IN VITRO EVALUATION OF THE ANTIMICROBIAL EFFECT OF *Nepeta cataria* AND *Basella alba* AGAINST CLINICALLY RESISTANT *Acinetobacter baumannii* IN NAIROBI, KENYA

A Research Thesis submitted Pan African University Institute for basic Sciences, Technology and Innovation, in partial fulfilment of the requirement for the Degree of Master of Science in Molecular Biology and Biotechnology.

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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 work to God, my Lord and Saviour.

I dedicate this thesis to my uncle, NOUBATOUR Adoumtar to have believed in me, my parents, DJONOUMA Asta and MOROMBAYE Mekonbé, this thesis is the motive of your pride, my husband, DANEMBAYE Ndoum-Madje Emmanuel and lovely daughter DANEMBAYE Virginie Taryam who always were great source of inspiration and permanent support for the accomplishment of this thesis. Also, to my brothers and sisters, for their assistance and prayers and my friends and relatives for all kind of support and encouragement.

Thank you and may God bless you all.

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ABBREVIATIONS AND ACRONYMS

ABC: *Acinetobacter baumannii–Acinetobacter calcoaceticus* complex

AKUH: Aga Khan University Hospital

AME: Aminoglycoside Modifying enzymes

BACP *Basella alba* (*B. alba*) Colorant Powder)

C. alba*: Carpolobia alba*

CNS: Central Nerve System

CRAB Carbapenem-resistant *Acinetobacter baumannii*

CTX: Cefotaximase

ICU: Intensive Care Units

JKUAT: Jomo Kenyatta University of Agriculture and Technology

LC_{50}: Lethal Concentration that kill 50% of the population

MBC: Minimal Bacteria Concentration

MBL: metallo-β-lactamases

MDRAB: Multidrug-Resistant *Acinetobacter baumannii*

MIC: Minimal Inhibitory Concentration

MHA: Mueller-Hinton Agar

PAUISTI: Pan African University - Institute for basic Science, Technology and Innovation

PCR: Polymerase Chain Reaction

PER-1: Period Circadian Regulator 1

TEM- Temoniera, the name of a patient from whom resistant bacteria were isolated

TSA: Tryptone Soya Agar

TSB: Tryptone Soya Broth

SHV- Sulphydryl Variable: a description of the biochemical properties of this β-lactamase

VEB-1: Vietnamase Extended spectrum Beta-lactamase 1

ABSTRACT

Acinetobacter baumannii once considered a low-category pathogen, has emerged as an obstinate infectious agent. It has displayed stubbornness to last resort antimicrobials, high prevalence of infections in the hospital setting and significantly increased rate of community-acquired infections over the past decade. To overcome this problem, knowledge of the antibiotic resistance mechanisms, and prospective treatment options of *A. baumannii* is important. The purpose of this study was to determine the potential antimicrobial activity of *Nepeta cataria* and *Basella alba* against *Acinetobacter baumannii* isolates. *Nepeta cataria* and *Basella alba* leaves were collected from Ol Donyo Sabuk National Park and Botanical garden JKUAT respectively. The leaves were dried under shade at room temperature for 30 days. Methanolic and aqueous plant extraction was done at PAUSTI Molecular Biology and Biotechnology Laboratory. Extracts were subjected to toxicity testing using brine shrimp and preliminary phytochemical screening. 30 anonymous stocked *Acinetobacter baumannii* isolates from different clinical sources were obtained from Aga Khan University Hospital in Nairobi. They were inoculated into appropriate media and identified according to standardized protocols. The antimicrobial activity of the plants extracts was determined by well diffusion method described by (CLSI, 2017). The antibiotic susceptibility testing was done for all bacterial isolates using the Kirby-Bauer disc diffusion technique. Molecular investigation involved the DNA extraction and the PCR amplification was performed using OXA-23, OXA-24 and NDM primers. From the phytochemical screening, the methanolic plant extracts revealed more phytochemicals as compared to aqueous extracts and most of them were detected from *Nepeta cataria* compared to *Basella alba* plant extracts.

The methanolic plant extracts had MIC values 60 mg/ml when tested against *Acinetobacter baumannii*. The results recorded from this study demonstrated that methanolic extract of *Nepeta cataria* and *Basella alba* had greater antimicrobial effect against *Acinetobacter baumannii* than aqueous extracts. *Nepeta cataria* extracts showed greater antimicrobial effect than *Basella alba* extracts. The antibiotic susceptibility analyses of the 30 isolates revealed that 100% were resistant to cefotaxime followed by the resistance to ampicillin (93.3%), piperacillin (80%), amikacin (60%), ciprofloxacin (67%), gentamicin (50%), imipenem (56.6 %) and minocycline (37%). Molecular analysis revealed that all the 30 *Acinetobacter baumannii* isolates were 100 % positive for OXA-23, 17 % for NDM-1 and only 10% for the OXA-24. The detected antimicrobial activity of the two plants justify their potential use as an alternative treatment to *Acinetobacter* infections. Further studies on animal models are required to authenticate its use. Further tests on the potential activity of these plants on other microbes is important to curb the rising antimicrobial resistance.

CHAPTER ONE

1.0 INTRODUCTION

1.1. General Background information

Acinetobacter species are ubiquitous gram-negative coccobacilli, are widespread in nature, water and soil. More than 20 species of *Acinetobacter* have been characterized but only few species including *A. baumannii*, *A. calcoaceticus* and *A. lwoffii* play a significant role in nosocomial infections (Shamsizadeh *et al.*, 2017). *Acinetobacter* species has arisen as significant and challenging human pathogen as it is the causal agent of numerous kinds of infections. Most often related to endotracheal tubes or tracheostomies, endocarditis, meningitis, septicaemia, skin, wound infections, UTI and bacteraemia peritonitis in patients receiving peritoneal dialysis (Chandra *et al.*, 2017). The intrinsic resistance of this pathogen to many drugs is characterized by, its ability to acquire the determinants of antibiotic resistance through horizontal gene transfer, to almost all available antibiotics (Sheck *et al*., 2017). *A. baumannii* has been involved in infections with increased mortality rates. The virulence of *A. baumannii* can be enhanced by the occurrence of multiple antimicrobial resistance, resulting in difficulties when determining the therapeutic options to treat the infection. It has been reported that multidrug-resistant *A. baumannii* induces a fulminant infection following the treatment of a surgical wound (Xiao *et al.*, 2017). The clinical manifestations of *A. baumannii* are non-specific and present as a trans-maculopapular rash affecting the skin and soft tissue. *A. baumannii* bacteraemia is polymicrobial and is often associated with *K. pneumoniae* (Ehlers *et al.*, 2012). This pathogen possesses different antimicrobial resistance mechanisms, which include the enzymatic inactivation of β-lactam antibiotics through the production of enzymes (Lowings *et al.*, 2015). This contribute to the difficult the treatment of *A. baumannii* infections. Due to their fewer side effects, better patient tolerance, and relatively less expense than synthetic drugs Plant-based drugs are more accepted. Plants are also the source of most natural and synthetic drugs.

Biomolecules of plant origin can serve as alternatives for the control of resistant human pathogens. For instance, *Nepeta* species (*Labiateae*) are used in traditional medicine in many countries and have large ethnobotanical effects (Teixeira *et al*., 2011). *Basella alba* is an important medicinal plant in ethnoveterinary (Sushila *et al*., 2010).

1.2. Statement of the Problem

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is a major nosocomial pathogen in ICUs. Infections caused by CRAB are considered difficult to treat and control. It was observed an emergence of CRAB in Kenyan hospitals mainly from ICUs. The multi-drug resistant strains become particularly problematic leading to increased mortality, longer hospital stays and higher hospital costs. To overcome these multi-drug resistant strains, a successful treatment approach with an active compound is required and which provided an effective eradication of these Gram-negative organisms. Traditionally and for these reasons, people especially in developing countries use the herbal preparations to treat diseases without testing the efficacy and the toxicity of these herbal plants.

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1.3. Justification and Significance of the study

Multi-resistant strains can be found in environment, in any habitat and at any time, making chances for contact high and permanent. Carbapenem-resistant *A. baumannii* pose a significant threat to hospitalized patients, as therapeutic options are scarce. Because only few effective antibiotics are available, clinicians often face serious challenges when treating patients with MDRAB. Therefore, a deep understanding of the resistance mechanisms used by MDRAB can shed light on the possible strategies to combat the dissemination of antimicrobial resistance. To address this problem effectively, knowledge of species identification, clinical manifestations, and virulence factors is essential.

The choice was focused on *Nepeta cataria* and *Basella alba* according to their antimicrobial effect previously reported and their availability in Kenya. Since, therapeutic options are limited for multidrug-resistant *Acinetobacter* infection, the aim of this study was to look at active compounds by determining the potential safety and efficacy of these medicinal plants used as antimicrobial against *Acinetobacter baumannii*. The results of this study validated the usage of the herbs.

1.4. Research questions

1. Is there any potential antimicrobial activity of *Nepeta cataria* and *Basella alba* against *Acinetobacter baumannii* isolates?
2. What is the susceptibility profile of *Acinetobacter baumannii* isolates implicated in nosocomial infections in Aga Khan Healthcare in Kenya?
3. What is the molecular basis of antimicrobial resistant to *Acinetobacter baumannii* isolates?

1.5. Objectives

1.5.1 General Objective

To determine the antimicrobial potential of *Nepeta cataria* and *Basella alba* against clinically resistant *Acinetobacter baumannii* isolates.

1.5.2 Specific Objectives

1. To determine the susceptibility of *Acinetobacter baumannii* to *Nepeta cataria* and *Basella alba* extracts
2. To determine the susceptibility profiles of *Acinetobacter baumannii* isolates to commonly used conventional drugs
3. To determine the molecular basis of resistant *Acinetobacter baumannii* clinical isolates

1.6. Scope of the Study

The main scope of this study was to emphasize the antimicrobial resistance of prescript treatment of nosocomial diseases specially in the case of *A. baumannii* and to determine the potential antimicrobial activity from plants in Nairobi, Kenya. Therefore, this study helped to determine the molecular characteristics of *A. baumannii* gene resistance stocked isolates from clinical sources and looked at the alternative that could guide to overcome the problem of resistance in case of *Acinetobacter* infections.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Introduction

The genus *Acinetobacter* is a major cause of nosocomial infections that has been listed; associated with various epidemics and it has become a widespread concern in a variety of hospitals worldwide (Almasaudi, 2015). *A. baumannii* is a gram-negative coccobacillus that has been described as a dangerous pathogen by the Infectious Disease Society of America. It mostly infects people with compromised immune systems. It is almost exclusively isolated from hospital environments that differentiate it from other species of the genus *Acinetobacter*. It is a rapidly emerging pathogen in the healthcare setting, where it causes infections that include bacteraemia, pneumonia, meningitis, urinary tract infection and wound infection (Bassetti *et al.*, 2008). Antibiotics are important medicines. They help fight infections that are caused by bacteria. Multidrug-resistant organisms (MDROs) are defined as microorganisms, predominantly bacteria, that are resistant to one or more classes of antimicrobial agents (Siegel *et al.*, 2006). Before 2008, *A. baumannii* was uncommon among pathogens recovered at Aga Khan University Hospital, Kenya, and it had remained susceptible to several antibiotics (Revathi *et al*., 2013). *A. baumannii* has become an emerging pathogen in intensive care units (ICUs) because it has developed resistance to broad-spectrum antibiotics (Chen *et al*., 2017). The overall prevalence of nosocomial infections in community-acquired bacteraemia in Kenyan hospital intensive care units due to *A. baumannii* accounted for 10% (Chaudhary, 2012 ; Huber *et al.*, 2014).

2.2. Taxonomic status of the genus *Acinetobacter*

The genus *Acinetobacter* is now defined as belonging to subclass ɤ-Proteobacteria, family Moraxellaceae, with a DNA rich in G + C content (39–47%) (Bergogne-Bérézin and Towner, 1996).

They are aerobic Gram-negative coccobacilli commonly present in soil and water as free-living saprophytes. Some species are also common commensals of skin, throat and secretions of healthy people. The genus *Acinetobacter* comprises 38 validly named species. Clinical microbiological laboratories usually divide the genus into "complexes" with *A. baumannii*– *A. calcoaceticus* complex (ABC) being the most clinically relevant. Approximately 25 different "genomic species" have been identified based on DNA-DNA hybridization studies. *A. baumannii* is genomic species number 2 (Alsan and Klompas, 2010). In 1986 *A. baumannii* was taxonomically classified (Bouvet and Grimont, 1986).

Acinetobacter are grouped into three main complexes:

i. *A. calcoaceticus-baumannii* complex, which is glucose oxidizing and non-haemolytic;

ii. *A. lwoffii*, which are non-haemolytic

iii. *A. haemolyticus*, which is haemolytic.

The full classification of *A. baumannii* is listed in Figure 2.1 (Euzeby, 2008)

Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Pseudomonadales*
Family	*Moraxellaceae*
Genus	*Acinetobacter*
Species	*A. baumannii* *A. baylyi* *A. beijerinckii* *A. bouvetii* *A. calcoaceticus* *A. gerneri* *A. grimontii* *A. gyllenbergii* *A. haemolyticus* *A. johnsonnii* *A. junnii* *A. lwoffii* *A. parvus* *A. radioresistens* *A. schindleri* *A. soli* *A. tandoii* *A. tjernbergiae* *A. towneri* *A. ursingii* *A. venetianus* 14 species are still unnamed

***Figure 2.1**: Nomenclature of Acinetobacter baumannii*

2.3. *Acinetobacter baumannii* description

A. baumannii is ubiquitous in nature and has been recovered from soil, water, animals, and humans. *Acinetobacter* species are normal inhabitants of human skin and are frequently isolated from the throat and respiratory tract of hospitalized patients. It is a typically short, almost round and can be an opportunistic pathogen in humans. It affects people with compromised immune systems and is becoming increasingly important as a hospital-derived infection. *A. baumannii* has also been identified as an ESKAPE pathogen (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa*) a group of pathogens with a high rate of antibiotic resistance that are responsible for the majority of nosocomial infections (Moradi *et al.*,2015). Community-acquired infections like skin and soft tissue infections in relation to trauma are also caused by the genus *Acinetobacter*. The bacterium *A. baumannii* is the most clinically important of the bacteria belonging to this genus (Odsbu *et al.*, 2018).

2.4. Epidemiology of *Acinetobacter baumannii* infections

Acinetobacter baumannii causes of disease and nosocomial infections includ septicemia, bacteremia, ventilator-associated pneumonia, wound sepsis, endocarditis, meningitis, and urinary tract infections (Bae *et al.*,2012). It is an opportunistic human pathogen that predominantly infects critically ill patients. Once thought to be benign, *A. baumannii* is now considered a global threat in the health-care setting, mainly due to its propensity to acquire multidrug, extensive drug and even pandrug-resistant phenotypes at previously unforeseen rates (Harding *et al.*, 2017). The genus *Acinetobacter* consists of aerobic, Gram-negative bacilli that comprise 33 differentiated species, of which only ten have been named. The term '*A. calcoaceticus*– *A. baumannii*' complex is often used to group the most clinically relevant species (Bassetti *et al.*,2008). Two key factors contribute to the significant and ubiquitous dissemination of *A. baumannii* in hospitals. These are the extent of its antimicrobial resistance and its environmental resilience.

Resistance has significantly increased in Africa (including piperacillin-tazobactam, ceftriaxone, cefepime, amikacin, meropenem, and levofloxacin resistance). In Europe resistance to including piperacillin-tazobactam, ceftriaxone, ceftazidime, levofloxacin, amikacin, minocycline, meropenem, and cefepime resistance has been reported (Cases, 2014). *Acinetobacte*r infection prevalence is variable depending on the geographical localization and the patient's socio-economic status. In an international study in ICUs, the *Acinetobacter* infections rate was 19.2% in Asia; 17.1% in Eastern Europe; 14.8% in Africa; 13.8% in Central and South America; 5.6% in Western Europe; 4.4% in Oceania and 3.7% in North America. It was 15% in South African HIV-positive patients and 13% in Canadian burn care units (Uwingabiye *et al*., 2016). Limited data is available on resistant East African *A. baumannii* isolates (Huber *et al*., 2014 (Okinda and Revathi, 2012). *Acinetobacte*r was most commonly isolated from ICU (51.41%); followed by medicine ward and surgical wards. 34% of the isolates were from endotracheal tubes which were followed by sputum (14%), pus (13%), blood (10%), suction catheter (7%) and urine (5%) (Talukdar *et al.*, 2018). The epidemiologic source of these infections has yet to be definitively identified; possible sources include skin colonization prior to injury, traumatic inoculation with soil during combat, or nosocomial transmission. Studies have found that as many as 17% of healthy U.S.–based soldiers do have skin colonization with ABC complex; however, their strains differ from those recovered from injured soldiers (Alsan and Klompas, 2010). *A. baumannii* has a high propensity to cause nosocomial outbreaks, possibly due to its persistence in the hospital environment and its remarkable ability to acquire drug resistance (Talukdar *et al.*, 2018). Several studies indicate significantly increased resistance rates in epidemic *A. baumannii* strains compared with sporadic strains (Huber *et al.*, 2014). Interestingly, *A. baumannii*, the most important nosocomial *Acinetobacter* sp., has been rarely found on human skin (0.5 and 3%) and in human feces (0.8%) (Manchanda *et al.*, 2010).

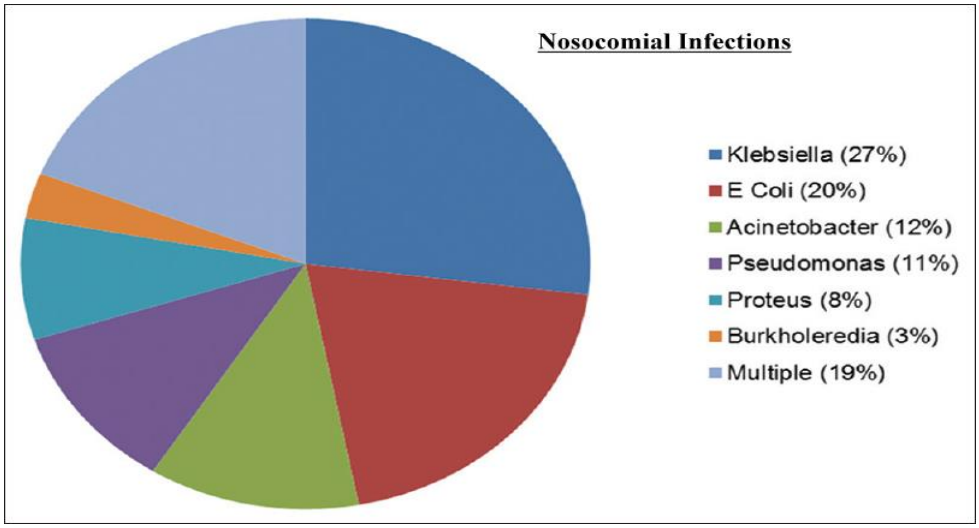

Figure 2.2*: Most common microorganisms causing nosocomial infections. (Choudhuri et al., 2017)*

2.5. Pathology of *Acinetobacter baumannii* infections

In case of *A. baumannii* infection, the pathological changes that occur depend on the organ system (Burke, 2003). The pathological changes observed in patients with pneumonia are indistinguishable from those caused by other non cavitating aerobic gram-negative bacilli that cause nosocomial pneumonias. Similarly, *A. baumannii* urinary tract infections are clinically indistinguishable from catheter-associated bacteraemia caused by other aerobic gram-negative bacilli (Burke, 2003). Most commonly, *A. baumannii* infections manifest as ventilator- associated pneumonia or central-line-associated bloodstream infections. Less frequently, *A. baumannii* causes infections in the skin and soft tissues and at surgical sites as well as catheter-associated urinary tract infections (Harding *et al.*, 2017). Since *Acinetobacter* colonization is common and treatment difficult, it is important to distinguish a colonization and an infection, with treatment reserved for true infections (Kanafani and Kanj, 2018).

2.6. Routes of transmission of *Acinetobacter* infections

The most common isolated *Acinetobacter* spp. from human samples is *A. baumannii* (Mohammadi *et al.*, 2017). *A. baumannii* is mainly transmitted by direct contact with infected persons or indirect contact with contaminated environments. However, airborne route also plays an important role in transmission of *A. baumannii* infections in hospitals (Shamsizadeh *et al.*, 2017). *A. baumannii* has been associated with severe infections; possible sources have been recognized in the skin, environment and food. In the hospital setting, digestive tract colonization is common, with rates as high as 41% in patients in the intensive care unit (ICU) (Bassetti *et al.*, 2008). This community-acquired pneumonia has most frequently been reported in tropical Australia and Asia and typically affects adults with compromised immune function (i.e., alcoholism, diabetes, renal failure) during the rainy season. The disease is characterized by a fulminant clinical course, secondary bloodstream infections, and a mortality rate of 40% to 60% (Alsan and Klompas, 2010). Other problems that rarely occur are soft tissue infections and complicated skin, abdominal infections and central nervous system (CNS) infections. The skin of patients and medical personnel is involved in the transmission of *A. baumannii* strains and in some outbreaks; molecular typing has identified the epidemic strain on the skin of the patients (Ehlers *et al.*, 2012). The mortality rate within the hospitals is high, with a 23% mortality recorded for hospitalized patients and a 43% mortality rate among patients in intensive care (Falagas *et al*., 2006).

2.7. Laboratory Diagnosis Techniques of *Acinetobacter* Species

Acinetobacter is oxidase negative, indole negative, catalase positive, and haemolytic. *Acinetobacter* is easily isolated in standard cultures due to its ability to use various sources of nutrition (Kanafani and Kanj, 2018). There are automated methods and biochemical kits available today to quickly and accurately identify an isolate at the species level.

These methods are phenotypic, utilizing biochemistry and assimilation tests usually done manually to identify the genus and species of bacteria in less time (Camp and Tatum, 2010). The most common and widespread detection methods include characterization via phenotypic system and commercial phenotypic methods (e.g., 16S rRNA gene amplification) (Kanafani and Kanj, 2018).

2.8. Conventional drugs used against *Acinetobacter baumannii* infections

A. baumannii is intrinsically multidrug resistant. Relatively few antibiotics are active against this organism (Figure 2.3). Carbapenems are classified as a subgroup of the β-lactam antibiotics that can bind to the penicillin-binding proteins of bacteria and inhibits cell wall synthesis and results in cell death. In general, first-, second-, and third-generation cephalosporins, macrolides, and penicillin have little or no anti-*Acinetobacter* activity, and their use may predispose to *Acinetobacter* colonization that should be avoided (Ndegwa, 2011). Some strains are sensitive to cefepime, ceftazidime, and sulbactam-containing beta-lactam/beta-lactamase–inhibitor drugs. A study done in East Africa had reported that all CRAB isolated were resistant to β-lactams, except for one cefepime-intermediate isolate. With respect to fluoroquinolone antibiotics, there was a higher resistance rate to ciprofloxacin (100%) than to levofloxacin (43.8%). With regard to aminoglycosides, the resistance rates were 68.8% for gentamicin and 37.5% for amikacin (Revathi *et al*., 2013). Combination therapy is often discussed and suggested, but data proving lower failure rates or lower rates for the development of resistance are inconclusive. Specifically, *A. baumannii* has a remarkable capacity to survive in unfavourable conditions. The emergence of isolates resistant to all commonly prescribed anti-microbial drugs have become more difficult to treat *Acinetobacter* infections estimated as a recent phenomenon that started in the 1970s (Bassetti *et al.*, 2008). In an article published by Giamarellou *et al.*, in 1986 the findings shown that imipenem was a promising drug for the treatment of hospital infection.

Almost 25 years ago, *A. baumannii* was found to be resistant against antimicrobial drugs, such as aminopenicillins, cephalosporins, first- and second-generation cephalosporins, cephamycins, aminoglycosides, ureidopenicillins, chloramphenicol, and tetracyclines. Strains of *A. baumannii* have started to acquire resistance to newly developed antimicrobial drugs and become prevalent in many hospitals (Almaghrabi *et al.*, 2018). In the study carried out by (Huber *et al.*, 2014), all isolates were resistant to cefepime, ceftazidime, ticarcillin/clavulanic acid, cefotaxime/clavulanic acid, piperacillin/tazobactam, cefoxitin, ciprofloxacin, gentamicin, nitrofurantoin, Fosfomycin trometamol and trimethoprim/sulfamethoxazole. In addition, all isolates except one (AKUH AB008) showed resistance to meropenem and imipenem.

2.8.1. Aminoglycosides

Amikacin and tobramycin are the two agents that appear to retain activity against many *A. baumannii* isolates. As with all antimicrobial agents and multidrug-resistant pathogens, resistance is increasing, and susceptibility testing is required to ensure activity (Fishbain and Peleg, 2018).

2.8.2. Polymyxins.

They include colistin or polymyxin E and polymyxin B. They have been used for the treatment of highly drug-resistant Gram-negative bacteria. Colistin is most commonly used in the United States, and it is administered intravenously as a pro-drug known as colistimethate sodium (CMS). Colistin sulfate is used topically but, most importantly, is also the form that should be used in the laboratory for susceptibility testing. Current Clinical and Laboratory Standards Institute breakpoints for colistin are $\leqslant$2 µg/mL (susceptible) and $\geqslant$4 µg/mL (resistant) (Fishbain and Peleg, 2018).

2.8.3. Tigecycline.

Tigecycline is the first of a new class of antibiotics known as the glycylcyclines. It is a semisynthetic derivative of minocycline and inhibits the 30S ribosomal subunit.

Its advantage over other tetracycline antibiotics is its ability to evade the traditional tetracycline-specific resistance mechanisms—*tet*(A-E) and *tet*(K) efflux pumps and the *tet*(O) and *tet*(M) determinants that provide ribosomal protection—and therefore it has a broader spectrum of activity. (Manchanda *et al.*, 2010).

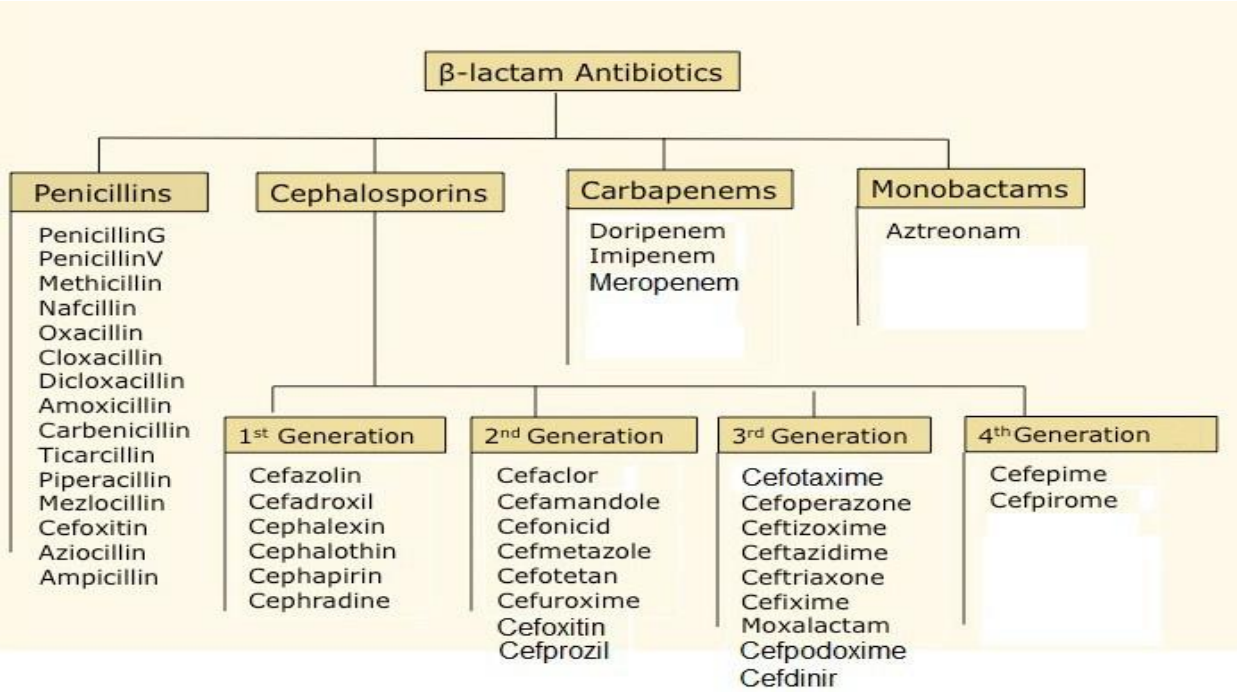

***Figure 2.3**: List of β-lactam antibiotics.* http://www.antibiotics.tips/2016/04/beta-lactam-antibiotics-list-classification-indications-contraindications.html

2.9. Alternative Medicines against *Acinetobacter baumannii* infections

Traditional medicine plants are likely to provide further new antibiotics in the future (Cheesman *et al.*, 2017). Numerous works have been done to isolate and characterize bioactive compounds from plant resources that are active against Gram-positive and Gram-negative bacteria, fungi and viruses. Miyasaki *et al*. (2013) reported that generally flavones, tannins and phenolic compounds are known to be active against *Acinetobacter*.

In recent decades, the pharmacological properties of numerous medicinal plants and opportunities in phytotherapy have been explored through research projects, reviews, and monographs. These studies confirm that medicinal plants offer new approaches to tackling diseases (Colalto, 2017). Plant products derived from barks, leaves, flowers, roots, fruits or seeds have been part of phytomedicines from the ancient of days (Ochwang *et al*., 2016).

2.9.1. Description and Antimicrobial Potential of *Nepeta cataria*

Figure 2.4: Nepeta cataria

Nepeta cataria, commonly known as catnip, catswort, and catmint is a species of genus of *Nepeta* in the family Lamiaceace. Catnip shares the same family with mint plant. It is a perennial herb that has square stem, opposite leaf arrangement. It bears many small purple-spotted white or pale lavender tubular flowers, which are tightly clustered at the end of the floral branches (Figure 2.4). The plant can grow up to about 3 feet high and blooms in summer. The leaves are heart-shaped with scalloped edges ranging from grey green to green color and are often crowded toward the top of the plant. The plant can be propagated from seeds or from root divisions (Chin *et al.*, 2005). Commonly known as catnip, plants of the *Lamiaceae* family are important ornamental, medicinal, and aromatic plants, many of which produce essential oils that are used in traditional and modern medicine, and in the food, cosmetics, and pharmaceutical industry. Various species of the genera *Hyssopus*, *Leonurus*, *Mentha*, *Nepeta*, *Origanum*, *Perovskia*, *Phlomis*, *Salvia*, *Scutellaria*, and *Ziziphora* are widespread throughout the world, are the most popular plants in traditional remedies, and are often used for the treatment of wounds, gastritis, infections, dermatitis, bronchitis, and inflammation (Mamadalieva *et al.*, 2017).

Typical member of the mint family of plants, *Nepeta* species are characterized by terpenoid-type compounds and phenolic constituents. The phytochemicals compounds exert several activities such as an antimicrobial, repellent, insecticide, larvicide, cytotoxic anticarcinogen, antioxidant, anticonvulsant, analgesic, anti-inflammatory agent, and antidepressant activities. This therefore reveals its importance in medicinal and agricultural fields. On the basis of numerous studies, the *Nepeta* genus demonstrates remarkable therapeutic effects against various diseases (Süntar *et al.*, 2017). The essential oil 1,8-cineole chemotype and 1,8-cineole in the plant extracted by steam distillation has showed moderate antimicrobial activity and significantly inhibited the activity of acetylcholinesterase enzyme. Cytotoxicity evaluation against three breast cancer cell lines showed a potent inhibitory activity (Kahkeshani *et al.*, 2017). It is supposed that the antigenotoxicity of *Nepeta rtanjensis* methanol extracts was caused by high presence of chlorogenic acid, rosmarinic acid and rutin, all known as efficient antioxidant bioactive compounds (Bosnjak-Neumuller *et al*., 2017). *Nepeta trachonitica* appears to have reasonable antioxidant activity and decent antimicrobial activity as indicated by the inhibition of the organisms' growth. The most susceptible species were *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 11229 among the organisms tested with the inhibition zone of 12.0 ± 1.24 mm. Ethanol extract of the plant has the highest effect on *Saccharomyces cerevisiae* but no effect on *Yarrowia lipolytica.* The HPLC-MS/MS analysis showed that at least 11 major phenolic compounds of *N. trachonitica* exist, the major ones being rosmarinic acid, chlorogenic acid and quinic acid. The obtained results suggest that *N. trachonitica* could be a promising source for food and nutraceutical industries because of its antimicrobial and antioxidant properties and phenolic compounds (Köksal *et al*., 2017).

2.9.2. Description and Antimicrobial Potential of *Basella alba*

Figure 2.5:Basella alba

Basella alba which is a fast-growing, soft-stemmed vine, reaching 10 m in length (Kumar *et al*., 2013). *Basella alba* is an important green leafy vegetable found commonly in the tropical regions of the world. The plant is used as a substitute for true spinach (i.e. *Spinacea oleracea L*.) and has great ethnomedicinal importance. Different studies have proved that the plant is rich in vitamin A, vitamin C, iron and calcium along with flavonoids, saponins, carotenoids, many amino acids and organic acids. It is commonly used from West Kenya (where it is called nderema), through Uganda to the Democratic Republic of Congo (baselle or epinard) and West Africa, where it is occasionally cultivated."(Birkett*et al.*, 2011). Various *in vivo* and *in vitro* studies revealed that the plants are enriched with active substances/principles having medicinal potential. The results from the previous study done by Deshmukh and Gaikwad, (2014) reported that major biological activities are exhibited by *Basella alba*. They included androgenic, antidiabetic, anti-inflammatory, antimicrobial, antioxidant, antiulcer, antiviral, CNS depressant, hepatoprotective and wound healing, properties. Besides these all the plant possesses a valuable ethnomedicinal importance and are used to cure digestive disorders, skin diseases, bleeding piles, pimples, urticaria, irritation, anemia, whooping cough, leprosy, aphthae, insomnia, cancer, gonorrhea, burns, headache, ulcers, diarrhea, liver disorders, bilious vomiting, sexual asthenia (Deshmukh and Gaikwad, 2014). *Basella alba* is an important medicinal plant in ethnoveterinary for treatment of retained afterbirth and anaplasmosis. It is administered in gonorrhea and balanitis. The mucilaginous liquid obtained from the leaves and tender stalks of this plant is a popular remedy for habitual headaches.

A decoction of the leaves is a good laxative for pregnant women and children (Sushila *et al.*, 2010). *Basella alba* (*B. alba*) colorant powder (BACP) has been characterized in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. BACP has been shown to exert potent anti-inflammatory activity by down-regulation of inflammatory mediators including nitric oxide (NO), prostaglandin E2 (PGE2), TNF-α, IL-1β, IL-6 and IL-12 and the blockage of IκB kinase (IKK)/IκB/nuclear factor-κ B (NFκB) activation cascade (Huang *et al.*, 2016). The treatment with *B. alba* extracts significantly lowers the levels of total cholesterol, LDL, and triglycerides and increases HDL and antioxidant enzymes (SOD and GPx) levels. Treatment with *B. alba* extract also significantly suppresses the aortic plaque formation and reduced the intima: media ratio. Previous *in vivo* study done on *B. alba* suggested its potential as an alternative therapeutic agent for hypercholesterolemia and atherosclerosis (Baskaran *et al.*, 2015). The findings of *B. alba* and *C. alba* show beneficial effects of extracts on male fertility, and suggest their protective effect against maneb-induced toxicity in male reproductive function (Manfo *et al.*, 2014).

2.10. Mechanisms of antibiotic resistance in *Acinetobacter baumannii* infections

Before the 1970s, *A. baumannii* infections could be treated with a range of different antibiotics, such as aminoglycosides, β-lactams, and tetracyclines, according to a report in the journal Clinical Microbiology Reviews (Ni *et al*., 2013). Today, however, some strains of *A. baumannii* are resistant to most antibiotics, including first-line antibiotics and carbapenems, which are often used only as a last resort. *A. baumannii* has many resistance mechanisms (including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects, and modifications of target sites). They can be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan drug-resistant (PDR) if resistant to three or more of potentially effective antimicrobial agents (Durante-Mangoni *et al*.,2014).

The accumulation of several resistance mechanisms in *A. baumannii* has gradually decreased the number of antibiotic classes available to treat *A. baumannii* infections in clinical practice (Lee *et al*., 2017). *A. baumannii* is an encapsulated gram-negative coccobacillus containing proteins, namely porins and efflux channels, on the outer cell membrane, which mainly contribute to their resistance mechanisms. However, as compared to other gram-negative bacteria, it has fewer and smaller porin channels, which thereby decrease its cell permeability and increase its antibiotic resistance. It was also discovered that the cell wall of the bacteria changes according to the environmental conditions, thus causing an increase in its thickness when it is placed in a very dry condition, thereby again providing extra resistance at high temperatures also (Singh *et al.*, 2013). Antibiotic resistance in *Acinetobacter spp*., particularly *A. baumannii*, is increasing rapidly. *A. baumannii* possesses two intrinsic b-lactamase genes, in addition to weak permeability and efflux systems, that together confer a natural reduced susceptibility to antibiotics. In addition, numerous acquired mechanisms of resistance have been identified in *A. baumannii*. The very high genetic plasticity of *A. baumannii* allows an accumulation of resistance determinants that give rise to multidrug resistance at an alarming rate (Bonnin *et al.*, 2011). The three main mechanisms of resistance are: enzymes inactivating antibiotics, reduced entry into the target site of bacteria, alteration of the target or cellular functions due to mutations. *A. baumannii* is labelled as MDR-Ab when it is resistant to more than two of the following five classes of antibiotics: Antipseudomonal cephalosporins (ceftazidime or cefepime), Antipseudomonal carbapenems (imipenem or meropenem), Ampicillin/sulbactam, Fluoroquinolones (ciprofloxacin or levofloxacin) and Aminoglycosides (gentamicin, tobramycin, or amikacin) (Singh *et al.*, 2013). *A. baumannii* strains can hydrolyze penicillins (benzylpenicillin, ampicillin, ticarcillin and piperacillin) because they possess an intrinsic class D oxacillinase belonging to the OXA-51- like group of enzymes.

These microorganisms may also break down antipseudomonal penicillins + β-lactamase inhibitors and cephalosporins through an AmpC cephalosporinase, as well as third-generation cephalosporins through the expression of TEM, SHV, CTX-M, GES, SCO, PER and VEB families of class A extended-spectrum β-lactamases (Figure 2.6). Management of *A. baumannii* infections may be very difficult for its unique capacity of acquiring several mechanisms of antibiotic resistance, as outlined above. Thus, (Durante-Mangoni *et al.*, 2014). Presence of resistance genes among in *Acinetobacter* spp. make them more prevalent in healthcare settings. This bacterium is resistant to many available antibiotics because it has been in contact with other gram-negative bacteria in hospital environments and exposed to extensive bombardment with antibiotics, so most strains of *A. baumannii* are resistant to ampicillin, tetracycline, rifampin, amoxicillin/clavulanic acid, macrolides, anti- staphylococcal penicillin, and wide range cephalosporins except for cefepime, ceftazidime, and chloramphenicol. Therefore, it can acquire resistance mechanisms from plasmids, integrons, transposons, and other gram-negatives, in addition to its inherent tendency to acquire resistance (Mohammadi *et al.*, 2017).

2.10.1. AmpC cephalosporinases resistance

The chromosome encoded cephalosporinase (AmpC type) is common to all strains of *A. baumannii*. Insertion sequences (ISs) have been found that increase production of the chromosomal β-lactamase of *A. baumannii* at 1200-bp sequence described by (Bonomo and Szabo, 2006).

2.10.2. Serine and metallo–β-lactamases (carbapenemases)

Carbapenemases are divided into three subclasses based on their hydrolysis characteristics (Frere *et al.*, 2005). The first carbapenemases were described as metalloenzymes and have one zinc atom in the active site. The second form of carbapenemases use serine at the active sites and are inactivated by clavulanic acid and tazobactam (Ehlers *et al.*, 2012).

The most problematic recent occurrence is the emergence of numerous OXA enzymes in *A. baumannii* that confer β-lactam resistance. The first description of a serine carbapenemase in *A. baumannii* was ARI-1 (OXA-23), a clinical isolate from a blood culture at the Royal Infirmary in Edinburgh, Scotland, in 1985. Although OXA carbapenemases may not robustly hydrolyze imipenem, their presence in an organism that may have an IS element that acts as a promoter can result in imipenem resistance (Bonomo and Szabo, 2006).

2.10.3. Other β-lactamases

Other β-lactamases have been reported in *A. baumannii* and they include the TEM-1 type, SHV type, CTX-M type, PER-1, and VEB-1 β-lactamases. Although they are important, but it is difficult to assess their impact on resistance in the presence of the AmpC cephalosporinase (Perez *et al.*, 2007).

2.10.4. Outer membrane protein (OMP [porin]) changes

Because of the reduction of transport into the periplasmic space via changes in porins or OMPs, access to penicillin-binding proteins is reduced. Since the β-lactam entering the periplasmic space is less, the weak enzymatic activity of the β-lactamase is increased. Many outbreaks of infection with imipenem-resistant *A. baumannii* are due to porin loss (Bonomo and Szabo, 2006). In a previous study the reduced expression of 2 porins and the presence of an OXA-derived β-lactamase were responsible for the carbapenem resistance of the epidemic nosocomial imipenem-resistant *A. baumannii* isolates (Bou *et al.*, 2000).

2.10.5. Aminoglycoside-modifying enzymes (AMEs)

AMEs catalyze the modification at −OH or $-NH_2$ groups of the 2-deoxystreptamine nucleus or the sugar moieties and can be acetyltransferases (AACs), nucleotidyltranferases (ANTs), or phosphotransferases (APHs). All 3 types of AMEs: the acetylating, adenylating, and phosphorylating AMEs have been identified in *A. baumannii*.

The combination of mutagenesis, which leads to continuous generation of new enzyme variants with ability to transfer at the molecular level as part of integrons, gene cassettes, transposons, or integrative conjugative elements and at the cellular level through conjugation, as part of mobilizable or conjugative plasmids, natural transformation or transduction results in the ability of this resistance mechanism to reach virtually all bacterial types (Bonomo and Szabo, 2006).

2.10.6. Quinolone resistance

Fluoroquinolones resistance is primarily caused by mutational alterations in target enzymes through stepwise mutations in the quinolone resistance-determining regions (QRDRs) of the DNA gyrase genes (*gyr*A and *gyr*B) and/or topoisomerase IV genes (*par*C and *par*E) (Hamed *et al.*, 2018). Modifications to DNA gyrase or topoisomerase IV through mutations in the *gyr*A and *par*C genes are responsible for quinolone resistance and they have been well described for *A. baumannii*. These mutations interfere with the transcription process by targeting the site binding (Peleg *et al.*, 2008).

2.10.7. Efflux pump antibiotic resistance

The natural role of efflux is to remove chemicals that could potentially disorganize the cytoplasmic membrane; however, from the point of view of antibiotic resistance, efflux pumps have a potent ability to actively expel β-lactams, quinolones, and even aminoglycosides. They usually have 3 components: the pump itself, which lies in the cytoplasmic membrane; an exit portal (porin channels traversing the outer membrane); and a linker lipoprotein between the two. An RND-type efflux pump has been described in *A. baumannii* (Bonomo and Szabo, 2006). From the previous studies, the proportion of this resistance developed by *A. baumannii* to resist to the conventional drugs were re-evaluated.

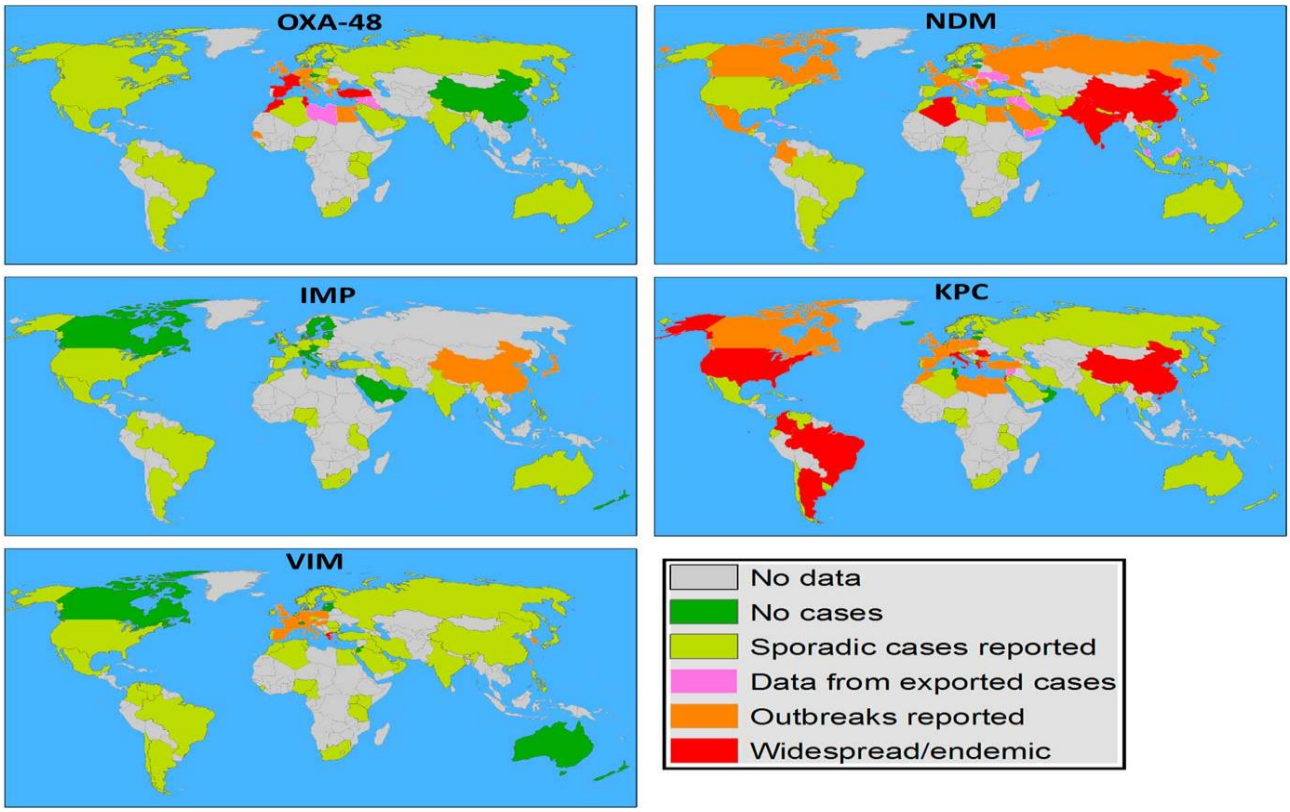

Figure 2.6: *Occurrence and geographic distribution of carbapenemase-producing Enterobacteriaceae (CPE) worldwide by resistance mechanism, based on literature review, 2015.* (Friedman *et al.*, 2017)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Study Site

Figure 3.1: A*: Ol Donyo Sabuk National Park (http://www.safarilodges.com/),* ***B:*** *Jomo Kenyatta University (https://international.uwo.ca/whe/internship/jkuat.html)*

The stocked isolates of initially identified as *A. baumannii* were obtained from Aga Khan University Hospital, a private, not for profit institution established in 1958, that provides tertiary and secondary level health care services in Nairobi. *Nepeta cataria* and *Basella alba* leaves were collected from Ol Donyo Sabuk National Park, near Thika town and Botanical garden JKUAT respectively. The laboratory work was carried out in PAUSTI Molecular Biology and Biotechnology laboratory.

3.2. Material transfer agreement

This study did not involve sampling patients from the hospital directly. All consecutive clinically significant *Acinetobacter* bacterial isolates had been collected and archived in AKUH laboratory. All isolates for use in this study were anonymously transferred, only the source of the samples was recorded.

The permission to move the *Acinetobacter* isolates was obtained through the collaboration with Aga Khan University Hospital (AKUH) Scientific and Ethical Review Committees (Appendices).

3.3. Study Design

This study was a cross sectional prospective laboratory-based study of stocked *A. baumannii* isolates at Aga Khan University Hospital Nairobi and an experimental research which evaluated the qualitative phytochemicals composition of *Nepeta cataria* and *Basella alba* and the assessment of the antimicrobial activity and toxicity of the extracts in PAUSTI Molecular Biology and Biotechnology laboratory.

3.4. Collection and Processing of Plant Materials

The *Nepeta cataria* leaves were harvested from *Ol Donyo Sabuk* National Park near Thika town, Kenya in February 2018. The town is in Kyanzavi Division, Machakos County. *Basella alba* leaves were harvested at Botanical Garden within Jomo Kenyatta University of Agriculture and Technology (JKUAT). The two plant materials were authenticated by the Department of Botany in JKUAT. The plant material were prepared as per the method described by Mboso *et al*. (2014). Collected and transported in the tissue bags, two kilograms of the mature leaves were briefly rinsed in clean water to remove dirt and properly dried under shade for thirty (30) days. The dried leaves were blended to a fine powder using a mortar and pestle.

3.4.1. Methanolic Plant Extraction

The methanol solvent was prepared using analytical grade methanol (Scharlau, GERMANY) and distilled water. The dilution was done in the 2 liters glass flask by mixing 1400 ml of the methanol in 600ml of the distilled water to get 1 liter of the methanol 70 %. Methanolic extraction of powdered leaves was prepared according to the method described by (Suguna *et al.*, 2015). One hundred and fifty grams (150 g) of the plant material was added into 750 ml of methanol 70 % at a ratio of 1:5 (w/v) in a flask and shaken vigorously.

The plant material was soaked in the solvent for 72 hours to allow extraction to take place. The mixture was filtered firstly using a sieve and Whatman No.1 filter paper. The extracts were concentrated at 50ºC using a vacuum rotary evaporator (Labtech EV311, DIAHANLABTECHCO., LTD) for 3 hours then frozen at -80º C until lyophilization. The final extracts obtained were weighed to obtain the percentage yield then stored at 4º C in the refrigerator until use.

3.4.2. Aqueous Plant Extraction

The powdered plant material was prepared according to Agaie and Onyeyili (2011) protocol. 150 g of the powdered leaves were stirred for five minutes into 750 ml of boiled distilled water which had been cooled to 55 ºC at a ratio of 1:5 (w/v) in a flask. The mixture was kept off the hot plate, for 30 minutes to allow it to infuse. The mixture was cooled and filtered using Whatman No.1 filter paper as described by Bamidele *et al.* (2010). The mixture was frozen and dried using a freeze dryer machine (FDL mr c Beijing Province, CHINA). The obtained extracts were weighed, the percentage yield was determined and then stored at 4°C for further analysis.

3.5. Phytochemicals screening

Qualitative screening of the crude extract for the following phytochemicals was done according to the standard protocols described by Mohammed *et al.* (2014) based on the qualitative chemical screening for identification of various classes of active chemical constituents.

3.5.1. Test for tannins

About 0.5 g of each methanol extract was boiled in 20 ml of water in their respective test tubes and then filtered. A few drops of 0.1% ferric chloride was added to each test tube. A brownish green or blue-black coloration indicates the presence of tannins.

3.5.2. Test for Phlobatannins

An aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid. A deposition of a red precipitate indicates the presence of Phlobatannins.

3.5.3. Test for Saponins

50 g of powdered sample to 400 ml of distilled water was added in a conical flask and boiled for 5 min. The mixture was filtered when still hot and 5 ml of sterile distilled water added to a test tube containing equal amounts of cooled filtrate. The test tube was shaken vigorously for 30 seconds and then allowed to stand for 30 min. Formation of honey comb froth indicates the presence of saponins.

3.5.4. Test for Flavonoids

5 ml of dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4. Formation of yellow color indicates the presence of flavonoids.

3.5.5. Test for Steroids

2 ml of acetic anhydride was added to 0.5 g of the methanol extract with 2 ml H_2SO_4. The change in colour from violet to blue or green indicates the presence of steroids.

3.5.6. Test for Essential oils

0.1 ml of 2 M sodium hydroxide was added to 2 ml of the extract, followed by a small quantity of 2 M hydrochloric acid and shaken. A white precipitate indicates the presence of essential oils.

3.5.7. Test for Phenols

2 ml of Iron III chloride was added to 2 ml of the extract. A deep bluish-green solution indicates the presence of phenols.

3.6. Brine Shrimp Lethality Assay

In vivo Brine Shrimp Lethality Assay (BSLA) of the methanolic and aqueous extracts of two plants was determined. Brine shrimp eggs were bought from Aqua-pet shop in Nairobi. They were dissolved in artificial seawater then transferred in to a hatching chamber with a covered and light part for hatching (4 days). After 78 hours, the shrimps were mature as nauplii (*Artemia salina*) and were ready for the assay. The brine shrimp lethality bioassay was carried out on the *Nepeta cataria* and *Basella alba* crude extract using the standard procedure (Manivasagam *et al.*, 2010). Using a magnifying glass and pipette, 10 nauplii were added to the previously labelled test tubes containing 9 ml of salted water and 1 ml of the working plant extract solution at different concentrations (600, 400, and 200 mg/ml) by serial dilution from the stock solution (Sarah *et al.*, 2017). Each concentration was tested in triplicate. Pure methanol was used as a positive control while artificial seawater was used as a negative control (Janackovic *et al.*, 2016). These tubes were incubated uncovered at room temperature for 24 hours. The number of dead nauplii was counted after 24 hours and recorded. (Sarah *et al.*, 2017). The percentage mortality was calculated by dividing the number of dead nauplii by the total number and then multiplied by 100%. (Olowa and Nuñeza, 2013). The toxicity was reported as LC_{50}, representing the concentration in micrograms per microliter that caused 50% larval mortality, respectively, in 24 h (Klocucar *et al.*, 2006).

$$\text{Percentage of Death (\%)} = \frac{(\text{Total naupii} - \text{Alive naupii})}{\text{Total naupii}} \times 100\%$$

3.7. Subculture and Identification of the Bacteria Isolates

In this present study, the bacteria were archived isolates from different clinical sources (Table 3.1). Majority of the bacterial isolates (80 %) were from wound swabs, blood and tissue aspirates). The rest of the isolates were from abscess aspirates, penicle swab, urine and ascites fluid.

The bacteria specimens were inoculated into appropriate media according to standardized protocols. Preliminary identification was done by sub culturing on 5% Sheep Blood agar and Brain Heart Infusion agar. The bacterial were incubated at 37 °C for 24 hours and they appeared colourless and non-haemolytic. Confirmation of the bacteria to species level was done using standardized biochemical reactions (API, bioMérieux SA, Marcy l'Etoile, France) (Pritsch *et al.*, 2017).

Table 3.1*:Clinical sources of Acinetobacter baumannii isolates*

Specimen Source	**Number of Isolates No. (%)**
Wound Swab	9 (30)
Blood	8 (26.7)
Tissue Aspirates	7 (23.3)
Abscess Aspirates	2 (6.7)
Penicle Swab	2 (6.7)
Urine	1 (3.3)
Ascites fluid	1 (3.3)
Total	**30**

3.8. Antimicrobial susceptibility testing of the crude Planty extracts

Fresh and pure colonies of *A. baumannii* were used for the susceptibility testing. Bacteria were sub-cultured on Blood agar and Brain Heart Infusion agar overnight at 37 ˚C. The fresh overnight cultures were diluted into the sterile normal saline to make 0.5 MacFarland standard inoculum. This was then inoculated on Mueller Hinton agar plates to determine the antibacterial activity of *Basella alba* and *Nepeta cataria* extract.

600 mg, 400mg and 200 mg of methanolic and aqueous extracts of *Basella alba* and *Nepeta cataria* were weighed and stored in well labelled sterile centrifuge tubes for further dilution. To obtain the working solution, the methanolic and the aqueous extract were diluted with distilled water (Janackovic *et al.*, 2016). The antimicrobial activity of the plants extracts was determined by well diffusion method described by CLSI, (2017). Using sterile swabs, the bacterial suspension of *A. baumannii* was spread on the plates. Six millimeters well was cut in the plates with the sterile cork borer (Idris *et al.*, 2013). Using pipette, 100 µl of the extract preparation was dispensed into each well at different concentration. The plates were incubated overnight at 37 °C. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the extract was recorded by measuring the zone of inhibition formed around the discs expressed in millimeter (Suguna *et al.*, 2015).

3.9. Determination of the Minimum Inhibitory Concentration

Based on CLSI guidelines, MICs was determined by using the broth microdilution method (CLSI, 2017). 9 ml of the nutrient broth (Oxoid Ltd., Basingstoke, United Kingdom) was dispensed into the test tubes and 1 ml of the stock working solution was added to the first tube and diluted serially to the next tubes up to 10^{-9}. The negative control was prepared without adding the plant extracts while for the positive control, 1 ml 70 % methanol was added. The bacterial suspension was added to all the test tubes followed by overnight incubation at 37 ºC. For the 96 well plates, the serial dilution was performed with 50µl of reverse gradient dilution of the plant extract and 50µl of the test organism suspension as described by (Elshikh *et al.*, 2016). The plates were covered with plate sealing tape and incubated at 37 ºC for 24 hours (Andrews, 2001).

3.10. Antibiotic susceptibility testing

After the sub-culture of *A. baumannii*, the antibiotic susceptibility testing was done for all bacterial isolates using the Kirby-Bauer disc diffusion technique (Bauer, 2017). Antibiotic sensitivity pattern with antibiotic disks (Mast, UK) imipenem (10 µg), ampicillin (10 µg), piperacillin (100 µg), ceftazidime (30 µg), ciprofloxacin (30 µg), amikacin (30 µg), gentamicin (10 µg), and tigecycline (15 µg) were assessed using disc diffusion test (Table 3.2). The bacterial suspension was adjusted to 0.5 McFarland and spread over the Mueller-Hinton agar (MHA) plates using a sterile cotton swab according to the recommendations and definitions of the manufacturers and (CLSI, 2017) guidelines. A pair of forceps was sterilized on fire and used to remove and disposed the antibiotics on the labelled plates. After incubation at 37 °C for 24 h, the diameter of inhibition was measured and interpreted as resistant, intermediate or sensitive for different antibiotic disc used. The MDR *Acinetobacter spp* have defined whether the isolates are resistant to three antibiotics different to ceftazidime, such as ciprofloxacin, gentamicin, and imipenem (Castillo, 2017). The distance of inhibition was measured using a calliper and the result classified as Sensitive, Intermediate or Resistant (Otieno *et al.*, 2018).

***Table 3.2**: List of antibiotics used*

GROUP OF ANTIBIOTICS	**EXAMPLE OF ANTIBIOTIC**
Aminoglycoside	Gentamicin
Carbapenem	Imipenem
Cephalosporin	Ceftazidime,
Glycylcycline	Tigecycline
Penam	Amikacin, Piperacillin
Quinolone	Ciprofloxacin
Tetracycline	Minocycline

3.11. Determination of resistance Genes

DNA extraction and detection of carbapenemase resistant genes was were performed on all the thirty *A. baumannii* isolates. From the Nutrient agar plates, singles colonies were sub-cultured on Nutrient broth at 37ºC. After 24 hours, the inoculum was transferred to 15 ml centrifuge, equilibrated and centrifuge at 11000 times gravity (x g) for 30 seconds at 4 ºC. The supernatant was discarded, and the pellet used for genomic material extraction according to 5min Cell/Virus DNA Extraction kit (Bio Factories, USA) protocol (Appendices). The DNA obtained from fresh culture was stored in the labelled Eppendorf tubes at -20 ºC. Conventional PCR assays were performed for the detection of β-lactamase genes using primers listed in (Table 3.3). PCR was carried out with 1 µl of the template DNA (50 ng), 4µl of the 5x FIREPol® Master Mix (Solis BioDyne,) ready to load, 0.4 µl concentrations of each primer (10 µM) and nuclease-free water up to a total volume of 20 µl. The reaction mixture was prepared in ice to prevent nuclease activity and nonspecific priming. PCR amplification was carried out using gradient melting temperature (± 3) on a ProFlex PCR System (Thermo Fisher Scientific, Waltham, USA) under the conditions shown in (Table 3.3). A negative control (nuclease-free water) was included for all PCR assays and the PCR products obtained at standard conditions as positive control. PCR products were separated by electrophoresis agarose gel. 1.5 % Agarose gel was prepared to contain 5 µl Red fluorescent dye and warmed in the microwave for 1.30 min before poured into the tank with including combs by Cleaver apparatus (Cleaver Scientific Ltd., UK). Once set, the combs were removed and using a micropipette 5 µl of the PCR product were slowly loaded into the wells. A volume of 5 µl of DNA ladder was loaded into the first and last wells as positive control and used as size markers. Mounted into the electrophoresis tank and filled with Tris-base, acetic acid EDTA (TBE) electrophoresis buffer, the separation was done at 60 volts for 1 ½ h. The visualization of the bands was done on a UV trans-illuminator (UVITEC, Cambridge).

***Table 3.3**: Specific carbapenem resistance genes detected by Polymerase Chain Reaction. OXA-23/24(Oxacillinase 23/24), NDM-1 (New Delhi metallo-β-lactamase 1)*

Primers	**Sequences**	**PCR Amplification**	**Size (bp)**
OXA-23	**F**-5'_GAT CGG ATT GGA GAA CCA GA_3´ **R**-5'_ATT TCT GAC CGC ATT TCC AT_3´	95 °C 5 min, 94 °C 1 min, 55 °C 1min, 72 °C 1min 35 cycles, 72 °C 10 min	501
OXA-24	**F**- 5'_ATG AAA AAA TTT ATA CTT CCT ATA TTC AGC_3´ **R**- 5'_TTA AAT GAT TCC AAG ATT TTC TAG C_3´	95 °C 5 min, 94 °C 1 min, 53 °C 1min, 72 °C 1min 35 cycles, 72 °C 10 min	825
NDM	**F**- 5'_ GGT TTG GCG ATC TGG TTT TC _3´ **R**- 5'_ CGG AAT GGC TCA TCA CGA TC _3´	95 °C 5 min, 94 °C 1 min, 61 °C 1min, 72 °C 1min 35 cycles, 72 °C 10 min	621

3.12. Statistical Analysis

The primary data obtained was analysed using Statistical Package for the Social Sciences (SPSS) to determine the significance (P-value) with values given in means ± SD for each measurement. Graphs were established using GraphPad Prism and Microsoft Excel.

3.13. Ethical issues and Permits

This study did not involve directly sampling patients from the hospital. All isolates transferred for use in this study contained only laboratory numbers; no names of patients were contained in these records. Permission to carry out the study was obtained from the collaboration with Aga Khan University Hospital (AKUH).

CHAPTER FOUR

4.0. RESULTS

4.1. Percentage yield of *Nepeta cataria* and *Basella alba* extract

The percentage yield of *Nepeta cataria* from methanolic extraction was (4.03 %) and (3.11 %) from aqueous extraction. However, there was no significant differences (P>0.05) between the plant extract yields from the two solvents (Table 4.1).

***Table 4.1**: Determination of solvent extraction yield of Nepeta cataria extracts*

Solvent used *N. cataria*	Extraction yield %	Mean ± SD	P value
Methanol 70%	4.01	4.03 ± 0.11	0.087
	3.93		
	4.14		
Water	2.33	3.11 ± 0.70	
	3.67		
	3.33		

For *Basella alba*, the percentage yield from methanolic extraction and aqueous extraction was (4.63 %) and (1.93%) respectively (Table 4.2). However, there were significant differences (P< 0.05) between the plant extract yields from the two solvents.

***Table 4.2**: Determination of solvent extraction yield of Basella alba extract*

Solvent used *B. alba*	Extraction yield %	Mean ± SD	P value
Methanol 70%	4.77	4.63 ± 0.21	0.001
	4.39		
	4.73		
Water	2.07	1.93 ± 0.14	
	1.93		
	1.79		

4.3. Phytochemical analysis of *Nepeta cataria* and *Basella alba* extract

The phytochemical screening in the present study showed the presence of various phytochemical constituents from the methanolic and aqueous *Nepeta cataria* and *Basella alba* extracts. From the methanolic extract of *Nepeta cataria*, the phytochemicals present included phenols, tannins, saponins, flavonoids and essential oils while in the aqueous extraction. All the phytochemicals detected in the methanolic extracts were present except flavonoids. *Basella alba* methanolic extract revealed the presence of phenol, phlobatannin, saponin, flavonoid and steroid whereas saponin was the only phytochemical detected in the aqueous plant extract (Table 4.3).

Table 4.3*: Qualitative phytochemicals analysis of Nepeta cataria and Basella alba extracts.*

		Phytochemicals						
Solvent	Plants	Tannins	Saponins	Phenols	Oils	Flavonoids	Steroids	Phlobatanins
70% Methanol	*N. cataria*	+	+	+	+	+	-	-
	B. alba	-	+	+	-	+	+	+
Water	*N. cataria*	+	+	+	+	-	-	-
	B. alba	-	+	-	-	-	-	-

The (+) signify presence of phytochemicals and (-) signify absence of phytochemicals.

4.4. Brine Shrimp Lethality Assay

4.4.1. Hatching Shrimp

In the artificial seawater, 10 % of the brine shrimp eggs hatched in 48 hours. At the optimum period of day 5 the viability of the nauplii reduced and only 45 % were still alive. The percentage of hatched eggs was recorded in (Figure 4.1).

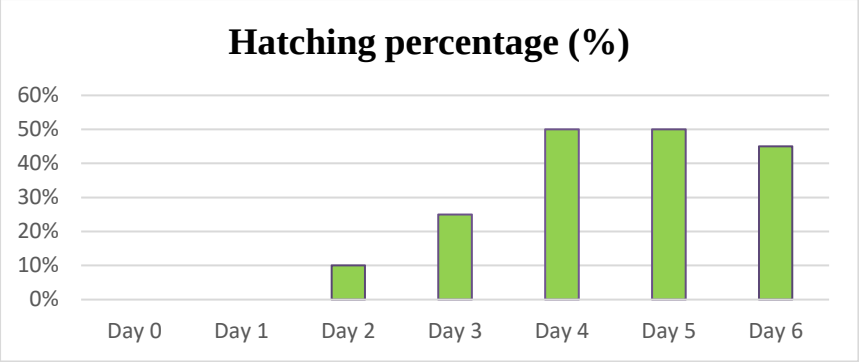

***Figure 4.1**: The percentage estimation of hatching shrimp based on days*

4.3.2. Toxicity testing

After 78 hours, 10 nauplii were inoculated in the five (05) test tubes containing a solution of 600 mg/ml, 400 mg/ml, 200mg/ml, distilled water and methanol in triplicate. After 24 hours, the number of larvae was counted. The death percentage and lethal concentration (LC_{50}) was determined using statistical analysis (Klocucar *et al.*, 2006).

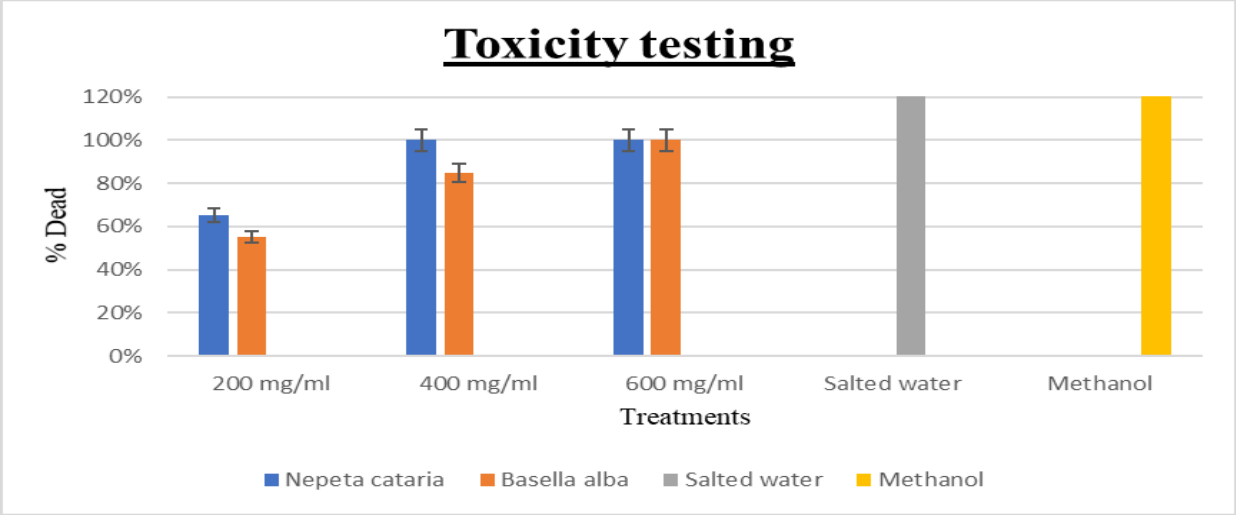

***Figure 4.2**: Toxicity testing of the plant extracts using brine shrimp eggs*

All the hatched eggs died in the positive control and the methanolic *Nepeta cataria* and *Basella alba* extracts. The methanolic extracts of *Nepeta cataria* and *Basella alba* were responsible for 100 % of nauplii at 600 mg/ml, 400 mg/ml and 200 mg/ml. 65 % of death was recorded for *Nepeta cataria* aqueous extract at 200 mg/ml and 100 % for 400 and 600 mg/ml.

For aqueous *Basella alba*, it was 55 % for 200 mg/ml; 85 % for 400 mg/ml and 100 % for the 600 mg/ml. The concentration of the aqueous extracts that kills 50% of the test nauplii during the observation period the LC50 was less than 200 mg/ml (Figure 4.2).

4.5. Subculture and Identification of *Acinetobacter baumannii*

On Sheep Blood agar, *A. baumannii* were recognisable by their smooth colony morphology, were non-haemolytic and were colourless. And on the Brain Heart Infusion, the isolates were creamy colour colonies (Plate 4.3).

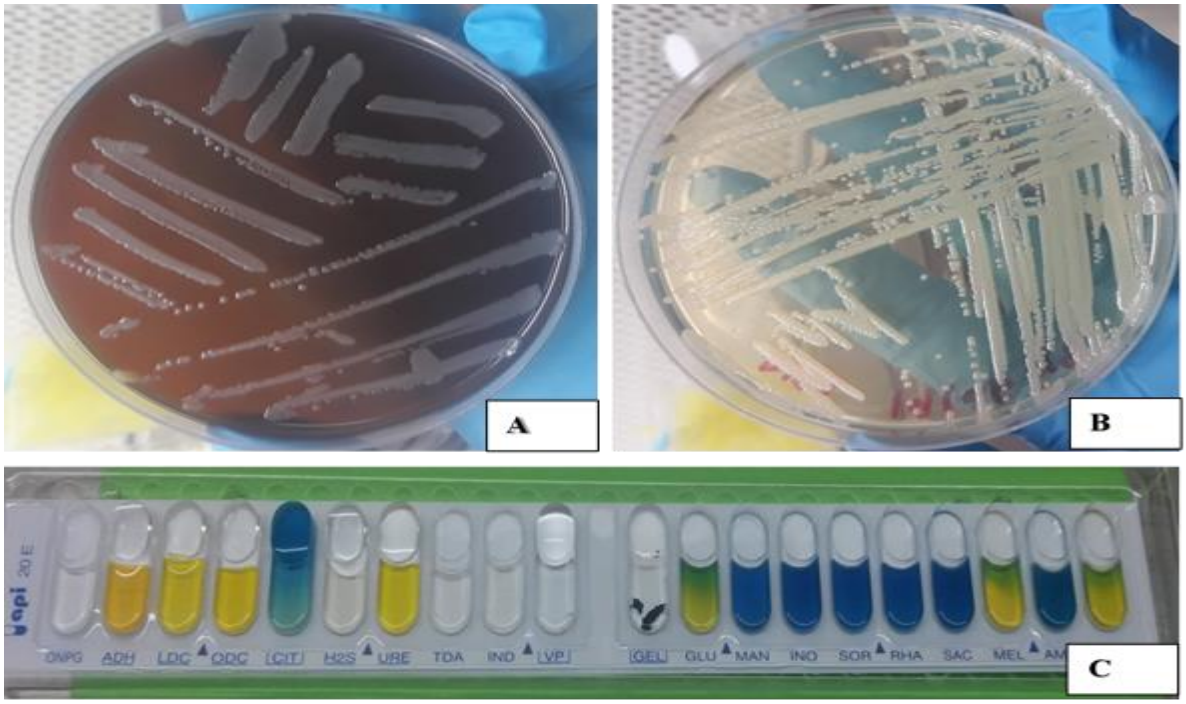

Plate 4.1*: Acinetobacter baumannii isolates sub-cultured on Blood agar (A), Brain Heart Infusion agar (B) and identification using Gallery API 20E kit.*

Using fresh single colonies, the identification kit used to confirm *A. baumannii* isolates were recorded and the results generated a code that can be recorded using the identification chart supplied by the manufacturer (Table 4.4). The results from the API 20E test showed that all the 30 isolates were identified as *A. baumannii*.

***Table 4.4**: Reaction results based on colours changes using API 20E*

Reagents	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
Reaction after 24 hrs	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+

The (+) signify colour changes and (-) signify no colour changes.

4.6. *In vitro* antimicrobial activity of the crude Planty extract

All the three concentrations (600 mg/ml, 400 mg/ml and 200mg/ml) of methanolic plant extract of the two plants demonstrated higher antimicrobial activity compared to the aqueous extracts. Methanolic *Nepeta cataria* extract showed greater antimicrobial activity than those of *Basella alba* extract at the same concentrations (Plate 4.1).

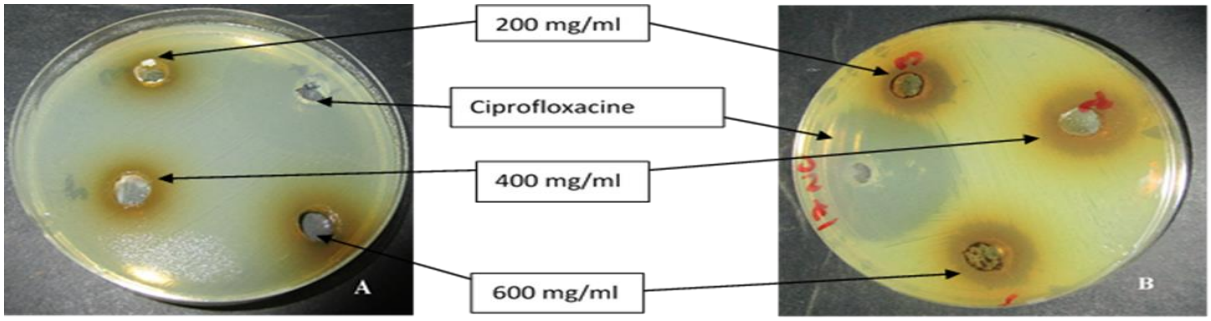

***Plate 4.2**: Antimicrobial activity of methanolic extract of Basella alba (A) and Nepeta cataria (B) against Acinetobacter baumannii.*

From the aqueous extract preparation, *Nepeta cataria* shown greater antimicrobial activity at 600 mg/ml (30 %) and 400 mg/ml (3.33 %). However, no zone of inhibition was recorded at 600 mg/ml, 400 mg/ml and 200mg/ml for *Basella alba* aqueous extracts (Plate 4.2).

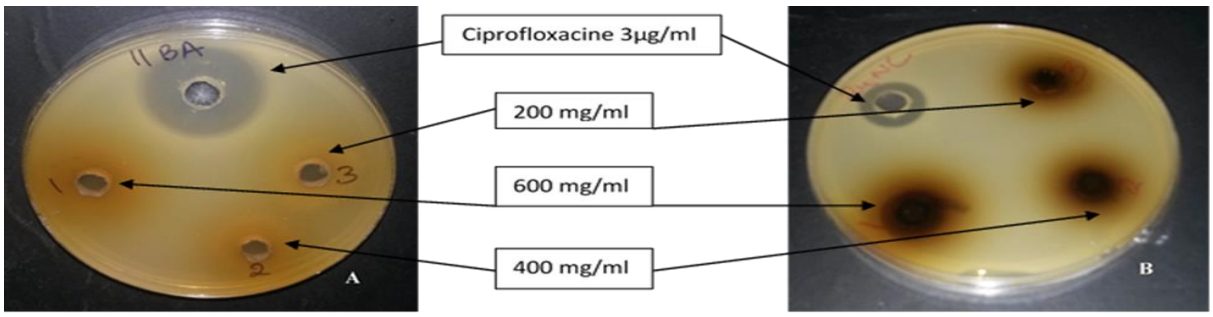

***Plate 4.3**: Antimicrobial activity of aqueous extract of Basella alba (A) and Nepeta cataria (B) against Acinetobacter baumannii.*

Methanolic plant extracts demonstrated higher antimicrobial activity compared to aqueous extracts in the two plants used in this study.

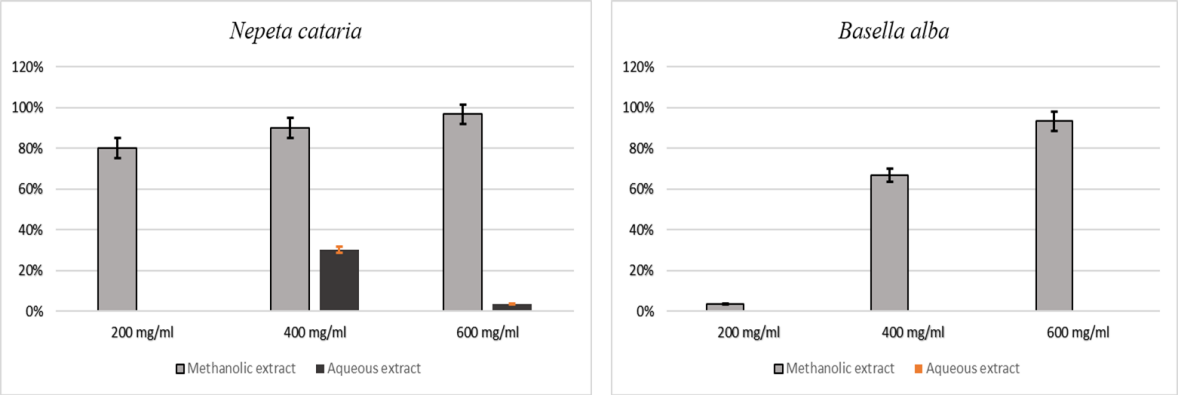

***Figure 4.3**: The percentage of sensitivity (response) of methanolic and aqueous extracts of Nepeta cataria and Basella alba*

4.7. Determination of the Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration of *Nepeta cataria* and *Basella alba* crude extracts were recorded based on the bacterial growth and only in the first tube where there was not bacterial growth at the concentration of 60 mg/ml for *Nepeta cataria* and *Basella alba* (Plate 4.5).

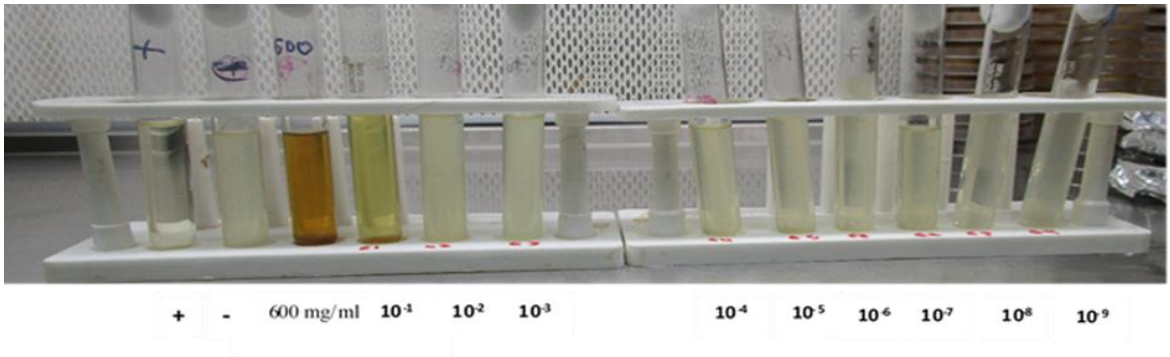

***Plate 4.4:** Determination of MIC using macro-dilution (test tubes)*

It was reported in the present study that this Minimum Inhibitory Concentration was 60 mg/ml using the 96 wells plate (Plate 4.6).

Plate 4.5: *Determination of MIC using micro two-fold dilution (96-wells)*

4.8. Antibiotic susceptibility testing

The antibiotic susceptibility testing was performed according to the CLSI recommendations on the thirty *A. baumannii* isolates plates shown in (Plate 4.4).

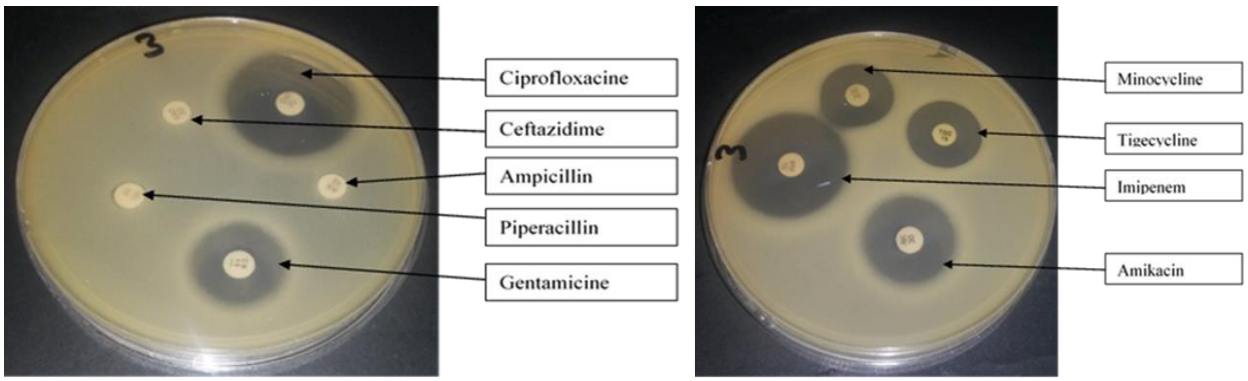

Plate 4.6: *The antibiotic susceptibility testing of Acinetobacter baumannii on Mueller-Hinton plate*

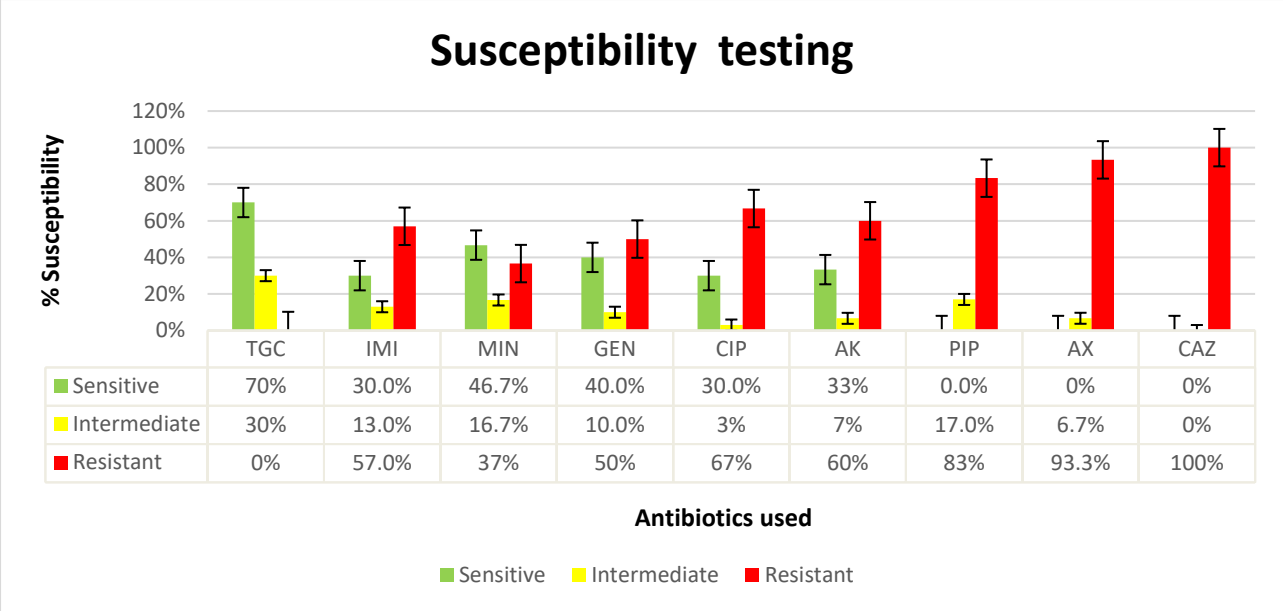

Figure 4.4: *The antibiotic susceptibility testing of Acinetobacter baumannii isolates based on the zone of inhibition of each drug used*

The antibiotic resistance profile of *A. baumannii* isolates was determined using nine (9) antibiotics based on the diameter of inhibition that classified an antibiotic as sensitive, resistant or intermediate. This revealed that the isolated strains were most commonly resistant to ceftazidime (100%) and ampicillin (93.3%), followed by piperacillin (83%), ciprofloxacin (67 %), amikacin (60 %), imipenem (56.6 %), gentamicin (50 %) and minocycline (37 %). However, *A. baumannii* isolates were sensitive at 70 % and intermediate at 30 % to tigecycline (Figure 4.4).

4.9. Determination of genes of resistance

In accordance with the DNA Extraction kit procedure, the results confirmed the presence of DNA from the 30 isolates used in this study with detection of bands viewed under UV light (Plate 4.7). PCR experiments were carried out using specific primers for the genes encoding D β-lactamase (OXA-23-like, OXA-24-like, NDM-1-like) as described previously from whole-cell DNA.

According to our findings, the amplification of the resistance genes was positive for OXA-23, OXA-24 and NDM-1 at 100 %, 10 %, 17 % respectively among the 30 MDR isolates of *A. baumannii* (Plate 4.8).

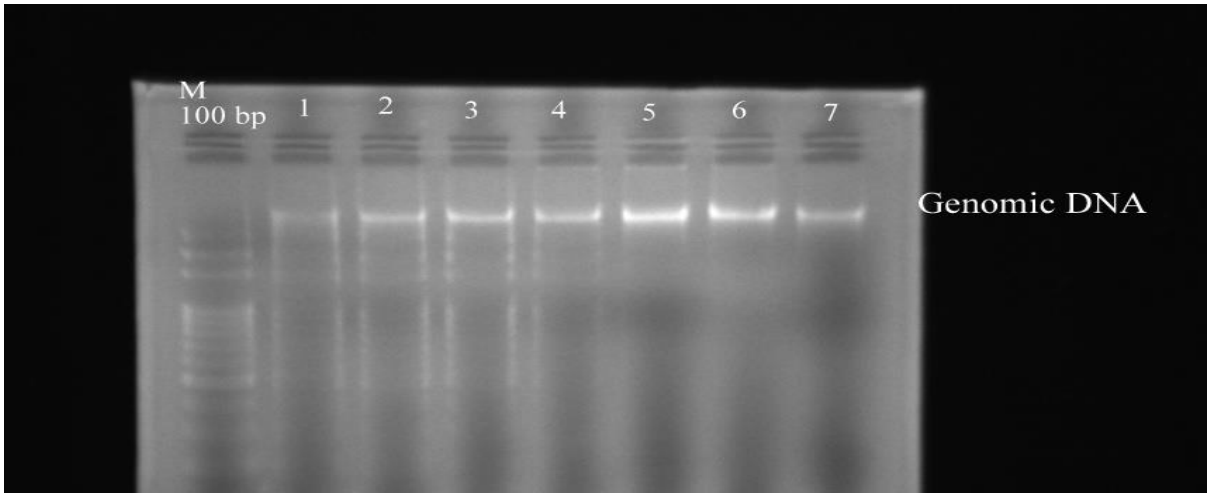

Plate 4.7*: Gel image of representative DNA products from clinically strains of A. baumannii isolates. M – Molecular ladder of 100 bp 1,2,3… samples*

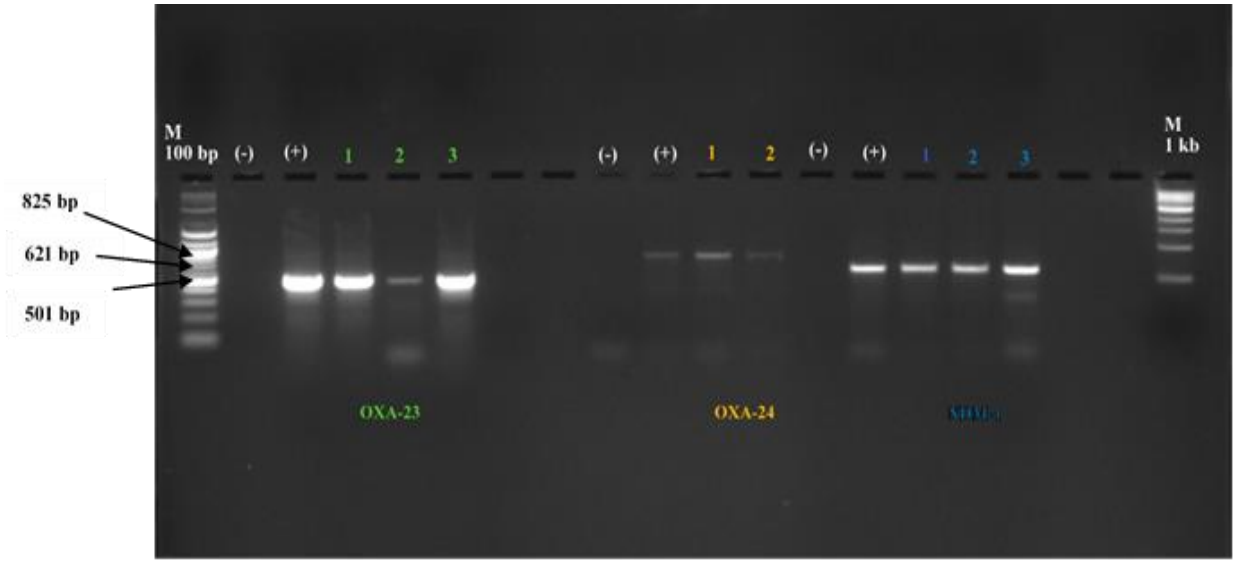

Plate 4.8: *Characterisation of carbapenemase genes.Gel Electrophoresis of Acinetobacter baumannii samples. M – Molecular ladder of 100 bp; Green: OXA-23, Yellow: OXA-24 and Blue: NDM-1; (-) negative control and (+) positive control.*

CHAPTER FIVE

5.0. DISCUSSION, CONCLUSION AND RECOMMANDATIONS

5.1. DISCUSSION

5.1.1. Percentage yield of *Nepeta cataria* and *Basella alba* extract

From traditional knowledge, plants have been used since time immemorial for their antimicrobial traits shown by the various secondary metabolites (phytochemicals) synthesized and deposited in specific parts or all parts of plant. The present study is one of the few studies that has evaluated the antimicrobial activity of the *Nepeta cataria* and *Basella alba* leaves and the findings were that methanolic extract of the two plants had greater activity than the aqueous extract. On the same way, a previous study reported methanol to be the most effective solvent enabling maximum separation of the different phytochemicals, and preliminary analysis of the extracts revealed the presence of secondary metabolites in leaves of *Basella alba* (Suguna *et al.*, 2015; Edewor *et al.*, 2011). In 2010 Das *et al.*, reported that the choice will also depend on the targeted compounds to be extracted. Initial screening of plants for possible antimicrobial activities typically begins by using the crude or alcohol extractions and can be followed by various organic solvent extraction methods. Using different parts of the plant, the study carried out by Adegoke and Ojo, (2017) reported that the leaf fraction of *Basella alba* had the highest percentage yield (24.30%) than *Basella alba* fruit fraction with a yield of 11.13% with methanol used as extraction solvent. Water is a universal solvent, used to extract plant products with antimicrobial activity. Clarkson *et al.*, (2004) reported that this inactivity of aqueous extracts may have been because the crude extracts were not prepared according to the traditional methods, which in some cases involved boiling for several hours. After obtaining the extracts in the crude form, the percentage yield of the extracts was calculated and the results showed that maximum percent yield is obtained in methanol extract (85.36%), followed by distilled water (78%), ethyl acetate (62.44%) and acetone (62.48%) (Dhawan and Gupta, 2016).

The results obtained by Iloki-Assanga *et al.* (2015) proved that methanol was the most effective solvent for isolation of phenolic compounds, whereas much lower yields were obtained from samples extracted with acetone.

5.1.2. Phytochemical analysis of *Nepeta cataria* and *Basella alba* extract

The phytochemical screening in the present study showed the presence of various phytochemical constituents from the methanolic than the aqueous extracts of *Nepeta cataria* and *Basella alba* (saponins, tannins phenols…). A good solvent is characterized by its optimal extraction and its capacity in conserving the stability of the chemical structure of desired compounds (Thouri *et al.*, 2017). Previous studies showed presence of flavonoid, phenol, steroids and essential oils in methanolic extract of *Nepeta cataria* (Adiguzel *et al.*, 2009; Edewor, T. I. and Usman, 2011; Süntar *et al.*, 2017). Phenols and flavonoids have previously been detected in aqueous extracts of *Nepeta cataria* (Adegoke and Ojo, 2017; Deshmukh and Gaikwad, 2014). Other studies report presence of phenols, saponins, tannins and oils from *Nepeta cataria* leaf methanolic extracts whereas tannins, oils and phenols were detected in aqueous extracts (Kumar *et al.*, 2013; Nostro *et al.*, 2000; Seladji *et al.*, 2014). A previous study done by Ta *et al.*, (2014) reported the presence of tannins, saponin, glycoside, flavonoids, alkaloids and phenols from the ethanolic and aqueous *Basella alba* extracts. The result of the crude methanolic extracts of *Basella alba* indicated the presence of tannin, terpene, steroids, saponins, anthraquinone, in the leaf and stem extracts of the plant, and carbohydrate in the stem (Oyewole and Owolabi, 2012). Baskaran *et al.*, (2015) carried out the phytochemical analysis using freeze–dried methanolic *Basella alba* extract revealed qualitatively presence for flavonoids, phenolics, saponins, tannins, alkaloids, triterpenes, and steroids. However, this results contradicts Azad *et al.*, (2013) findings on the presence of flavonoids and tannins from aqueous *Basella alba* extract.

The findings in this study agree with earlier study which also found that not all phytochemicals are present in all plant parts and that those present differ according to the type of the extracting solvent used (Suganthi and Tamilarasi, 2015).

5.1.3. Toxicity testing

Using methanolic *Nepeta cataria* and *Basella alba* extract, all the hatched eggs were dead almost in the same way as the positive control. From the plant materials, *Basella alba* showed the lowest rate of toxicity than *Nepeta cataria* whether 70 % methanol or water were used. 65 % of death was recorded for *Nepeta cataria* aqueous extract at 200 mg/ml and 100 % for 400 and 600 mg/ml. For aqueous *Basella alba*, it was 55 % for 200 mg/ml; 85 % for 400 mg/ml and 100 % for the 600 mg/ml. It could be explained by the fact that using water, active compounds were not easily extracted from *Basella alba* than *Nepeta cataria*. As described *Basella alba* is a vegetable popular in India and Africa due to the nutritional properties. The concentration of the extracts that kills 50% of the test nauplii during the observation period from this study reported that the LC_{50} value for the aqueous extract of the two plants was less than 200 mg/ml. Although, there was non-significant difference between the aqueous extracts and the sea water, the aqueous extract had the least mortality of 25 % while the organic extraction had the highest mortality of 100 %. The explanation could be based on the fact that most traditional herbal medicines are prepared using water as a solvent because it is not or less toxic as reported in previous study (Ohikhena *et al.*, 2016). Other authors reported from their study that methanolic extract of *Basella alba* leaf did not cause liver and muscle damage indicating that it is safe for consumption and no clinical sign or toxic effect was observed in live pups throughout the study (Nantia e*t al*., 2012; Baskaran *et al.*, 2015). The phonological age of the plant, percentage humidity of the harvested material, geographical location, climatic conditions, soil condition, time of harvest, and the method of extraction are possible sources of variation for the chemical composition, toxicity and bioactivity of the extracts (Khan *et al.*, 2011).

From the results recorded by Innocent *et al.*, the brine shrimp test results indicate that the plant extracts tested had LC_{50} values above 100 µg/ml which suggests that they are practically non-toxic. As traditional medicines, most of the extracts are prepared as decoctions, which, in a way is mirrored on the ethanol extracts, the results of which suggest that the way they are used poses no threat of acute toxicity.

5.1.4. *In vitro* antimicrobial activity of the crude Planty extract

From the two solvents used for plant material extraction, the methanolic extract showed a highest antimicrobial activity against *A. baumannii*. Crude extract of methanolic *Nepeta cataria* exhibited an antimicrobial activity by presenting a zone of inhibition around the wells at 600, 400 and 200 mg/ml. From the aqueous extract, the zone of inhibition at 600 mg/ml was only recorded for *Nepeta cataria*. On the basis of the results reported by it was concluded that the methanolic extract of Basella alba leaves had an activity with an inhibition zone at 10 ± 0.53mm. The results obtained from other studies revealed that the aqueous extracts of *Nepeta cataria* possessed potential antibacterial activity. Results obtained in an earlier study revealed that the aqueous extracts of *Nepeta cataria* possessed potential antibacterial activity as compared to its antifungal activity (Bandh *et al.,* 2011). This study agrees with previously studies done which reported that methanolic extract showed antimicrobial activity against all the test microorganisms except for *S. aureus* and *S. typhi* (Bandh *et al.,* 2011; Khan *et al.,* 2011). The antimicrobial activity observed for *Nepeta cataria* in the study done by Adiguzel *et al.*, (2009) was attributed to the presence of main components in the essential oil. For *Basella alba* aqueous extracts tested at 600, 400, 200 mg/ml concentration, no zone of inhibition was recorded against *A. baumannii* contrary to the methanolic extract which showed the greater activity at 200 and 400 mg/ml. Kumar *et al.*, (2013) reported a significant growth inhibition on human cancer cell lines and momentous zone of inhibition for microorganisms studied using the methanolic extract from *Basella alba* leaves.

The methanolic extracts exhibited marked antimicrobial activity against gram positive and gram-negative bacteria and fungi. *Basella alba* showed good inhibitory activity against *A. niger*. This mild activity of the aqueous was also reported in another study (Adhikari *et al.*, 2012). Interestingly, as the findings of this current study the results reported by Toyang *et al.*, (2012) that the solvents dichloromethane and methanol extracted compounds showed the highest degree of activity against seven microbial strains than aqueous extracts.

5.1.5. Determination of the minimum inhibitory concentration (MIC)

The values of MIC varied with plant samples from 10^{-1} to 10^{-9} mg/ml. From the macro dilution using test tubes, there was bacterial growth in all the tubes inoculated. Using the 96 well plates for the methanolic extract of both plants, the screening was revealed that *Nepeta cataria* and *Basella alba* methanolic extracts were the most sensitive to *A. baumannii* at the lower MIC value of 60 mg/ml where there was not bacterial growth. The antibacterial screening of the *Nepeta cataria* showed that methanolic extracts exhibited an antibacterial activity against *S. aureus*, *K. pneumoniae* and *S. typhii* with MIC of 0.1 mg/ml and inactivity against *P.s mirabilis*, *S. dysenteriae*, *E. coli* and *P. aeruginosa* (Edewor *et al.*, 2011). Moreover, the results obtained by different authors vary widely because of that there are no standard criteria for the evaluation of the plant activity. The MIC and MBC were 50mg/ml for *P. aeruginosa* and *E. coli* of *Basella alba* of both the leaf and stem (Oyewole *et al.*, 2012). The essential oils of *Nepeta cataria* inhibited the growth of the examined bacteria at concentrations of 0.125-2 µL/ml (Zomorodian *et al.*, 2013). However, only few literatures are available on the evaluation of the aqueous and methanolic activity of the *Nepeta cataria* and *Basella alba* extract, it is difficult to give a good comparison of this data with some variables such as environmental and climatic conditions of the plant and the method of extraction and antimicrobial screening.

5.1.6. *Acinetobacter baumannii* sub-culture and identification

From the study carried out by Maina *et al.*, (2014) using API20E did perform reasonably well in the laboratory giving an exact profile in 87.5% of the isolates tested, a nearest profile in 12%, and <0.5% having no profile. The API20E system is still useful for identifying certain organisms that may not have a panel in the automated system (Maina *et al.*, 2014; Kilic *et al.*, 2008).

5.1.7. Antibiotic susceptibility testing

*Acinetobacte*r infection prevalence is variable depending on the geographical localization and the patient's socio-economic status. Today, however, some strains of *Acinetobacter baumannii* are resistant to most antibiotics, including first-line antibiotics and carbapenems, which are often used only as a last resort. *A. baumannii* has many resistance mechanisms. The antibiotic resistance profile of *Acinetobacter* isolates was determined using nine (9) antibiotics, and this revealed that the isolated strains were most commonly resistant to ceftazidime (100%) and ampicillin (93.3%), followed by piperacillin (83%), ciprofloxacin (67 %), amikacin (60 %), imipenem (56.6 %), gentamicin (50 %) and minocycline (37 %). On the other hand, there was no resistant recorded using tigecycline with 20 % intermediate sensitivity for all the 30 isolates. As for the study done by Nandekar *et al.,* (2018), only one *A. baumannii* isolate was resistant to tigecycline, whereas 33.33% of the isolates were intermediate to tigecycline. A previous study results recorded the sensitivity rates for netilmicin, tigecycline, sulbactam, amikacin, and meropenem at 66.6%, 50%, 36.6%, 30% and 10%, respectively (Altun *et al.*, 2014). On the same way, the study carried out by Chen *et al*.(2017) reported that *A. baumannii* was more sensitive to the tigecycline and colistin groups compared with the six other groups of antibiotics. All the *A. baumannii* strains were sensitive to colistin, and only a small fraction was resistant to tigecycline. However, nearly 26.7% of the strains demonstrated intermediate resistance to the glycylcycline antibiotic tigecycline.

The most active compounds against these isolates were polymyxin B (99.3% susceptible) and tigecycline (99.0% susceptible). Overall, the isolates showed approximately 52 % susceptibility to imipenem and meropenem; these antimicrobials were followed in coverage by tobramycin (43.9% susceptible) (Gur *et al.*, 2018). The incidence of *A. baumannii* strains resistant to carbapenems was 76.8%. Resistance to the other tested antimicrobials was as follows: ampicillin/sulbactam, 60.7%; cefepime, 96.4%; quinolones, 91.1%; amikacin, 21.4%; polymyxin B, 8.9%; and tigecycline, 7.1% was reported.(Godoy *et al*., 2017). Recent studies have indicated that, the resistance to imipenem was 76.19% throughout the hospital and 87.7% in the ICUs (Uwingabiye *et al.,* 2016). This rate is lower than that noted in India, where the resistance to imipenem reached 89.6 % (Jaggi *et al.,* 2012) and higher than those of previous studies in Morocco: 23.8 % (Elouennas *et al.*, 2003), 42.6% (Lahsoune *et al.,* 2007) and those obtained in the United States, where the resistance to imipenem was 51% (Queenan *et al.*, 2012). In addition, the susceptibility of *A. baumannii* to carbapenems is < 37.0% and its susceptibility to minocycline is 47.8%, while the incidence of extensively drug-resistant *A. baumannii* is 60.1% (Xiao *et al.,* 2017). In another study Farivar AS *et al.*, (2006) results showed that the isolated bacteria had 100% resistance to tetracyclin, 95.2% to gentamicin, amikacin, and 90.5% to ceftazidime hence contradicting the present study which found that *A. baumannii* isolates were resistant to gentamicine at 50 % but 100 % to ceftazidime.

5.1.8. Determination of genes of resistance

The different results from the previous studies can be attributed to assessment of different hospitals all over the world that show numerous geographical differences have been observed in molecular epidemiology of carbapenemase genes. According to the findings of the present study, the amplification of the resistance genes OXA-23, OXA-24 and NDM-1 were 100 %, 10 %, 17 % respectively among the 30 MDR isolates of *A. baumannii*. Only one isolate harbouring *bla*NDM-1 was detected from the first report done in East Africa (Revathi *et al.*, 2013).

From their study Godoy *et al.*, (2017) also recorded that most of the isolates were OXA23 positive and MDR. The results of the present study agree with earlier study carried out by Joshi *et al*., (2017) which also found that the *bla*OXA-23 was present in all isolates but others class D β-lactamase genes, including *bla*OXA-24 and *bla*OXA-58, markers of carbapenem resistance in *A. baumannii*, were not detected in analyzed isolates. In the same way, previous studies had reported similar findings concerning *bla*OXA-23 *bla*OXA-24 genes (Ning *et al.*, 2017; Rolain *et al.*, 2016; Shamsizadeh *et al.*, 2017). The *bla*OXA-23 gene has been disseminated worldwide, recent reports revealed that the frequency of OXA-23-producing *A. baumannii* strains is significantly high with resistant to carbapenems, OXA-23 β-lactamases mostly found in the isolates (Al-Agamy e*t al.*, 2017; Al Atrouni *et al.*, 2016; Mugnier *et al.*, 2010). The current study reported five isolates of *A. baumannii* harbouring the *bla*NDM-1 gene while within one hospital in Ethiopia Pritsch *et al.*, (2017) had detected three from the isolates of *A. baumannii*. Recently in Turkey, Heydari, *et al.*, (2018) detected from tracheal aspirate and respiratory sample two isolates that were *bla*NDM-1 positive while in our study all the isolates positive to *bla*NDM-1 were isolated from wound swab samples. However, previous study done in Qatar by (Rolain *et al.*, 2016) did not detected New Delhi metallo-β-lactamase (NDM) in the studied isolates even though Qatar's population structure is composed of different nationalities, including many expatriates coming from the Indian subcontinent. In addition, a 2017 study done Cairo, Egypt showed that out of 56 *A. baumannii* isolates, *bla*NDM-1 was identified in 13 (59.1%) isolates (Gomaa *et al.*, 2017).

5.2. CONCLUSION

Based on the general objective of this study which was to Evaluate the potential antimicrobial activity of those two plants, the following conclusions can be made:

1. Many active compounds were extracted from methanolic extracts. The *in vitro* antimicrobial susceptibility testing was greater using methanolic extract than aqueous with MIC was 60 mg/ml and a LC_{50} less than 200 mg/ml
2. The susceptibility of *A. baumannii* to the *Nepeta cataria* and *Basella alba* extracts using nine (9) conventional drugs recorded that the isolates were sensitive at 70 % to tigecycline.
3. Results of the amplification of *bla*OXA-23like, *bla*OXA-24like and *bla*NDM-like genes using PCR were 100 %, 10 %, 17 % respectively among the 30 MDR isolates of *A. baumannii* used in this study.

5.3. RECOMMANDATIONS

From the findings of this present study, the following recommendations will be suggested for eventual future studies for the extensive use of *Nepeta cataria* and *Basella alba* to overcome the antibiotic resistance:

1. Screening the extracts of *Nepeta cataria* and *Basella alba* should be used against more other organisms
2. Further evaluation for *in vivo* toxicity can be carried out of the most active crude extracts
3. The phytochemical screening of the extracts should be investigated for pharmacological studies.

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APPENDICES

MEDIA PREPARATION

Blood agar

1. Suspend 28g of the media in 1 litre of distilled water.

2. Heat to boiling to dissolve the media completely

3. Sterilize by autoclaving at 15 Ibs pressure (121ºC) for 15 minutes.

4. Cool the media to 45-50ºC then dispense.

Brain Heart Infusion agar

1. Suspend 52g of the media in 1 litre of distilled water.

2. Heat to boiling to dissolve the media completely

3. Sterilize by autoclaving at 15 Ibs pressure (121ºC) for 15 minutes.

4. Cool the media to 45-50ºC then dispense.

Nutrient Agar

1. Suspend 28g of the media in 1 litre of distilled water.

2. Heat to boiling to dissolve the media completely

3. Sterilize by autoclaving at 15 Ibs pressure (121ºC) for 15 minutes.

4. Cool the media to 45-50ºC then dispense.

Nutrient Broth

Typical Formula: ‘Lab – lemco’ powder 1.0, Yeast Extract 2.0, Peptone 5.0, Sodium chloride 5.0

1. Dissolve 13g of the broth in to one litre of distilled water
2. Mix well then distribute in to the final containers
3. Sterilize by autoclaving at 121^0c for 15 minutes

Mueller-Hinton agar

1. Suspend 28g of the media in 1 litre of distilled water.
2. Heat to boiling to dissolve the media completely
3. Sterilize by autoclaving at 15 Ibs pressure (121ºC) for 15 minutes.
4. Cool the media to 45-50ºC then dispense.

1.5 % Agarose gel

1. Suspend 1.5 g of the media in 100 ml of distilled water.
2. 5 µl of the Red fluorescent dye was added
3. Warm in the microwave for 1 minute 30 seconds.
4. Cool to 45-50ºC then dispense into the tank

DNA extraction using 5min Cell/Virus DNA Extraction Kit

Lysate preparation

1. Transfer cell in culture media to a microtube and centrifuge for 30 seconds.
2. Remove supernatant with pipetting or gentle vacuum.
3. Closed the cap and loosened the pellet by scraping the tube for 5-6 times over an uneven surface such as a microcentrifuge tube rack.
4. Add 400 ul of Cell/Virus DNA solution and vortex for 30 seconds.

Procedure

- All centrifugation in 13,500 rpm at ambient temperature.

1. Transfer all lysate to column
2. Add 350 ul of 96-100% isopropanol to column. Close the cap and vortex for 10 seconds.
3. Centrifuge for 15 seconds or more as required.
4. Discard the flow through. Reassemble the spin column with its collection tube.
5. Apply 700 ul of DNA Washing Solution I and centrifuge for 15 seconds. Discard the flow through.
6. Apply 400 ul of DNA Washing Solution II and centrifuge for 30 seconds. Discard the flow through.
7. Replace the collection tube with a clean microcentrifuge tube.
8. Add 100 ul of DNA Elution Buffer to column.
9. Close the cap and vortex for 15 seconds.
10. Centrifuge for 1 minute for elution

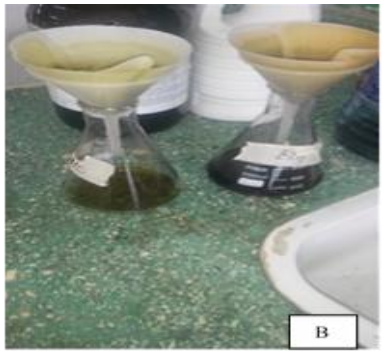
FIGURES

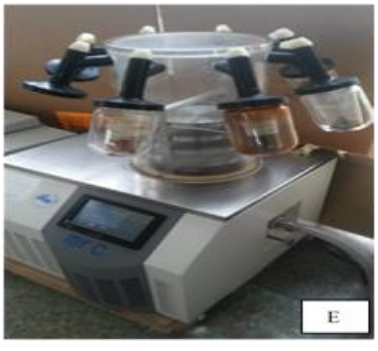
Nepeta cataria

Appendices 1: Nepeta cataria plant in the nature and powdered leaves

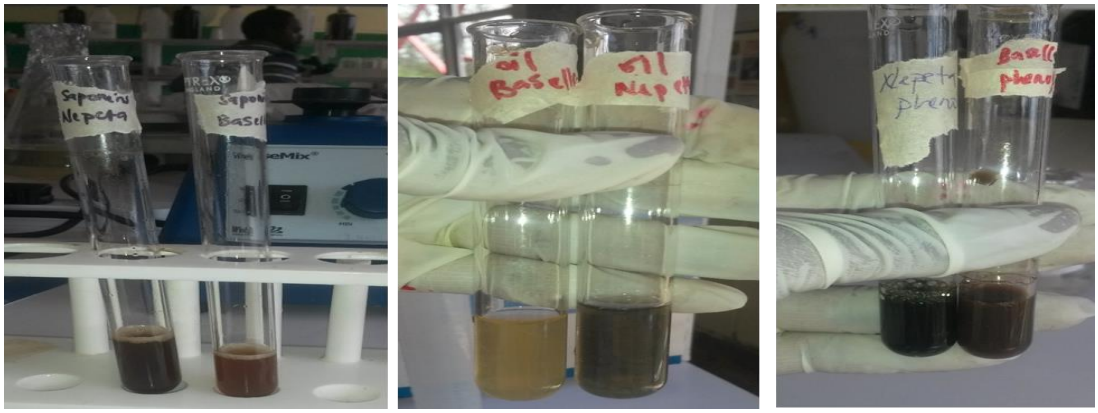
Basella alba

Appendices 2: Basella alba plant in the nature and powdered leaves





Appendices 3: Steps of plant material extraction



Appendices 4: Phytochemical screening from methanolic (black) and aqueous (red) extracts of Nepeta cataria and Basella alba