A comparative study of antibacterial properties of *Elephantorrhiza elephantine* (Muzezapasi) and *Zanthoxylum chalybeum* (Mukundanyoka) against those of commercial drug Azithromycin on *Escherichia coli*.

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Abstract

A study was carried out at the Midlands State University during the month of September 2016, to compare the antibacterial properties of *Elephantorrhiza elephantine* (Muzezapasi) and *Zanthoxylum chalybeum* (Mukundanyoka), against those of the commercial drug Azithromycin on *Escherichia coli*. Plant extracts were purchased, sun dried and ground into fine powders. Azithromycin tablets were also purchased. *Escherichia coli* bacteria were isolated from the University toilets. Confirmatory tests were used to verify *Escherichia coli*. The three treatment drugs were then prepared into percentage solutions of 1%, 5%, 10%, 25% and 40% for *E. elephantine* and *Z. chalybeum* alone. A control was also prepared with sterile distilled water. Filter paper discs of a diameter of 6mm were prepared and embedded with the treatment drug percentage solution. Mueller hinton agar was inoculated with *E.coli* and the treated filter paper discs were placed on the media and incubated for 18 hours at 37°C. Zones of inhibition (ZOI) were measured in millimetres. Comparisons on the effectiveness of the drugs were then done and the results showed that the standard drug Azithromycin was the most effective. Azithromycin had the lowest inhibitory effect of 12mm at 1% concentration and a highest inhibitory effect of 27mm at 25%. Its general inhibitory effect increased by an average of 15mm from 1% to 25%. Azithromycin's most significant increase was 10mm between 1% and 5%. *Elephantorrhiza elephantine* was more effective than *Zanthoxylum chalybeum* at lower percentage concentrations as it showed some inhibitory effect at 1% and 5% of 7mm and 8mm respectively and *Zanthoxylum chalybeum* showed no inhibitory effect at all at the two lowest concentrations. *Elephantorrhiza elephantine* had an average total inhibitory effect of 15mm. Its most significant inhibitory effect occurred between 25% and 40% at 7mm zone of inhibition diameter. *Zanthoxylum chalybeum* inhibition effect started at 10% concentration and its most significant increase was between 25% and 40% at 8mm zone of inhibition diameter, which was similar to that of *Elephantorrhiza elephantine*. *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* had the same average effectiveness of 8mm at 25% concentration. The results concluded that the commercial drug is greatly more effective than the unstandardized plant treatments, meaning that its dosage must be less than that of the plant drugs. Azithromycin therefore can have different dosages starting from below 1% because its 1% concentration has significant antibacterial effectiveness. The two plants *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* have an insignificant difference in effectiveness maybe due to differences in active ingredients and mode of action on bacteria, and so their dosages may be the same and should be in concentrations higher than 25% as their effectiveness is significant from that point going to higher concentrations.

Dedication

This dissertation is dedicated firstly to the Almighty, who has blessed me enough to do this degree with minimum challenges. His grace and mercy have ushered me through this degree in a way that would not have been possible without Him. This dissertation is also dedicated to my parents and siblings and close friends who have supported me not only financially but emotionally too. They have given me words of wisdom and pushed me to work hard at all times.

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CHAPTER 1: Introduction

1.1 Background

Diarrhoea is the frequent passage of unformed, loose, soft or watery stools, usually three or more times in 24 hours plus vomiting accompanied by abdominal pain, leading to dehydration if fluids are not taken by the individual (Cairneross and Curtis, 2003). It is the most common clinical manifestation of gastrointestinal disease and can be caused by bacteria, viruses and protozoa found mainly in human faeces. These organisms are spread through the faecal-oral root. Transmission of vectors occurs in conditions of poor sanitation and poor supply of clean water. Hands can act as a vector for transmission of faecal pathogens either through direct person-person transmission, or by contaminating food that is later consumed (Cairneross and Curtis, 2003).

Diarrhoeal diseases are a common cause of morbidity especially in developing countries and the main cause of mortality among children under five years of age. Diarrhoeal diseases account for 19% of mortality in the five and under age group. Rotavirus infection is responsible for the most severe forms of diarrhoea, especially in children, and may account for up to 40% of cases in the developed countries and 25% in the developing world (Black *et al*., 2010). Although viral infections are the most severe they are combated by vaccinations while children are in their infancy.

Diarrhoea accounted for about 9% of all deaths among children under age five world-wide in 2015 (UNICEF Data, 2015). This translated into over 1400 young children dying each day, or about 530 000 children a year, despite the availability of simple effective treatment (UNICEF Data, 2015). Most deaths from diarrhoea occur among children less than two years of age living in South Asia and sub-Saharan Africa (UNICEF Data, 2015). Despite this heavy toll,

progress was made, reducing the death toll during the 2000 to 2015 year period, the total annual number of deaths from diarrhoea among children under five years decreased by more than 50%, from over 1.2 million to half a million (UNICEF Data, 2015), although these figures are still high.

Bacterial causal agents of diarrhoea include general causative agent *Escherichia coli* (O157:H7), *Vibrio cholerae*, non-typhoidal *Salmonella*, *Shigella* species and *Salmonella typhi*. Protozoans such as *Cryptosporidium parvum*, *Giardia lamblia* and *Entamoeba histolytica* have also been incriminated as serious causes of diarrhoea in Africa and other parts of the developing world (WHO, 1978). Cholera and dysentery are severe, sometimes life threatening forms of diarrhoea (Thapar and Sanderson, 2004).

Oral rehydration salts are the common treatment for diarrhoeal diseases as the salts and sugars in combination with water help water to be absorbed into the body. While antibiotics are not a necessary part of diarrhoea treatment, some of these drugs may reduce both the amount and duration of cholera-related diarrhoea. A single dose of first line drugs; ciproflaxin or azithromycin (Zithromax, Zmax) may be effective in severe cases (Kaushik *et al.*, 2010). Azithromycin is more suitable for toddlers. When used in conjunction with oral rehydration salts, these antibiotics increase survival by preventing further loss of minerals and decreasing the number of bacteria. In rural areas, however, traditional herbal medicines are used to treat these diarrhoeal diseases due to financial and/or transport problems.

In Zimbabwe, the leading diarrhoeal diseases are cholera, dysentery and typhoid. Outbreaks of these diseases that have occurred in the past have been attributed to poor hygiene and sanitation and lack of clean water (The Ministry of Health and Child Care Zimbabwe, 2014).

Water borne diseases as cholera, typhoid and dysentery account for a significant number of deaths in Zimbabwe, especially in urban high density areas such as Epworth and rural areas

where adequate sanitation and primary health care are a challenge (Ministry of Health and Child Care Zimbabwe, 2014). In the year 2010 in Zimbabwe, 455 deaths were caused by diarrhoeal diseases, which made diarrhoeal diseases the third highest cause of death after HIV/AIDS and coronary related diseases. Children under the age of five years accounted for about 60% of the total diarrhoeal disease deaths in the year 2010 (www.healthmetricsandevaluation.org).

A total of nine people died due to typhoid while over 300 cases were reported in 2014 in a fresh outbreak of the disease in Harare, Mutare and Chegutu (The Ministry of Health and Child Care Zimbabwe, 2014). The disease outbreak was attributed to councils' failure to provide safe drinking water in suburbs, some suburbs going for months without running water (The Ministry of Health and Child Care Zimbabwe, 2014). The erratic supply of running water resulted in some residents seeking alternative sources including unsafe boreholes. The Harare City Council and many other local authorities failed to deliver clean and safe drinking water resulting in outbreaks of water-borne diseases like diarrhoea and dysentery (The Ministry of Health and Child Care Zimbabwe, 2014). The cumulative figure for dysentery cases in the year 2014 was 24 430 and 76 deaths while the cumulative figure for diarrhoea was 358 391 and 573 deaths. In the year 2012, typhoid outbreak affected more than 1 500 people in the country (The Ministry of Health and Child Care Zimbabwe, 2014).

Access to medical health care is still limited for most urban and more so in the rural communities due to high costs of medicines and distance to medical facilities. This leads to significant numbers of people resorting to traditional medicines to treat their ailments, while others believe more in traditional healers than medical doctors and prefer natural remedies due to possible side effects of manufactured drugs. *Elephantorrhiza elephantina* and *Zanthoxylum chalybeum* are popular, commonly used remedies for diarrhoeal diseases in

places such as Southern Victoria Falls, Central Harare, Mutare, Bulawayo, Northern Lomagundi and Mtoroshanga areas for *Elephantorrhiza elephantina* and in the Victoria Falls Livingstone area and the Odzi region for *Zanthoxylum chalybeum* where the plants are found as availability of the active ingredient may be affected by season and extraction efficiency (Drummond, 1981).

Although it may be known that traditional herbs are effective in treating different ailments, the problem is that there is a gap in their efficacy knowledge, in terms of how they work in comparison to standardised drugs. The effective doses of these plants are not standardized and this may lead to under or over dosage. The efficacy and safety of *Elephantorrhiza elephantina* and *Zanthoxylum chalybeum* in terms of how they compare to each other and commercial drugs as well as determination of minimum inhibitory concentrations are still to be ascertained and research results to be published to the scientific and general communities.

1.2 Justification

Diarrhoeal diseases, especially in children under the age of five years, are a recurring problem and access to commercial drugs may be out of reach for many people, especially those in remote rural areas due to shortage of money and long distances to medical facilities. Traditional medicines such as *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* are rapidly gaining popularity in Zimbabwe. Scientific evaluation of plant products continues to provide new therapeutic agents to fight diseases, especially where pathogenic resistance to drugs is common.

The medicinal plants used by herbalists and common people in Zimbabwe have not been well documented, despite their widespread use. The threat of complete disappearance of the knowledge on herbal medicine from factors such as deforestation, lack of proper regulation and overexploitation warrants an urgent need to document the information. The purpose of

this study was to document information on medicinal plants used by traditional people in Zimbabwe towards the utilization of indigenous ethnobotanical knowledge for the advancement of biomedical research and development.

The present study seeks to scientifically evaluate the efficacy of two local plant species in treatment of common diarrhoeal diseases and compare their efficacies with that of a common first line defence drug, Azithromycin. This study may then help the medical industry by stimulating the local production of standardised, cheaper and more easily accessible drugs from the plants used in the research study. Importation of drugs from foreign companies can then be reduced and thus benefit the country economically. New drugs are always needed to widen treatment options can discover better and less harmful drugs. The study results will also benefit the community at large as they will know how these plants extracts work in comparison to each other and the commercial drug Azithromycin and better determine how much they should use when healing themselves in order to prevent under or unnecessary over doses.

1.3 Objectives

1.3.1 Main Objective

The study seeks to evaluate the antimicrobial properties of *Elephantorrhiza elephantine*, *Zanthoxylum chalybeum* and Azithromycin by comparing their efficacies in the treatment of diarrhoeal diseases through investigating their effect on the general causative bacteria, *Escherichia coli*.

1.3.2 Specific objectives

The specific objectives were:

To isolate and purify strains of *Escherichia coli,*

To determine minimum inhibitory concentrations for effective treatment against diarrhoeal pathogens using *Elephantorrhiza elephantine*, *Zanthoxylum chalybeum* and Azithromycin

To compare the efficacies of the plant extracts with that of commercially used Azithromycin *(Zithromax).*

CHAPTER 2: Literature Review

2.1 Phytomedicines for diarrhoeal diseases in Zimbabwe

Phytomedicine is defined as herbal preparations produced by subjecting plant materials to fractionation, purification, extraction, concentration or other biological or physical processes (WHO, 1978). These preparations may be produced for immediate consumption or as the basis for other herbal products.

Plant products may contain inert ingredients that aid the action of the active ingredients (WHO, 1978). Phytomedicines have been an important part of traditional health care system in most parts of the world for years through traditional healers and wise elders of communities. Today, the greater percentage, 80%, of world population depends on traditional medicine for their primary healthcare needs (WHO, 1978).

Phytomedicine has reduced poverty by increasing the economic well-being of communities and develops health system by increasing health coverage to the people. It has demonstrated its contribution to the reduction of excessive morbidity, mortality and disability due to diseases such as HIV/AIDS, sickle-cell anaemia, diabetes, malaria, tuberculosis, mental disorders and microbial infections. (WHO, 1978). When it comes to diarrhoeal diseases plant extracts are known to stimulate water adsorption or reduce electrolyte secretion (Palombo, 2006).

Some of the popular medicinal plants in Zimbabwe for treatment of diarrhoeal diseases are *Elephantorrhiza elephantine* (Muzezapasi, Mupangara or Ndolani/Ndorani) and *Zanthoxylum chalybeum* (Mukundanyoka). When using *E. elephantine* the medicine is made by infusing the root stock or ground stem in clean water. When using *Z. chalybeum* the medicine is prepared by infusion of the bark in clean water. The infusions are drunk as often as necessary until the patient is cured.

Elephantorrhiza elephantine: Family *Fabaceae* or *Leguminosae*

Subfamily *Mimosoideae*

Genus *Elephantorrhiza*

Species *elephantine* (Grobler, 2010).

It is found in Botswana, Mozambique, South Africa and Zimbabwe, where weather conditions are dry and hot, in a mixture of soils. Its thickness is about 8 centimetres and height 20-90 centimetres (Grobler, 2010). The shrub produces yellow flowers and seeds of about 22cm long for reproduction. Common names for *Elephantorrhiza elephantine* include Eland's Bean, Eland's Wattle and Elephant's Root. Synonyms for its scientific name are *Acacia elephantine* and *Acacia elephanthorhiza* (Grobler, 2010).

The rhizomes used as a general remedy for intestinal and abdominal complaints such as diarrhoea, dysentery and stomach-aches. It is also used to treat painful menstruation and as a relief for heart troubles. Externally, it is used to treat haemorrhoids, acne and to cure skin diseases. The root is steeped in water for 24 hours or longer, after which it is strained and ready for external use. For internal use, the infusion has to be boiled for 10 minutes first.

Its active ingredient is allicin, diallyl thiosulfinate. Allicins mode of action on bacteria is inhibiting RNA synthesis, (Fern and Morris, 2014).

In South Africa the Council for Scientific and Industrial Research (CSIR) and Afriplex studied commonly used plants to evaluate their efficacy on various problems and *Elephantorrhiza elephantine* was the first to be studied. *Elephantorrhiza elephantine* is commonly known as Elands *boontjie* (Afrikaans) and *Intolwane* (Nguni languages) is found in grassland areas over large parts of the country. The roots of this plant are commonly used by indigenous people for a wide range of ailments including diarrhoea and dysentery,

stomach disorders, acne haemorrhoids and perforated ulcers. The CSIR through its studies showed significant activity of the extracts and compounds against the enzyme steroid 5-alpha reductase. This steroid 5-alpha reductase enzyme converts testosterone to dihydrotestosterone (DHT). DHT is seen as a causative factor in the progression of prostatic hyperplasia and also male pattern baldness. The extract also showed potent anti-oxidant activity. Several formulations such as treatment shampoos and scalp massage serums for topical application were then developed from this plant with aid from a manufacturing company in South Africa known as Afriplex (The Council for Scientific and Industrial Research, 2011).

A was study done in South Africa to determine the most commonly used plants to treat ailments was done through a survey with local people and traditional healers. *Elephantorrhiza elephantine* was in the top five used plants in South Africa with it being mainly used in the Limpopo province, where the roots of the plant are crushed, boiled in water and taken orally (Mathabe *et al*., 2006).

Zanthoxylum chalybeum is a deciduous shrub or tree with a rounded but open crown; it can grow from 1.5 - 10 metres tall, the bole can be 15 - 40cm in diameter, with large woody spines to 2cm long. A popular traditional medicine in south east. Its active ingredient are the alkonoids and the mode of action is inhibiting cell functions of cell wall synthesis, cell membrane function, protein synthesis, nucleic acid synthesis and other metabllic processes (Fern and Morris, 2014).

Zanthoxylum chalybeum: Kingdom *Plantae*

Phylum *Tracheophyta*

Class *Magnoliopsida*

Order *Sapindales*

Family *Rutaceae*

Genus *Zanthoxylum*

Species *chalybeum* (Fern and Morris, 2014).

Zanthoxylum chalybeum is an important medicinal plant that is widely used in traditional medicine. Extracts are highly active against *Plasmodium falciparum.* In Kenya and Tanzania *Zanthoxylum chalybeum* has been harvested to near extinction for medicinal purposes. *Zanthoxylum chalybeum* is widely used in traditional medicine. In Kenya, Kokwaro, its native name, is used as a medicinal plant in a similar way as *Zanthoxylum chalybeum,* In Kenya, Tanzania and Uganda the fruits, stem bark and root bark are commonly sold in local markets (Katende *et al*., 1995). In Uganda *Zanthoxylum leprieurii* is used in a similar way as a medicinal plant as *Zanthoxylum chalybeum* (Engeu *et al*., 2008).

Stem bark or root bark infused water is widely taken to treat malaria, fevers and headache, sickle cell disease, respiratory tract ailments including colds and tuberculosis, skin diseases including ulcers, tumours and measles, intestinal problems including abdominal pain, diarrhoea, intestinal worms, bilharzia, amoebas, general body pain and vomiting. It is found in East tropical Africa, Ethiopia, Somalia, Tanzania, Rwanda, Uganda, Angola, Botswana, Zambia, Zimbabwe and Mozambique (Neuwinger, 2000).

In Somalia the leaves have been reported to be used against stomach pain and urinary retention. Bark extracts cure malaria. A decoction of the bark and roots is used as a remedy for malaria, generalized body pains, coughs, scorpion bites, snakebites, anaemia and body

swellings. It is also used as a gargle for treating toothache. The bark and root powder is mixed with oil and applied as a liniment for treating pains and sprains (Bbosa *et al*., 2014).

In Uganda *Zanthoxylum chalybeum* root-bark and to a lesser extent its leaves are used to treat malaria. Although the continued use of the root-bark has led to plant extinction due to the destructive method of harvesting the herb as opposed to the leaves. The ether and methanol extracts of *Zanthoxylum chalybeum* caused *Plasmodium falciparum* schizonts suppression at a lower concentration. These results continued use of the herb by the traditional herbalist and local communities in Uganda, in the treatment of malaria (Bbosa *et al*., 2014).

Another study was carried out in Uganda to evaluate *Zanthoxylum chalybeum* antibacterial and antifungal properties on *Staphylococcus aureus, Escherichia coli* and the fungus *Candida albicans* by macerating *Z. chalybeum* in petroleum ether, alcohol and water filtering and then concentrating the filtrate by rotating in low temperatures. Three techniques were used disc diffusion, well diffusion and plate count. The results showed no inhibition on all methods (Olila *et al*., 2001).

A survey in Marakwat Kenya for most popular plants and their uses barks and seeds of *Zanthoxylum chalybeum* was number two after the leaves of *Acacia lahai,* the *Z. chalubeum* was used for malaria treatment mainly and joint pain, rheumatism and as a pain killer (Kipkore *et al*., 2014)

In Asia *Zanthoxylum* species are known for its numbing effect and is commonly known as the toothache tree. In India, the leaf is used against fever, dyspepsia and bronchitis. In Manipur, India, the seed oil is applied against baldness and bark powder is used to treat toothache (Singh and Singh, 2004). The young stems are employed as a toothbrush in cases of toothache and bleeding gums, whereas the roots and bark are used to cure malaria. Though

generally eaten as a vegetable, the leaves of *Z. rhetsa* are also consumed to kill tapeworms and reduce infection (Chadha 2008).

2.2 Diarrhoeal diseases

Cholera is an acute, diarrhoeal illness caused by infection of the intestine by the bacterium *Vibrio cholerae*. The infection is often mild or without symptoms, but can sometimes be severe. Approximately one in 10 (5-10%) infected persons will have severe disease characterized by profuse watery diarrhoea, vomiting, and leg cramps. In these people, rapid loss of body fluids leads to dehydration and shock. Without treatment, death can occur within hours (Moy *et al*, 1991).

Typhoid fever is caused by *Salmonella typhi* bacterium. Shellfish taken from sewage-polluted areas are an important source of infection. Flies may cause human infection through transfer of the infectious agent to foods. Transmission can occur through eating raw fruit and vegetables fertilized by human excreta and through ingestion of contaminated poultry, milk and milk products. Pollution of water sources may lead to epidemics of typhoid fever when large numbers of people use the same source of drinking water. Typhoid fever symptoms are headache, generalized aches, poor appetite, and pains, fever and lethargy (Moy *et al*, 1991).

Dysentery is diarrhoea in which there is blood, pus and mucus usually accompanied by abdominal pain. There are two types of dysentery which both occur mostly in hot areas. The first type is amoebic dysentery. It is caused by a single celled, microscopic parasite that lives in the large intestine called *Entamoeba histolytica*. The second type, bacillary dysentery, is caused by the invasive bacterium *Shigella dysenteriae*. The main symptoms of dysentery are sudden onset of high fever and chills, loss of appetite, weight loss, abdomen pains, cramps, urge to pass stool, feeling of incomplete emptying, bloating, flatulence, fatigue, vomiting and dehydration (Moy *et al*, 1991).

2.3 Escherichia coli

Escherichia coli, also known as *E. coli*, are a gram-negative, facultative anaerobic, rod-shaped bacterium of the genus *Escherichia.* They are commonly found in the lower intestine of warm-blooded organisms, called endotherms. Most *E. coli* strains are harmless or cause relatively brief diarrhoea, but some serotypes such as *E. coli* O157:H7, which is most often associated with disease in humans can cause serious an food poisoning and intestinal infection in their hosts leading to severe abdominal cramps, bloody diarrhoea, fever, vomiting, loss of appetite, fatigue, as fluids and electrolytes (dehydration) occurs, making the patient feel sick and tired which can be life-threatening. The harmless strains form part of the normal flora of the gut and can benefit their hosts by producing vitamin K2, B vitamins and preventing colonization of the intestine with pathogenic bacteria. Escherichia *coli* is expelled into the environment through faecal matter. The bacterium is generally spread by contaminated food, such as milk, under cooked meat, unwashed fruit and water but also can be passed among humans where personal hygiene is lacking. Urban residences can become infected when a city or town water supply has not been properly treated with chlorine (Singleton, 1999).

A healthy adult will usually make a full recovery from *E. coli* O157:H7 infection within 5-7 days. However, young children, pregnant women, elderly individuals, and patients with weakened immune systems, such as patients with AIDS, those taking immunosuppressive medications and people receiving chemotherapy, are more susceptible to the illnesses. People with reduced stomach acid such as those who have decreased stomach acid, either due to stomach surgery or medicines that lower stomach acid, have a higher risk of infections. They can also develop potentially fatal haemolytic uremic syndrome (HUS), a complication of *E. coli* infection that causes a type of kidney failure (Ishii and Sadowsky, 2008).

2.4 Azithromycin

Azithromycin is an antibiotic useful in the treatment of bacterial infections and has been sold commercially since 1988 (Banić Tomišić, 2011). It has broad antibacterial activity and inhibits gram-negative bacteria, some gram-positive bacteria and many atypical bacteria. This antibiotic is widely used alone or in combination with other drugs to treat middle ear infections, strep throat, influenza, pneumonia, pharyngitis, traveller's diarrhoea, chlamydia, gonorrhoea, respiratory infections and gastrointestinal infections (Banić Tomišić, 2011).

Azithromycin belongs to the class of drugs known as macrolide antibiotics. It binds to the 50S subunit of the bacterial ribosome, thus inhibiting translation of mRNA, microbial protein synthesis. Nucleic acid synthesis is not affected (Banić Tomišić, 2011).

People suffering from typhoid are given an oral, single 500mg tablet dose of Azithromycin three times and day, those suffering from Dysentery receive 250mg of Azithromycin three times a day, and those suffering from cholera receive 500mg of Azithromycin for adults. Child dosages are 10-12 mg/kg of body weight taken once per day for 2-5 days depending on age and severity of disease and Azithromycin drug should not be used in children younger than 6 months of age (Medicne net, 2014).

The most common side effects are diarrhoea, nausea, abdominal pain and vomiting. Other side effects of nervousness, fatigue, skin reactions, such as hives, rash and itching, increased heart rate in patients with heart problems and inflammation of the colon lining; colitis, have also been reported. Less than 1% of patients stop taking the drug due to side effects (Medicne net, 2014).

This drug comes as a tablet, suspension, and extended-release suspension taken by mouth. It also comes as eye drops. A health care provider can also give this drug in an intravenous

form. Zithromax for oral suspension is supplied in a single dose packet containing azithromycin dehydrate equivalent to 1g azithromycin. The tablets are supplied as white, oval-shaped, film-coated tablets, with active ingredient azalide. Azithromycin contains the following inactive ingredients: sodium phosphate tribasic, dibasic calcium phosphate anhydrous, colloidal silicon dioxide, sodium croscarmellose, pregelatinized starch, magnesium stearate, sodium lauryl sulfate and an aqueous film coat consisting of hypromellose, lactose titanium dioxide, and triacetin, anhydrous; spray dried artificial banana flavour, spray dried artificial cherry flavour, and sucrose (Pfizer labs, 2009).

Assays using Azithromycin were run by dissolving azithromycin in 95% ethanol, then diluting with water and then running disc diffusion assays on the different bacteria. Azithromycin was shown to have significant antibacterial effects on aerobic gram-positive microorganisms such as *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus pneumoniae* and *Streptococcus pyogenes* and on aerobic gram-negative microorganisms such as *Haemophilus influenzae* and *Moraxella catarrhalis* as well as other microorganisms such as *Chlamydia trachomatis.* It was shown that most strains of *Enterococcus faecalis* and methicillin-resistant *Staphylococci* are resistant to azithromycin and that beta-lactamase production should have no effect on azithromycin activity (National Committee for Clinical Laboratory Standards, 1993).

Azithromycin has been shown to be active *in vitro* and in the prevention and treatment of disease caused by the following microorganisms: *Mycobacterium avium* complex consisting of: *Mycobacterium avium and Mycobacterium intracellulare.*

Minimum Inhibitory Concentrations (MIC) interpretation used was;

$\leq$ 2µg/mL is Susceptible, 4µg/mL is Intermediate and $\geq$ 8µg/mL is resistant.

MICs for the tested bacteria were; *Escherichia coli (*ATCC 25922) 2.0-8.0µg/mL *Enterococcus faecalis (*ATCC 29212) 1.0-4.0µg/mL and *Staphylococcus aureus (*ATCC 29213) 0.25-1.0µg/mL. Using a 15µg azithromycin disk the observed zone diameter results were interpreted by saying a Zone Diameter of ≥ 18mm is Susceptible, 14-17mm is Intermediate and ≤ 13mm is Resistant (National Committee for Clinical Laboratory Standards, 1993).

CHAPTER 3: Materials and Methods

Isolation of *Escherichia coli* and *in-vitro* susceptibility disc diffusion assays to compare *Elephantorrhiza elephantine*, *Zanthoxylum chalybeum* and Azithromycin antibacterial properties on *Escherichia coli* were carried out at the Midlands State University in the department of Biological sciences laboratories in the month of September 2016.

3.1 Isolation of bacterial strain, *Escherichia coli*

Isolates of the test organism *Escherichia coli* were obtained from MSU toilets by swabbing the toilet inner surfaces with sterile manufactured swabs. The swabs were then inoculated on MacConkey agar. MacConkey agar was prepared by weighing 2.5g of the agar powder and dissolving in 50ml of distilled water. The solution was then mixed and heated to boiling point until the powder was completely dissolved. The solution was then autoclaved, 121°C for 15 minutes, and after that, it was poured into petri dishes to cool and set. A total of two toilets were swabbed and each swab was inoculated on a separate plate of MacConkey agar and incubated at 37°C for 24 hours. After incubation, *Escherichia coli* was confirmed using macroscopic and biochemical tests which included catalase, oxidase and citrate. The gram staining technique was also used to confirm the shape and gram status of *Escherichia coli.*

3.2 Biochemical identification of *Escherichia coli*

3.2.1 Catalase test

A drop of 3% hydrogen peroxide (H_2O_2) was placed on clean slide using a dropper. An isolated bacterial colony from the MacConkey agar was placed in the drop of hydrogen peroxide by using a sterilised inoculation loop sterilised by dipping it in methylated spirit, flaming it until red hot and then allowing it to cool in the air. The drop was viewed for any effervescence.

3.2.2 Oxidase test

A manufactured oxidase test strip was inoculated with an isolated bacterial colony using a sterilised inoculation loop. The test part of the strip was viewed for any colour change.

3.2.3 Citrate test

Simmons citrate agar was prepared by weighing 0.6g of the agar powder and dissolving in 25ml of distilled water. The solution was then mixed and heated until the powder was completely dissolved. The solution was then autoclaved at 121°C for 15 minutes, and after that, it was poured into petri dishes to cool and set. An isolated bacterial colony from the MacConkey agar was placed on Simmons agar using a sterile inoculation loop. The Simmons agar was then incubated at 37°C for 24 hours and after incubation the plate was viewed for any colour change.

3.3 Microscopic morphological identification

3.3.1 Gram stain and microscopy

The suspected test organism from the biochemical tests was gram stained using the standard gram stain technique, Appendix 1.

3.4 Preservation of *Escherichia coli*

The identified colonies of *Escherichia coli* were then preserved by inoculating single colonies on MacConkey agar and Nutrient agar.

Nutrient agar was prepared by weighing 0.7g of the agar powder and dissolving in 25ml of distilled water. The solution was then mixed and heated to boiling point until the powder was completely dissolved. The solution was then autoclaved, 121°C for 15 minutes and after that, it was poured into petri dishes to cool and set.

3.5 Collection and processing of plant material extracts

The roots of *Elephantorrhiza elephantine* and the bark of *Zanthoxylum chalybeum* were collected from local vendors in Gweru and dried in direct sunlight for 7 days. The plant parts were then ground into fine powders using a hand held blender, a pestle and mortar.

3.5.1 Preparation of percentage solutions

Percentage solutions of 1%, 5%, 10%, and 25% were prepared for all the three treatment components and an additional percentage solutions of 40% for *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum*. Sterile distilled water was prepared by autoclaving distilled water at 121°C for 15 minutes.

An analytical balance was used to weigh ground powders of *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum*. Masses of 0.2g for 5%, 0.4g for 10%, 1g for 25% and 1.6g for 40% were obtained. The respective masses were then suspended in 4ml of sterile distilled water in 10ml containers and mixed by shaking vigorously. To prepare the 1% solutions a volume of 800µl of the prepared 5% solution was micro-pipetted and added to 3.2ml of sterile distilled water.

A concentrated Azithromycin tablet of 500mg was purchased from a pharmacist in Gweru. To prepare the desired the percentage solutions a quarter of the Azithromycin tablet was cut using a sterile scalpel. The separated piece of tablet was crushed into a powder by the scalpel and dissolved in pipetted 500µl of sterile distilled water in a 2ml Eppendorf tube to make a 25% solution. A 10% solution was made by pipetting 200µl of the 25% solution and topping it up to 500µl with 300µl sterile distilled water in a separate sterile 2ml tube. The 5% Azithromycin solution was prepared by pipetting 250µl of the 10% solution into a sterile 2ml

tube and adding 250µl sterile distilled water with a pipette. A 1% solution was prepared by pipetting 100µl of the 5% solution and pipetting 400µl of sterile distilled water.

The prepared percentage solutions were left for 24 hours before they were impregnated on to filter paper discs.

3.6 In-vitro susceptibility test of *Escherichia coli* to treatments

Bacterial strain *Escherichia coli* was tested for in-vitro susceptibility to the traditional herbs *Elephantorrhiza elephantine, Zanthoxylum chalybeum* and commercial Azithromycin the Kirby Bauer disc diffusion method.

Escherichia coli colonies were isolated from the nutrient agar by a prepared sterile cotton swab and used to inoculate the Muellar Hinton agar in a zigzag manner. Muellar Hinton agar was prepared by weighing 12.2g of the agar powder and dissolving in 320ml of distilled water. The solution was then mixed and heated to boiling point until the powder was completely dissolved. The solution was then autoclaved at 121°C for 15 minutes, and after that, it was poured into petri dishes to cool and set. Impregnated filter paper discs with the medicinal drug treatments under study were placed on the inoculated agar.

3.6.1. Preparation of filter paper disc

Filter paper discs of 6mm diameter were prepared by punching filter paper with a standard paper puncher. The filter paper discs were autoclaved in the standard conditions of 121°C for 15 minutes while wrapped in multipurpose paper and aluminium paper foil. In a lamina flow cabinet, the discs were then impregnated on sterile petri dishes with 20ul of each concentration of the 3 antibacterial solutions by using a pipette. After the discs were impregnated, they were dried by leaving them open in the laminar flow cabinet for about 30 minutes. When the discs were dry, they were placed on Muellar Hinton agar plates that had

been spread plate with *Escherichia coli*, by gently pressing the paper disc into the solid agar. The plates were then inverted and incubated at 37°C for 18 hours, where after the plates were viewed for zones of inhibition (ZOI) and the diameter of the zones recorded.

3.7 Petri dish set up

A total of three filter paper discs for each treatment, *(Elephantorrhiza elephantine, Zanthoxylum chalybeum* and Azithromycin) and for each concentration, (1%, 5%, 10% 25% and 40%) were made. There were five plates prepared, each for the five different concentrations. Each plate had one disc for all three treatments. Each plate was replicated a total of three times. The 40% *Elephantorrhiza elephantine and Zanthoxylum chalybeum* solutions had the third disc as the control which was impregnated with sterile distilled water. Therefore a total of 15 plates were used.

3.8 Measurement of inhibition zones

Inhibition zone diameters, the regions where *Escherichia coli* bacteria did not grow around the filter paper discs, were measured using a mathematical set clear ruler. The diameters of the rings were then recorded in millimetres.

3.9 Statistical Analysis

Statistical analysis of the data obtained from the zones of inhibition measurements was done using SPSS software. The data generated from the results all conformed to normality, (Normality 0.298<=p>=1, Appendix 5.1), and the data obtained conformed to the assumption of homogeneity of variance, (ANOVA p = 0.689, Appendix 5.2), therefore analysis method of 2 Way ANOVA was used as there were 2 independent variables of treatment drug and concentration of the drug. There was also one response variable of the diameter of bacterial zone of inhibition.

CHAPTER 4: Results

4.1 Macroscopic identification of *Escherichia coli* on MacConkey agar

The swabs that were inoculated on MacConkey agar from the toilets resulted in pink colonies growing on the agar. The colonies once streaked on the agar, separated into single colonies that were circular in shape with entire smooth edges, punctiform, tiny, in size, with a smooth and glistening surface, butyrous, buttery, and moist in texture. The colonies were umbonately elevated, raised in the center more than on the edges and had an opaque lighter pink pigmentation on their edges and a darker hot pink pigmentation in the middle. The colonies had a bright pink halo around them which demonstrated strong lactose fermentation (Appendix 2).

4.2 Bio-Chemical identification of bacterial strain, *Escherichia coli* confirmation

4.2.1 Oxidase test

The oxidase test strip which was exposed to the separated pink colonies from MacConkey agar did not show any colour change, indicating a negative result. The tested strip was compared to a non-tested strip visually to accurately confirm if any colour change had taken place, Figure 4.1.

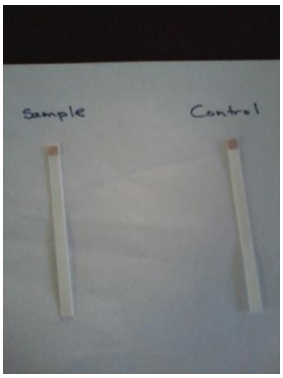

Figure 4.1 Oxidase test strip with non-tested oxidase strip as comparative control.

4.2.2 Catalase test

The catalase test on the pink colonies on MacConkey agar resulted in the hydrogen peroxide and the bacteria producing bubbles in effervescence, indicating a positive result. The bubbles which were produced were white in colour, Figure 4.2.

Figure 4.2 Effervescence produced from hydrogen peroxide and isolated pink colonies.

4.2.3 Citrate test

The pink colonies after being inoculated on citrate agar and incubation resulted in no colour change on the citrate agar, indicating a negative result. The citrate agar remained green in colour, Figure 4.3.

Figure 4.3 Citrate test

4.3 Gram stain morphological identification

The pink colonies from MacConkey agar after gram staining were viewed under the microscope and had a pink colour, indicating a gram negative result. The morphology of the bacteria was that of rods which were all separated individually.

4.4 Measurements of inhibition zones

The commercially used drug, Azithromycin showed the best results as all its zones of inhibition were greater than the traditionally used drugs at all concentrations. Azithromycin 40% represented the control test and it resulted in no zone of bacterial growth inhibition. *Elephantorrhiza elephantine* (Muzezapasi) showed to be more effective than *Zanthoxylum chalybeum* (Mukundanyoka) as it showed some inhibition at lower concentration, of 7mm at 1% concentration and 8mm at 5% concentration unlike *Zanthoxylum chalybeum* (Mukundanyoka) which showed no inhibition at those concentrations at all. At concentrations of 25% and 40%, both *Elephantorrhiza elephantine* (Muzezapasi) and *Zanthoxylum chalybeum* (Mukundanyoka) resulted in similar lengths of zones of inhibition, Figures 4.4 and 4.5.

Azithromycin showed a steady increase in zone of bacterial growth inhibition with the most significant increase being 10mm between 1% and 5% concentrations, Azithromycin's zones of bacterial inhibition ranged from an average of 12mm at 1% concetration to a highest of 27mm at 25% concentration, Figures 4.4 and 4.5.

Elephantorrhiza elephantine (Muzezapasi) showed the most significant increase from 25% concentration to 40% concentration of 7mm. The increases in those zones between 1%, 5% and 10% were that of 1mm. The 25% concentration had an anomaly of 1mm decrease in zone

diameter from the 9mm at 10% to 8mm. The diameters therefore had a range from 7mm at 1% to 15mm at 40% and an 8mm general increase, Figures 4.4 and 4.5.

Zanthoxylum chalybeum (Mukundanyoka) inhibition started at 10% drug concentration and from 25% to 40% concentration a drastic increase in bacterial growth inhibition was shown of about 8mm, similar with that of *Elephantorrhiza elephantine* (Muzezapasi) at the same concentrations. The final concentration had an average zone diameter of 16mm. The average general increase was 5mm, Figure 4.4 and 4.5.

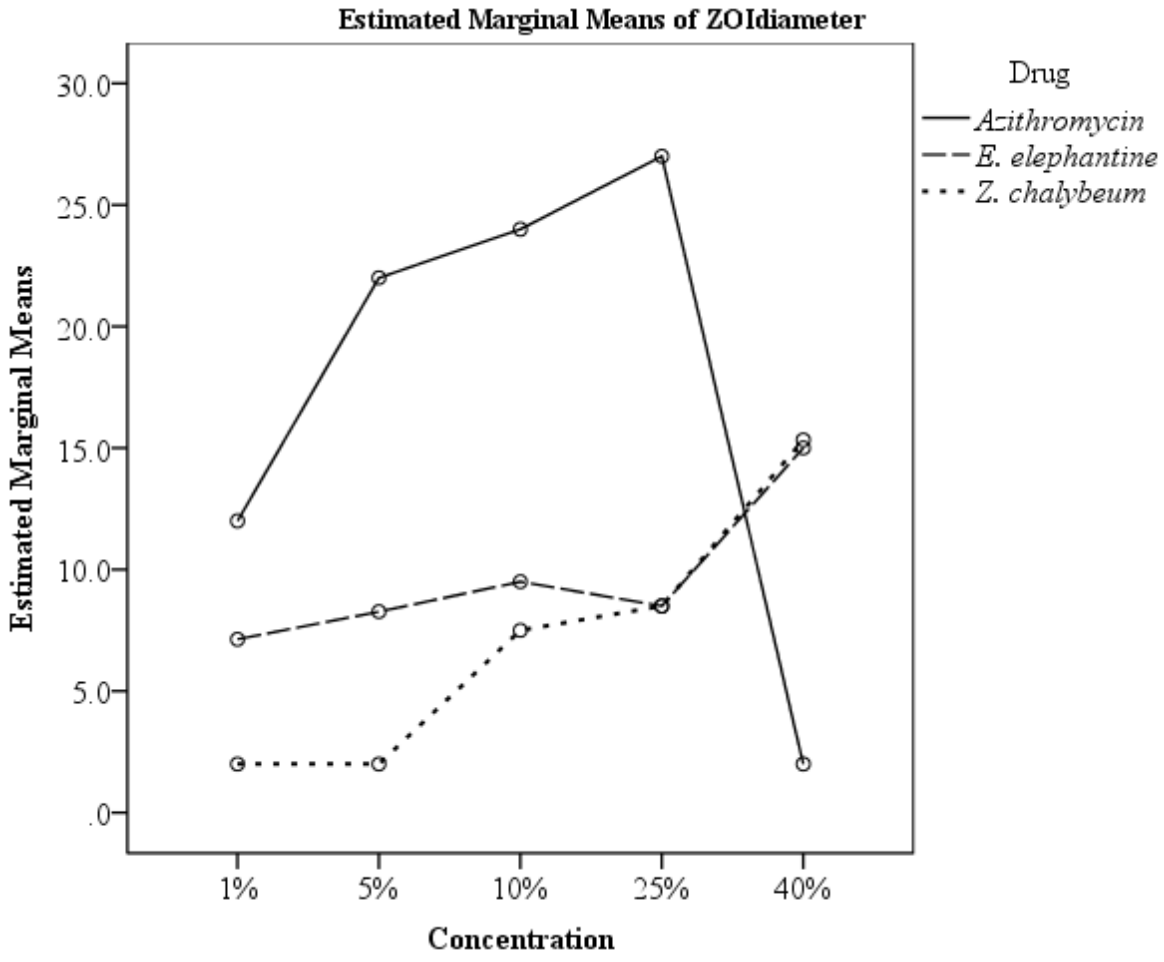

Figure 4.4 Comparative marginal mean graph of the three drugs

The error lines in the bars spanned across the bars for zones of inhibition that were less than 6mm. The rest of the lines spanned portions at the top of bars for all the other zone of inhibition representative bars, Figure 4.5.

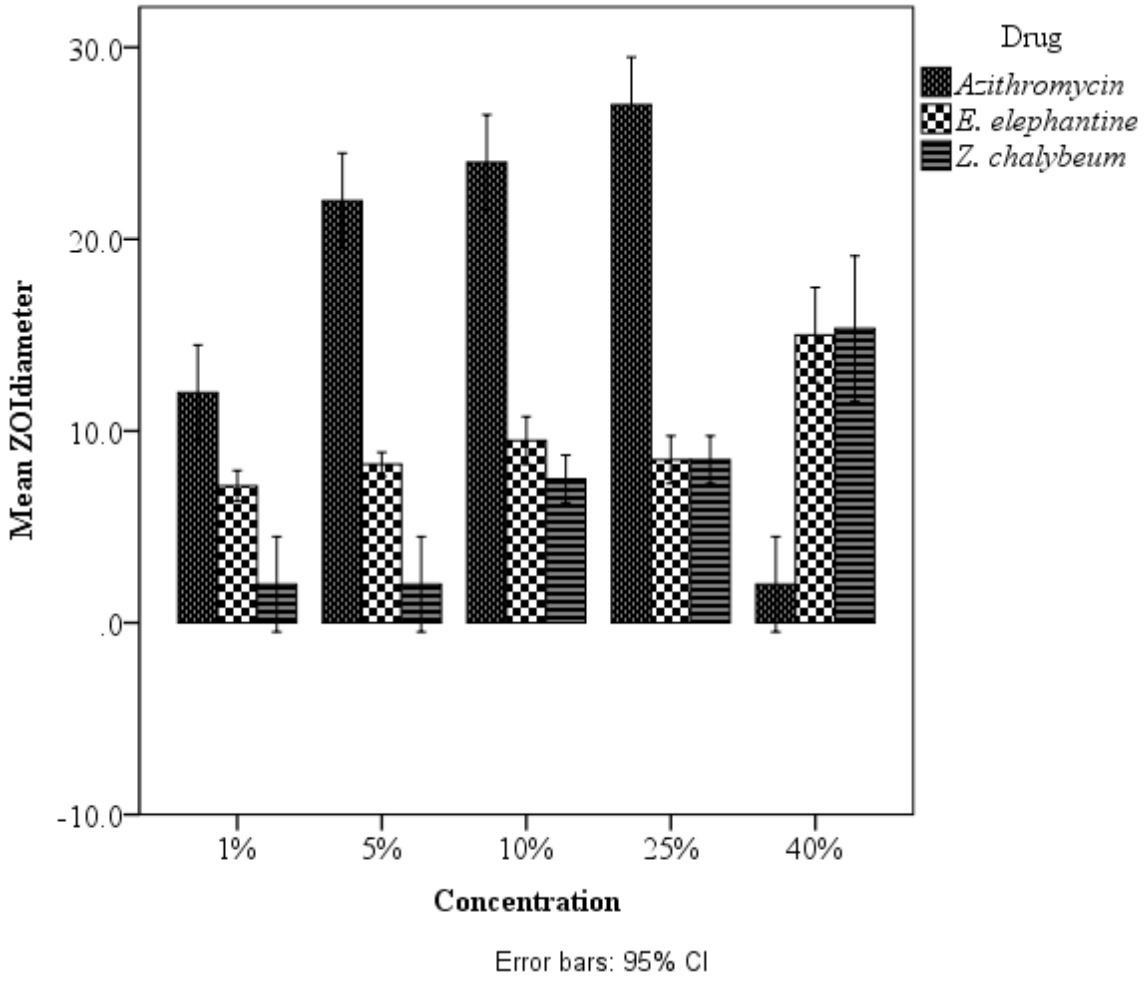

Figure 4.5 Drug mean Zone Of Inhibition (ZOI) comparative error bar graph

The type of drug and level of concentration have an effecton the length of zone of inhibition diameter, (ANOVA, p = 0.00, Appendix 5.2). The significant differences in concentration occurred where increases were greater than 2mm. The significant increases occurred at 5% − 25% for Azithromycin (ANOVA, p = 1.00, Appendix 5.2), 5%−40% for the 2 traditional drugs (ANOVA, p = 1.00, Appendix 5.2) and 10% - 25% for Azithromycin (ANOVA, p = 0.137, Appendix 5.2)

CHAPTER 5: Discussion

5.1 Macroscopic and Microscopic identification of *Escherichia coli*

The morphology of bacteria that grew on MacConkey agar from the toilet swabs correspond with the way that *E.coli* should be on MacConkey agar. The positive catalase test, negative oxidase test, negative citrate test also all comply with the expected results for *E. coli.* The microscopic results of pink coloured rods also correspond with the expected microscopic results for *E. coli.*

Therefore, with all the tests that were carried out complying with the expected results for *E.coli* it can be concluded that *E. coli* was the bacteria that was isolated and used in the study. Gram negative bacteria that grow on MacConkey agar and are catalase positive, oxidase negative are enterobacteriaceae. Those that are also citrate negative are *E. coli,* (Quinn *et al*., 1994)

5.2 Measurement of inhibition zones

The increase in zone of inhibition for Azithromycin was caused by the increase in concentration. As concentration increased, diameter of zone of inhibition (ZOI) also increased. At lower concentrations the drugs inhibitory effect decreases the further it diffuses away from the disc and so smaller zones of inhibition are expected as there is less concentration of the inhibitory agent, (Bauer *et al*., 1966). The most significant increase of 10mm ZOI diameter between 1% and 5% concentrations, could mean that between those two concentrations the active ingredients is significantly increased and thus the large increase in inhibitory effect. Azithromycins Minimum Inhibotory Concentration (MIC) is lower than 1%. Threshold values in literature are the MIC values (National Committee for Clinical Laboratory Standards, 1993). Azithromycins MIC is between 2 μg/ml and 8 μg/ml (National

Committee for Clinical Laboratory Standards, 1993). The increases from 5% to 10% and from 10% to 25% of 2mm and 3mm respectively could mean that no significant changes in active ingredient occurred and so slight changes were observed as concetrations were increased. The increase in *Elephantorrhiza elephantine* (Muzezapasi) zones of inhibition were also caused by increases the drug concentration. The results showed the most significant increase from 25% concentration to 40% concentration of 7mm, this could mean that between these two concentrations the antibacterial property is at high enough concentrations to make significantly large inhibitory zones. At the lowest run concentration of 1% a diameter 7mm zone of inhibition indicates an insignificant antibacterial effect. This is derived from the statistical analysis that showed that 2mm increases are the significant changes, (where ANOVA $p > 0.05$, Appendix 5.2). This, therefore, could imply that the thresholds for effective inhibitory effect start at a minimum of 8mm zone of inhibition. Zone diameters of less than 8mm can mean that the bacteria are resistant and from 9mm going up the bacteria are intermediate and at longer diameter lengths such as 15mm the bacteria are susceptible (National Committee for Clinical Laboratory Standards, 1993).

From the concentrations of 5% with ZOI 8mm, 10% with ZOI 9mm and 25% ZOI 8mm the lengths of the inhibition zones are all considered to be above the threshold. The minimum inhibitory concentration for *Elephantorrhiza elephantine* (Muzezapasi) would be between 1% and 5% if further studies are to be carried out, according to this assay 5% would be the determined minimum inhibitory concentration.

The 25% concentration had an average 8mm diameter zone, which was an anomaly of 1mm decrease in zone diameter from the previous lower percentage concentration. This could have been because between 10% and 25% there is no significant change in inhibitory effect, a difference of 1mm is insignificant, (ANOVA $p < 0.05$, Appendix 5.2).

The increase in *Zanthoxylum chalybeum* (Mukundanyoka) diameter of zone of inhibition was also due to the increase in concentration of the drug. Inhibition started at 10% drug concentration with 7mm ZOI although this was an insignificant inhibitory effect. The active ingredient and concentrations 10% and lower could not diffuse far away enough to cause any significant inhibitory effects. However, the concentration of 25% and a significant inhibitory length of 8mm and so, according this study, is the minimum inhibitory concentration as 8mm diameter is significant enough to qualify. The actual minimum inhibitory concentration could be below 10%. From 25% to 40% concentration a drastic increase in bacterial growth inhibition was shown of about 8mm, similar with that of *Elephantorrhiza elephantine* (Muzezapasi) at the same concentrations, meaning that the active antibacterial agent, allicin, is concentrated enough between the two concentrations to make greatly noticeable differences in inhibition. This could mean that these two plants have the same or very similar antibacterial properties. The final concentration had an average zone diameter of 16mm, which was not a significant difference from that of *Elephantorrhiza elephantine* (Muzezapasi) at 15mm, (ANOVA, $p < 0.00$, Appendix 5.2).

Elephantorrhiza elephantine therefore is more potent and effective than *Zanthoxylum chalybeum* as it has a lower minimum inhibition concentration, but the two plants at higher concentrations have a similar potency. Mode of action of active ingredient in the different drugs could also have an impact on the results obtained.

5.3 Mean bars

The mean bars show the possible error that could have been done while measuring the lengths of bacterial inhibition. Where the bars span the whole bar it shows that those results are insignificant, *Zanthoxylum chalybeum* (Mukundanyoka) at 1% and 5% as the true recordings were of zero at those two percentages. Azithromycin at 40% was representative of the control test and had recordings of zero thus the error bar spanned its whole bar length.

5.4 Recommendations

This study can be used as a baseline study to further studies can be done to better evaluate the antibacterial properties of *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum.* Further assays should be run to determine the actual MICs for the two plants, between 1% and 5% for *Elephantorrhiza elephantine* and between 10% and 25% for *Zanthoxylum chalybeum.* Azithromycin should have lower doses than the plants as it is more effective. *Elephantorrhiza elephantine* can have doses at low concentrations as it is effective to from 5%. Children can therefore be given milder *Elephantorrhiza elephantine* drug doses at its low concentrations which will be safer for them. Both *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* can have standardised similar doses from 25% as their effectiveness is similar at that concentration. Further studies for *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* at higher concentrations than 40% should be done to see if there are any changes in bacterial inhibitory activities. Standardised drugs should be manufactured for *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* and be administered for free in poor communities or be sold at more affordable prices than commercial drugs such as Azithromycin.

5.6 Conclusions

Elephantorrhiza elephantine is more effective than *Zanthoxylum chalybeum* as it has inhibitory effects at lower concentrations than 1%. Both *Elephantorrhiza elephantine* and *Zanthoxylum chalybeum* are scientifically effective in the inhibiting bacterial growth and have similar potencies and effectiveness at concentrations from 25% going up and so work similarly at high concentrations. Both plants are safe to use as their antimicrobial activities are within acceptable ranges under those of the commercial drug Azithromycin and so would pose no harm when taken to treat diarrhoeal diseases.

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Appendices

Appendix 1: Gram stain technique

The gram staining technique was done of smearing dropping a drop of water on a slide inoculating an isolated colony in the drop of water and heat fixing with a flame from a burner. The smear was the covered with primary stain crystal violet for about 1 minute and that was washed away with gentle running water. The smear was then rinsed with alcohol until all the purple colour was washed away from the smear. Then iodine was added to the stain for about 30seconds and washed away with gentle running water. The smear was then stained with counter stain, safranin, for about a minute. The safranin was then washed off using gentle running water. The slide was then blotted dry with multipurpose paper before it was placed on the microscope for viewing.

Appendix 2: Macroscopic *Escherichia coli* identification

Streaked swab coliforms on MacConkey agar.

Appendix 3: Pictures of the resultant drug effect on bacterial growth for all percentages

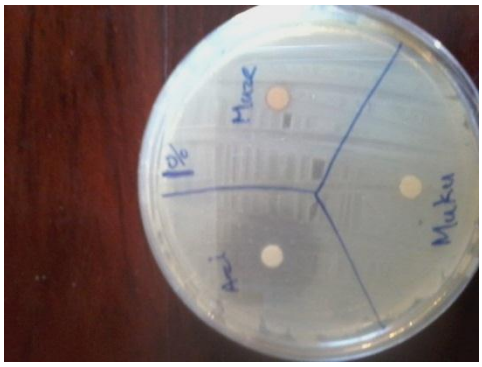

Figure 5. 1% zone of inhibitions

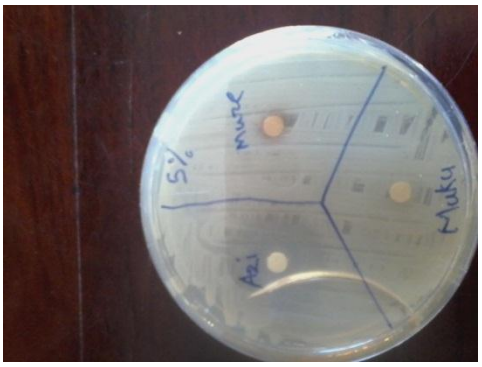

Figure 6. 5% zone of inhibitions.

Figure 7. 10% zone of inhibitions

Figure 8. 25% zone of inhibitions.

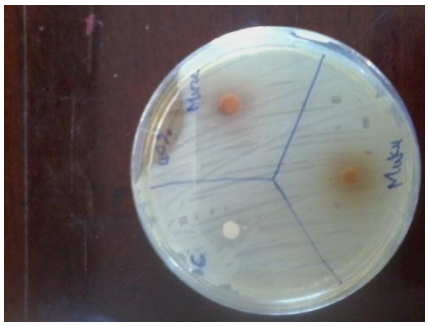

Figure 9. 40% concentration zone of inhibitions and control

Appendix 4: Raw data from diameters of inhibition zones.

Table 1: Raw data for zone of inhibition diameter lengths

Percentage concentration	Azithromycin diameter of zone of inhibition in mm	*Elephantorrhiza elephantine* (Muzezapasi) diameter of zone of inhibition in mm	*Zanthoxylum chalybeum* (Mukundanyoka) diameter of zone of inhibition in mm
1%	12.0	7.5	0
	13.0	7.0	0
	11.0	6.9	0
5%	21.0	8.0	0
	23.0	8.5	0
	22.0	8.3	0
10%	25.0	9.0	7.0
	24.0	10.0	8.0
	23.0	9.5	7.5
25%	27.0	9.0	8.0
	26.0	8.0	9.0
	28.0	8.5	8.5
40%	Control	16.0	17.0
	Control	14.0	14.0
	Control	15.0	15.0

Appendix 5: SPSS output

4.1 Tests of normality

Table 2: SPSS output tables

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	Df	Sig.
ZOIdiameter	1%	.175	3	.	1.000	3	1.000

a. drug = azithromycin, concentration = 1%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	5%	.175	3	.	1.000	3	1.000

a. drug = azithromycin, concentration = 5%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.

ZOIdiameter	10%	.175	3	.	1.000	3	1.000

a. drug = azithromycin, concentration = 10%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	25%	.175	3	.	1.000	3	1.000

a. drug = azithromycin, concentration = 25%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	1%	.328	3	.	.871	3	.298

a. drug = elephamuze, concentration = 1%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.

ZOIdiameter	5%	.219	3	.	.987	3	.780

a. drug = elephamuze, concentration = 5%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	10%	.175	3	.	1.000	3	1.000

a. drug = elephamuze, concentration = 10%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	25%	.175	3	.	1.000	3	1.000

a. drug = elephamuze, concentration = 25%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.

ZOIdiameter	40%	.175	3	.	1.000	3	1.000

a. drug = elephamuze, concentration = 40%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	1%	.175	3	.	1.000	3	1.000

a. drug = zanthochamuku, concentration = 1%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	5%	.175	3	.	1.000	3	1.000

a. drug = zanthochamuku, concentration = 5%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.

ZOIdiameter	10%	.175	3	.	1.000	3	1.000

a. drug = zanthochamuku, concentration = 10%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	25%	.175	3	.	1.000	3	1.000

a. drug = zanthochamuku, concentration = 25%

b. Lilliefors Significance Correction

Tests of Normality[a]

	Concentration	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
ZOIdiameter	40%	.253	3	.	.964	3	.637

a. drug = zanthochamuku, concentration = 40%

b. Lilliefors Significance Correction

4.2: 2 Way ANOVA

Levene's Test of Equality of Error Variances[a]

Dependent Variable: ZOIdiameter

F	df1	df2	Sig.
.772	14	30	.689

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + concentration + drug + concentration * drug

Multiple Comparisons

Dependent Variable: ZOIdiameter

Tukey HSD

(I) drug	(J) drug	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
azithromycin	Elephamuze	7.720*	.3197	.000	6.932	8.508
	Zanthochamuku	10.333*	.3197	.000	9.545	11.122
elephamuze	Azithromycin	-7.720*	.3197	.000	-8.508	-6.932
	Zanthochamuku	2.613*	.3197	.000	1.825	3.402
zanthochamuk	Azithromycin	-10.333*	.3197	.000	-11.122	-9.545

u	elephamuze	-2.613*	.3197	.000	-3.402	-1.825

Based on observed means.

The error term is Mean Square(Error) = .767.

*. The mean difference is significant at the .05 level.

Table 3: Multiple Comparisons

Dependent Variable: ZOIdiameter

(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1%	5%	-3.711*	.4128	.000	-4.908	-2.514
	10%	-6.622*	.4128	.000	-7.819	-5.425
	25%	-7.622*	.4128	.000	-8.819	-6.425
	40%	-3.733*	.4128	.000	-4.931	-2.536
5%	1%	3.711*	.4128	.000	2.514	4.908
	10%	-2.911*	.4128	.000	-4.108	-1.714
	25%	-3.911*	.4128	.000	-5.108	-2.714
	40%	-.022	.4128	1.000	-1.219	1.175
10%	1%	6.622*	.4128	.000	5.425	7.819
	5%	2.911*	.4128	.000	1.714	4.108
	25%	-1.000	.4128	.137	-2.197	.197
	40%	2.889*	.4128	.000	1.692	4.086
25%	1%	7.622*	.4128	.000	6.425	8.819
	5%	3.911*	.4128	.000	2.714	5.108
	10%	1.000	.4128	.137	-.197	2.197
	40%	3.889*	.4128	.000	2.692	5.086

	1%	3.733*	.4128	.000	2.536	4.931
	5%	.022	.4128	1.000	-1.175	1.219
40%	10%	-2.889*	.4128	.000	-4.086	-1.692
	25%	-3.889*	.4128	.000	-5.086	-2.692
		-3.733*	.4128	.000	-4.797	-2.669
1%	40%					
5%	40%	-.022	.4128	1.000	-1.086	1.042
10%	40%	2.889*	.4128	.000	1.825	3.953
		3.889*	.4128	.000	2.825	4.953
25%	40%					

Based on observed means.

The error term is Mean Square (Error) = .767.

*. The mean difference is significant at the .05 level.

b. Dunnett t-tests treat one group as a control, and compare all other groups against it.