# EVALUATION OF ANTIBACTERIAL ACTIVITY OF PHYT$^{EXPONENT}$ PREPARATION ON SELECTED PRIORITY BACTERIAL STRAINS AT HIGHER CONCENTRATIONS.

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## DECLARATION

I declare that this research project is my original work and to the best of my knowledge it has never been presented in any academic institution in award of degree or any form of qualification whatsoever. Where work of other people has been included, relevant acknowledgement to that work has been accorded to the text and reference quoted.

**Sign…….** **Date……**

**Terry Wangui Kuria**

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**Supervisors' approval**

This research project has been submitted for examination with my approval as university supervisor

**Sign……..** **Date……..**

**Dr. Epaphrodite Twahirwa (MKU)**

## DEDICATION

I dedicate this study to my dear parents, Dr. Fred and Nelly Maina, my sister Anne Omoko and my brother Daniel Omoko for their encouragement and unwavering support.

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I am grateful to God for the gift of life and good health, His guidance throughout my life and this study and the gift of wisdom and understanding throughout the course of my 5 year degree program and the research project.

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## OPERATIONAL DEFINITION OF TERMS

**WHO:** World Health Organization

***A. baumanii:*** *Acinetobacter baumanii*

***H. influenzae:*** *Haemophilus influenzae*

***S. enteritidis:*** *Salmonella enteritidis*

**Facultative anaerobe:** an organism that makes ATP by aerobic respiration if oxygen is present, but is capable of switching to fermentation if oxygen is absent.

## ABSTRACT

*H. influenzae, A. baumanii* and *S. enteritidis* are pathogenic micro-organisms known to cause disease in human beings. They have developed resistant strains over the years thus made it difficult to treat these bacterial infections. They are part of the list of pathogens published by WHO that urgently need new antibiotics. Thus, this study aims to find an alternative to the conventional antibiotics in which the bacterial strains have developed resistance. Antibiotic resistance brings about treatment failure, increased cost of treatment due to prolonged hospitalization and use of expensive antibiotics. The conventional antibiotics are also known for their side effects which lead to non-compliance in most patients thus increasing the rate of development of resistance. The evaluation of antibacterial activity of plant sources has increased in order to come up with alternatives for the treatment of bacterial infection. Phyt$^{Exponent}$ is a poly-herbal preparation with five herbs that have shown to have significant in vitro antibacterial activity. The disc diffusion assay technique is an example of agar diffusion method used for susceptibility testing and assay for potency. It is the most used technique in most microbiology laboratories to test for susceptible micro-organism to certain antibacterial agents. Using the disc diffusion technique, Phyt$^{Exponent}$ demonstrated increased antibacterial activity against the test micro-organisms as its concentration increased.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background information

Antibiotics are substances produced or derived from certain microorganisms that are used to inhibit the growth of or kill harmful bacteria in the body. They are one of the most important drug classes since bacterial infections are a common occurrence worldwide. Antibiotics, also known as anti-bacterials, are widely used in the treatment of bacterial infections, as prophylactics during surgeries such as caesarian section, hip or knee replacement and organ transplants among others(national Instituties of Health, 2020). They have also been used in the prophylaxis of infections such as tuberculosis and pneumonia in people living with HIV and AIDS(Levin, 1965). With this wide range of applications of antibiotics, there is need for such drugs that are therapeutically effective, cost effective and with minimal side effects to encourage patient compliance.

The discovery of antibiotics about 90 years ago, by Alexander Fleming who discovered Penicillin in the 1920s, revolutionized the field of medicine. Before the discovery of antibiotics, people died from minor infections such as strep throat (a throat infection caused by *Streptococcus pyrogens*), respiratory infections and surgeries were riskier too. After the discovery of antibiotics and development of new ones other than Penicillin, the life expectancy increased, surgeries got a lot safer and people recovered from minor and even more severe bacterial infections. The applications for the antibiotics were expanded to what we currently used them for today.

However, with the broadened applications for antibiotics came with its own set of challenges. Antibiotics are no longer as effective as they used be. Certain micro-organisms have developed resistance to these drugs thus the bacterial infections are not cleared from the body. This is known as antimicrobial resistance. It is now a worldwide concern. Antimicrobial resistance has been brought about by a number of factors such as overuse of antibiotics, misuse such as using antibiotics to treat viral infections e.g. common cold, non-compliance, wrong diagnosis, frequent use

of broad spectrum antibiotics and self medicating among others(Iredell, 2019). Increased production of counterfeit or substandard medicines into the market has also led to the increased development of resistance in the society. There has also been increased irrational use of veterinary antibiotics whereby the domestic animals which are a source of food are treated with antibiotics without proper diagnosis and most farmers do not observe the withdrawal period. Thus, traces of these antibiotics are present in the meat consumed by human beings thereby exposing them to small amounts of these agents leading to development of resistant bacterial strains. This has led to an increase in mortality rate due to drug-resistant bacterial infections worldwide at an alarming rate. Therefore, there is a need to develop new antibiotics.

The World Health Organization (WHO) recently published a list of twelve families of drug resistant bacterial strains that urgently require new antibiotics("Who Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed," 2017). They are divided in terms of their priority, priority 1 being critical, priority 2 is high and priority 3 is of medium priority. The bacterial strains with critical priority include; *Acinetobacter baumanii*, carbapenem resistant, *Pseudomonas aeruginosa* and *Enterobacteriaceae* which are all carbapenem resistant. The bacterial strains with high priority include *Enterococcus faecium* Vancomycin resistant, *Staphylococcus aureas* methicillin resistant, *Helicobacter pylori* clarithromycin resistant and *Salmonellae* fluoroquinolone resistant. The medium priority bacterial strains include; *Streptococcus pneumonia* penicillin non-susceptible, *Haemophilus influenzae* ampicillin resistant and *Shigella* spp fluoroquinolone resistant. The list is a tool to ensure that the research and development of antibiotics responds to the urgent public health needs. It is a bid to guide and promote the research and development of new antibiotics.

Nonetheless, the development of new antibiotics has proven to be a formidable challenge. This is due to the prolonged period of development that takes about 10-15 years and also it is an expensive process that can cost over one billion dollars(Rolain et al., 2016). It is not given that the drug will pass and often than not, these new drug molecules fail the research and development process. Moreover, no

new drug class of antibiotics has been discovered and developed for several years now, there has only been improvement of the already existing drug classes. In addition, when these new antibiotics are developed, they are viewed as drugs of last resort to minimize the development of antibiotic resistance. They are used sparingly and not sold in large volumes therefore; there are no significant returns on the resources used in the research and development process(El-Dairi & House, 2019). This discourages the pharmaceutical and R&D companies from developing new antibiotics. Most big pharma companies have hung their towel on antibiotic research and now medium-sized pharmaceutical companies are dominating antibiotic research and development but lack of investments and funding poses a great challenge.

In as much as the promotion of research and development of new antibiotics is essential in the prevention of continuous growth of antibiotic resistance and death caused by resistant micro-organisms, this cannot be enough to solve the problem. This is because it is guaranteed that the micro-organisms will find a way to resist these drugs. Therefore other approaches must be employed to prevent or minimize incidences of antibiotic resistance. These approaches include; better prevention of infections, appropriate use of existing antibiotics in both humans and animals and also rational use of new antibiotics. The society does not simply need a large number of new antibiotics but what it actually needs is antibiotics that have the ability to treat serious infections and provide significant improvements in safety and efficacy over existing therapies and provide treatment options where there is currently clinical failure.

Therefore, there is need for innovation in coming up with new products with sufficient antibacterial activity, vaccines and diagnostics and better prevention, control and surveillance. Complementary and alternative medicine has been an area of interest in sourcing new products with sufficient antibacterial activity. Several plants, herbs and plant derived products are being studied for their antibacterial activity and this could be a possible solution to the drastic increase in antibiotic resistance.

## 1.2 Problem Statement

Bacterial infections are a major cause of death in hospitals and healthcare facilities worldwide(Gannon, 2000). The bacteria enter the body through wounds, open injuries, surgical incisions, catheters and implant devices. Hospital acquired infections are the leading cause of death(Iredell, 2019). The bacterial strains in the critical priority list are majorly gram negative, hospital acquired, and multi-drug resistant strains. In Europe, there are about 37,000 deaths due to hospital bacterial infections per year and about 98,000 deaths per year in the United States. Worldwide, there is an additional 110,000 deaths per year due to serious bacterial infections(Baylor, 2017).

The development of antibiotic resistance makes treating these infections more difficult and surgeries and chemotherapy for cancer patients carry a greater risk. Antibiotic resistance leads to lengthy hospital stays, long-term disability and more preventable death. This in turn results in a hurting economic growth. Most people will spend more money in re-hospitalization, long hospital stays and using expensive antibiotics to try and treat these antibiotic resistant micro-organisms, thus healthcare will be a heavy financial burden in most households(Brien, 2014).

The increasing development of antibiotic resistance threatens the capability to treat minor and common bacterial infections such as tuberculosis and gonorrhea, subvert major medical advances such as surgeries, chemotherapy for cancer patients and management of preterm babies. ABR also threatens the ability to reach global health goals such as a decrease in child mortality and improvement of maternal health. About 214,000 newborns die of sepsis worldwide per year due to the hospital acquired multi-drug resistant bacterial infections. These resistant bacterial strains spread silently as more than 60% of the world's population carry resistant strains in their normal flora. ABR is no longer a future threat, rather it is a present crisis(Iredell, 2019).

The development of antibiotics has demonstrated to be an alarming challenge due to the long period involved (about 10-15 years) and the extremely high cost of research and development. The challenge of developing new antibiotics, the high

cost of the new antibiotics and the side effects associated with them poses a great challenge in solving the antibiotic resistance crisis. Currently, the clinical pipeline of new antibiotics is wilted. With the WHO publishing the priority list for the pathogens, about 32 antibiotics are in development with only six categorized as innovative. This demonstrates that there is an innovation gap in the field of antibiotic research and development and it is time it is explored in order to deal with the ever growing antibiotic resistance(El-Dairi & House, 2019).

### 1.3 Justification

There is an increased need for innovative ideas and new sources of products with sufficient antibacterial activity to deal with the challenge of developing new antibiotics, antibiotic resistance, increased cost of treating bacterial infections and the side effects associated with the existing antibiotics. In recent years there has been an increase in review and investigations of plant sources with antibacterial activity. Herbal products have been used in the management of various diseases such as digitalis (digoxin) in the treatment of heart attack, quinine and artemisinin in the treatment of malaria. About 25-50% of current pharmaceuticals are plant derived thus; plants are a valuable source of pharmaceutical agents. The interests of plants in antibiotic resistance is also based on the fact that plant extracts have many molecules with different mechanisms which can act synergistically against bacteria, making resistance difficult like in the classic combination of drugs(Nascimento et al., 2000).

Thus herbal products can be studied for the treatment of bacterial infections. An example of such a herbal product is Phyt$^{Exponent}$ which is marketed as an immunomodulator. It is a poly-herbal preparation with five components in which their in vitro antibacterial activity has been studied. Such a preparation may come in handy in the treatment of drug-resistant strains of bacteria, lower treatment cost since there will be no treatment failure and will have fewer side effects compared to the conventional antibiotics.

## 1.4 Hypothesis

There is increased antibacterial activity of Phyt$^{Exponent}$ against *Acinetobacter baumanii, Haemophilus influenzae* and *Salmonellae enteritidis* at higher concentrations.

## 1.5 Objectives

### 1.5.1 General Objective

To evaluate antibacterial activity of Phyt$^{Exponent}$ against certain micro-organisms at higher doses.

### 1.5.2 Specific Objectives

I. To evaluate the antibacterial activity of Phyt$^{Exponent}$ against *Haemophilus influenza* at higher concentrations.
II. To evaluate the antibacterial activity of Phyt$^{Exponent}$ against *Acinetobacter baumanii* at higher concentrations.
III. To evaluate the antibacterial activity of Phyt$^{Exponent}$ against *Salmonella enteritidis* at higher concentrations.

## 1.6 Significance of the Study

This study aims to find alternatives to conventional antibiotics from plant sources that have been associated with development of resistance, high cost and side effects which discourage the compliance of the patient. This will greatly improve treatment outcomes and could potentially be the solution to the increased development of resistance.

## 1.7 Limitations of the Study

In vitro susceptibility testing and in vivo studies have demonstrated discrepancies in the results. This is because the agar diffusion systems cannot include all the biologic variables found within the human body. In vitro studies only involves the diffusion of the antibiotic agent from the disc into the agar whereas in vivo studies involve quite a lot of biological processes that include absorption, distribution, metabolism, excretion and also pharmacodynamic processes. This may influence the antibacterial activity of the agent in the study. Thus in as much as the antibacterial agent in the study has significant in vitro antibacterial activity against the test micro-organisms, in vivo studies are also crucial to ensure the agent still

has significant antibacterial activity in biological processes. The disc diffusion method may indicate in vitro susceptibility of certain agents for some organisms despite the lack of therapeutic efficacy in actual practice, for example *Salmonella typhi* susceptibility to aminoglycosides and *Enterococcus* susceptibility to cephalosporins.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Disk Diffusion Assay Technique

This is a type of agar diffusion antimicrobial susceptibility testing. The broth dilution technique was the first method for susceptibility testing, and it is still the gold standard today. The disk diffusion method was created in response to the time-consuming characteristics of the broth dilution assay method (Jorgensen & Ferraro, 2009). This approach was widely utilized in microbiology laboratories, and each one tweaked it to fit their needs. As a result, the test findings were highly variable. W.M. M Kirby and colleagues from the University Of Washington School Of Medicine and the King County Hospital introduced a single disk approach for antimicrobial susceptibility testing in 1956. Later on, Kirby and A.W. Bauer reviewed the literature on antimicrobial susceptibility testing extensively and published their findings. This publication led the WHO to establish a committee to standardize the procedure, thus this procedure was referred to as the Kirby Bauer technique.(Hudzicki, 2012)The Clinical Laboratory Standards Institute (CLSI) is currently at the helm of updating and modifying the original procedure. The purpose of the antimicrobial susceptibility testing is to determine the sensitivity or resistance of the pathogenic micro-organisms to the different antimicrobial agents. This assists health care practitioners in selecting appropriate treatment options in relation to the causative pathogen. With the development of antimicrobial resistance, it is essential to carry out susceptibility tests in order to avoid treatment failure and promote the development of multidrug resistant bacterial strains.

The disk diffusion assay method involves the use of Mueller Hinton agar on plates or petri dishes inoculated with the test micro organisms and 6 mm filter paper disks impregnated with the different concentrations of the antimicrobial agent. This is then incubated at specified temperature conditions and time. The interpretation of the results involves observing the presence of zones of inhibition adjoining the disc. Two to five concentrations of the antimicrobial agents are often used to determine the most appropriate concentration of the drug to use. The 6 mm disks impregnated with the different concentrations of the antimicrobial is placed after the inoculation

of the test pathogenic micro-organism onto the agar. After the placement of the disks, the plates are incubated to allow for the diffusion of the antimicrobial into the agar(Hudzicki, 2012).

The rate of diffusion is influenced by the solubility and diffusion profiles of the drug and the molecular weight of the drug. The rate of extraction of the antimicrobial from the disk onto the agar is more rapid than the rate of diffusion through the agar. The diameter of the zones of inhibition is measured and it suitably sets out the antimicrobial potency of the compound. The advantage of using the disk diffusion assay method is that it is an inexpensive, simple method as it does not require any special equipment. It is also flexible in the selection of disks for testing. The method also allows for visibility of growth, correct inoculum, mixed cultures and other abnormalities. The results of the susceptibility testing in disk diffusion are easily interpreted since only the diameters of the zones of inhibition are measured(Balouiri et al., 2016).

### 2.2 Phyt$^{Exponent}$

Phyt$^{Exponent}$ is a poly herbal preparation consisting of five components; *Alium sativum, Triticum repens*, *Echinacea purpurea, Viola tricolor* and *Matricaria chamomile.* This preparation is manufactured in Belgium and marketed as an immunomodulator. An immunomodulator is a substance that modifies the response of the immune system or its functioning. Phyt$^{Exponent}$ has been studied for treatment of inflammatory disorders, blood pressure, oxidative stress and as an immunity booster. Due to its immunomodulating activity, it is a possible herbal preparation for the treatment of bacterial infections. Some of its components, for example *Alium sativum,* have demonstrated in vitro antibacterial activity.

#### 2.2.1 *Alium sativum*

*Alium sativum* commonly referred to as garlic, is a plant with extensive medicinal and culinary properties. It has been used in most households, and continues to be used as a food spice. Garlic has also been used traditionally as concoctions in the management of flu and colds(Rouf et al., 2020). Other medicinal properties of garlic include; antifungal, antiviral, antibacterial, antihistamine, expectorant and

antiseptic properties. It has also demonstrated protective action against fat induced increase in serum cholesterol and fibrinolytic activity of the plasma fibrinogen.

The component in garlic responsible for its antibacterial activity is allicin, especially in its pure form. It has demonstrated antibacterial activity against gram positive and gram negative bacteria as well as multidrug resistant *Escherichia coli.* It has also shown significant antibacterial activity against *Streptococcus mutans, Salmonella typhi, Escherichia coli, Klebsiella pneumonia* and *Bacillus cereus*(Viswanathan et al., 2014). The aqueous allicin rich extract demonstrated more activity as compared to the garlic oil. Allicin works by inhibiting RNA synthesis as the primary target and also blocks the Acetyl CoA formation thereby blocking DNA and protein synthesis(P. Saravanan, V. Ramya, H. Sridhar, V. Balamurugan, 2010). The thiosulfinates present in garlic are responsible for most of the health benefits of *Alium sativum.*

### 2.2.2 *Triticum repens*

*Triticum repens* also known as couch grass, has been widely used in traditional medicine for the treatment of urinary tract pain and spasms as well as a diuretic. It is usually referred to as a weed but its other common names include; dog grass, common couch and quick grass among other names. It has had applications such as incense where the rhizomes were broken up and used as incense when other options were not available and also used as medicine for the management of fever and inflammatory skin conditions externally. It was used as a syrup, tea or cold maceration in water for internal purposes and also applied topically for external purposes(Al-Snafi, 2015).

The pharmacological effects of *Triticum repens* have been tested in in vitro and in vivo studies. These effects include; hypolipidaemic effects where the test was done in specific diabetic mice with high lipid levels and also normal lipid levels and it significantly decrease the lipid levels on these test animals. The hypoglycemic effects were also tested on specific diabetic mice and demonstrated to balance the blood sugar in about two weeks without any effect on insulin secretion. The *T. repens* extract also showed to decrease motility in the test mice on a Rota rod. The

extract also acts as a diuretic due to the presence of mannitol in the plant that acts as an osmotic diuretic. *T. repens* also has antimicrobial activity in addition to its diuretic effects, thus used to flush out infections in the urinary tract(Widy-tyszkiewicz et al., 2012).

### 2.2.3 *Echinacea purpurea*

*Echinacea purpurea* is also known as the purple coneflower or the red sunflower. It is commonly used in the treatment and management of respiratory infections. Traditionally, it has been used in the treatment of wound infections, snake and spider bites, colds and headaches. It has significant immunostimulating effects by activating phagocytosis and release of the tumor necrosis factor (TNF). *E. purpurea* contains three major chemical components which include; polysaccharides, caffeic acid derivatives and lipophilic alkamides. Polysaccharides with high molecular weight such as heteroxylan, alkylamides and chicoric acid glycosides all have the potential to activate phagocytosis and arabinogalactan also a polysaccharide has the potential to induce the release of TNF.

The pharmacological effects seen with *E. purpurea* include; immunostimulation, antioxidation by reducing the production of arachidonic acid and prostaglandin E2, anti-inflammatory, antiviral especially against the Influenza virus and antifungal activity against *Candida spp* and *Saccharomyces cerevisiae*. It also has significant activity in the treatment of upper respiratory tract infections(Chiellini et al., 2017).

### 2.2.4 *Viola tricolor*

*Viola tricolor* is commonly referred to as the wild pansy or the pansy plant which has three colored flowers and an alternate leaf arrangement. It was traditionally used in the treatment of mild seborroeic conditions such as nursing infants with seborrhea of the scalp. It has also been used in the treatment of numerous skin conditions such as acne, eczema, pruritus and impetigo. In vitro studies on its antibacterial activity have been carried out and it revealed remarkable activity against *Candida albicans, Staphylococcus aureas, Staphylococcus epidermidis* and *Bacillus cereus.* It also showed moderate activity against *Klebsiella pneumonia, E.*

*coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*(Witkowska-Banaszczak et al., 2005).

### 2.2.5 *Matricaria chamomilla*

*Matricaria chamomilla*, popularly known as chamomile, is a herb commonly used in the food industry as a flavor in tea, in the cosmetic, perfumery and aromatherapy as candles. Its effects are often calming and most people find themselves taking chamomile tea just before bed to help calm them into sleep(Singh et al., 2011). Its preparations have demonstrated various pharmacological effects which include; antidiarrheal and antispasmodic where it was used traditionally to treat infantile colic though the amount of chamomile tea required for effectiveness discouraged its use. It has also shown usefulness in the treatment of skin conditions such as radiation dermatitis and eczema. Its chemical components such as the flavonoids, chamazulene and bisabolol have shown anti-inflammatory activity. *M. chamomilla* has also demonstrated estrogenic activity where the ethanol extract with the flavonoid apigenin has shown weak in vitro estrogenic and progestational activity and the aqueous extract has demonstrated non proliferative activity on the cells of cervical cancer and antiestrogenic activity on breast cell tissue. It has also been useful in the management of radiation and chemotherapy induced mucositis in cancer patients(Hameed et al., 2018). In vitro studies of its antibacterial activity have revealed both gram positive and gram negative bactericidal activity in select bacterial strains.

## 2.3 The Test Micro-organisms

### 2.3.1 *Acinetobacter baumanii*

This is a pleomorphic, non-motile, aerobic gram negative bacillus. This pathogen is medically important due to the number of increasing infections it causes and also the increased spread of multidrug resistant strains of this pathogen(Howard et al., 2012). It is one of the pathogens published by the WHO that require new antibiotics urgently as of critical priority(“Who Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed,” 2017). It is mostly found in the hospital setting where patients hospitalized long term are at a greater risk. It also causes a high

incidence of infection among immunocompromised patients such as people living with HIV and AIDS, transplant patients and cancer patients.

It was first isolated in 1911, by Beijerick, a Dutch microbiologist from soil using minimal media enriched with calcium acetate. It was previously recounted as *Micrococcus calcoaceticus.* It was described as strictly aerobic, non-fastidious, non-fermenting, oxidase negative and catalase positive bacteria. The genus *Acinetobacter* originated from the Greek word 'akinetos' which means non-motile. This characteristic and name differentiated it from the motile micro-organisms of the genus *Achromobacter*. The genus *Acinetobacter* contains more than 50 species which are non-pathogenic thus does not cause disease.

*Acinetobacter baumanii* explicitly targets moist body tissues such as the mucous membranes or exposed areas of the skin and soft tissues brought about by injuries or an accident. In as much as this pathogen is widely associated with the skin, it is rarely found in the skin's normal flora. The *A. baumanii* infections are most likely found in patients on long term hospitalization who either have been fitted with artificial devices such as sutures, prosthetics, catheters and ventilators or those who have undergone antibiotic therapy or dialysis in the past ninety days. Another risk group is the soldiers who are members of the armed forces who have been deployed to war zones such as Afghanistan and Iraq. The environment of such conflict zones comprises of dry and sandy conditions which are ideal for the growth and prevalence of the pathogen *A. baumanii.* In addition to colonizing artificial devices such as catheters, the pathogen also colonizes the intravenous fluids and irrigating solutions. *Acinetobacter baumanii* causes various types of infections such as endocarditis, bacteremia, urinary tract infections, pneumonia both community acquired and hospital acquired, meningitis where a larger number of cases occur in patients who are recovering from neurosurgical procedures and osteomyelitis which occurs mainly in military patients sustaining trauma that is war-related(McConnell et al., 2013).

Treatment of *A. baumanii* has involved antibiotics such as Cephalosporins, penicillins, polymyxin E and B, carbapenems and sulbactam among

others(Fishbain & Peleg, 2010). It has currently developed multidrug resistance thus the pathogen is not as sensitive to antibiotics as it was earlier in the 1970s.

### 2.3.2 *Haemophilus influenzae*

This is a small, pleomorphic, facultative anaerobic, gram negative coccobacillus of the family Pasteurellaceae. This pathogen was first described incorrectly by Richard Pfeiffer in 1892 during the influenza pandemic. He thought that the bacteria *H. influenzae* caused the influenza though it was later established that it was in fact caused by a virus(Sawyer, 1978). This bacterium is classified according to the presence or absence of the polysaccharide capsule. Some bacterial strains of this group have a polysaccharide capsule and these strains are further serotyped into six different types according to the differences in the biochemical nature of the capsule. The most virulent of these strains is the *H. influenzae* type b (Hib)(Influenzae et al., n.d.). Other strains do not possess the polysaccharide capsule thus referred to as non-encapsulated or non-typeable *H. influenzae* (NTHi). The NTHi strains cause more invasive disease now as compared to Hib due to the discovery of routine vaccination. Hib was the most common cause of fatal bacterial infection in children younger than 5 years before the discovery of the routine Hib vaccine in 1993.

*H. influenzae* is facultative anaerobic which means it can produce ATP in the presence or absence of oxygen. For aerobic growth which involves the presence of oxygen, hemin (X) and Nicotinamide adenine Dinucleotide (NAD) (V) factors must be present in the chocolate agar in which *H. influenzae* is classically grown or cultured. Hib has the ability to live and survive in the nose and throat of healthy people without causing disease. It is usually spread through infected droplets of respiratory secretions in sneezes and coughs(King, 2012). Thus it can be transmitted by both symptomatic and asymptomatic individuals. The groups of people most at risk of getting the H. influenzae infection include children under the age five and patients suffering from other medical conditions such as HIV and AIDS, sickle cell disease, hyposplenism which involves a non-functioning spleen,

a patient who has undergone bone marrow transplant or a patient receiving chemotherapy for the treatment of cancer.

The pathogen *H. influenzae* can lead to various infections in different parts of the body. This includes meningitis where the pathogenic micro-organism invades the spinal fluid and parts of the brain causing an infection. It cause pericarditis where the pathogen infects the lining that surrounds the heart, cellulitis where it affects the skin and soft underlying tissues, osteomyelitis, septic arthritis, epiglottitis, pneumonia and sepsis which can be fatal. The diagnosis usually involves culturing of the patient's blood or spinal fluid. Samples from the nose and throat are not usually used since this micro-organism can be present even in healthy individuals. One is declared ill from an *H. influenzae* infection if the pathogen is isolated from other areas such as the lung, spinal fluid or blood. The treatment has involved various antibiotics and corticosteroids though in recent years this pathogen has developed resistant strains thus listed as a medium priority pathogen by the WHO in the urgency of new antibiotics.

### 2.3.3 *Salmonella enteritidis*

This is a rod-shaped facultative anaerobic, non-spore forming gram negative bacteria which belongs to the *Enerobacteriaceae* family. It has the ability to generate ATP by aerobic respiration when oxygen is available and through fermentation or electron acceptors when oxygen is not available. It is mainly motile and is a chemotroph thus capable of acquiring their energy from redox reactions using organic sources. The *Salmonella spp.* was named after an American veterinary surgeon called Daniel Elmer Salmon.

*S. enteritidis* is mostly found in the intestines of animals and also widely distributed in the environment. It is predominantly transmitted through the consumption of contaminated food such as meat, eggs and milk. It is the most prevalent cause of gastroenteritidis which is a form of food poisoning(Salmon, 2021). Its symptoms include fever, diarrhea and abdominal cramps which often occur 12-72 hours after exposure to the pathogen. Most individuals recover without needing treatment whereas other individuals may suffer from severe diarrhea and the infection may

spread from the gastrointestinal tract into the bloodstream and eventually other body issues which may lead to death, thus such individuals would require treatment with antibiotics. Individuals can get infected by either eating or drinking contaminated food and water respectively or being in contact with infected animals, their faeces or their environment.

The groups of people most at risk are children younger than five years, unbreastfed infants who are younger than 12 months, individuals taking certain medications such as proton pump inhibitors, antacids and $H_2$ antagonists which reduce the level of stomach acid and generally infants, individuals older than 65 years of age and patients who are immunocompromised. Development of multidrug resistance in certain *Salmonella spp.* is a major public health problem and this has been brought about by irrational antibiotic use in both human beings and animals. This has greatly limited the treatment options in severe infection. Thus it has been listed as a high priority pathogen in the development of new antibiotics by the WHO.

### 2.4 Summary

The list published by the WHO on the twelve resistant bacterial strains that urgently require new antibiotics has led to an increase in research and in vitro studies of herbal products amongst other sources of antibiotics in order to solve the increasing antibiotic crisis worldwide. Phyt$^{Exponent}$ is a poly-herbal preparation with five components that have potential antibacterial activity thus a great candidate for a novel antibacterial agent. The disc diffusion technique has been used for several years in numerous microbiology laboratories. It is often updated and improved to meet current scientific and microbiological trends. *A. baumanii, H. influenzae* and *Salmonella enteritidis* are examples of bacteria which have developed some resistant strains to either certain antibiotics or to multiple antibiotics. They are part of the list published by WHO on the bacterial strains that urgently require new antibiotics. Thus the purpose of this study is to investigate whether these bacterial strains are susceptible to Phyt$^{Exponent}$.

# CHAPTER THREE

# RESEARCH DESIGN AND METHODOLOGY

## 3.1 Source of Herbal Preparation

The Phyt$^{Exponent}$ poly-herbal preparation was bought from Maendeleo Pharmacy Nairobi. It was then transported to Mount Kenya University as per the guidelines given by the manufacturer. It was stored according to the manufacturer's guidelines by the supervisor Dr. Epaphrodite Twahirwa.

## 3.2 The Test Bacterial Strains

The test bacterial strains are three. They include; *Haemophilus influenzae*, *Acinetobacter baumanii* and *Salmonella enteritidis.* The stock cultures were obtained from Kenya Medical Research Institute and working cultures of these bacterial strains prepared from the stock culture.

## 3.3 Reagents and Equipment

The reagents needed for the study were; normal saline to prepare the inoculum, ethanol to maintain aseptic conditions at the working station and Ciprofloxacin 30mcg/ml which will serve as the positive control. About twenty petri dishes or plates will be needed to hold the culture media and inoculum in order to carry out the study. An autoclave will be needed to carry out the sterilization process and a ruler to measure the zones of inhibition.

## 3.4 Aseptic Techniques

Aseptic conditions were achieved by use of protective clothing in the laboratory which includes laboratory white coat, latex gloves and closed shoes. The working bench was sterilized using ethanol. The glassware was sterilized using the autoclave and the airborne contamination was minimized by lighting three spirit lamps which served as a source of heat.

## 3.5 Preparation of Culture Media

During the practical at the laboratory, the working station was maintained clean using 70% ethanol and cotton wool. The beakers, round bottomed flask and pipette dispenser tips were sterilized in an autoclave at 121$^{o}$C for 1 hour. The purchased petri dishes that I was to use came pre-sterilized thus did not require additional

sterilization. In the preparation of the culture media, I first wore latex gloves then measured 6.5g of Nutrient Agar from High Media and mixed with 100ml of water then stirred the mixture for 10 minutes in a water bath. I then used distilled water to top up upto the 500ml mark of the volumetric flask. The prepared agar was then sterilized through autoclaving for 1 hour then allowed to cool for 20 minutes carefully to ensure it does not cool into solidification. The agar media was then poured onto 20 petri dishes till the bottom of the petri dishes were completely covered and allowed to cool until a solid surface was formed. The media did not completely fill the petri dishes to avoid wastage.

### 3.6 Preparation of Inoculum

A well isolated colony was taken and inoculated into sterile normal saline. The saline tube was then vortexed to create a uniform solution. The turbidity was then adjusted to 0.5 McFarland Standard which comprises of BaCl and $H_2SO_4$.

### 3.7 Susceptibility Test

#### 3.7.1 Disk Diffusion Assay

This is a culture-based microbiology assay for susceptibility testing. It is a simple and practical technique and has been well-standardized. It is easy to perform, economical and adaptable to clinical practice. It is not a quantitative method but a qualitative method that allows us to investigate susceptible or resistant micro-organisms. Pure isolates of *A. baumanii, H. influenzae* and *S. enteritidis* were sub cultured onto nutrient agar. Disks of diameter 6 mm containing 30mcg/ml of antibiotic was placed and inoculated on the Nutrient Agar and the zones of inhibitions measured after 24 hours. Additional 6 mm-diameter disks containing different concentrations of Phyt$^{Exponent}$ was also placed on dried and inoculated on Nutrient Agar and the zones of inhibitions measured after 24 hours.

### 3.8 Ethical consideration

This study was conducted as per the ethical guidelines set out by the Scientific Research Ethics Review Committee of Mount Kenya University.

### 3.8 Data Analysis

The data was collected by measurement of the zones of inhibition using a ruler. The data was recorded and stored in tables and photographs. The results were then expressed as a mean ±SEM (Standard Error of the Mean) since the study was carried out in triplicate. The statistical analysis was then carried out using the one way ANOVA analysis using Graph pad software.

# CHAPTER FOUR

## DATA ANALYSIS, PRESENTATION AND INTERPRETATION

### 4.1 Results and Data presentation

The data was collected after 24 hours of incubation at 37$^{o}$C by measuring the diameter of the zones of inhibition observed for every test micro-organism. The data was then recorded in a table and the mean ± SEM calculated. The results obtained from the study are in Table 1 which clearly indicates that all the three test bacterial strains were susceptible to Phyt$^{Exponent}$ and its antibacterial activity increased with increase in its concentration. The size of the diameter of the zones of inhibition surrounding the disc was analyzed using the De Almeida Alves *et al* (2000) criteria to determine the level of antibacterial activity of Phyt$^{Exponent}$ at the various concentrations.

**Table 4. 1Mean zones of inhibition with different Phyt concentrations**

| **Conc. of Phyt$^{Exponent}$ in µl** | ***H. influenzae*** | ***A. baumanii*** | ***S. enteritidis*** |
|---|---|---|---|
| | **Mean (mm) ±SEM** | **Mean (mm) ±SEM** | **Mean (mm) ±SEM** |
| 10 | 11.500 ±0.5 | 12.000 ±0.577 | 10.333 ±0.882 |
| 20 | 16.333 ±0.333 | 15.333 ±0.333 | 14.333 ±0.882 |
| 30 | 19.667 ±0.333 | 20.000 ±0.000 | 20.000 ±1.155 |
| 40 | 24.000 ±0.577 | 23.333 ±0.333 | 25.000 ±0.000 |
| 50 | 26.333 ±0.333 | 25.333 ±0.882 | 29.667 ±0.333 |
| Cipro 30mcg/ml | 35.000 ±0.577 | 36.667 ±0.333 | 38.667 ±0.667 |

**Figure 4. 1 Zones of inhibition in *Acinetobacter baumanii***

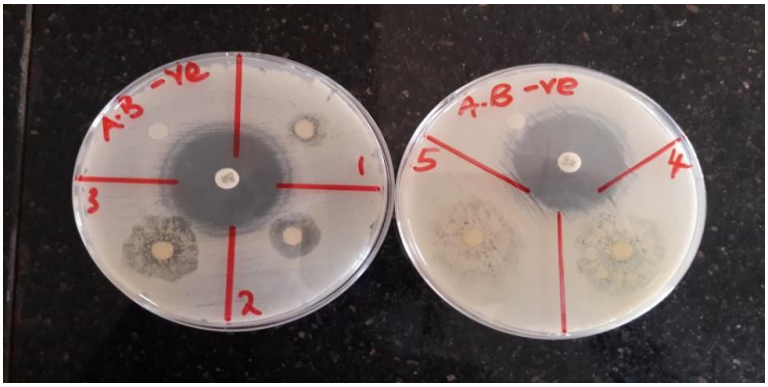



**Figure 4. 2 Zones of inhibition *Haemophilus influenzae***

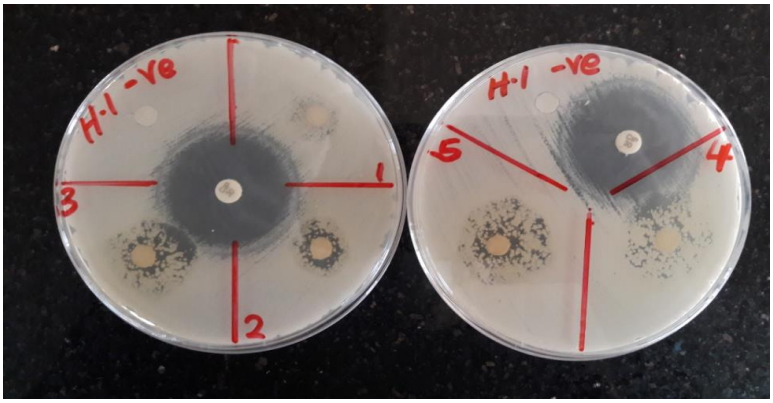
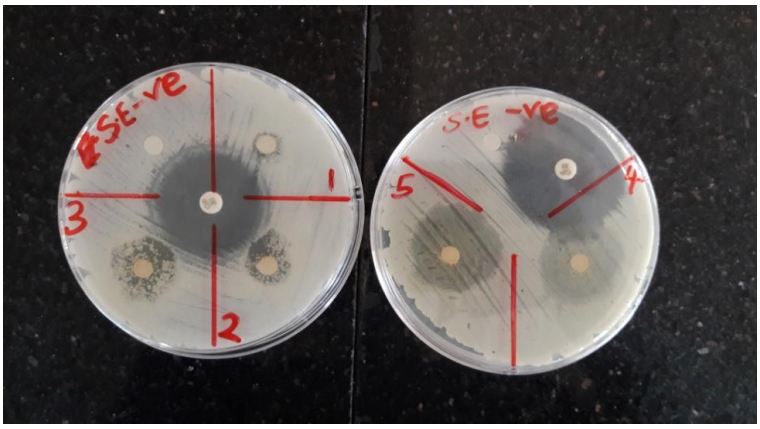



**Figure 4. 3 Zones of inhibition *Salmonella enteritidis***



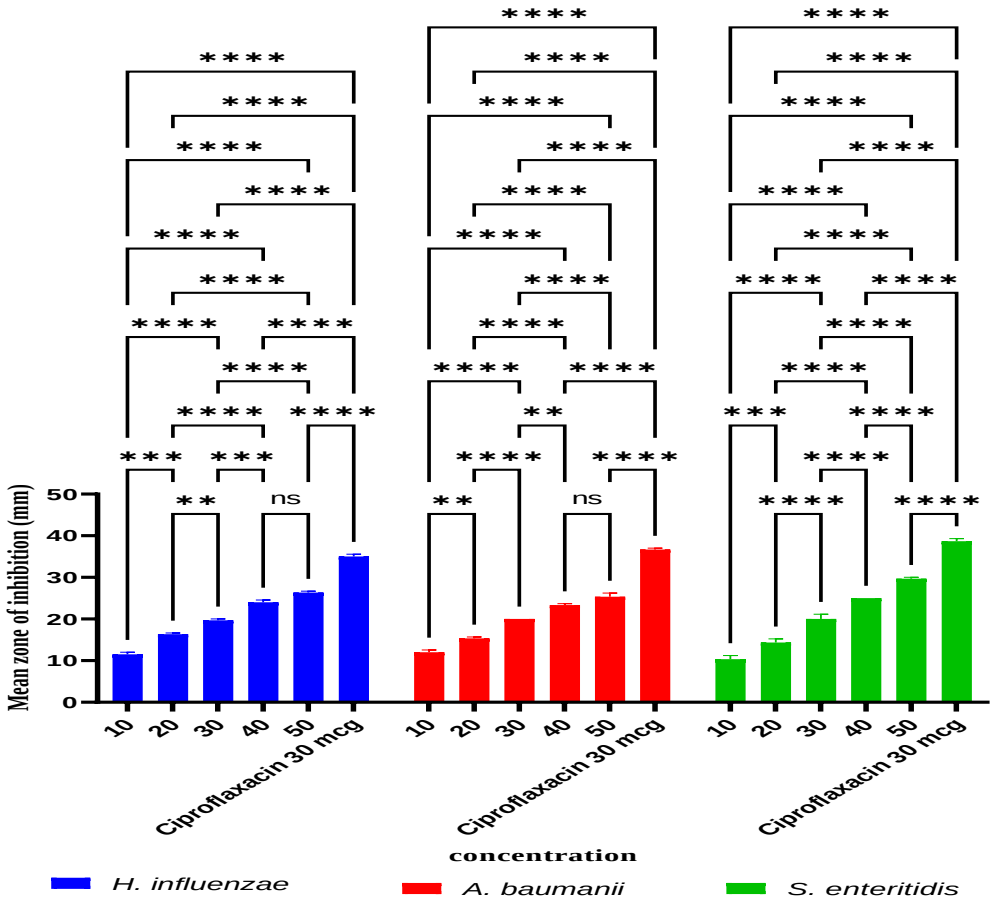

## 4.2 Discussion

This study aimed to evaluate whether the antibacterial activity of Phyt$^{Exponent}$ increased with increase in its concentration. The practical involved the susceptibility testing of different concentrations of Phyt$^{Exponent}$ against *H.*

*influenzae*, *A. baumanii* and *S. enteritidis*. The different concentrations of Phyt$^{Exponent}$ were determined by increasing the volume of Phyt$^{Exponent}$ i.e.; 10µl, 20µl, 30µl, 40µl and 50µl. A standard disc of Ciprofloxacin was used as the positive control.

As the concentration of Phyt$^{Exponent}$ was increased, the zones of inhibition of the various micro-organisms also increased. The level of antibacterial activity of the different concentrations was based on the De Almeida Alves *et al.* (2000) criteria, where zones < 9mm showed the preparation or extract was inactive, zones of 9-12mm showed partially active preparations, zones of 13-18mm demonstrated active preparations and zones > 18mm showed very active preparations.

**Figure 4. 4 Graph Presentation of Mean Zones of Inhibition for each test**



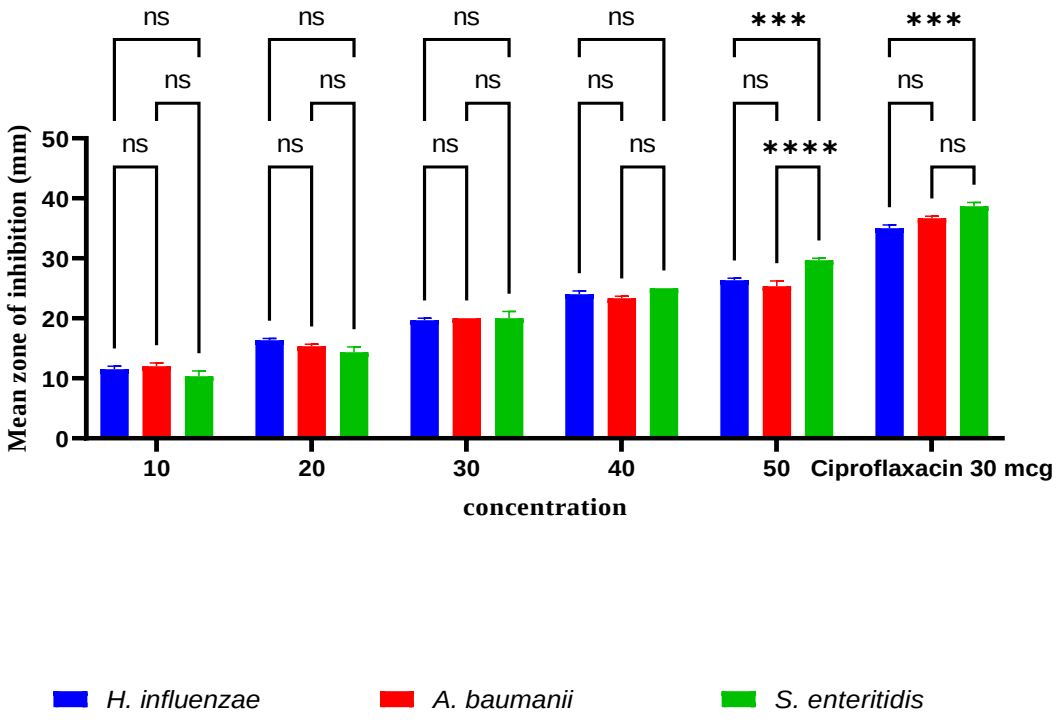

According to figure 4 which is a graph presentation, at 10µl of Phyt$^{Exponent}$ there was partial activity against *H. influenzae* and at 20µl the preparation was active. At 30-

50µl of PhytExponent, these concentrations demonstrated very active antibacterial activity against *H. influenzae.* The antibacterial activity of Phyt[Exponent] against *H. influenzae* showed comparable activity to Ciprofloxacin at higher concentrations.

The antibacterial activity of Phyt[Exponent] against *A. baumanii* demonstrated partial activity at 10µl, active at 20µl and very active at 30-50µl of Phyt[Exponent.] The antibacterial activity of Phyt[Exponent] against *A. baumanii* was also comparable to the positive control, Ciprofloxacin 30mcg/ml at higher concentrations. Thus *A. baumanii* was susceptible to the poly-herbal preparation.

In the evaluation of antibacterial activity of Phyt[Exponent] against *Salmonella enteritidis,* it was partially active at 10µl, active at 20µl and very active at 30-50µl. The antibacterial activity of the poly-herbal preparation was also comparable to Ciprofloxacin used as the positive control. Therefore, this showed that *S. enteritidis* was susceptible to Phyt[Exponent].

**Figure 4. 5 Mean Zones of Inhibition of each test pathogen at each Phyt conc**



As shown in figure 5, the difference in antibacterial activity of Phyt$^{Exponent}$ against all the three test bacterial strains at each concentration was not significant. Thus, this demonstrated that PhytExponent had similar antibacterial activity against all the test micro-organisms. The antibacterial activity at 50µl of Phyt$^{Exponent}$ was quite comparable to that of Ciprofloxacin 30mcg/ml. Therefore, this shows that Phyt$^{Exponent}$ has sufficient in vitro antibacterial activity.

The pharmacological or therapeutic effects of medicinal plants are determined by the phytochemical components of the plant. Phyt$^{Exponent}$, as explained earlier, is a poly herbal preparation with five herbs which in phytochemical studies have shown to have alkaloids, phenols, saponins, tannins, terpenoids, flavonoids, glycosides and steroids(Moriasi et al., 2021). These phytochemicals are responsible for the antibacterial activity of this poly-herbal preparation. Thus an increase in concentration of these constituents increases the antibacterial activity of Phyt$^{Exponent}$.

# CHAPTER FIVE

# CONCLUSION AND RECOMMENDATION

## 5.1 Conclusion

In conclusion, according to the findings from this study, it is clear that Phyt$^{Exponent}$ has significant antibacterial activity. Its antibacterial activity against the select bacterial strains increases as the concentration of the poly-herbal preparation is increased. *A. baumanii, H. influenzae* and *S. enteritidis* are all susceptible to Phyt$^{Exponent}$.

## 5.2 Recommendations

From this study, the recommendations that can be made are;

i. In vivo studies of antibacterial activity of Phyt$^{Exponent}$ can be evaluated.
ii. Evaluate the antibacterial activity of Phyt$^{Exponent}$ against *Acinetobacter baumanii* carbapenem-resistant.
iii. Evaluate the antibacterial activity of Phyt$^{Exponent}$ against *Haemophilus influenzae* ampicillin-resistant.
iv. Evaluate the antibacterial activity of Phyt$^{Exponent}$ against *Salmonella enteritidis* fluoroquinolone-resistant.

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