

**COMPARATIVE ANALYSIS OF PHYTOCONSTITUENTS, CAFFEINE
LEVELS, ANTI- BACTERIAL ACTIVITIES AND EVOLUTIONARY
ANALYSIS OF *ACACIA NILOTICA* (SUBALATA) AND *COFFEA ARABICA*
VARIETIES**

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**Comparative Analysis of Phytoconstituents, Caffeine Levels, Anti- bacterial
Activities and Evolutionary Analysis of *Acacia nilotica* (subalata) and *Coffea
arabica* Varieties**

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**A thesis submitted in partial fulfillment for the Degree of Master of Science Degree
in Bioinformatics and Molecular Biology in the Jomo Kenyatta University of
Agriculture and Technology**

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

I dedicate this thesis to my grandmother for her role in mentoring me through my entire life as her advice keep on lingering in my mind. Secondly, to my family for their continued love, support and encouragement. Thirdly to my uncle who denied himself the comfort of life to ensure that I got to where I am today by educating me.

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ABBREVIATION AND ACRONYMS

ANOVA	:	Analysis of Variance
CDC	:	Centre for Disease Control
CRF	:	Coffee Research Foundation
cpDNA	:	Chloroplast Deoxyribonucleic acid
DCM	:	Dichloromethane
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
HPLC	:	High Performance Liquid Chromatography
JKUAT	:	Jomo Kenyatta University of Agriculture and Technology
MEGA	:	Molecular Evolutionary Genetic Analysis
MICs	:	Minimum Inhibitory Concentrations
MLE	:	Maximum Likelihood Estimation
mtDNA	:	Mitochondrial Deoxyribonucleic acid
NCBI	:	National Centre for Biotechnology Information
nDNA	:	Nuclear Deoxyribonucleic acid
PAD	:	Photodiode Array Detector
Ppm	:	Parts per million
<i>RbcL</i>	:	Ribulose-1, 5-Bisphosphate Carboxylase
Rubisco	:	Ribulose 1, 5 Bisphosphate Carboxylase/Oxygenase
SPSS	:	Statistical Packages for Social Scientists
ZI	:	Zone of inhibition
MUSCLE	:	Multiple sequence comparison by log-expectation

- ATCC** : American type culture collection
- UTI** : Urinary tract infection
- HIV** : Human immunodeficiency virus.
- matK** : Maturase kinase
- HCL** : Hydrochloric acid
- Gent** : Gentamycin
- CFU** : Colony forming unit
- FeCl₃** : Iron (III) chloride

ABSTRACT

Acacia nilotica (subalata) is small to big tree (2.5 – 15m) in the family Fabaceae. The species has a relatively wide ecological distribution in Asia and Africa. In Africa it is majorly distributed in southern and eastern regions. In Kenya, it grows in Coast, Eastern, Rift valley, Nyanza and Western regions. The prolonged use of *Acacia nilotica* extracts to make coffee like concoction by the locals whereas no scientific work has been done to compare the caffeine levels, phytoconstituents and evolutionary relatedness with *Coffea arabica* varieties. Therefore the aim of this work was to investigate phytoconstituents, caffeine levels, anti-bacterial activity and evolutionary analysis of *Acacia nilotica* (subalata) in comparison with *Coffea arabica* varieties. Methanol, ethanol and water extracts of stem bark of *Acacia nilotica* and *Coffea arabica* varieties were used to screen for phytochemicals while methanol and ethanol extracts were screened for anti-bacterial activity. Gentamycin and dimethyl sulfoxide were used as positive and negative controls respectively. Methanol and ethanol extracts were used for HPLC profiles on caffeine content. Using disc diffusion technique, organic extracts were screened against *Pseudomonas aeruginosa* and results showed that methanol extract had the highest activity. The minimum inhibitory concentrations (MICs) value was in a range between 5 and 12.5 mg/ml. The HPLC profiles displayed peak areas with same retention time; *Acacia nilotica* (7.452), Batian 27 (7.476) and Ruiru 11 (7.474) extract against retention time for standard caffeine (7.476) hence the samples have caffeine content. Ruiru 11 had the highest caffeine content (161.75 ± 39.16) followed by Batian 27 (127.39 ± 14.83) and *Acacia nilotica* (subalata) (68.91 ± 17.39). They are statistically significant ($p < 0.05$). Phylogenetic trees showing the evolutionary relationship between *A. nilotica* and *Coffea arabica* generated from MEGA using ribulose-1, 5-Bisphosphate Carboxylase (*rbcL*) gene sequences from Genbank revealed that *A. nilotica* and *C. arabica* are monophyletic.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Medicinal plant products have secondary metabolites which are chemical compounds that have pharmacological and biological activity (Daffre *et al.*, 2008). These plants are used by rural residence worldwide since time immemorial to date as herbal medicine to treat or control diseases. Almost 88 % of the world's population depends on traditional medicine (Doughari *et al.*, 2009). The chemical compounds found in these plants are called phytoconstituents. The word 'phyto' means plant in Greek. Therefore the phytoconstituents in plants acts as protection against invading microbial infections. The various phytochemicals that are of medicinal value include tannins, alkaloids, saponins, anthraquinones, terpenes, phenolics, flavonoids among others (Liu, 2003).

The plant kingdom have been a source of antimicrobial agents for centuries, and more than 30% of pharmacological drugs are derived from plants, (Banso (2009); Murugesan *et al.*, 2011).The emergence of drug resistance has become a global problem and most communities have resorted to using plant extracts to cure various ailments either by chewing or taking it as a concoction.

Acacia nilotica is one of the plants that have been used extensively by rural dwellers in Kenya in treatment or management of diseases. They use either whole plant or their parts (flowers, leaves, bark or roots) to treat or control various diseases. Although this plant have been reported to contain valuable phytochemicals (Banso 2009), no work has been carried out to compare phytochemicals, caffeine content, *anti-Pseudomonas aeruginosa*

and evolutionary relationship in *Acacia nilotica* (subalata) with *Coffea arabica* varieties. The data will provide an insight as to whether the extensive use of *Acacia nilotica* (subalata) as a medicinal plant and as a beverage possess health risks to human or can be used as coffee alternative in semi-arid and arid areas.

This plant is among 1380 species spread across the world. The plant thrives well in tropical and subtropical regions. It grows in arid and semi-arid areas. In Africa, it is found in North West countries such as Senegal, Northern Africa, South West and East Africa. In East Africa, *Acacia nilotica* subspecies subalata is found in Ethiopia, Sudan and Kenya (Rajvaidhya *et al.*, 2012).

Acacianilotica (subalata) in Kenya is found in the Rift Valley, Northeastern and some parts of Coastal region. The specific regions are; Marsabit, Baringo, Voi, Nandi, Turkana, Central, Masai, Nakuru, Kilifi, Samburu, Meru, Kwale, Kitui, Embu, Garissa, Narok, Wajir and Mandera (Rajvaidhya *et al.*, 2012).

On the other hand, coffee consists of two main species; coffee Arabica and Robusta. Arabica is of high quality, mild and much favored for blending whereas Robusta tends to be bitter than Arabica. In Kenya, Arabica is grown in rich volcanic soils found in the highlands. Coffee is grown in regions such as Murang'a, Kericho, Nyeri, Embu, Kiambu, Molo, Bomet and Nanyuki.

Both *Acacia nilotica* and *Coffea arabica* share the same taxonomic division and class. However Acacia belongs to the order fabace, and family fabaceae. On the other hand Coffee belongs to order gentianales, family rubicae. These plants share conserved genes

found in the nuclear (nDNA), mitochondrial (mtDNA) genomes, and chloroplast genome (cpDNA) (Chase *et al.*, 2007).

Plastid-encoded ribulose-1, 5-bisphosphate carboxylase (*rbcL*) gene is the most common gene among cpDNA genes used to provide sequence data for plant phylogenetic analyses (Chase *et al.*, 1993; Donoghue *et al.*, 1993). The function of the ribulose-1, 5-bisphosphate carboxylase (*rbcL*) gene is to code for the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase (RUBISCO) (Duvall & Morton, (1996)).

1.2 Statement of the problem

Acacia nilotica (subalata) is reported to treat ear, eye infection, skin rashes, painful urination and as a beverage by the Pokots (Aloush *et al.*, 2006). It is also used to treat cancer, diarrhea and diabetes (Kalaivani & Methew, 2010 & Del, 2009). It makes coffee like concoction for medicinal use. There is no scientific knowledge on comparison of inhibitory activities between *A. nilotica* (subalata) and *Coffea arabica* varieties extracts on the microorganism that cause the above mentioned ailments. Also there is no scientific facts on comparison of phytoconstituents, caffeine levels and evolutionary relatedness between *A. nilotica* (subalata) and *Coffea arabica* varieties. Because of prolonged use of *A. nilotica* (subalata) extracts to make coffee like concoction and treat diseases by locals whereas no scientific work has been done to compare the caffeine levels, phytoconstituents and evolutionary relatedness of *A. nilotica* (subalata) and *Coffea arabica* varieties can cause long term health effects. Therefore due to the taxonomic relatedness of these two plants, it is important to study and evaluate their antibacterial

activity, phytochemistry and evolutionary relationship in order to provide scientific evidence of their closeness.

1.3 Justification

For a long time, *A. nilotica* (subalata) has been exploited by local communities as a medicinal plant in Kenya. The plant is extremely overexploited for charcoal which are sold to urban areas and even exported to neighboring countries such as Uganda. Some organizations have planted this species in farms across the vast plains. This shows that *A. nilotica* (subalata) has a high economic value and therefore, there is an urgent need to tap on this resource and protect it. The findings from the study will be used to sensitize the locals and government to conserve this species and ban the charcoal business. Also educating the masses on the correct drug administration to avoid overdose or under dose as this will result to drug resistance as it takes a long time to test and market a drug yet drug resistance is an urgent issue. The results from the study will assist pharmaceutical companies to make new antibiotics. The findings will also give us the evidence supporting the local knowledge pertaining use of *A. nilotica* (subalata) to treat diseases and to make coffee like concoction (beverage). This research is expected to sensitize the locals to grow *A. nilotica* (subalata) in large scale to provide raw material for pharmaceutical industries, paper industry and fodder for their livestock and also as coffee alternative. This will increase income and improve livelihood of the locals. Therefore, this research is vital as it is aimed to validate and compare antibacterial activity, phytochemical constituents and

caffeine levels of plants under study which can act as an alternative source of antibacterial medicines to counteract the constant and ever increasing drug resistance.

1.4 Research questions

1. What are the phytoconstituents and concentration of caffeine in *Acacia nilotica* (subalata) and *Coffea arabica*?
2. Do *Acacia nilotica* (subalata) stem bark and *Coffea arabica* extracts possess anti-bacterial activity?
3. What is the evolutionary relatedness between *Acacia nilotica* and *Coffea arabica*?

1.5 Objectives

1.5.1 General Objective

To evaluate comparative phytoconstituents, caffeine levels, anti-bacterial activity and phylogenetic relatedness of *Acacia nilotica* subalata subspecies and *Coffea arabica* varieties (Batian 27 and Ruiru 11).

1.5.2 Specific objectives

1. To determine and quantify the phytoconstituents and evaluate the caffeine levels in *Acacia nilotica* (subalata) and *Coffea arabica*.
2. To establish the anti-bacterial activity of *Acacia nilotica* subalata's stem bark and *Coffea arabica* varieties extracts against *Pseudomonas aeruginosa*.
3. To compare their evolutionary relationship between *Acacia nilotica* and *Coffea arabica*.

1.6 Null hypothesis

There was no difference in quality and quantity of phytochemicals, levels of caffeine, anti-bacterial activity and evolutionary relationship of *Acacia nilotica* (subalata) and *Coffea arabica*.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Acacia nilotica* (subalata)

2.1.1 Taxonomy and ecology of *Acacia nilotica*

The Brenan (1983) taxonomy of *Acacia nilotica* subspecies subalata is as follows; domain (*eukaryota*), kingdom (*plantae*), phylum/division (*spermatophyta*), subphylum (*angiospermae*), class (*dicotyledonae*), order (*fabace*), family (*fabaceae*), subfamily (*mimosoideae*). There are over 1380 acacia species worldwide. In Africa there are about 130 species (Rajvaidhya *et al.*, 2012).

Acacia nilotica (subalata) grows well in riverine alluvial soil and black cotton soil. It grows on saline and alkaline soils. *Acacia nilotica* grows thrives well in sub-tropical to tropical climatic conditions (Godghate *et al.*, 2014). This plant can withstand high temperature of up to 50 ° C but adequate moisture is needed for full growth and development. The average annual rainfall ranges from 250-1500 mm (Bargali & Bargali, 2009).

2.1.2 Economic importance of *Acacia nilotica*

Acacia nilotica subspecies subalata is a source of fodder, timber, gum and fuel, and also have pharmacological effects (Bargali & Bargali, 2009). The major primary use of *Acacia nilotica* subsp. subalata is wood fuel (Ali *et al.*, 2012). Its charcoal burns well without too much smoke and sparks. *Acacia nilotica* subsp. subalata wood is used to make pulp in paper industry (Ali *et al.*, 2012) while its timber makes good furniture. Leaves and pods

are used as fodder for animals and production of biogas (Ali *et al.*, 2012). This plant has common names from various tribes and subtribes in Kenya, Uganda, and Sudan. For instance in Kenya: Kipsigis (chebitet), Masai (chebiwa), Tugen (chebiwo), Turkana (ekapelimen), Samburu (ikoloriti), Rendille (illgiti), Luhya (inyanya), Pokot (kopka), Swahili (mgunga), Kamba (muthi), Giriama (muhegakululu), Somali (twerr); Sudan: Lutoho (asit); Tanzania: Mbulu (bariomot), Sukuma (dubilo), Masai (elarai); Uganda: Suk (kapka). (<http://www.Acacia local names/index.html>).



Plate 1.1: (a) A mature whole plant and (b) stem of *Acacia nilotica* (subalata) in its natural habitat; source (Photo courtesy of George Kisoi at Koloa in Baringo County, November; 2014).

2.1.3 Health benefits of *Acacia nilotica*

Banso A (2009) studied the antimicrobial activity of ethanolic extracts of the stem bark against *Streptococcus viridans*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Shigella sonnei* using the agar diffusion method and found the minimum inhibitory concentration of the stem bark extract of the plant ranged between 35 and 50 mg/ml. *Acacia nilotica* is also known to contain active ingredient used for treatment of human diseases including cancer, diabetes, diarrhea, and headache, (Kalaivani & Methew, 2010). Its bark is used extensively as anti-diarrhoea, dysentery and bleeding piles (Del, 2009). The stem bark powder have been used against tumors of ear, eye and testicles, (Kalaivani & Methew, 2010). Methanol extract from the bark has been reported to be anti-oxidant, (Agrawal *et al.*, 2010).

Acacia nilotica has been used for the treatment of infectious bacteria including *Escherichia coli* (Rajvaidhya *et al.*, 2012). It has also showed antifungal activity of methanolic extracts and aqueous extract of *A. nilotica* with percentage inhibition ranging from 34.27 ± 1.45 to 93.35 ± 1.99 . Methanol extracts of *Acacia nilotica*, showed highest antibacterial activity against *B. subtilis*. and *Staphylococcus aureus* with inhibition zone 15 ± 0.66 mm, (Mahesh B. and Satish S. (2008))

2.2 *Coffea arabica*

2.2.1 Taxonomy and Ecology of *Coffea arabica*

Coffee belongs to kingdom *plantae*, division *tracheophyta*, class *dicotyledonae*, order *gentianales*, family of *Rubicae*, genus *Coffea* and species *arabica* (Farah, 2012). It is

grown in more than 50 countries in three continents (Asia, Australia and Africa). In Africa it is grown in countries such as Kenya, Ethiopia, Rwanda and Uganda. There are seventy types of coffee varieties but only two are of economic importance namely Arabica and Robusta. In Kenya, coffee arabica is grown in the highlands such as Kiambu, Nyeri, Limuru, Muranga, Meru whereas Robusta coffee grows in low lying regions such as Baringo (Mureithi, 2008). *Coffea arabica* has many varieties which include Ruiru 11, SL34, 38, Batian 27 and 28 (Mureithi, 2008). The pictures of *Coffea Arabica* varieties; Ruiru 11 (a) and Batian 27 (b) are shown in plate 2.



Plate 1.2: (a) Ruiru 11 and (b) Batian 27 with mature berries respectively. Source (Photo courtesy of George Kiso at CRF in Kiambu County, November; 2014).

Coffee is a plant which grows in areas with temperatures ranging of between 15-24°C and 24-30°C for Arabica and Robusta respectively. The plant needs an annual rainfall of between 1500-3000 mm and thrives well in volcanic red earth soil (Mureithi, 2008). The pattern for rainy and dry periods is very important for growth, budding and flowering of coffee plants. The chemical compounds found in coffee are used by the plant as a natural

defense against herbivores because it produces toxic substance that protects the plant berries (Mureithi, 2008).

2.2.2 Economic importance of *C. arabica*

Coffee is an important beverage with over 2.25 billion cups consumed daily (Farah, 2012). Unfortunately, consumption takes place in developed countries whereas its production (90%) takes place in developing countries (Farah, 2012). It is the second most valuable commodity in the world after petroleum with 25 million farmers in 50 countries cultivating coffee (Dimins *et al.*, 2011). There are over 70 varieties of coffee but only two are of major economic value. These are Arabica and Robusta where the former accounts for almost 75% and Robusta accounts for the rest of the world's consumption (Dimins *et al.*, 2011). Coffee earns Kenya foreign exchange, creates employment, improved standard of living and it has led to development of roads and schools (Mureithi, 2008).

2.2.3 Health benefits of *C. arabica*

Caffeine is among the phytochemicals found in coffee which has been shown to possess health benefits (Durak & Dziki, 2014). Coffee consumption lowers the risk of Type Two diabetes, liver disease and cancer (Kang *et al.*, 2011). *Coffea arabica* is rich in alkaloids, flavonoids and amino acids (Daruk & Dziki, 2014). The content of phytochemicals in coffee varies depending on species, climate, soil composition, season, age, zone, agricultural practice and maturity (Wanyika *et al.*, 2010).

Studies suggest that low to moderate intake of caffeine leads to increase in alertness, heartbeat, learning capacity and decrease in fatigue leading to clearer flow of thoughts,

whether in tablet form or not (Wanyika *et al.*, 2010). High to acute caffeine consumption is associated with high blood cholesterol and coronary, urinary excretion of calcium (Wanyika *et al.*, 2010). During lactation, it may cause irritability and wakefulness in a breastfed baby. Other studies suggests that long term consumption of caffeine leads to most of these acute effects to disappear. This is because of metabolic adaptations in the body and other compounds that can counteract these acute effects of high consumption of caffeine. It stimulates the central nervous system as an adenosine receptor antagonist (Wanyika *et al.*, 2010)

According to Jansen (2006), Arabica coffee produce 100% world's best coffee thus it is more expensive than Robusta coffee beans. To achieve specific and desirable aroma the two types of coffee are blended in different proportions to produce a wide variety of taste and aroma of coffee beverage (Jansen, 2006).

2.3 Plant secondary metabolites

Plant compounds have been a constant source of valuable substitutes than synthetically antimicrobial agents (Banso, 2009). These phytochemicals have been detected by accurate methods of screening. The procedures have revealed that many substances that were believed to have been rare are now found almost all the plants. Phytochemicals are classified into primary metabolites and secondary metabolites (Wadood *et al.*, 2013). The primary metabolites are amino acids while secondary metabolites include alkaloids, volatile essential oils, phenols and phenolic glycosides, resins, oleosins, steroids, flavonoids and terpenes (Banso, 2009). Phytochemical screening of the stem bark of *Acacia nilotica* revealed that *Acacia nilotica* contains terpenoids, alkaloids, saponins

and glycosides. There were absence of steroids and tannins from this plant (Banso, 2009). *Acacia nilotica* contains gallic acid, catechin, chlorogenic acid, 4-diol, androstene steroid, (Singh *et al.*, 2009). The bark contains epicatechin, dicatechin, quercetin, gallic acid, sucrose and catechin- 5-gallate (Mitra and Sundaram, 2007). There are several factors which affect the presence and concentration level secondary metabolites. These include season, altitude, age of the plant, climate, soil type, soil P^H, time of collection that is evening or morning and health condition of the plant, (Adedapo *et al.*, 2005).

2.4 *Pseudomonas aeruginosa*

2.4.1 Taxonomy and description

Belongs to kingdom (Bacteria), Phylum (*Proteobacteria*), Class (*Proteobacteria*), Order (*Pseudomonadales*), Family (*Pseudomonadaceae*), Genus, (*Pseudomonas*), species (*Pseudomonas aeruginosa*), (<http://www.textbookofbacteriology.net/pseudomonas.html>)

Pseudomonas aeruginosa is an opportunistic pathogen found in soil, water, plants and animals, including humans. *Pseudomonas aeruginosa* is a Gram negative, rod-shaped bacterium (Talbot *et al.*, 2006, Kiska & Gilligan, 2003). It is known to be highly resistant to antibiotics (Kiska & Gilligan, 2003). It is common in hospital environments. These bacteria are identified by its odor which is grape like and smells sweet *in vitro* (Chamot *et al.*, 2003). It produces the blue-green pigment pyocyanin, a redoxactive phenazine, which kills both mammalian and bacterial cells via the generation of reactive oxygen intermediates (Pollack *et al.*, 2000). *Pseudomonas aeruginosa* often causes infection such

as pneumonia, urinary tract infections (UTIs) and bacteremia (bacterial infection of the blood) to people with immunodeficiency (Gaynes & Edwards, 2005).

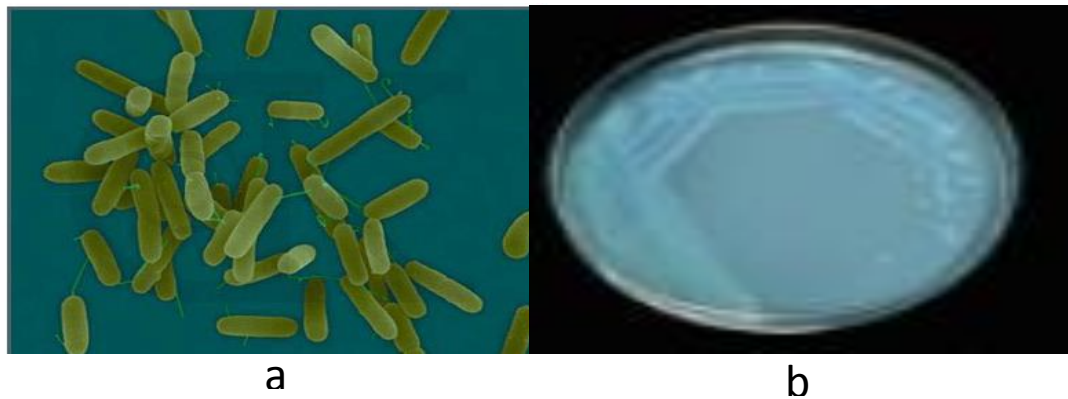


Plate 1.3. *Pseudomonas aeruginosa* (a) morphology and (b) on nutrient agar. Source; (<http://www.microbiologyinpictures.com/pseudomonasaeruginosa>)

2.4.2 Transmission and risk factors

Pseudomonas aeruginosa is spread through improper hygiene, unclean hands of healthcare workers or via contaminated medical equipment especially in hospital set up. Therefore its reservoirs include; respiratory equipment, sinks, food, taps, mop, showers, soaps, irrigation fluids and eye drops (<http://emedicine.medscape.com/article>).

Common hospital-associated *Pseudomonas aeruginosa* infections include bloodstream infections, pneumonia (lung infection), urinary tract infections, and surgical wound infections. These infections typically affect people, who are ill in the hospital and are immune deficiency, especially patients on long-term treatments, (Savina, 2015). Loss of the integrity of a physical barrier to infection for instance, skin and mucous membrane as

a result of burns or surgery makes most infections to take advantage (Janeczko, 2013). When one is hospitalized, he or she has a higher risk of a serious, life-threatening *P. aeruginosa* infection. For instance if one has surgical wounds or burns, or being treated with a breathing machine (mechanical ventilator, urinary or intravenous catheters) (Gaynes & Edwards, 2005).

Exposure to contaminated water may cause mild *Pseudomonas aeruginosa* infections in healthy people outside of healthcare environment (Bitsori *et al.*, 2012). For example, inadequately chlorinated hot tubs and swimming pools, *Pseudomonas aeruginosa* can cause ear, eye infections (most common in children) and skin rashes (Aloush *et al.*, 2006). This bacterium can infect any part of the body including the liver, brain, bones, and sinuses. These infections are a serious problem in patients with cancer, cystic fibrosis, HIV and severe burns where fatality rate is near 50% (Aloush *et al.*, 2006).

2.4.3 Epidemiology and symptoms of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the most common bacterial species causing disease, according to the Centers for Disease Control and Prevention (CDC, 2013). In the United States, there were an estimated 51,000 healthcare-associated *Pseudomonas aeruginosa* infections each year, according to the (CDC, 2013). This translates to about 8 % of all healthcare-associated infections reported (CDC, 2013). It is the fourth most commonly isolated nosocomial pathogen.

Symptoms of *Pseudomonas aeruginosa* vary based on the type of infection which include;

Bloodstream infections: Their symptoms include the following; fever and chills, body aches, light-headedness, rapid pulse and breathing, nausea and vomiting, diarrhea, decreased urination, very low blood pressure, called hemodynamic shock, which leads to failure of other organs such as heart, kidneys, and liver (Van, 2007 & Micek *et al.*, 2005). Pneumonia (lungs): fever and chills, difficulty breathing, cough, sometimes with yellow, green, or bloody mucus. Urinary tract infections: strong urge to urinate frequently, painful urination, cloudy or bloody urine (Van, 2007 & Micek *et al.*, 2005). Wound infections: inflamed wound site, fluid leakage from wound. Ear infections: ear pain, hearing loss, dizziness and disorientation (Van *et al.*, 2007).

2.4.4 Treatment and prevention

Mild, water-related *P. aeruginosa* infections are generally treated easily with certain antibiotics such as imipenem and imikacin. Treating severe hospital-associated *Pseudomonas aeruginosa* infections is becoming more difficult, because some bacterial strains have shown resistance to nearly all classes of powerful antibiotics, including: Aminoglycosides, Cephalosporin, Fluoroquinolones and Carbapenems which are antibiotics of last resort (Ratjen *et al.*, 2010 & Fuentefria, 2011).

Cleaning equipment in hospitals should be sterilized as this can help prevent infection. Also one should avoid hot tubs and swimming pools that are outside healthcare settings that are poorly cared for. Shower with soap after getting out of the water. Drying your ears after swimming can also help prevent swimmer's ear (Marcus, 2015).

2.5 Ribulose-1, 5-Bisphosphate Carboxylase (*rbcL*) gene for phylogenetic analysis

Ribulose-1, 5-Bisphosphate Carboxylase (*rbcL*) gene is among other conserved genes such as maturase kinase (*matK*) found in chloroplast genome used in phylogenetic tree construction and determination of phylogenetic relatedness. Because of its complexity and repetitive properties, nuclear genome is used in systematic botany less frequently (Shinwari *et al.*, 2014). The mitochondrial genome is used at the species level due to its rapid changes in its structure, size, configuration, and gene order. On the other hand, chloroplast genome is well suited for evolutionary and phylogenetic studies. It is a relatively abundant component of plant total DNA. Secondly, contains primarily single copy genes. Thirdly, extensive background for molecular information on the chloroplast genome is available (Doebley *et al.*, 1990).

Ribulose-1, 5-bisphosphate carboxylase (*rbcL*) was the first gene that was sequenced from plants (Zabta *et al.*, 2014). It is a single copy gene approximately 1430 base pairs in length, free from length mutations except at the far 3' end, and has a fairly conservative rate of evolution. It encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO). This enzyme Ribulose-1, 5-Bisphosphate Carboxylase (Rubisco) is responsible for fixation of carbon dioxide in the Calvin Cycle, Michael (1993). In green algae and land plants, the genetic information for the small subunit is encoded in the nuclear genome, typically in a small multigene family (Meagher *et al.*, 1989 & Palmer, 1991).

The ribulose-1, 5-bisphosphate carboxylase (*rbcL*) contains high substitution rates within the species and is emerging as potential candidate to study plant systematics and evolution

(Sathishkumar *et al.*, 2008). It has long been evident that molecular sequences contain useful information about evolutionary history. The ribulose-1, 5-bisphosphate carboxylase (*rbcL*) gene has ideal size, high rate of substitution, large proportion of variation at nucleic acid and protein level at first and second codon position, low transition/transversion ratio and the presence of mutationally conserved sectors. These features of *rbcL* gene are exploited to resolve genus and species level relationships. Polymorphism of chloroplast DNA especially ribulose-1, 5-bisphosphate carboxylase *rbcL* has been used to study the phylogeny of various plants (Sathishkumar *et al.*, 2008). The sequence data of the ribulose-1, 5-bisphosphate carboxylase (*rbcL*) gene are widely used in the reconstruction of phylogenies throughout the seed plants and flowering plants and more than 10,000 ribulose-1, 5-bisphosphate carboxylase (*rbcL*) gene sequences are already available in GenBank (Newmaster *et al.*, 2006 and Chase *et al.*, 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental site, collection and preparation

Acacia nilotica (subalata) sample was collected from Baringo County where random and purposive sampling was carried out as the plant is scattered across Koloa area in Baringo County. Only plants with dark to grey scaly stems, between 15- 17 m in height and 2-3 m in diameter were sampled (Iman *et al.*, 2007). Plants with these qualities belong to the same age. The plant samples were packed in a clear air tight paper bags and brought to the laboratory. On the other hand *Coffea arabica* ripe berries (Batian 27 and Ruiru 11) were randomly picked from Coffee Research Foundation farm at Githunguri in Kiambu County. The *Coffea arabica* varieties have a uniform age because they were planted at the same time. During dry season there is high temperatures which leads to reduction in level of photosynthesis and damaging effect of solarisation which affects volatile metabolites. High humidity leads to accumulation of phenolic compounds. High concentrations of flavonoids, alkaloids, saponins and phenols are as a result of low temperature stress (Soni *et al.*, 2015). Therefore the best time and season for sample collection is during a wet season and in the morning.

The mode of collection involved chopping a healthy plant's stem bark. The bark of *Acacia nilotica* was separated from the stem using a knife. Samples were then brought to the laboratory for identification and authentication in the Department of Botany JKUAT based on whether the leaves are densely hairy, whether the flowers are globular, height and colour of the pods. Height of the coffee, size of the berries, colour of the leaves and

the number of stems were some of the characteristics used for identification. The barks were sun dried under a tree shade for five days. Then they were ground using an electric mill grinder, weighed and their weights recorded. *Coffea arabica* berries (Batian 27 and Ruiru 11) samples were picked randomly from three different areas in Coffee Research Foundation farm, sorted, pulped and fermented in a slab at 18°C for 3 days. They were washed and soaked in water for 1 day. After soaking, final washing was carried out. Drying, parchment, hulling, roasting was done. After which, grinding was done and the powder weighed and packaged into air tight bags and brought to the laboratory for analysis.

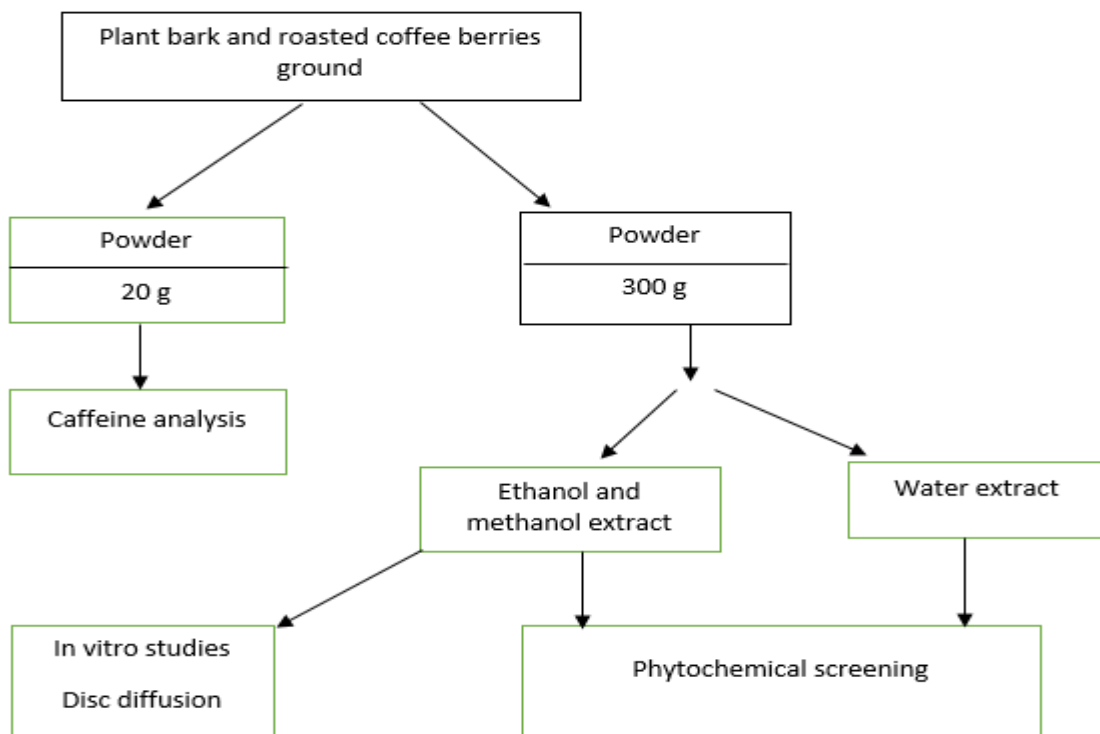


Figure 3.1: Experimental design for the phytochemical analysis, caffeine analysis and in vitro studies

3.2 Extract preparation

3.2.1 Organic and water extract

Approximately 30 g of powdered *Acacia nilotica* stem bark and *Coffea arabica* were mixed separately with 300 ml of methanol, ethanol and water respectively and left to stand for five days. Methanol and ethanol solvents dissolve most of the secondary metabolites and enhancing their release from cellular matrix (Jakhetia *et al.*, 2010). Filtration was done for organic extracts and the filtrates were concentrated using a rotavapour (BuchiR200) at 60 °C. Rotavapour is equipment that works by removing low boiling organic chemicals from a mixture of compounds. Water extracts were filtered and freeze dried at 4 °C. The extraction was done singly, (Raaman, 2006).

3.3 Qualitative phytochemical analysis

Phytochemical analysis of organic (ethanol, and methanol) and water solvent extracts of *Acacia nilotica* subsp. Subalata (bark) and coffee samples were carried out using standard procedures (Dey *et al.*, 2012, Deshpande, 2013, Jigna *et al.*, 2007, Akinyeye *et al.*, 2014, Ejikeme *et al.*, 2014, Raaman, 2006, Wadood *et al.*, 2013) as follows:

3.3.1 Detection of saponin

About 50 mg of *Acacia nilotica* subsp. Subalata and *Coffea arabica* both water and organic extract were dissolved in 30 ml of distilled water and were shaken for 15 minutes. Formation of a layer of foam indicated the presence of saponin (Ejikeme *et al.*, 2014).

3.3.2 Detection of tannins

About 0.05 g of *Acacia nilotica* subsp. *subalata* and *Coffea arabica* both water and organic extract were dissolved in 5 ml of distilled water and 5 % ferric chloride was then added. The presence of a dark green colour indicated the presence of tannins (Raaman, 2006).

3.3.3 Detection of flavonoids

About 50 mg of *Acacia nilotica* subsp. *subalata* and *Coffea arabica* both water and organic extract were dissolved in 10ml distilled water and 5ml of dilute ammonia were added followed by addition of 1 ml of concentrated Sulphuric acid. Formation of a yellow colour indicated the presence of flavonoids (Raaman, 2006).

3.3.4 Detection of alkaloids

A drop of Wagner's reagent was added side by side of the test tube containing few milliliters of filtrate. A precipitate reddish brown to orange colour infers the presence of alkaloid (Deshpande, 2013).

3.3.5 Detection of carbohydrates

Few drops of Benedict's reagent were added to 1 ml of the test solution (filtrate) and boiled in water bath for approximately 2 minutes. The formation of reddish brown precipitate indicated the presence of carbohydrate (Deyet *al.*, 2012).

3.3.6 Detection of phenolic compounds

Few drops of 10% lead acetate solution were added to 0.01g of *Acacia nilotica* subsp. *Subalata* extract and *Coffea arabica* powder. A white precipitate indicated the presence of phenolic compounds (Akinyeye *et al.*, 2014).

3.3.7 Detection of steroids and triterpenoids

To 2 ml of the *Acacia nilotica* subsp. *Subalata* extract and *Coffea arabica*, a few drops of concentrated sulphuric acid were added. The mixture was shaken and allowed to stand for 3 minutes. A red color in lower layer indicated the presence of steroids and the formation of yellow color at the top layer confirmed the presence of triterpenoids (Wadood *et al.*, 2013).

3.4 Quantitative phytochemicals analysis

The quantitative estimation of different phytochemicals of *Acacia nilotica* ssp *subalata*'s stem bark and coffee varieties were performed in triplicates according to the following standard protocols adapted from those reported by Okwu and Ukanwa, 2007, Poornima and Ravishankar, 2009, Gupta *et al.*, 2013, Aliyu, 2008, Hussain *et al.*, 2011, Abdel *et al.*, 2007 as follows.

3.4.1 Determination of total flavonoids

Approximately 5g of sample was extracted repeatedly with 50 ml of 80% aqueous methanol at room temperature and filtered using Whatman filter paper no. 42. The filtrate was transferred into a crucible and evaporated to dryness and finally weighed. The flavonoid content was determined by subtracting the weight of the empty crucible from the weight of the crucible with the contents after evaporating it to dryness. The value was divided by the original amount of sample and was converted to percentage (Okwu & Ukanwa, 2007). The percentage of flavonoids was calculated as follows:

$$\% \text{ Flavonoids} = (\text{weight of flavonoids}) / (\text{weight of sample}) \times 100.$$

3.4.2 Determination of total alkaloids

Approximately 4g of the sample was extracted with 200 ml of 20% ethanolic acetic acid for 10 hours, filtered and the volume reduced to a quarter of the original volume using a water bath(60°C). Concentrated Ammonium hydroxide was added drop wise to the extract till precipitation occurred. The whole solution was allowed to settle. The precipitate was collected by filtration, dried in an oven and weighted (Poornima & Ravishankar, 2009 and Gupta *et al.*, 2013). The percentage alkaloids were calculated as:

$$\% \text{ Alkaloids} = (\text{weight of alkaloids}) / (\text{weight of sample}) \times 100.$$

3.4.3 Determination of total saponins

Approximately 25g of sample was extracted with 250 ml of 20% aqueous ethanol (80ml ethanol and 20ml water) in a water bath for 4 hours at 55°C, and later filtered. The residue was re-extracted with 200 ml of 20% ethanol. The two extracts were combined and concentrated to 40ml in a water bath at 90°C then transferred into a separating funnel. The sample was extracted thrice using 20ml of diethyl ether each. The ether layer was discarded and the aqueous layer extracted thrice using n-butanol. The extract was washed twice with 10 ml of 5% aqueous sodium chloride and evaporated in a water bath, dried in oven and later weighed (Aliyu, 2008). The percentage of saponins was expressed mathematically as:

$$\% \text{ Saponins} = (\text{weight of saponins}) / (\text{weight of sample}) \times 100.$$

3.4.4 Determination of total phenols

Approximately 5g of sample was extracted twice with 100 ml of n-hexane for four hours. Exactly 100 ml of diethyl ether was added and heated for 15 min each. The extract was

cooled and filtered using a separating funnel. Exactly 50 ml of 10% sodium hydroxide solution was added twice and shaken well. The organic layer was aspirated off and washed thrice with 25 ml deionized water. The aqueous layer was acidified to a pH of 4 by adding 10% hydrochloric acid solution (10ml HCl and 90ml water). The extract was further extracted twice using 50 ml dichloromethane (DCM). Later the organic layer was collected, dried and weighed (Hussain *et al.*, 2011). The following formula was used in calculations:

$$\% \text{ Phenols} = (\text{weight of phenols}) / (\text{weight of sample}) \times 100.$$

3.4.5 Determination of total tannins

Approximately 0.06g of sample was extracted using 3 ml of methanol, shaken for 1 min and filtered. Exactly 3 ml of methanol was quickly added and the contents poured into the funnel. The filtrate volume was made up to fifty milliliters using distilled water and analyzed in an hour for total tannins. Three milliliters of 0.1 M FeCl₃ in 0.1 N HCl and three milliliters of 0.008 M K₃[Fe(CN)₆] were added to the extract. The absorbance was measured after ten minutes at 720 nm in a spectrophotometer using water as blank. A standard was prepared using tannic acid to get 1000 ppm and measured (Abdel *et al.*, 2007). The percentage of tannins was calculated as follows:

$$\text{Tannic acid (mg/(100 g))} = (\text{concentration of tannic acid} \times \text{extract volume} \times 100) / (\text{aliquot volume} \times \text{weight of sample}) \times 100.$$

3.5 Determination of caffeine levels

3.5.1 Standard solutions preparation

Caffeine stock solution of 1000ppm (1 ppm=1mg/L) was prepared by weighing 0.1 g of caffeine and transferring it to a 100ml volumetric flask. It was filled to the mark using the mobile phase- water, acetic acid, methanol (59.5 ml, 0.5 ml and 40 ml). Working standards of 10, 20, 40, 60, 80 and 100 ppm of the caffeine stock solution was prepared by diluting portions of the 1000 ppm solution using methanol: water (40 ml: 59.5 ml) containing 0.5% acetic acid (Shrestha *et al.*, 2016).

3.5.2 Sample preparation and analyte determination

Exactly 2g of *Acacia nilotica* subalata subsp. and *Coffea arabica* samples were weighed in triplicates and transferred into 250 ml beakers where 100 ml of boiling distilled water was added. This was left to stand for 5 min while stirring. The solution was cooled and filtered into conical flask. Five milliliters of the filtrate was pipetted into 50 volumetric flasks and made to the mark using the mobile phase. Samples and standards were then run on the High Performance Liquid Chromatography (HPLC) system. The conditions of the HPLC were as follows; Column, Reverse phase – ODS, 250 × 4.6 mm, flow rate (1 ml/min), photodiode array detector (PAD) was set at 278 nm, pressure (150 khf/cm²), mobile phase - water, acetic acid, methanol (59.5, 0.5 and 40) ml and sample volume of 10 µl (Eloff, 2004). Calibration curve of peak areas versus concentration of the standards was plotted and caffeine content of various samples was calculated by using regression equation of the best line of fit.

3.6 Anti-bacterial Assays

3.6.1 Inoculation of microorganism

The anti-bacterial properties of *Acacia nilotica* subalata and coffee arabica varieties (Batian 27 and Ruiru 11) were investigated against *Pseudomonas aeruginosa* (ATCC 10145) obtained from the Department of Medical Laboratory Science of Jomo Kenyatta University of Agriculture and Technology (JKUAT). The nutrient agar medium was prepared by dissolving 28 g of nutrient agar in 1000 ml of hot distilled water and allowed to cool. The mixture was then sterilized by autoclaving at 120 °C for about 15 min at 15 psi pressure and cooled to 50 °C (Aoko, 2009). The medium was dispensed into Petri - dishes to yield a depth of 4 mm and isolated colonies were aseptically transferred to nutrient agar in the Petri-dishes and incubated at 37 °C for 72 hours (Bamidele *et al.*, 2014).

3.6.2 Preparation of test samples

To determine the inhibitory concentration of the plant extract, various concentrations (15.0 mg/ml, 12.5 mg/ml, 10.0 mg/ml, 7.5 mg/ml, 5.0 mg/ml and 2.5 mg/ml) were tested. The stem bark of *Acacia nilotica* subalata and *Coffea arabica* varieties both methanol and ethanol extracts powder were weighed and dissolved in 10 % DMSO to prepare different concentrations of 15.0 mg/ml, 12.5 mg/ml, 10.0 mg/ml, 7.5 mg/ml, 5.0 mg/ml and 2.5 mg/ml. This was carried out by a serial dilution method using equation, $c_1v_1 = c_2v_2$, where c = concentration and v = volume (Aoko, 2009).

3.6.3 Disc diffusion bioassay

The ethanol and methanol extracts of *Acacia nilotica* (subalata) stem bark and *Coffea arabica* powder were screened for anti-*pseudomonas aeruginosa* activity by using disc diffusion method (Zaidan *et al.*, 2005). Inoculum suspension (10^8 CFU/MI) was spread over the nutrient agar surface by sterile collection swab. The 6mm disc was sterilized at 120 °C for about 15 min then loaded with 100 µg/µl gentamycin and 10 % DMSO(10µl) as a positive and negative control (used as comparison with the extracts). Extract solutions of *Acacia nilotica* subalata and *Coffea arabica* varieties at 15.0 mg/ml, 12.5 mg/ml, 10.0 mg/ml, 7.5 mg/ml, 5.0 mg/ml and 2.5 mg/ml concentrations each at volumes of 10µl were impregnated in the discs. The impregnated discs were dried for 5 min and dispensed onto the surface of inoculated plates with sterile forceps. The plates were labeled and incubated at 37 °C for 3 days (Zaidan *et al.*, 2005). The zone of inhibition (ZI) was measured (if any) using a ruler in millimetres. All experiment was performed in triplicate.

3.7 Data analysis and presentation

Statistical analysis of data from HPLC, phytochemicals, antibacterial activity was analyzed using analysis of variance. SPSS was used to compare concentration of caffeine, extracts from the three plants to find out whether these plants exhibited growth inhibition of the tested organism. Once differences were identified, ANOVA was further used to compare the treatments with the positive and negative control (Gentamicin and 10% DMSO) respectively to find out whether if the treatments had any bioactivity comparable to the positive control. Calibration curve of peak areas versus concentration of the

standards obtained from HPLC was plotted and caffeine content of various samples was calculated by using regression equation of the best line of fit. Ms. Excel 2007 was used to determine mean inhibition zones and Micro-soft Word 2007 was used to draw tables. The level of significance used in analysis of the data was 0.05 ($p < 0.05$) with 95% confidence interval.

3.8 Phylogenetic analysis

3.8.1 Data collection and sequence analysis

The entire coding region of *rbcL* sequences of 13 different species belonging to both generic Acacia and Coffee and *Thiotrichales bacterium* (outgroup) were obtained from taxonomy database of National Centre for Biotechnology information (NCBI), www.ncbi.nlm.nih.gov/GenBank.

The data analysis was carried out for the plant species *Acacia nilotica* and *Coffea arabica*. The protein sequence of the chloroplast ribulose-1, 5-bisphosphate carboxylase *rbcL* gene of *Acacia nilotica* and *Coffea arabica* sequences are available in Genbank. In this process, sequence is assigned on the basis of its similarity to a set of reference (identified) sequences (Ross *et al.*, 2008). The related sequences were retrieved from the GenBank database to determine the phylogenetic analysis of *Acacia nilotica* and *Coffea arabica*. Multiple sequence alignment was done using MUSCLE in MEGA offline software that performs optimum alignment for sequences (Shinwari *et al.*, 2014). The sequences that had variable numbers of Indels in the *rbcL* gene were not included in data analysis (Dwivedi B & Gadagkar S 2009). Aligned sequences were edited using JALVIEW

software. The phylogenetic tree was constructed using MEGA 7 tool and tree analysis were conducted using maximum likelihood methods (Dwivedi B & Gadagkar S, 2009).

3.8.2 Phylogenetic data analysis

Data for phylogenetic analysis that is basic sequence statistics including amino acid frequencies, transition/transversion (ns/nv) ratio and variability in different regions of sequences were computed by Molecular Evolutionary Genetics Analysis (MEGA) (Kumar *et al.*, 2016). The sequence data was analyzed by Maximum Likelihood Estimation (MLE) (Thorne *et al.*, 1991) using MEGA. Distances were calculated using Neighbor-join method. Bootstrapping analysis was done. Various clades were determined by MEGA (Shinwari *et al.*, 2014).

CHAPTER FOUR

RESULTS

4.1 Phytochemical analysis

4.1.1 Qualitative analysis

The results of qualitative phytochemical screening of water, methanol and ethanol extracts of stem bark of *Acacia nilotica* ssp. subalata and coffee arabica varieties (Batian 27 and Ruiru 11) are shown in the Table 4.1. The three extracts for the plant samples contained secondary metabolites such as alkaloids, flavonoids, saponins, carbohydrates, phenols, steroids and triterpenoids. However tannins were absent in the stem bark extract of *Acacia nilotica* ssp. subalata. Saponins, carbohydrates, steroids and triterpenoids were absent in the ethanol extract from three plant extracts. Whereas, carbohydrates were absent in both water and methanol extracts of Ruiru 11. Steroids and triterpenoids were absent in both methanol and ethanol extract of Batian 27, however flavonoids was absent in ethanol extract.

Table 4.1: Phytochemical profile of *Acacia nilotica* (subalata), Batian 27 and Ruiru

11

Secondary metabolites	ANSBWE	ANSBME	ANSBEE	PBWE	PBME	PBEE	PRWE	PRME	PREE
Tannins	-	-	-	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	-
Alkaloids	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	-	+	+	+
Steroids	+	+	+	+	-	-	+	+	-
Triterpenoids	+	+	+	+	-	-	+	+	-
Carbohydrates	+	+	-	+	+	+	-	+	-
Phenols	+	+	+	+	+	+	+	+	+

KEY:

ANSBWE-*Acacia nilotica* ssp Subalata water extract, ANSBME- *Acacia nilotica* ssp Subalata methanol extract, ANSBEE- *Acacia nilotica* ssp Subalata ethanol extract, PBWE- Processed Batian 27 water extract, PBME- Processed Batian 27 methanol extract, PBEE- Processed Batian 27 ethanol extract, PRWE- Processed Ruiru 11 water extract, PRME- Processed Ruiru 11 methanol extract, PREE- Processed Ruiru 11 ethanol extract. (-) and (+) indicates absence and presence of a secondary metabolites respectively.

4.1.2 Quantitative analysis

Quantitative phytochemical analysis of three extracts prepared from *Acacia nilotica* subalata subspecies, Batian 27 and Ruiru 11 are shown in the Table 4.2 and Figure 4.1. The three plant extracts contained saponins, tannins, phenols, flavonoids and alkaloids. *Acacia nilotica* subalata subsp. extract has the highest amount of saponins, alkaloids and flavonoids but the lowest contents of phenols and tannins. Ruiru 11 and Batian 27 had the highest amount of phenols and tannins respectively.

Table 4.2: Quantitative phytochemical analysis of saponins, alkaloids, flavonoids, phenols and tannins. Values are percentage mean \pm standard deviation (n=3)

Phytochemicals	Concentration in %		
	<i>Acacia nilotica</i>	Batian 27	Ruiru 11
Alkaloids	25.13 \pm 0.81	16.93 \pm 0.31	17.00 \pm 0.92
Saponins	11.60 \pm 0.72	5.23 \pm 0.40	4.47 \pm 0.12
Flavonoids	4.93 \pm 1.17	11.87 \pm 0.42	12.00 \pm 0.60
Phenols	14.00 \pm 0.18	22.00 \pm 0.08	26.00 \pm 1.09
Tannins	0.53 \pm 0.05	2.52 \pm 0.11	2.82 \pm 0.16

4.1.3 Caffeine Analysis

Graphical representation of quantitative phytochemical analysis and determination of caffeine levels of *Acacia nilotica* ssp. subalata in comparison with Batian 27 and Ruiru 11 (Figure 4.1 and 4.2). Ruiru 11 had the highest contents of phenols while *Acacia nilotica* ssp. subalata has the lowest. The opposite was true for the alkaloid content (Figure 4.1)

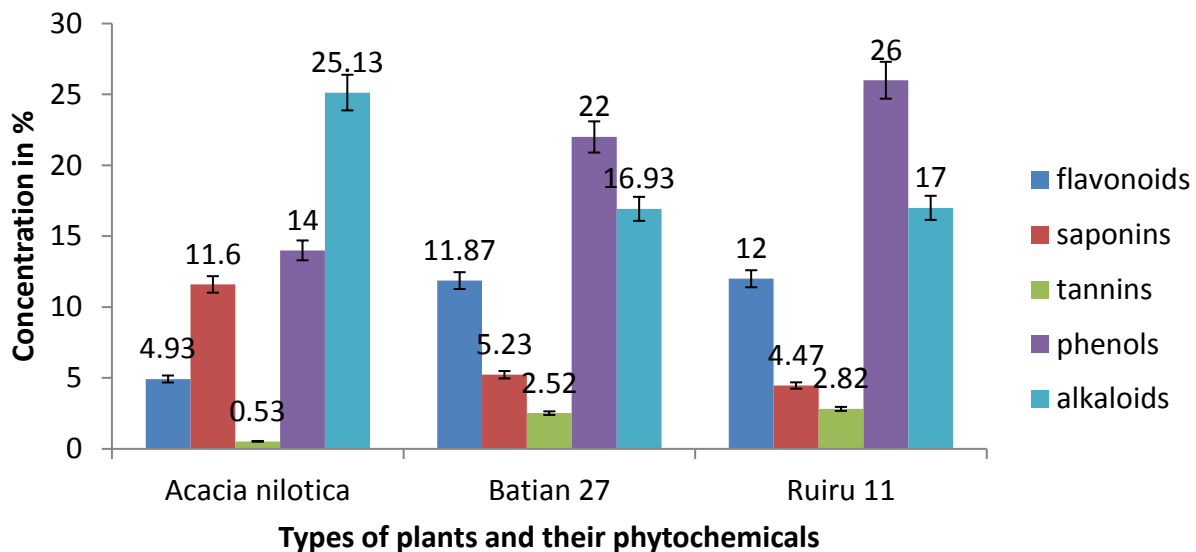


Figure 4.1: Concentrations of various phytochemicals in *Acacia nilotica* ssp. subalata, Batian 27 and Ruiru 11. Results are mean concentration \pm SD of the three replicates.

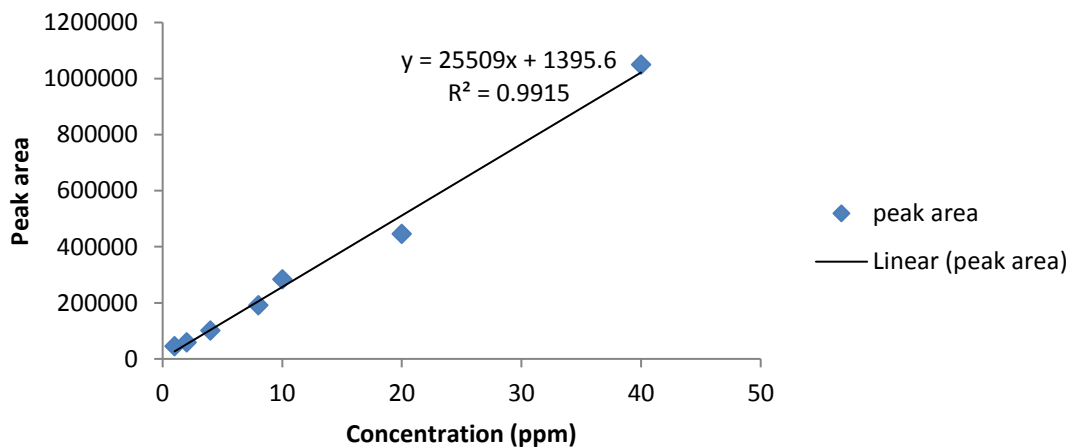


Figure 4.2: Determination of caffeine levels in *Acacia nilotica* ssp. subalata, Batian 27 and Ruiru 11 using HPLC. Caffeine calibration curve.

The results for chromatogram for the three samples and a standard (figure 4.3, 4.4, 4.5 and 4.6) had peak areas with same retention times which indicated presence of caffeine in *Acacia nilotica* in comparison with *Coffea arabica* varieties and the standard caffeine. The retention time of the *Acacia nilotica* was (7.452), Batian 27 (7.476), Ruiru11 (7.474) extract and standard caffeine (7.474).

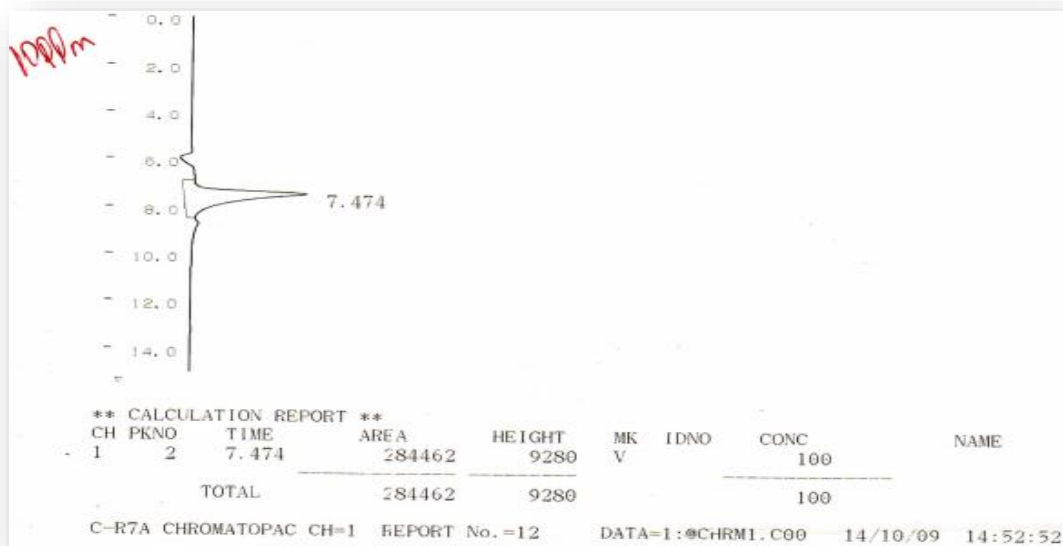


Figure 4.3:A chromatogram of standard solution of caffeine of 10 ppm

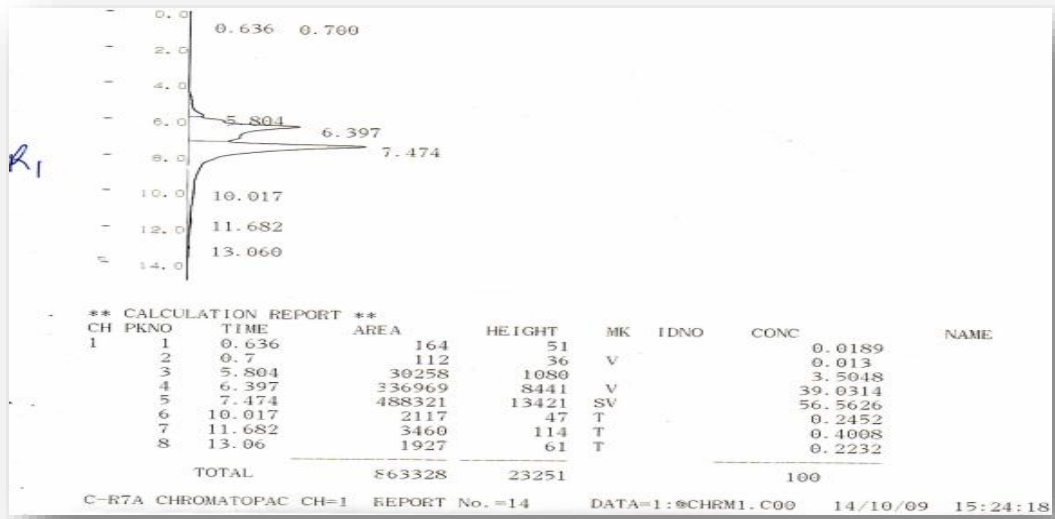


Figure 4.4: A chromatogram of sample solution of Ruiru 11

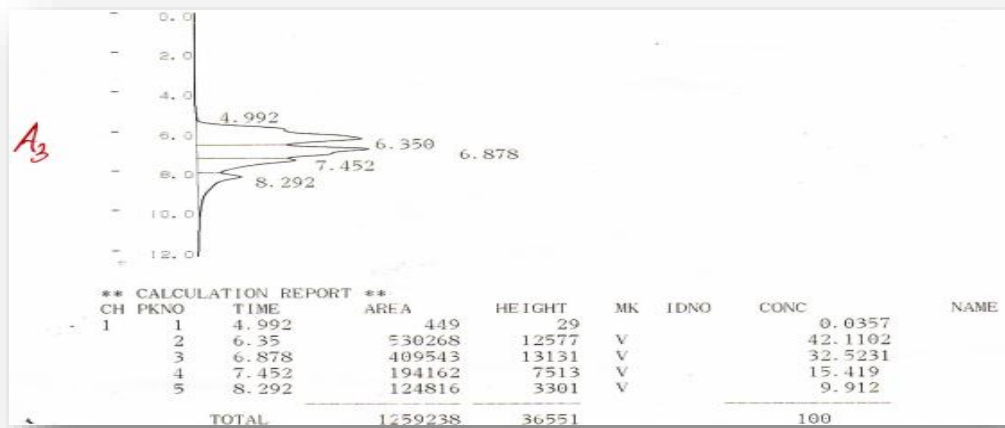


Figure 4.5: A chromatogram of sample solution of *Acacia nilotica* (Subalata)

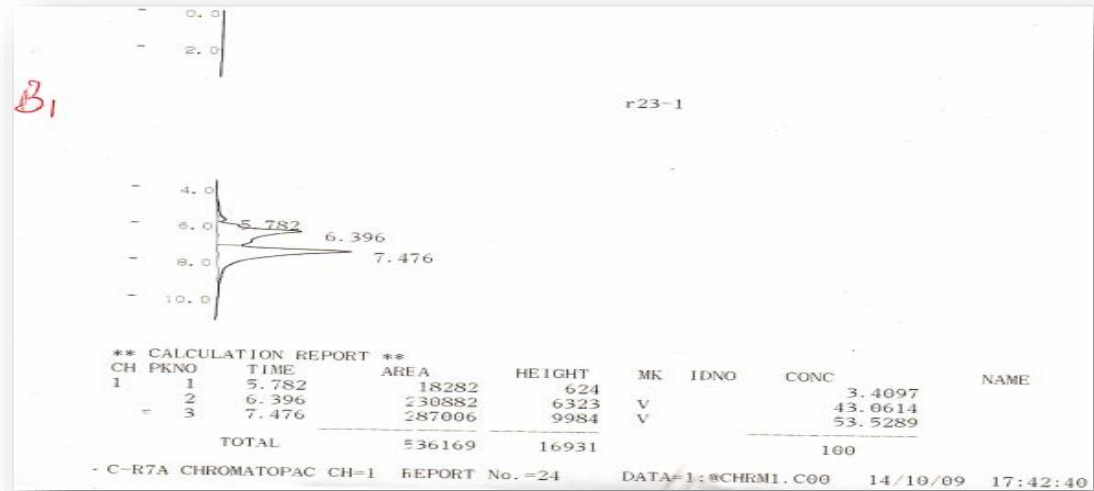


Figure 4.6: A chromatogram sample solution of Batian 27

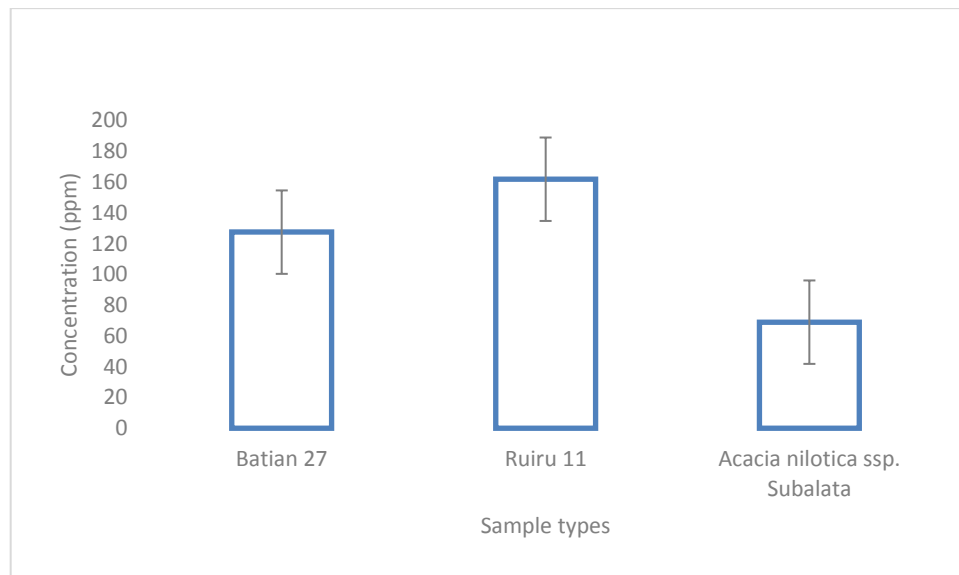


Figure 4.7: Comparative caffeine levels in *Acacia nilotica* ssp. Subalata, Batian 27 and Ruiru 11. Results are mean concentration \pm SD of the three replicates.

Determination of caffeine concentration (Figure 4.7 and Table 4.3) reveals that Ruiru 11 had the highest concentration of 161.75 ± 39.16 ppm followed by Batian 127.39 ± 14.83 ppm. However, *Acacia nilotica* ssp. subalata had the lowest concentration of 68.91 ± 17.39 ppm.

Table 4.3: Caffeine concentration (ppm) in *Acacia nilotica* ssp. Subalata, Batian 27 and Ruiru 11. Values are mean \pm SD of the three replicates.

	Mean \pm SD
<i>Acacia nilotica</i> ssp. subalata	68.91 ± 17.39
Batian 27	127.39 ± 14.83
Ruiru 11	161.75 ± 39.16

The amount of caffeine in *Acacia nilotica* ssp. subalata, Batian 27 and Ruiru 11 and their P. values were determined (Table 4.5). Their amounts ranged between 0.34 ± 0.09 % to 0.81 ± 0.20 % with Ruiru 11 having the highest concentration of 0.81 ± 0.20 % and *Acacia nilotica* ssp. subalata having the lowest value of 0.34 ± 0.09 %.

Table 4.4: Percentage levels of caffeine in *Acacia nilotica* ssp. Subalata, Batian 27 and Ruiru 11. Results are percentage mean \pm SD of the three replicates.

Samples	Concentration %
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<i>Acacia nilotica</i> ssp. Subalata	0.34 ± 0.09
Batian 27	0.64 ± 0.07
Ruiru 11	0.81 ± 0.20

4.2 Antibacterial activity of the crude extracts

Antibacterial activity of ethanol and methanol extracts of the three plants against *Pseudomonas aeruginosa* are summarized in Table 4.6. The results for *Acacia nilotica* (Subalata) and Batian 27 methanol crude extracts showed activity against the selected microorganism with the exception of Ruiru 11 extract and Batian 27 ethanol extract which did not show any inhibitory activity against *Pseudomonas aeruginosa*. The standard drug (gentamycin) had activity against *Pseudomonas aeruginosa*. The antibacterial activity of both ethanol and methanol extracts of *Acacia nilotica* (Subalata) increases with an increase in extract concentrations with inhibition zones of 11.3 and 12.6 mm at a concentration of 15 mg/ml.

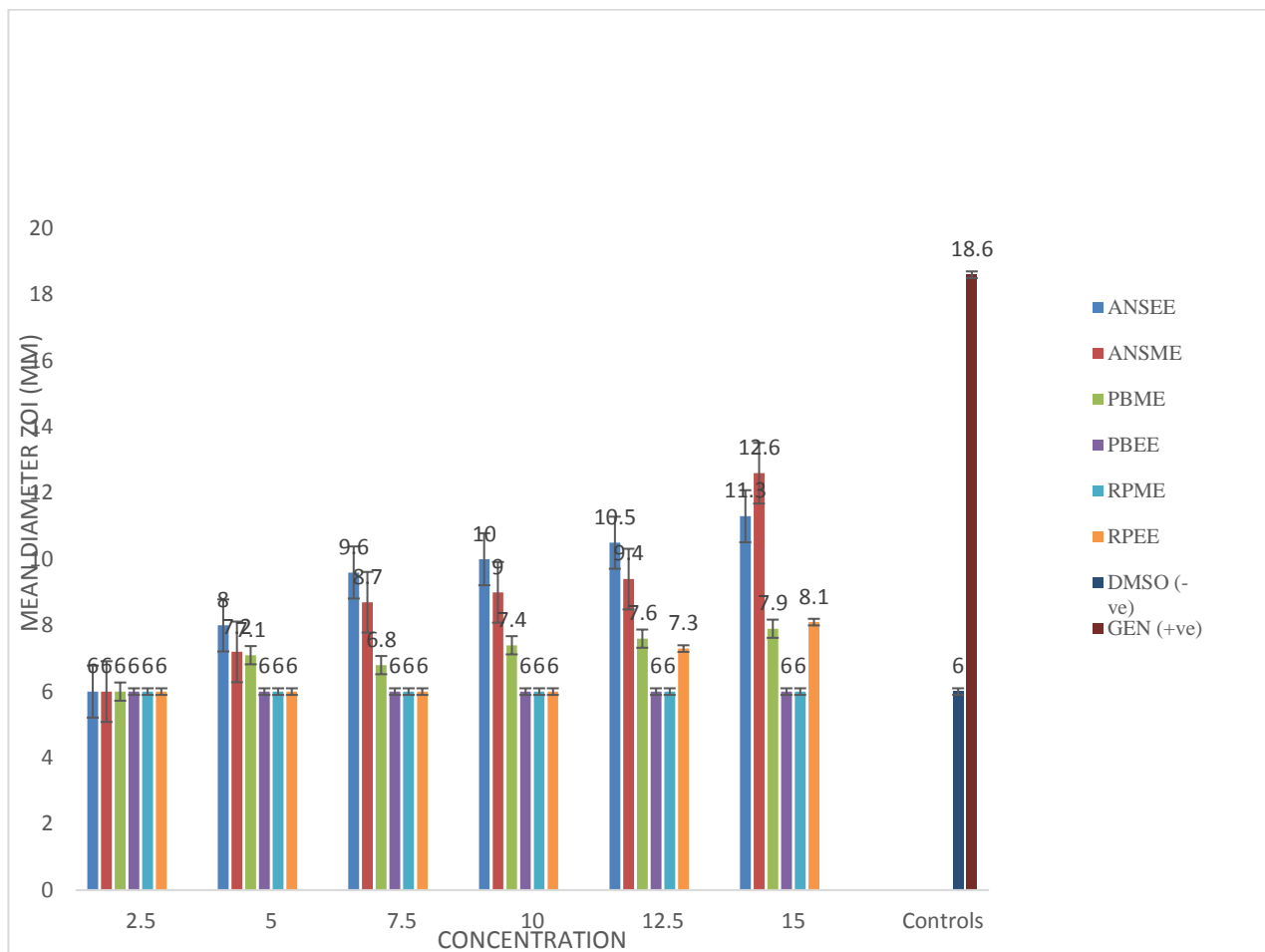


Figure 4.8: Comparison of zone of inhibition of both methanol and ethanol extract of *Acacia nilotica* and *Coffea arabica* varieties (Batian 27 and Ruiru 11) on *Pseudomonas aeruginosa*. Results are mean \pm SD of the three replicates.

KEY

ANSEE- water extract, PBME- Processed Batian 27 methanol extract, PBEE- Processed Batian 27 ethanol extract, PRME- Processed Ruiru 11 methanol extract, PREE- Processed Ruiru 11 ethanol extract. *Acacia nilotica* ssp subalata ethanol extract, ANSME- *Acacia nilotica* ssp Subalata methanol extract, PBWE- Processed Batian 27 water extract.

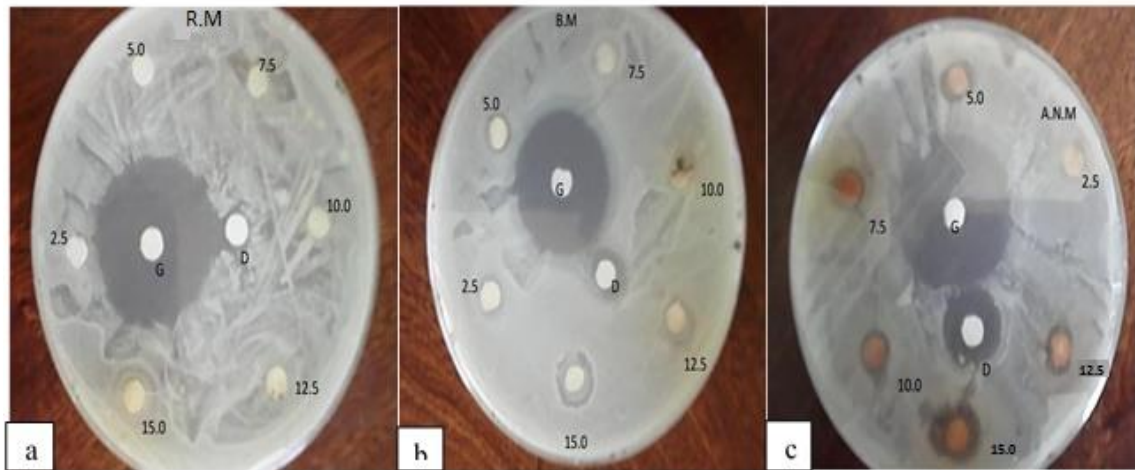


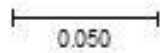
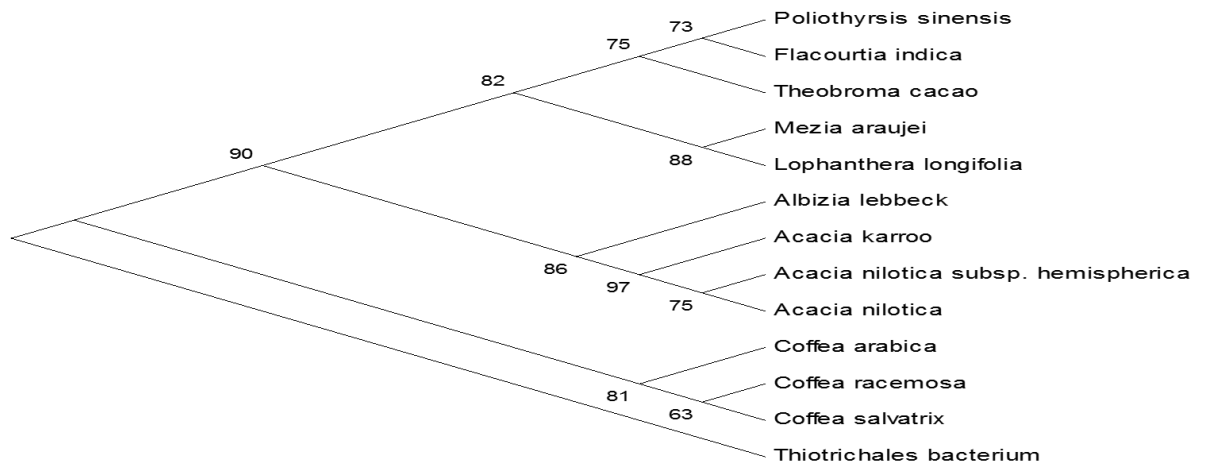
Plate 4.1. Diagrams with zone of inhibition (ZOI) of different concentrations of plant extracts from the three specimens of study. Diagram b and c shows plant extract with inhibitory activity. There is a clear halo around drug disc in each diagram.

KEY

Diagrams a, b and c shows ethanol and methanol extracts of *Acacia nilotica*, Batian 27 and Ruiru 11 inhibitory activity. The values between 2.5 and 15.0 represent different concentrations. R.E-Ruiru 11 ethanol extract, B.M-Batian 27 methanol extract and A.N.M- *Acacia nilotica* methanol extract. Discs G and D in each case were impregnated with gentamycin (positive control) and 10 % DMSO (negative control) respectively. There is an interchange such that at concentration of 12.5 should have been 15.0 and vice versa for *Acacia nilotica* methanol extract

4.3 Phylogenetic analysis

The figure 4.5 shows part of a data set used to construct phylogenetic trees for *Acacia nilotica* and *Coffea arabica*. The data are the aligned sequences of large subunit of



Divergence time in millions of years (Myr)

Figure 4.10. Maximum Likelihood tree (ML) rooted on *Thiotrichales bacterium* as outgroup showing the relationship of between *Acacia nilotica* and *Coffea arabica* with the related taxa

The phylogenetic tree is based on the protein sequence of *rbcL* gene. The numbers at the branches are confidence values based on Felsenstein's bootstrap method. B = 1000 bootstrap replications. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown in figure 4.5 (>50%) next to the branches. The scale bar represents the branch length measurement in the number of substitutions per site.

CHAPTER FIVE

DISCUSSION

5.1 Phytochemical screening

Phytochemical screening of bioactive constituents showed that methanol extracts had more secondary metabolites, followed by ethanol and water. Flavonoids, phenols and alkaloids were present in the three plant extracts. *Acacia nilotica* (subalata) had more components revealed than Batian 27 and Ruiru 11. Saponins were absent in Ruiru 11 ethanol extract. The presence of these saponins, alkaloids, flavonoids, steroids, triterpenoids, phenols and carbohydrates in *Acacia nilotica* (subalata) organic extracts in this study probably explained their inhibitory activity in comparison with both Batian 27 and Ruiru 11 organic extracts which had no inhibitory activity.

Phytochemical screening of *Acacia nilotica* (subalata) revealed the presence of secondary metabolites: phenols, saponins, alkaloids, flavonoids, triterpenoids and steroids. The pharmacological properties of these groups of chemical compounds have been extensively reported and documented (Banso, 2009; Deshapande &Kadam, 2013; Tenguria *et al.*, 2012; Ango *et al.*, 2012; Teke *et al.*, 2013). For example, flavonoids have anti-oxidant, anti-cancer, anti-fungal, anti-viral, anti-allergic and detoxification activities (Adedapo, 2013). Tannins in aqueous, ethanol and methanol extracts of *Acacia nilotica* (subalata) were absent. Similar results for the absence of tannins in aqueous and organic extracts of *Acacia nilotica* had been recorded in other species of genus *Acacia* (Banso, 2009; Godghate *et al.*, 2014).

Water extracts were rich in flavonoids, alkaloids, saponins, triterpenoids and steroid (Kisoi *et al.*, 2016). This justifies the use of water to prepare concoction or beverage by the locals in Pokot. It is well known that herbal medicinal products usually contain more than one plant or active constituents and their therapeutic efficacy is not provided by a single compound. Some of these compounds act synergistically to modify the bioavailability and efficacy of the active constituent. For instance, tannins have been considered in traditional medicine to treat various diseases, and their synergistic effects with various antibiotics, such as carbenicillin and tetracycline, have been shown to be beneficial against antibiotic resistant bacteria (Dusane *et al.*, 2015; Okuda and Ito, 2011).

Phytochemical analyses of Batian 27 and Ruiru 11 revealed the presence of tannins, flavonoids, alkaloids and phenols in water, ethanol and methanol extracts while saponins was absent in ethanol extracts of Ruiru 11 which was consistent with Guanalan *et al.*, (2012) findings.

The results of quantitative phytochemical analysis of *Acacia nilotica* ssp. subalata bark and coffee varieties extract showed significant amount of phenols, tannins, alkaloids and flavonoids which correlates with several studies by (Alam *et al.*, 2007 & Nayeem *et al.*, 2011). The differences in terms of quantity and phytochemical presence among *Acacia nilotica* (subalata), Batian 27 and Ruiru 11 in Baringo County and Kiambu County can be attributed to growing conditions, altitude, soil chemistry and processing techniques (Wanyika *et al.*, 2010).

5.2 Caffeine analysis

The chromatographic conditions allowed for the separation of the component of the extracts shown by the peaks. In this study we were looking for presence of caffeine in *Acacia nilotica* and compared with that of Batian 27 and Ruiru 11. This was determined by the retention time of the extract against retention time for standard caffeine. The retention time of *Acacia nilotica* were (7.452), Batian 27 (7.476), Ruiru 11 (7.474) extracts and standard caffeine (7.476). From the data it is clear that the retention time of standard caffeine and the three samples are the same hence the samples have caffeine content. Ruiru 11 had the highest caffeine content followed by Batian 27 and *Acacia nilotica* (subalata). They are statistically significant ($p < 0.05$).

The chromatograms were run for 20 min and typical retention time for aqueous components is between 0 – 20 min (Chang *et al.*, 2008). Ruiru 11 had the highest caffeine content (161.75 ± 39.16) followed by Batian 27 (127.39 ± 14.83) and *Acacia nilotica* (subalata) (68.91 ± 17.39).

5.3 Anti-bacterial activity

The use of *Acacia nilotica* (subalata) by locals in Pokot to treat skin rashes, eye and ear infection caused by *Pseudomonas aeruginosa* were supported by the results of this study. From the results, *Acacia nilotica* (subalata) has moderate to strong activity (zone of inhibition were between 8.0 mm-12.6 mm). A zone of inhibition ≥ 9 –15 mm is an indication of strong antimicrobial activity (Rani & Khullar, 2004). Against *Pseudomonas aeruginosa*, *Acacia nilotica* (subalata) had strong antibacterial activity with a zone of

inhibition of 11.3 mm for ethanol and 12.6 mm for methanol extract. It could be possible that the alkaloids found in the stem bark played a vital role in inhibitory effect (Nedi *et al.*, 2004; Mariita, 2006). Batian 27 methanol extract had poor activity (zones of inhibition of 6.8 mm-7.9mm). Ruiru 11 extracts had poor inhibitory effect at high concentration of 12 mg/ml and 15 mg/ml with inhibition zones of 7.3 mm and 8.1 mm respectively against *Pseudomonas aeruginosa*, possibly due to the antagonistic effects of the phytochemicals (Ruttoh, 2009; Cherotich, 2015).

Acacia nilotica (subalata) ethanol and methanol crude extracts had activity against *Pseudomonas aeruginosa* compared to Batian 27 and Ruiru 11 extracts which show poor inhibitory activity. This can be ascribed to the effect of the processing technique coffee berries undergo which could result to loss of active ingredients. This is not the case in *Acacia nilotica* (subalata) as it does not undergo a lot of processing technique. Also, single compound of extracts may not show inhibitory activity but more than one compound may exhibit synergistic effect thus causing inhibitory activity (Jembere & Hassanali, 2001). In this case components from *Acacia nilotica* (subalata) extracts have inhibitory activity compared to the Batian 27 ethanol and Ruiru 11 extracts.

Traditional practitioners mostly utilize water as the main solvent for active metabolites from medicinal plants, but based on the results of this study, methanol extracts of *Acacia nilotica* (subalata) showed the highest degree of anti-bacterial activity followed by ethanol. Other findings of ethanol extracts having the least anti-bacterial activity as compared to other organic extracts have been reported earlier (Allero & Afolayan, 2006). This may be due to better solubility of the active components in this organic solvent (De

Boer *et al.*, 2005) hence methanol stands as the most effective solvent for extraction of anti-bacterial metabolites from *Acacia nilotica* (subalata). The better efficacy of methanol was further clearly supported by minimum inhibitory concentration results in the study. In addition, all the extracts exhibited concentration dependent activity at tested concentrations; higher activity was observed at high concentration (15 mg/ml).

Methanol extract of *A. nilotica* (subalata) exhibited the strongest inhibitory activity against *Pseudomonas aeruginosa* (12.6 mm) compared to other extracts tested. This is in agreement with previous findings on methanol extracts of *Acacia nilotica* (Mahesh & Satish, 2008). Methanol extracts of *A. nilotica* had a minimum inhibitory concentration value of 5.0 mg/ml on *Pseudomonas aeruginosa* which is similar to previous findings using *A. nilotica* (10 mg/ml) by Banso, 2009. At higher concentrations, *A. nilotica* (subalata) extracts were more active against *Pseudomonas aeruginosa* as shown by the least minimum inhibitory concentration value of 5 mg/ml when compared to Batian 27 and Ruiru 11 extracts. Therefore, these strongly support the traditional utilization of the plant by locals in Pokot in Baringo County in management of various ailments.

5.4 Phylogenetic Relatedness

The results from phylogenetic analysis revealed that *Acacia nilotica* were phylogenetically related with *Coffea arabica*. It also showed that *Coffea arabica* were closely related to *C. salvatrix* and *C. racemose*. All the trees that were inferred from the *rbcL* gene sequence of *Acacia nilotica* and *Coffea arabica* and related taxa demonstrated a distinct lineage; thus, could distinguish the species of *A. nilotica* and *C. arabica* and show their relatedness. The sequences generated from *rbcL* also indicated that *Acacia*

nilotica and *coffea arabica* are monophyletic. The evolutionary analysis on the basis of *rbcL* proved that *Acacia nilotica* ssp. *Subalata* and *Acacia nilotica* ssp. *hemispherica* are closely related as they form the sister groups. Figure 4.5 shows *rbcL* sequences alignment part of the chloroplast -encoded Rubisco, large subunit of Ribulose-1,5-Bisphosphate Carboxylase (*rbcL*) of 13 different species where majority of the sequences were conserved.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Traditional knowledge can lead to useful medicinal plants used to manage and treat various disease conditions including infections caused by pathogens. This study was designed to evaluate phytoconstituents, caffeine levels, anti-bacterial and evolutionary analysis of *Acacia nilotica* (subalata) in comparison with *coffea arabica* varieties. Most of the screened phytochemicals were present among the three plants. The quantities of five compounds believed to have inhibitory activity varied across the three plants. This can be attributed to factors such as geographical location, rainfall, soil type and plant type. Using the bacteria *Pseudomonas aeruginosa*, anti-bacterial study was carried out using the single extraction and disc diffusion techniques. The results from bioassay revealed presence of inhibitory activity from *Acacia nilotica* (subalata) extracts whereas Batian 27 methanol extract showed low activity. The zone of inhibition varied suggesting the varying degree of efficacy and different constituents of the plants on *Pseudomonas aeruginosa*. Of the three screened plants, *Acacia nilotica* (subalata) was active against the test organism. The significant activity observed in this study could be attributed to the interaction of one or more secondary metabolites. The chromatograms were analyzed and the peak areas with same retention times indicated presence of caffeine. Absorption was at 278nm with total run time of 20 min. Ruiru 11 had the highest caffeine content followed by Batian 27 and *Acacia nilotica* (subalata). The results on caffeine levels may be useful for the evaluation of dietary information and can be concluded that this plant has the

potential to be sources of natural antioxidant nutrients. Phylogenetic tree analysis revealed that *Acacia nilotica* and *Coffea arabica* are monophyletic as they share a common ancestor though distantly related. *Acacia nilotica* have higher boot strap values than *Coffea arabica* making the evolutionary sense between the two genus. Therefore this study provides some basic information on the activity of *Acacia nilotica* (subalata) against *Pseudomonas aeruginosa* compared to Batian 27 and Ruiru 11.

6.2 Recommendations

- 1) This study forms a basis for further phytochemical and pharmacological studies to isolate and characterize the bioactive principles in order to understand their modes of action hence development of new anti-microbial drugs
- 2) Further work is needed to carry out using *in vivo* models to determine the levels of toxicity on the crude extracts and active pure compounds to determine their potential for safe use.
- 3) Because only one part of the plant was used, studies should be carried out to study different parts of the same plant and compare their activities.
- 4) A detailed comparison of caffeine content in *Camellia sinensis*, *Acacia nilotca*, *Coffea arabica* varieties and *Coffea robusta* varieties needs to be carried out.
- 5) Study the evolutionary analysis of *Camellia sinensis*, *Acacia nilotca*, *Coffea arabica* varieties and *Coffea robusta* varieties

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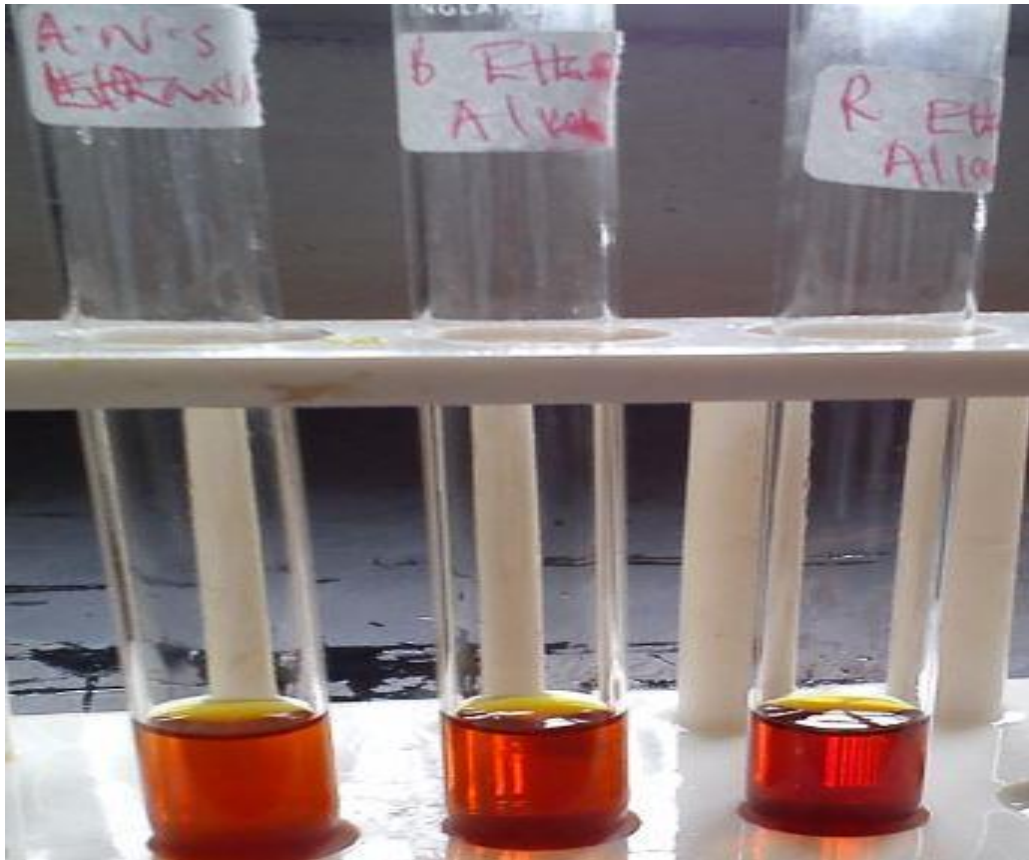
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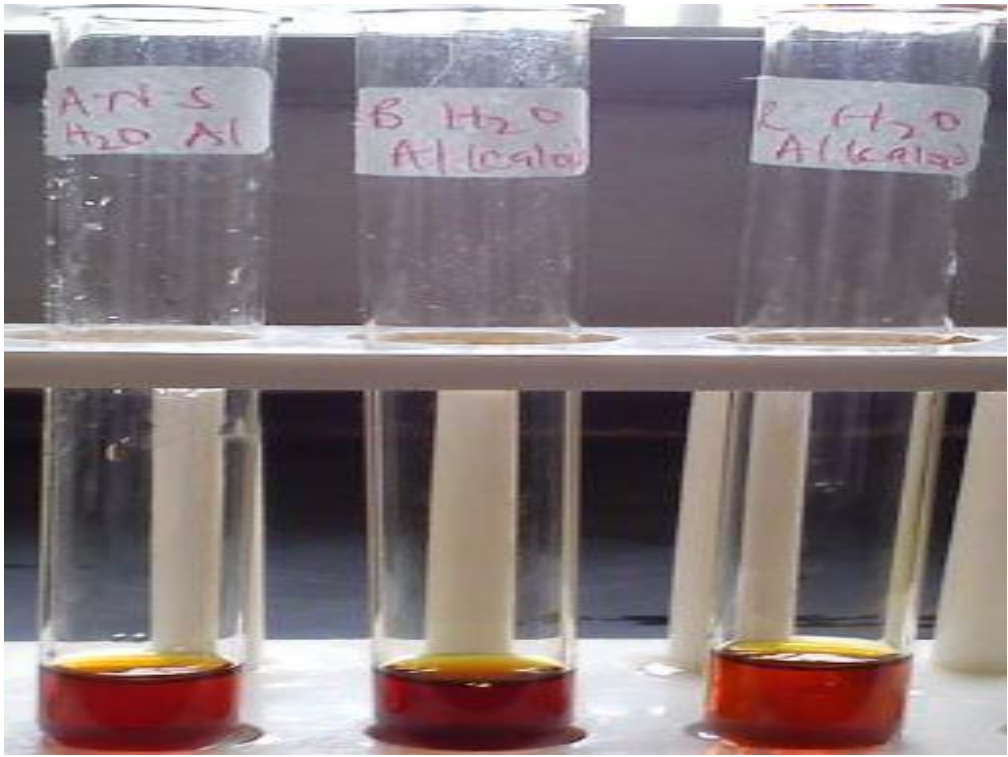
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APPENDICES

Appendix I: Presence of alkaloids in *Acacia nilotica*, Batian 27 and Ruiru 11 ethanol extract



AppendixII: Presence of alkaloids in *Acacia nilotica*, Batian 27 and Ruiru 11 water extract



Appendix III: Presence of saponin in *Acacia nilotica*, Batian 27 and Ruiru 11 Methanol extract



Appendix IV: Presence of saponin in *Acacia nilotica*, Batian 27 and Ruiru 11 ethanol extract



Appendix VI. Accession numbers of sequences retrieved from GenBank.

<u><i>rbcL</i></u>	
KC417042.1	AJ402991.1
KC417043.1	AM235003.1
KC417041.1	X81095.5
GQ981898.1	HQ247539.1
KR529342.1	JX572420
AF344502.1	JX572421
KP793075.1	
