CHEMICAL COMPOSITIONS AND ANTIMICROBIAL ACTIVITY OF *Amaranthus hybridus, Amaranthus caudatus, Amaranthus spinosus* AND *Corriandrum sativum.*

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A thesis submitted to the Graduate School in partial fulfilment for the requirement of the degree of Master of Science in Biochemistry of Egerton University.

EGERTON UNIVERSITY

OCTOBER, 2008.

DECLARATION AND RECOMMENDATION

DECLARATION

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

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RECOMMENDATION

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DEDICATION

To

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ABSTRACT

Different communities in Kenya use a wide variety of indigenous plants for food. About 850 species of plants are used for food. To this group belong *Amaranthus* species that has important nutrients and a rich array of biologically active secondary metabolites. *Corriandrum sativum* is a leafy spice/ herb that contain volatile oils from which it derives its flavour and aroma and also antimicrobial properties. Essential oils from fresh leaves of *C. sativum* were extracted by hydro distillation using a modified Clevenger–type system, analysed by gas chromatography–mass spectroscopy (GC–MS) and evaluated for antimicrobial activity. Subsequently, minimum inhibitory concentration was determined by serial dilution of the oil. Powdered samples of *A. hybridus, A. caudatus* and *A. spinosus* were sequentially extracted using hexane, ethyl acetate, dichloromethane and methanol. The resultant extracts were subjected to chemical and antimicrobial activity. Part of the samples underwent protein extractions which were tested for antimicrobial activity. The oil was obtained in 0.04% w/w yield with twenty seven components. The oil was dominated by aldehydes (56.07%), and alcohols (41.33%). The major constituents were 2E-decenal (15.88%). decanal (14.29%), 2E-decen-1-ol (14.22%) and n-decanol (13.64%) Oxygenated monoterpene present was linalool which was 0.32% of the oil. The oil showed activity against *Escherichia coli,* S*taphylococcus aureus*, *Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhi, Bacillus spp* and *Candida albicans* though inactive against *Pseudomonas aeruginosa*. All the *Amaranthus* species showed the presence of flavonoids, steroids, terpenoids and cardiac glycosides which are known to have antimicrobial activity. The extracts showed antimicrobial activity against *E. coli, S. aureus*, *K. pneumoniae, P. mirabilis, P. aeruginosa, S. typhi, Bacillus spp* and *C. albicans*. Serial dilution of the active extracts was done to concentrations of 20%, 25%, 33% and 50% for MIC analysis. The MIC's of the active fractions from *Amaranthus* species against the tested organisms varied between 129 mg/ml and 755 mg/ml. The antimicrobial properties of these plants which have been used by mankind for centuries without any signs of toxicity can be used in the traditional herbal medicines which play a very important role in primary care systems in the developing world and are becoming increasingly popular in the developed world.

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LIST OF ABBREVIATIONS

ATCC	-	American type culture collection
CLSI		Clinical and laboratory standards institute
GC-MS	-	Gas chromatography combined with mass spectrophotometer
KEMRI	-	Kenya Medical Research Institute
MIC		Minimum inhibitory concentration
MTBE	-	Methyltert-butylether
WHO	-	World health organization

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Nature has been a source of medicinal agents for thousands of years. Today, despite advances in pharmacology and synthetic organic chemistry, this reliance on natural products, particularly on plants, remains largely unchanged (Trevor, 2001). Approximately 80% of the world's population currently depends on traditional system of health care that incorporates natural products many of which are plants (Foster, 1999). In Kenya, 850 species of plants are used for food. These include fruits, cereals, legumes, leafy vegetables, tubers, roots and many non-foods such as gums and additives. About 1,000 species in Africa are used as leafy vegetables. Top of the list are well known vegetables such as the cowpea (*Vigna unguiculata*), *Amaranthus*, Spider plant, Rosele (*Hibiscus sabdariffa*) and a range of *Solanum* species (Maundu *et al*., 1999).

Spices and herbs have been used for centuries by many cultures to enhance the flavour and aroma of food, preserving foods and for their medicinal value (Jones, 1996). Scientific experiments since the late 19th century have documented properties of some spices, herbs and their components (Emamghoreishi *et al*., 2005). *C. sativum* is a spice of the family Apiaceae known to produce essential oils that relieve minor digestive problems and externally for haemorrhoids and painful joints (Burst, 2004; Fragiska, 2005). Although chemical and pharmacological studies of *C. sativum* essential oils have been carried out, most of the studies have used mainly the seeds/fruits (Lo Cantro *et al*., 2004). However, pharmacological studies on *C. sativum* leaves, the part of the plant most consumed by humans is limited. At the same time, available studies have assessed antimicrobial activity against few microorganisms (Wong and Kitts, 2006).

Plants have limitless ability to synthesize aromatic substances as secondary metabolites of which at least 12000 have been isolated (Cowan, 1999). In many cases, these substances serve as plant defence mechanisms against attack by microorganisms and herbivores. These substances include terpenoids that give plants their odours, quinones and tannins that are responsible for plant flavour (Cowan, 1999). Essential oils have been used for a long time in traditional medicine and in therapeutics (Bhattacharjee *et al.,* 2005; Matasyoh *et al*., 2007). The antimicrobial activity of essential oils has been well recognized for many years and their

preparations have wide applications as naturally occurring antimicrobial agents in pharmacology, medicine and clinical microbiology (Consentino *et al*., 1999; Hammer *et al.,* 1999; Wong and Kitts, 2006). Indeed aromatic plants have traditionally been used to extend the shelf life of food, showing inhibition against bacteria, fungi and yeast (Alves *et al*., 2000; Sartoratto *et al*., 2004).

Extraction of bioactive compounds from plants permits the demonstration of their physiological activity (Foster, 1999). It also facilitates pharmacological studies of more potent drug with reduced toxicity. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be active. These complementary components give the plant as a whole safety and efficacy much superior to that of its isolated and pure active components (Shariff, 2001). The information obtained from ethno medicine is therefore being put on a scientific basis and it is very important to investigate the pharmacological and phytochemical aspects of different preparations from plant sources (Hostettman and Marston, 1996).

Fungi and bacteria cause important human diseases especially in the tropical regions. Despite the existence of potent antibiotic and anti-fungal agents, resistant or multi-resistant strains are continuously appearing imposing the need for a search and development of new drugs from non-microbial sources. Antimicrobials have enormous therapeutic potential and are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are associated with synthetic antimicrobials (Iwu *et al.,* 1999; Chopra, 2007).

With over 40,000 plant species and over 1000 ethnic groups, Africa has both the cultural and plant diversity needed to invigorate its economy (Maundu *et al*., 1999). The high cultural diversity offers the continent a high choice of indigenous knowledge and related practices such as food processing techniques and recipes. In spite of this great potential and the wide range of opportunities, indigenous knowledge has not been effectively used to reduce current widespread malnutrition and poverty in Africa (Maundu *et al*., 1999). Africa has the potential in alleviating the health and economic status of its people using some of the work done on traditional leafy vegetables.

In an effort to discover new therapeutic compounds many research groups screen plant extracts to detect secondary metabolites with relevant biological activities. This study too is aimed at screening and analysing the extracts from *A. hybridus*, *A. caudatus* and *A. spinosus* and

the essential oils from *C. sativum* that could be useful for development of new tools for the control of infectious diseases.

1.2 Statement of the Problem

Most of the deaths of children and the elderly are caused by infectious and parasitic diseases such as diarrhoea, respiratory infections, typhoid and measles. In developing countries, these diseases remain a public health problem where for instance an estimate of 60 million cases and 500,000 deaths annually are caused by typhoid fever alone (WHO, 2002). Due to inadequate health facilities and the high cost of synthetic drugs, it is necessary to seriously look into the role of indigenous plants/herbs and spices. Scientific efficacy of combating diseases using plants/herbs will not only provide cheap drugs to Africa but also eradicate the problem of increased bacterial and viral resistance associated with the use of synthetic antimicrobials.

1.3 Objectives of the Study

1.3.1 Main objective:

To extract and characterise phytochemical extracts from *A. hybridus, A. caudatus and A. spinosus* and the essential oils of *C. sativum* and assess their antimicrobial activities.

1.3.2 Specific objectives

1. To assess the chemical composition of *A. hybridus*, *A. caudatus*, *A. spinosus* extracts and the essential oils of *C. sativum.*
2. To assess the antimicrobial activity of the phytochemicals from *A. hybridus*, *A. caudatus*, *A. spinosus* extracts and the essential oils of *C. sativum.*
3. To determine the minimum inhibitory concentrations of *A. hybridus*, *A. caudatus and A. spinosus* extracts and the essential oils of *C. sativum.*

1.4 Research Justification

The poorest countries in the world are in need of inexpensive and effective treatments for diseases. WHO (2002) estimates that one-third of global population lacks regular access to essential drugs and that in the poorest parts of Africa this figure rises to over 50%. One possible alternative source of medicine lies on traditional herbs/natural products used. The main problem facing the use of traditional medicinal herbs and spices is the lack of proof that active components contained in these plants are useful, safe and effective. Scientific rationalisation of traditional edible vegetables and spices against disease causing microbes becomes a priority to

show that they can be used in management of various ailments. This proof is required for a wider acceptability of use by the public. Naturally occurring substances form a significant base of raw materials for chemical and pharmaceutical industries as starting points for a series of their products.

1.5 Hypothesis

The extracts from *A. hybridus, A. caudatus and A. spinosus* and the essential oils of *C. sativum* have antimicrobial activity.

1.6 Expected Outputs

1. Appropriate realisation of the chemical constituents of *A. hybridus, A. caudatus, A. spinosus* extracts and the essential oils of *C. sativum.*
2. Establishment of the antimicrobial activity and of *A. hybridus, A. caudatus, A. spinosus* extracts and the essential oils of *C. sativum.*
3. Establishment of the minimum inhibitory concentration of *A. hybridus, A. caudatus, A. spinosus* extracts and the essential oils of *C. sativum.*
4. The research findings will be used in Master of Science thesis and publications.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Amaranthus species*

Amaranth is a common name for any flowering plants with blossoms that do not readily fade when picked. Most of the *Amaranthus* are found in the tropics. They are herbs or shrubs with simple leaves, and flowers in heads or spikes. The spikes are sometimes several centimetres long. Amaranths belong to the family Amaranthaceae. *Amaranthus* locally named "Terere" is cultivated in several areas of the world including South America, Africa, India, China and the United States (He *et al*., 2002). In Kenya, their leaves are eaten as green vegetables. In Nigeria, *Amaranthus* leaves combined with condiments are used to prepare soup (He *et al.,* 2002). These leaves boiled and mixed with a groundnut sauce are eaten as salad in Mozambique (Oliveira, 1995) or pureed into a sauce and served over (farinaceous) vegetables in West Africa (Martin, 1999).

2.1.1 *A. hybridus*

Figure 1-A photograph of *A. hybridus*

A. hybridus (Figure 1) is an annual plant growing to 0.6 m in height. The flowers are monoecious with individual flowers being either male or female, but both sexes can be found on the same plant. The edible parts are the leaves and stems. It is used in the treatment of intestinal bleeding, diarrhoea and excessive menstruation (He and Corke, 2003). It prefers light (sandy), medium (loamy) and heavy (clay) well-drained fertile soil in a sunny position. The plant prefers acid, neutral and basic (alkaline) soils (Facciola, 1990). *A. hybridus* should not be given inorganic fertilizers. Nitrates are implicated in stomach cancers, blue babies and some other health problems. It is inadvisable, therefore to eat this plant if it is grown inorganically (Facciola, 1990).This species has the potential through crossbreeding of imparting early maturity to the white seeded grain amaranths. *A. hybridus* is of uncertain origin, it grows wild in cultivated fields and waste places (Foster and Duke, 1990).

2.1.2 *Amaranthus caudatus*

Figure 2- A photograph of *Amaranthus caudatus*

A. caudatus is an annual plant growing to 2m by 0.45 m (Figure 2). The leaves are mild flavoured and rich in vitamins and minerals are eaten raw or cooked or added to soup. It is used in the treatment of stranguary and is applied externally to scrofulous sores (Larkcom, 2001). *A. caudatus* is domesticated mostly for its grain. It prefers light (sandy), medium (loamy) and heavy (clay) well-drained fertile soil in a sunny position. The plant prefers acid, neutral and basic (alkaline) soils (Facciola, 1990).

2.1.3 *Amaranthus spinosus*

Figure 3-A photograph of *Amaranthus spinosus*

A. spinosus is an annual plant growing to 0.6 m that is widely distributed in the humid zone of the tropics including Kenya (Figure 3). The flowers are monoecious with individual flowers being either male or female but both sexes can be found on the same plant and are wind pollinated. The Leaves and stems are eaten raw or cooked. The plant has pharmacological properties and has anthriquinone derivatives, cardiac glycosides and saponins (Assiak *et al.,* 2001). Leaf extracts are used in the treatment of menstrual disorders in man (Ayethan *et al.,*

1996) and is recommended for eruptive fevers. The leaves are also considered a good emollient, lactogogue and specific for colic. Externally, the bruised leaves are applied locally for treatment of eczema (Leyel, 1987). It grows on Roadsides, waste places and cultivated fields. It prefers light (sandy), medium (loamy) and heavy (clay) well-drained fertile soil in a sunny position. The plant prefers acid, neutral and basic (alkaline) soils (Facciola, 1990).

2.2 *Corriandrum sativum*

C. sativum (Figure 4) is a spice of the family Apiaceae. The leaves are variously referred to as coriander leaves, cilantro in the United States and dhania in Kenya. Within the Indian Subcontinent, Britain and China it is referred to as parsley or Mexican parsley (Fragiska, 2005). It grows to 50 cm tall. The leaves are variable in shape, broadly lobed at the base of the plant, slender and feathery higher on the flowering stems.

Figure 4: A photograph of *C. sativum*.

The flowers are borne in small umbels, white or very pale pink, asymmetrical, with the petals pointing away from the centre of the umbel longer about 5-6 mm than those pointing to the middle of the umbel only 1-3 mm long (Herklots, 1989). *C. sativum* has been recommended for dyspeptic complaints, loss of appetite, convulsion, insomnia and anxiety (Burst, 2004; Emamghoreishi *et al*., 2005).

2.3 Antimicrobial Drug Resistance

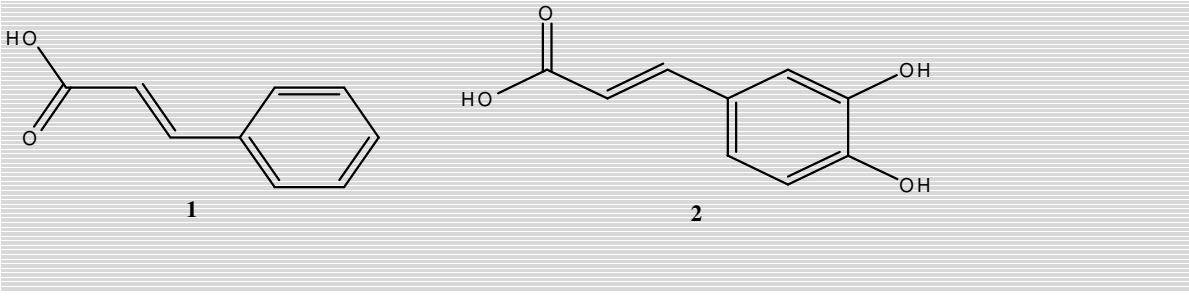
The wide-scale use of antimicrobials has led to microbial drug resistance, an adaptive response in which microorganisms become able to tolerate an amount of drug that would ordinarily be inhibitory. When infections become resistant to first-line antimicrobials, treatment has to be switched to second or third line drugs which are nearly always much more expensive and sometimes more toxic as well (Jukes *et al*., 2000).

2.4 Antimicrobial Compounds from Plants

Plants in general are primary producers on which all other members of the ecosystem depend. They have an almost limitless ability to synthesize aromatic substances most of which are phenols or their derivatives. Most are secondary metabolites of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total (Cowan, 1999). Antimicrobial phytochemicals can be divided into several categories. They include:

2.4.1 Phenols and polyphenols

Some of the simplest bioactive phytochemicals are phenols and phenolic acids, which consist of a single, substituted phenolic ring. Cinnamic (**1**) and caffeic (**2**) acids are common representatives of a wide group of phenyl propane-derived compounds which are in the highest oxidation state.

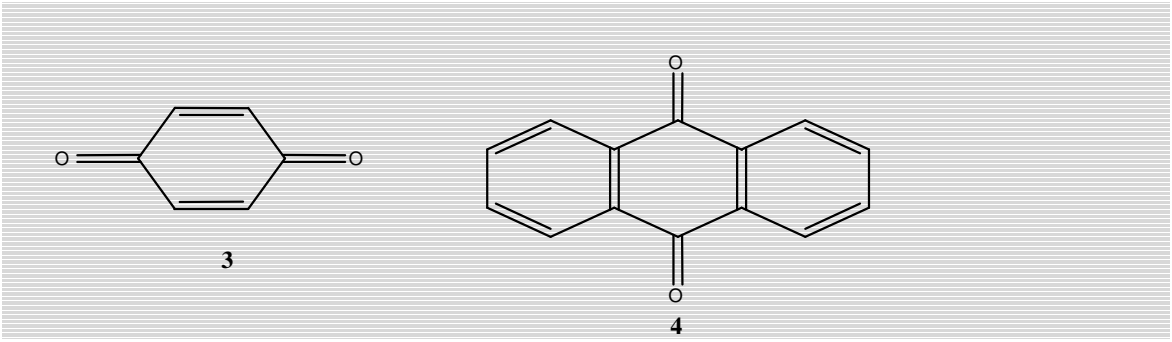


The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms (Cowan, 1999). The mechanisms responsible for

phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Mason and Wassermann, 1987).

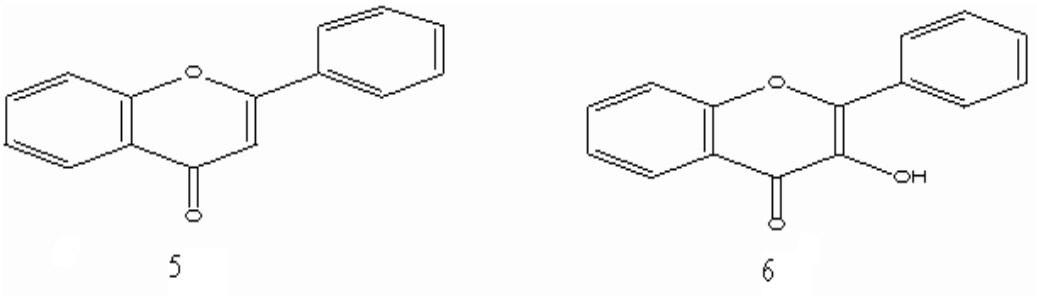
2.4.2. Quinones

Quinones (**3** and **4**) are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. Quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al*., 1996) often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes. Quinones also render substrates unavailable to the micro organism (Cowan, 1999).



2.4.3 Flavones, flavonoids and flavonols

Flavones (**5**) are phenolic structures containing one carbonyl group as opposed to the two carbonyls in quinones. The addition of a 3-hydroxyl group yields a flavonol (**6**) (Fessenden and Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon *et al*., 1983), they have been found *in vitro* to be effective antimicrobial substances against a wide array of micro organisms.



Their activity is due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids disrupt microbial membranes (Tsuchiya *et al*., 1996).

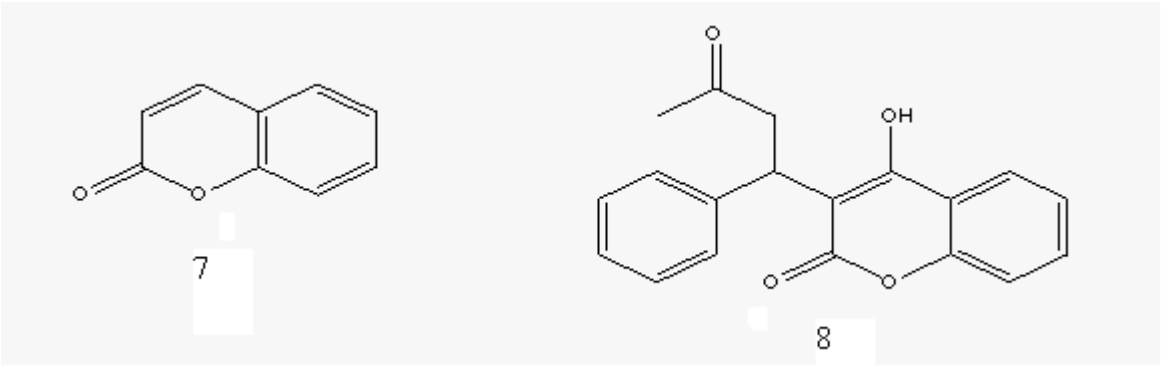
2.4.4 Tannins

They are divided into two groups, hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid usually as multiple esters with D-glucose while the more numerous condensed tannins, often called proanthocyanidins are derived from flavonoid monomers. Tannins are formed by condensations of flavan derivatives which have been transported to woody tissues of plants or polymerization of quinone units (Cowan, 1999).

Many human physiological activities such as stimulation of phagocytic cells, host-mediated tumour activity and a wide range of anti-infective actions are assigned to tannins (Cowan, 1999). One of their molecular actions is to complex with proteins through non-specific forces such as hydrogen bonding and hydrophobic effects as well as by covalent bond formation. They are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al*., 1996) leading to inactivation of the protein and loss of function. This is related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins.

2.4.5 Coumarins

Coumarins (**7**) are phenolic substances made of fused benzene and α-pyrone rings (O'Kennedy and Thornes, 1997). Their fame has come mainly from their antithrombotic (Thastrup *et al.,* 1985), anti-inflammatory (Piller, 1975) and vasodilatory (Namba *et al.,* 1988) activities. Warfarin (**8**) is a particularly well-known coumarin which is used both as an oral anticoagulant and interestingly as a rodenticide (Keating and O' Kennedy, 1997).



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During subsequent *in vivo* tests on rabbits, the coumarin-spiked water supply was inadvertently given to all the animals in the research facility and was discovered to be a potent contraceptive agent when breeding programs started to fail (Thornes, 1997). Its estrogenic effects were later described (Cowan, 1999). Coumarins have been found to stimulate macrophages (Casley-Smith and Casley- Smith, 1997), which have an indirect negative effect on infections. More specifically, coumarin has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Cowan, 1999) but was found ineffective against leprosy (Thornes, 1997). Hydroxy Cinnamic acids, related to coumarins are inhibitory to gram-positive bacteria (Fernandez *et al*., 1996).

2.4.6 Terpenoids and essential oils

The fragrance of plants is carried in *quinta essentia* or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure (Cowan, 1999). Essential oils are volatile plant oils (steam distillable), which are composed chiefly of terpenoids: mono-, sesqui- and di-terpenes plus various alcohols, ketones and aldehydes with commonly occurring aromatic compounds arising from the phenylpropanoid pathway (eugenol and safrole). In some species, alkanes, aliphatic alcohols and ketones may be obtained (Williams, 1996). They are also considered as a complex mixture of various aromatic chemicals. Each of these constituents contributes to the beneficial or adverse effects of the oil (Buchbauer, 1993). The volatile oils are more or less modified during the preparation process (Buchbauer, 1993).

Terpenenes or terpenoids are active against bacteria (Scortichini and Pia Rossi. 1991; Tassou *et al*., 1995), fungi (Taylor, 1996), viruses (Sun *et al*., 1996), and protozoa (Vishwakarma, 1990). The mechanism of action of terpenes involves membrane disruption by the lipophilic compounds. Accordingly, Mendoza *et al*., (1997) found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity. Food scientists have found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Aurelli *et al.,* 1992).

The composition of essential oils is dependent on such characteristics as the geographic character of the location from which the plant is obtained, seasonal variations and climate, production techniques and purity, the effect of plant maturity at the time of harvesting and the

existence of chemotypic differences can also drastically affect the composition suggesting that ecological condition and/or physiological states could interfere with the presence of biologically active compounds in the plant (Cornu *et al*., 2001). These variations are of distinct importance for the study of these natural products because the value of an essential oil in aromatherapy has to be related to its chemical composition (Lahlou, 2003).

2.4.7 Lectins and polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 (Balls *et al*., 1942). They are often positively charged and contain disulfide bonds (Zhang and Lewis, 1997). Their mechanism of action may be the formation of ion channels in the microbial membrane (Terras *et al*., 1993; Zhang and Lewis, 1997) or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Sharon and Ofek, 1986). Recent interest has been focused mostly on studying anti-HIV peptides and lectins, but the inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthus*, has long been known (De Bolle *et al*., 1996). Antimicrobial proteins and peptides in plants have most commonly been discovered in seeds, where they accumulate to high levels and may also function as storage proteins. Homologues of the seed proteins have been found subsequently at much lower concentrations in vegetative and floral tissues (Bohlmann *et al.,* 1988; Terras *et al*., 1995).

CHAPTER THREE

METHODOLOGY

3.1 Collection and Identification of Plants

The plants under study were collected from different sites of Rift Valley, Nyanza and Western provinces of Kenya. The regions have short rains in the months of October/November when material collection was done hence their availability. Domesticated *A. caudatus* and *C. sativum* were collected from plots in Egerton University-Nakuru District which is at an altitude of 2127m. Wild *A. spinosus* were collected from Ahero-kisumu. *A. hybridus* sample were obtained from Shinyalu-Kakamega District which is at an altitude of 1535m. All the test species were local landraces. The samples were randomly collected. For each species 50 samples were collected and only the vegetative part was used in this study. Interplant variations were accounted for by the standard error of the mean (SE). This error was due to extraneous factors such as heterogeneity of the soil (soil moisture, soil fertility), Climate factors, plant status, harvesting season, plant competition and phenology. Taxonomic identification was done at the Department of Biological Sciences, Egerton University.

3.2 Collection and Identification of Test Micro organisms

The test microorganisms were obtained from Kenya Medical Research Institute (KEMRI) and maintained in the microbiology laboratory at the Department of Animal Science of Egerton University. The test bacteria of American Type Culture Collection (ATCC) included: *Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. Clinical isolates used were: *Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhi* and *Bacillus spp*. The anti-fungal activity was evaluated on the clinical isolate of the *Candida albicans*.

3.3 Isolation of Essential Oils from *Corriandrum sativum*

Fresh leaves of *C. sativum* weighing 250g were subjected to hydro-distillation (Figure 5), using modified Clevenger-type apparatus for a minimum of 4 hours (Matasyoh *et al.,* 2007). The resultant mixture of steam and essential oil was passed through a Lie-big condenser which is connected to a continuous flow of cold water. Essential oils are less dense than water and were separated as an upper layer floating on the distillation water. The oil was then collected by decanting into sample bottles and dried using anhydrous sodium sulphate. The procedure was

repeated until a reasonable amount of oil was obtained which was used for analysis and antimicrobial tests. The dried oil was weighed and the percentage yield calculated.

Figure 5: Photograph of hydro-distillation apparatus

3.4 Preparation and Solvent Extraction of *A. caudatus, A. spinosus* and *A. hybridus*

The stems and leaves of the *A. caudatus, A. spinosus* and *A. hybridus* were dried under a shade to a constant weight for two weeks (Figure 6). The dried material was then ground to a fine powder from which 500g was weighed and sequentially extracted with 2 × 1L of distilled hexane, ethyl acetate, dichloromethane and methanol after soaking for 24 hours. These are solvents of varying polarity with hexane being the least polar, ethyl acetate and dichloromethane with intermediate polarity and methanol the most polar. The extracts of dichloromethane and methanol were filtered through a thin layer of active charcoal on a Buchner funnel fitted to a vacuum pump to remove the chlorophyll matter. All the resultant extracts were then subjected to rotary evaporation to remove the solvents giving crude extracts of the respective solvents. The crude extracts of each solvent were subjected to thin layer chromatography (TLC) with silica gel as the stationary phase and the mobile phases being the extraction solvents. The 4 crude solvent extracts of *A. caudatus, A. spinosus* and *A. hybridus* were introduced to different solvent systems to identify a good resolution solvent which was used for column separation and purification.

A 20cm by 20cm TLC plate was carefully cut into small pieces of 2.5cm by 6cm. A line was drawn using a pencil 1cm from the bottom of the plate. The different plant extracts were then spotted 10mm apart using micropipettes. The spots were concentrated by air drying. The spotted plates were then developed on various solvent systems that were already saturated with the vapours of the solvent systems. The plates were then withdrawn and the solvent fronts marked using a pencil. These plates were dried using a blow drier and sprayed with anisaldehyde reagent using a spray gun in a fume chamber. This was followed drying using the blow drier. Analysis of the spots was done using ultra violet (UV) light at a wavelength of 254 nm. The crude extracts were then separated and purified into visualised colour bands using column chromatography.

A column on a retort stand was filled with silica gel slurry of 60g silica gel in 100% hexane. The slurry was introduced carefully with no air bubbles. The column was allowed to run with excess solvent to avoid cracking of the gel. After proper settlement of the gel on the column, laboratory sand was slowly on top for easy sample introduction using a Pasteur pipette. The sample extract was allowed to flow past the sand before introduction of more elution solvent. The fractions were collected according to their colour bands as visualised on TLC plate. The collected extract fractions were then subjected to analytical TLC where the fractions visualised

with similar spots were mixed. The same procedure was then repeated for ethyl acetate, dichloromethane and methanol crude extracts of *A. caudatus*. The same treatment was done for the *A. spinosus* and *A. hybridus* crude extracts. All the sample fractions were underwent phytochemical and functional group analysis. Antimicrobial activity was done against the test microorganisms.

3.5 Preparation and Extraction of Plant Proteins from *Amaranthus* species

The stems and leaves of the *A. caudatus, A. spinosus* and *A. hybridus* were dried under a shade to a constant weight for two weeks (Figure 6). The dried material was then ground to a fine powder and the proteins extracted with ice-cold extraction buffer (10m*M* NaH_2PO_4, 15m*M* Na_2HPO_4, 100m*M* KCL, 2 m*M* EDTA, 1m*M* thiourea) (Terras *et al*., 1995). A portion of the extract was added to 10ml of ice-cold extraction buffer in tubes and the tubes placed on a shaking platform for 2 hours after which they were centrifuged at 5000g for 10 minutes. In order to remove secondary metabolites and other non-protein molecules that might contribute antimicrobial activity, the supernatants were loaded to a 5cm bed of Sephadex G-100 column, equilibrated in 10m*M* sodium phosphate and eluted with 3.5 ml 10m*M* sodium phosphate. Elutes were collected by volume of 1ml.The elutes were subjected to ninhydrin regent to test for the presence proteins on the vegetative parts of *A. caudatus, A. spinosus* and *A. hybridus.* Antimicrobial proteins and peptides in plants have most commonly been discovered in seeds, where they accumulate to high levels and may also function as storage proteins. Homologues of the seed proteins have been found subsequently at much lower concentrations in vegetative and floral tissues (Bohlmann *et al.,* 1988; Terras *et al*., 1995). Antimicrobial activity was done against the test microorganisms.

3.6 Antimicrobial Assay

3.6.1 Screening for antimicrobial activity of the phytochemicals and essential oil

The antimicrobial activity of the phytochemicals and essential oils was tested according to the Clinical and Laboratory Standards Institute (CLSI) (2007). The test organisms were inoculated in culture nutrient broth and then incubated for 4-6 hours at 37 ^{o}C. To standardize the bacterial inoculums for the susceptibility test, a barium sulphate standard equivalent to McFarland No. 0.5 standards or its optical equivalent was used; McFarland No. 0.5 standard gives cell density of $1.5x10^{8}$/ ml.

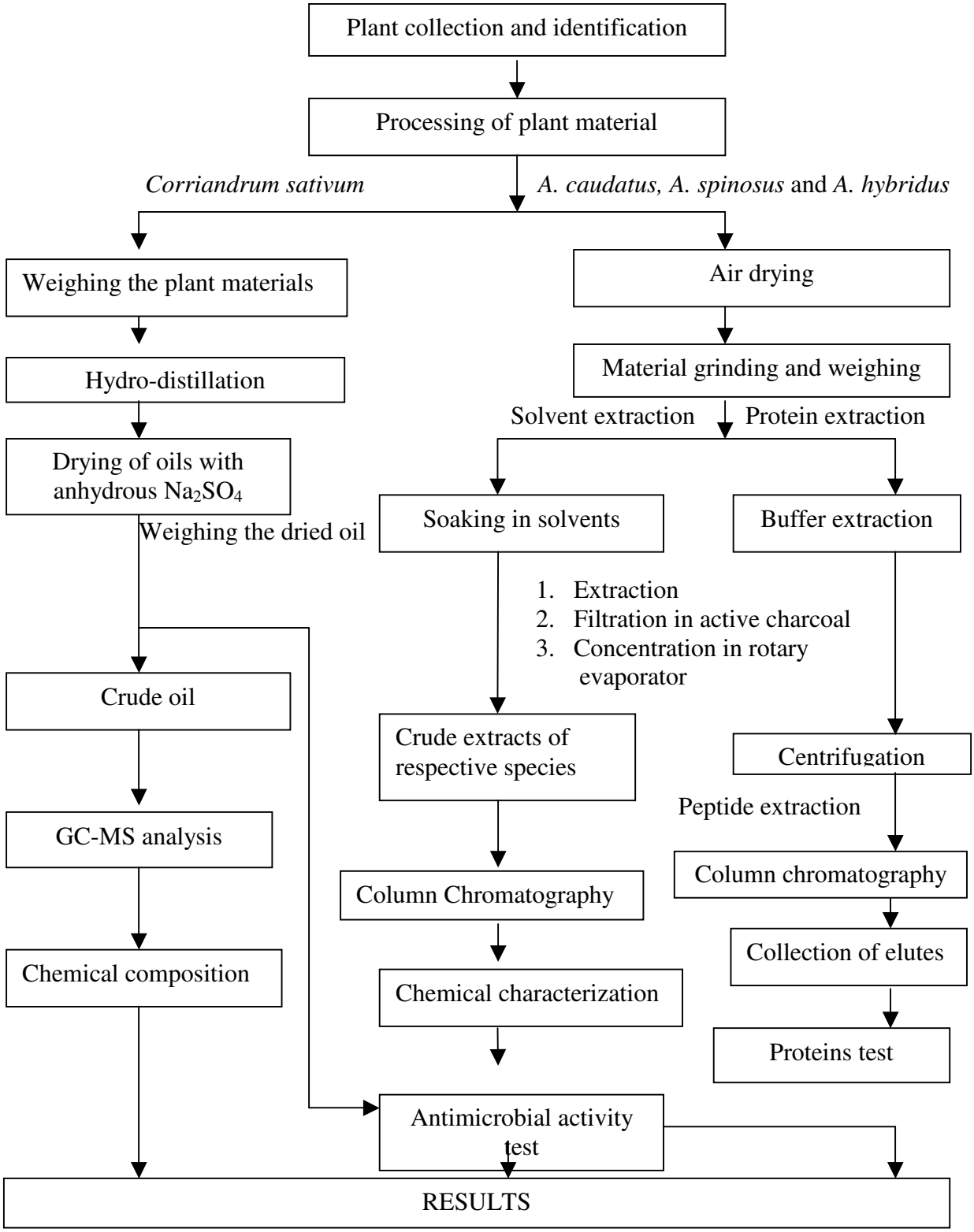


Figure 6: Summary of methodological procedure

The susceptibility of the test organism was done using disc diffusion technique using filter paper discs (CLSI, 2007). Antibiotic discs impregnated with the phytochemical and essential oil were placed on bacterial and fungal culture during the log phase growing on appropriate solid media. Anti-bacterial activity assay was done on Mueller Hinton agar while anti-fungal activity on Sabouraud dextrose agar (SDA). The media were reconstituted using distilled water and sterilized by autoclaving at 121 ^{o} C for 15 minutes then dispensed into petri dishes aseptically and left to solidify and then stored in the refrigerator at 4 ^{o} C.

Sensitivity discs containing antibiotics to which the organisms are sensitive were used as positive controls. Chloramphenicol for bacteria and nystatin for fungi were used as positive standard control where as sterile distilled water was used as the negative control. The cultures were incubated at 37 ^{o} C for 18-24 hours. Growth inhibition caused by extracts/essential oil was compared with that of the standard sensitivity discs. The freshly grown microbial cultures were inoculated on solid media. Blank sensitivity discs prepared from filter paper were divided into three bijoux bottles and sterilized in the oven by air-drying at 160 ^{o} C for one hour. Test phytochemicals and essential oil were impregnated into sterile blank discs by soaking in the samples for 24 hours.

The discs with *A. caudatus, A. spinosus* and *A. hybridus* phytochemical were then removed from the samples and placed a glass plate to allow the solvents to evaporate and the test chemicals adsorbed in the discs. Essential oil discs were also removed to allow them to dry. They were then aseptically into the inoculated petri dish. All these procedures were done in triplicate. The individual petri dish was covered to avoid any possible evaporation or contamination. The inoculated plates were incubated at 4 ^{o} C for at least 2 days to allow the phytochemicals and the essential oil to diffuse into the media and then transferred to 37 ^{o} C for 24 hours before the activity was determined. The activity of the test oil and phytochemicals was established by the presence of measurable zones of inhibition in mm. The discs used absorbs 0.01ml of the sample hence the concentration of each sample extract was established. The minimum inhibitory concentration (MIC) of the oil and phytochemicals were determined by serial dilution.

3.6.2 Minimum inhibitory concentrations

Phytochemicals and essential oil that presented inhibitory properties *in vitro* in the screening activity were evaluated for their MIC using the gel diffusion test. Approximately $1.5x10^{8}$ cells of

freshly grown microbial suspension was uniformly spread in the sterile Sabouraud dextrose agar dishes for fungi and Mueller Hinton agar dishes for bacteria using sterile cotton swabs. Serial dilutions of the *A. caudatus, A. spinosus* and *A. hybridus* active fraction extracts were carried out using the respective solvent used for extraction. The essential oil serial dilutions were carried out using 10% Tween 80 in distilled sterile water which was used also as the negative control. Each serial dilution of the extracts was impregnated into blank sterile sensitivity disc.

The discs with respective dilutions were placed on a Muller Hinton agar and Sabouraud dextrose agar plates to which the sensitive micro organism has been inoculated. Sterile distilled water was used as the negative control. The disc with phytochemicals and essential oil were placed at a distance of 3cm diameter from each other. The plates were incubated at 4^{o} C for 2 days to allow the extract to diffuse into the media, then transferred to 37 ^{o} C for 18-24 hours and checked for growth and inhibition. Zone of inhibition caused by the different dilutions of the phytochemicals and essential oil were measured in mm. The smallest concentration of the plant extract that inhibited growth was taken to be the MIC. The anti-bacterial or anti-fungal activity was established by the presence of measurable zones of inhibition (mm) after 24 h of incubation at 37 ^{o} C detected visually (Figure 7). The same procedure was repeated for the standard antimicrobial (Chloramphenicol) and its MIC for each test organism determined.

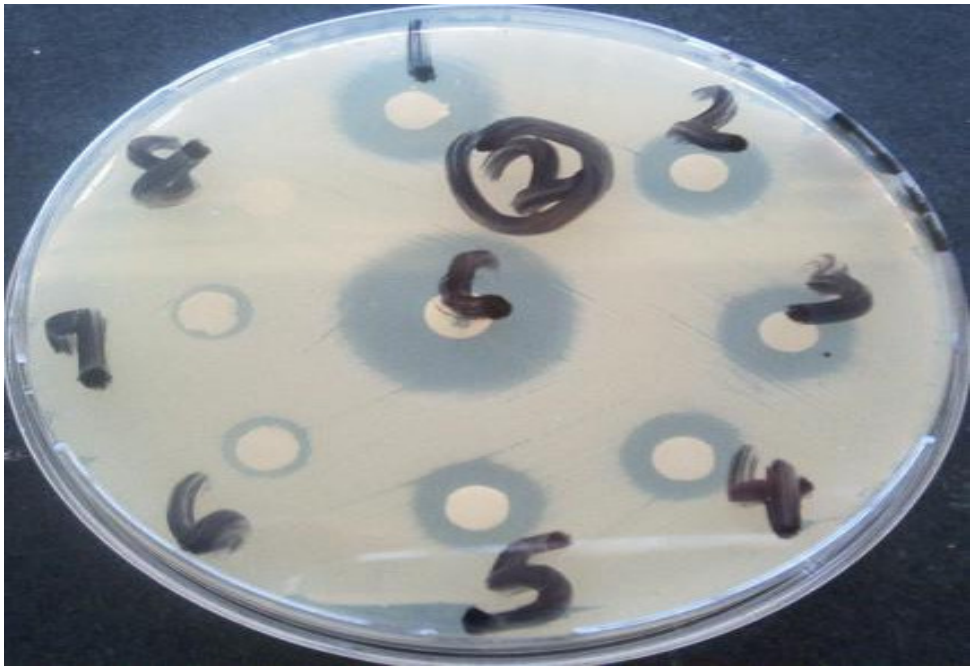


Figure 7: A photograph of a MIC plate (Essential oil of *C. sativum* on *S. aureus*).

3.7 Phytochemical and Chemical Screening

3.7.1 Phytochemical tests

Each of the extraction solvent is known to be associated with isolation of particular phytochemicals from the plant material. Various phytochemical tests were carried out to determine the class of compounds in the active fractions. These were identified by characteristic colour changes using standard procedures (Faraz *et al.*, 2003).They included:

a) Tests for tannins/phenols

To about 1ml of the sample extract was added a few drops of 0.1% ferric chloride. A positive test result was indicated by appearance of a brownish-green or blue-black colouration.

b) Test for phlobatannins

About 2ml of sample extract were boiled with 1% aqueous hydrochloric acid. A positive test result was indicated by deposition of a red precipitate.

c) Test for flavonoids

About 5ml of dilute aqueous ammonia solution was added to 1ml portion of the sample extract to 1ml of concentrated sulphuric acid was slowly added. A positive test result was indicated by the formation of a yellow colouration that disappeared instantly or on standing.

d) Test for steroids

About 2 ml each of acetic anhydride and concentrated sulphuric acid was added to about 2ml of the sample extract. A positive test result was confirmed by the change of colour from violet to blue or green.

e) Test for terpenoids

About 5ml of the sample extract was mixed with 2ml of CH_3CL and 3ml of concentrated H_2SO_4 added slowly to form a layer. A positive test result was indicated by the presence of a red colouration at the interface.

f) Test for cardiac glycosides

About 5 ml of the sample extract was mixed with 2 ml of glacial acetic acid containing one drop ferric chloride solution. To which 1 ml of concentrated sulphuric acid was slowly under laid to the sample mixture. A positive test result was indicated by the presence of a brown ring at the interface.

3.7.2 Chemical tests

Chemical tests were performed to determine the functional groups in the active fractions. These were identified by characteristic colour changes using standard procedures (Trease and Evans, 1983). Various chemical tests coupled with other methods of identification have been used to identify functional groups present in samples extracts (Vishnoi, 1979; Solomon's, 1996).

a) Sodium hydroxide test

Sodium hydroxide test was used to test for the presence of phenolic groups. Solubility of the sample in the solution is considered a positive test. Phenols dissolve in aqueous NaOH to give a water soluble sodium phenoxide. This reaction is also used to differentiate phenols from alcohols as alcohols with six carbons and more are not soluble in aqueous NaOH. 1ml of the sample fraction was placed in a test tube containing about 2ml of sodium hydroxide and the tube shaken to enhance solubility.

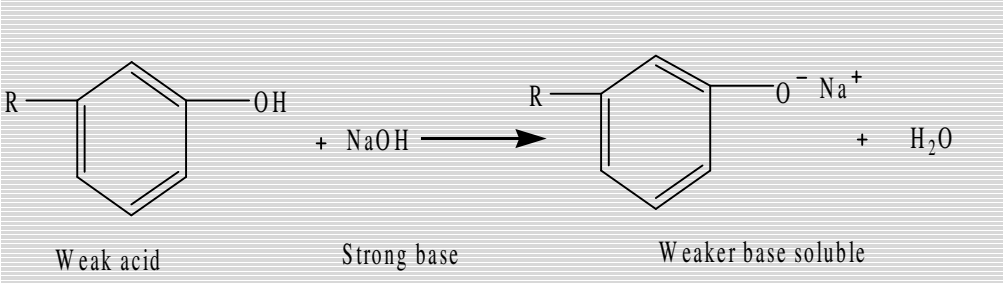


Figure 8: Sodium hydroxide test reaction

b) Sodium hydrogen carbonate test

Sodium hydrogen carbonate test was used to test for the presence of carboxylic acid groups. The sample fraction was added to approximately 5ml of cold saturated $NaHCO_3$. Vigorous evolution of CO_2 with brisk effervescence or solubility indicated the presence of COOH group in the sample fraction (Vishnoi, 1979).

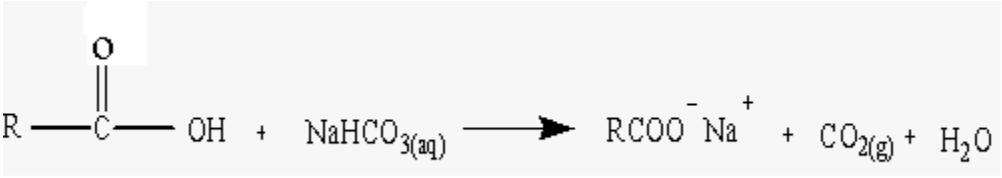
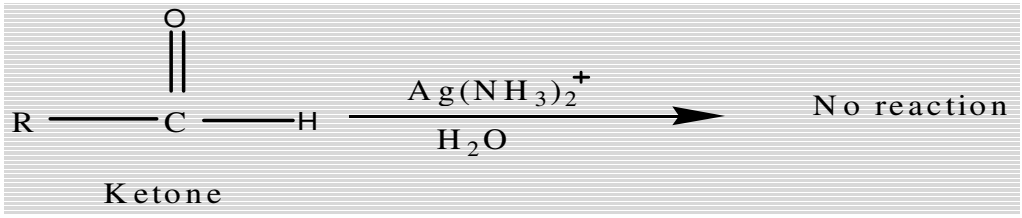
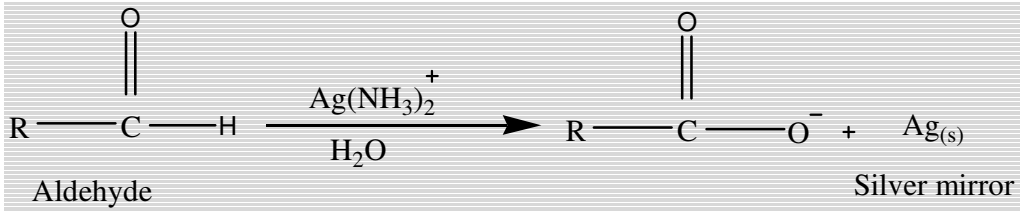


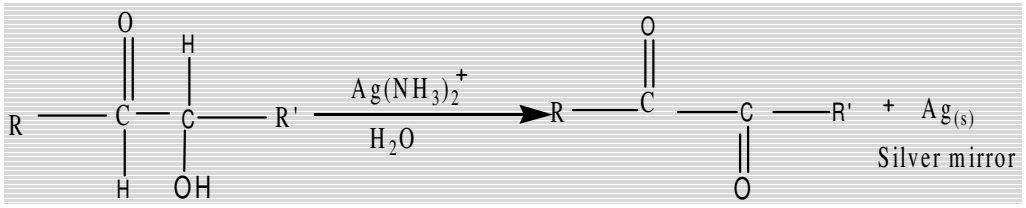
Figure 9: Sodium hydrogen carbonate test reaction

c) Tollen's test

Tollen's test was used to differentiate between aldehydes and ketones. Aldehydes undergo oxidation easily in Tollen's reagent. Tollen's reagent which is silver nitrate in aqueous ammonia oxidizes aldehydes to carboxylate ions. Silver was reduced from +1 oxidation state [Ag $(NH_3)_2$] $^+$to metallic silver, which sticks on the walls of the test tube to form the silver mirror. If not deposited on the walls, it is deposited at the bottom as a grey or black precipitate. The reaction gives negative result with all ketones except–Hydroxy ketone. The sample fraction was added to 2ml of Tollen's reagent. The test tube was then put in a water bath for 5 minutes and the observations recorded.







Hydroxy ketone

Figure 10: Tollen's test reaction

d) Bromine water test

The bromine water test was used to determine the unsaturation of the C-C bonds which are not aromatic by decolourisation of the bromine solution. Any colour was considered a positive indication of unsaturation (Vishnoi, 1979).

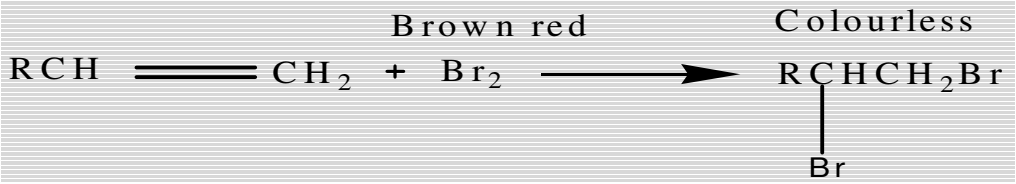


Figure 11: Bromine water test reaction

e) Lucas test

Lucas test was used to determine the presence of primary, secondary and tertiary alcohols. Formation of a green precipitate was a positive result. If the reaction took place very fast, tertiary alcohols were present and if moderate, secondary alcohols are present. Where no reaction occurred primary alcohols were present or absent.

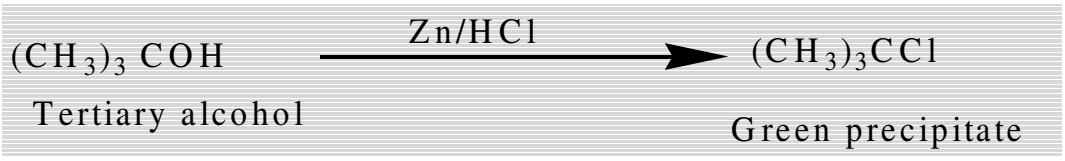


Figure12: Lucas test reaction

3.8 Chromatographic Analysis

Analysis of the crude essential oil was carried out by gas chromatography hyphenated with a mass spectrometer (GC-MS). The essential oil sample was diluted in Methyltert-butylether (MTBE) (1:100) and analysed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS ('Resets') (30m x 0.25 mm internal diameter, 0.25µm film thickness) fused-silica capillary column. Helium (at 0.8 ml/min) was used as a carrier gas. The sample was injected in the split mode at a ratio of 1:10 to 1:100. The injector was kept at 250 ^{o} C and the transfer line at 280 ^{o}C. The column was maintained at 50 ^{o} C for 2 minutes and then programmed to 260 ^{o} C at 5 ^{o} C /minute and held for 10 minutes at 260 ^{o}C. The MS was operated in the EI mode at 70 eV, in m/z range 42-350. The compounds were identified by comparing their retention indices and mass spectra with those found in library of mass spec data (Adams, 1995) and supplemented by Wiley and QuadLib 1607 GC-MS libraries. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization, all relative response factors being taken as one while their Kovat indices (KI) were obtained from library of mass spec data (Adams, 1995).

3.8.1 Determination of Kovat Indices (KI)

Relative retention values were determined in accordance with Kovat's method (Adams, 1995). The basis of this retention index-I is the finding that within a homologous series of *n*-alkanes (*n*-paraffin) a linear relationship exists between the logarithmic of the adjusted retention time and the number of carbon atoms in the compound. The retention of a compound to be investigated is then related to that of *n*-alkanes and one defines as follows:

The retention time of a substance is equal to 100 times the carbon number of a hypothetical n-paraffin with the same retention time as the substance of interest (Kellner *et al*., 1998).

In accordance with the definition, the *n*-alkanes have an index of 100 times the relevant carbon number at every temperature on all separation columns, for example *n*-hexane, 600 or *n*-octane, 800. In order to determine the retention time, the substance being examined is chromatographed in a mixture, which contains at least two *n*-alkanes. In so doing, the retention times of these *n*-alkanes must encompass the retention time of the compound of interest.

Normally, a graphical procedure is not required in determining retention indices. Instead, retention data are derived by interpolation from a chromatogram of a mixture of the solute of interest and two or more alkane standards. Retention index for a normal alkane is independent of temperature and column packing (Skoog and Leary, 1992) (Figure 13). Calculation of the index is undertaken on the basis of the equation:

$$I = 100y\left(\frac{\log t_{RX} - \log t_{Rz}}{\log t_{R(z+y)} - \log t_{Rz}}\right) + 100z$$

With t_{Rx}, t_{Rz}, $t_{R(z+y)}$ retention times relevant for the substance being examined, x, for the *n*-alkane with the carbon number z, and for the *n*-alkane with the carbon number (z+y), with y being the number of additional carbon atoms compared to z (Kellner *et al.,* 1998).

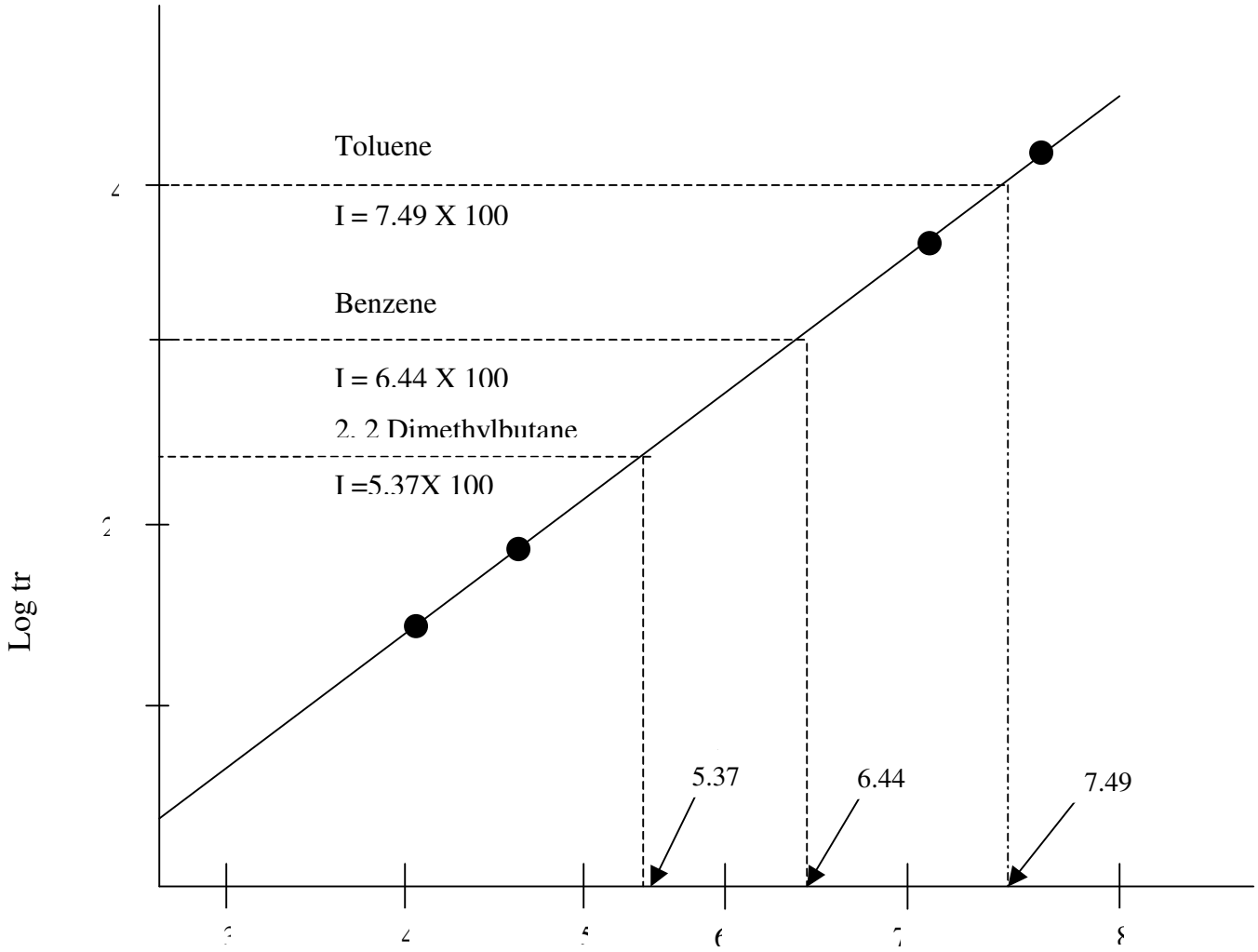


Figure13: Graphical illustration of the method of determination of retention indices

3.9 Statistical Analysis of the Antimicrobial results

The results were expressed as means ± standard error (SE). The assays were carried out in triplicates and student's t-test was used to compare the means.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Phytochemical Results for *A. caudatus, A. spinosus* and *A. hybridus*

The phytochemical screening of the extracts of *A. caudatus, A. spinosus* and *A. hybridus* revealed that tannins, phlobatannins and saponins were only present in the most polar solvent, methanol (Table 1). Tannins were present only in *A. caudatus* methanol sample fraction. Both *A. caudatus* and *A. hybridus* had phlobatannins in their methanol extracts.

Table 1: Phytochemical results for *A. caudatus*, *A. spinosus* and *A. hybridus*

Phytochemical Constituent	Fraction	Hexane			Ethyl acetate			Dichloromethane			Methanol		
		A. hybridus	*A. caudatus*	*A. spinosus*	*A. hybridus*	*A. caudatus*	*A. spinosus*	*A. hybridus*	*A. caudatus*	*A. spinosus*	*A. hybridus*	*A. caudatus*	*A. spinosus*
Tannins	1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
	3	-ve	-ve	-ve	0	0	0	0	0	0	0	0	0
Phlobatannins	1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
	2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	3	-ve	-ve	-ve	0	0	0	0	0	0	0	0	0
Saponins	1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
	2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
	3	-ve	-ve	-ve	0	0	0	0	0	0	0	0	0
Flavonoids	1	-ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
	2	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
	3	+ve	+ve	+ve	0	0	0	0	0	0	0	0	0
Steroids	1	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	3	+ve	+ve	+ve	0	0	0	0	0	0	0	0	0
Terpenoids	1	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
	2	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve
	3	+ve	-ve	+ve	0	0	0	0	0	0	0	0	0
Cardiac	1	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
glycosides	2	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
	3	+ve	-ve	+ve	0	0	0	0	0	0	0	0	0

+ve- Represents the presence of the tested phytochemical in the sample fraction

-ve- Represents the absence of the tested phytochemical in the sample fraction

Saponins were present only in *A. spinosus* methanol extract (Table 1). The similarity in phytochemical composition of *A. caudatus, A. spinosus* and *A. hybridus* was the presence of steroids in all the sample fractions of hexane, ethyl acetate, dichloromethane and methanol. Flavonoids, terpenoids and cardiac glycosides were distinctively absent in the ethyl acetate and dichloromethane sample fractions of *A. spinosus* as shown in Table 1. The fractions analysed for hexane, ethyl acetate, dichloromethane and methanol extracts were those that could be purified.

4.2 Chemical Tests for *A. caudatus, A. spinosus* and *A. hybridus*

The chemical tests revealed the presence different classes of alcohols in all the fractions of hexane, ethyl acetate, dichloromethane and methanol (Table 2).

Table 2: Chemical tests for *A. caudatus*, *A. spinosus* and *A. hybridus*

Chemical test	Fraction	Hexane			Ethyl acetate			Dichloromethane			Methanol		
		A. hybridus	*A. caudatus*	*A. spinosus*	*A. hybridus*	*A. caudatus*	*A. spinosus*	*A. hybridus*	*A. caudatus*	*A. spinosus*	*A. hybridus*	*A. caudatus*	*A. spinosus*
NaOH	1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve
	3	-ve	-ve	-ve	0	0	0	0	0	0	0	0	0
$NaHCO_3$	1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve
	2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
	3	-ve	-ve	-ve	0	0	0	0	-ve	0	0	+ve	0
Tollen's	1	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	2	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve
	3	+ve	+ve	+ve	0	0	0	0	0	0	0	0	0
Br_2 water	1	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
	2	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
	3	+ve	+ve	+ve	0	0	0	0	0	0	0	0	0
Lucas	1	+++	+++	+	+++	+++	++	+++	+++	++	++	++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++
	3	+++	+++	+++	0	0	0	0	0	0	0	0	0

+Ve- Represents the presence of the tested functional group in the sample fraction

-Ve- Represents the absence of the tested functional group in the sample fraction

+-Primary alcohols ++-Secondary alcohols +++-Tertiary alcohols

Depending on the rate of reaction, the presence of OH group in the sample fractions was classified as primary, secondary and tertiary alcohols. Tertiary alcohols dominated the fractions of the hexane and ethyl acetate extracts. Secondary alcohols were dominant in the *A. spinosus* and *A. hybridus* dichloromethane and methanol extract fractions. Carboxyl groups were present in the methanol extracts of *A. spinosus* and *A. caudatus.* However, phenolic compounds were present in the methanol fractions of *A. caudatus* which were a clear indication that their presence depended on the polarity of the extraction solvent (Table 2). All the analysed fractions of *A. caudatus, A. spinosus* and *A. hybridus* showed the presence of an unsaturation except the dichloromethane extracts of *A. spinosus*. Aldehydes were the common functional group for *A. caudatus, A. spinosus* and *A. hybridus.*

4.3 Protein Test Results for *A. hybridus A. caudatus, A. spinosus* and *A. hybridus*

The crude protein extract of *A. hybridus A. caudatus, A. spinosus* and *A. hybridus* showed the presence of proteins on different fractions collected. The column elutes were collected by volume of 1ml. For each species 4 fractions were collected. The test was done in duplicates. For *A. hybridus* the first two fractions had proteins. *A. caudatus* fourth fraction was the only fraction with proteins (Table 3). The *A. spinosus* crude protein extract showed the presence of proteins on ninhydrin reagent on its second fraction.

Table 3: Protein test results for *A. caudatus, A. spinosus* and *A. hybridus*

Fraction	*A. hybridus*		*A. caudatus*		*A. spinosus*	
	1st test	2nd test	1st test	2nd test	1st test	2nd test
1	+	+	-	-	-	-
2	+	+	-	-	+	+
3	-	-	-	-	-	-
4	-	-	+	+	-	-

+ - Represents the presence of the tested functional group in the sample fraction

- - Represents the absence of the tested functional group in the sample fraction

4.4 Antimicrobial Activity Protein Extracts for *A. caudatus, A. spinosus* and *A. hybridus*

The protein extract fractions showed a broad spectrum activity to the test microorganisms. In *A. hybridus* weak activity was observed. An inhibition zone of 9.3 ± 0.6 mm (Table 4) was

observed on *K. pneumoniae*. They were resistant to *S. Aureus*, *Bacillus spp, E. coli, P. aeruginosa, P. mirabilis* and *S. typhi.* The second fraction of *A. spinosus* showed activity to only as in Table 4. *Bacillus spp* showing an inhibition zone of 13.5±1.2 mm. *K. pneumoniae and S. typhi and C. albicans* were susceptible to the fourth fraction of *A. caudatus* (Table 4).

Table 4: Antimicrobial activity protein fractions from *A. caudatus*

	Fraction	Inhibition zones in mm Microorganism						
Amaranthus spp.		***E. coli***	***S. typhi***	***K. pneumoniae***	***P. mirabilis***	***S. aureus***	***Bacillus spp***	***C. albicans***
A. hybridus	1	0	0	9.3 ± 0.6	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
A. caudatus	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	4	0	18.0±1.7	17.7±2.0	0	0	0	10.0±1.2
A. spinosus	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	32.7±1.5	0
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
[a]STD		48.3±1.7	33.7±0.9	37.7.±1.5	34.3±2.3	24.3±2.3	24.3±2.3	
Nystatin								22.0±1.2

[a]STD-The positive control for bacteria-Chloramphenicol

4.5 Antimicrobial Activity of *A. caudatus, A. spinosus* and *A. hybridus* Solvent Extracts

The fractions showed activity to both gram-negative and gram-positive bacteria (Table 5a). There was a marked activity of the *A. hybridus* hexane extract on *E. coli, S. typhi* and *P. aeruginosa.* The extract fractions however, lacked anti-fungal activity against *C. albicans* as shown in Table 5a. All the *A. spinosus* fractions were inactive to all the test microbes except the fourth fraction of hexane on *S. typhi* which gave an inhibition zone of 13.0 ± 1.2 mm. The extract fractions from *A. caudatus* showed activity against *S. aureus, Bacillus spp, S. typhi* and *P. Mirabilis.* All the species did not have activity on the test fungus *C. albicans.*

Table 5a: Antimicrobial activity of *A. caudatus, A. spinosus* and *A. hybridus*

Microorganism	*Amaranthus spp*		Fraction	Inhibition zones in mm						MIC (mg/ml)	
				Extract concentration % (w/w)				[b]STD	[a]STD	E	[a]STD
Gram-negative				100	50	33	25				
E. coli	***A. hybridus***	Hexane	1	17.5±0.7	15±1.4	11±1.4	7.5±0.7	0	48.3±1.7	453	25
ATCC 25922		Ethyl acetate	1 & 2	0	0	0	0	0	48.3±1.7	0	25
		Dichloromethane	1 & 2	0	0	0	0	0	48.3±1.7	0	25
		Methanol	1 & 2	0	0	0	0	0	48.3±1.7	0	25
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	48.3±1.7	0	25
		Ethyl acetate	2	11.0±1.7	0	0	0	0	48.3±1.7	162.6	25
		Dichloromethane	1 & 2	0	0	0	0	0	48.3±1.7	0	25
		Methanol	1 & 2	0	0	0	0	0	48.3±1.7	0	25
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	48.3±1.7	0	25
		Ethyl acetate	1 & 2	0	0	0	0	0	48.3±1.7	0	25
		Dichloromethane	1 & 2	0	0	0	0	0	48.3±1.7	0	25
		Methanol	1 & 2	0	0	0	0	0	48.3±1.7	0	25
S. typhi	***A. hybridus***	Hexane	1	11.0±1.4	9.0±1.4	0	0	0	33.7±0.9	755	25
KEMRIc		Ethyl acetate	2	11.0±1.7	9.0±1.4	0	0	0	33.7±0.9	200	25
		Dichloromethane	1	9.5±1.7	7.5±3.3	0	0	0	33.7±0.9	344	25
		Methanol	1	17.5±2.0	15.0±1.4	0	0	0	33.7±0.9	0	25
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	33.7±0.9	0	25
		Ethyl acetate	1 & 2	0	0	0	0	0	33.7±0.9	0	25
		Dichloromethane	1	11.0±1.7	9.0±1.4	0	0	0	33.7±0.9	665	25
		Methanol	1	13.0±1.2	11.0±1.7	0	0	0	33.7±0.9	259.3	25
	A. spinosus	Hexane	3	13.0±1.2	11.0±1.7	0	0	0	33.7±0.9	129	25
		Ethyl acetate	1 & 2	0	0	0	0	0	33.7±0.9	0	25
		Dichloromethane	1 & 2	0	0	0	0	0	33.7±0.9	0	25
		Methanol	1 & 2	0	0	0	0	0	33.7±0.9	0	25
K. pneumoniae	***A. hybridus***	Hexane	1	13.0±1.2	11.0±1.7	0	0	0	37.7±1.5	566	22.5
KEMRIc		Ethyl acetate	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
		Dichloromethane	1	17.5±2.4	15.0±1.4	9.5±1.7	0	0	37.7±1.5	258	22.5
		Methanol	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	37.7±1.5	0	22.5
		Ethyl acetate	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
		Dichloromethane	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
		Methanol	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	37.7±1.5	0	22.5
		Ethyl acetate	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
		Dichloromethane	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
		Methanol	1 & 2	0	0	0	0	0	37.7±1.5	0	22.5
P. mirabilis	***A. hybridus***	Hexane	1 2 & 3	0	0	0	0	0	34.3±2.3	0	_
KEMRIc		Ethyl acetate	1 & 2	0	0	0	0	0	34.3±2.3	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	34.3±2.3	0	_
		Methanol	1 & 2	0	0	0	0	0	34.3±2.3	0	_
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	34.3±2.3	0	_
		Ethyl acetate	1 & 2	0	0	0	0	0	34.3±2.3	0	_
		Dichloromethane	1	13.5±1.5	11.0±1.7	9.5±1.5	0	0	34.3±2.3	449	_
		Methanol	1 & 2	0	0	0	0	0	34.3±2.3	0	_
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	34.3±2.3	0	_
		Ethyl acetate	1 & 2	0	0	0	0	0	34.3±2.3	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	34.3±2.3	0	_
		Methanol	1 & 2	0	0	0	0	0	34.3±2.3	0	_
P.aeruginosa	***A. hybridus***	Hexane	1 & 2	15.0±1.4	13.0±1.4	10.5±0.9	0	0	24.3±2.3	755	_
ATCC 27853		Ethyl acetate	1 & 2	0	0	0	0	0	24.3±2.3	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	24.3±2.3	0	_
		Methanol	1 & 2	0	0	0	0	0	24.3±2.3	0	_
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	24.3±2.3	0	_
		Ethyl acetate	1 & 2	0	0	0	0	0	24.3±2.3	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	24.3±2.3	0	_
		Methanol	1 & 2	0	0	0	0	0	24.3±2.3	0	_
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	24.3±2.3	0	_
		Ethyl acetate	1 & 2	0	0	0	0	0	24.3±2.3	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	24.3±2.3	0	_
		Methanol	1 & 2	0	0	0	0	0	24.3±2.3	0	_

Microorganism	*Amaranthus spp*		Fraction	Inhibition zones in mm						MIC (mg/ml)	
				Extract concentration % (w/w)				[b]STD	[a]STD	E	[a]STD
Gram-negative				100	50	33	25				
Gram-positive	***A. hybridus***	Hexane	1 2 & 3	0	0	0	0	0	37.7±1.5	0	31.3
S. aureus		Ethyl acetate	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
ATCC 25923		Dichloromethane	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
		Methanol	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	37.7±1.5	0	31.3
		Ethyl acetate	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
		Dichloromethane	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
		Methanol	1	15.0±1.2	13.5±1.5	11.0±1.7	7.5±3.3	0	37.7±1.5	155.6	31.3
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	37.7±1.5	0	31.3
		Ethyl acetate	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
		Dichloromethane	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
		Methanol	1 & 2	0	0	0	0	0	37.7±1.5	0	31.3
Bacillus spp	***A. hybridus***	Hexane	1 2 & 3	0	0	0	0	0	32.7±1.5	0	26.3
KEMRI c		Ethyl acetate	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
		Dichloromethane	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
		Methanol	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	32.7±1.5	0	26.3
		Ethyl acetate	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
		Dichloromethane	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
		Methanol	**1**	13.5±1.5	9.5±1.5	7.5±3.3	0	0	32.7±1.5	194.5	26.3
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	32.7±1.5	0	26.3
		Ethyl acetate	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
		Dichloromethane	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
		Methanol	1 & 2	0	0	0	0	0	32.7±1.5	0	26.3
Fungus	***A. hybridus***	Hexane	1 2 & 3	0	0	0	0	0	22.0±1.2	0	_
C. albicans		Ethyl acetate	1 & 2	0	0	0	0	0	22.0±1.2	0	_
KEMRI c		Dichloromethane	1 & 2	0	0	0	0	0	22.0±1.2	0	_
		Methanol	1 & 2	0	0	0	0	0	22.0±1.2	0	_
	A.caudatus	Hexane	1 2 & 3	0	0	0	0	0	22.0±1.2	0	_
		Ethyl acetate	1 & 2	0	0	0	0	0	22.0±1.2	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	22.0±1.2	0	_
		Methanol	1 & 2	0	0	0	0	0	22.0±1.2	0	_
	A. spinosus	Hexane	1 2 & 3	0	0	0	0	0	22.0±1.2	0	_
		Ethyl acetate	1 & 2	0	0	0	0	0	22.0±1.2	0	_
		Dichloromethane	1 & 2	0	0	0	0	0	22.0±1.2	0	_
		Methanol	1 & 2	0	0	0	0	0	22.0±1.2	0	_

Inhibition zones in mm was given as the mean of triplicate results ± the standard error

E-Represents the extract fraction

[a]**STD-** Chloramphenicol as the positive control

[b]**STD-** Sterile distilled water as the negative control

NB: Control experiment showed no inhibition. Chloramphenicol and nystatin were used as standards.

4.6 Minimum Inhibitory Concentrations for *A. caudatus, A. spinosus* and *A. hybridus*

Serial dilution of the active extracts was done to concentrations of 20%, 25%, 33% and 50% for MIC analysis. The concentrations of the active fractions were as follows:

Table 5b: The concentrations of the active fractions

Concentration	*Amaranthus spp.*	Solvent extract
0.4%,	*A. hybridus*	Ethyl acetate
0.23%	*A. hybridus*	Hexane
0.08%,	*A. caudatus*	Methanol
0.05%	*A. hybridus*	Dichloromethane
0.04%	*A. spinosus*	Hexane
0.04%	*A. caudatus*	Ethyl acetate
0.02%	*A. caudatus*	Dichloromethane

The minimum inhibitory concentration of the *Amaranthus* species active fractions varied with the type of micro organisms (Table 5a). The gram-negative bacteria had higher minimum inhibitory concentrations than the gram-positive bacteria. The MICs for *A. hybridus* varied between 200mg/ml and 755mg/ml. The *A. caudatus* methanol extract fraction on the other hand had much lower MIC concentration against the gram-positive *S .aureus* and *Bacillus spp.* of 155.6mg/ml and 194.5 mg/ml respectively. *A. spinosus* had 129mg/ml against the gram-negative *S. typhi* as in Table 5a.

4.7 T-Test for Active fractions of *A. caudatus, A. spinosus* and *A. hybridus*

4.7.1 T-Test results for active fractions from *A. spinosus*

The extract fractions of *A. spinosus* had anti-bacterial activity against *S. typhi* and *Bacillus spp*. However, it lacked anti-fungal activity against *C. albicans*. Their activity against these test microorganisms however showed a significant difference to the conventional standard antibiotic chloramphenicol (Table 6).

Table 6: T-Test results for active fractions from *Amaranthus spinosus*

Extract /standard	Fraction	Micro-organism	Mean	SD	T-value	Sign.
Hexane	4	*S. typhi*	12.0	2.0	11.8	0.007*
[a]Standard			33.7	1.5		
Protein	2	*Bacillus spp*	13.5	0.7	35	0.018*
[a]Standard			32.7	1.5		

P<0.05 *- significant difference in activity [a]Standard-Chloramphenicol

4.7.2 T-Test results for active fractions of *A. hybridus*

The extract fractions of *A. hybridus* had antimicrobial activity against *E. coli, S. typhi P. mirabilis* and *K. pneumoniae*. Their activity against these test microorganisms however showed a significant difference as to the conventionally used standard antimicrobial chloramphenicol. Hence, the antimicrobial activity of *A. hybridus* extracts at P<0.05 was significant as shown in Table 7. Comparing the means of inhibition zones of the active extracts with that of the positive control chloramphenicol using t-test shows clearly that, there is a significant difference though the extracts exhibit antimicrobial activity against the tested microorganisms of interest. This was true also for the protein extract of *A. hybridus* (Table 7).

Table 7: T-Test results for active fractions of *A. hybridus*

Extract /standard	Fraction	Micro-organism	Mean	Standard deviation	T-value	Sign.
Hexane	1	*E. coli*	14.7 ± 1.8	3.1	15	0.004*
[a]Standard			48.3 ± 1.7	2.9		
Hexane	1	*S. typhi*	16.3 ± 0.9	1.5	13	0.006*
[a]Standard			33.7 ± 0.9	1.5		
Ethyl acetate	2	*S. typhi*	10.7 ± 1.8	3.0	11	0.008*
[a]Standard			33.7 ± 0.9	1.5		
Dichloromethane	1	*S. typhi*	12.0 ± 1.2	2.0	12	0.007*
[a]Standard			33.7 ± 0.9	1.5		
Hexane	2	*P. mirabilis*	12.0 ± 1.2	2.0	15.58	0.004*
[a]Standard			24.3 ± 2.3	4.0		
Protein	2	*K. pneumoniae*	9.3 ± 0.6	1.2	14	0.005*
[a]Standard			41.7 ±.1.6	2.9		

P<0.05 * significant difference in activity [a]Standard–Chloramphenicol

4.7.3 T-Test results for active fractions of *A. caudatus*

The extract fractions of *A. caudatus* had antimicrobial activity against *E. coli, S. typhi P. mirabilis, K. pneumoniae* and *C. albicans*. Their activity against these test microorganisms however showed a significant difference to the conventionally used standard antibiotic chloramphenicol for bacteria and nystatin for the fungus *C. albicans*. Hence, the antimicrobial activity of *A. caudatus* extracts at P<0.05 was significantly different to those of the standards chloramphenicol and nystatin as illustrated in Table 8.

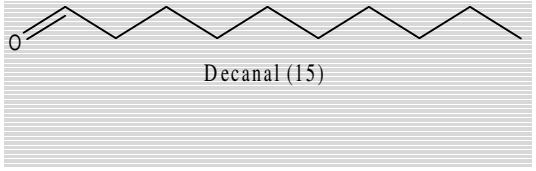
Table 8: T-Test results for active fractions of *Amaranthus caudatus*

Extract /standard	Fraction	Micro-organism	Mean	Standard. Deviation	T-value	Sign.
Methanol	1	*S .aureus*	9.7	2.5	24	0.002*
[a]Standard			37.7	2.5		
Methanol	1	*Bacillus spp*	12.0	2.0	62	0.000*
[a]Standard			32.7	1.5		
Ethyl acetate	1	*S. typhi*	10.0	2.0	71	0.000*
[a]Standard			33.7	1.5		
Dichloromethane	3	*S. typhi*	12.0	2.0	13	0.006*
[a]Standard			33.7	1.5		
Methanol	1	*S. typhi*	14.0	5.9	16	0.004*
[a]Standard			33.7	4.0		
Dichloromethane	3	*P. mirabilis*	7.7	1.5	7	0.022*
[a]Standard			34.3	4.0		
Methanol	1	*P. mirabilis*	18.3	2.5	5	0.035*
[a]Standard			34.3	2.5		
Protein	4	*K. pneumoniae*	17.7	2.1	8	0.015*
[a]Standard			37.7	2.5		
Protein	4	*S. typhi*	18	1.0	13	0.006*
[a]Standard			33.7	1.5		
Protein	4	*C .albicans*	10.0	1.0	3	0.097
[a]Standard			22.0	7.9		
P<0.05	*- significant difference in activity			[a]Standard-Chloramphenicol		

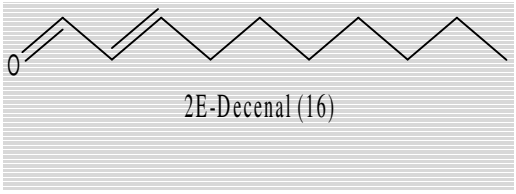
4.8 *Corriandrum sativum*

4.8.1 Chemical composition and active constituents of *Corriandrum sativum*

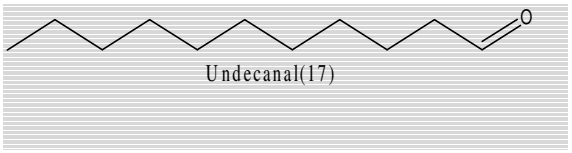
The colourless essential oil of *C. sativum* with a characteristic smell was obtained in 0.04% w/w yield equivalent to 0.212 g obtained from 554 g of fresh leaves. The chemical constituents

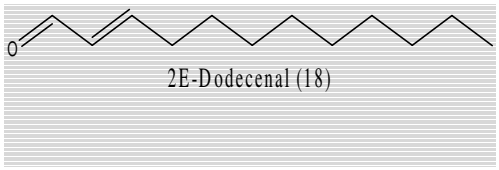


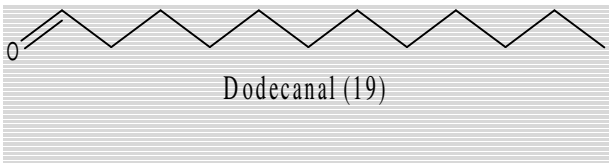
Decanal (15)

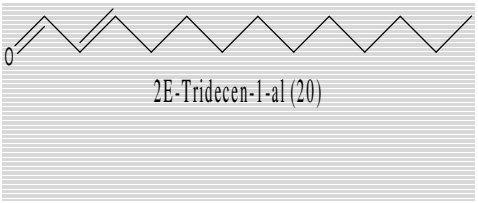


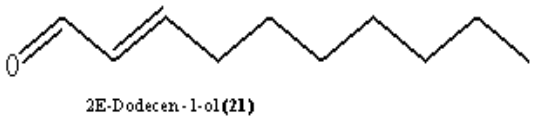
2E-Decenal (16)

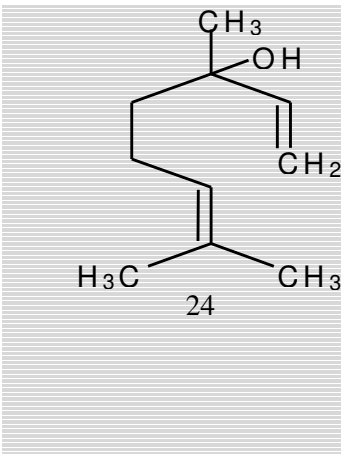


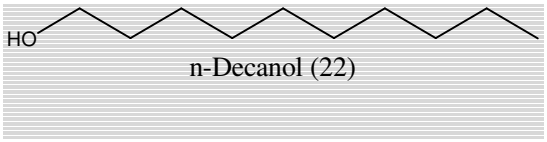


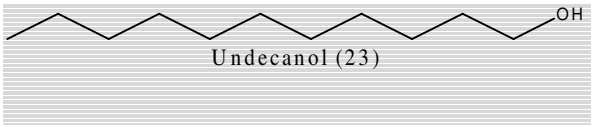












identified by GC-MS analysis, their retention times and relative amounts are summarized in Table 9. The compounds identified were 24 out of 27 peaks detected. The oil was dominated by aldehydes and the alcohols making 56.07% and 36.26% respectively. The major part of this oil was composed of volatile terpene hydrocarbons and a small portion by their oxygenated derivatives. The oxygenated monoterpenes accounted for only 1.09% of the essential oil. Considering components with concentrations more than 3%, the major components were: decanal (14.29%) (**15**), 2E-decenal (15.88%) (**16**), undecanal (3.23%) (**17**), 2E-dodecanal (6.23%) (**18**), dodecanal (4.36%) (**19**), 2E-tridecen-1-al (6.75%) (**20**), 2E-decen-1-ol (14.22%) (**21**), n-decanol (13.64%) (**21**) and undecanol (3.37%) (**22**). Oxygenated monoterpene present was linalool (**24**) which was 0.32% of the oil.

Table 9: Chemical composition of essential oil of *Corriandrum sativum*

	Compound	KI	Concentration %	Method of identification
	Monoterpenes			
1	α –Pinene	920	0.04	RI, GC-MS
2	Linalool	1096	0.32	RI, GC-MS
		Total	**0.36%**	
	Aldehydes			
3	n-Octanal	996	0.8	RI, GC-MS
4	Nonanal	1101	0.54	RI, GC-MS
5	2E-Hexanal	1195	0.12	RI, GC-MS
6	Decanal	1204	**14.29**	RI, GC-MS
7	2E-Decenal	1261	**15.88**	RI, GC-MS
8	Undecanal	1305	**3.23**	RI, GC-MS
9	Dodecanal	1406	**4.36**	RI, GC-MS
10	2E-Dodecenal	1464	**6.23**	RI, GC-MS
11	Tridecanal	1507	0.63	RI, GC-MS
12	2E-Tridececen-1-al	1567	0.56	RI, GC-MS
13	Tridecanal	1609	1.16	RI, GC-MS
14	2E-Tridecen-1-al	1669	**6.75**	RI, GC-MS
15	3-Dodecen-1-al	1774	0.91	RI, GC-MS
		Total	**55.46%**	
	Alcohols			
16	Octanol	1067	0.15	RI, GC-MS
17	Nonanol	1170	0.38	RI, GC-MS
18	2E-Decen-1-ol	1268	**14.22**	RI, GC-MS
19	n-Decanol	1272	**13.64**	RI, GC-MS
20	Undecanol	1362	**3.37**	RI, GC-MS
21	trans-2-Undecen-1-ol	1368	2.12	RI, GC-MS
22	n-Undecanol	1372	2.38	RI, GC-MS
		Total	**36.26%**	
	Alkanes			
23	Nonane	860	1.21	RI, GC-MS
24	n-Decane	992	0.25	RI, GC-MS
		Total	**1.46%**	
	TOTAL		**92.74%**	
	KI-Kovat index		**RI–Retention index**	

4.8.2 Antimicrobial activity of the essential oil of *C. sativum*

The oil showed a broad spectrum activity against the test micro organism (Table 10). It had more activity on the gram-positive bacteria and the fungus than to the susceptible gram-negative bacteria. The oil however was ineffective to *Pseudomonas aeruginosa*.

Table 10: Antimicrobial activity of the essential oil of *Corriandrum sativum*

Micro organism	Inhibition (mm)							MIC mg/ml	
								EO[b]	STD[a]
Gram-negative	Essential oil (µg x 10^2)								
	65	32.5	21.7	16.3	13	10.8	STD[a]		
E. coli	14.0±0.6	12.0±0.6	9.0±0.6	0	0	0	48.3±1.7	163	25
ATCC 25922									
S. typhi	11.0±3.3	9.0±3.3	8.0±3.3	7.5±3.3	0	0	33.7±0.9	130	25
KEMRI[c]									
K. pneumoniae	22.0±1.2	17.0±0.9	13.0±0.6	0	0	0	41.7±1.7	163	22.5
KEMRI[c]									
P. mirabilis	19.0±0.6	13.7±0.3	0	0	0	0	34.3±2.3	217	_
KEMRI[c]									
P. aeruginosa	0	0	0	0	0	0	24.3±2.3	0	_
ATCC 27853									
Gram-positive									
S. aureus	30.7±0.6	21.7±0.9	16.0±1.2	11.0±0.9	10.0±0.6	0	37.7±1.5	108	31.3
ATCC 25923									
Bacillus spp	33.0±0.4	21.0±0.9	18.5±0.7	13.0±0.3	8.0±0.6	0	32.7±1.5	108	26.3
KEMRI[c].									
Fungus									Nystatin
C. albicans	21.0±1.7	17.0±0.9	13.0±0.6	0	0	0	22.0±1.2	163	_
KEMRI[c]									

a Chloramphenicol-30µg b Essential oil c Clinical isolates from Kenya Medical Research Institute (KEMRI).

NB: Control experiment showed no inhibition. Chloramphenicol and nystatin were used as standards.

4.8.3 Minimum inhibitory concentration of the essential oil of *Corriandrum sativum*

The minimum inhibitory concentrations for the oil varied according to the type of micro organism. The MICs for the gram-negative bacteria were higher than those of the gram-positive bacteria. The MICs for the gram-negative bacteria were between 130mg/ml and 217mg/ml. The gram-positive bacteria had similar minimum inhibitory concentration of 108mg/ml. The fungus on the other hand had a higher concentration than that of the gram-positive bacteria. All the concentrations were much higher in comparison to the positive standard antimicrobial chloramphenicol (Table 10). The active ingredients composed 92.74% of the oil which was obtained at 0.04% (w/w).

4.8.4 T-Test for the essential oil of *Corriandrum sativum*

There was a significant difference in antimicrobial activity of the oil and the standard chloramphenicol to the gram-negative bacteria. The activity of the oil was statistically not significant to the gram-positive bacteria and the fungus *C. albicans* (Table 11).

Table 11: T-Test results of antimicrobial activity of the essential oil of *C. sativum*

E. oil /standard	Micro-organism	Mean	S. deviation	T-value	Sign.
Gram-negative					
Essential oil	*E. coli*	14.0	1.0	19	0.003*
[a]Standard		48.3	2.9		
Essential oil	*S. typhi*	11.7	0.5	21	0.002*
[a]Standard		33.7	1.5		
Essential oil	*K. pneumoniae*	22.0	2.0	22	0.002*
[a]Standard		41.7	2.9		
Essential oil	*P. mirabilis*	19.0	1.0	41	0.001*
[a]Standard		34.3	4.0		
Essential oil	*P. aeruginosa*	0	0	0	0*
[a]Standard		24.3	2.0		
Gram-positive					
Essential oil	*S .aureus*	30.7	1.2	0.2	0.885
[a]Standard		37.7	2.5		
Essential oil	*Bacillus Spp*	33.7	2.1	0.9	0.461
[a]Standard		32.7	1.0		
Fungus					
Essential oil	*C .albicans*	21.0	2.0	0.23	0.840
Nystatin		22.0	2.1		

P<0.05 *- significant difference in activity [a] **Standard** - Chloramphenicol

4.9 Discussion

Different communities in Kenya use a wide variety of indigenous plants for food. About 850 species of plants are used for food. To this group belong *Amaranthus* species that has important nutrients and a rich array of biologically active secondary metabolites. In Kenya, the rural poor population are the major consumers of indigenous vegetables with the middle class and the rich shunning from such traditional food. The plants in this study were obtained from Shinyalu in Kakamega district of the Western province, Ahero Kisumu and Egerton University in Nakuru district of the Rift valley province of Kenya. The districts have a high agricultural potential and these plants are grown organically in small scale as part of the practiced mixed farming. The objective of the study was to determine the role of indigenous vegetables and spices in primary health care. These vegetables are usually cooked along with a spice to add aroma and flavour hence their study with *C. sativum.* The health benefits were studied by testing the antimicrobial activity of *A. hybridus, A. caudatus, A. spinosus* and *C. sativum.*

Although chemical and pharmacological studies of *C. sativum* essential oils have been carried out, most of the studies have mainly used the seeds/fruits (Lo Cantro *et al*., 2004). However, pharmacological studies on *C. sativum* leaves, the part of the plant most consumed by humans is limited. At the same time, available studies have assessed antimicrobial activity against few microorganisms (Wong and Kitts, 2006). *Amaranthus* species has a wider mode of consumption varying from community to community. It is eaten as salad in Mozambique (Oliveira, 1995) or pureed into a sauce and served over (farinaceous) vegetables in West Africa (Martin, 1999). In Kenya, their leaves are eaten as green vegetables. For the observed activity to be retained these plants should be taken raw as salads. The medicinal value of these vegetables adds value to their nutritional composition. Naturally occurring substances form a significant base of raw materials for chemical and pharmaceutical industries as starting points for a series of their products. The active components contained in these plants are useful, safe and effective. Natural products have not been associated with antimicrobial drug resistance (Bhattacharjee *et al.,* 2005). This therefore eliminates concerns of continued use of these vegetables in different quantities.

The phytochemicals found to be present in *A. hybridus, A. caudatus* and *A. spinosus* included tannins, phlobatannins, steroids, terpenoids, cardiac glycosides saponins and flavonoids. These phytochemicals are known to be biologically active hence the observed antimicrobial activity

against the test microbes. It is well established that some plants contain compounds that inhibit microbial growth (Cowan, 1999). These plant compounds have different structures and different action when compared with antimicrobials conventionally used to control microbial growth and survival (Nascimento *et al.,* 2000). The potential antimicrobial properties of plants are related to their ability to synthesise by secondary metabolism several chemical compounds of relatively complex structures with antimicrobial activity. These phytochemicals include: flavonoids, tannins, coumarins, glycosides, terpenes, steroids, organic acids and phenylpropanes (Kessler *et al*., 2003).

Screening for different phytochemicals in *Amaranthus* showed that this genus is rich in an array of phytochemicals. Synergistic interaction of these phytochemicals could contributed to the observed antimicrobial activity. The phytochemical analysis of the *A. hybridus* extracts revealed the presence of tannins, phlobatannins, flavonoids, steroids, terpenoids and cardiac glycosides. However saponins were absent. These extracted compounds are known to be biologically active (Nascimento *et al.,* 2000). Tannins have been found to form irreversible complexes with proline-rich proteins resulting in the inhibition of the cell protein synthesis. Apart from antimicrobial activity exhibited by tannins, they also react with proteins to provide the typical tanning effect. Medicinally, this is important for the treatment of inflamed or ulcerated tissues (Mota *et al.,* 1985). Tannins are stable and potent antioxidants (Trease and Evans, 1983). Herbs that have tannins as their main component are used for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003).

Flavonoids present in *A. hybridus, A. caudatus* and *A. spinosus* are a group of polyphenolic compounds. Polyphenolics are known for free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Biological actions of these compounds are related to their antioxidant activity (Kessler *et al*., 2003). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process (Kessler *et al*., 2003).

Most phytochemicals of *A. spinosus* were present in the extract fractions of extreme polarities; hexane and methanol with hexane being the least polar and methanol the most polar solvent used. Saponins and cardiac glycosides were present in the *A. spinosus* have

pharmacological properties. This was in agreement with the Assiak *et al* (2001) report where leaf extracts of *A. spinosus* contained cardiac glycosides and saponins. Its extracts are used in the treatment of menstrual disorders in man (Assiak *et al.,* 2001). Saponins help to reduce the level of LDL cholesterol circulating in the body and are useful in the human diet for controlling cholesterol. However, some saponins including those produced by the soap berry are poisonous if swallowed and can cause urticaria or skin rash in many people (Assiak *et al.,* 2001).

The solvent extract fractions of *A. spinosus* had no activity on the test microorganisms of interest except on *S. typhi*. The yield collected for this active fraction was 0.04% (w/w). This activity was attributed to the presence of flavonoids, steroids, terpenoids and cardiac glycosides in the sample fraction. It had a MIC of 566mg/ml. *A. caudatus* was dominated by all the screened phytochemicals. The methanol fraction was rich in tannins, phlobatannins, flavonoids, steroids, terpenoids and cardiac glycosides were active on *S. typhi*, *S. aureus* and *Bacillus spp*. The yield collected for this active fraction was 0.08% (w/w). The ethyl acetate extract showed anti-bacterial activity on *E. coli*. Dichloromethane extract rich in flavonoids, steroids and cardiac glycosides had anti-bacterial activity on *S. typhi* and *P. mirabilis*. The yield collected for these active fractions were 0.04% (w/w) and 0.02% (w/w) respectively.

Cardiac glycosides are an important class of naturally occurring drugs whose actions are beneficial on the heart. Cardiac steroids are widely used in the modern treatment of congestive heart failure and for treatment of arterial fibrillation and flutter (Nascimento *et al.,* 2000). They are composed of the sugar/glycoside and the non-sugar/aglycone or steroid moieties and two classes have been observed in nature. These are the cardenolides that have an unsaturated butyrolactone ring and the bufadienolides having α-pyrone ring (Consentino *et al.,* 1999).

Antimicrobial proteins and peptides in plants have most commonly been discovered in seeds, where they accumulate to high levels and may also function as storage proteins. Homologues of the seed proteins have been found subsequently at much lower concentrations in vegetative and floral tissues (Bohlmann *et al.,* 1988; Terras *et al*., 1995). Vegetative protein extracts from *Amaranthus caudatus, A. spinosus* and *A. hybridus* showed a favourable antimicrobial activity against *E. coli, K. pneumoniae, S. typhi, P. mirabilis, S. aureus, Bacillus spp* and *Candida albicans.* The protein extracts of *A. hybridus* however showed a broad spectrum activity against both the gram-positive and the gram-negative bacteria except *Pseudomonas aeruginosa* that was

resistant to the protein extract fractions of *A. hybridus*. This case was similar to the *A. caudatus* protein extract which had activity against *Klebsiella pneumoniae, Salmonella typhi* and *Candida albicans*.

The MICs for *A. hybridus* was higher for the hexane extract against the gram-negative bacteria compared to the ethyl acetate and dichloromethane extracts of *A. hybridus* and *A. caudatus* against the same sensitive gram-negative bacteria which can be attributed to their differences in their structure. The gram-negative bacteria posses a lipopolysacharide that is important in the pathogenesis of some diseases. The gram-negative bacteria lipopolysacharide layer which hinder absorption of hydrophobic molecules and thus limit entry of such molecules (Consentino *et al*., 1999; Deferera *et al*., 2000 and Bhattacharjee *et al*., 2005). The *A. caudatus* methanol extract fraction on the other hand had much lower MIC concentration against the gram-positive *S. aureus* and *Bacillus spp.* that was comparable to the hexane extract fraction of *A. spinosus* against the gram-negative *S. typhi*. The MIC for *Amaranthus* spp. extracts against the gram-negative bacteria ranged between 200-755 mg/ml and 194 mg/ml for the gram-positive bacteria.

The isolated essential oil from *C. sativum* was rich in aldehydes and alcohols as was revealed by GC-MS analysis The activity of the *C. sativum* oil on the other hand would be expected to relate to the respective composition of the plant volatile oil, the structural configuration of the chemical constituents of the oil, their functional groups and possible synergistic interactions of the components. A correlation of the antimicrobial activity of essential oil, percentage composition and chemical structure of the components, functional groups and configuration has been done and a number of observations in this study have been suggested (Ultee *et al*., 2002).

Aldehydes which formed the major component of the oil are known to possess powerful antimicrobial activity. The aldehydes present in the oil were 56.07% of the oil composition. It has been proposed that an aldehyde group conjugated to a carbon double bond is a highly electronegative arrangement, suggesting that increase in electro negativity increases the anti-bacterial activity that may explain the observed biological activity (Ultee *et al*., 2002). Such electronegative compounds interfere with biological processes involving electron transfer and react with vital nitrogen compounds such as proteins and nucleic acids, and therefore inhibit the growth of the micro organism (Pattnaik *et al.,* 1997).

The oil had alcohols known to possess bactericidal rather than bacteriostatic activity against vegetative cells (Sartoratto *et al.,* 2004). Alcohols exhibit activity against micro-organism by potentially acting as either protein denaturing agents or solvent dehydrating agents (Bhattacharjee *et al.,* 2005). Minor monoterpene alcohol, linalool (19) exhibits wide range of anti-bacterial and anti-fungal activity (Pattnaik *et al.,* 1997). Linalool is known to inhibit spore germination and fungal growth. The inhibition of sporulation is due to respiratory suppression of aerial mycelia. Since alcohols are known to possess bactericidal rather than bacteriostatic activity against vegetative cells, the tertiary alcohol linalool is active against the test micro-rganisms potentially acting as a protein-denaturing agent or as a solvent dehydrating agent (Knobloch *et al.,* 1989). Previous studies have also shown that secondary alcohols such as 2-octanol, *L*-menthol and tertiary alcohols like linalool possesses a markedly lower anti-fungal activity as compared to primary alcohols (Knobloch *et al.,* 1989).

α-Pinene, which was found in appreciable amounts in the oil of *C. sativum* exhibit anti-fungal activity (Magiatis *et al.,* 1999). Therefore the remarkable anti-fungal activity of *C. sativum* oil could be due to the presence of α-pinene since it is known that some components of the essential oil exhibit higher antimicrobial activity than other compounds. However, interaction between essential oil components has been documented to play an important role in the determination of antimicrobial activity (Consentino *et al.,* 1999).

Other terpene hydrocarbons such as n-decane have less antimicrobial activity than phenols and alcohols when used alone. This is because they lack a hydroxyl group, which is thought to play an important role in the antimicrobial activity (Ultee *et al*., 2002). Hydroxyl groups in phenols are related to their toxicity to the micro organism, with evidence that increased hydroxylation results in increased toxicity (Consentino *et al.,* 1999). In addition highly oxidized phenols have more inhibitory action against microbial growth (Cowan, 1999). The mechanism for phenolic toxicity to micro organism is by either enzyme inhibition by the oxidized compounds through reaction with sulfhydryl side groups in the enzyme or through non-specific interaction with the proteins thus resulting in their inactivation (Geissman, 1963).

The activity of the *C. sativum* oil varied with concentration and kind of bacteria. Although the concentrations of the oil were generally in the range of 100 times more than the standard antimicrobials, they showed marked anti-bacterial and anti-fungal activities as evidenced by their

zones (Table 10). This difference in concentrations of the essential oil and the standard antimicrobials can be explained in terms of the fact that the active components in the oil compromise of only a fraction of the oil used. It is important to note that if the active components were isolated and purified, they would probably show higher antimicrobial activities than observed here.

Research has shown that gram-negative bacteria are less susceptible to essential oils than gram-positive bacteria because of their outer membrane surrounding the cell membrane which restricts diffusion of hydrophobic compounds through its lipopolysacharide covering (Sartoratto *et al.,* 2004). This explains why the gram-positive bacteria *Bacillus spp* and *S. aureus* and fungus *Candida albicans* were more susceptible than the gram-negative bacteria. Among the gram-negative bacteria, the *C. sativum* oil was much active against *K. pneumoniae* and *P. mirabilis* but resistant to *P. aeruginosa. P. aeruginosa* has also been observed to be resistant to the essential oil of other plants such as *Salvia verbanica* (Tawfeq, 2000), *Achillea holosericea* (Magiatis *et al*., 1999) and *Stachys species* (Skaltsa *et al*., 2003). The best activity was however, observed for the gram-positive bacteria. The minimum inhibitory concentrations of the gram-positive bacteria were therefore lower than those of the gram-negative bacteria.The MIC of the oil for gram-negative bacteria ranged from 130-217mg/ml and 108 mg/ml for both gram-positive bacteria. The MIC for the fungus *C. albicans* was 163 mg/ml. The MIC values for chloramphenicol ranged from 22.5- 31.3 mg/ml. In general, the oil showed greater anti-bacterial activity than anti-fungal activity (Table 10)

The chemical composition of the essential oil was, however, different from that observed in Tunisia plant materials (Msaada *et al*., 2007). Indeed in the Tunisia study the predominant aldehyde was 2E-dodecenal while in the current study was 2E-decenal (16). A confirmation that the composition of essential oils is dependent on such characteristics as the geographic character of the location from which the plant is obtained, seasonal variations and climate, production techniques and purity, the effect of plant maturity at the time of harvesting and the existence of chemotypic differences can also drastically affect the composition suggesting that ecological condition and/or physiological states could interfere with the presence of biologically active compounds in the plant (Cornu *et al*., 2001).

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

From this study the following conclusions were made:

- The vegetables of *A. hybridus, A. caudatus* and *A. spinosus* contain phytochemicals with antimicrobial activity and can be used in management of various ailments. Naturally occurring substances form a significant base of raw materials for chemical and pharmaceutical industries as starting points for a series of their products. The compounds would get into the arsenal of pharmaceuticals and form the structural basis or lead compounds to formulate new and more potent antimicrobial drugs from natural products that are readily available.
- The vegetables of *A. hybridus, A. caudatus* and *A. spinosus* are rich in biologically active phytochemicals especially the steroids, terpenoids and cardiac glycosides thus are of medicinal value.
- Traditional vegetables are accessible and technologies for utilizing them are available within the communities. Production and utilization can be achieved with minimum technological and financial inputs hence a major source of income for local people especially women as they are the major producers, processors and sellers thus reducing the current widespread poverty levels.
- The essential oils of *C. sativum* are rich in aldehydes and alcohols that are responsible for their antimicrobial activity.
- The essential oils of *C. sativum* are more active to the gram-positive bacteria and fungus than is to the gram-negative bacteria.
- *C. sativum* oil has a high concentration of medicinal ingredients which would reduce the concentration costs of the extracts.

The discovery of medicinal value of these plants would safeguard against the drawbacks of widespread drug resistance should these plants go into pharmacognostic development

5.2 Recommendations

These plants are useful, safe and effective in management of various ailments. Therefore the following recommendations were made.

- Research could be carried out on isolation and identification of individual compounds using separation and spectroscopic techniques such as UV, IR and NMR in *A. hybridus, A. caudatus* and *A. spinosus* and the essential oils of *C. sativum.* Subsequent analysis of anti-microbial activity because the compounds would get into the arsenal of pharmaceuticals and form the structural basis/ lead compounds to formulate new and more potent antimicrobial drugs from natural products that are readily available.
- A comparative study could be carried out on antimicrobial activity of the aqueous extracts of *A. hybridus, A. caudatus* and *A. spinosus* to establish the degree of loss of activity on boiling.
- Essential oils could have greatest potential use as food preservatives since they have been known to inhibit bacteria, fungi and yeast, therefore research on this could be carried out.

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