HPLC QUANTIFICATION AND ANTIMICROBIAL ACTIVITY TRENDS OF ALLYL THIOSULPHINATE UPON CHROMATOGRAPHIC FRACTIONATION OF *ALLIUM SATIVUM* EXTRACTS

BY

OPONDO FLORENCE ATIENO

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MAY, 2017.

DECLARATION

DECLARATION BY THE CANDIDATE

I declare that this thesis is my own original work and has not been presented in any other university for award of a degree of Master of Science in Analytical Chemistry.

Signature…….....Date…….

Opondo Florence Atieno

MSC/ACH/08/14

DECLARATION BY THE SUPERVISORS

This research thesis has been submitted for examination with our approval as University supervisors

Signature…….……Date……..……..

Dr. Were L. L. Munyendo

Department of Chemistry and Biochemistry

Moi University

Signature……..Date…….....

Dr. Patrick S. Musyoki

Department of Chemical Sciences & Technology

Technical University of Kenya

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DEDICATION

This thesis is dedicated to my Husband Patrick Ochieng’ to my children William Ong’injo, Raphael Junior and Abigael Achieng’, to my parents Eunice Oguda and Raphael Nyayieka and to all my siblings.

ABSTRACT

Allium sativum extracts have continually been used as herbal therapy for a variety of bacterial, fungal and viral infections. There is however reports of contraindications leading to adverse effects like ulcerations associated with use of these garlic herbal concoctions as medication. The main purpose of this study was therefore to quantitatively determine allicin and alliin by HPLC and investigate antimicrobial activity trends of allyl thiosulphinate (allicin) upon chromatographic fractionation of *Allium sativum* (garlic) constituents. The objectives were to develop and validate HPLC analysis method for quantification of allicin and alliin, to determine allyl thiosulphinate content in garlic obtained, to evaluate antimicrobial activity trends of *Allium sativum* extracts on chromatographic fractionation and model the antimicrobial trends. Experimental research design was adopted for this study using garlic juice. Two garlic bulbs sample categories were obtained, one from Kenyan cultivation regions while the other purchased from importing commercial stores which were separately blended to afford garlic juice for analysis. Extraction was performed using the soxhlet and maceration methods with ethanol and methanol solvents. Synergistic trend was evaluated using Disc-diffusion assays for antibacterial, antifungal activities of crude extracts, column chromatography fractions and HPLC eluents while determining their content of diallyl thiosulfinate and allylsulfinyl-L-alanine through High Performance Liquid Chromatographic (HPLC) quantification of allicin and alliin in the fractions of *Allium sativum* extracts. Extractions yielded up to 0.9% and 1.3% for the ethanol and methanol solvents respectively. The HPLC method developed and validated for determination of allicin displayed high accuracy and precision from the percent recoveries of 96.09$\pm$1.09% and Relative standard deviations ranging from 1.63 to 4.01 for intraday and 0.08 to 3.40 for inter day precision statistical assays. Inter- and intra-day consistency was depicted. Bioassays reported inhibition zones of 10mm to 18mm and finally 5mm (antibacterial) while 12mm to 28.4mm and finally 5mm (antifungal) from crude extract to column fraction and finally HPLC eluents respectively. In conclusion, antimicrobial trends indicate a rise and later a drop in activity moving from crude extract to column fraction and finally HPLC eluents. The developed and validated HPLC method is substantial for satisfactory performance in ethno pharmacovigilance of traditional medicine agents. Use of Garlic concoctions should be checked for standardization of constituents as these determines the efficacy.

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ACRONYMS

ATCC	American Type Culture Collection
ALLICIN	Allyl thiosulphinate
ALLIIN	Allyl sulphenic acid
ATR	Attenuated Total Reflectance
C_{18}	Octahedral carbon
FTIR	Fourier Transform Infrared
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization of technical requirements for registration of pharmaceuticals for Human use
KEMFRI	Kenya Marine and Fisheries Research Institute
LOD	Limit of detection
LOQ	Limit of quantitation
MANOVA	Multivariate Analysis of Variance
M.I.C	Minimum Inhibitory Concentration
NCCLS	National Committee for Clinical Laboratory Standards
R_F	Retardation factor
R^2	Coefficient of Determination
RSD	Relative standard deviation
R_T	Retention time
SPSS	Statistical Package for Social Scientists
TLC	Thin Layer Chromatography

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

The use of alternative medicine in developing countries is continually increasing which has given medicinal value to plants (Lawson and Koch, 1996). Garlic, of the family Liliaceae is one of these plants which is reported to have had an important dietary and medicinal role for centuries. Garlic (*Allium sativum*) is a bulbous perennial food plant whose name comes from an English word ‘garleac´, meaning "spear leek” (Curtis *et al.*, 2004).

Garlic contains organosulfur compounds that possess antibacterial, antioxidant and anti-inflammatory activities (Block *et al.*, 1986; Chen *et al.*, 2009; and Hassan *et al.*, 2007). It is most often used as a seasoning or a condiment, and is believed to have some medicinal value notably against hypertension. Crushed or finely chopped garlic yields allicin, a powerful antibiotic and anti-fungal compound (Ross *et al*., 2001).

It also contains alliin, ajoene, enzymes, vitamin B, minerals, and flavonoids (Nikolic *et al.*, 2004). Garlic was found to exhibit antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria, including multi drug resistant enterotoxicogenic strains of *Escherichia coli*; antifungal activity, particularly against *Candida albicans*; antiparasitic activity, including some major human intestinal

protozoan parasites such as *Entamoeba histolytica* and *Giardia lamblia*; and antiviral activity (Curtis *et al.*, 2004; Chen *et al.*, 2009 and Ross *et al.*, 2001).

Most of these prophylactic and therapeutic effects are ascribed to specific oil- and water soluble organosulfur compounds, which are responsible for the typical odour and flavour of garlic (Nikolic *et al.*, 2004). Historically, *Allium sativum* had been found to possess many therapeutic properties including antimicrobial, antineoplastic, anti-cardio vascular, anti -hypertensive, anti-hyperlipidaemia, anti-diabetic, immuno-stimulatory and hypoglycaemic activities (Nemeth and Piskula, 2004; Lawson and Koch, 1996).

It has also been used for centuries to fight infections in several instances; Egyptians used it to treat diarrhoea; the ancient Greeks used it to treat intestinal and extra-intestinal diseases, while the ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever (Onyeagbu *et al*., 2004).

In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhoea, otitis media and respiratory tract infections (Jiben *et al*., 2006). Although several studies have confirmed that garlic has antimicrobial properties (Reuter *et al*., 1996), not much work has been carried out to investigate this antimicrobial activity.

One such successful research, among a few others, was reported by Onyeagbu *et al*., 2004 on the antimicrobial activity of aqueous extracts of *Zingiber officinale Roscoe and Citrus aurantifolia Linn* and the garlic blends, on some beer spoilage organisms.

The application of blends was based on the concept of the effectiveness of combination therapy (synergism) in the treatment of certain bacterial infections involving drug resistant organisms (Songsungkan and Chanthai, 2014).

Garlic has even been noted to act synergistically with antibiotics (Rahman, 2007). Synergic treatment of ailments has been a long aged practice in both orthodox and traditional medicines. In Sub-Saharan Africa, especially West Africa, including Ghana and Nigeria, herbal medicines are believed to be more effective when taken in combination (Jiben *et al*., 2006).

In particular, herbal medicines taken orally are combined and immersed in locally brewed gin for a short period of time for effective extraction of the active ingredients of the herbs. The gin extracts of the herbs are taken in homes, or sold in wine bars as medicines against some ailments (Josling, 2010).

The aim in this study was to ascertain synergistic trends for the antimicrobial activity of garlic extract constituents against known bacterial and fungal pathogens through fractionation of the crude extractions and quantification of major phytocompounds. This therefore entailed development and validation of a HPLC method for this analysis.

1.2 Problem statement

Garlic extracts have continually been used as herbal therapy for a variety of bacterial, fungal and viral infections (Ilic *et al.*, 2011). However several researchers have highlighted safety issues regarding ingestion of garlic extracts (Li *et al*., 2007; Freeman and Kodera, 1995). The literature reviewed cautions against use of garlic herbal medications thereby leaving a gap in knowledge regarding appropriate exploitation of *Allium sativum* extracts.

If the contraindications are not studied even other effects patients as topical garlic burns, anaphylaxis and platelet dysfunction could continue to be magnified. Additionally the practice of traditional medicine in Kenya is rapidly increasing. Meanwhile at national, provincial, and district levels, in developing countries, there is now a critical mass of self-alleged alternative medicine clinicians (Onyeagbu *et al.*, 2004; Jiben *et al*., 2006).

These poorly regulated and administrated capacities with their resources have been realized to be an impediment at the community level rather than a supporter of effective health management. This problem is mostly aggravated by lack of appropriate methodologies and tools for the standardization of traditional medicine products.

1.3 Objectives

1.3.1 General objective

To quantitatively determine allicin and alliin by HPLC and study the antimicrobial activity trends of Allyl thiosulphinate upon chromatographic fractionation of *Allium sativum* constituents.

1.3.2 Specific objectives

i. To develop and validate HPLC analysis method for quantification of allicin and alliin in garlic extracts.
ii. To determine allicin and alliin content in garlic obtained from garden and supermarket.
iii. To evaluate antibacterial activity trends of *Allium sativum* extracts on chromatographic fractionation.
iv. To evaluate antifungal activity trends of *Allium sativum* extracts on chromatographic fractionation.
v. To model antimicrobial activity trends with chromatographic fractionation.

1.4 Justification of study

The effectiveness of aqueous garlic extracts against a number of bacterial has been known for centuries (Ilic *et al*., 2011). Several researchers have demonstrated that aqueous garlic extract has an inhibition against the growth of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeroginosa* (Songsungkan and Chanthai, 2014; Nemeth and Piskula, 2007; Onyeagbu *et al*., 2004).

However Freeman and Kodera in 1995 argued that although garlic possesses little safety issues there are isolated cases of topical garlic burns. Garlic allergy has also been attributed to the protein allinase which has induced immunoglobulin E (IgE) mediated hypersensitivity (Lee *et al*., 2009; Qing-Yang *et al*., 2002).

As a result the literature caution against use of garlic extracts as herbal therapy (Josling, 2010) hence the current study focused on antimicrobial activity trends for selected bioactivities of allicin upon fractionation of *Allium sativum* extracts to demystify this and other related concepts on garlic use induced contraindications.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Allium sativum is one of the edible plants which has generated a lot of interest throughout human history as a medicinal plant (Lawson and Koch, 1996). A wide range of microorganisms including bacteria and fungi have been shown to be sensitive to crushed garlic preparations (Han *et al*., 1995). Chemical analyses of garlic cloves have revealed an unusual concentration of sulphur-containing compounds (Thompson and Ali, 2003). Pure allicin chemically known as allyl thiosulphinate is a volatile molecule that is poorly miscible in aqueous solutions and which has the typical odor of freshly crushed garlic (Ilic *et al.*, 2011).

The name garlic may have originated from the Latin word 'all' meaning pungent. Cultivated practically throughout the world, garlic appears to have originated in central Asia and then spread to China, the Near East, and the Mediterranean region before moving west to Central and Southern Europe, Northern Africa (Egypt) and Mexico (Chen *et al.*, 2009). Garlic has been used for thousands of years for medicinal purposes. Sanskrit records show its medicinal use about 5,000 years ago, and it has been used for at least 3,000 years in Chinese medicine.

The Egyptians, Babylonians, Greeks, and Romans used garlic for healing purposes. In 1858, Pasteur noted garlic's antibacterial activity, and it was used as an antiseptic to prevent gangrene during World War I and World War II. Garlic's current principal medicinal uses are to prevent and treat cardiovascular disease by lowering blood pressure and cholesterol, as an antimicrobial, and as a preventive agent for cancer (Boon and Smith, 2004; Ross, 1999).

The amounts of alliin and allicin present in different strains of garlic have been studied by numerous investigators. Considerable variations have been reported, ranging from 2.8 to 7.7 mg/gram found in Romanian red (Block *et al.*, 1986). The transformation of alliin into the biologically active allicin molecule upon crushing of a garlic clove is extremely rapid, being complete in seconds. This very efficient organization ensures that the clove defense mechanism is only activated in a very small location and for a short period of time, whereas the rest of the alliin and alliinase remain preserved in their respective compartments and are available for interaction in case of subsequent microbial attacks (Shen *et al*., 2002).

Moreover, since massive generation of allicin could also be toxic for the plant tissues and enzymes, its production is very limited and with short-lived reactivity, which is confined to the area where the microbial attack takes place. This ensures minimal or restrains any potential self-damage to the plant (Ilic *et al.*, 2011).

The other active constituents include several complex sulfur-containing compounds mainly diallyl disulfides that are rapidly absorbed, transformed and metabolized. Pooled data from numerous randomized trials suggest that garlic lowers total cholesterol concentrations by approximately 10% and favourably alters HDL/LDL ratios. Randomized trials also support garlic's effectiveness as a mild antihypertensive (Ankri and Mirelman, 1999).

2.2 Biological activity of *Allium sativum* constituents

2.2.1 Antibacterial activity

The antibacterial properties of crushed garlic have been known for a long time. Analysis of steam distillates of crashed garlic performed over a century ago showed a variety of allyl sulfides isolated and identified the compound responsible for the remarkable antibacterial activity as an oxygenated sulfur compound termed allicin from the Latin name of the garlic plant *Allium sativum* (Benavides *et al*., 2007; Chen *et al.*, 2009 and Tsao *et al.*, 2001).Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria including species of *Escherichia, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Bacillus, and Clostridium* (Londhe *et al.*, 2011). Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic (Vinnalon, 2001).

Garlic extracts are also effective against *Helicobacter pylori*, the cause of gastric ulcers. Garlic extracts can also prevent the formation of *Staphylococcus enterotoxins* A, B, and C and also thermo nuclease (Ilic *et al.*, 2011).

Interestingly, various bacterial strains resistant to antibiotics such as methicillin resistant *Staphylococcus aureus* as well as other multidrug-resistant enterotoxicogenic strains of *Escherichia coli, Enterococcus, Shigella dysenteriae, S. flexneri*, and *S. sonnei* cells are also found to be sensitive to allicin (Thompson and Ali, 2003).

On the other hand, other bacterial strains such as the mucoid strains of *Pseudomonas aeruginosa*, *Streptococcus hemolyticus* and *Enterococcus faecium* were found to be resistant to the action of allicin. The reasons for this resistance are unclear. It is assumed that hydrophilic capsular or mucoid layers prevent the penetration of the allicin into the bacteria, but this has to be studied more in depth.

A synergistic effect of allicin against *M. tuberculosis* was also found with antibiotics such as streptomycin or chloramphenicol (Lee *et al.*, 2009). A very interesting aspect of the antibacterial activity of allicin is the apparent inability of most bacteria to develop resistance to it because the mode of action is completely different from that of other antibiotic substances. It has been proposed that the development of resistance to beta lactam antibiotics is 1000-fold easier than development of resistance to allicin (Lawson and Hughes, 1992).

2.2.2 Antifungal activity

Garlic extracts also have a strong antifungal effect and inhibit the formation of mycotoxins like the aflatoxin of *Aspergillus parasiticus.* Allicin has been shown to be the main component of *Allium sativum* responsible for the inhibition of fungal growth.

A concentrated garlic extract possess potent in vitro fungi static and fungicidal activity against three different isolates of *Cryptococcus neoformans* (Amagase *et al.*, 2001). Pure allicin has antimicrobial activity against species of Candida, Cryptococcus, Trichophyton, Epidermophyton, and Microsporum at low concentration (minimal inhibitory concentrations of allicin were between 1.57 and 6.25 µg/mL) (Ilic *et al*., 2011; Rose *et al*., 2001; Onyeagbu *et al*., 2004). Allicin also inhibits both germination of spores and growth of hyphae (Nemeth and Piskula, 2007).

Garlic is grown all over the world and is used in various forms as food, spices and medicine. Garlic is an indigenous herb of Western Asia and Mediterranean where it has been cultivated for centuries. The major garlic growing countries include Korea, China, India, USA, Spain, Argentina and Egypt among which China is by far the largest producer (Rose *et al.*, 2001).

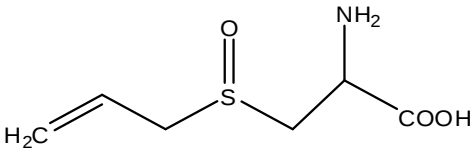
2.3 Biochemical conversions of *Allium sativum* constituents

2.3.1 Sulphoxides

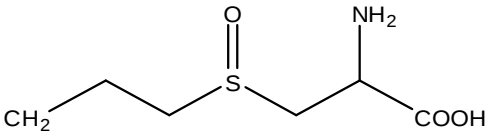
Garlic contains high levels of sulfur, zinc, phosphorous and potassium; moderate levels of selenium, vitamin A, and vitamin C; and low levels of iron, manganese, calcium, magnesium, sodium, and B-complex vitamins.

In addition to these, about 33 sulfur compounds and amino acids that include alanine, arginine, aspartic acid, asparagine, histidine, leucine, methionine, phenylalanine, praline, serine, threonine, tryptophan, and valine have been identified and isolated (Agarwal, 1996).

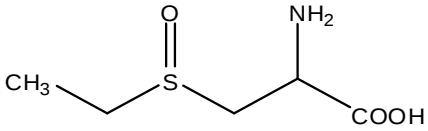
One unique constituent group of allium plants is S-Alkenyl–cysteine sulfoxides (ACSOs) that are responsible for their typical odor and flavors (Harris *et al.*, 2001). These sulfoxides include S–Methyl–L–cysteine sulfoxide (Methiin), S–Allyl–L–cysteine sulfoxide (Alliin), S–propyl–L–cysteine sulfoxides (propiin), S–Propenyl–L–cysteine sulfoxide (Isoalliin), S–Ethyl-L-cysteine sulfoxide (Ethiin) and S–n–Butyl–L–cysteine sulfoxide (Butiin).



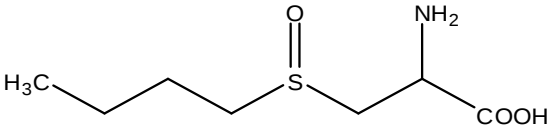
S- Allyl-L- cysteine sulfoxide



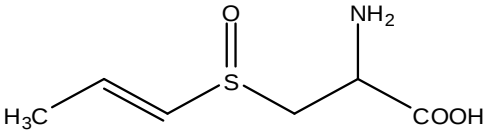
S-Propyl- L-cysteine



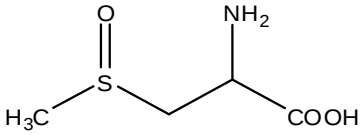
S- Ethyl- L-cysteine sulfoxide



S-n- Butyl-cysteine sulfoxide



S-Propenyl- L- cysteine sulfoxide



S- Methyl- L-cysteine sulfoxide

Figure 2.1: Sulphoxides in the garlic cloves (Ilic *et al*, 2011)

In the clove, alliinase is found closely associated with a lectin (Block *et al.*, 1986). Significant homology has been reported between the garlic and onion alliinases, although alliin was not detected in the latter species. Garlic cloves are odor-free until crushed. Cross-section studies have indicated that the substrate alliin and the enzyme alliinase are located in different compartments (Amagase *et al.*, 2001; Benavides *et al.*, 2007).

This unique organization suggests that it is designed as a potential defense mechanism against microbial pathogens of the soil. Invasion of the cloves by fungi and other soil pathogens begins by destroying the membrane which encloses the compartments that contain the enzyme and the substrate. This causes the interaction between alliin and alliinase that rapidly produces allicin and which in turn inactivates the invader. The reactive allicin molecules produced have a very short half-life, as they react with many of the surrounding proteins, including the alliinase enzyme, making it into a quasi-suicidal enzyme.

The enzyme responsible for the lysis is alliinase, or alliin-lyase, a pyridoxal 5-phosphate-dependent glycoprotein consists of two subunits (Vinnalon, 2001). Alliinase is present in unusually high amounts in garlic cloves: at least 10% of the total protein content (10 mg/g Generation of allicin in a garlic clove. The gene coding for the enzyme has been cloned, and upon translation, found to consist of 448 amino acids with a protein molecular mass of 51.45 kDa and together with a carbohydrate content of 5.5–6%, gives 55 000 kDa. Alliinase has 10 cysteine residues, all of them in S-S bridges, and their reduction, or the removal of the pyridoxal coenzyme factor, renders the enzyme inactive.

2.3.2 Allyl thiosulphinate (allicin)

2.3.2.1 Allicin transformation

The structure of allicin shown in scheme 1 was elucidated by Ilic and his colleagues who showed that allicin could be synthesized by mild oxidation of diallyl disulphide. Alliin was found to be the stable precursor that is converted to allicin by the action of an enzyme termed alliinase which is also present in the cloves (Iberl *et al.*, 1990).

Only one isomer of alliin ((+)-Sallyl- L-cysteine-sulfoxide) was found to be present, which in itself had no antimicrobial activity. Under the influence of alliinase, allicin is produced by enzymatic transformation of Alliin [(+)-(S)-allyl-L-cysteine-sulfoxide]. Alliin and alliinase are found in separate parts of garlic clove therefore this chain reaction is initiated only after crushing the cells. The alliin complex with the enzyme alliinase is then formed in the presence of water. The Unstable alliin-alliinase complex is further subjected to dehydration by pyridoxal phosphate and transformed to allyl sulfonic acid, pyruvic acid and ammonia.

2.3.2.2 Mechanism of action of allicin

Inhibition of certain thiol-containing enzymes in the microorganisms by the rapid reaction of thiosulfinates with thiol groups was assumed to be the main mechanism involved in the antibiotic effect (Mukherjee *et al.*, 2009). In 2002 Miron and other researchers demonstrated the ability of allicin to react with L-cysteine to form the sulfur thiolation product S-allylmercaptocysteine. The identification of this thiolation product was proven by nuclear magnetic resonance as well as by mass spectroscopy.

In 1998 Rabinkov *et al*., argued that “The main antimicrobial effect of allicin is due to its interaction with important thiol-containing enzymes”, on this note allicin was found to strongly inhibit the cysteine proteinases, alcohol dehydrogenases, as well as the thioredoxin reductases which are critical for maintaining the correct redox state within the amoeba parasites.

Inhibition of these enzymes was observed at rather low concentrations (< 10 µg/mL). Interestingly all these three enzymes could be reactivated with thiol containing compounds such as mercaptoethanol and glutathione (Rabinkov *et al*., 1998).

Allicin also irreversibly inhibited the well-known thiol-protease papain, the $NADP^+$-dependent alcohol dehydrogenase from thermo anaerobium brockii, and the NAD^+-dependent alcohol dehydrogenase from horse liver. This suggests that RNA polymerase could also be a target for allicin.

The condensation product of allicin, ajoene, which has a similar oxygenated sulfur group, has been shown to inhibit the proliferation of *Trypanosoma cruzi*, possibly by inhibition of phosphatidyl choline biosynthesis. Ajoene was also recently shown to inhibit phosphatidyl choline biosynthesis in the human pathogenic fungus *Paracoccidioides brasiliensis* (Lawson and Hughes, 1992).

2.3.2.3 Degradation of allicin

Allicin is an oily liquid, bright yellow in color, with a characteristic garlic odor, very unstable, thus its degradation occurs readily even at room temperature (Ilic *et al.*, 2011). Allicin can disintegrate under the influence of various factors. In previous studies (Ilic *et al.*, 2011; Block *et al*, 1986)), allicin degradation under the influence of temperature was monitored by FTIR spectrometry. A band in the IR spectrum originating from S=O valence vibrations at 1087 cm^{-1} (indicative of allicin) was chosen to monitor the allicin degradation (Ilic *et al.*, 2011).

2.3.2.4 Allicin transformation products

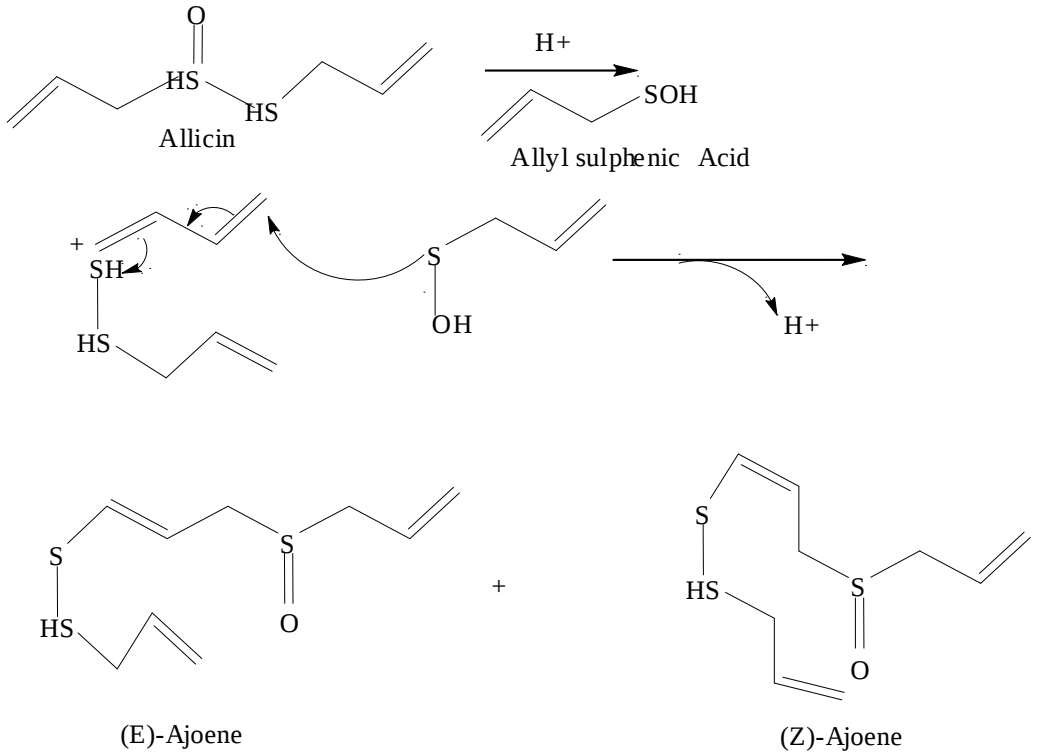


Figure 2.2 Allicin transformation product (Ilic *et al.*, 2011)

,

Ajoenes ((E) - and (Z)-4, 5, 9-trithiadodeca-1, 6, 11-triene-9-oxides) are chemically more Stable than allicin. These degradation products of allicin are commonly found in chloroform and oil extracts of garlic, or in garlic powder mixed with water (Yoshida *et al*., 1999). They are found in form of (E) and (Z) isomers, whereby (E)-ajoene is usually present in twice as high quantities .The degradation mechanism of allicin to (E)- and (Z)-ajoene was described by Block *et al* in 1986.

2.3.3 Allyl sulphonic acid (alliin)

Allyl sulfonic acid is unstable and very reactive at room temperature. With the elimination of water, two molecules of allyl sulphonic acid condense spontaneously to allicin as shown below. Allicin can also be synthesized in the laboratory from allyl disulphide in the presence of hydrogen peroxide as shown below.

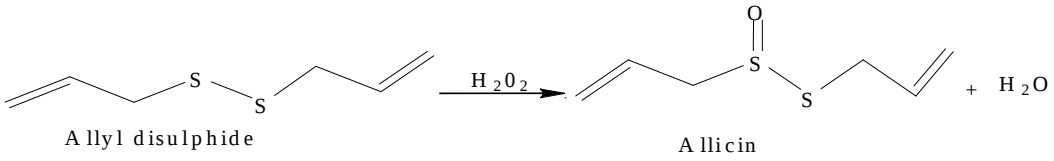


Figure 2.3: Laboratory synthesis of allicin (Ilic *et al*., 2011)

2.4 Medicinal applications of Allium sativum constituents

The medicinal value of garlic has been explored since antiquity (Boon and Smith, 2004; Ross, 1999). It has been used additionally to treat several conditions such as cold, coughs, hypertension, high blood pressure; rheumatism, diarrhoea, and snake bite. Its traditional medicinal use varies from one culture to another. For instance, in East Asia, hot water garlic extract has been used as an aphrodisiac and also to treat asthma.

In England, hot water garlic extract is taken orally for diabetes (Nemeth and Piskula, 2007). Garlic bulb is chewed together with other leaves in Ethiopia to treat stomachache (Onyeagbu et al., 2004). In Guatemala, hot water extract of dried bulb is used externally for ring worm, fungal diseases of the skin, and skin diseases and irritations from leukorrhea, vaginitis, and infections of the skin mucosa (Rose et al, 2001). Water extract garlic is used in Japan to promote hair growth (Shen et al., 2002).

In Nigeria, garlic is dried and soaked in juice of citrus aurantifolia and a pinch of copper sulfate and taken orally to treat convulsions in children (Onyeagbu *et al*., 2004).

Numerous pharmacological functions and clinical trials over the years have confirmed the biological functions and the health benefits of garlic. For instance, recent epidemiological studies had shown that consumption of large amount of garlic is associated with reduced cancer risk in humans, mostly stomach and colon cancer (Benavides *et al*, 2007). Furthermore, studies have also shown the ability of garlic to reduce chemical carcinogens in different animals.

Other well studied benefits of garlic include; antimicrobial, antithrombotic, antioxidant, improving immune- system, anticardiotoxic and anti–ischemic effects, antiaging, antibacterial, anticytotoxic, antifatigue, antifungal, antihypertensive, antihypotensive, antihypothermic, antimutagenic, antitoxic, and antiprotozoan activities (Ross, 1999). In addition, there have been studies on the inhibition of acetylcholinesterase, acid phosphatase, adenosine deaminase, adherence (bacteria to host cells), aflatoxin production, and alanine aminotransferase, as well as alamine aminotransferase and alkaline phosphatase stimulation (Yoshida *et al*., 1999).

Extensive investigation into garlic and its medicinal value has led to an improvement in the quality and yield of fresh garlic production. The countless benefits of garlic no doubt have given rise to the production of several garlic food supplements ranging from tablets to powder and are based on either allicin content or on the potential to produce allicin (Miron *et al.*, 2002). This consequently has initiated a host of investigations to ascertain or debunk the claims that they have the same essential ingredients as raw garlic.

One of such investigations has revealed that though garlic powder and granules can serve as important food supplement, if stored for a long time, the ingredients present in fresh garlic are often lost. In addition, because alliinase is irreversibly deactivated at the pH level in human stomach, if garlic powder is taken directly there would only be an insignificant amount of allicin that can be produced inside the human body (Chen *et al.*, 2009).

Based on this knowledge, garlic capsules coated with materials that can resist human stomach conditions in order to prolong the shelf life and protect alliinase activity through the stomach have been commercially produced. In this way, allicin can be released only in the intestine and consequently decrease the characteristic odor and after taste (Li et *al*., 2009). Many other studies had been done to find out the pharmacological benefits of these food supplements, but not all of them can assumed equivalence in their composition and in their biological response (Diego *et al.*, 2007).

2.4.1 Pharmacological effects of ajoene

Ajoenes have antimicrobial effect (they affect a great number of bacteria, viruses, fungi and other parasites). They participate in the inhibition of in vitro growth of Helicobacter pylori, a bacterium responsible for gastric diseases such as gastric ulcer and stomach cancer (Ross, 1999).

The mechanism is based on interaction with sulfhydryl group in microorganism's enzyme. The antifungal effect of ajoene was proven in both in vivo and in vitro conditions against many pathogen fungi.

The mechanism of their effect as the antifungal agents has not yet been explained. It is considered to be related with a selective inhibition of phosphatidyl choline biosynthesis in lower eukaryotes (Ankri and Mirelman, 1999).

2.5 Analytical Techniques for Allium sativum constituents

In 1973, Freeman and McBreen developed the first method for analysis of volatiles of onions and garlic based on a rapid Spectrophotometric determination (Miron *et al*., 2002). Twenty years later, many other methods were proposed (Miron *et al*., 2002). Currently, a number of analytical methods have been used to determine S-alkenylcysteine sulfoxides in garlic.

These include; capillary electrophoresis, Spectrophotometric methods, gas chromatography, micellar electrokinetic capillary chromatography, thin layer chromatography, high performance ion-pair chromatography, and high performance liquid chromatography (Lanzotti, 2006). They can be categorized into direct methods that allow determination of S-alkenylcysteine sulfoxides content before their enzymatic hydrolysis to form allicin or indirect methods that are employed in the determination of diverse products arising from the enzymatic reaction (Han *et al.*, 1995).

Some of the above named techniques involve a single step sample preparation, whereas others are associated with several steps during which the analyte is derivatized (Kubec *et al*., 1999).

2.5.1 Spectrophotometric Method

Spectrophotometric methods employ light to measure the chemical concentration of analytes. This absorption should be distinguishable from that due to other substances in the sample. The absorption by a sample is proportional to the total amount of material that absorbs the incident light that is referred to as chromophore. This is defined by the Beer – Lambert Law;

$$A = \varepsilon bc \quad \text{.....Equation 2.1}$$

Where ε, molar absorptivity is a constant that is a property of the material itself as well as the wavelength of the measurement; b refers to the length of the path through which the light travels in the sample; and c, the molar concentration of the material that absorbs the light (Han *et al*., 1995). Alliin content in garlic extract can be indirectly determined using this method. This determination is based on the principle that alliin in presence of allinase produces allicin that reacts rapidly with free thiol groups through a thiol–disulfide exchange reaction. Such exchange reaction can cause a shift in the optical absorption of a thiol-containing chromophore, and therefore the molar concentration of alliin can be calculated from the difference in absorbance according to the equations adapted from Miron *et al* in 2002.

[Alliin] = [absorbance without alliin] – [absorbance with alliin] x dilution x [ε]-1

.....Equation 2.2

Where ε =molar absorptivity

Miron and his co-workers used this method in their assay for allicin, alliin, and alliinase. In their recent work, they used 4-mercaptopyridine (4-MP) rather than the previously used 2-nitro-5-thiobenzoate (NTB) because the former is a commercially available chromogenic thiol. Their determinations were based on the reaction of 4-MP (which has maximum absorption at 324 nm) with the activated disulfide bond of thiosulfinates -S(O)-S- to form 4-allylmercaptothiopyridine, which has no absorbance in this region. The structure of the 4-allylmercaptothiopyridine was confirmed by mass-spectrometry. The difference in absorbance obtained was thus used to calculate the content of the analyte. This method though indirect, was reported to be sensitive, fast, and non-costly and gives highly efficient throughput assay of alliin, allicin, and alliinase in garlic extracts (Han *et al*., 1995).

2.5.2 Gas Chromatography

Gas chromatography (GC) involves the separation of components of vaporized sample as a result of partitioning between a gaseous mobile phase (called carrier gas) and a liquid or solid stationary phase. The carrier gases commonly used are helium, nitrogen, or hydrogen gas. The choice of carrier gas depends on the detector and the desired separation efficiency and speed (Harris *et al*., 2001). The liquid stationary phase is a non-volatile liquid bonded to the inside of the column or to a fine solid support. This is used in gas-liquid partition chromatography. The solid stationary phase is used in gas-solid adsorption chromatography in which the analyte is adsorbed directly on solid particles of stationary phase.

The essential elements of gas chromatography include a regulated supply of carrier gas, a device for vaporizing the sample (injector), a thermostatic oven in which the column is housed, a detector, and a data processor (Poole and Schuette, 1984). When a volatile liquid or gaseous sample is injected through a septum into a heated port, it is rapidly evaporated and the vapor is carried through a hot column by carrier gas causing separation of components of sample to take place. The column is kept hot enough to provide sufficient vapor pressure for analyte to be eluted in reasonable time.

The separated components flow through a detector and their responses are recorded by a recording device and processed (Harris *et al*., 2001). The columns used are generally open tubular columns and packed columns. The open tubular columns are used in wide range of analyses. It is made up of fused silica (SiO_2) and coated with polyimide for support and protection from atmospheric moisture. An open tubular column has the advantage of better separation efficiencies and greatly improved sample detectability for a given analysis time than any packed columns. It also gives greater sensitivity and shorter analysis time.

Open tubular columns however have disadvantages that include more demanding of instrument performance, less forgiving of poor operator technique, and possess a lower sample capacity than the packed columns (Harris *et al*., 2001). Packed columns are made up of fine particles of solid support coated with nonvolatile liquid stationary phase, or the solid itself may be the stationary phase.

The packed column offers a greater sample, gas chromatography gives excellent resolution and has mass identification capabilities (Block *et al.*, 1986). For this reason gas chromatography and gas chromatography-mass spectrometry (GC-MS) have been widely used in the characterization of allium volatiles. Gas chromatography determination (GC-FPD) of alliin in garlic and garlic products involves derivatizing alliin with trifluoroacetic acid anhydride (TFAA) followed by GC analysis using a short packed column. (Poole and Schuette, 1984).

This method was found inadequate because trifluoroacetic derivative was unstable and consequently decomposed after it was exposed to sunlight for 15 minutes. This was thus, a major limitation of their method. Another limitation that was also reported was poor column resolution that renders the method unsuitable for routine work.

In their work published in 1986, Block *et al* emphasized that gas chromatography as typically performed with high injector and column temperature present an erroneous picture of the composition of room temperature extracts of Alliin species and that HPLC provides a reliable quantitative and measure of what is actually present in the species. Arnault and his team also reiterated that gas chromatograph–mass spectrometer (GC-MS) does not seem to be a suitable method for the analysis of true garlic flavors as it gave artifacts. They proposed high performance liquid chromatography as a preferable alternative (Arnault *et al.*, 2003).

Highly sensitive and reproducible determination of S-alkenylcysteine sulfoxides (including minor derivative) can be analysed using gas chromatography (Han et *al.*, 1995).

The method was based on isolation of amino acid fraction by ion-exchange chromatography followed by derivatization with ethyl chlorofomate at ambient temperature and reduction of derivatized S-alkenylcysteine sulfoxides by sodium iodide. They reported that all preliminary attempts to analyze S-alkenylcysteine sulfoxides by GC immediately after derivatization failed. They used two different capillary columns, various temperature programs, and injector temperatures and realized that under all the conditions there was a substantial decomposition of S-alkenylcysteine sulfoxides similar to what had been described in connection with GC determination of glucosinolates having a sulfoxide moiety in the side chain. They attributed this to the presence of the highly polarized and extremely labile sulfoxide group and suggested that the best way to analyze S-alkenylcysteine sulfoxide by GC is removing this group prior to the injection, hence the use of sodium iodide in their method.

Even though this method offered outstanding sensitivity, excellent resolution capacity, accuracy, and reliability, time requirements are a serious drawback for use in routine analysis. In addition, the method is unable to resolve between S-alkenylcysteine and their sulfoxides (Han *et al.*, 1995)).

2.5.3 Thin Layer Chromatography

Thin layer chromatography (TLC) involves movement of a mobile phase through a thin layer of sorbent (coated on an inert, rigid background such as aluminium, plastic, or glass) by capillary action.

The separation of sample is a result of the differences in migration of sample components in the direction the mobile phase travelled. It is measured in terms of retention factor or retention index, R_F given as:

$$R_F = \frac{\text{Distance travelled by spot from origin}}{\text{Distance travelled by solvent from origin}} \quad \text{……..……..Equation 2. 3}$$

Thin layer chromatographic method of analysis of garlic constituents has been developed involving the use of ninhydrin detection reagent to optimize the differentiation between cysteine sulfoxides and other amino acid derivatives (Miron *et al*., 2002). The shortcoming of this method has been reported to be enzymatic degradation of alliin due to preparation of sample extract by homogenizing garlic. This allows the interaction between alliin and allinase that otherwise are present in separate components in the intact cells (Kanaki and Rajani, 2005).

In 2005 Kanaki and Rajani published a proposed HPTLC method for the analysis of garlic and its formulation for its alliin content. This method involves densitometric evaluation of alliin after resolving it by HPTLC on silica gel plates with n–Butanol: acetic acid: water (6:2:2 v/v) as the mobile phase.

After derivatizing the resolved bands with ninhydrin reagent, the peak areas were recorded at 540 nm in densitometric evaluation. They found the relation between the concentration of alliin and the corresponding peak area to be linear within the range of 250 to 1500 ng/ spot. They recommended this method for use in routine quality control of garlic and its formulation due to its good precision, specificity, sensitivity, and accuracy (Kanaki and Rajani, 2005).

2.5.4 High Performance Liquid Chromatography

High performance liquid chromatography has been used widely in analysis of diverse varieties of samples since most compounds are not sufficiently volatile for gas chromatography. Its advantages include; high speed resolution, sensitivity (femtograms–nanograms), good reproducibility, recovery, accuracy, precision, and ease of automation.

It has proven to be a reliable technique for quantitative and qualitative determination of sulfoxides in allium extracts (Block *et al.*, 1986). For this reason a number of HPLC techniques have been developed and have been used in the determinations of organosulfur compounds in allium species.

In 2002, Miron and his coworkers published a report on a rapid HPLC method suitable for routine analysis of sulphoxides. This method involves the use of 3 μm particle Hypurity Elite C_{18} column of dimension 150 x 3mm, an ultra-violet detector operated at 208 nm, and a gradient elution involving the use of mobile phase consisting of (a) 20 mM sodium dihydrogenphosphate, 10 mM heptanesulfonic acid, 85 % orthophosphoric acid and (b) acetonitrile, 20 mM sodium dihydrogenphosphate, 10 mM heptanesulfonic acid.

They used eluents containing an ion–pairing reagent to ensure a sufficient separation between alliin and the more retained dipeptide at very low pH. Arnault *et al*. reported that their method that was without derivatization allowed simultaneous quantification of alliin, allicin, as well as dipeptides and requires no particular sample preparation.

The method also yielded good linearity for each compound and gave a run time of 30 minutes.

However, the sensitivity of the method was weaker compared to a pre-column derivatization. An alternative method for the determination of sulfoxides involving one step sample preparation procedure followed by normal phase and reversed phase HPLC techniques to determine the sulfoxides has been advanced (Ichakawa *et al*, 2006).

Alliin, isoalliin, methiin, cycloalliin, and γ-L-glutamyl-S-methyl-L-cysteine were determined by normal phase HPLC using an aminopropyl-bonded column, whereas γ-L-glutamyl-S-(2-propenyl)-L-cysteine and γ-L-glutamyl-S-(trans-1-propenyl)-L-cysteine were separated on an octadecylsilane column. They reported overall recoveries of 97.1%-102.3% and relative standard deviation values of intra- and interday precision lower than 2.6% and 4.6% respectively.

The advantages of their method include specificity, speed, and ease of use. They confirmed that the method was useful for chemical and biological studies of garlic and its preparations.

In 2007, Diego *et al* developed and validated a reversed phase HPLC assay for quantitative determination of allicin in garlic powder and tablets. Their chromatographic separation was performed on a RP-C18 column of dimensions 124 mm x 4mm. They used a mobile phase made up 50:50 methanol: water, a flow rate of 0.5 mL/min, and ultra-violet detection at 220 nm.

The method also involved the use of ethyl paraben as internal standard. Diego and his colleagues reported that the method was linear for allicin concentrations of 5.0-60.0 μL/mL and gave relative standard deviation for precision to be less than 6.14% with accuracy above 89.11% (Diego *et al.*, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

In this study materials included all the samples, chemicals, apparatus and microorganisms that were used during the bioassay and chemical analysis. The extraction process was done at the Kenya Marine and Fisheries Research Institute in Kisumu on a Soxhlet system model 345 from England. The 0.45µm microbibre filters and Whattman thimbles were sourced from Ultra lab in Nairobi while Chloroform, ethyl acetate, hexane, ethanol, methanol all HPLC grade were sourced from Gelsap Laboratory supplies in Nairobi. Alliin standard, allyl chloride and sodium sulphide were all purchased from Sigma Aldrich manufacturing company in Germany. Distilled deionised water was purchased from Microbiologics Laboratories in Kisumu. Kechap blender model 104 was purchased from Nakumatt mega city in Kisumu.

Incubator Omex Model was accessed at the Microbiology Laboratory at Kenya marine and Fisheries Research Institute, Kisumu. Refrigerator Sanyo model from Japan was also availed in the same laboratory. Petri dishes, syringes and needles and gloves were purchased from Ultra Lab in Nairobi while amoxicillin clavulanate susceptibility testing discs and fluconazole susceptibility testing discs were purchased from Cypress diagnostics in Belgium.

Mueller Hinton agar, tryptic soy agar, Baerd- parker, Plate count agar, nutrient broth and Sabroud dextrose agar were all purchased from Itech Laboratory suppliers in Nairobi. Ninhydrin reagent and Silica gel for column chromatography were all purchased from Ultra Lab Supplies in Nairobi.

The HPLC analytical work was done at Moi University Chemical Engineering Laboratory on a LC-10AT Liquid Chromatography System with SPD-10A UV-Vis detector Shimadzu, Japan), using Phenomenex^{TM} ODS C_{18} column (250 mm x 4.6mm, 5 µm) and 20µL variable loop injector. Other instruments included UV-VIS spectrophotometer (Shimadzu), Rotary evaporator system and Vacuum filtration system and bath sonicator system Shimadzu from Japan.

Characterisation of synthetic allicin was done at The University of Nairobi, Chemistry Research Laboratory using IR Affinity-1S Fourier transform infrared spectrophotometer (Shimadzu) equipped with Quest Attenuated Total Reflectance (ATR) crystal.

In this study two sampling sites were adapted. Sampling point A, gardens based in Kimalel Location in Baringo County Kenya and sampling point B garlic samples purchased from Nakumatt supermarket in Eldoret town. Sample A were cloves freshly obtained from the garden while sample B were garlic cloves packed in ventilated sample bags.

Gram-positive *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeroginosa* ATCC 27853 and gram-negative *Escherichia coli* ATCC 25922 were purchased from Microbiologics. Fungal yeast *Candida albicans* ATCC 10231 was also from Microbiologics in Canada.

3.2 Methods

3.2.1 Sample processing and extraction

Samples from the garden were cleaned to remove soil particles and then aerated to reduce moisture content. Samples from the supermarket were cleaned and aerated at room temperature to prevent rotting. The fresh garlic cloves were stored at 24°C and used within 2 weeks. Garlic juice was produced aseptically from peeled cloves using a juice extractor. The fresh garlic paste (50 ml) was immediately centrifuged at 4000 ×g for 10 min at 24°C awaiting extraction.

Soxhlet and maceration methods were adopted for extractions to compare yields. For soxhlet, 25g of garlic paste was extracted with 100 ml of analytical grade methanol and ethanol as solvents using both garden collected and purchased samples separately. Extractions were done for up to one and a half hours over a heating mantle in the soxhlet apparatus. The maceration method of extraction was achieved by soaking 200g of garlic paste from both garden collected and purchased samples separately in a hermetically sealed container with 800ml methanol and ethanol as solvents for 24 hours.

This was followed by filtration through 0.45µm microbibre filters to exhaustively remove solids from the crude garlic extracts. Rotaevaporation was then done to remove excess solvent. The samples were coded as shown in table 3.1 below.

Table 3.1: Extraction of allicin from garlic cloves

Sample	**Extraction method**	**Solvent**	**Code**
GA	Soxhlet	Ethanol	GA0101
GA	Soxhlet	Methanol	GA0201
GA	Maceration	Ethanol	GA0301
GA	Maceration	Methanol	GA0401
SA	Soxhlet	Ethanol	SA0101
SA	Soxhlet	Methanol	SA0201
SA	Maceration	Ethanol	SA0301
SA	Maceration	Methanol	SA0401

*SA- Garlic samples from Nakumatt supermarket, Eldoret
*GA- Garlic samples from Baringo

Immediately after extraction the samples were refrigerated at 0˚C awaiting the fractionation process. Percentage yield was then calculated using equation 3.1 adopted from Ilic *et al*., 2011.

$$\%\ yield = \frac{\text{Total weight of extract obtained}}{\text{Total weight of } \textit{Allium sativum} \text{ cloves extracted}} \times 100$$ .....Equation 3.1

(Ilic *et al*, 2011)

3.2.2 Fractionation of crude garlic extract

Column chromatography was used to separate the constituents of crude garlic extracts while thin layer chromatography was used to identify the fractions for pooling based on R_F values of the various column eluents. 2g of crude garlic extract was used in the

fractionation process. The column was packed by suspending 60g of 120 mesh silica gel in 100ml of eluting solvent attaining a column length of 40cm. Normal phase chromatographic gradient elution starting with 50ml of hexane before increasing polarity stepwise with chloroform to ethyl acetate and finally methanol in increasing ratio of 5ml was then performed. Eluents of 5ml each were collected in test tubes.

Thin layer chromatography was immediately performed on the column eluents. The elution solvent for thin layer chromatography was prepared by mixing methanol, chloroform and ethyl acetate in the ratio 2:1:1 v/v/v. Ninhydrin spray consisting of ninhydrin- Cd acetate was prepared and used for developing the chromatograms while UV lamp at 254nm and 324nm was used for visualization of the spots. After drying the sprayed chromatograms at 105˚C the R_F values were determined.

The fractions having similar R_F values were pooled together affording four fractions at the end of the thin layer chromatography. The four fractions were further concentrated through rotaevaporation of the solvents after which the fractions were stored in the refrigerator at temperatures below 0˚C awaiting UV, HPLC analysis and bioassay.

3.2.3 HPLC method development and validation

Alliin standard was purchased from Sigma Aldrich while allicin standard was synthesized in the laboratory.

3.2.3.1 Synthesis of allicin standard

The synthesis of allicin was based on a previously published method by Block *et al*., (1986). 30g of sodium disulphide was reacted with 200ml of 0.06407M allyl chloride at 40˚C and 760mmHg pressure to produce diallyl disulphide and sodium chloride. Hexane was added in the solution mixture to dissolve diallyl disulphide and the mixture placed in the oven at 30˚C for 3 hours. Sodium chloride solution was precipitated using ammonium chloride while diallyl disulphide was recovered at 40˚C by washing the solution in hexane.

A separatory funnel was then used to separate diallyl disulphide at the top from the sodium chloride solution at the bottom. Diallyl disulphide was collected as a yellowish liquid with a characteristic strong garlic smell. The diallyl disulphide liquid was then oxidized using hydrogen peroxide (30% v/v) in a reflux glass apparatus for four hours. 5g of allicin was recovered after rotaevaporation of the hexane solvent. This was followed by purification by column chromatography using hexane, ethyl acetate, chloroform and methanol as the mobile phase via a gradient elution. After the purification process 4g allicin was recovered and used as a standard for UV and HPLC analyses of allicin extracts.

Characterization of the synthesized allicin standard was performed on a FTIR spectrophotometer Shimadzu IR affinity IS-I equipped with Quest ATR Happ-Genzel apodization. A thin film of synthesized allicin (0.1mm) was thoroughly mixed with KBr to develop pellets and the FTIR spectrum obtained on over a scan range of 4000cm^{-1} to

600cm^{-1}. UV- VIS spectrophotometer was also used to confirm the λ_{max} by scanning from 190-600nm. The obtained spectra were compared to literature values for confirmation.

3.2.2.2 Chromatographic conditions optimization

The HPLC analysis was performed on a reversed phase analytical column at 25˚C. The UV detector was set at 224nm for allicin analysis and 340nm for alliin analysis. The mobile phase was selected by using different ratios of methanol: water ranging from 1:1, 1:2 1: 3, 2: 3, 2:1 and ratio 3:2.

During the analysis the flow rate used were 1mL.min^{-1}, 0.75mL.min^{-1} and 0.5mL.min^{-1}. At 1mL.min^{-1} the HPLC system went into error mode because the column used was not able to give resolution. Isocratic elution was used with a few modifications. Isocratic step gradient was employed by decreasing the volume of water in the mobile phase from 1:1, 2:1 and 3:1. Two pressure systems were involved in the HPLC system. The pump pressure A and pump pressure B were set at a maximum of 15.0 MPa and a minimum of 2.0 MPa. The column pressure was set at 8.2 MPa.

Two sets of injection volumes namely, 10µL and 20µL of alliin and allicin were injected into the sample loop and loaded into the column.

3.2.3.3 Calibration curves for allicin and alliin

Stock solution for alliin standard was prepared by dissolving 10mg of the standard alliin ($C_6H_{11}NO_3S$) in 10ml of methanol: HCL mixture in the ratio (90:10) to inhibit conversion of alliin to allicin, while the stock solution for synthesized allicin as prepared by dissolving 4g of the synthesized allicin in 10ml of methanol: water mixture in the ratio (1:1) v/v. The stock solutions were then serially diluted to give standard solutions of concentrations: 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml and 160µg/ml respectively for calibration curve construction for alliin and allicin.

The five different concentrations for allicin standard and alliin standard were analysed using the HPLC Shimadzu model with a UV/VIS detector. Two calibration curves were developed by plotting the area under the HPLC chromatograms against concentration of standard allicin and alliin in µg/mL.

3.2.3.4 HPLC method validation

The HPLC method was developed and validated according to ICH guidelines (ICH, 2003). The column was thermostated at 25˚C and detection was carried out at 340nm for alliin and 224nm for allicin. The mobile phase consisted of methanol: water (50:50 v/v) to which 1ml acetic acid was added. Two standard curves were developed for alliin in order to establish linearity and range.

Stock solution of alliin standard 10mg/ml in a mixture of methanol and HCL was diluted to get two sets of standard solutions in the range 10-320 µg/mL and 20-120 µg/mL. Coefficient of determination (R^2) was then utilized to select the best range.

Relative standard deviation (RSD) was used to establish precision while the accuracy by determination of analytical recoveries which entailed intra-day and inter-day evaluations by analysis of alliin standard and synthesized allicin at six concentration levels namely 10 µg/mL, 20 µg/mL, 40 µg/mL, 80µg/mL and 160 µg/mL in alliin and allicin standard samples (n=18) on the same day and on three consecutive days.

The limits of quantification and detection were calculated by the simultaneous analysis of alliin and allicin samples and using standard deviations of pre-determined analytical recoveries. The Limit of detection and Limit of quantitation was calculated using the following equations according to the ICH standards and guidelines.

$LOD = 3.3 \times \sigma/s$……...……..……Equation 3.2

$LOQ = 10 \times \sigma/s$ …….……....Equation 3.3

Where

σ- Standard deviation

s – Slope of the calibration curve

Experimental conditions were purposefully altered to determine the robustness of the HPLC method. Effect of flow rate on peak resolution was changed gradually from 0.5ml.min^{-1}, 0.75ml.min^{-1} and 1mml.min^{-1} while the mobile phase remained the same.

Effect of injection volume on the resolution was also studied at 10µL, 15µL and 20µL. The column was thermostated at 15˚C, 25˚C and 50˚C.

3.2.4 HPLC quantification of allicin and alliin

The developed and validated HPLC method was used to quantify allicin and alliin in *Allium sativum* crude extracts, column fraction B_4 and HPLC eluents to monitor antimicrobial trends with fractionation.

3.2.4.1 Determination of allicin and alliin in crude garlic extracts

Freshly prepared crude garlic extracts were analysed in the HPLC Shimadzu Model at 224nm. Stock solution was prepared by dissolving 1ml of crude garlic extract in 100ml of mobile phase that consisted of methanol: distilled water in the ratio 1:1 v/v. Different dilutions were prepared by diluting 6.25ml, 12.5ml, 25ml and 50ml of the stock solution in 100ml volumetric flask using the mobile phase. 20µL of sample was injected into the sample injection loop and the sample loaded into the luna^R column.

3.2.4.2 Determination of allicin and alliin in column chromatography fraction B_4

Stock solutions of B_4 extracts were prepared by diluting 1ml of sample in 10ml of methanol. Different dilutions of B_4 extracts were prepared by diluting 6.25ml, 12.5ml,

25ml and 50ml of the stock solution in 100ml volumetric flask using methanol: distilled water mobile phase in the ratio 1:1. 20µL of sample was injected into the sample injection loop and the sample loaded into the luna^{R} column. The analysis was done at 224nm wavelength and quantification of allicin and alliin done by using the pre-developed calibration curves.

3.2.5 Evaluation of antibacterial and antifungal activity trends

The microorganisms were chosen according to the National Committee for Clinical Laboratory Standards, 2010 (NCCLS) protocols. Bioassay was performed using gram positive *Staphylococcus aureus*, *Pseudomonas aeruginosa* and gram negative *Escherichia coli* while Fungal yeast *Candida albicans* was used in the fungal bioassays.

The *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *Candida albicans* strips were pilled and the microorganisms recacitated by pinching the lipophilic liquid to mix with the microbes at the bottom of the strips. The crystals were crashed using hands and tapped on the bench to mix the liquid with the microorganisms. The swab was removed and inserted inside pre warmed nutrient agar tubes in triplicate.

The inoculated microorganisms were then incubated for 18 hours at 37˚C. 0.5 McFarland standard was prepared by diluting 0.05ml of 1% $BaCl_2$ in 9.95ml of 1% H_2SO_4 and used to adjust the optical density of the colony forming units. UV spectrophotometer was used

to confirm the optical density of the appropriately. The microbes while the McFarland standard was used to adjust optical density surface of MacConkey agar plates, were uniformly cultured with *Escherichia coli*; Mueller Hinton agar plates were inoculated with *Staphylococcus aureus* while antibacterial activity of garlic extracts against *Pseudomonas aeruginosa* on Baerd-Parker agar plates. Antifungal effects of garlic extracts was bio assayed by inoculation of freshly growing strains of *Candida albicans* on Sabroud agar plates.

The unopened ATCC cultured microbial strips were stored under refrigeration at 4˚C. The disc diffusion technique using the Kirby-Bauer method was applied in testing the ATCC cultures for their antimicrobial sensitivities. The sensitivity discs used for the test were punched from Whatman No. 1 filter paper (5 mm in diameter). After sterilization, the discs were placed on the inoculated agar plates and impregnated with 25µL solution of different concentrations of crude garlic extracts, column fraction B_4 and HPLC eluents.

Inoculated plates containing *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were incubated at 37˚C for 18 hours while *Candida albicans* inoculated plated plates were incubated at 40˚C for 24 hours.

After incubation the plates were observed for zones of inhibition followed by calculation of the mean zones of inhibition (mm).

Antimicrobial susceptibility testing discs containing amoxicillin clavulanate purchased from Cypress diagnostics in Belgium were used as positive control on the bioassay for *Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa* while antifungal susceptibility testing discs containing fluconazole from the same supplier were used as positive control for *Candida albicans*. During bioassay 1% Dimethyl sulphoxide was used as the negative control for both antibacterial and antifungal activity of the crude garlic extracts and column fraction B_4.

The minimum inhibitory concentration (MIC) was determined by preparing a standard inoculum for *Escherichia coli* ATCC 25922 *Pseudomonas aeroginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 into tryptone soya broth (Oxoid, CM129) and incubating at room temperature overnight. The overnight broth culture (0.1 mL) was diluted with 1 mL of distilled deionised water in the ratio of 1:100 to give a final dilution of 10^{-3} of the standard inoculum (Ankri and Mirelman, 1999) after which the dilution susceptibility test technique (Muschietti *et al*., 2005) was applied.

Six different dilutions of garlic juice and column fraction B_4 in 1% dimethyl sulphoxide was prepared in the following ratios; 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. The concentrations of the different dilutions were calculated based on the ratios.

The minimum inhibitory concentration (MIC) of the extracts was determined for *Escherichia coli and Candida albicans* by diluting the extracts in Mueller Hinton agar (MHA) media, (NCCLS, 2010). Petri dishes, controls and with different concentrations of garlic juice (ml/ml), were inoculated with *Escherichia coli, Pseudomonas aeroginosa, Staphylococcus aureus and Candida albicans by* using the Steer's replicator and incubated at 37˚C for 18 hours for the bacteria and at 40˚C for 24 hours for the fungal yeast.

The concentration that inhibited visible growth of each strain (MIC) was recorded, and the MIC 90% was calculated. The presence or absence of growth for each concentration was recorded at the end of incubation.

The lowest concentration of the extracts resulting in no growth after 18 hours of incubation for the bacteria and 24 hours of incubation for the fungi was taken as the MIC.

3.2.5 Antibacterial and antifungal activity trends modeling with fractionation

Concentrations of alliin and allicin were monitored in garlic extracts, column fractions and HPLC eluents and compared with their antibacterial/antifungal activity. A model was devised by plotting zone of inhibition zones against the samples.

3.3 Data analysis

Qualitative and quantitative statistical analysis was used to evaluate certainty of the data collected. Analysis of variance using Multi variate analysis of variance (MANOVA) was used to establish whether variation of bioactivity was significant with regards to method of extraction, solvent used and fractionation process. F values were calculated and minimal significance was evaluated in the variation of bioactivity of different microorganisms that were bio assayed during the research process. Statistical Package for Social Scientists (SPSS) version 21 was then used to establish the significance of variability between and within different groups (Type of sample, extraction method and solvent) with bioactivity as the dependent variable to establish the significance at 0.05 confidence level.

CHAPTER 4

4.0 RESULTS AND DISCUSSION

4.1 Sampling

Sampling site A was from Kimalel location in Baringo County with GPS coordinates 0˚28’0.01’’ N 35˚58’ 0.01’’ E. Sampling point B was Nakumatt Eldoret.

4.1.1 Sample processing

Percentage yield for each extraction of 25g of blended *Allium sativum* juice using soxhlet extraction and 200g using maceration were determined. Comparison of the average yields for the different extraction methods using two different solvents revealed that *Allium sativum* cloves extracted using maceration method in methanol had the highest yield of 1.3% followed by ethanolic extraction using maceration method. Lowest yield was recorded in soxhlet extraction method using ethanol as the solvent with a percentage yield of 0.24%. The percentage yield of crude extracts of the *Allium sativum* were evaluated and tabulated as shown in table 4.1 below:

Table 4.1: Percentage yield for crude *Allium sativum* extracts

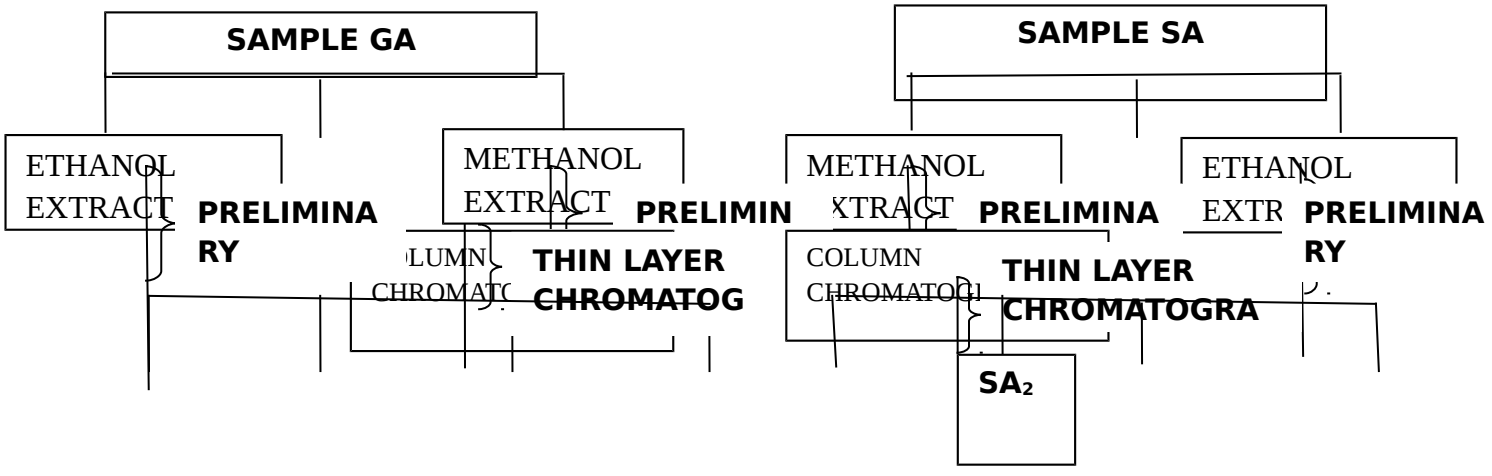
Sample	solvent	extraction method	extract weight (g)	% yield
GA	Methanol	Soxhlet	0.08	0.32
GA	Ethanol	Soxhlet	0.06	0.24
GA	Methanol	Maceration	2.4	1.2
GA	Ethanol	Maceration	1.6	0.8
SA	Methanol	Soxhlet	0.10	0.4
SA	Ethanol	Soxhlet	0.09	0.36
SA	Methanol	Maceration	2.8	1.3
SA	Ethanol	Maceration	1.8	0.9

* SA- Garlic samples from Nakumatt supermarket, Eldoret.
*GA- Garlic samples from Baringo.

The highest percentage yield for allicin was 1.3% in Chinese samples using methanol as the solvent. The lowest percentage yield for allicin production was 0.24% in garden samples using soxhlet method of extraction in ethanol solvent. From the extraction process methanol gave more allicin yield as compared to ethanol solvent extraction. Maceration method of extraction was more preferred due to stability of allicin at room temperature which led to higher yields. The method also provided more time for enzymatic conversion of alliin to allicin.

4.1.2 Fractionation of crude garlic extracts

The data was developed in the scheme presented in figure 4.1 below:



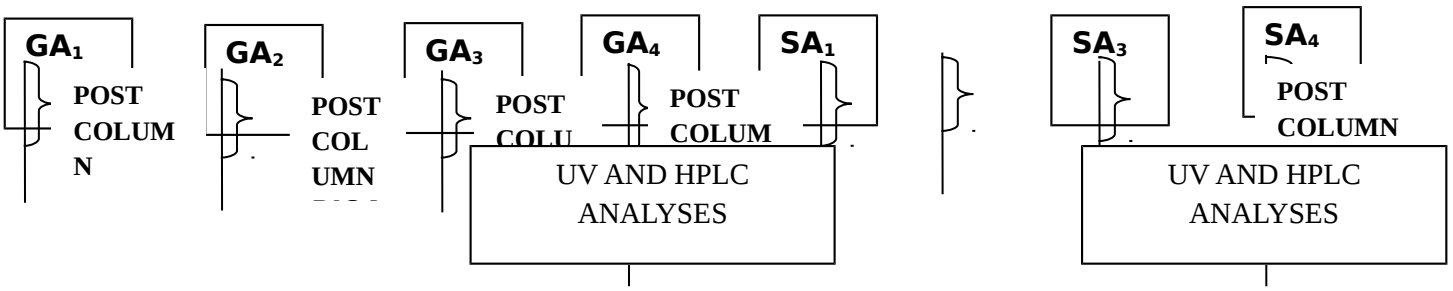


Figure 4.1: Schematic process from extraction to HPLC analysis

All column chromatography fractions were analysed by TLC and compounds having similar R_F values were combined to form one sample. Four fractions were identified by comparing the R_F values obtained with values from the literature under the same experimental conditions (Rahman, 2007). The results are presented in table 4.2.

Table 4.2: Comparison of experimental and literature R_F values for allicin and other allyl disulphides

Fraction	Extract	Isolates	Experimental R_F values	Literature R_F values	Spot color
B_1	Hexane	2-Vinyldithiin	0.67	0.68	Orange

B_2	Ethyl acetate	Diallyl disulphide	0.52	0.53	Yellow
B_3	Chloroform	Ajoene	0.67	0.68	Orange
B_4	Methanol	Allicin	0.53	0.55	Yellow

4.1.3 HPLC method development and validation

4.1.3.1 Synthesized allicin standard

For the structural characterization of synthesized allicin, FTIR spectroscopy was employed to identify the functional groups. The FTIR spectrum is represented in figure 4.2 below:

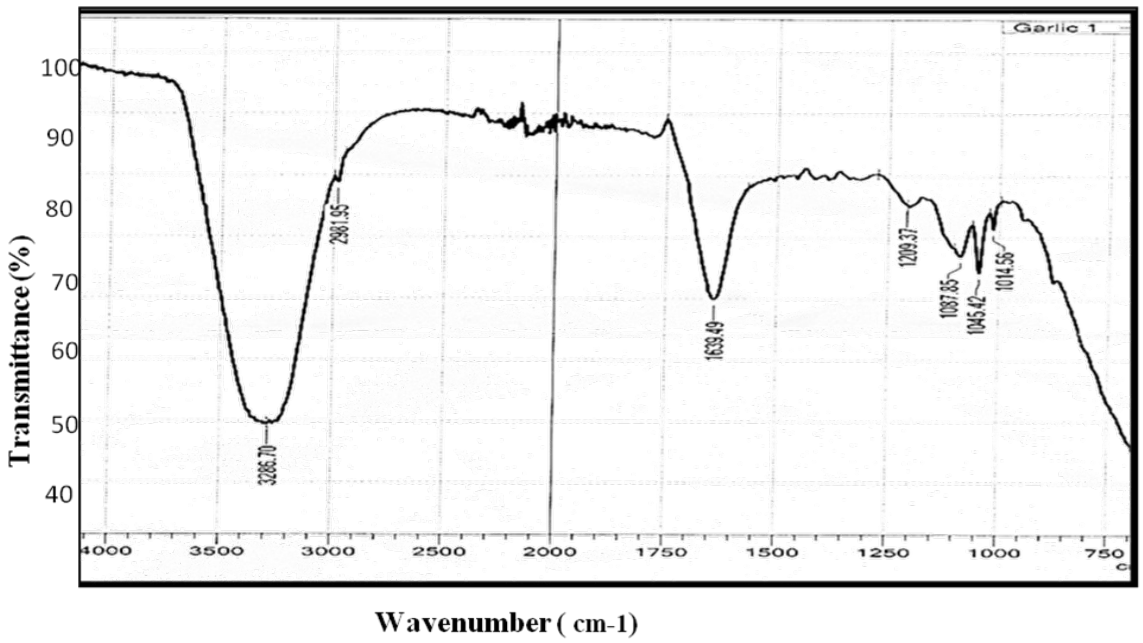


Figure 4.2: FTIR spectrum for synthesized allicin

FTIR 0.1mm thin layer method on KBr plates gave medium intensity band at 1639.49 cm^{-1} originating from stretching of terminal C=C bond valence vibrations. This was confirmed by medium intensity at 2981.95 cm^{-1} resulting from aliphatic (C-H) stretching symmetric of $=CH_2$. The broad intensity band at 3286.70 cm^{-1} was attributed to O-H stretching vibrations due to presence of water in the allicin sample.

At 1087.85 cm^{-1} strong, stretching valence vibrations of S=O group characteristic of allyl thiosulfinate was observed (Trifunschi *et al*., 2015). This was confirmed by 1209.37 cm^{-1} (S-S) stretching of a single thiol group and 1045.42 cm^{-1} (C-S) stretching vibrations in the finger printing region. The observed wave numbers compared well with reported values in previous studies (Trifunschi *et al*., 2015; Block *et al*., 1986; Qing-Yang *et al*., 2002

and Rajam *et al*., 2013). The FTIR spectrum for synthesized allicin was in agreement with FTIR spectrum (figure 4.3) adopted from Trifunschi *et al*., 2015.

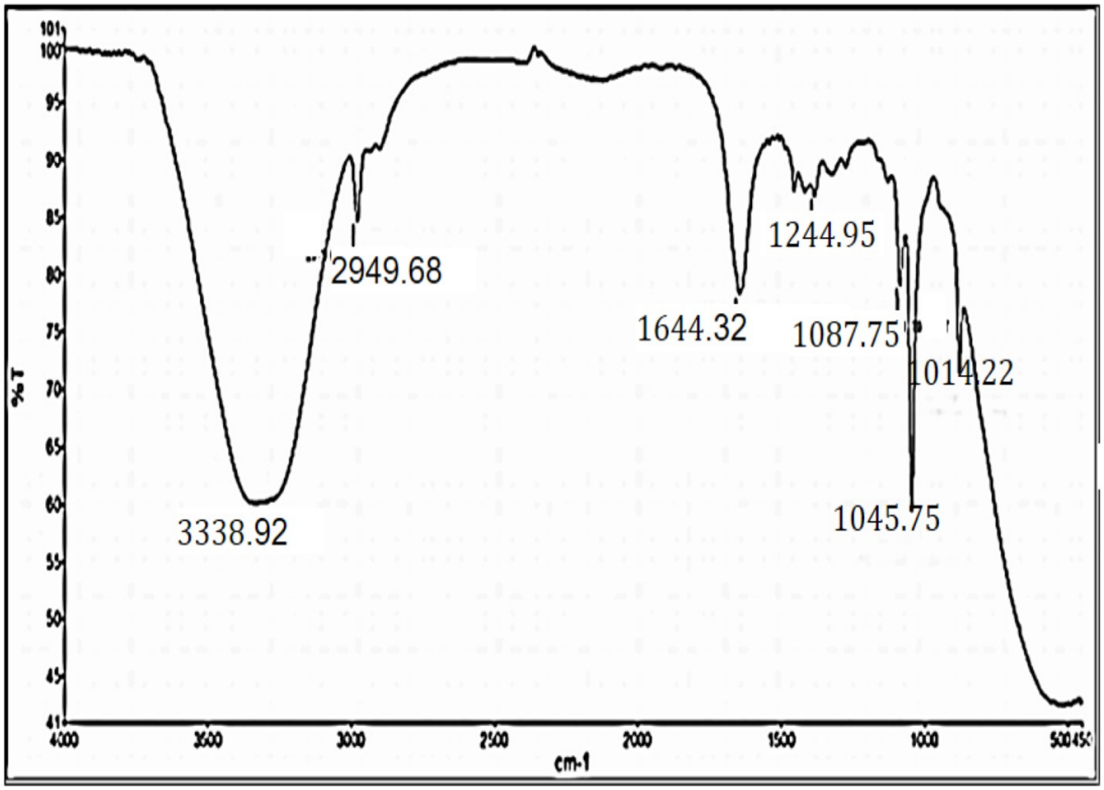


Figure 4.3: FTIR spectrum for allicin standard (source: Trifunschi *et al*., 2015)

Using the developed HPLC method, garlic samples were qualitatively identified using the retention times R_T values (Table 4.3). Synthesized allicin was eluted from the analytical column at R_T 9.253±0.004 while Crude garlic extract and Column fraction B_4 were eluted at R_T values of 9.647±0.040 and 9.373±0.020 respectively.

Table 4.3: Retention times (R_T) for allicin

Sample	**R_T±SD (min)**
Synthesized allicin	9.253±0.004
Crude garlic extract	9.647±0.040
Column fraction B_4	9.373±0.020

The values were in agreement with Ilic *et al*., 2011 and Block *et al*., 1986 who recorded retention times of 9.3 and 9.5 respectively.

4.1.3.2 chromatographic conditions optimization

The HPLC analysis was performed on a reversed phase analytical column at 25˚C. The UV detector was set at 224nm for allicin analysis and 340nm for alliin analysis. The mobile phase of methanol: water ratio 1: 1 was identified as the best ratio for preparation of the mobile phase .Glacial acetic acid was added in the solvent system to enhance peak resolution.

The optimum flow rate for the HPLC system was 0.5mLmin^{-1} the low flow rate led to decreased longitudinal diffusion along the column length. This led to high resolution.

The pump pressure A and pump pressure B were set at a maximum of 15.0 MPa and a minimum of 2.0 MPa. The column pressure was set at 8.2 MPa. These optimum pressure conditions were developed to optimize the system and increase the efficiency of the two pumps to maintain stable flow. The sample volume indicated on the column and sample injection system was 20µL, hence for all the HPLC analyses 20µL sample was injected in the sample injection loop.

Reversed phase HPLC method was developed and validated for the quantitative and qualitative analysis of alliin and allicin. Rational selection of mobile phase composition was done with methanol: distilled water in the ration 1:1 (v/v) being identified as the best mobile phase for both analytes. Glacial acetic acid was used to enhance the peak resolution. UV spectrum was used to qualitatively determine the wavelength that gave highest absorbance as 324nm for alliin and 224nm for allicin.

Consequently reversed phase HPLC analyses for alliin and allicin were performed at the two predetermined wavelengths. Standard alliin gave well resolved peak at retention time of 2.721$\pm$0.0005 minutes while allicin extracts produced well resolved peaks at a retention time of 9.253$\pm$0.004 minutes. Variation of the flow rate from 7.5mL min^{-1} to 1.0mL min^{-1} resulted in shortening of the retention time for alliin from 2.5 minutes to 2.2 minutes and 8.9 minutes to 8.3 minutes for allicin.

Reduction of the flow rate from 7.5mL min^{-1} to 5mL min^{-1} increased the retention times of both alliin and allicin with the same proportionality; however the peak area for all the flow rates remained the same. Robustness of the validated HPLC method was therefore confirmed and hence it could be used for qualitative and quantitative analysis of alliin and allicin.

4.1.3.3 Calibration curves

Calibration curves were prepared for quantitative analysis of allicin and alliin each independently. Linearity was evaluated for the curve for alliin standard in the range of 10µg/L to 320µg/L. This fitted a straight line with the equation y= 5054.4x-14895 and R^2 value of 0.9986. The data was used to generate the calibration curve represented by figure 4.4 below:

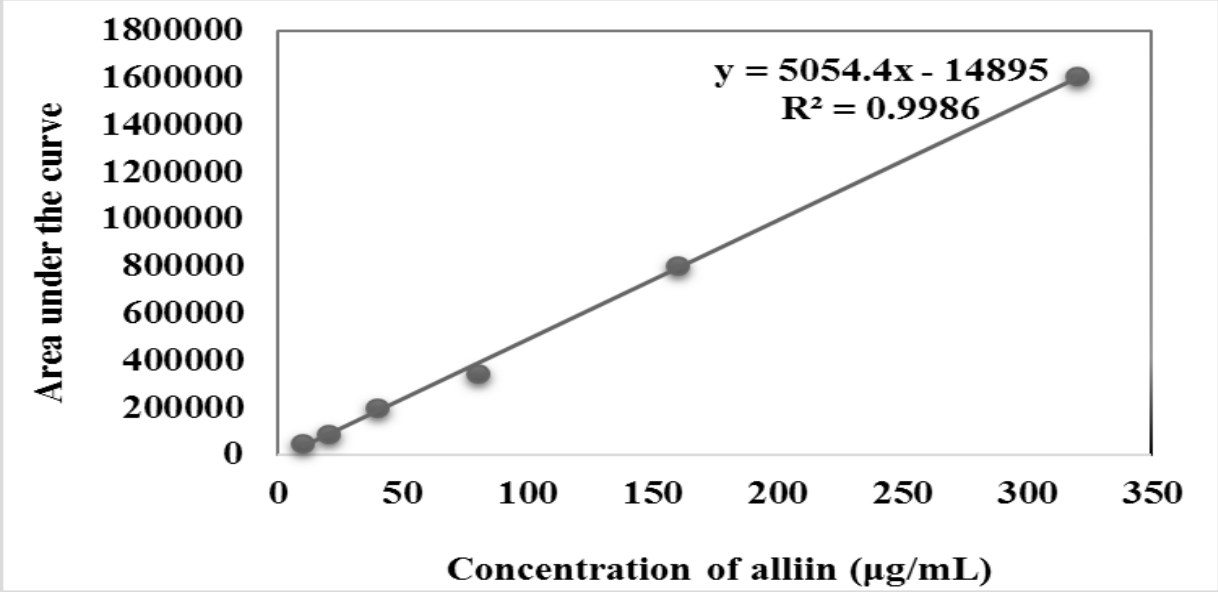


Figure 4.4: Calibration curve for alliin standard S_1

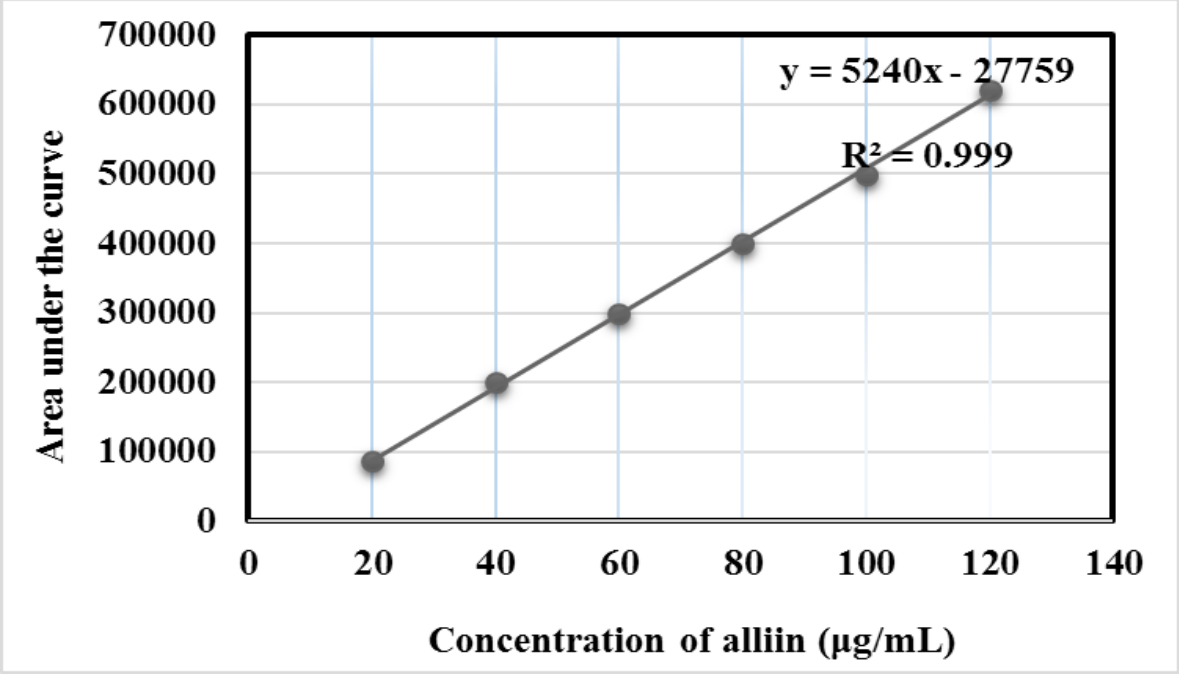


Figure 4.5: Calibration curve for alliin standard S_2

The calibration curve prepared from concentration range 20µg/L to 120µg/L gave a high value of R^2=0.999 and was therefore selected for quantitative analysis of alliin with the equation of the line in the form y= 5240x-27759 being adopted in the calculation of the concentration of alliin in crude garlic extracts and the chromatographic fractions.

Calibration curve for the synthesized allicin standard was established for the concentration range 10g/ml to 120µg/ml. This fitted in a straight line with the equation

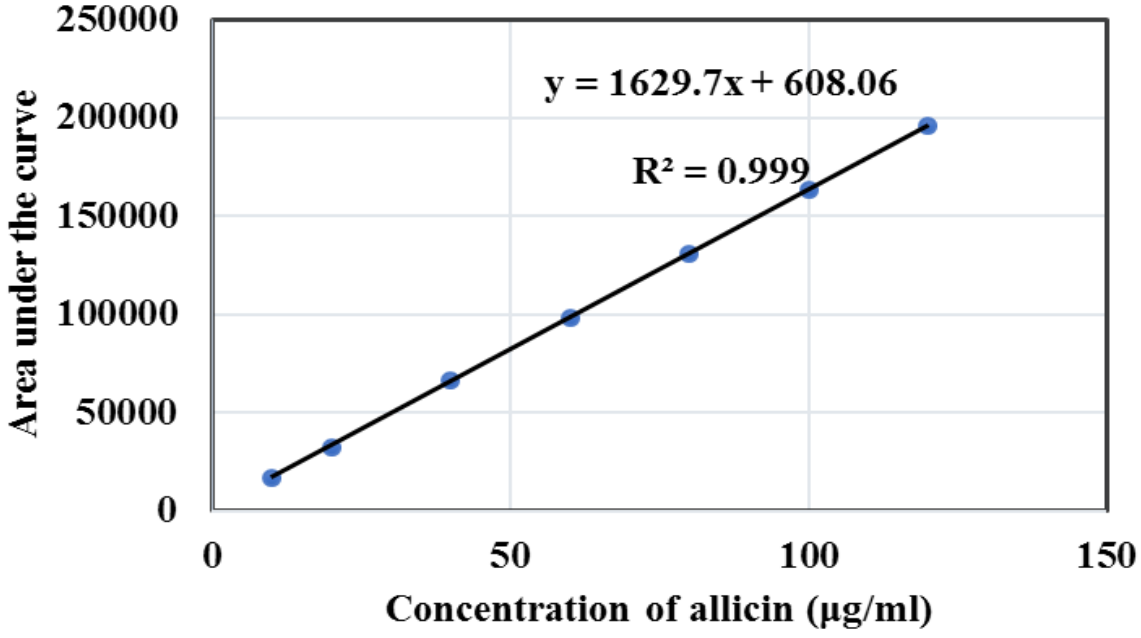


Figure 4.6: Calibration curve for allicin standard

A line of best fit was developed where y represented the area under the curve and x represented concentration of synthesized allicin in µg/L. The calibration curve was then used to quantify allicin concentration in crude garlic extracts, column fractions and HPLC eluents.

4.1.3.4 HPLC method validation

4.1.3.4.1 Precision and Accuracy

Precision expressed as %RSD for alliin standard ranged from 0.008% to 0.327% (table 4.4) and 0.081% to 3.398% for synthesized allicin (table 4.5). This indicated that variation of analysis between different concentrations was not significant hence allowing for high reproducibility for the developed HPLC method.

Intraday and interday recoveries for alliin standard and synthesized allicin were used to evaluate the accuracy of the developed HPLC method tabulated in table 4.4 and table 4.5 below:

Table 4.4: Recovery results for intraday and interday analysis of alliin standard

Conc. µgmL^{-1}	**Day1**	**Day2**	**Day3**	**Mean**	**S.D**	**RSD**	**RSD%**
20	98.95	98.85	99.39	99.06	0.082533	**0.000833**	**0.0833**
40	99.52	99.71	99.46	99.56	0.106562	**0.001070**	**0.1070**
60	98.52	99.23	99.18	98.98	0.323557	**0.003269**	**0.3269**
80	98.94	99.16	99.57	99.22	0.261066	**0.002631**	**0.2631**
100	99.19	99.30	99.11	99.20	0.077889	**0.000785**	**0.0785**
120	99.34	99.33	99.35	99.34	0.008165	**0.000082**	**0.0082**
Mean	99.08	99.26	99.34				
S.D	0.124667	0.077587	0.029667				
RSD	**0.001258**	**0.000782**	**0.000299**				
RSD%	**0.1258**	**0.0782**	**0.0299**				

Table 4.5: Recovery results for intraday and interday analysis of synthesized allicin standard

Conc. µgmL^{-1}	**Day1**	**Day2**	**Day3**	**Mean**	**S.D**	**RSD**	**RSD%**
10	94.37	86.83	90.96	90.72	3.0828	**0.03398**	**3.398**
20	94.11	93.35	97.52	94.99	1.8134	**0.01909**	**1.909**
40	95.91	94.04	95.58	95.18	0.8150	**0.00856**	**0.856**
60	97.33	96.46	97.90	97.23	0.5921	**0.00609**	**0.609**
80	97.30	97.49	97.43	97.41	0.0793	**0.00081**	**0.081**
100	98.61	97.89	98.85	98.45	0.4079	**0.00414**	**0.414**
120	97.52	98.79	99.62	98.64	0.8636	**0.00876**	**0.876**
Mean	96.45	94.98	96.84				
S.D	1.5766	3.8067	2.669				
RSD	**0.01634**	**0.04008**	**0.02756**				

RSD%	**1.634**	**4.008**	**2.756**

Accuracy expressed as recoveries for intra and inter day analysis ranged from 98.98% to 99.56% for alliin standard (table 4.4) and 90.72% to 98.64% for synthesized allicin (table 4.5) at all the concentration levels. Alliin standard analysis recorded R.S.D values of 0.0833%, 0.1070%, 0.3269%, 0.2631%, 0.0785% and 0.0082% respectively for the six different concentrations that were analysed while synthesized allicin precision analysis gave R.S.D values ranging from 0.08% to 3.4%.

The high R.S.D values for synthesized allicin was attributed to the instability of the thiosulphinate group in the structure of allicin (Miron *et al*., 2007). Variations of the validated chromatographic parameters did not result in significant change in the values of the peak areas.

4.1.3.4.3 Limit of detection and limit of quantitation analysis

LOD and LOQ results obtained for the analysis of the alliin standard and the synthesized allicin were tabulated and represented in table 4.6 below.

Table 4.6: Limit of detection and limit of quantitation analysis of alliin standard and synthesized allicin standard

Parameter	Alliin standard	Synthetic allicin
Calibration range	20-120 µg/mL	10-140 µg/mL
Limit of detection	4.868 x 10-^{5} µg/mL	2.21 x 10^{-3} µg/mL

Limit of quantitation	1.475 x 10^{-4} µg/mL	6.69 x 10^{-3} µg/mL

The limit of detection that was determined by the developed HPLC method was 4.868 x 10-^{5} µg/mL for alliin standard and 2.21 x 10^{-3} µg/mL for synthesized allicin (table 4.6). Analysis of alliin standard recorded at low standard deviation of 0.08 as compared to analysis of synthesized allicin that gave a high standard deviation value of 1.09.

4.1.4 Quantification of allicin and alliin in crude garlic extracts and column fractions

The concentration of allyl thiosulphinate and other allyl disulphides was calculated from the calibration curves developed from HPLC analysis of alliin standard and synthesized allicin. The data is represented in table 4.7 below:

Table 4.7: Calculation of the concentration of allyl thiosulphinate

Sample	**Concentration of allicin (µg/ml)**	**Concentration of alliin (µg/L)**
Garlic juice	0.01	2350
Crude ethanolic extract SE-GA	117.218	1800.118
Crude methanolic extract SE-GA	118.240	1752.411
Crude ethanolic extract ME-GA	206.411	1664.140
Crude methanolic extract ME-GA	3852.434	311.267
Crude ethanolic extract SE-SA	204.119	1782.254
Crude methanolic extract SE-SA	206.748	1599.289
Crude ethanolic extract ME-SA	221.154	1700.517
Crude methanolic extract ME- SA	4066.22	320

Column fraction B_4	4638.56	90
Column fraction B_3	330.27	80
Column fraction B_2	310.81	70
Column fraction B_1	220.17	60
HPLC eluent	10	0

The developed and validated HPLC method was used to quantitatively calculate the concentration of allyl thiosulphinate and other diallyl disulphides in garlic juice, crude garlic extracts, column fractions and HPLC eluents. This is in agreement with the alliin studies that were carried out by Apawu *et al*., in 2009.

The use of the validated reversed phase HPLC method in the quantification of allyl thiosulphinate in garlic cloves was successful and the method proved to be accurate, precise, efficient and robust. The developed and validated HPLC method is substantial for satisfactory performance in ethno pharmacovigilance of traditional medicine agents.

4.1.5 Antibacterial and antifungal activity

4.1.5.1 Antimicrobial testing

Freshly growing microbial strains were quantified using UV spectrophotometer by analysing the optical densities of ATCC of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The data was tabulated as shown in table 4.8 below:

Table 4.8: Quantification of microorganisms

Microorganism	CFU (X 10^6/mL)	Wavelength **(nm)**	Optical density
Escherichia coli	<300	600	0.129
Staphylococcus aureus	<300	300	0.087
Pseudomonas aeruginosa	<300	300	0.052
Candida albicans	<300	600	0.140

The optical densities were adjusted to 0.5 McFarland standard by use of 1% $BaCl_2$/1% H_2SO_4. The optical densities for the four microorganisms ranged from 0.052 to 0.140 (Table 4.9). The range for the optical density was optimum for the bioassay of garlic extracts as adopted from NCCLS, 2013.

The antibacterial effects of crude garlic extracts on *Staphylococcus aureus*, *Pseudomonas aeruginosa and Escherichia coli* were bio assayed using the disc diffusion method. Antibacterial Activity Index adopted from Hassan *et al*., 2007 was used to evaluate antimicrobial activity of the garlic extracts (Table 4.9).

From the table it is evident that the highest growth inhibition zones were observed in *Escherichia coli* with bioactivity of 5+ in *Allium sativum* maceration extracts while no bioactivity was observed in the three bacteria when soxhlet extracts were bioassayed.

Table 4.9: Mean inhibition diameters for bacterial strains

Sample	**Extraction method**	**Solvent**	**Concentration (µg/ml)**	**Mean Zone of inhibition diameter (mm)**		
				Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***
Amoxicillin clavulanate	-	-	2500	5+	3+	0
Dimethyl Sulphoxide	-	-	Control	0	0	0
SA	Soxhlet	Ethanol	204.190	0	1	0
GA	Maceration	Methanol	3852.434	0	5+	0
		Ethanol	118.240	1+	1+	1+
GA	Maceration	Methanol	4638.555	2+	5+	1+
SA	Maceration					
SA	soxhlet	methanol	206.748	0	1+	0
GA	Soxhlet	Methanol	206.411	0	1+	0
SA	Maceration	Ethanol	221.154	2+	4+	2+
GA	Soxhlet	Ethanol	117.218	0	0	0

Antibacterial Activity Index:
0 = zone of inhibition <8mm (no inhibition)
1+ = 8 - 9 mm
2+ = 10 -14 mm
3+ = 15 - 19 mm
4+ = 20 – 24 mm
5+ = 25 mm and above

Antifungal activity of different crude garlic extracts were bioassayed using *Candida albicans*. The bioactivity represented by growth inhibition zones in mm (table 4.10)

indicated the antifungal activity of the extracts. Bioactivity of 5+ in terms of growth inhibition zone was observed in the maceration extracts while no activity was observed in the soxhlet extracts.

Table 4.10: Mean inhibition diameters for *Candida albicans*

Sample	Extraction method	Solvent	Mean zone of inhibition diameter (mm)
			Candida albicans
DMSO	Control	-	0
Fluconazole	Standard drug	-	5+
SA	Soxhlet	Ethanol	0
GA	Maceration	methanol	3+
	Maceration	Ethanol	0
GA	Maceration	Methanol	5+
SA			
SA	soxhlet	methanol	0
GA	Soxhlet	Methanol	0
SA	Maceration	Ethanol	0
GA	Soxhlet	ethanol	0

Antifungal Activity Index:

0 = zone of inhibition <8mm (no inhibition)
1+ = 8 - 9 mm
2+ = 10 -14 mm
3+ = 15 - 19 mm
4+ = 20 – 24 mm
5+ = 25 mm and above

Antibacterial and antifungal activity of the four column fractions were assayed using the disc diffusion method. ATCC cultured strains of *Staphylococcus aureus*, *Pseudomonas*

aeruginosa, *and Escherichia coli* were used to evaluate antibacterial activity *while Candida albicans* was used for the antifungal assays of the fractions.

Column fractions B_1, B_2, B_3 and B_4 were used for the antibacterial assays for *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *and Escherichia coli*. Amoxicillin clavulanate was used as a positive control while dimethyl sulphoxide acted as the negative control as shown in table 4.11 below.

Table 4.11: Antibacterial activity of pure column fractions

Sample	**Mean Zone of inhibition diameter (mm)**		
	Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***
Amoxicillin clavulanate	5+	3+	0
Dimethyl sulphoxide	0	0	0
B_1	0	1+	0
B_2	0	1+	0
B_3	0	2+	0
B_4	0	3+	0
HPLC eluent	0	0	0

Antifungal activity of the four column fractions and HPLC eluents against *Candida albicans* were expressed in inhibition zones (table 4.12) with fluconazole as the positive control and dimethyl sulfoxide as negative control.

Table 4.12: Antifungal activity of column fractions

Sample	**Solvent**	**Mean zone of inhibition diameter (mm)**
		Candida albicans

Fluconazole	-	5+
Dimethyl sulfoxide	-	0
B_1	Hexane	0
B_2	Ethyl acetate	0
B_3	Chloroform	2+
B_4	Methanol	4+
HPLC eluent	Methanol : water (50:50)	0

From the growth inhibition zones represented by tables 4.12 and 4.13 it is evident that allicin exhibited high antimicrobial activity against *Escherichia coli* with a growth inhibition zone of 3+ when column fraction B_4 was screened for bioactivity. This data was in agreement with Ilic *et al*., 2011 who recorded inhibition zones for *Escherichia coli* of 4+ under the same experimental conditions.

Low antimicrobial activity of 5mm was observed when column fraction B_4 was bioassayed against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

This difference in activity between gram positive and gram negative bacteria could be attributed to the mode of action of allyl thiosulphinates which depends on the sulfur and oxygen elements in the compounds (Miron *et al*., 2007).

4.1.5.2 Minimum Inhibitory concentration

Table 4.13 shows the minimum inhibitory concentrations obtained using the crude garlic extracts concentrations against the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli and Candida albicans*.

Table 4.13: Minimum inhibitory concentrations for the microbial strains

Concentration of Allicin (µg/mL)	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
72.477	Nil	Nil	Nil	Nil
144.955	5.00mm	5.00mm	5.09mm	5.11mm
289.910	5.25mm	6.02mm	5.2mm	5.2mm
579.820	5.84mm	6.15mm	5.28mm	8.87mm
1159.639	8.82mm	9.29mm	8.60mm	10.18mm
2319.278	9.11mm	9.85mm	9.20mm	11.10mm

The assayed bacteria recorded a minimum inhibition concentration of 1159.639 µg/mL while in Candida albicans the minimum inhibition concentration was 579.820 µg/mL. Using the disc diffusion method it was evident that allicin exhibited inhibition activity against both gram positive and gram negative bacteria.

The highest inhibitory activity for the crude garlic extract was recorded in *Candida albicans* at 28.6mm for Chinese samples extracted using methanol as solvent under maceration method. The minimum inhibition concentration was 1159.639µg/L for ally thiosulphinate in *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* while for *Candida albicans* minimum inhibition concentration was 579.820µg/L.

4.1.5 Antimicrobial trends for antibacterial and antifungal activity

Antimicrobial trends were developed for antibacterial activity against *Escherichia coli* and antifungal activity against *Candida albicans*. Data from concentration of allicin in

garlic juice, crude garlic extracts, column fractions and HPLC eluents represented by figure 4.7 was used to develop the relationship between fractionation of allicin and antibacterial activity against *Escherichia coli*. The trend line gave a polynomial of order four with R^2 value of 1. In statistics polynomial regression analysis is always used to explain nonlinear phenomena such as growth rate of tissues (Yin- Wen *et al*., 2010). In the current study results from growth inhibition zones and the different fractions that were bioassayed fitted into a polynomial quadratic equation of the form $y= -4x^3 = 25.5x^2 – 43.5x+ 27$ with coefficient of determination ($R^2= 1$).

This showed that there was a nonlinear relationship between concentration of allicin in the different fractions and growth inhibition against *Escherichia coli*.

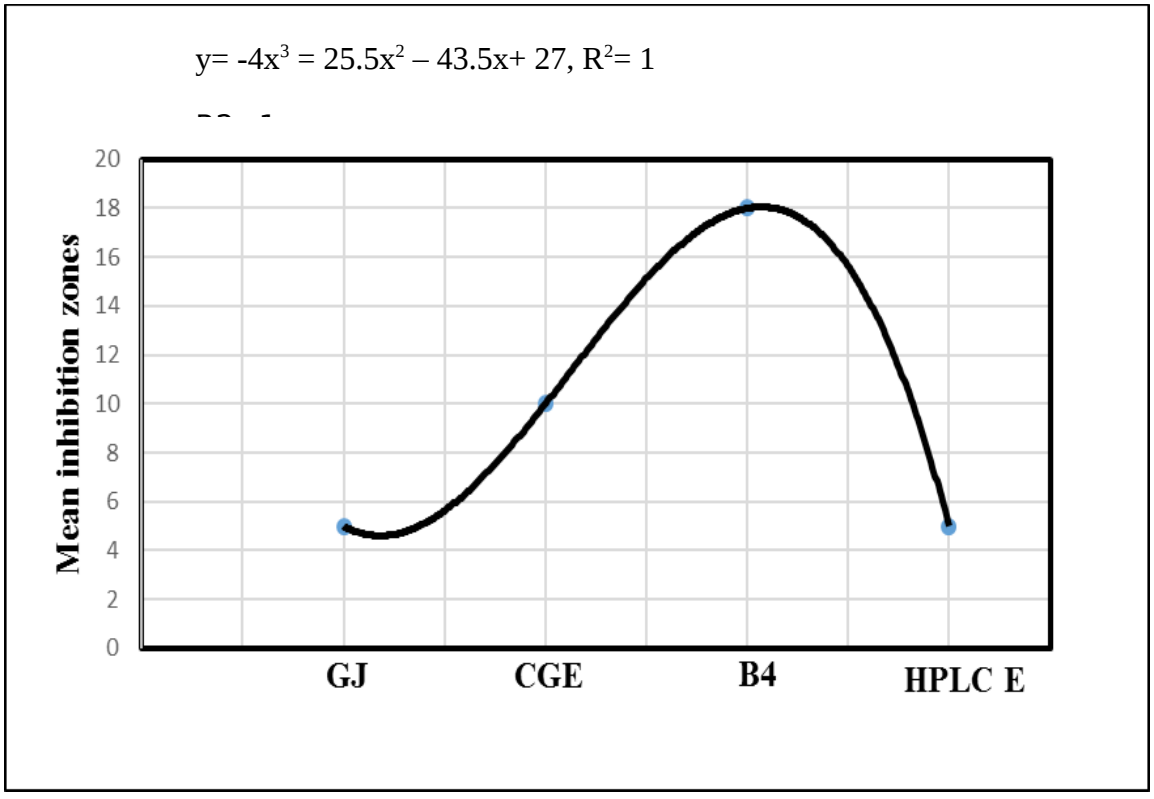


Figure 4.7: Antibacterial activity against *Escherichia coli*

KEY

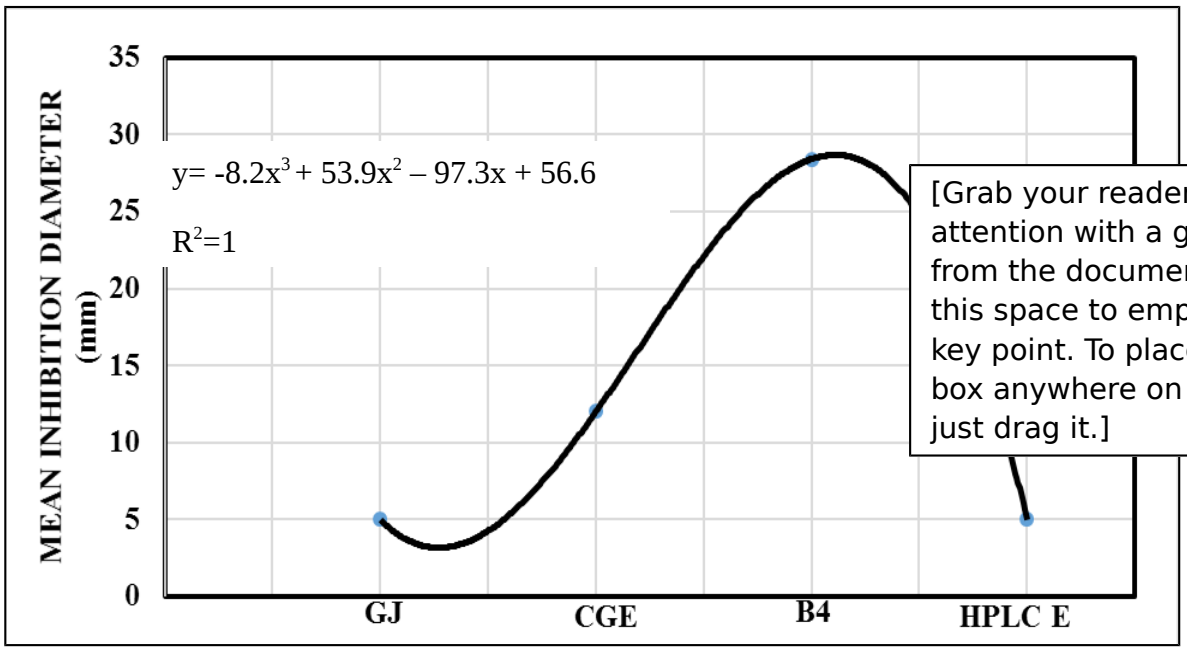
GJ – Garlic juice

CGE- Crude garlic extract

B_4- Column fraction B_4

HPLC- HPLC eluents

Data from the antifungal activity of allicin on *Candida albicans* was also used to develop the relationship between fractionation of allyl thiosulphinates and antifungal activity. The trend line gave a polynomial of order four (Figure 4.8). This multiple linear regression gave an equation of the form ($Y= -8.2x^3 + 53.9x^2 – 97.3x + 56.6$, $R^2=1$) which explained the nonlinear relationship between concentration of allicin and growth inhibition against *Candida albicans*.



[Grab your reader's attention with a great quote from the document or use this space to emphasize a key point. To place this text box anywhere on the page, just drag it.]

Figure 4.8: Antifungal activity against *Candida albicans*

KEY

GJ – Garlic juice

CGE- Crude garlic extract

B_4- Column fraction B_4

HPLC- HPLC eluents

From previous studies (Ilic *et al*., 2011 and Miron *et al*., 2007) allicin has been proven to possess both antifungal and antibacterial activity however in the current research it was demonstrated that the synergy of the constituents in *Allium sativum* extracts was highly dependent on the extraction method and fractionation process. Maceration was identified as the best extraction method for allicin while methanol was able to elute pure allicin due to its high polarity.

During the purification process four fractions were identified. The methanol extract gave the highest inhibition zone of 28.6mm for *Candida albicans* while the chloroform extract gave the highest inhibition zone of 18mm in *Escherichia coli.*

A comparison of the bioactivity in crude garlic extract with the pure column fraction showed there was a reduction in activity with fractionation. From figures 4.7 and 4.8 it is

evident that synergistic trends indicate a rise and later a drop in activity moving from crude extract to column fraction and finally HPLC eluents. As the sample changes from GJ to B4, the mean inhibition zones of diameter increases and starts to drop between B_4 and HPLCE.

4.1.7 Statistical data analysis

Multi Variate Analysis of Variance (MANOVA) was performed with the aid of Statistical Package for Social Scientists (SPSS) version 21. The independent variable was the different concentrations of allicin while the dependent variable was the growth inhibition zones for the bacteria and fungi that were bio assayed during this study.

Allicin concentration and *Escherichia coli* have a strong positive correlation of 0.872 and the probability value of 0.010 (which is <0.05) which implied that the correlation was statistically significant. In the next microorganism *Candida albicans*, there was statistical significance ($p< 0.05$) positive correlation ($r = 0.871$). However growth inhibition zones for *Pseudomonas aeruginosa* and *Staphylococcus aureus* do not correlate with the

concentration levels of allicin. This is because their probability values (0.218 and 0.239) respectively were all > 0.05. The statistical results are shown in Appendices XIII, XIV.

Multivariate analysis of Variance revealed that there was a statistically significant difference in the four microorganisms that were bio assayed based on the concentration of allicin $F(4, 2) = 270.852$ $p < 0.005$; Wilk's $\Lambda = 0.002$ partial $\eta^2 = 0.998$. From the analysis table represented on Appendix XV it was concluded that concentration of allicin has a statistically significant effect on *Escherichia coli* $F(1,5) = 15.943$; $p < 0.05$; partial $\eta^2 = 0.761$ and *Candida albicans* ($F(1,5) = 15.655$; $p<0.05$ partial $\eta^2 = 0.758$) as opposed to *Pseudomonas aeruginosa* $F(1,5) = 1.989$; $p > 0.05$; partial $\eta^2 = 0.285$ and *Staphylococcus aureus* $F(1,5) = 1.788$; $p > 0.05$; partial $\eta^2 = 0.263$.

Multi variate regression analysis on the data was used to establish the effect on concentration of allicin on the four microorganisms. From the table represented by Appendix XV it is evident that the concentration of allicin extracts significantly affected the growth inhibition zones for *Escherichia coli* and *Candida albicans*. (p- values< 0.05 while the effect of allicin concentration on growth inhibition zones for *Pseudomonas aeruginosa* and *Staphylococcus aureus* was statistically insignificant as indicated by p-values (0.218 and 0.239) which are all > 0.05.

Further statistical analysis of the means of the percentage yield on the two garlic samples that were analysed showed that there was statistical significance of 0.001 at Pearson's moment correlation of 0.999. This implied that there was no significant difference between the yields of the two garlic samples. From the table on Appendix XVII it is clear that the concentration of the extracts of Allicin have a statistically significant effect on

both *Escherichia coli* and *Candida albicans*. These are shown by the following respective statistics: (F 1, 2) = 28.361; $p < .05$; partial $\eta^2 = 0.934$) and (F 1, 2) = 68.712; $p < .05$; partial $\eta^2 = 0.972$)

The T- test table on Appendix XVIII shows that the two zones of inhibition for *Escherichia coli* and *Candida albicans* represented on the antimicrobial trends (figure 4.7 and figure 4.8) are not statistically significant to fractionation by the fact that their p-values are all > 0.05. However a positive correlation exists of 0.934 and 0.972 for Escherichia coli and *Candida albicans* respectively.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The HPLC optimum conditions were gradient elution, column temperature of 25 ° C, pump pressure of 8.2Mpa, mobile phase of methanol: water (50: 50 v/v) and a sample volume of 20 µL at a flow rate of $0.5mLmin^{-1}$.

During extraction garlic sample code 0401 gave the highest yield while sample 0101 gave the lowest yield. Hence maceration was the best extraction method while methanol was the preferred solvent.

The crude garlic extracts, column fractions and HPLC eluents were tested for antimicrobial activity where by highest antimicrobial activity of 28.6mm was observed in column fraction B_4 – (column fraction that eluted in methanol: water ratio 25:25 v/v) against *Candida albicans* while low antimicrobial activity of 5mm was observed in the same column fraction against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Antimicrobial trends are dependent on fractionation whereby optimum activity was observed in the fractions of intermediate polarity.

5.2 Recommendations

The developed HPLC method was accurate and robust, however the limit of quantification was slightly high therefore an additional method like GC-MS is recommended to elucidate concentration of allicin in parts per trillion.

Use of Garlic concoctions should be checked for standardization of constituents as these determines the efficacy hence more research work is recommended in the development of a commercially available allyl thiosulphinate standard to improve on the quantitative analysis of allyl thiosulphinates.

The current research validated the antimicrobial activity of allyl thiosulphinate especially against *Candida albicans* however issues of toxicity should be carried out in order to improve the medicinal values of allicin and reduce the contraindications.

Further research work is recommended to model a mathematical expression to be used for predicting efficacy.

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APPENDICES

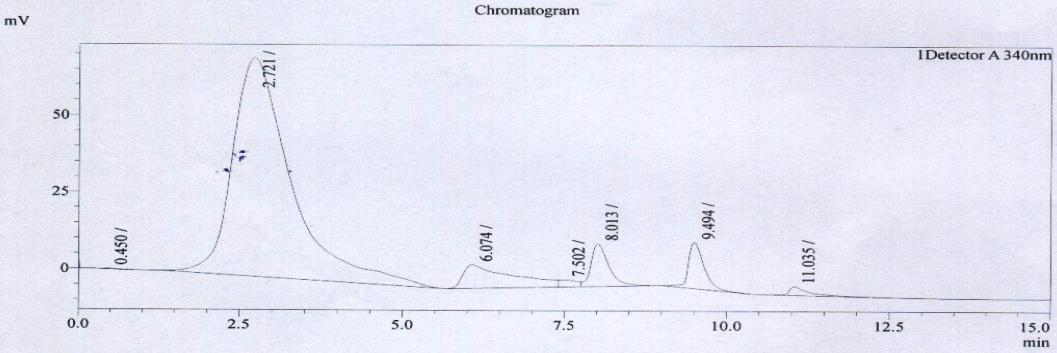
APPENDIX I: HPLC ANALYSIS OF ALLIIN STANDARD

MOI UNIVERSITY
SCHOOL OF ENGINEERING
Department of Chemical & Process Engineering

Sample Information

Acquired by	: System Administrator
Sample Name	: Alliin
Sample ID	: Standard 10
Tray#	: 1
Vial#	: 1
Injection Volume	: 10
Data File	: A Std 4.lcd
Method File	: A std 1.lcm
Batch File	:
Report Format File	: Moi University LC Report 2.lsr
Date Acquired	: 5/24/2016 1:25:38 PM
Date Processed	: 5/24/2016 1:40:39 PM

Chromatogram



Peak Table

Detector A 340nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	0.450	2564	241	0.000			
2	2.721	4455418	71194	0.000			
3	6.074	408283	7671	0.000			
4	7.502	42253	2297	0.000		V	
5	8.013	262792	13833	0.000		V	
6	9.494	254516	15054	0.000			
7	11.035	59471	2831	0.000			
Total		5485296	113121				

Analyst Florence Ugoob Sign ..... Date 24/05/2016

Checked By ..... Sign ..... Date .....

Approved By ..... Sign ..... Date .....

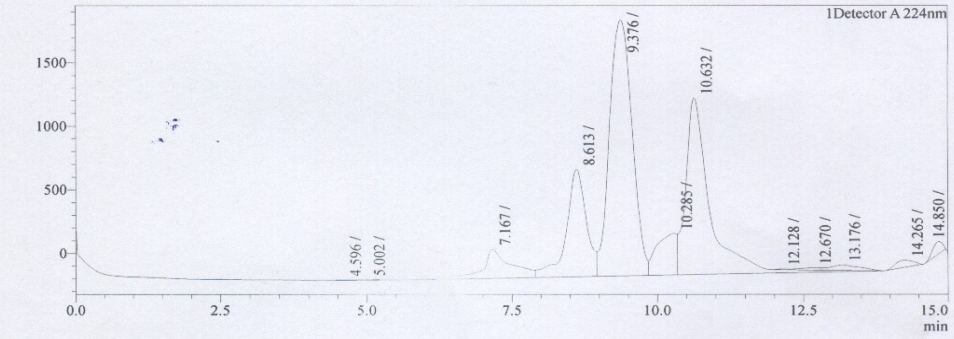
APPENDIX II: HPLC ANALYSIS OF GARLIC METHANOL CRUDE EXTRACT

MOI UNIVERSITY
SCHOOL OF ENGINEERING
Department of Chemical & Process Engineering

Sample Information

Acquired by	: System Administrator
Sample Name	: Alliin
Sample ID	: Standard 10
Tray#	: 1
Vial#	: 1
Injection Volume	: 20
Data File	: A Std 25.lcd
Method File	: ALLICIN EXTRACT1103.lcm
Batch File	:
Report Format File	: Moi University LC Report 2.lsr
Date Acquired	: 5/26/2016 1:07:13 PM
Date Processed	: 5/26/2016 1:22:14 PM

Chromatogram



Peak Table

Detector A 224nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	4.596	17967	1616	0.000			
2	5.002	87690	2505	0.000		V	
3	7.167	7846508	230589	0.000			
4	8.613	20326716	844038	0.000		V	
5	9.376	54523945	2013235	0.000		V	
6	10.285	7818272	326148	0.000		V	
7	10.632	40062896	1385701	0.000		SV	
8	12.128	90673	6820	0.000		T	
9	12.670	431507	17582	0.000		TV	
10	13.176	1534285	43447	0.000		TV	
11	14.265	1235324	55951	0.000			
12	14.850	1188185	94606	0.000			
Total		135163969	5022239				

Analyst Florence Opondo Sign ..... Date 26/05/2016

Checked By ..... Sign ..... Date .....

Approved By ..... Sign ..... Date .....

APPENDIX III: RETENTION TIMES FOR ALLIIN AND ALLICIN

Compound	Sample	Retention time (R_T)			Average	SD
Alliin	Alliin standard	2.721	2.720	2.721	2.721	0.0005
	Crude garlic extract	2.800	2.926	2.867	2.864	0.05
	Column fraction B_4	2.724	2.739	2.711	2.725	0.01
Allicin	Synthesized allicin	9.253	9.259	9.247	9.253	0.004
	Crude garlic extract	9.696	9.597	9.649	9.647	0.040
	Column fraction B_4	9.376	9.392	9.353	9.373	0.020

APPENDIX IV: UV-VIS SPECTRUM ANALYSIS

SAMPLE	WAVELENGTH	ABSORBANCE
ALLICIN	536	0.271
	453	0.612
	418	0.008
	357	1.419
	224	3.154
ALLIIN	536	1.063
	448	1.391
	366	1.602
	340	2.517
	284	0.104

APPENDIX V: ANTIBACTERIAL ASSAYS

Sample	Extraction method	Solvent	Concentration (µg/ml)	Mean Zone of inhibition diameter (mm)		
				Staphylococcus aureus	***Escherichia coli***	***Pseudomona aeruginosa***
Amoxicillin clavulanate	-	-	2500	28	18	5
Dimethyl sulphoxide	-	-	control	5	5	5
SA	Soxhlet	Ethanol	204.190	5.10	7.55	5.0
GA	Maceration	methanol	3852.434	5.20	24.6	5.10
GA	Maceration	Ethanol	118.240	8.59	9.30	8.56
SA	Maceration	Methanol	4638.555	12.71	26.8	9.83
SA	Soxhlet	methanol	206.748	7.70	9.20	6.50
GA	Soxhlet	Methanol	206.411	7.20	8.37	7.14
SA	Maceration	Ethanol	221.154	13.5	22.4	10.42
GA	Soxhlet	ethanol	117.218	5.00	5.46	5.0

APPENDIX VI: ANTIFUNGAL ASSAYS

Sample	Extraction method	Solvent	Concentration of allicin(µg/mL)	Mean zone of inhibition diameter (mm)
				Candida albicans
DMSO	**Control**			
Fluconazole	**Standard drug**	**-**	**5000**	**28.9**
SA	Soxhlet	Ethanol	204.190	9.5
GA	Maceration	Methanol	3852.434	18.5
GA	Maceration	Ethanol	118.240	6.4
SA	Maceration	Methanol	4638.555	28.6
SA	soxhlet	methanol	206.748	6.7
GA	Soxhlet	Methanol	206.411	6.8
SA	Maceration	Ethanol	221.154	7.3
GA	Soxhlet	Ethanol	117.218	6.2

APPENDIX VII: MINIMUM INHIBITORY CONCENTRATION FOR SA0101

Minimum inhibitory concentration for ethanol extract

Concentration	*Staphylococcu*	*Escherichia*	*Pseudomonas*	*Candida albicans*

(µg/mL)	*s aureus*	*coli*	*aeruginosa*	
72.477	Nil	Nil	Nil	Nil
144.955	5.00mm	5.00mm	6.09mm	5.11mm
289.910	5.25mm	6.02mm	5.2mm	5.2mm
579.820	5.84mm	6.15mm	5.28mm	6.87mm
1159.639	5.82mm	6.29mm	9.60mm	6.18mm
2319.278	6.1mm	6.85mm	6.20mm	7.10mm

Minimum inhibitory concentration for methanol extract

Concentration in ratios	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
72.477	5mm	5mm	5mm	5mm
144.955	6mm	7mm	6mm	7mm
289.910	7mm	7mm	7.2mm	8mm
579.820	8.0mm	8.1mm	8.07mm	8.8mm
1159.639	9.6mm	9.7mm	9.6mm	10.2mm
2319.278	11.1mm	11.2mm	11.15mm	12.45mm

Minimum inhibitory concentration for ethanol extract in garden samples

Concentration in ratios	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
72.477	5mm	5mm	5mm	5mm
144.955	5mm	6mm	5mm	6mm
289.910	5.36mm	6.04mm	5.3mm	6.2mm
579.820	5.57mm	6.12mm	5.63mm	6.47mm
1159.639	5.92mm	6.27mm	5.99mm	6.86mm
2319.278	6.38mm	6.56mm	6.40mm	7.41mm

APPENDIX VIII: MINIMUM INHIBITORY CONCENTRATIONS OF GARDEN SAMPLES

Minimum inhibitory concentration for methanol extracts using garden samples

Concentration in ratios	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
72.477	5mm	5mm	5mm	5mm
144.955	6.4mm	7mm	6.2mm	7mm
289.910	7.7mm	7.5mm	7.1mm	8.4mm
579.820	8.1mm	8.4mm	7.8mm	8.9mm
1159.639	9.6mm	9.9mm	9.6mm	10.8mm
2319.278	11.8mm	12.6mm	11.2mm	14.3mm

APPENDIX IX: ANTIBACTERIAL BIOASSAYS OF PURE COLUMN EXTRACTS

Bioassay was performed on the B_1, B_2, B_3 and B_4 column extracts. The data was presented in the tables below:

Sample	**Concentration of (µg/ml)**	**Mean Zone of inhibition diameter (mm)**		
		Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***

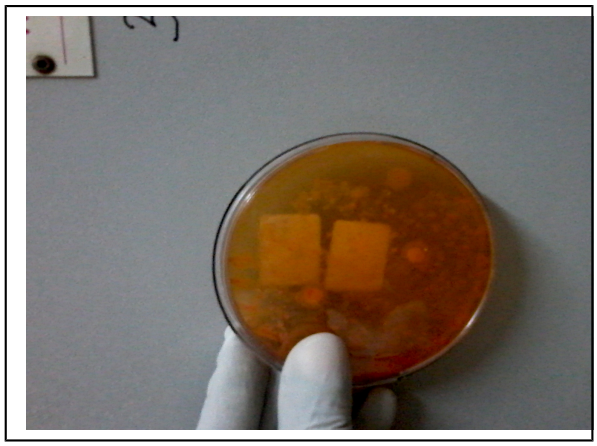
Amoxicillin clavulanate	40	28	18	5
Dimethyl sulphoxide		5	5	5
B_1	89.22	5	9	5
B_2	90.37	5	8	5
B_3	114.269	7	11	6
B_4	3812.672	7	18	5

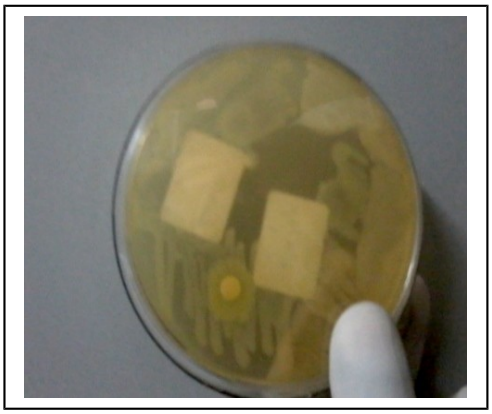
APPENDIX X: ANTIFUNGAL ACTIVITY OF THE PURE COLUMN EXTRACTS

Sample	**Solvent**	**Concentration of allicin(µg/mL)**	**Mean zone of inhibition diameter (mm)**
			Candida albicans
B_1	Hexane	89.22	5
B_2	Ethyl acetate	90.37	7

B_3	Chloroform	114.269	12
B_4	Methanol	3812.672	20

APPENDIX XI: INHIBITION ZONES FOR BACTERIA AND FUNGI





Inhibition zone for *Escherichia coli*

Plate showing no inhibition zone for *Staphylococcus aureus*

Inhibition zone for *Candida albicans*

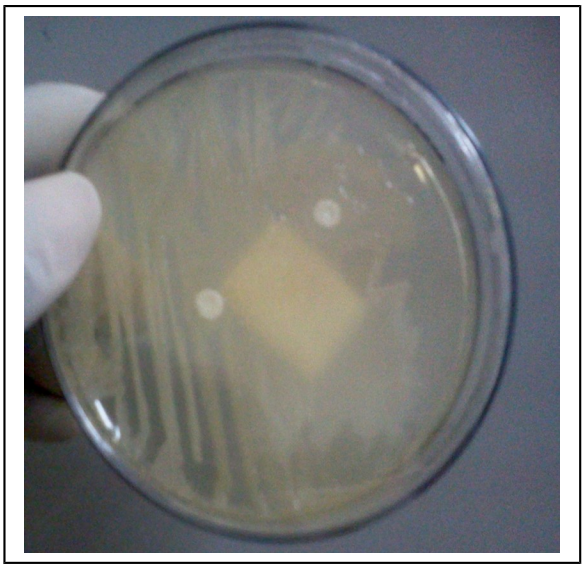


Plate showing no inhibition zone for *Pseudomonas aeruginosa*

APPENDIX XII: ACCURACY RESULTS FOR ALLIIN STANDARD

Conc. µgmL^{-1}	Day1	Day2	Day3
20	98.97	99.22	99.77
	99.13	98.34	99.18
	98.76	98.99	99.23
40	102.48	100.17	99.65
	98.62	99.55	99.42
	97.46	98.60	99.32
60	99.26	99.81	98.84
	97.24	98.95	99.22
	99.07	98.93	99.47
80	99.17	99.65	99.89
	98.21	98.54	99.16
	99.45	99.28	99.67
100	99.46	98.97	99.14
	99.23	99.37	99.02
	98.88	99.56	99.17
120	99.53	99.69	99.78
	99.13	98.79	99.01
	99.36	99.53	99.27

APPENDIX XIII: PEARSON MOMENT CORRELATIO

Correlations

		Concentration	*Escherichia coli*	*Candida albicans*
Concentration	Pearson Correlation	1	.872*	.871*
	Sig. (2-tailed)		.010	.011
	N	7	7	7
Escherichia coli	Pearson Correlation	.872*	1	.994**
	Sig. (2-tailed)	.010		.000
	N	7	7	7
Candida albicans	Pearson Correlation	.871*	.994**	1
	Sig. (2-tailed)	.011	.000	
	N	7	7	7
Pseudomonas aeruginosa	Pearson Correlation	.533	.769*	.709
	Sig. (2-tailed)	.218	.043	.074
	N	7	7	7
Staphylococcus aureus	Pearson Correlation	.513	.699	.644
	Sig. (2-tailed)	.239	.081	.118
	N	7	7	7

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		Pseudomonas aeruginosa	*Staphylococcus aureus*
Concentration	Pearson Correlation	.533	.513
	Sig. (2-tailed)	.218	.239
	N	7	7
Escherichia Coli	Pearson Correlation	.769*	.699
	Sig. (2-tailed)	.043	.081
	N	7	7
Candida albicans	Pearson Correlation	.709	.644
	Sig. (2-tailed)	.074	.118
	N	7	7
Pseudomonas aeruginosa	Pearson Correlation	1	.969**
	Sig. (2-tailed)		.000
	N	7	7
Staphylococcus aureus	Pearson Correlation	.969**	1
	Sig. (2-tailed)	.000	
	N	7	7

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

APPENDIX XIV: REGRESSION ANALYSIS

Parameter Estimates

Dependent Variable	Parameter	95% Confidence Interval		Partial Eta Squared
		Lower Bound	Upper Bound	
Escherichia coli	Intercept	3.703	9.538	.872
	Concentration	.001	.004	.761
Candida albicans	Intercept	2.060	12.805	.717
	Concentration	.002	.008	.758
Pseudomonas aeruginosa	Intercept	4.300	7.650	.944
	Concentration	.000	.001	.285
Staphylococcus aureus	Intercept	4.253	6.806	.961
	Concentration	.000	.001	.263

The linear relationships of each of the dependent variables with the independent variable are as follows:

Escherichia coli= 0.03 Concentration + 6.620

Candida albicans = 0.05 Concentration + 7.433

Pseudomonas aeruginosa = 0.01 Concentration + 5.975

Staphylococcus aureus = 0.00 Concentration + 5.529

APPENDIX XV: MULTIVARIATE ANALYSIS OF VARIANCE

Descriptive Statistics

	Mean	Std. Deviation	N
Escherichia coli	8.8571	4.87950	7
Candida albicans	11.5143	8.92327	7
Pseudomonas aeruginosa	6.4286	1.61835	7
Staphylococcus aureus	5.8571	1.21499	7

Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df
Intercept	Pillai's Trace	1.000	1932.617[a]	4.000	2.000
	Wilks' Lambda	.000	1932.617[a]	4.000	2.000
	Hotelling's Trace	3865.233	1932.617[a]	4.000	2.000
	Roy's Largest Root	3865.233	1932.617[a]	4.000	2.000
Concentration	Pillai's Trace	.998	270.852[a]	4.000	2.000
	Wilks' Lambda	.002	270.852[a]	4.000	2.000
	Hotelling's Trace	541.704	270.852[a]	4.000	2.000
	Roy's Largest Root	541.704	270.852[a]	4.000	2.000

a. Exact statistic
b. Design: Intercept + Concentration

a. R Squared = .761 (Adjusted R Squared = .714)
b. R Squared = .758 (Adjusted R Squared = .710)
c. R Squared = .285 (Adjusted R Squared = .142)
d. R Squared = .263 (Adjusted R Squared = .116)

APPENDIX XVI: TESTS OF BETWEEN – SUBJECTS EFFECTS

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F
Corrected Model	*Escherichia coli*	108.750[a]	1	108.750	15.943
	Candida albicans	362.097[b]	1	362.097	15.655
	Pseudomonas aeruginosa	4.472[c]	1	4.472	1.989
	Staphylococcus aureus	2.333[d]	1	2.333	1.788
Intercept	*Escherichia coli*	232.060	1	232.060	34.019
	Candida albicans	292.505	1	292.505	12.646
	Pseudomonas aeruginosa	189.023	1	189.023	84.070
	Staphylococcus aureus	161.889	1	161.889	124.074
Concentration	*Escherichia coli*	108.750	1	108.750	15.943
	Candida albicans	362.097	1	362.097	15.655
	Pseudomonas	4.472	1	4.472	1.989
	Staphylococcus aureus	2.333	1	2.333	1.788
Error	*Escherichia coli*	34.107	5	6.821	
	Candida albicans	115.651	5	23.130	
	Pseudomonas aeruginosa	11.242	5	2.248	
	Staphylococcus aureus	6.524	5	1.305	
Total	*Escherichia coli*	692.000	7		
	Candida albicans	1405.800	7		
	Pseudomonas aeruginosa	305.000	7		

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F
	Staphylococcus aureus	249.000	7		
Corrected Total	*Escherichia coli*	142.857	6		
	Candida albicans	477.749	6		
	Pseudomonas aeruginosa	15.714	6		
	Staphylococcus aureus	8.857	6		

Tests of Between-Subjects Effects

Source	Dependent Variable	Sig.	Partial Eta Squared
Corrected Model	*Escherichia coli*	.010	.761
	Candida albicans	.011	.758
	Pseudomonas aeruginosa	.218	.285

Tests of Between-Subjects Effects

	Staphylococcus aureus	.239	.263
Intercept	*Escherichia coli*	.002	.872
	Candida albicans	.016	.717
	Pseudomonas aeruginosa	.000	.944
	Staphylococcus aureus	.000	.961
Concentration	*Escherichia coli*	.010	.761
	Candida albicans	.011	.758
	Pseudomonas aeruginosa	.218	.285
	Staphylococcus aureus	.239	.263
Error	*Escherichia coli*		
	Candida albicans		
	Pseudomonas aeruginosa		
	Staphylococcus aureus		
Total	*Escherichia coli*		
	Candida albicans		
	Pseudomonas aeruginosa		
	Staphylococcus aureus		
Corrected Total	*Escherichia coli*		
	Candida albicans		
	Pseudomonas aeruginosa		
	Staphylococcus aureus		

APPENDIX XVII : F-TEST

Multivariate Tests[b]

Effect		Value	F	Hypothesis df	Error df
Intercept	Pillai's Trace	1.000	7298.006[a]	2.000	1.000
	Wilks' Lambda	.000	7298.006[a]	2.000	1.000
	Hotelling's Trace	14596.012	7298.006[a]	2.000	1.000
	Roy's Largest Root	14596.012	7298.006[a]	2.000	1.000
Concentration of Allicin	Pillai's Trace	1.000	149632.573[a]	2.000	1.000
	Wilks' Lambda	.000	149632.573[a]	2.000	1.000
	Hotelling's Trace	299265.145	149632.573[a]	2.000	1.000
	Roy's Largest Root	299265.145	149632.573[a]	2.000	1.000

a. Exact statistic

b. Design: Intercept + Concentration of Allicin

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F
Corrected Model	*Escherichia coli*	105.556[a]	1	105.556	28.361
	Candida albicans	355.182[b]	1	355.182	68.712
Intercept	*Escherichia coli*	97.576	1	97.576	26.217
	Candida albicans	103.098	1	103.098	19.945
Concentration of Allicin	*Escherichia coli*	105.556	1	105.556	28.361
	Candida albicans	355.182	1	355.182	68.712
Error	*Escherichia coli*	7.444	2	3.722	
	Candida albicans	10.338	2	5.169	
Total	*Escherichia coli*	474.000	4		
	Candida albicans	1000.560	4		
Corrected Total	*Escherichia coli*	113.000	3		
	Candida albicans	365.520	3		

a. R Squared = .934 (Adjusted R Squared = .901)

b. R Squared = .972 (Adjusted R Squared = .958)

Source	Dependent Variable	Sig.	Partial Eta Squared
Corrected Model	*Escherichia coli*	.033	.934
	Candida albicans	.014	.972
Intercept	*Escherichia coli*	.036	.929
	Candida albicans	.047	.909
Concentration of Allicin	*Escherichia coli*	.033	.934
	Candida albicans	.014	.972
Error	*Escherichia coli*		
	Candida albicans		
Total	*Escherichia coli*		
	Candida albicans		
Corrected Total	*Escherichia coli*		
	Candida albicans		

APPENDIX XVIII: ONE SAMPLE T-TEST

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Escherichia	4	9.5000	6.13732	3.06866
Candida	4	12.6000	11.03812	5.51906

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Escherichia	3.096	3	.053	9.50000	-.2658	19.2658
Candida	2.283	3	.107	12.60000	-4.9641	30.1641