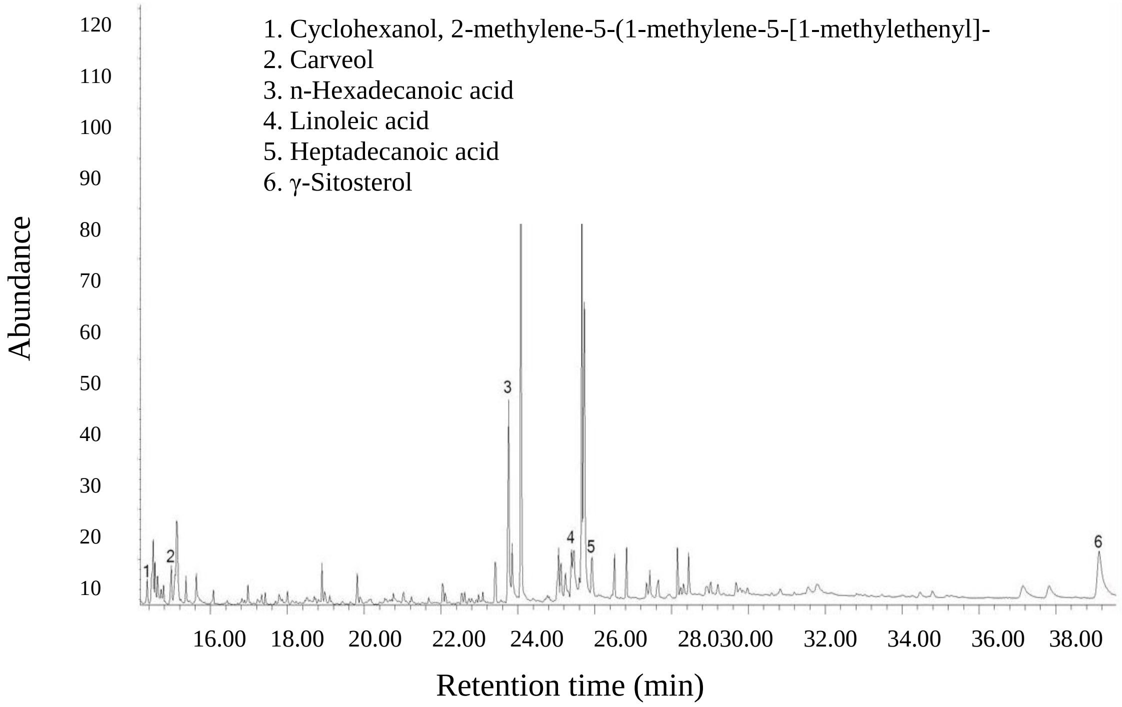
GC-MS Chromatogram obtained from ginger juice

Appendix 1: Cont'd

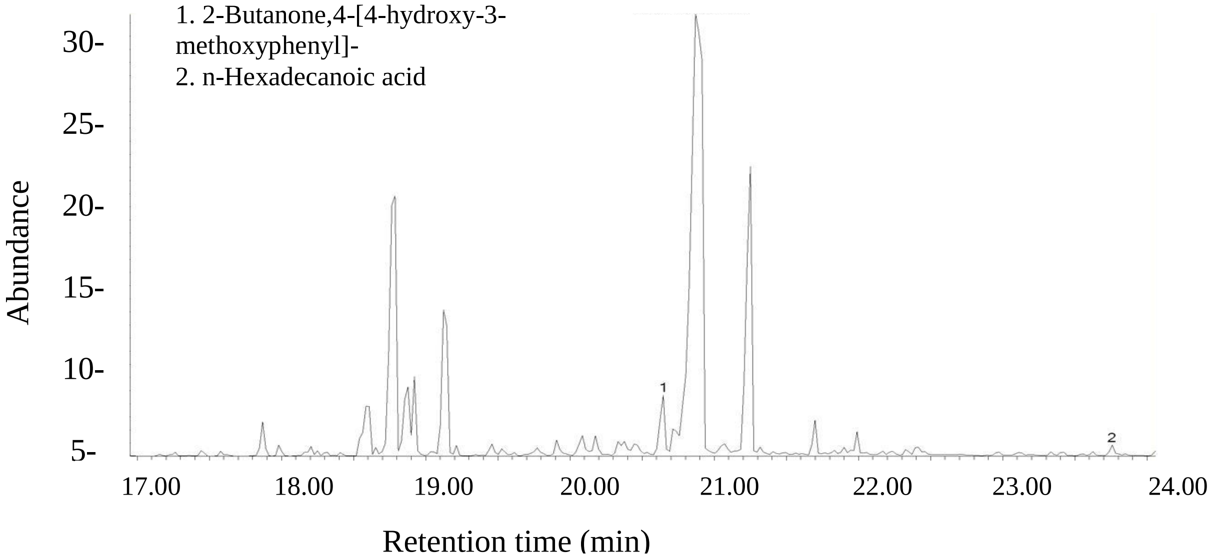


GC-MS Chromatogram obtained from turmeric juice

Appendix 2: GC-MS Chromatogram obtained from active methanol extract blends

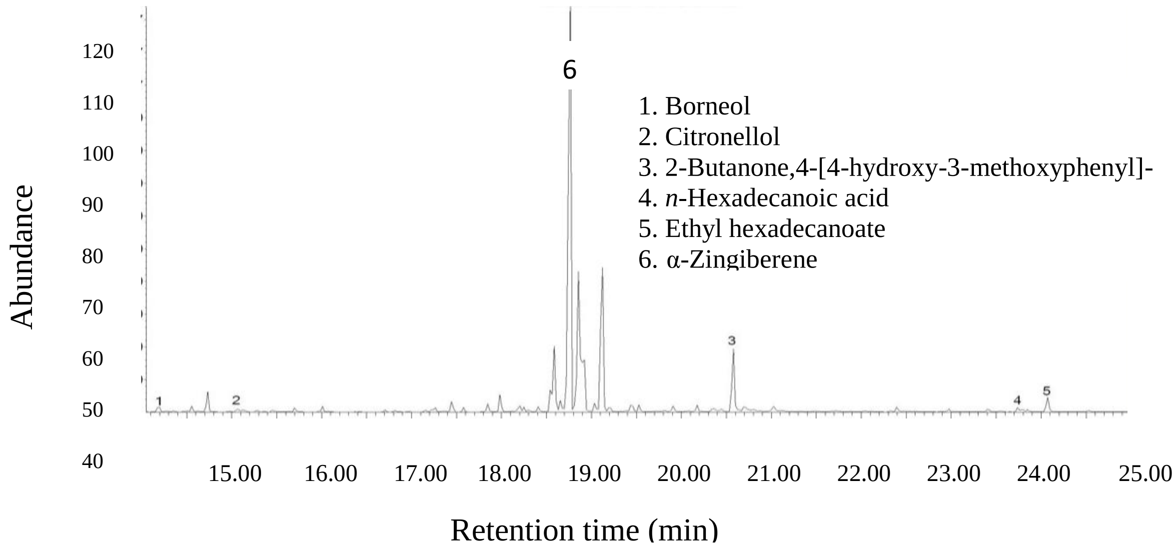


GC-MS chromatogram obtained from lemon methanol extract

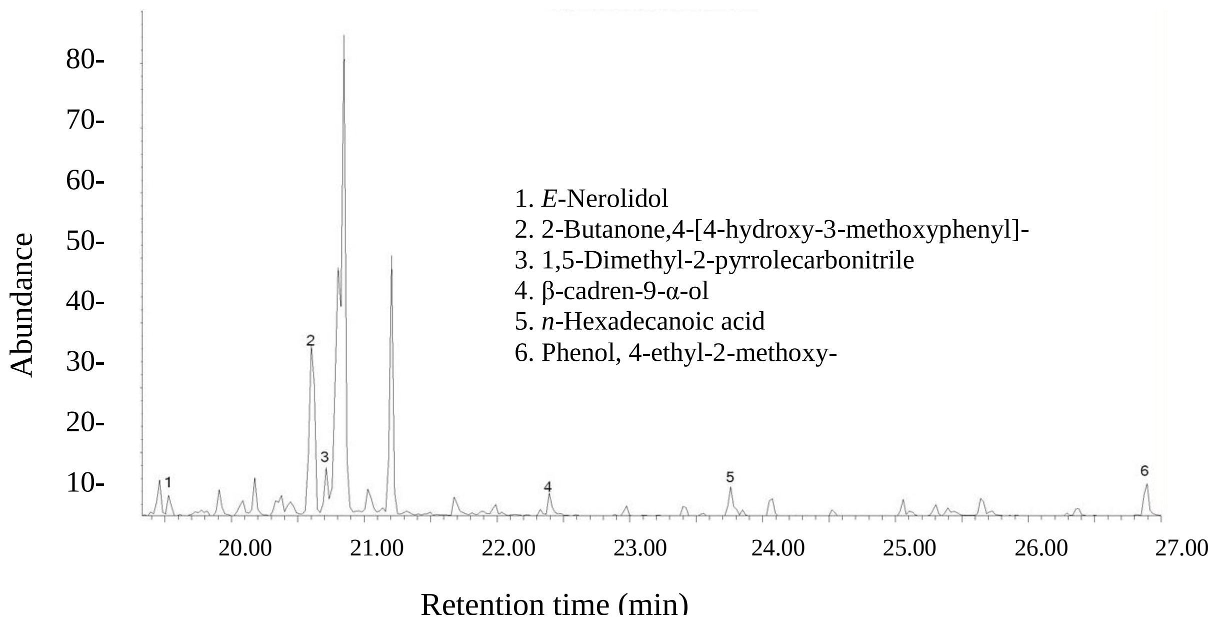


GC-MS chromatogram obtained from garlic/ginger/turmeric methanol extract blend

Appendix 2: cont'd

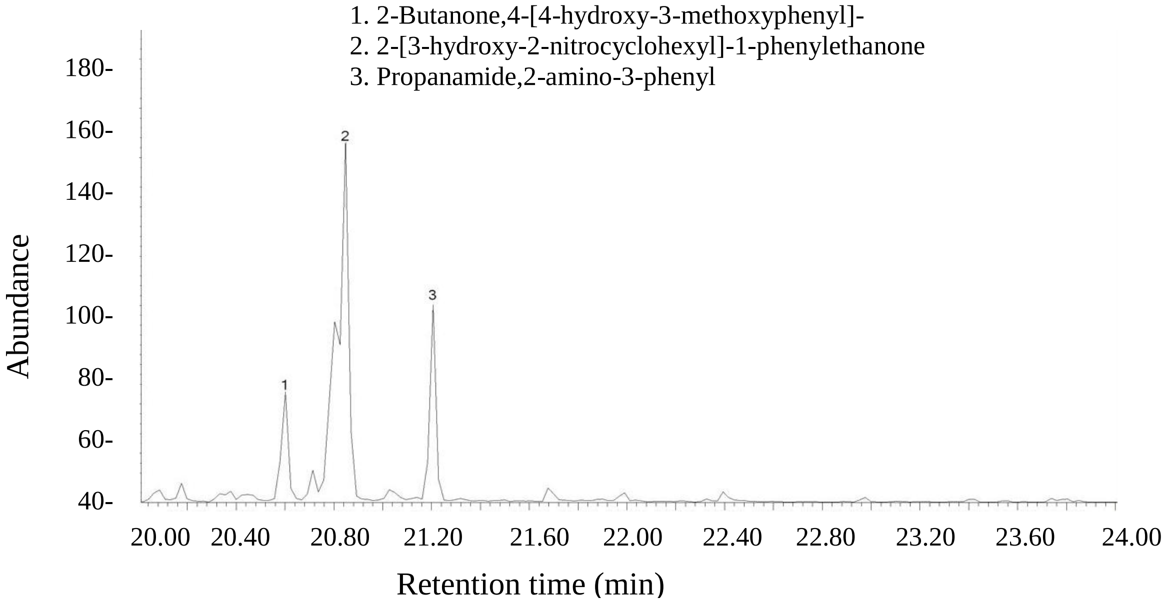


GC-MS chromatogram obtained from lemon/ginger methanol extract blend

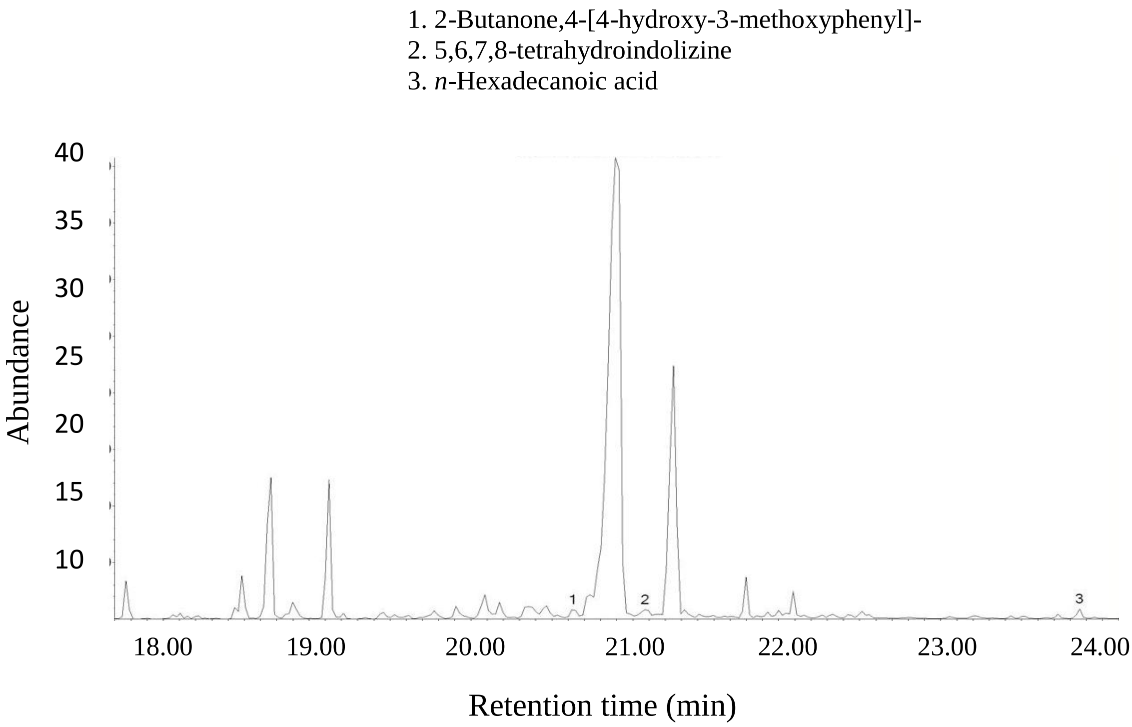


GC-MS chromatogram obtained from lemon/ginger/turmeric/garlic methanol extract blend

Appendix 2: cont'd

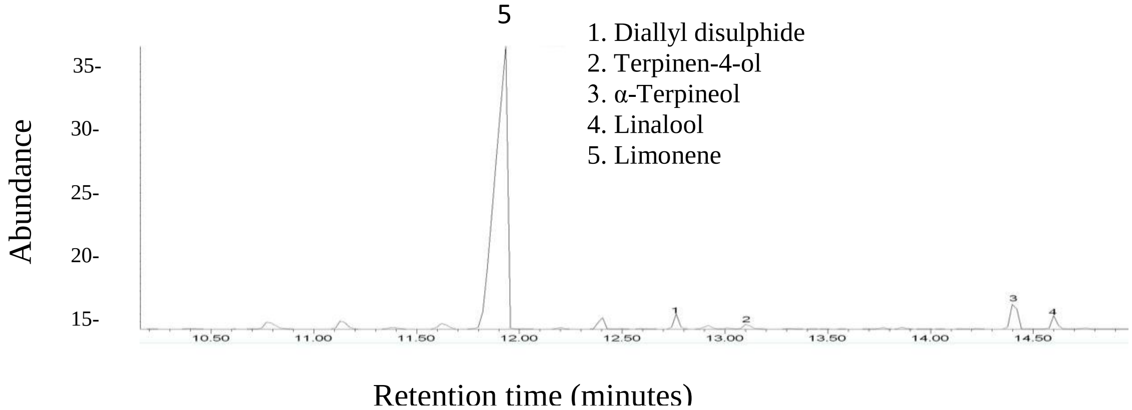


GC-MS chromatogram obtained from ginger/lemon/turmeric methanol extract blend

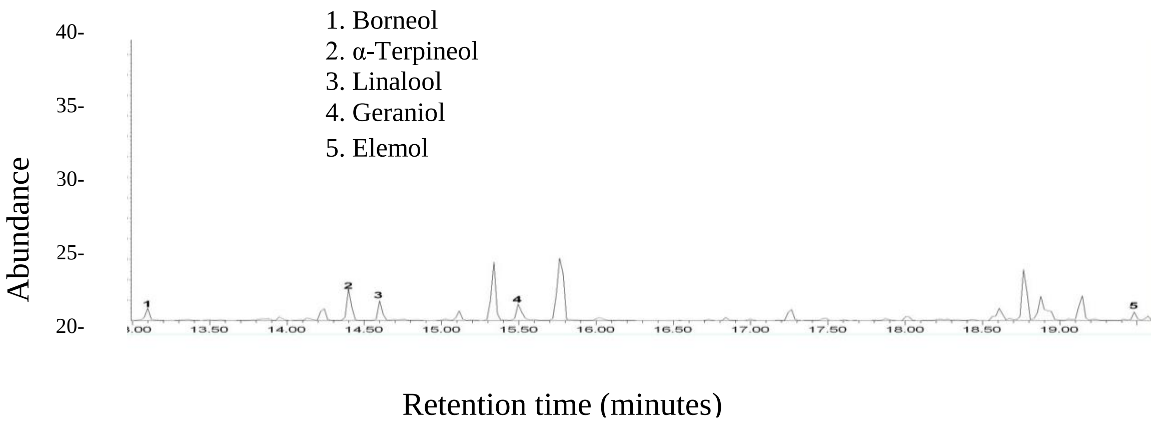


GC-MS chromatogram obtained from lemon/garlic/turmeric methanol extract blend

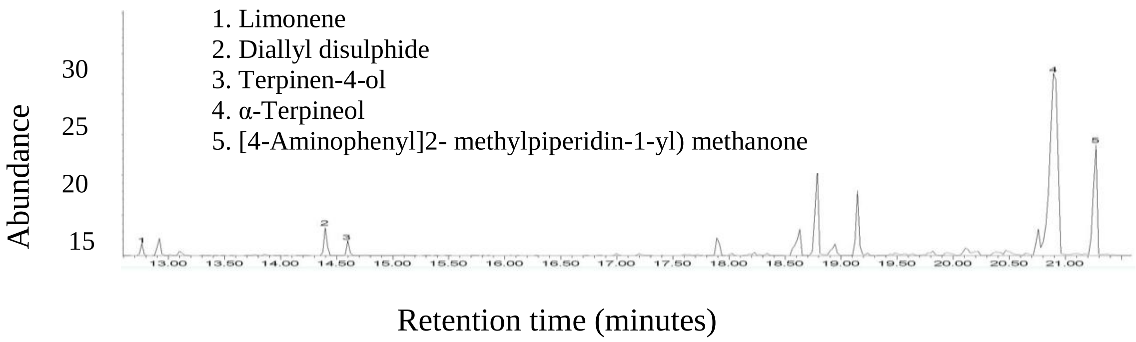
Appendix 3: GC-MS Chromatogram obtained from essential oil blends



GC-MS chromatogram obtained from lemon/garlic oil



GC-MS chromatogram obtained from lemon/ginger essential oil blend



GC-MS chromatogram obtained from lemon/garlic/turmeric oil blend