**PHYTOCHEMICAL COMPOSITION AND ANTIBACTERIAL EFFECT OF *Sida cuneifolia* Vollesen ON SELECTED PATHOGENIC BACTERIA**

**BY**

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# DECLARATION

**Declaration by the candidate**

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# DEDICATION

To my Mother, Winnie Cheruiyot, sister, Brenda Chepngetich and my friends

## ABSTRACT

During the last decade, the use of traditional medicine has escalated globally. With the increase in antibiotic resistance, there is need for increased search for new plants that possess antimicrobial properties. *Sida cuneifolia* is a woody shrub that is used traditionally to treat several ailments caused by enterobacteriaceae and *Staphylococcus spp*, yet its medicinal properties are not well researched. Conventional treatment of enteric and opportunistic infections is by use of antibiotics but of late these causal microorganisms have developed resistance to most antibiotics. The objectives of this study were to determine the efficacy of *S. cuneifolia* plant parts against *E. coli, Shigella, S. aureus* and *Salmonella typhi*, evaluate the best extraction solvent and to determine the various phytochemicals found in the plant, in the lab. The Kirby baueur disk method was used to determine the efficacy of the extract. The phytochemicals in the plant were determined using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Leaves were the most effective plant part against the pathogens followed by the stems. Generally, ethanol extracts exhibited higher activity against *S. aureus, S. typhi* and *Shigella* compared to the aqueous extracts. However, leaf aqueous extracts had the highest antimicrobial activity against *S. typhi*. The roots and stem ethanol extracts exhibited the lowest minimum inhibitory concentration (MIC) of 0.001 µg/ml each against *S. typhi*. The leaves ethanol extracts had a MIC of 0.01 µg/ml against *Shigella* and *S. aureus.* This was similar to the leaf aqueous extracts against *S. typhi,* stem ethanol extracts against *Shigella* and root ethanol and aqueous extracts against *S. aureus* and *Shigella.* The TLC results indicated that all the tested phytochemicals were present in the leaf, root and stem ethanol extracts except alkaloids in the stem. The alkaloids and flavanoids were absent in the aqueous extracts. Further analysis using HPLC showed caffeic acid, gallic acid and flavanoles 4’OH,5’OH as the common phytochemicals present in all the extracts. *Sida cuneifolia* has antimicrobial activities against *S. aureus, S. typhi* and *Shigella*. Ethanol leaf extracts of the plant may be exploited for treatment of infections caused by the three microorganisms.

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## LIST OF ABBREVIATIONS

AIDS – Acquired Immunodeficiency Virus

ATCC - American Type Culture Collection

CMR - Centre of Microbiology Research

CTMDR - Centre for Traditional medicine and Drug Research

CLSI - Clinical and Laboratory Standards Institute

CYP - Cytochromes P450

DMSO - Dimethyl sulphoxide

EAEC - Enteroaggregative *E. coli*

EIEC - enteroinvasive *E.coli*

ETEC - Enterotoxigenic *E.coli*

FDA - Food and Drug Administration

GC - Gas Chromatography

GCMS - Gas chromatography Mass Spectrophotometry

GRAS - Generally Recognized as Safe

HCW - Healthcare workers

HIV - Human Immunodefiency Virus

HP - Hewlett Packard

HPLC - High Performance Liquid Chromatography

IZD - Inhibition zone diameter

JICA - Japanese International Corporation Agency

KEMRI - Kenya Medical Research Institute

LPS - Lipopolysaccharide

MDR - Multi drug resistant

MHA - Müller-Hinton agar

MIC - Minimum inhibitory concentration

MRSA - methicillin-resistant *S. aureus*

MS - Mass Spectrophotometer

PBP - penicillin binding proteins

RIZD - Relative inhibition zone diameter

STEC - Shiga-toxin *E. coli*

TLC - Thin Layer Chromatograph

WHO - World Health Organization

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Background Information

During the last decade, the use of traditional medicine has expanded globally and has gained popularity with time. It has not only continued to be used for primary health care of the poor in third world countries, but has also gained popularity in countries where conventional medicine is the main method of treatment in the health care system (Zhang, 2000). A major part of the total population in developing countries still use traditional medicine obtained from plant resources. Estimation by the World Health Organization (WHO) have shown that more than 80% of the 4 billion world population lives in rural areas, and most of these people rely on herbal medicine as their primary health care source. The study on properties and uses of medicinal plants is increasing and eliciting growing interests. In recent years the interest to evaluate plants possessing antibacterial activity for various diseases has grown (WHO, 2003).

Plants are known to be rich sources of antibacterial agents because they produce a wide array of bioactive molecules. These phytochemicals are thought to have evolved with time as chemical defense against predation or infection, and are obtained by extraction using organic and inorganic solvents (Modi *et al*., 2012).

*Sida cuneifolia,* the plant in this study, is used as herbal remedy, and has been reported to be useful in the management of 12 disease conditions. The plant is used to disinfect the umbilical cord, wounds and in clearing stomach contents of postpartum mothers and their infants in Busoga region, Uganda (Ogwal, 1996; Nalubega, *et al*., 2013). The Sabaot people who live around Mt. Elgon in Kenya chew the roots to cure sore throat (Okello *et*

*al*., 2010). One or more species of *Sida* are likely to be found in most areas of Kenya except for very dry deserts (Vollesen, 1986).

The human gut is the natural habitat for a large bacterial community, but not all these bacterial populations have been described (Eckburg *et al.,* 2005). Major functions of the gut micro flora include metabolic activities that salvage energy and absorbable nutrients, important trophic effects on intestinal epithelia and on immune structure and function, and protection of the colonized host against invasion by invading microbes (Guarner, 2006). Gut flora is also an essential factor in disorders such as organ failure, colon cancer, and inflammatory bowel diseases. Nevertheless, bacteria are also useful in promotion of human health. Probiotics and prebiotics are known to have a role in prevention or treatment of some diseases (Guarner & Malagelada, 2003).

Enteric bacteria are facultative anaerobic, gram-negative rods that are, if motile, peritrichously flagellated. Morphologically, they are rods. This is an important bacterial group because of the fact that they inhabit the intestinal tracts of humans and other animals. Most enteric bacteria are active fermenters of glucose and carbohydrates (Tortora *et al*., 2006). Because of the clinical importance of enterics, there are many techniques to isolate and identify them. Enteric bacteria have fimbriae that help them adhere to surfaces or mucous membranes. Specialized sex pili aid in the exchange of genetic information between cells, which often includes antibiotic resistance. Enterics, like many bacteria, produce proteins called bacteriocins that cause the lysis of closely related species of bacteria. Bacteriocins may help maintain the ecological balance of various enteries in the intestines (Wilson *et al*., 2002).

*Escherichia coli* is an intestinal micro flora of humans. It can be a very dangerous pathogen (Kaper *et al*., 2004). It is one of the most diverse microorganisms in the human intestinal tract. Though it is normally harmless, certain strains can be pathogenic. The pathogenic strains produce toxins that cause gastrointestinal disturbances, collectively termed *E. coli* gastroenteritis. The cause of most cases is a strain usually referred to as Enterotoxigenic *E. coli* (ETEC). ETEC produces an enterotoxin that causes a watery diarrhea resembling mild cases of cholera. Enteroaggregative *E. coli* (EAEC) is another strain that is increasingly being recognized as a cause of diarrhea (Nataro & Kaper, 1998).

*Shigella flexneri* is a Gram-negative, aerobic, bacteria in the genus *Shigella* (Ewing, 1949), causing diarrhea in humans (Zychlinsky *et al.,* 1992). There are four species of *Shigella* i.e. *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* that cause shigellosis. *Shigella spp*. causes dysentery by invading the colonic mucosa. The pathogen multiplies within colonic epithelial cells, causing cell death. It spreads laterally infecting and killing adjacent epithelial cells, causing destruction and bleeding in the end (Kotloff *et al*., 1999). Shigellosis, the disease is characterized by loose stool with blood and mucus, accompanied with fever, and abdominal pains. It is also associated with complications such as haemolytic uraemic syndrome (Sur *et al.*,, 2004). *Salmonella* t*yphi,* a Gram negative bacteria causes typhoid fever (Crump.*et al.*, 2004). Typhoid incidence globally is about 200,000 deaths a year (Dougan & Baker, 2014). Symptoms of typhoid are abdominal discomfort, fever, and headache, constipation accompanied by diarrhea,

malaise and rash. Human beings are the only known natural reservoir. *Salmonella typhi* is transmitted through ingestion of water or food that has been contaminated by feces or urine of the pathogen carriers (Bhunia *et al*., 2009).

*Staphylococcus aureus* is one of the causative agents of nosocomial infections. In sub-Saharan Africa, bacteraemia is one of the major causes of illness and death in children. The overall risk of nosocomial bacteraemia is 5·9 per 1000 admissions (Aiken *et al*., 2011). One major concern is methicillin-resistant *S. aureus* (MRSA), which is now an issue though it was rare a decade ago (Cushnie & Lamb, 2005). The increasing resistance of this bacterial pathogen to various antibiotics has led to complications in the treatment of *S aureus* infections (Wertheim *et al*., 2005). It has been known to be more resistant then Gram-negative bacteria (Dorman & Deans, 2000). With the rising antibiotic resistance, bacterial isolates have shown multiple resistance patterns to different combination of antibiotics. This may result to a negative effect on antibiotic therapy for infectious diseases and the need for further investigation for new plants that have antimicrobial properties (Akhter *et al*., 2014).

Phytochemicals are bioactive compounds that promote health. These substances range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, processed foods and beverages. They have a tremendous impact on the health care system and may provide medical health benefits including the prevention or treatment of diseases and physiological disorders (Raskin *et al*., 2002). Majority of foods, such as whole grains, beans, fruits, vegetables and herbs contain

phytochemicals. These phytochemicals, either alone or in combination, have tremendous therapeutic potential in curing various ailments. They have anti-inflammatory, anti-allergic, antioxidants, antibacterial, antifungal, antispasmodic and chemo-preventive effects (Prakash *et al*., 2012). In this study, the efficacy of *Sida cuneifolia* crude extracts in controlling the growth of selected enteric bacterial pathogens was determined using different plant parts and different solvents for extraction.

## 1.2 Statement of the Problem

Diarrheal diseases caused by pathogenic bacteria are a major public health problem in Africa, where they cause the death of 1.8 million children less than 5 years annually (Ahs *et al*., 2010). *E. coli, Salmonella typhi* and *Shigella* are major causative agents for diarrheal diseases and together with *Staphylococcus aureus* cause food poisoning (Mead *et al.,* 1999). Diarrhea causes 16 % of deaths among children below five years in Kenya and in most rural public health facilities and is the third leading cause of outpatient cases (Njuguna & Muruka, 2011). Treatment has mainly been by oral rehydration therapy and use of antibiotics such as quinolones, macrolides, ampicillin and third-generation cephalosporins (Hof, 2003; Citron *et al.,* 2007). Recently, antimicrobial resistance has been reported for *E. coli, Salmonella typhi*, *Shigella* and *Staphylococcus aureus* because of abuse of these drugs and misdiagnosis, (Rowe *et al.,* 1997; Wiener *et al.,* 1999; Sivapalasingam *et al.,* 2006; Ben-David, *et al.,* 2008), hence, the need for development of novel methods for treatment.

*Staphylococcus aureus* infections spread from patient to patient, are a significant problem in hospitals and they are responsible for food poisoning outbreaks (Forbes & Schaberg,

1983; Vitale *et al.,* 2015). Use of appropriate oral antibiotics like amoxicillin/ clavulanate, cephalexin, clindamycin, is used in the treatment of *S. aureus* infections. One of the strains of *S. aureus*, methilicin resistant *S. aureus* (MRSA) is a worldwide problem in health. It is highly resistant to conventional antibiotics (Muto *et al.,* 2003; Ben-David, *et al.,* 2008), with its occurrence in hospitals being relatively high in Nigeria, Kenya, and Cameroon having its rates ranging from 21-30% (Kesah *et al.,* 2003).

With the development of resistance to antibiotics by many strains of *E. coli, Salmonella typhi*, *Shigella* and *Staphylococcus aureus,* there is need for alternative methods of treatments. Thus, need for fast and rapid research on plants with medicinal properties (Alanis, 2005). The present study sought to establish the efficacy of *S. cuneifolia*, for the control of *Escherichia coli, Salmonella typhi*, *Shigella* and *Staphylococcus aureus.*

## 1.3 Justification

Enterics are known to cause human and animal health problems. Antimicrobial resistance is becoming increasingly important in the treatment of enteric infections, particularly those due to *Shigella*, enterotoxigenic *E. coli* and *Salmonella typhi*. *Staphylococcus aureus* is known to develop high levels of resistance to antimicrobial agents, is responsible for wound and skin infections, respiratory infections and food poisoning. The rate of antimicrobial resistance is highest in the developing world, where the use of antimicrobial drugs is relatively unrestricted. Diarrhea that is caused by enteric pathogens is one of the most common causes of mortality in infants in developing countries. Of greatest immediate concern is the need for an effective, inexpensive antimicrobial that can be used safely as treatment for small children with dysentery due to the four bacteria. Herbal medicines may offer alternative treatment options. Natural antibiotics act in a

unique mechanism – in addition to their bactericidal action. They stimulate the immune system, exhibit anti-inflammatory and anti-septic properties that inhibit the growth of bacteria as well as scavenge them. The increasing cases of antibiotic resistance calls for the need to develop alternative medicine for the control of pathogenic bacteria.

*Sida cuneifolia* has been used by many communities to treat a number of ailments yet its action has not been elucidated scientifically. There is need to further investigate the antimicrobial activity of *S. cuneifolia* against *Salmonella typhi, Shigella, Esherichia coli* and *Staphylococcus aureus* as an alternative to conventional drugs and hopefully tackle the emerging issue of antibiotic resistance. Traditionally, water is used as the main solvent during extraction as it is readily available and safe to use. However there is need to explore the performance of other solvents and determine their efficacy. The present study compared the efficacy of ethanol and aqueous extracts of *S. cuneifolia* against bacterial pathogens.

### 1.4 Objectives

#### 1.4.1 Broad objectives

To explore *S. cuneifolia* as a potential source of antibiotics in the treatment of bacterial infections

#### 1.4.2 Specific objectives

1. To determine the efficacy of *S. cuneifolia* crude extracts and its different plant parts in the control of *E. coli, S. aureus, Shigella* and *S. typhi.*
2. To evaluate the performance of aqueous and ethanol extracts against *E. coli, S. aureus, Shigella* and *S. typhi.*
3. To determine the phytochemicals present i*n S. cuneifolia.*

## 1.5 Hypothesis

**Null hypothesis**

1. *S. cuneifolia* crude extracts are not effective in the control of *E. coli, S. aureus, Shigella* and *S. typhi*.
2. Aqueous and ethanol solvents do not differ in their efficiency against *E. coli, S. aureus, Shigella* and *S. typhi.*
3. There is no difference in the efficacy of roots, leaves and stem in the control of pathogenic bacteria.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Overview

The World Health Organization (WHO) and Kenya government have recognized the role of herbal and traditional medicinal practice in primary health management due to the observation that 80% of the population is rural communities consult traditional healers prior to seeking conventional medical services. The traditional herbal practice therefore remains one of the most sought after methods of treatment (Agbor & Naidoo, 2016). Moreover, through the ethnobotanical field surveys, over 95 plant species that are being used by herbalists have been identified (Chinsembu & Hedimbi, 2010). It has also been reported that 100% of the plants are used by other herbalists in East Africa to treat human and animal ailments while 30% of the species had received phytochemical and pharmacological evaluations, thus confirming their therapeutic values (Ogoche, 2014).

It is also known that traditional healers have been using plants to prevent or cure diseases and western medicine has tried to evaluate and develop these methods. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have reported to have antimicrobial properties *in vitro* (Cowan, 1999).

The phytochemicals present in plants are known to contain defensive and curative activity (Borokini & Omotayo, 2012). There is for instance a long history on plant use in the improvement of dental health and promotion of oral hygiene. There are many species of medicinal plants belonging to various families that are being used traditionally to

control and cure a variety of dental problems by the population. Therefore there is need for proper documentation of traditional knowledge that may be helpful to promote further research in various areas of medical science (Bhardwaj & Bhardwaj, 2012).

A study conducted in Mbeere, Eastern Kenya reveled that most herbalists perform treatment as their full time job carried on from their fore fathers (Kareji, 2013). The herbalists' were found to be Christians and had average formal education. They always recommended a concoction of herbs for treatment to the patients. Typhoid, pneumonia and candidiasis are some of the common diseases found in the area treatable with a total of 5, 4 and 4 plants respectively (Maregesi *et al*., 2007).

HIV/AIDS is a disease characterized by life-threatening opportunistic infections. As the formal health sector struggles to confront this epidemic, new medicines from traditional sources are needed to complement control efforts. A survey conducted in four districts in Uganda identified 103 plant species of medicinal value; plant parts frequently used were leaves, 33% stem bark, 23% and root bark, 18%. Approximately, 80% of traditional medicines were administered orally in variable doses. The role of these plants in the management of opportunistic infections warrants further investigation as they may have a role in Uganda's public health approach to HIV/AIDS control (Lamorde *et al*., 2010).

Herbal medicine is used to treat both human and livestock ailments as observed in studies conducted in various parts of Kenya, Uganda and Tanzania (Bekalo *et al.,* 2009). Ejobi *et al.,* (2007) reported that the highest number of important plants used, were found in

Kenya, (81 species) plants of importance followed by Tanzania with 50 and Uganda with 24. Livestock diseases and conditions traditionally treated included diarrhea, pneumonia, eye infections, heart water, mastitis, and wounds (Ejobi *et al.,* 2007).

The people of Kopsiro Division in Bungoma County are known to rely a lot on plants to manage human ailments. An ethnobotanical study carried out to determine plants traditionally used for medicinal therapy by the Sabaot commuity identified 107 species belonging to 56 families to be of medicinal value to the locals. Roots were the most frequently used plant part (47.3%), followed by the bark (23.35%) and leaves (22.75%). Traditional medicine offered cheap, convenient and accessible health solutions that fitted the traditional lifestyle of the community as compared to conventional medicine (Okello *et al.,* 2010).

Water is the universally used solvent in extraction of plant materials for treatment by herbalists (Cowan, 1999). Traditionally, plants are soaked in hot water or prepared as tincture, that is, plants in traditionl alcoholic solutions,or inhaled via steam from boiling suspensions of the plant parts. Most amateur herbalists usually prepare traditional medicines and extract healing compounds from plants using water.

## 2.2 *Sida cuneifolia*

### 2.2.1 Taxonomy

*Sida cuneifolia* is likely to be found in the plains of Ethiopia, Zaire, Uganda, Tanzania and Kenya which are dominated by tropical grassland vegetation. The plant is found in

most of these areas, except for very dry deserts. *Sida cuneifolia* is a shrub that has very tough woody stems. It can grow a meter or more tall if left undisturbed. The leaves are notched at the tip with small serrations in some populations, but the *S. cuneifolia* specimen has smooth margins; flowers are yellow with distinct petals and five sepals. *Sida cuneifolia* belongs to the Kingdom: Plantae, Phylum; Magnoliophyta Class: Magnoliopsida Order: Malvales Family: Malvaceae, genus; *Sida*, species *cuneifolia* (Vollesen, 1986).

### 2.2.2 Ethnobotanical studies

An ethnobotanical study in Central Uganda identified various ethno botanical uses of *S. cuneifolia.* Sixty two percent of the respondents reported to use it as a herbal remedy (Nalubega *et al.,* 2013). The plant was reported to be useful in the management of 12 disease conditions and it also found use in prevention of miscarriages, expulsion of retained placenta in mothers, disinfectant of umbilical cord wounds and clearing stomach contents of postpartum mothers and their infants in Busoga region, Uganda (Ogwal, 1996). Its tough and fibrous stems are used for cleaning or brushing teeth (Vollesen, 1986).. The Sabaot people who live around Mt. Elgon in Kenya chew the roots of *S. cuneifolia* (Kupchuwet) to cure sore throat (Okello *et al*., 2010). The plant is also important in ethno veterinary for it was used to treat retained placenta in cattle in Kweneng District of Botswana (Moreki *et al.,* 2012). In Ethiopia, about half of the women used *S. cuneifolia* (Guftee) to clean utensils used for milking, storing and processing milk. The active ingredients were thought to act as preservatives in keeping butter for longer periods (Fita *et al.,* 2005).

### 2.2.3 Plant mode of action

Plants have shown the ability to inhibit pathogen growth. Crude plant extracts have been observed to work like conventional drugs in several ways. For example, inhibiting growth as well as killing of pathogenic bacteria thus treating ailments and diseases. They target cell-wall biosynthesis enzymes and substrates, cell membranes, bacterial protein synthesis, and bacterial nucleic acid replication and repair, which act via an anti-metabolite mechanism (Hancock, 2005).

Plants just like the quinolone class of antimicrobials might target DNA–topoisomerase complexes. They are derivatives of nalidixic acid, a byproduct of chloroquine that has been used to treat urinary tract infections. They interfere with the maintenance of chromosomal topology by targeting topoisomerase II and topoisomerase IV, trapping these enzymes at the DNA cleavage stage and preventing strand rejoining (Hooper, 2001).

Like other antibiotics, *S. cuneifolia* may contain plant-acidic polysaccharides that inhibit DNA dependent transcription by binding with the β-subunit of a DNA bound and actively transcribing RNA polymerase. The β-subunit is found in the path that’s formed by the RNA polymerase DNA complex, where newly synthesized RNA strand come from. This specifically requires RNA synthesis not to have gone beyond the addition of two ribonucleotides. This is because of the drugs ability to inhibit RNA strand initialization (Wilson, 1997; Kozlov *et al.,* 2013).

A bacterial cell is enclosed by layers of peptidoglycan that is made up of a covalently cross linked polymer matrix. This layer of the cell wall gives the cell mechanical strength that is important to the cell so as to survive environmental conditions that change prevailing osmotic pressures (Typas *et al.,* 2012). Effective treatment using *S. cuneifolia* extracts as a cell wall synthesis inhibitor can bring about changes in the cell shape and size. Coumarins a chemical found in plants is known to affect the cell wall by inhibiting the C-glucose in the cell wall. The phytochemicals can also block the cross linking of peptidoglycan units by inhibiting the peptide bond formation reaction. The plant in study has glycopeptides that act as steric inhibitors of peptidoglycan maturation and reduce the mechanical strength of the cell. In addition, it may inhibit the transport and synthesis of individual peptidoglycan units in use, thus affecting structural integrity by inserting themselves into the cell membrane and inducing membrane depolarization (Hara *et al.,* 1973; Kohanski *et al.,* 2010).

Plant extracts are also able to disrupt the process of mRNA translation. Tannins bind to proline rich protein and interfere with protein synthesis. This process occurs over three stages that involve the ribosome (Yadav & Agarwala 2011). The ribosome is composed of two ribonucleoprotein subunits, the 50S and 30S. These drugs are the widest classes of antibiotics divided into two: the 50S inhibitors and 30S inhibitors. The 50S ribosome inhibitors work by physically blocking either initiation of protein translation or translocation of peptidyl tRNAs (Ermolenko *et al*., 2007). The 30S ribosome inhibitors work by restricting the access of aminoacyl tRNAs to the ribosome, they also bind the 16S rRNA component of the 30S ribosome subunit. They can also interfere with the

stability of peptidyl tRNA binding to the ribosome by inhibiting elongation. This promotes tRNA mismatching, which can result in protein mistranslation (Kohanski *et al*., 2010).

**2.3 Phytochemical Composition in Different Plant parts**

The chemotaxonomic study approach is based on plants having similar constituents in different families and the screening of specific parts of a plant such as the seeds, barks, roots, leaves and other plant parts (Igoli *et al,*. 2005)**.** Different plant parts have different constituents in terms of phytochemicals present; this brings about varied results in their antimicrobial activity. The most used plant part are the leaves and roots (Akinmoladun *et al*., 2007). Leaves of plants contain compounds with high specific peroxyl radicals scavenging activities that provide antimicrobial properties (Caldwell. 2003).

In some plants the leaf paste is externally applied on healing wounds, sores, itch and cutaneous diseases. Leaves are also used on bone fractures, fever, ringworm, skin diseases, throat infection and wounds. Twigs can be used as toothbrushes (Harsha *et al.,* 2002).

Roots are considered important in obstruction of stomach and incipient dropsy. They are veterinary medicines for animal diseases, and antidotes in case of poisoning. Roots are used against gastric complaints, to increase lactation, and against whooping cough. In Nigeria, the roots of *S. cuneifolia* are boiled with water and taken as tea for constipation and against white vaginal discharge in ladies (Selvam, 2008), and in Kweneng District of Botswana, it was found that 57.14% of medicine men used the stems of different

medicinal plants for their therapeutic properties and 40.48% used the roots for treatment of human ailments (Moreki *et al*., 2012). It has been found that roots are rich in free and bounded anthraquinones, but the quantities differ significantly, the anthraquinone content is in higher quantities in other plant parts but less in leaves (Yadav *et al*., 2010).

**2.4 Extraction of crude plant extracts**

The extraction process is a major player in the role of determining the concentration of constituents in plants. The extraction method and compounds extracted and isolated are very important in relation to the antimicrobial activity of the plant extract (Singh, 2010). Scientific screenings of plants for antimicrobial activities begins by using crude aqueous or alcohol extractions that can then be followed by other organic extraction methods. Nearly all identified compounds from plants that are active against microorganisms are aromatic or saturated organic compounds. They are most often obtained through initial ethanol or methanol extraction (Cowan, 1999).

It has been shown that ethanol extracts have a significantly higher antibacterial activity than the aqueous extracts (Bukar *et al*., 2013). The ethanolic extracts of leaves are known to produce an impressive antibacterial activity against organisms (Tula *et al.,* 2012). Ethanol as a solvent in extraction has also shown the highest antioxidant activity and phenol content than in water extraction (Mao *et al.,* 2006), though it has been observed that water and ethanol produce the same results in terms of susceptibility when tests were done with garlic (Tagoe *et al.,* 2010). Water extracts of *Cassia alata* exhibited a higher antibacterial activity than ethanol extracts (Somchit *et al.,* 2003). Hot water used during

extraction on dried leaves has been seen to give phenolic compounds its anti-oxidant and radio-protective properties, of which 70% of the extracted phenolic compounds, triterpenoids and other compounds show anti-inflammatory and analgesic activities (Porwal *et al.,* 2012). When water is used as a solvent in crude extracts, it strongly increases the activities of CYP, a series of microsome hemoproteins in the liver called cytochrome P450 (CYP). The CYP plays an important role in the metabolic oxygenation of a variety of lipophilic chemicals such as drugs, pesticides and pollutants. And ethanol is known to decrease the level of CYP isoforms (Tang *et al.,*2006). Many studies do not prefer the use of aqueous method of extraction, unless extraction of water-soluble compounds, such as polysaccharides like starch and polypeptides, including fabatin and various lectins, which are commonly more effective as inhibitors (Ncube *et al*., 2008). Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents. (Cowan, 1999).

## 2.5 Enteric bacteria

Enteric bacteria belong to the Domain; bacteria, kingdom; eubacteria, phylum; proteobacteria, class; gammaproteobacteria, order; enterobacteriales, family; enterobacteriaceae. They are gram-negative rods which are facultative anaerobic, and flagellated (Tortora *et al.,* 2006). The human large intestine contains a micro biota; which are generically complex and metabolically diverse. The primary function of these is to store energy from carbohydrate not digested in the upper gut. Storage of energy is through fermentation and absorption of the major products, short chain fatty acids, which is about 40–50% of the total energy of the carbohydrate. Bacteria are known to grow in

the presence of carbohydrate substrates such as non-starch polysaccharides. Intestinal bacteria also have a role in the synthesis of vitamins B and K and the metabolism of bile acids, other sterols (Cummings & Macfarlane, 1997). Members of the *Enterobacteriaceae* family are known to cause human, plant, and animal health problems. Diarrhea, caused by *Enterobacteriaceae,* is known to be one of the most common causes of morbidity and mortality among infants and children in developing countries. Pathogenic *E. coli* is the most frequently identified pathogen. Among pathogenic *E. coli*, the prevalence of Enteroaggregative *Escherichia coli* (EAEC) is the highest. Other major causes of diarrhea are *S. aureus*, *Salmonella* species, *B. cereus*, *C. jejuni*, *C. perfrin* and *Shigella* species. (Cho *et al.,* 2008) Diarrhea is caused by a range of bacterial pathogens, including enteric bacteria. It can be detected using several conventional diagnostic methods, such as culture, biochemical tests, and enzyme-linked immunosorbent assay (ELISA). The main pathogens include pathogenic *Aeromonas, Campylobacter jejuni, Campylobacter coli, Salmonella, Shigella,* enteroinvasive *E. coli* (EIEC), *Vibrio,* and *Yersinia* (Liu *et al*., 2012).

There has been an increasing trend of resistance to antibiotics used in the treatment of infections caused by *Enterobacteriaceae*. This has led to difficulties in management of the infections thus resulting to increased morbidity, mortality and high cost of health care (Reuben *et al.,* 2013). Resistance arises spontaneously due to point mutations that result in amino acid substitutions within the topoisomerase subunits as is in the case of *E.coli* with some plant extracts (Hopkins *et al.,* 2005). Another source of resistance by *E. coli* is the production of an exopolysaccharide matrix or glycocalyx, distinguishing

characteristics of biofilms. One of its functions is to prevent the access of antibiotics to the bacterial cells. The biofilm can limit the transport of antimicrobial agents to the cells within the biofilm (Mah & O'Toole, 2001). Overuse of this important class of antimicrobial in both human and veterinary medicine should be avoided to prevent an increase in the occurrence of resistant in *E. coli* (Hopkins *et al.,* 2005).

### 2.5.1 *Escherichia coli*

One of the most diverse microorganisms in the human intestinal tract is *E. coli.* It belongs to the genus *Escherichia* and species *coli.* It is very common making it easy to culture. It is normally harmless, but certain strains can be pathogenic. The pathogenic strains of *E. coli* have specialized fimbriae that allows them to bind to certain intestinal epithelial cells. They also produce toxins that cause gastrointestinal disturbances, collectively termed *E. coli* gastroenteritis. The cause of most cases is a strain usually referred to as *E. coli.* Enterotoxigenic *E. coli* (ETEC), though not invasive, it produces an enterotoxin that causes a watery diarrhea resembling mild cases of cholera. Enteroaggregative *E. coli* (EAEC) is another strain that is increasingly being recognized as a cause of diarrhea, though after Enterotoxigenic Escherichia coli (ETEC). There is also Shiga toxin, termed Shiga-toxin *E. coli* (STEC) (Tortora *et al*., 2006). A rare *E. coli* serotype, which is apparently transmitted by undercooked meat, is known to have caused an illness which was characterized by severe cramps and abdominal pain, initially watery diarrhea followed by grossly bloody diarrhea, and little or no fever (Riley *et al*., 1983). One *E. coli* strain is known to cause 73,000 illnesses. Forty-nine states in the United States of America reported 350 outbreaks, representing about 8,598 cases, 17% hospitalizations,

4% hemolytic uremic syndrome cases, and 0.5% deaths. The *E. coli* transmission routes were found to be 52% foodborne, 21% unknown, 14% person-to-person, 9% waterborne, 3% animal contact, and 0.3% laboratory-related (Rangel *et al*., 2005). Vertical transmission from mother to infant is also possible and *E. coli* O and H antigens have been found in maternal and infantile cultures of babies with *E. coli* meningitis (Sarff *et al*., 1975).

Cattle are considered to be the most important source of human infections with *E. coli*. The organism has also been reported in sheep, goats, water buffalos, and deer, but has not been reported in pigs and poultry (Caprioli *et al.,* 2005). Livestock rearing has become commercial, and cattle are being grain-fed rather than pastured. The diet affects the pH of the rumen promoting the colonization of the animal gut with STEC, which is acid resistant. About 2-3% of domestic cattle carry STEC, which contaminate the carcass during slaughter finding its way to the meat (LeJeune *et al.,* 2001).

### 2.5.2 *Shigella*

*Shigella* species are important pathogens responsible for diarrhoeal diseases and dysentery occurring all over the world. The morbidity and mortality due to shigellosis are especially high among children in developing countries. Shigellosis is caused by *Shigella spp*. a global human health problem (Sack *et al.,* 1997). About 165 million cases of *Shigella* diarrhea that occur annually, 99% occur in developing countries, and in developing countries 69% of episodes occur in children under five years of age. Moreover, of the 1.1 million deaths attributed to *Shigella* infections in developing countries, 60% of deaths occur in children under-five. Travelers from developed to

developing regions are at an increased risk of contracting shigellosis (Niyogi, 2005). Four species of *Shigella* i.e. S. *dysenteriae, S. flexneri, S. boydii* and *S. sonnei* are known to cause shigellosis. These species are subdivided into serotypes on the basis of O-specific polysaccharide of the LPS. *Shigella spp*. causes dysentery by invading the colonic mucosa. *Shigella* multiplies within colonic epithelial cells, causing cell death. It spreads laterally infecting and killing adjacent epithelial cells, causing mucosal ulceration, inflammation and bleeding (Kotloff *et al*., 1999). Shigellosis is characterized by the passage of loose stools mixed with blood and mucus and accompanied by fever, and abdominal cramps. It is also associated with complications such as haemolytic uraemic syndrome (Sur *et al.,* 2004).

Transmission and spread of shigellosis usually occurs via contaminated food and water or through person-to-person contact (Crim *et al*., 2014). Diagnosis of *Shigella* in the laboratory is achieved by culturing the stool samples using selective or differential media. *Shigella spp*. is a highly fragile organism and considerable care must be exercised in collecting faecal samples, transporting them to the laboratories by using appropriate media for isolation (Humphries & Linscott, 2015). Antimicrobials are the solution in therapy against cases of shigellosis. With the emergence of drug resistance globally, the choice of antimicrobials for treating shigellosis is limited. Although single doses of ciprofloxacin and norfloxacin has reported to work, they are currently less effective against *S. dysenteriae* type 1 infection. Currently, there are no vaccines against *Shigella* infection. Though, there is live and subunit parenteral vaccine candidates under development (Klontz & Singh, 2014). With the increasing resistance to antibiotics and

high mortality, shigellosis is now a serious problem. As *Shigella* is associated with poor hygiene, simple interventions like washing hands with soap and water can be very effective in checking the spread of the disease (Khan, 1982).

### 2.5.3 *Salmonella typhi*

*Salmonella typhi* belongs to the genus *Salmonella* species *S. enterica* and sub species *S enterica* serovar *Typhi*, which is known to cause of typhoid and is host specific restricted to humans. Typhoid is a common disease in low class areas with limited public health infrastructure. Typhoid incidence globally is difficult to estimate because the current diagnosis method is culture of *Salmonella enterica* serovar t*yphi* from clinical samples. Estimates of about 200,000 deaths a year have been made globally, but morbidity is significantly higher, with more than 20 million new cases per year (Dougan & Baker, 2014). Early symptoms of typhoid are an onset of fever, abdominal discomfort, headache, and loss of appetite, constipation accompanied by diarrhea, dry cough, malaise and rash. The case fatality is about 10% if no treatment is administered and less than 1% if antibiotics are given. Human beings are the only known natural reservoir. Transmission is through ingestion of water or food that have been contaminated by feces or urine of carriers (Bhunia *et al.,* 2009). Prevention is based on access to safe water, chlorination of drinking water and hygienic food handling practices. Also the construction and expansion of water distribution networks with proper sewage systems can aid in the control of typhoid. Hygiene education, mass vaccination campaigns, and/or the identification of carriers within or outside the households of patients are also an important in the curbing of the spread of the disease (Vollaard *et al.,* 2004).

*Salmonella enterica* serovars Typhi isolates from human patients has displayed different levels of resistance to quinolones or fluoroquinolones that are commonly used in the treatment of typhoid (Baucheron *et al.,* 2014). The multidrug resistance especially fluoroquinolone resistance has severely limited therapeutic options in high occurrence areas, of which has increased from 84.7% to 91.7%. Cefixime- and ceftriaxone-resistant *S.* Typhi have also been isolated from children, and the occurrence of the two cases of resistance is alarming (Qamar *et al.,* 2014). There is emergence of a specific haplotype, H58, which is well adapted to transmission in modern settings and is able to resist antimicrobial chemical agents more efficiently than other *S. typhi*. Evidence from genomics and functional studies using the mouse and in vitro cell systems, together with clinical investigations, have shown the mechanisms that justify the pathogenesis of human typhoid and host restriction (Dougan & Baker, 2014).

## 2.6 *Staphylococcus aureus*

*Staphylococcus aureus* is a gram-positive coccal bacterium that is a member of the Firmicutes, and is frequently found in the respiratory tract, digestive tract and on the skin, and is not always pathogenic (Ezaki *et al.,* 2006). *Staphylococcus aureus* is one of the major human pathogen as first proposed by Sir Alexander Ogston. He noted that it was the major cause of wound and skin infections, respiratory infections such as sinusitis, and food poisoning. *Staphylococcus aureus* is known to cause impetigo bullosa, cellulitis, septic arthritis, epidural abscess, septic shock, food borne gastroenteritis among others (Blot *et al.,* 1998). *Staphylococcus aureus* pathogenic strains are responsible for

infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. One of the strains of *S. aureus*, Methilicin Resistant *S. aureus* (MRSA) is a worldwide problem in health. One major part of this problem of resistance and spread is been due to the spread of resistant strains from patient to patient with the main carriers being contaminated healthcare workers in hospitals and are called nosocomial infections (Forbes & Schaberg, 1983; Ben-David, *et al.,* 2008). Medical equipment used in hospitals and other healthcare facilities are also pathogen vehicles when contaminated, portable equipment carried by HCWs, such as stethoscopes, tourniquets, sphygmomanometer cuffs, and others, can highly be contaminated. Patients who spend long periods on hospital beds, surrounded by devices, and environmental surfaces which can harbor microorganisms are prone to infection, thus, making the environment a key player in the transmission of antimicrobial-resistant pathogens between patients (Muto *et al.,* 2003). Recently, cases of MRSA have been reported in people living in so called healthy communities with no particular established risk factors for MRSA acquisition noted in the community, these infections are usually referred to as community acquired (Vandenesch *et al.,* 2003).

*Staphylococcus aureus* infections remain a significant problem in hospitalized patients because of emergence of resistance (Forbes & Schaberg, 1983). These pathogens can easily share genes of antimicrobial resistance (Shapiro *et al*., 2001). Resistance involves mutation of a bacterial gene on the chromosome or transfer of a resistance gene from other organisms (Ito *et al*., 2003). Transfer is usually by conjugative mobilization where it can be by conduction where plasmids physically combine with conjugative plasmids by

recombination between regions of homology or mobilization by donation where the plasmid encodes proteins that produce a single strand nick at the origin of transfer, which can occur if the conjugative part is encoded by the genome of a co-resident conjugative plasmid with the copies of the mobilized plasmid only, without the conjugative plasmid may appear in the transcojugants (Projan & Archer, 1989). Vancomycin resistance can also be transferred from *E. faecalis* to *S. aureus* at a relatively high frequency in vivo but rarely occurs in vitro (Weigel *et al*., 2003).

Some models that accounts for drug resistance in S. aureus include; Plasma Membrane Mechanisms, this is where the plasma membrane of drug resistant cells appears different from that in cells that are susceptible to the drug. Changes such as these can significantly influence on the trans-membrane partitioning of the drug by a non-channel diffusion-mediated process (Simon & Schindler, 1994). ATP-Driven Drug Efflux Model is another model which is the widely used to explain Multi Drug Resistant (MDR) cases. The peptidoglycan protein uses ATP to power a molecular pump that then removes chemotherapeutic molecules from the cell. The pump either transports the drugs out of the cytosol or serves as a "flippase" to expel them from the bilayer (Li *et al*., 2015). Nuclear Mechanisms brings about resistance whereby, the main target for chemotherapeutic agents is the DNA. Drug resistance is achieved in cells by preventing drug accumulation in the nucleus thus no binding to the DNA inactivating DNA template in transcription and replication and other associated nuclear proteins. Drug resistance can also be through modifications in; mechanisms of drug translocation across the nuclear envelope, binding sites on the genetic material, DNA repair mechanisms; and efficiency

of mRNA export and protein import into the nucleus (Longley & Johnston, 2005). Exocytosis also contributes to drug resistance as the drugs that accumulate in the exocytotic pathways, are trapped into sequestered transport compartments then expelled from the cell. This in turn would decrease the drug concentration in the cytoplasm, leading to drug resistance (Chen *et al.,* 2007). Finally compartmentalization is where there are changes in the ability of internalized drugs to reach critical concentrations at intranuclear sites; this will seriously limit the effectiveness of drug therapy and lead to drug resistance. Chemotherapeutic drugs primarily accumulate in the acidic compartments of the cell, the Trans Golgi and lysosomal compartments (Das *et al*., 2013).

Control of MRSA has been successful in areas that follow strict transmission-based control methods that involve active surveillance cultures to identify colonized patients and putting strict barriers of precautions for patients infected with MRSA. In several northern European countries, the prevalence of MRSA is low (Bronzwaer *et al.,* 2002). For instance in Denmark, the prevalence was at a peak of 33% in the 1960s, but went down to less than 1% for 25 years after introduction of policies to control transmission. A similar situation is found in Finland and the Netherlands where it has been maintained at a rate lower than 0.5%. These countries ensure there is strict surveillance of cultures from patients and hospital staff to identify unrecognized colonization, setting up of strict barriers, cohort nursing, and quarantine of infected patients until confirmation that they are free from MRSA. It is also thought that successful control of MRSA is because of their low rate of antibiotic use than prevention of transmission (Muto *et al.,* 2003).

## 2.7 Phytochemicals

Herbs are known to contain potent antioxidant compounds that provide significant protection against chronic diseases. These compounds commonly referred to as phytochemicals have antimicrobial, antiviral or antitumor activity (Winston, 1999). Plant foods are known to contain a lot of phytochemicals and non-nutrients that have health benefits. Nuts, fruits, whole grains, and vegetables contain phenolic compounds, terpenoids, pigments, and other natural antioxidants that offer protection from and treatment of diseases (Liu, 2004). Some foods and herbs have antimicrobial activity like garlic, soybeans, cabbage, and vegetables. Citrus fruits are also known to provide a lot of vitamin C, folic acid, potassium, and pectin that contains active phytochemicals (Saraf & Kaur, 2010). Phytochemicals in grains are also known to reduce the risk of cardiovascular disease and cancer (Winston 1997). Volatile essential oils of commonly herbs used in cooking, spices, and herbal teas inhibit mevalonate synthesis and thereby suppress cholesterol synthesis and tumor growth (Winston 1999). Mode of action of these phytochemicals include; being cofactors of enzymatic reactions, that is they are substrates for biochemical reactions, they also act as inhibitors to enzymatic reactions by binding to and eliminating undesirable constituents in the intestine. Others are compounds that enhance the absorption and stability of essential nutrients taken in. Some phytochemicals allow selective growth factors for beneficial gastrointestinal bacteria fermentation substrates for beneficial bacteria in the gut and selective inhibitors of harmful intestinal bacteria (Dillard & German, 2000).

### 2.7.1 Phenolics

Phenolics are compounds that consist of one or more aromatic rings with one or more hydroxyl groups. They are widely distributed in plants and are the most abundant secondary metabolites in plants. They range from simple molecules such as phenolic acids to highly polymerized substances such as tannins (Bravo, 1998). Plant phenolics are generally involved in defense against aggression by pathogens and parasites. They are ubiquitous in all plant organs and are therefore an integral part of the human diet. Phenolics are the main constituents of plant foods in fruits, vegetables, cereals and legumes, also beverages such as tea, coffee, beer and wine (Carlsen *et al.*, 2010). Anthocyanins, one of the six groups of a large group of plant polyphenol constituents known as flavonoids, are responsible for the orange, red, blue and purple colors in fruits and vegetables. Plant phenolics include phenolics acids, flavonoids, tannins and the less common stilbenes and lignans (Pandey & Rizvi, 2009). Flavonoids are the most abundant polyphenols in the foods we take. Flavonoid are divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central C ring (Tsao, 2010). Some of the most common flavanoids include quercetin, a flavonol common in onion, broccoli, and apple; catechin, a flavanol found in tea and several fruits; naringenin, the main flavanone in grapefruit; cyanidin-glycoside, an anthocyanin abundant in berry fruits; and daidzein, genistein and glycitein, the main isoflavones in soybean (Mortazaeinezhad *et al.,* 2014). Phenolic acids can be divided into two classes: benzoic acid derivatives like gallic acid, and cinnamic acid derivatives, coumaric, caffeic and ferulic acid. Caffeic acid is the most abundant phenolic acid in many fruits and vegetables, most often esterified with quinic acid as in

chlorogenic acid, which is the major phenolic compound in coffee (Škerget *et al.*, 2005). Another common phenolic acid is ferulic acid, which is present in cereals. Tannins are another major group of polyphenols in our diets and usually subdivided into two groups; hydrolysable tannins, compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins and condensed tannins, oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions (Dai & Mumper, 2010).

Flavonoids are most abundant chemical in photosynthetic cells that are mainly found in vegetables, fruit, seeds, nuts, stems, flowers and tea. For many years, these compounds have been used as a solution to human ailments. These products need more research, and the compounds have been isolated and identified. Flavonoids are known to possess antifungal, antiviral and antibacterial activity (Cushnie & Lamb, 2005). Tannins, another group of phytochemicals are known to be toxic to fungi, bacteria and yeasts. Mechanisms of action that tannins possess that promote antimicrobial activity include; inhibition of extracellular bacterial enzymes, deprivation of the substrates required for growth and action against microbial metabolism through inhibition of oxidative phosphorylation. Iron deprivation is another mechanism that has been brought up. Plants detoxify tannins to avoid destruction of its cells by synthesizing tannin complexing polymers, oxidation, tannin biodegradation or synthesis of siderophores (Scalbert, 1991). Terpenoids on the other hand have been found to contain antimicrobial, cytotoxic, leishmanicidal, and

aldose reductase inhibitory activities. Its compounds have been able to indicate significant antimicrobial activity against Gram-positive bacteria (Tincusi *et al*., 2002). Saponins are another group of phytochemicals, they are a steroid or triterpenoid glycosides and are common in a lot of plants that are important in human and animal nutrition. Saponins are known to have membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic abilities. They also impair the digestion of protein and the uptake of nutrients in the gut, causing hypoglycemia. Saponins can kill protozoans and mollusks and are antioxidants (Francis *et al.,* 2002). Alkaloids are one of the most common phytochemical, they are one of the largest groups of phytochemicals in plants have health effects on humans which has led to the development of powerful pain killer. Alkaloids biological properties that make them lethal are their toxicity against cells of foreign organisms (Igbinosa *et al*., 2009). The increase in antibiotic resistance is real and this poses a great challenge in the management of bacterial infections. Some plants have the ability to inhibit the growth of pathogenic bacteria. The scientific validation of the plant species may help in discovering new drugs to tackle new, reemerging and resistant pathogens. The aim of the current study is therefore to assess the antibacterial activity of crude extracts of *S. cuneifolia.*

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study Area

The study was conducted in Nandi County (Figure 3.1), Kenya. The county is located between latitude $0^0$34’N and $0^0$65’S; and between longitude $34^0$45’E, and $35^0$25’E and with an altitude of 2,000 meters above sea level (Wandili *et al.,* 2013). The area has a cool and moderate wet climate, with an average mean annual rainfall between 1,200 to 2,000 mm in two seasons and mean temperature of $18^0$C-$22^0$C. The soils are well-drained and moderately fertile. Sandy and clay loams are the main soil types found in the County with a few areas having humic nitosols that are generally suitable for production of various crops (KFS, 2010).

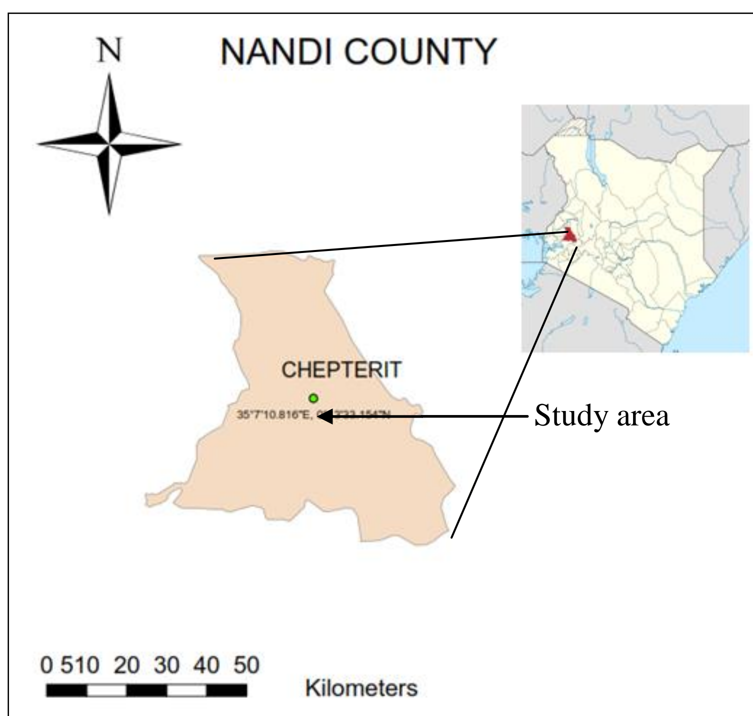


**Figure 3.1: Map of the study area where *S. cuneifolia* was collected (Source: Author, 2016)**

### 3.2 Collection and processing of plant samples.

*Sida cuneifolia* plants (Plate 1) were collected from Chepterit village, Kapng'etuny Chemundu ward of Chesumei Sub County of Nandi County (Figure 3.1). The plants were uprooted using a hoe and the plant parts separated into stems, leaves and roots, chopped into small pieces and packed in clear plastic re-sealable bags and transported in a dry zipper storage bags to KEMRI, CTMDR. At KEMRI, the plants were dried in an oven to a moisture content of 13%. The plant material was ground into fine powder, weighed (Appendix II, a) and stored for other subsequent procedures that followed. Plant identification followed Agnew and Agnew (1994) and voucher specimens were deposited at the University of Eldoret herbarium.

**Plate 1:** ***Sida cuneifolia*** **(Source: Author, 2016)**

### 3.3 Preparation of Plant extracts

Crude extracts from the stored fine powder were prepared using hot water and ethanol. The crude extracts were later to be reconstituted to attain the desired concentration was prepared by dissolving 50 g of the ground samples into 100 ml of the respective solvents (Usman & Osuji, 2008).

#### 3.3.1 Ethanol extraction

Fifty grams of the ground plant parts were placed in a 250 ml conical flask and 100 ml of 70 % ethanol added into the flask and shaken well. The mixture was left to settle for 24 hours. After which, the samples were filtered using 6 mm filter paper (Whatman No. 1) (Appendix II, b). The filtrate that was obtained was transferred into a round-bottomed flask. The flask was attached to a rotary evaporator until the ethanol evaporated leaving a thick paste (Appendix II, c). The paste was transferred to a vial and left to dry in front of a fan until a solid was obtained indicating that all the ethanol had evaporated from the paste (Appendix II, d). The solid was stored in a refrigerator at $2^0$C - $4^0$C for other processes that followed.

#### 3.3.2 Aqueous extraction

Fifty grams of the ground samples were mixed with 100 ml of distilled water in a 1 litre conical flask and the mixture shaken until completely dissolved. The flask was placed in a shaking water bath at 70±1 $^0$C for one and a half hours. After incubation in a water bath, the mixture was removed and filtered using surgical cotton wool in a glass funnel. The filtrate was left to cool and transferred into a 250 ml round bottom flask. The filtrate

was put in a round bottomed flask then inserted into a shallow tray containing acetone and dry ice to freeze dry, and coat on the flask. The sample was freeze-dried using Modulyo K4 freeze dryer (EDWARDS) so as to completely eliminate any water through vacuum until it was completely dry (Appendix II, e). The dried sample was removed from the flask, weighed into vials and refrigerated at $2^{0}$C - 4 $^{0}$C for future use.

### 3.3.3 Source of bacterial isolates

Pure isolates of the selected enteric pathogen; *S. aureus* (ATCC 25923), *Shigella* (Clinical isolate), *S. typhi* (Clinical isolate) and *E. coli* (ATCC 25922) were obtained from the Centre of Microbiology Research laboratory at the JICA-KEMRI culture collections and then sub cultured to make fresh populations. Clinical isolates strains of the pathogens were obtained from patients at the Kenyatta National Hospital where KEMRI is based. *S. aureus* (ATCC 25923), a quality control strain for the CAMP test, assay of wood smoke concentrate, evaluation of Mueller Hinton agar, examination of dairy products, media testing, Clinical and Laboratory Standards Institute (CLSI) disk diffusion, and for Abbott and Autobac products. (*Staphylococcus aureus subsp. Aureus* ATCC® 25923™). *E. coli* (ATCC 25922), does not produce verotoxin. This organism is also a CLSI control strain for antimicrobial susceptibility testing. It is used for media testing, as a negative control for LT toxin production. Used in susceptibility disc testing of neomycin, colistin, kanamycin, cephalexin, gentamicins, cefamandole, cephalothin, tetracycline, cephaloglycin, cephaloridine [cephalomycin], nalidixic acid, and Chloramphenicol (*Escherichia coli* ATCC® 25922™).

### 3.2.4 Preparation of media

Müller-Hinton agar (MHA) (OXOID, UK) was used to culture the pathogens. The agar was prepared according to manufacturer's instructions then sterilized at 121 $^{0}$C for 15 min. The pH was adjusted to 7.1 with sterile 1N NaOH, then allowed to cool to 50–55 $^{0}$C and poured into sterilized culture dishes (Baker *et al.,* 1985).

Bacterial suspensions of the four pathogens were made by picking the sub cultured pathogens from the primary cultures and introducing it in sterile distilled water using a sterile plastic disposable loop in a screw cap test tube. The test tube was then shaken to help in the even distribution of the inoculums in the tube. Using sterile swabs, the bacterial suspensions were then transferred by streaking onto the media that had been prepared and then spread evenly. The plates were then left to dry (Chandrasekaran & Venkatesalu, 2004).

New stock cultures were then prepared for the next day's work and other subsequent runs in replications.

### 3.3.5 Antimicrobial bioassays

Bioassay tests were done at the Mycology Laboratories, Center for Microbiology Research (CMR) - KEMRI. The sensitivity testing of the extracts were determined using Kirby Bauer disk method. The extracted solids were first reconstituted by using 1ml of DMSO in 1g of the extract to obtain a concentration of 1g/ml before running antimicrobial assay (Okeke *et al.,* 2001).

Sterile 6 mm paper disks were impregnated with 15 µl of the reconstituted extract. The controls were also impregnated on the discs (Appendix III). All the extract impregnated and control discs were transferred using a sterile forceps and placed on the prepared culture media. The culture media was incubated at 37 $^{0}$C for 24 hours, after which the plates were removed and examined for any antibacterial activity caused by the extracts.

The diameter of the zones of inhibition, where there was absence of growth, was measured using a ruler and recorded in mm. Chloramphenicol (30 µg disc $^{-1}$) that was used as the standard antibacterial agent, was treated the same way as the crude extract. The bioassay tests were done triplicates; blank (DMSO), extract, and standard (Chloramphenicol). The antimicrobial activity in terms of percentage was calculated by applying the RIZD (Relative Inhibition Zone Diameter) expression shown below (Haniffa & Kavitha, 2012).

$$\%RIZD = \frac{[IZD\ Sample - IZD\ Negative\ Control\ (D)]}{IZD\ Standard\ (Chloramphenicol)} \times 100$$

D: IZD of plain disc with DMSO

%RIZD: Percentage of Relative Inhibition Zone Diameter

IZD: Inhibition zone diameter (mm)

The equation above compensates the possible effect of the solvent blank also the negative control (water), extract, standard which is also the positive control (Chloramphenicol). The resulting IZD of the samples are either higher than or equal to the IZD of the blanks. Therefore, the obtained percentages are positive. The test is considered negative (-) when

the IZD of the sample is equal to the IZD of the blank.

### 3.3.6 Minimum inhibitory concentration (MIC)

Any plant part extracted by any of the two extracting solvents that exhibited an inhibition zone had its MIC done. The MICs were determined using serial dilutions through serial dilutions from the original crude extract in a micro titter plate (Appendix IV, a). The other respective wells of the plate had the 100 µl of the reconstituting solution which is DMSO added to it. Concentrations were made of 1000 µg/ml, 10 µg/ml, 0.01 µg/ml, 0.001 µg/ml and 0.0001 µg/ml. The solutions in the micro titter plates were impregnated onto sterile paper discs and placed on plates that had been inoculated with the bacterial cultures (Appendix IV, b). The dilutions from the wells on the discs were subjected to bioactivity test using disc diffusion method to determine the zones of inhibition. (Ogutu *et al.,* 2012).

## 3.4 Phytochemical tests

The possible phytochemical compounds that are present in *S. cuneifolia* are tannins, terpenoids, flavanoids, saponins and alkaloids. They were tested for their presence after bioassay tests were run and showed activity. The extracts obtained from aqueous and ethanol was used to test for the phytochemicals present-and each test was performed in triplicates. Thin layer chromatography (TLC) was used to determine tannins, terpenoids, alkaloids and flavanoids. Saponins were determined separately by the Frothing test. The phytochemicals were further tested using High Performance Liquid Chromatography (HPLC), Hewlett Packard (HP) model 5988A MS coupled to a HP 5980 GC equipped with a HP-1 fused silica capillary column to tests extracts.

### 3.4.1 Thin Layer Chromatography (TLC)

Phytochemical compounds that were present in the aqueous and ethanol extracts of *S. cuneifolia* were tested on silica gel plates via thin layer chromatography (TLC) to determine the presence of tannins, terpenoids, alkaloids and flavanoids. A system of solvents constituting ethanol and petether in a ratio of 7:3 was prepared. The silica gel plate was placed into a beaker containing the solvent system (Appendix V, a). The solvent moved up via capillary action and resulting bands were visualized in a UV chamber (Appendix V, b) (Bigoniya *et al.,* 2013). Saponins were determined separately by the Frothing test (Onwukaeme & Asonye, 2007).

#### 3.4.1.1 Tannins test

The TLC plate was sprayed using ferric chloride-potassium ferricyanide reagent. A blue or green color on spots on the plate indicated the presence of tannins (Appendix V, c) (Pratt & Miller, 1984).

#### 3.4.1.2 Terpenoids tests

The plates were sprayed with 1% vanillin sulphuric acid reagent and heated gently to confirm the presence of terpenoids. If the spots on the TLC plate turned purplish, it indicated presence of Terpenoids (Appendix V, c) (Dimitriadis & Williams, 1984).

#### 3.4.1.3 Flavonoids test

The TLC plate was put over a steam bath (40–50 $^0$C) for 2 min; then put on a bottle that had dilute ammonia to expose the plate to ammonia fumes. A yellow coloration indicated positive test for flavanoids (Appendix V, c) (Kaur & Arora, 2009).

#### 3.4.1.4 Alkaloids test

TLC plate and the plate was sprayed with Dragendorff's reagent which is composed of potassium bismuth iodide prepared from basic bismuth nitrate ($Bi(NO_3)_3$), tartaric acid, and potassium iodide (KI). If the spots turned orange, it would indicate presence of alkaloids (Appendix V, c) (Kumar *et al*., 2007).

#### 3.4.1.5 Saponin test

The extracts obtained were transferred into a test tube, 20 ml of water added and shaken vigorously for 15 minutes then was left to stand for 10 minutes and the result noted. A thick persistent froth of about 1 centimeter thick indicated the presence of saponins (Appendix VI) (Onwukaeme *et al*., 2007).

### 3.4.2 High Performance Liquid Chromatography (HPLC)

The column temperature was initially held at 200 $^0$C for 0.8 min, then increased at 10 $^0$C/min to 250 $^0$C, and then held at 250 $^0$C for 24 min: the helium flow-rate was 10 mL/min. The ion source of the MS was operated at 250 $^0$C and the transfer line at 280 $^0$C. Electron impact (EI) ionisation was carried out at 70 eV, and quantitative determination was based on the total ion current corrected for the detector response of each individual alkaloid. The mass range from 125 to 450 atomic mass units (amu) was scanned at a rate

of 2.6 scans/s. The samples were then run so as to identify the possible phytochemicals that may be present in the extracts.

### 3.5 Data analysis

The experiment for the antibacterial activity of the plant was carried out using the factorial design with three replicates. Data collected from the zones of inhibition in millimeters (mm) from the crude extracts of individual plant parts had their mean values of the three replicates and standard error (S.E) calculated. The values were calculated using Statgraphics and STATA by three way ANOVA to determine the relationships. The confidence level considered was 95% and the means obtained were separated using Tukey's Honesty Significance Test (THST).

# CHAPTER FOUR

# RESULTS

## 4.1 Effectiveness of *S. cuneifolia* plant parts against selected enteric pathogens

The results on the effects of *S. cuneifolia* extracts on the test pathogens and the relationship between the microorganism, plant parts and solvents used are summarized in Table 4.1 and Appendix VI. The zone of inhibition of the test microorganisms differed significantly ($p<0.05$) depending on the plant part and solvent used. The interactions between the microorganism and the plant part (Microorganism x Plant part), microorganism and the solvent (Microorganism x Solvent) and the plant part and solvent (Plant part x Solvent) also had a significant effect on the zone of inhibition ($p<0.05$). The interaction between microorganism, plant part and solvent used was not significant ($p>0.05$).

**Table 4. 1: Effects of the plant parts and their interactions on the zone of inhibition**

| Source of variation | F ratio | P value | Effect |
|---|---|---|---|
| Microorganism | 64.64 | 0.0000 | S |
| Plant part | 1383.90 | 0.0000 | S |
| Solvent | 12.05 | 0.0008 | S |
| Microorganism x Plant part | 76.69 | 0.0000 | S |
| Microorganism x Solvent | 3.11 | 0.0311 | S |
| Plant part x Solvent | 3.31 | 0.0146 | S |
| Microorganism x Plant part x Solvent | 1.00 | 0.4586 | NS |

*S: Significant at p<0.05 and NS: Not Significant at p>0.05*

The mean zones of inhibition of *S. cuneifolia* extracts against enteric pathogens are shown in Table 4.2. Aqueous extracts had significantly lower zones of inhibition compared to the ethanol extracts.

There was no inhibition against *E. coli* (Plate 2), for all aqueous extracts. The highest zone of inhibition obtained when water was used as a solvent was that of leaf extracts against *S. typhi* (8.6 mm). For stems with water as the solvent, the highest zone of inhibition was against *S. typhi* and *Shigella* (6.6 mm). The highest zone of inhibition for roots with water as a solvent was against *Shigella* and *S. typhi* (7.3 mm) (Plate 3).

All ethanol extracts did not show any zone of inhibition against *E. coli*. The highest zone of inhibition recorded was with stem ethanol extracts against *Shigella* (9 mm). For the leaf ethanol extracts, the highest zone of inhibition was against *Shigella* (8.6 mm), while for the root ethanol extracts, the highest zones of inhibition was against *Shigella* and *S. aureus* (8.3 mm).

**Table 4. 2: Mean zones of inhibition in mm of *S. cuneifolia* extracts against enteric pathogens**

| | **Aqueous** | | | **Ethanol** | | | **Controls** | |
|---|---|---|---|---|---|---|---|---|
| | **Stem** | **Leaves** | **Roots** | **Stem** | **Leaves** | **Roots** | **Chloramphenicol** | **DMSO** |
| *E. coli* | 6±0.0 [a] | 6±0.0 [a] | 6±0.0 [a] | 6±0.0 [a] | 6±0.0 [a] | 6±0.0 [a] | 21.6±0.57 [b] | 6±0.0 [a] |
| *S. aureus* | 6 ±0.0 [a] | 6±0.0 [a] | 6.3±0.57 [b] | 8±1.73 [cd] | 7±1.0 [c] | 8.3±1.15 [de] | 27±1.0 [f] | 6±0.0 [a] |
| *S. typhi* | 6.6±1.15 [ab] | 8.6±0.57 [cf] | 7.3±1.52 [bc] | 8.3±0.57 [ce] | 7.6±1.15 [bd] | 7.3±1.52 [bc] | 12±1.0 [g] | 6±0.0 [a] |
| *Shigella* | 6.6±0.57 [b] | 7±0.0 [bc] | 7.3±1.52 [cd] | 9±1.0 [g] | 8.6±0.57 [f] | 8.3±1.15 [e] | 23.6±1.52 [h] | 6±0.0 [a] |

*Means denoted with the same letters within the same rows are not significantly different at P<0.05*

*6 mm indicates there was no zone of inhibition*

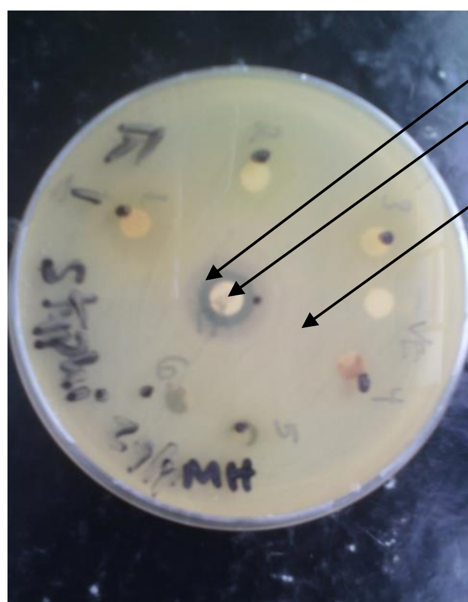

**Plate 2: Zone of inhibition of ethanol extract against *S. typhi* plate**

**Plate 3: Zone of inhibition of ethanol extract against *E. coli***

*a – Zone of inhibition, b – Impregnated paper disc, c – Bacterial colony*

The percentage Relative Inhibition Zone Diameter of *S. cuneifolia* extracts against bacterial pathogens is shown in Figure 4.1. Aqueous leaf extracts had the highest (RIZD) of 22.22% against *S. typhi*. It was followed by the stem ethanol extracts against *S. typhi* with a %RIZD of 19.44%. Roots aqueous extracts had the lowest %RIZD of 1.23% against *S. aureus* followed by stem aqueous extracts against *Shigella* (Plate 4) with a %RIZD of 2.81% and by leaf ethanol extracts against *S. aureus* (Plate 5) with a %RIZD of 3.7% (Figure 4.1).

*Escherichia coli* had the lowest % RIZD of 0 for all the crude extracts. *Salmonella typhi* exhibited the highest %RIZD with all crude preparations. This was significant for leaf

aqueous extract, stem and leaf ethanol extracts (22.22, 19.4 and 13.9 respectively). *Shigella* had the second highest %RIZD with all the crude plant part preparations, which was significant for stem, leaf and root extracts (12.67, 11.26 and 9.85 respectively).

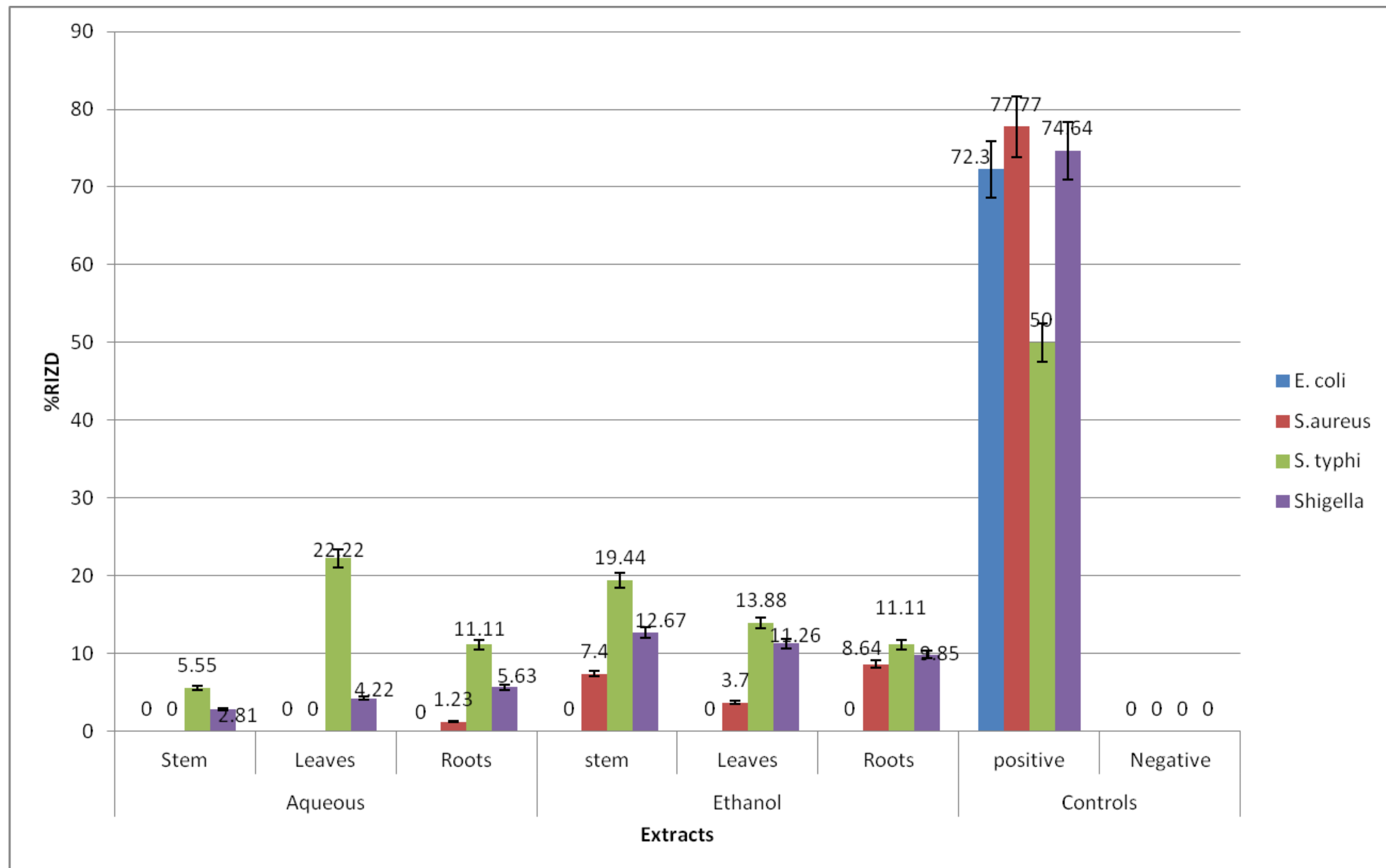


**Figure 4. 1: %RIZD of *S. cuneifolia* extracts against bacterial pathogens**

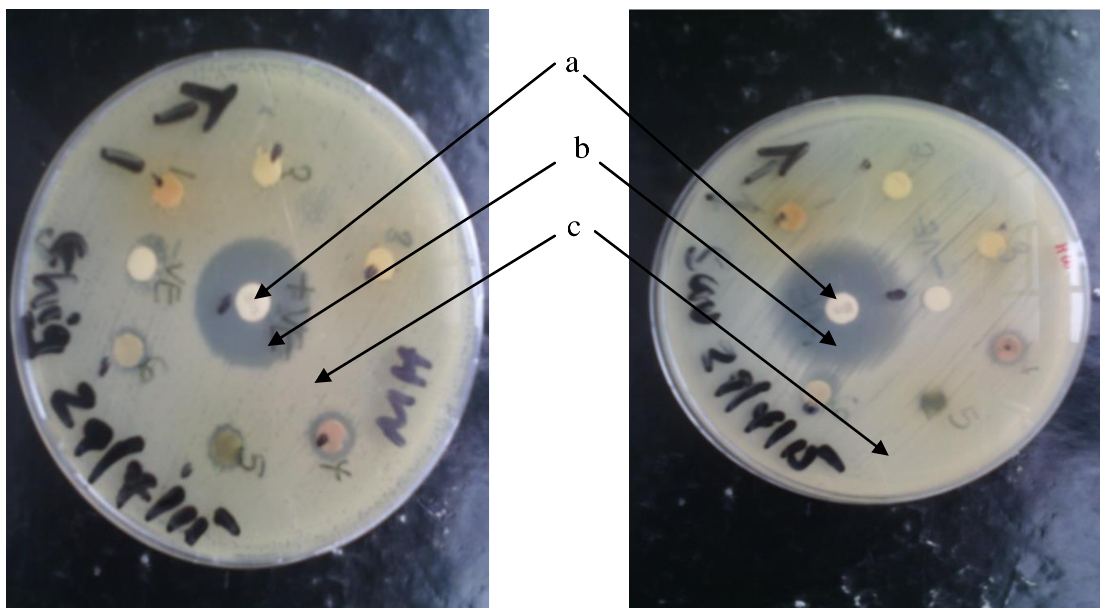


**Plate 5: Zone of inhibition of aqueous extracts against *Shigella***

**Plate 4: Zone of inhibition of aqueous extracts against *S. aureus***

*a – Zone of inhibition, b – Impregnated paper disc, c- Bacterial colony*

## 4.4 Effectiveness of *S. cuneifolia* plant parts

Table 4.3 shows the zone of inhibition of microbes obtained with each plant parts irrespective of the solvent used. The leaves had a significantly higher %RIZD of 18.05% against *S.typhi* followed by the stems (12.5%) against *S. typhi* and roots (11.11%) against *S. typhi*. The rest had RIZD% below 10% with leaves showing the lowest %RIZD of 1.85% against *Staphylococcus aureus*.

**Table 4. 3: Mean RIZD% of *S. cuneifolia* plant parts against enteric pathogens**

| | Stem | Leaves | Roots |
|---|---|---|---|
| *E. coli* | $0\pm0.0^{a}$ | $0\pm0.0^{a}$ | $0\pm0.0^{a}$ |
| *S.aureus* | $3.70\pm0.86^{b}$ | $1.85\pm0.5^{a}$ | $4.93\pm0.86^{c}$ |
| *S. typhi* | $12.5\pm0.86^{b}$ | $18.05\pm0.86^{c}$ | $11.11\pm1.52^{a}$ |
| *Shigella* | $7.74\pm0.78^{c}$ | $7.74\pm0.28^{a}$ | $7.74\pm1.33^{b}$ |

*Means denoted with the same letters in the same row are not significantly different at $P<0.05$*

**4.5 Efficacy of solvent for extraction**

Table 4.4 shows the general efficiency of the two solvents used for extraction irrespective of the plant part. There was no activity of the crude extracts against *E. coli*. Ethanol extracts had the highest activity against all microorganisms with an RIZD of 14.81% for *Salmonella typhi* followed by 11.26% for *Shigella* and 6.58% for *S. aureus.* For aqueous extracts there was no significant difference observed against the microorganisms. The highest mean activity was recorded against *Salmonella typhi* with a RIZD of (12.96%) followed by *Shigella* (4.22%) and lastly *Staphylococcus aureus* (0.411%) (Table 4.4).

**Table 4. 4: Comparison of the activities between the two solvents against the bacterial pathogens**

| | Aqueous | Ethanol |
|---|---|---|
| *E. coli* | 0±0.0[a] | 0±0.0[a] |
| *S.aureus* | 0.41±0.19[a] | 6.58±1.29[b] |
| *S. typhi* | 12.96±1.08[a] | 14.81±1.08[b] |
| *Shigella* | 4.22±0.69[a] | 11.26±0.96[b] |

*Means denoted with the same letters are not significantly different at P<0.05*

**4.6 Minimum inhibitory concentration**

Table 4.5 shows Minimum inhibitory concentration in mg/µl of *S. cuneifolia* extracts against the various pathogens. MICs were done on extracts that showed activity. The roots and stem ethanol extracts had the lowest MIC of 0.001 µg/ml against *Salmonella typhi*. The leaf, stem and root ethanol extracts came second with MICs of 0.01 µg/ml against *Shigella.* Similar results were obtained with the leaf and root ethanol extracts against *Staphylococcus aureus* and the leaf and root water extracts against *Salmonella typhi* and *Shigella* respectively. (Table 4.5).

**Table 4. 5: Results of MIC of the plant parts and different solvents in µg/ml**

| | AQ | | | ETH | | |
|---|---|---|---|---|---|---|
| | Stem | Leaves | Roots | Stem | Leaves | Roots |
| *S. aureus* | - | - | 1000 | 10 | 0.01 | 0.01 |
| *Shigella* | 1000 | 10 | 0.01 | 0.01 | 0.01 | 0.01 |
| *S. typhi* | 10 | 0.01 | 1000 | 0.001 | 10 | 0.001 |

## 4.6 Phytochemicals present in *S. cuneifolia*

### 4.6.1 Phytochemicals obtained from Thin Layer Chromatography (TLC)

Table 4.6 shows the phytochemicals present in the plant. Terpenoids, tannins, alkaloids, flavanoids and saponins were tested using TLC. The root and leaf ethanol extracts had all the tested phytochemicals. The stem ethanol extracts had all phytochemicals except alkaloids. Aqueous extracts had terpenoids, tannins and saponins but no alkaloids and flavanoids.

**Table 4. 6: Phytochemicals obtained using TLC**

| | Aqueous | | | Ethanol | | |
|---|---|---|---|---|---|---|
| | Stem | Leaves | Roots | Stem | Leaves | Roots |
| Terpenoids | P | P | P | P | P | P |
| Tannins | P | P | P | P | P | P |
| Alkaloids | A | A | A | A | P | P |
| Flavanoids | A | A | A | P | P | P |
| Saponins | P | P | P | P | P | P |

P – Present, A – Absent.

### 4.6.2 Phytochemicals obtained from High Performance Liquid Chromatography

Table 4.7 shows the various phytochemicals precisely as they were found via High Performance Liquid Chromatography (HPLC) through GCMS. Caffeic acid, gallic acid, flavanoles 4’OH, 5’OH were the common phytochemicals present among the extracts. Ethanol leaf extracts had the highest number of phytochemicals found most of medicinal importance. Other phytochemicals obtained are listed in Appendix VII.

**Table 4. 7: Phytochemicals obtained from High Performance Liquid Chromatography**

| Aqueous | | | Ethanol | | |
|---|---|---|---|---|---|
| Stem | Leaves | Roots | Stem | Leaves | Roots |
| Caryophyline | caffeic acid | caffeic acid | Apigenin | caffeic acid | caffeic acid |
| Silychristin | gallic acid | Cryptopine | benzenedicarboxylic acid | gallic acid | Cryptopine |
| Benzenetriol | hydroxycaffeic acid | ferulic acid | Flavanoles 4’OH, 5’OH | hydroxycaffeic acid | ferulic acid |
| Apigenin | Octadecanoic acid,methyl ester | flavones 4'-OH,5-OH | Carvone | Octadecanoic acid,methyl ester | flavones 4'-OH,5-OH |
| Limonene | *p*-coumaric acid | protocatechuic acid | Caryophyline | *p*-coumaric acid | protocatechuic acid |
| Gallic acid | vanillic acid | Protopine | Coniilne | vanillic acid | Protopine |
| Guaiol | Cholesterol. | Xylitol | Guaiol | Flavanoles 4’OH, 5’OH | Xylitol |
| Hexadecanoic acid,methylester | cinnamic acid | Octadecanoic acid,methyl ester | Hexadecanoic acid,methylester | Carvacrol | Octadecanoic acid,methyl ester |
| Khellin | Eucolyptol | Glycerin | Caffeic acid | Cineol | Glycerin |
| Carvone | flavones 4'-OH,5-OH | gallic acid | Limonene | ferulic acid | gallic acid |
| Methyl tetradecanoate | gentisic acid | Borynl Acetate | Methyl tetradecanoate | Hexadecanoic acid,methylester | Adlumine |
| Octadecanoic acid,methyl ester | Glycerin | caphyllene oxide | Octadecanoic acid,methyl ester | homogentisic acid | Bicuculline |
| Silybin | Kaempferol | cinnamic acid | Silybin | Linalool | Hexadecanoic acid,methylester |
| Silydianin | myri- cetin | Cinnamyl Acetate | Silychristin | Luteolin | Fumariline |

**Table 4.7 Continued: Phytochemicals of medicinal importance obtained from High Performance Liquid Chromatography**

| Aqueous | | | Ethanol | | |
|---|---|---|---|---|---|
| Stem | Leaves | Roots | Stem | Leaves | Roots |
| Flavanoles 4’OH, 5’OH | Phenol-2,4-Bis (1,1-dimethyl) | gentisic acid | Visnagin | methylchavicol | Stylopine |
| Caffeic acid | protocatechuic acid | *p*-coumaric acid | Gallic acid | protocatechuic acid | Undecane |
| | Quercetin | Phenol-2,4-Bis (1,1-dimethyl) | Silydianin | Quercetin | Methyl tetradecanoate |
| | α cadinene | vanillic acid | | sesquiterpenes | |
| | Naphthalene | Dihydrosanguinarine. | | Thujene | |
| | | | | Thymol | |
| | | | | Methyl tetradecanoate | |

# CHAPTER FIVE

# DISCUSSION

## 5.1 Effectiveness of *S. cuneifolia* the plant

The antibacterial activity of aqueous and ethanol-based extracts on leaves, stem and roots of *S. cuneifolia* were tested on *E. coli, S. typhi, Shigella* and *S. aureus.* All the extracts showed varying degrees of antibacterial potential with the roots ethanol extracts displaying the highest zone of inhibition. Also the antibacterial activity of all extracts depended largely upon the solvent used in extraction. Ethanol-based extracts displayed significantly higher activity than the aqueous extracts. In the current study, there was proof that this plant may have the ability to work against *S. typhi, Shigella* and *S.aureus*, probably the same way conventional drugs work.

*Escherichia coli* was the most resistant bacterial pathogen for it showed no zone of inhibition against the extracts. Resistance arises spontaneously due to point mutations that result in amino acid substitutions within the topoisomerase subunits and this may have been in the case with *E. coli* (Ito *et al.,* 2003; Hopkins *et al.,* 2005). Another source of resistance by *E. coli* might be the production of an exopolysaccharide matrix or glycocalyx, distinguishing characteristics of biofilms. One of its functions is to prevent the access of antibiotics to the bacterial cells. The biofilm can limit the transport of antimicrobial agents to the cells within the biofilm (Mah & O'Toole, 2001; Hopkins *et al.,* 2005).

In the present study *Staphylococcus aureus* was the second most resistant pathogen for it had the second lowest zone of inhibition. *Staphylococcus aureus* infections are a

significant problem in hospitalized patients. The pathogen can easily gain resistance by sharing genes for antimicrobial resistance (Forbes & Schaberg, 1983; Shapiro *et al.,* 2001). Sharing of genes is usually by conjugative mobilization or by conduction where plasmids physically combine with conjugative plasmids by recombination between regions of homology or mobilization (Projan & Archer, 1989). Other models that might account for drug resistance by the *S. aureus* and *E. coli* to several plant part extracts include plasma membrane mechanisms, the plasma membrane of drug resistant cells appear different from the cells that are susceptible to the drug thus making the cell not be recognized by the antibiotic (Simon & Schindler, 1994). The second is the ATP driven drug efflux model where the peptidoglygan protein uses ATP to power a molecular pump that then removes drugs out of the cytosol and cell (Li *et al*., 2015). Nuclear mechanisms bring about resistance by preventing drugs that inactivate transcription and replication of DNA from accumulating in the nucleus thus no binding to DNA and associated nuclear proteins. Resistance can also be through modifications in; mechanisms of drug translocation across the nuclear envelope; binding sites on the genetics material; DNA repair mechanisms; and efficiency of m RNA export and protein import into the nucleus (Longeley & Johnston, 2005).

Exocytosis is another model that explains resistance where drugs accumulate in exocytotic pathways; the drugs are trapped into exocytosis compartments then expelled from the cell. This decreases the drug concentration in the cytoplasm (Chen *et al.,* 2007). Compartmentalization also brings resistance by changes in the ability of internalized drugs to reach critical concentrations at intranuclear regions; thus seriously limiting the

effectiveness of drug therapy and leads to drug resistance. Drugs accumulate in the acidic compartments of the cell, the trans golgi and lysosomal compartments (Das *et al.,* 2013).

### 5.2 Efficiency of *S. cuneifolia* plant parts against pathogens

This study showed that leaves exhibited a high activity against *S. aureus* with both aqueous and ethanol extracts. This is probably because a high concentration of phytochemicals was also found in the leaves thus a high antimicrobial activity (Chaparro *et al.,* 2013). Crude preparations of whole plant parts which contain both the active and non-active components have been known to have higher efficacy than semi-crude or pure plant substances (Agrawal *et al.,* 2012). Previous studies by Nalubega *et al*., (2011) on the stem bark of *S. cuneifolia* showed activity against *S. aureus,* though this study has gone ahead and proved further that leaves are superior to the stem. Plants have phytochemicals that are highly concentrated in the roots as Chaparro *et al*., (2013) found out, and this probably explains why all the root extracts exhibited antimicrobial activity. The absence of some of the phytochemicals in the aqueous extracts like; alkaloids and flavanoids may be the reason for lower inhibitory or no effect. These phytochemicals may have not have been not extracted or they were lost during boiling of the plant material. These active plant compounds are volatile and can be lost during boiling (Oguekea *et al.,*2007).

### 5.3 Effectiveness of solvent for extraction

In this study, ethanol performed better than water as a solvent. The effectiveness of different crude extracts of the same plant material depends on the solvent used for

extraction of the phytochemical compounds, though the extracts from the same plant material may not be the same in their antioxidant concentrations and activities (Das *et al.,* 2010).

Ethanol extracts showed significantly higher inhibition than the aqueous extracts. Ethanol as a solvent works better than water as it is more polar and can dissolve both polar and non-polar substances (Wolfenden *et al.,* 1981). It has been known that higher concentration of phytochemicals is favoured by an increase in polarity of the solvent used. When ethanol and water are used in extraction, ethanol is therefore more efficient than water for extracting total phenolics. Extracts obtained by high polarity solvents are considered to be more effective radical scavengers than those less polarity solvents. A change in solvent polarity alters its ability to dissolve antioxidant compounds (Turkmen *et al.,* 2006).

Phytochemicals yield extracted are known to increase for water content of ethanol from 10% to 30%, and remain constant for water content from 30% to 60%. The concentration of phenols in ethanol extracts decreases when the water content is above 50% (Spigno *et al.,* 2007).

### 5.4 Phytochemicals present in *S. cuneifolia*

In the present study, phytochemicals present were Terpenoids, tannins and saponins in all the extracts alkaloids and flavanoids were absent in aqueous extracts and ethanol stem extracts. The absence of alkaloids and flavanoids is probably the reason for low or no inhibitory effect of the aqueous extracts. Possibly most of the active antimicrobial

compounds were not extracted or the volatile compounds were lost during boiling of the plant material (Oguekea *et al.,* 2007). Furthermore, ethanol extracts had a higher antibacterial activity compared to the aqueous extracts because of their high antioxidant and phenol content (Mao *et al.,* 2006; Bukar *et al.,* 2013).

It is further shown that other phytochemicals such as Gallic acid, Caffeic, Flavanoles 4'OH, 5'OH, propolis, Ferulic acid. The main groups of phytochemicals that were found are; Phenolics and Phenols, hydroxycinnamic acids, and flavanoids. Gallic acid, a simple phenolic acid is widely distributed in angiosperms and is also found in some green algae. It is a well-known antioxidant, and has been previously reported to possess antibacterial and antifungal activities (Chanwitheesuk *et al.,* 2007; Tula *et al.,* 2012). Caffeic acid is the phenylpropenoid commonly found in plants and has been demonstrated to have medicinal properties. It is an antioxidant agent and delays the onset of lipid peroxidation, thus lengthening of the lag phase (Nardini *et al.,* 1998). Flavanoles 4'OH, 5'OH are also antioxidants whose activity is related to the free radical-scavenging ability, by breaking the chain of reactions triggered by free radicals affecting the bacterial cell (Tripoli *et al*., 2005).

Other phytochemicals that were present such as propolis and some of its cinnamic and flavonoid components are known to uncouple the energy tranducing cytoplasmic membrane and to inhibit bacterial motility. These effects on the bioenergetic status of the membrane may contribute to the antimicrobial action of propolis and its observed synergism with selected antibiotics (Mirzoeva *et al.,* 1997). p-Coumaric acid one of the hydroxycinnamic acids is generally an effective inhibitor against *S. aureus* (Herald &

Davidson, 1983). Tannic acid is known to have antibacterial properties against *S. aureus* (Akiyama *et al.,* 2001). Luteolin isolated from *S. cuneifolia* has also showed antimicrobial activity (Tshikalange *et al.,* 2005). Quercetin, a flavonol found in vegetables and fruit has shown multiple biological effects as an antioxidant and free radical-scavenger and also exhibit antibacterial properties against bacteria (Bravo & Anacona, 2001). Ferulic acid has a wide spectrum of antimicrobial activity. It has antimicrobial activity towards Gram-positive bacteria, Gram-negative bacteria including some human gastrointestinal microflora like *Shigella* with its antimicrobial mechanism is credited to its inhibition of arylamine N-acetyltransferase in the bacteria (Ou & Kwok, 2004).

Carvacrol present is a monoterpenic phenol found in aromatic plants, categorized as Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration (FDA) (Marchese *et al*., 2006), which is used as a flavoring agent in sweet foods. It acts on the cytoplasmatic membrane interacting with the lipid bilayer of bacterial cytoplasmic membrane causing a loss of integrity, collapse of the proton motive force allowing leakage of cellular material such as ATP, ions and nucleic acid bringing about growth inhibition of pathogens (Nostro *et al.,* 2012). And thus the presence of all these phytochemicals in *Sida cuneifolia* has contributed to its antimicrobial activity against the pathogens.

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusion

1. Despite the resistance of *E. coli*, the plant can be considered for the treatment of issues related to the other three enteric pathogens: *S. typhi, S. aureus* and *Shigella.*
2. Among all the three plant parts tested; roots, leaves and stems, the leaves were the most effective plant part in inhibition of bacterial growth, thus it should be considered first when preparing medicinal remedies.
3. Ethanol was the most effective solvent for extraction of the active components and preparation of crude extracts for medicinal purposes.
4. The phytochemicals present in *Sida cuneifolia* are diverse and of medicinal importance though not all are present depending on the method and solvent used during extraction.

## 6.2 Recommendations

Based on the findings from this study, the following recommendations were made;

1. There is need for sensitization of folklore medicine practitioners about the use of leaves in the treatment of ailments caused by *S. typhi, S. aureus* and *Shigella.*
2. The study only determined the antimicrobial activity, other studies such as toxicity and mode of action should be established.
3. There can be further purification of the crude extracts and/or preparation of concentrated concoctions so that the medicinal effect can be more pronounced.

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# APPENDICES

## Appendix I: Preparation and extraction of crude extracts

a. Ground plant parts

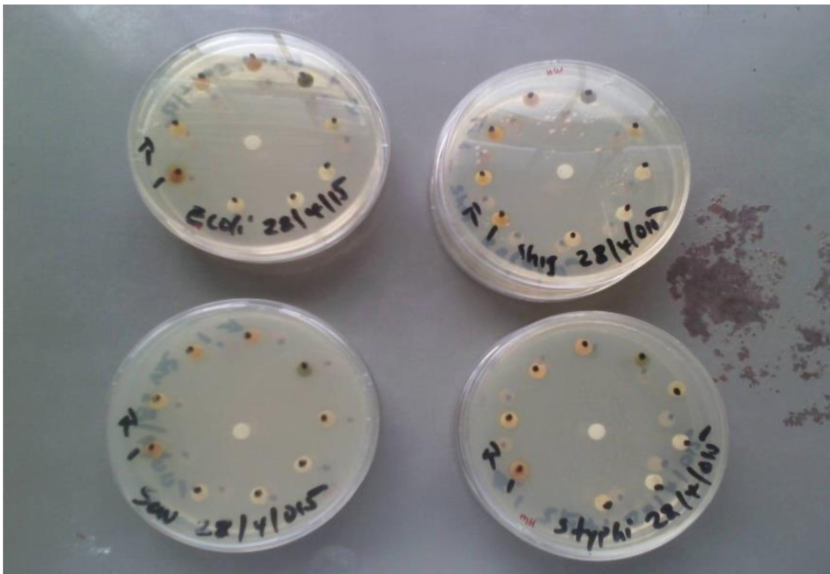
b. Filtering of ethanol extracts.

c. Removal of excess ethanol using a rotor evaporator

d. Ethanol extracts left to dry in front of a fan to expel any remaining ethanol

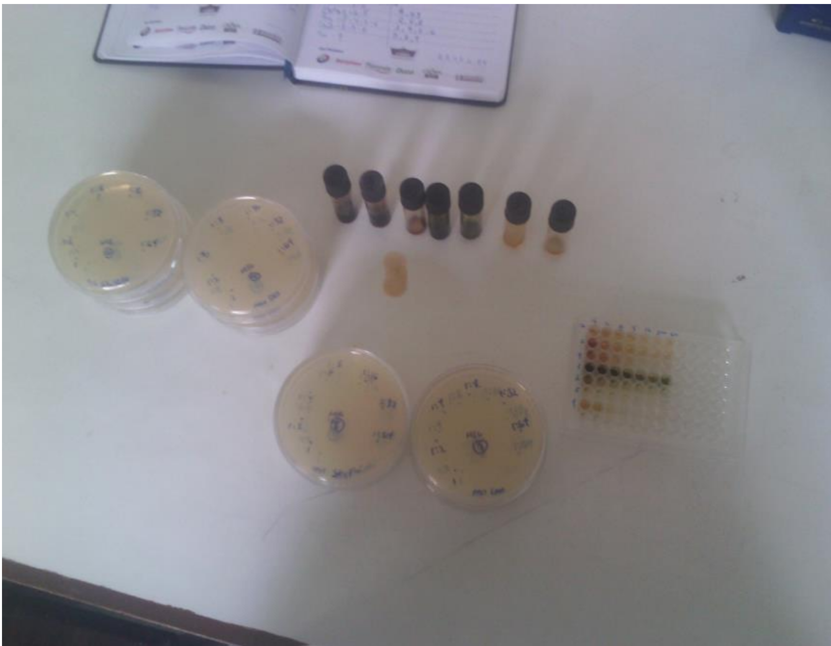
e. Freeze drying of aqueous plant extracts

**Appendix II: Kirby bauer disks on the various cultures**



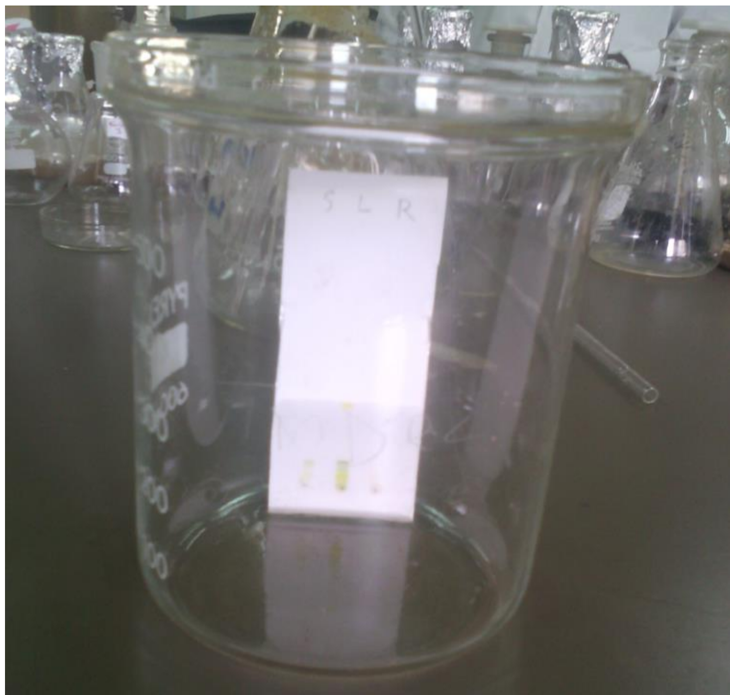
**Appendix III: Running MIC tests**

a. Preparation of microtitter plates for MIC

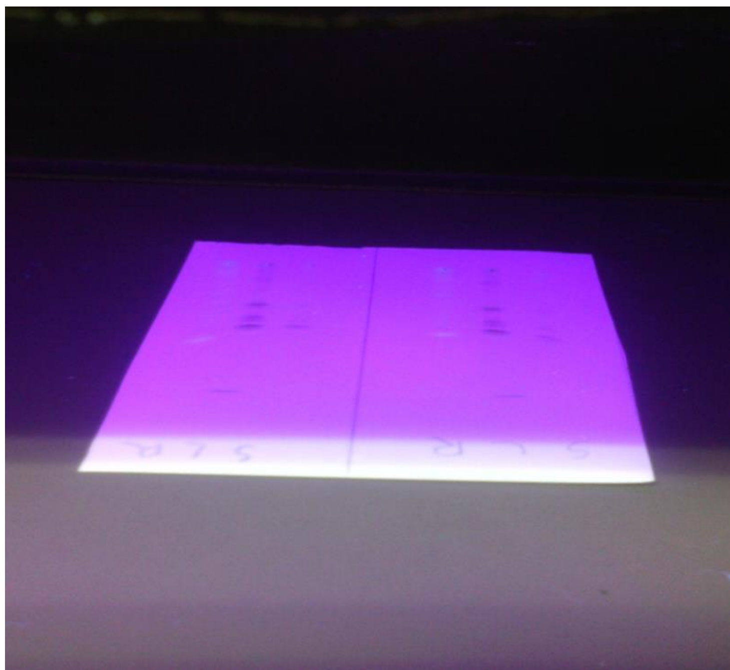


b. Cultures made for MIC

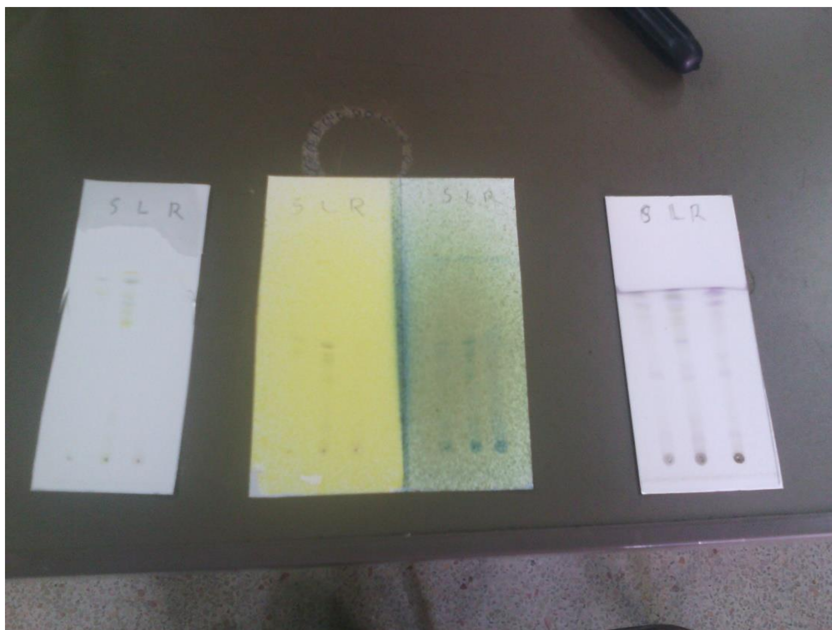
**Appendix IV: Running TLC tests**



a. TLC paper inserted in a chromatography system so as to allow separation of bands



b. TLC plate being viewed under Ultra Voilet before treatment with reagents.




c. TLC plates displaying bands after treatment with reagents

**Appendix V: Test for saponins being run**

**Appendix VI: ANOVA table showing effect and interaction of all the factors in terms of zones of inhibition**

| *Source* | *Sum of Squares* | *Df* | *Mean Square* | *F-Ratio* | *P-Value* |
|---|---|---|---|---|---|
| Microorganism | 137.367 | 3 | 45.7889 | 64.64 | 0.0000 |
| Plant part | 3921.05 | 4 | 980.262 | 1383.90 | 0.0000 |
| Solvent | 8.53333 | 1 | 8.53333 | 12.05 | 0.0008 |
| Microorganism x Plant part | 651.883 | 12 | 54.3236 | 76.69 | 0.0000 |
| Microorganism x Solvent | 6.6 | 3 | 2.2 | 3.11 | 0.0311 |
| Plant part x Solvent | 9.38333 | 4 | 2.34583 | 3.31 | 0.0146 |
| Microorganism x Plant part x Solvent | 8.48333 | 12 | 0.706944 | 1.00 | 0.4586 |
| ERROR | 56.6667 | 80 | 0.708333 | | |
| TOTAL (CORRECTED) | 4799.97 | 119 | | | |

**Appendix VII: Results of phytochemicals obtained from High Performance Liquid Chromatography via Gas Chromatography Mass Spectroscopy**

File : D:MassHunter/GCMS/1/data/TOXICOLOGY/2015023006.D60m, 0.25mm ID, 1.4µm Rtx®-1301 (cat.# 16016)

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- Instrument: 5977-GOVT CHEM
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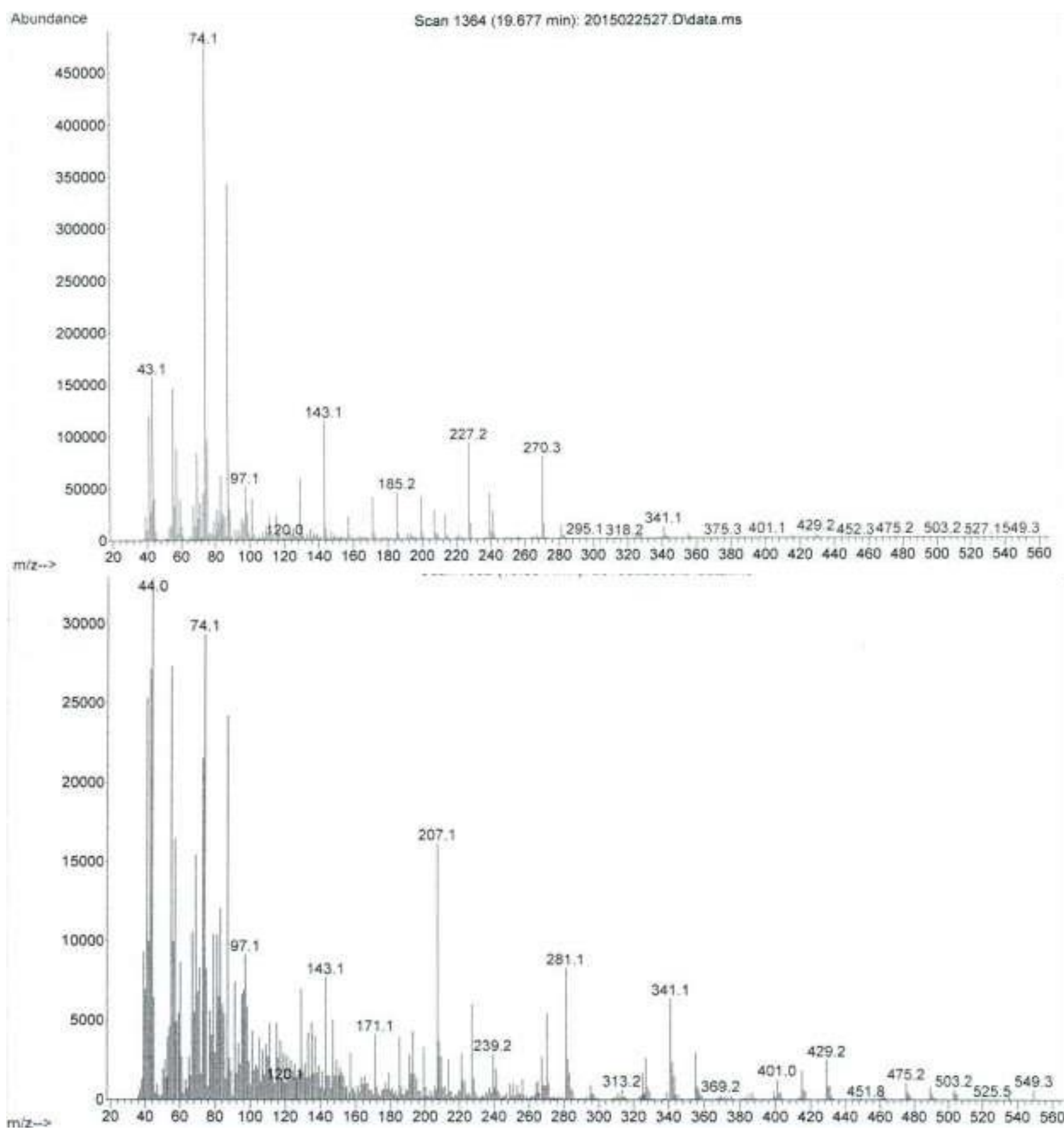
- Vial Number 2



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AMDIS GC/MS Analysis Report Library: C:/NIST11/AMDIS32/LIB/NISTEPA.MSL Number of Identifications:31

RT(min) Hits Chemical Name.

1. cinnamic acid
2 *p*-hydroxybenzoic acid
3 vanillic acid
4. gentisic acid
5. *p*-coumaric acid
6. protocatechuic acid
7. *p*-coumaric acid
9. hydroxycaffeic acid
10, gallic acid
11, caffeic acid
12. 3-nitro-phthalic acid.
14. quercetin
15 kaempferol
16. myri- cetin
17 Phenol
18 naphthalene
19. 2,3-dihydrobenzofuran
20 Phenol-2,4-Bis (1,1-dimethyl)
21. flavones 4'-OH,5-OH
22.7-di-O-glucoside
23. 5-hydroxy- 3,6,7,3',4'-pentamethoxy flavones
24. Eucolyptol
25. α cadinene
26. Glycerin
27.Orcinol
28. 9,12-octadecadienoic acid (Z,Z)-,methyl ester
29. 9-Octadecanoic acid (Z)-,methyl ester
30 Octadecanoic acid,methyl ester
31.Chole

sterol.

QA/QC

Instrument type :Quadrupole
Scan Direction High to low
High noise level.Median signal noise(Noise level)Threshold=940.8
Background (low vs. high retention time
Median low RT S/N=171, high
RT S/N=1316

This report consists of 3 page

AMDIS GC/MS Analysis Report- Tue May 12 - 8:14:22 2015

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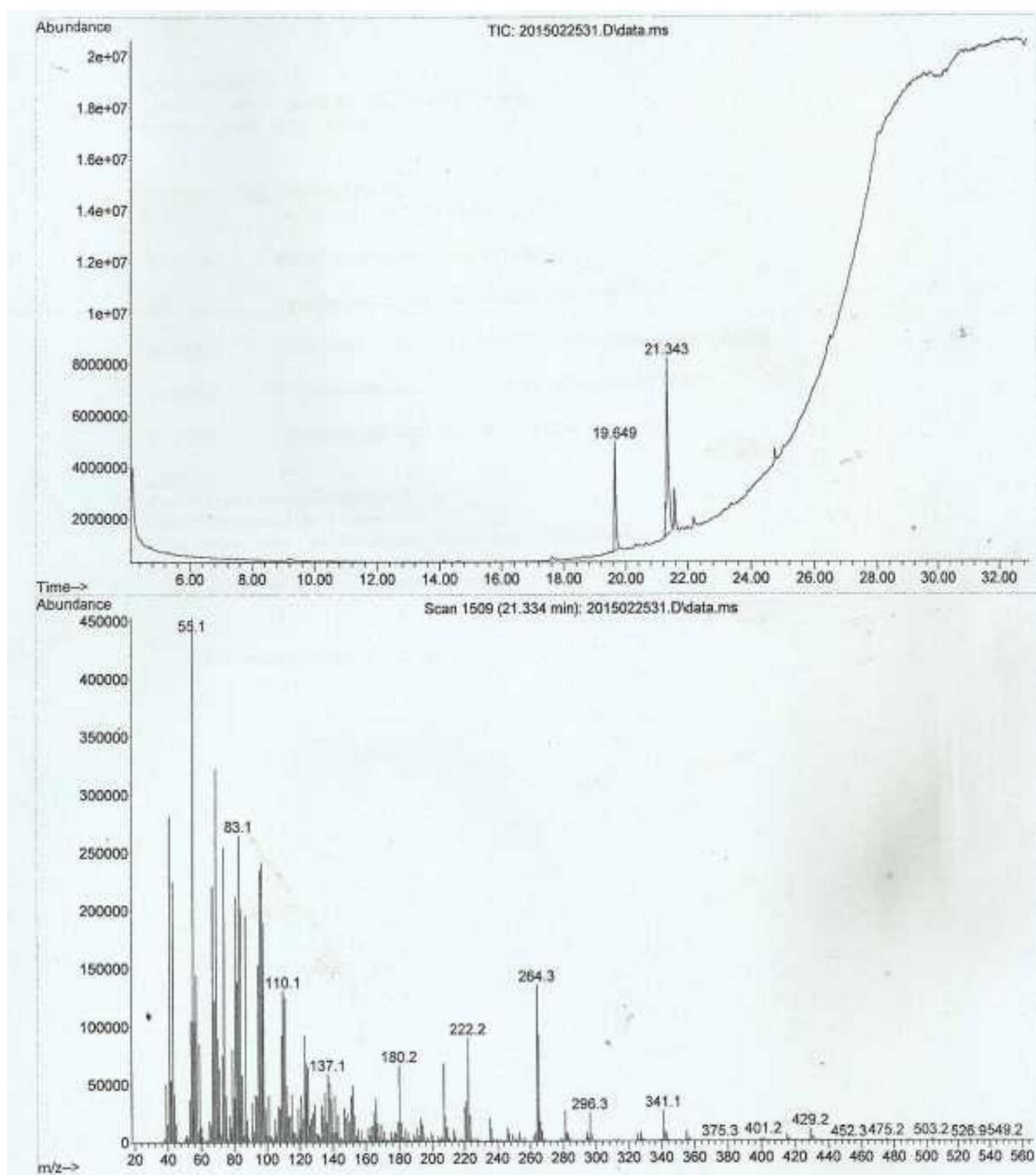
- Sample Name: Sample 2.
- Vial NUMBER: 3



AMDIS GC/MS Analysis Report

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Number of Identifications:40

RT(min) Hits Chemical Name.

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2 *p*-hydroxybenzoic acid
3 vanillic acid
4. gentisic acid
5. *p*-coumaric acid
6. protocatechuic acid
7. *p*-coumaric acid
8.4-Pyranone,.
9. alpha-d-Galactofuranoiside
10. methyl 2,3,5,6-tetra-O-methyl
11. Glycerin
12. Xylitol
13. N, N-Dimethylglycine (
14.5-Hydroxymethylfurfural
15 3-Deoxy-d-mannoic lactone,
16. 3,4-dihydroxy-5-[(3,4,5-trihydroxyphenyl)
17 gallic acid

18. ferulic acid
19.caffeic acid
20.(+)-Catechin
21.(−)-epicatechin.
22 Phenol-2,4-Bis (1,1-dimethyl)
23. flavones 4'-OH,5-OH
24.7-di-O-glucoside
25. 5-hydroxy- 3,6,7,3',4'-pentamethoxy flavones
26. Eucolyptol
27. α cadinene
28.α-pinene
29.caphyllene oxide
30.n hexadecanoic acid
31Borynl Acetate
32.Cinnamyl Acetate
33.protopine
34.cryptopine
35.fumarophycine
36.Dihydrosanguinarine.
37. 9,12-octadecadienoic acid (Z,Z)-,methyl ester
38. 9-Octadecanoic acid (Z)-,methyl ester
39.Octadecanoic acid,methyl ester
40.Bis (2-ethylhexyl) phthalate

QA/QC

Instrument type :Quadrupole
Scan Direction High to low
High noise level.Median signal noise(Noise level)Threshold=940.8
Background (low vs. high retention time
Median low RT S/N=171, high RT S/N=1316

This report consists of 3 page

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  (cat.# 16016)
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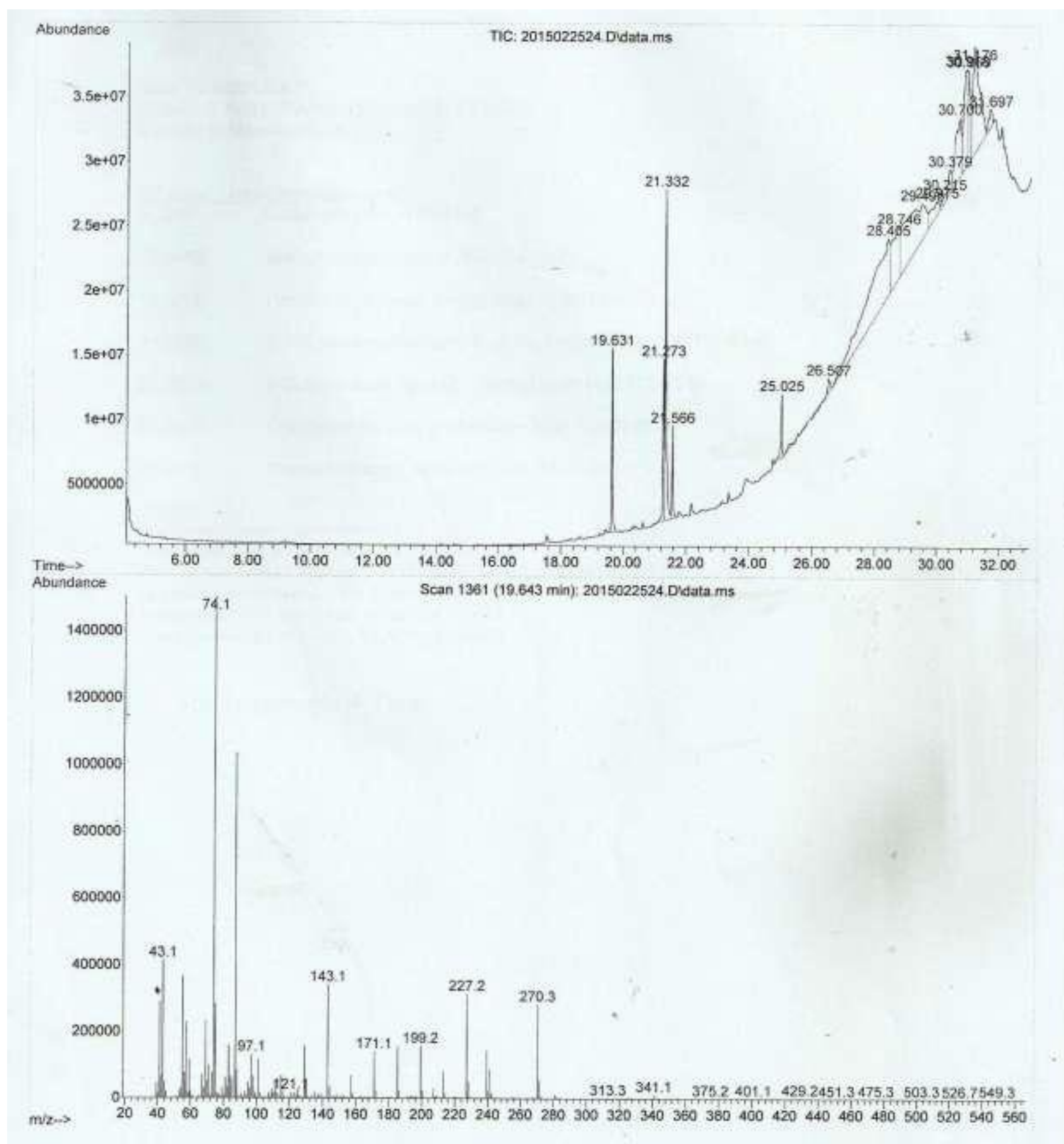
- Acquired: 12 May 2015 8:34
- Instrument: 5977-GOVT CHEM
- Sample Name: Sample 3.
- Vial NUMBER: 4



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AMDIS GC/MS Analysis Report

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Number of Identifications:6

RT(min) Hits Chemical Name.

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2.cryptopine
3. stylopine
4 adlumine
5. parfumine
6. *p*-coumaric acid
7. protocatechuic acid
8. *p*-coumaric acid
9. fumariline
10. bicuculline
11. phthalideiquiniline
12. Xylitol
13. Glycerin
14. 3-Deoxy-d-mannoic lactone,
15. 3,4-dihydroxy-5-[(3,4,5-trihydroxyphenyl)
16. gallic acid
17. ferulic acid
18.caffeic acid
19.(+)-Catechin
20.(−)-epicatechin.
21 Phenol-2,4-Bis (1,1-dimethyl)
22. flavones 4'-OH,5-OH
23.7-di-O-glucoside
24.Undecane
25. Methyl tetradecanoate
26. Hexadecanoic acid,methylester
27. 9,12-octadecadienoic acid (Z,Z)-,methyl ester
28. 9-Octadecanoic acid (Z)-,methyl ester
29.Octadecanoic acid,methyl ester

QA/QC

Instrument type :Quadrupole
Scan Direction High to low
High noise level.Median signal noise(Noise level)Threshold=940.8
Background (low vs. high retention time
Median low RT S/N=197, high
RT S/N=1615

This report consists o f 3 page

AMDIS GC/MS Analysis Report- Tue May 12 - 8:34:52 2015

File : D:MassHunter/GCMS/1/data/TOXICOLOGY/2015023006.D60m, 0.25mm ID, 1.4µm Rtx®-1301 (cat.# 16016)

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- Instrument: 5977-GOVT CHEM
- Sample Name: Sample 4.
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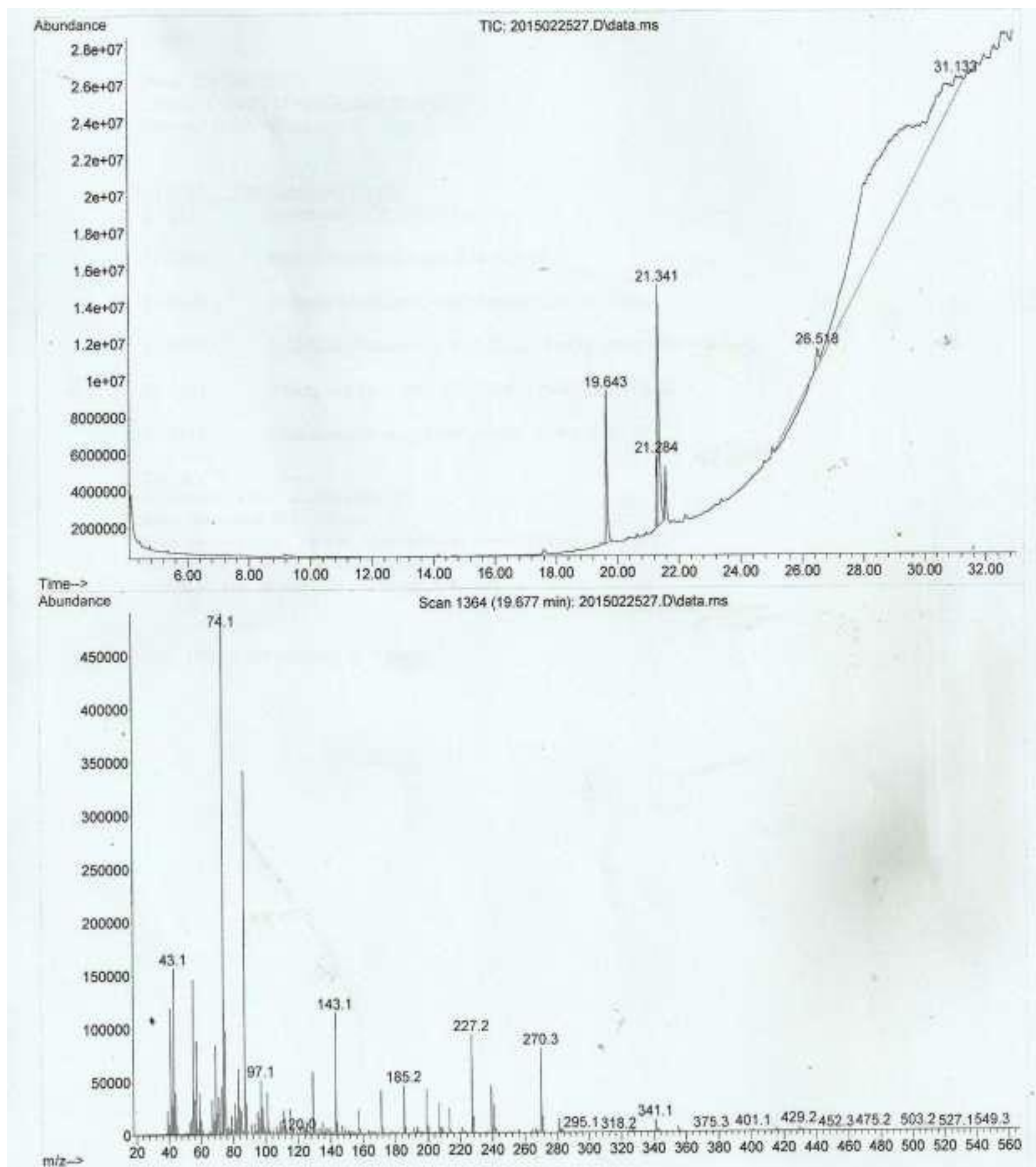

AMDIS GC/MS Analysis Report

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Number of Identifications:38

RT(min) Hits Chemical Name.

1. α-pinene
2.α-farnesene
3. β-cubebene
4 β-myrecene
5. β-caryophyline
6. *p*-propyl anisol
7. protocatechuic acid
8. *p*-coumaric acid
9. cineol
10. caffeic acid
11. ferulic acid
12.(+)-Catechin
13. thujene
14.α-terpinene
15.paracymene
16.linalool
17. carvacrol
18.thymol
19.sesquiterpenes
20.methylchavicol
21.carvacrol
22.quercetin
23.apigenin
24.luteolin
25.naringenin
26.gallic acid
27.p-hydroxybenzoic acid
28.vanillic acid
29.homogentisic acid
30.protocatechuic acid
31.hydroxycaffeic acid
32.3-nitro-phthalic acid
33Methyl tetradecanoate
34. Hexadecanoic acid,methylester
35. 9,12-octadecadienoic acid (Z,Z)-,methyl ester
36. 9-Octadecanoic acid (Z)-,methyl ester
37.Octadecanoic acid,methyl ester
38.Bis (2-ethylhexyl) phthalate

QA/QC

Instrument type :Quadrupole
Scan Direction High to low
High noise level.Median signal noise(Noise level)Threshold=940.8
Background (low vs. high retention time
Median low RT S/N=197, high
RT S/N=1615

This report consists of 4 page

AMDIS GC/MS Analysis Report- Tue May 12 - 8:44:06 2015

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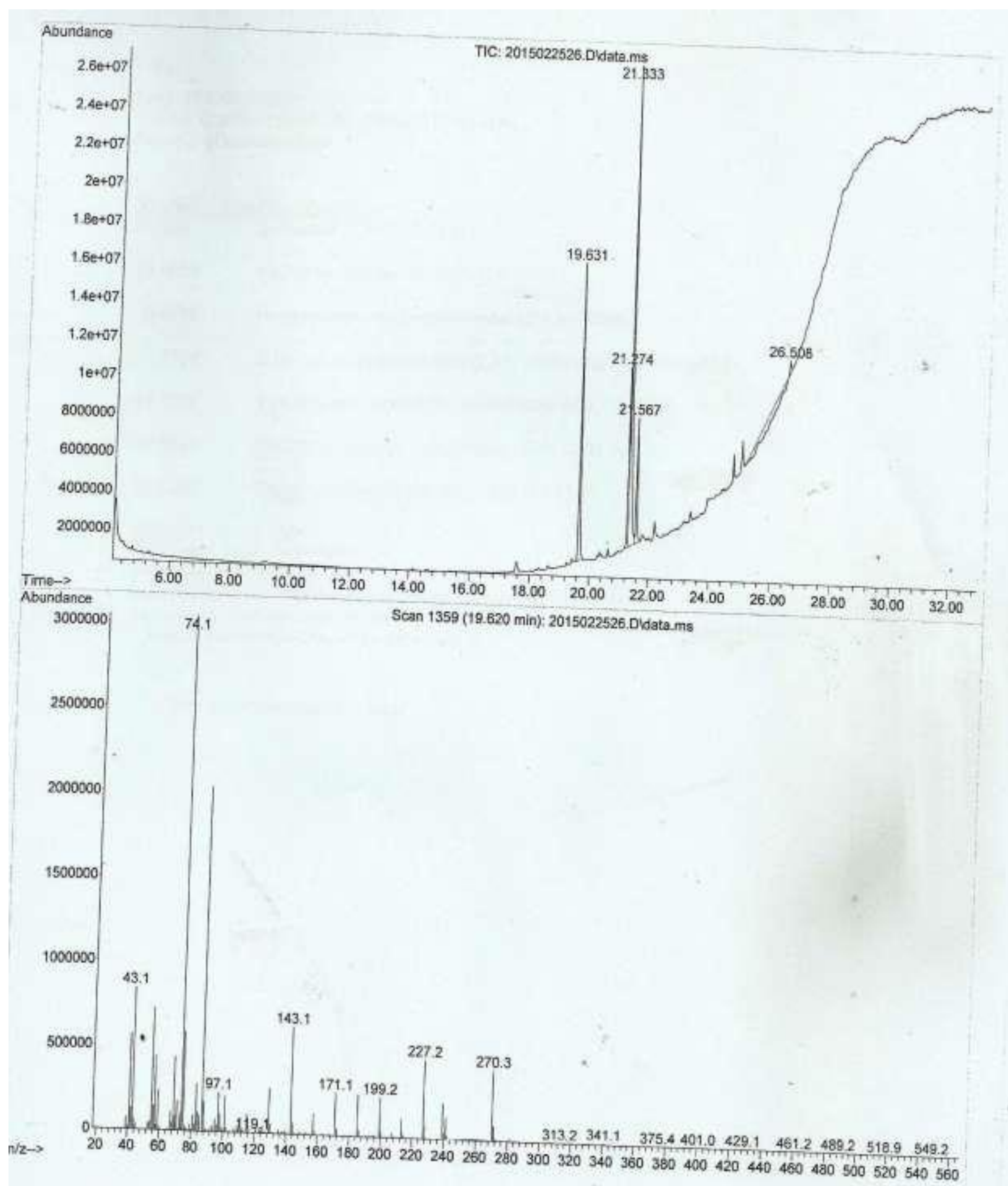


AMDIS GC/MS Analysis Report

AMDIS GC/MS Analysis Report

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Library: C:/NIST11/AMDIS32/LIB/NISTEPA.MSL

Number of Identifications:24

RT(min) Hits Chemical Name.
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2. β-cubebene
3 β-myrecene
4. caryophyline
5.guaiol
6.n-hexadacadienoic acid
7.9,12-octadecadienoic acid (z,z)
8.squalene
9.benzenedicarboxylic acid
10.benzenetriol
11.carvone
12.limonene
11.apigenin
12.silybin
13.silydianin
14.silychristin
15.visnagin
16.khellin
17.coniilne
18. Undecane
19. Methyl tetradecanoate
20. Hexadecanoic acid,methylester
21. 9,12-octadecadienoic acid (Z,Z)-,methyl ester
22. 9-Octadecanoic acid (Z)-,methyl ester
23.Octadecanoic acid,methyl ester
24.Bis (2-ethylhexyl) phthalate

QA/QC

Instrument type :Quadrupole
Scan Direction High to low
High noise level.Median signal noise(Noise level)Threshold=940.8
Background (low vs. high retention time
Median low RT S/N=197, high RT
S/N=1615
This report consists of 3 pages

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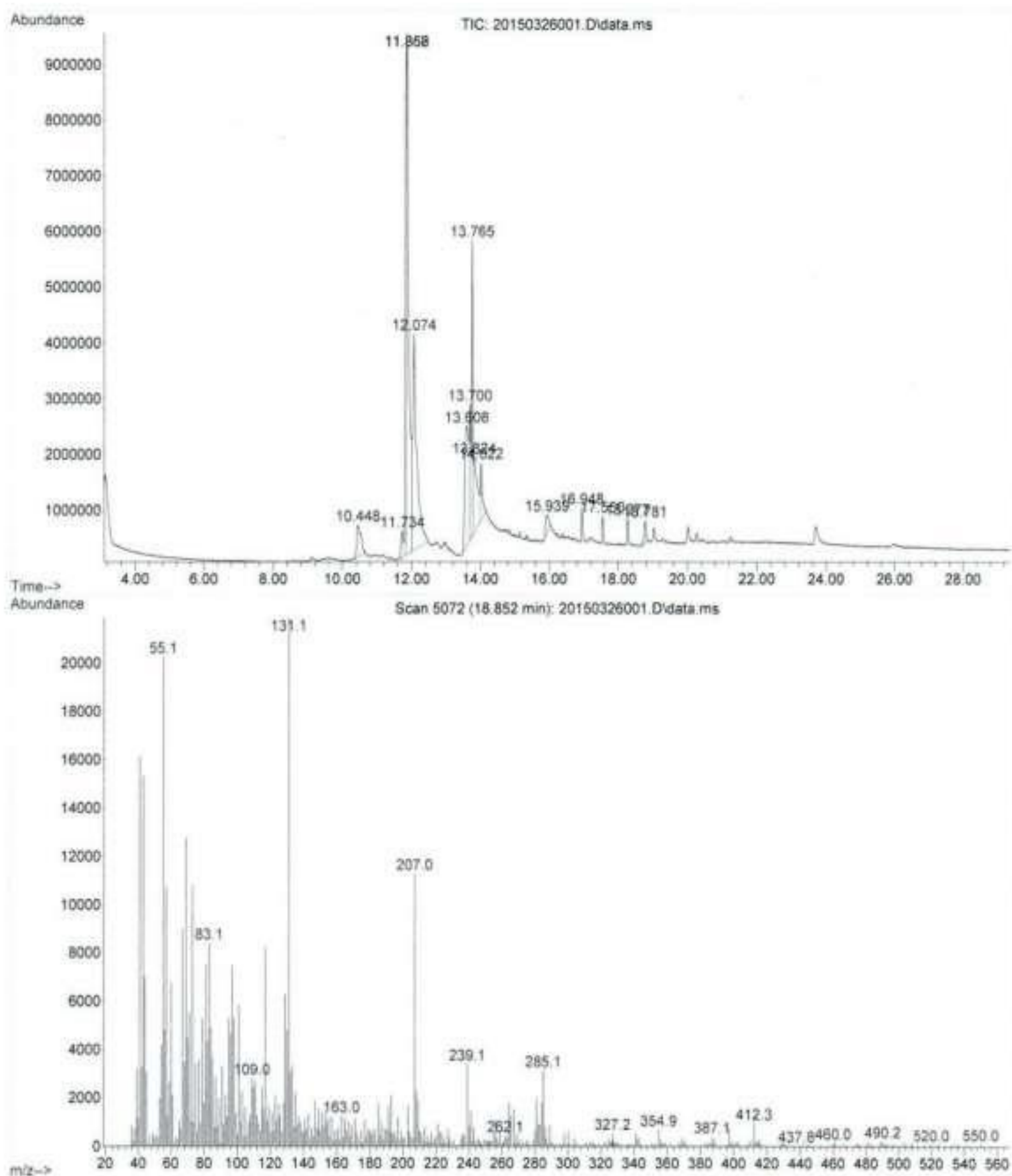
- Instrument: 5977-GOVT CHEM
- Sample Name: Sample 6.
- Vial NUMBER: 7



AMDIS GC/MS Analysis Report

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Library:

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Number of Identifications:32

RT(min) Hits Chemical Name.

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3 α-pinene
4.pectin
5.limonene
6.asparagine
7.daucol
8.isobutyric acid
9.asarone
10.Anisic acid
11.ferulic acid
12.lucernic acid
13.trifoline
14.inuline
15.urginin
16.cardiotonic
17. 3-nitro-phthalic acid.
18. quercetin
19 ambelline
20. undulatine
21 Phenol
22. gentisic acid
23. ferulic acid
24. *p*-hydroxybenzoic acid
25.solanine
26 Undecane
27. Methyl tetradecanoate
28. Hexadecanoic acid,methylester
29. 9,12-octadecadienoic acid (Z,Z)-,methyl ester
30. 9-Octadecanoic acid (Z)-,methyl ester
31.Octadecanoic acid,methyl ester
32.Bis (2-ethylhexyl) phthalate

QA/QC

Instrument type :Quadrupole

Scan Direction High to low

High noise level.Median signal noise(Noise level)Threshold=940.8

Background (low vs. high retention time

Median low RT S/N=171, high RT S/N=1316

This report consists of 3 page. AMDIS GC/MS Analysis Report- Tue May 12 - 9:04:22 2015