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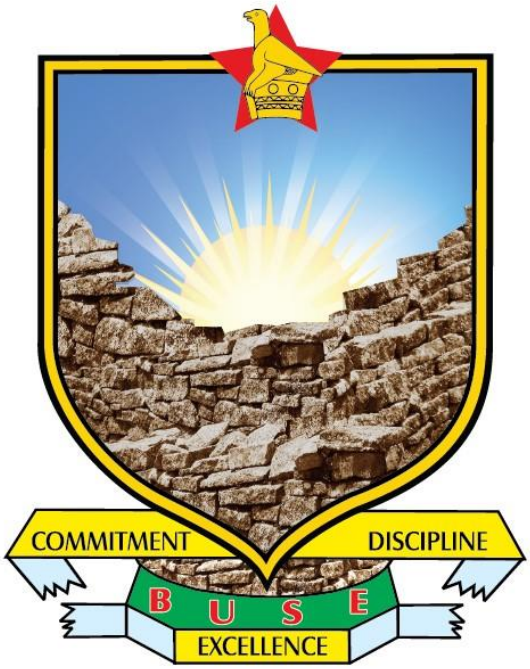

Investigating the antimicrobial activity of *Myrothamnus flabellifolius* plant extract against *Staphylococcus aureus*.

BY

BRIAN, R. MUGWENHI (B212786B)

A research project submitted in partial fulfilment of the requirements for the Bachelor of Science Honours Degree in Biological Sciences.

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APROVAL FORM

The undersigned certify that they have read and recommended to Bindura University of Science Education for acceptance of research project titled "Investigating the antimicrobial activity of *Myrothamnus flabellifolius* plant extract against *Staphylococcus aureus"* submitted by Brian R. Mugwenhi (B212786B).

Signature of Student:  Date: 16/06/2025

Signature of Supervisor: pmunosiyei Date: 09/09/2025

Signature of Chairperson:  Date: 11/09/25

DECLARATION

I, Mugwenhi Brian Richard (B212786B) declare that this research project herein is my own work and has not been plagiarized from another source(s) without acknowledgement of the respected author(s) either electronically or otherwise.

Signature:  Date: 16/06/2025

Supervisor

I, Pias Munosiyei declared that I have supervised this thesis and I am satisfied that it can be submitted to the Biological Sciences Department, Faculty of Science and Engineering at Bindura University of Science Education.

Signature: pmunosiyei Date: 09/09/2025

DEDICATION

I dedicate this thesis to family and friends.

ACKNOWLEDGEMENTS

To begin with, I want to express special thanks to the Almighty Jehovah for guidance and protection throughout this research project. I also want to give a thankful hand to my supervisor Mr. P Munosiyei for his support and help, he stood by me and made sure I understand what I was doing. Additionally, I would like to extent my appreciation to Mr. Katsande and his staff members of Astra Biological Sciences Laboratory who assisted me with all laboratory technical aspects for this research project. Finally, I want to thank my parents and guidance who helped and supported in all life aspects for the successful conclusion of this research project.

ACRONYMS

ANOVA- Analysis of Variance

MBC- Minimum Bactericidal Concentration

mg- milligrams

MIC- Minimum Inhibitory Concentration

ml- millilitre

mm- millimetre

MRSA- Methicillin Resistant *Staphyloccocus aureus*

MSA- Mannitol Salt Agar

SPSS- Statistical Package for the Social Sciences

TSA- Tryptic Soy Broth

ZOI- Zone of Inhibition

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ABSTRACT

The rapid emerging of specific bacterial types that are resistant to standard antibiotics is causing a global challenge for the effective use of current antimicrobial therapies, enforcing the need for the exploration of alternative novel antimicrobial agents that include traditional plants. It serves as a medicinal herb that is native to Southern Africa, well known for its resilience to extreme weather desiccation and its traditional uses in treating infections and inflammatory conditions. This research study is based on analysis of antimicrobial potentials of *M. flabellifolius* leaf plant extracts against *S. aureus*. The main aims were to identify and characterize the phytochemicals found in the plant's leaves, use of disk diffusion method to assess the antimicrobial effectiveness of the extracts, finding the minimum concentrations that inhibit or kill bacteria. Preliminary chemical-based screening of bioactive compounds showed the presents of phytochemicals which include flavonoids, tannins, phenolics as well as terpenoids. Solvent extraction techniques were done whereby methanol was the best extraction solvent with extraction mean yield percentages of 14.0, 13.4 and 11.8. Ethanol had mean extraction yield percentages of 8.5, 6.0 and 1.16 whilst aqueous obtained 5.0. Methanolic extract had the most antibacterial activity against *S. aureus* shown at all concentrations (12.5mg/ml, 25mg/ml, 50mg/ml, 100mg/ml). At 100mg/ml mean ZOI for methanol was 20.0mm, ethanol had 17.0mm and 13.0mm for aqueous. Methanol extract had MIC of 25mg/ml, ethanol extract had 50mg/ml and aqueous extract had 100mg/ml. Methanol extract had 50mg/ml MBC, ethanol extract had 100mg/ml and aqueous extract was not bactericidal. Alamycin and pure distilled water were involved as controls for positive and negative tests. Data analysis of the results has revealed the effectiveness of the extracts by showing p value(s) of 0.000 that is below the alpha significance level of 0.005. Hence these findings showed that *M. flabellifolius* has antimicrobial activity against *S. aureus* and holds a potential future as a natural novel agent of antibiotics, particularly against gram positive bacteria.

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CHAPTER 1.

1.1 BACKGROUND.

Morden scientists have increased the focus and awareness concerning the application of medical herbs to manage and cure infections caused by different pathogens. The use of plant material to treat pathogenic infections comes with a variety of consumer-based advantages. Natural plant-based medicines are rarely accompanied by side effects, inexpensive, safe and effective to use (Beuchat, 2002). The application of medicinal plants plays a crucial role in supporting many lives, especially from developing countries such as Zimbabwe and others from Asia and Africa. These plants are also beneficial not only for medical purposes but they promote the cultural security and traditional rituals of local people. Nipa (2011), outlined that plants play an undeniable role in their relationship and deeply connection to cultural beliefs of local people hence making their use a friendly application. Globally, an estimate of about 75 to 90% rural populations sorely depends on certain plants as the only available health care system and this excludes western nations (Suntar *et al.*, 2010).

Myrothamnus flabellifolius (Mufandichimuka) is a wild bush like plant commonly known as the resurrection plant, this plant has been in use for ages throughout human history for managing other conditions such as depression as well as wound healing. The plant grows on sandstones or rocks and it dries up during the whole summer season and seizes all the physiological processes. In recent studies, it has been discovered that *Myrothamnus flabellifolius* contains some antimicrobial effects against certain microorganisms (Quave & Horswill, 2014). This research focuses on investigating the antimicrobial effect of *Myrothamnus flabellifolius* plant extract on a specific type of bacteria which is *Staphylococcus aureus*. *Staphylococcus* is a pathogenic bacterium species responsible for a large number of infections, it can easily develop resistance for common antibiotics (Lee *et al.*, 2006).

1.3 Problem Statement

According to WHO (1999), Despite the availability of many antimicrobial agencies to tackle microbial infections, *S. aureus* remains one of the most notorious bacteria contributing to much of the infections being spread in hospitals. This project will be focusing on the need to engage a new antimicrobial agent that can help tackle resistance *S. aureus*.

1.4 Aim of the study

The aim of this work is to evaluate the effects of *Myrothamnus flabellifolius* leaf plant extracts against *Staphylococcus aureus* so as to observe if the plant extracts can act as an antimicrobial agent.

1.5 Justification

This research is considered relevance because it will help to gain insight on the antimicrobial activity of *Myrothamnus flabellifolius* towards *Staphylococcus aures*. The plant materials have been traditionally used for controlling some human ailments which includes depression, anxiety and wound healing but never approved for pharmaceutical uses on the *Staphylococcus aureus* antimicrobial management. Scientific confirmation of the antimicrobial effect of *Myrothamnus flabellifolius* against *Staphylococcus aureus* will help in the scientific research fields for the phytopharmaceutical efforts to contain and manage the emerge of a large number of antimicrobial resistance bacteria.

1.6 Objectives.

1. To extract and characterise *Myrothamnus flabellifolius* plant extracts using ethanol, methanol and water.
2. To analyse the antimicrobial effect of *Myrothamnus flabellifolius* extracts on *S. aureus* using disk diffusion method.
3. To determine the MIC and MBC of *Myrothamnus flabellifolius* extracts against *S. aureus*.

1.7 Research Questions

1. Which solvent is most efficient in extracting leaf plant material of *Myrothamnus flabellifolius* and what bioactive elements the plant leaves possess?
2. Which solvent extract of *Myrothamnus flabellifolius* has the most antimicrobial activity against S. aureus?
3. Which extract concentrations have the minimum inhibitory and minimum bactericidal activities?

1.8 Hypothesis.

- H_1: *Myrothamnus flabellifolius* plant extracts exhibit antimicrobial activity against *S. aureus*.

- Ho: *Myrothamnus flabellifolius* plant extracts does not have antimicrobial effect on *S. aureus*.

1.9 Significance

This research will significantly add and contribute to the efforts of discovering new antimicrobial agencies from natural herbal sources all across Africa. The study will also produce evidence for the traditional use of *Myrothamnus flabellifolius* in treating infections within Zimbabwe and Africa as a whole. As there is an emergency need for novel antibiotic drugs because of the rapid evolving of multi-drug resisting pathogens, this study focuses on exploiting medicinal effects of *Myrothamnus flabellifolius* on *S. aureus*. Hence the potential to avoid the outbreaks and growth of resistant bacterial strains. Additionally, pharmaceutical medicines have been confirmed to be very expensive in most of developing countries, so it can be wise to exploit and asses the medicinal potentials of plants that are readily available to manage a variety of infections.

1.10 Limitations of the study

There was absence and shortages of some reagents for further phytochemical screening of other phytochemicals of *Myrothamnus flabellifolius*. Also, there was shortages of other bacterial strains hence the use of *S. aureus* as it is one of the few viable bacteria found at the Astra laboratory. Extraction solvents were also limited per person hence subjecting the study to extraction errors.

1.11 Delimitations

The study focuses only on the leaf extracts of the plant hence neglecting other plant parts such as the barks and roots which maybe found to contain different phytochemicals and bioactive compounds. This study is also delimited to focus on only *S. aureus*, neglecting other bacterial strains such as *Escherichia coli* which may also exhibit relevant characteristics.

CHAPTER 2: LITERATURE REVIEW

2.1 Antimicrobial Drug Resistance

First time antibiotics were discovered between the period of 1920s and 1940s, whereby Alexander Fleming discovered penicillin in 1928. These were the early days of antibiotics and the discoveries changed a whole narrative on the treatment of bacterial infections. Antibiotic golden ages stretched from 1940s to 1960s where there was now a rapid development of new antibiotics that were effective on dealing with previously deadly bacterial infections. From this age, antibiotics became a cornerstone of medicine. From around 1970s to 1980s, the antibiotics started to be misused and this influenced initial stages of antimicrobial resistances (Ventola, 2015). The antimicrobial resistance problem continued to emerge up until 1990s to date. The rising antimicrobial resistance rates and the emerging of new bacterial resistance mechanisms resulted in a worldwide health crisis which promotes the necessity of novel antibiotics or alternative treatments (Suntar *et al.*, 2010).

Antimicrobial resistance has become a major health threat worldwide, with an estimate over 1 million deaths every year (WHO, 2014). If left alone, the issue of antimicrobial resistance and associated deaths may rise with a 70% increase by 2050, reaching almost 2 million direct fatalities and over 8 million related deaths (O'Neill, 2016). Methicillin resistance staphylococcus aureus (MRSA) is an important contributor to antimicrobial resistance related global deaths with an increase of 130% deaths between the period of 1990 to 2021. Also, gram negative bacteria especially the ones resistant to carbapenems have a huge threat on human health with deaths increase rates of 127 000 in 1990 to 216 000 in 2021 (CDC and Prevention, 2019)

According to WHO (2014), there are fifteen antibiotic resistance bacterial families that have a critical threat to global human health. The bacteria are classified into three main groups that are critical, high and medium groups, these groups are in the order of priority (WHO, 2014). On the critical priority list of resistant bacteria, there are gram-negative bacteria that have acquired the ability to resist last resort antibiotics and can spread resistance to other bacterial species. High priority bacteria include those with a gram-positive structure like *Staphylococcus*, which are responsible for severe bacterial infections (WHO, 2014). Bacteria causing common infections are classified in the medium priority, they cause treatable infections

but they require special attention due to the rapid spread of resistance, these include *Streptococcus pneumoniae* that is resistant to macrolides (Madigan *et al.*, 2015; Ryan *et al.*, 2022)

Other than medicinal plants, there are also some natural sources of antimicrobial sources that includes microorganisms themselves, these include fungi and bacteria, for example, the production of penicillin from fungi of the genus *Penicillium* such as *Penicillium chrysogenum* (Katzung et al., 2018). Also, vancomycin and streptomycin are produced by bacteria *Amycolatopsis orientalis* and *Streptomyces griseus* respectively (Ryan et al., 2022). The production of antibiotics from natural resources has also its own environmental or ecological advantages as they do not risk the activities of microorganism involved in geochemical cycles (Prescott *et al.*, 2017). Antimicrobial resistance causes lethality effects that can be comparable to those of global virus pandemics such as COVID-19. Although COVID-19 has rapid lethal effects, antimicrobial resistance has long term effects that are potentially more trajectory due to the ability of making antibiotics ineffective. Additionally, COVID-19 patients are prone to secondary bacterial infections which are hard to cure or manage mainly because of the antimicrobial resistance (CDC and Prevention, 2019).

2.2 *Staphylococcus aureus*

Staphylococcus aureus bacterial cells are also referred to as 'staph', the bacteria are usually found on human skin and in the nostrils of health individuals. S. aureus is generally not harmful to the hosts up until it becomes opportunistic pathogen due to the influence of certain conditions. The bacteria are usually responsible for a variety of pathogenic infections that ranges from minor skin conditions to a wide range of lethal chronic conditions. Skin infections include boils, impetigo, cellulitis and other severe infections resulting in diseases such as endocarditis and osteomyelitis. The capacity of S. aureus to develop resistance towards many antibiotics is now a major global concern. The continuous emerging of a type of S. aureus that is very stubborn and resistant to methicillin is also a bigger threat to huma health (Norton, 1986).

2.3 Plant extracts antimicrobial types, uses and advantages.

With approximately 390 000 plant species (*Angiosperms*) supporting the whole earth, in which about 15 000 to 23 000 species are consumed by humans and animals, leaving about 39 000 to 58 500 species for medicinal purposes (Madigan et al., 2015). These statistics give evidence that only about 10% to 15% of plant species are used as traditional medicines or they contain potential medicinal properties, hence the need to conserve many plant species (Cowan, 1999).

Use of medicinal plants have gained popularity on a global scale, although it is most common in developing countries, the use of plant-based remedies has also gained its way into developed countries including America and Europe (Ali *et al.*, 2019). Plants with healing properties such as Ginko Biloba, St. John's Wort and Turmeric are evidently used in developed countries (WHO, 1999). On a justified note, there is adequate evidence to state that plant antimicrobial medications and remedies have saved lives especially in developing countries (Davies & Davis, 2010; Evans, 2009; Quave & Horswill, 2014).

2.4 Description of Myrothamnus flabellifolius.

The *Myrothamnus flabellifolius*, widely referred as the resurrection plant. The plant is characterized by a woody shrub that is native to the arid regions of southern Africa, including Zimbabwe (Matotoka & Masoko, 2024). The plant belong to the Myrothamnaceae family commonly known for its unique characterization of the ability to survive extreme dehydration and revive suddenly upon rehydration. *Myrothamnus flabellifolius* grows to a height of about 0.2 to 1.2 meters, the plant possesses an extensive root system that can penetrate rocky crevices hence an access to moisture from minimal soil depths. During hot and dry seasons, *M. flabellifolius* shows a desiccated and lifeless appearance, but it rapidly rehydrates and regains its green coloration within hours of rainfall (Matotoka & Masoko, 2024). *Myrothamnus flabellifolius* is widely used in folk medicine across southern Africa, also the leaves are usually prepared into teas.

Figure 1 Myrothamnus flabellifolius plant, A. fresh & B. dry

2.5 Phenolic Compounds.

These are compounds or molecules that are obtained from plants and have antioxidant and antimicrobial characteristics. Phenolic compounds use different mechanisms to establish their antibacterial activities towards most gram-positive bacteria. The mechanisms include the disruption of cell membrane, the inactivation or denaturation of proteins, biofilm formation inhibition, antioxidant activity modulation and quorum sensing interference. Plants have phenolics in various parts such as fruits, leaves and even in the trunks Katerere *et al.*, 2003). For the cell membrane disruption, phenolic compounds connect with bacterial membrane whereby disrupting the structure and function of the membrane. The result of this action is the change in bacterial cell membrane permeability thereby causing a profound leakage of important nutrients (Katerere *et al*., 2003). Phenolic compounds have a tendency of binding to bacterial proteins and this results in the denaturation of the proteins hence affecting processes such as DNA replication. Also, phenolic compounds can affect the quorum sensing of bacteria hence inhibiting biofilm formation (Ncube *et al.*, 2019). As the data of Ncube *et al.*, (2019) suggested, *Mangifera indica* phenolic extract showed promising antimicrobial effect towards *Staphylococcus aureus* and *Staphylococcus epidermidis*. Also, observations by Liu *et al.*, (2018), illustrated that phenolic extracts from *Hibiscus acetosella* had antimicrobial effects on *S. aureus* and *P. aeruginosa*. Phenolics extracted from *Scutellaria baicalensis* root showed great antimicrobial activities towards some viral and bacterial isolates (Katerere *et al.*, 2003)

2.6 Alkaloids.

Alkaloids can be known as a group of naturally occurring plant compounds and they often posses important biological characteristics. As of to date, there are a variety of identified alkaloid compounds and some of them have been used in traditional medicine since ages ago, but modern scientists are still exploring them for modern drug development (Liu *et al.*, 2018). Alkaloids have shown promise in fighting against microorganism such as bacteria, fungi and viruses. They depend on the use of different mechanisms such as enzyme inhibition, protein synthesis inhibition and DNA binding. Alkaloids can also destroy the structure and function of bacterial cells and disrupt microbial metabolism processes. Examples of antimicrobial alkaloids include quinine and berberine, which hinders growth of most microbes (Mabhiza *et al.*, 2013). As observed by Mabhiza *et al.* (2013), alkaloid extracts of *Callistemon citrinus* leaves demonstrated quality negative growth rates on *Staphylococcus* and *Pseudomonas* species. The author has believed that the alkaloids have interfered with the bacterial cell membranes and affect bacterial metabolism and growth. Also, alkaloids from *Doryphora aromatica* leaf extracts, specifically phaeantharine trifluoroacetate has exhibited antimicrobial

efficacy on *S. aureus* obtaining an MIC value of 9.9µm (Liu *et al.*, 2018). In the study by Pech-Puch *et al.* (2016), alkaloid compounds from *Agelas citrina* has also exhibited a good fight against many bacteria inclusive of *Staphylococcus*.

2.7 Saponins.

Saponins are a group of plant phytochemicals that most plants possess, they have a soap-like characteristic and traditional practitioners have used them for hundreds of years and have also muchly contributed to the development of modern medicine. Saponins have shown to have antimicrobial effects through several mechanisms that include permeabilization of microbial cell membranes promoting the leakage of important ions and molecules (Mthembu *et al.*, 2015). Inhibition of enzyme activities and disruption of cell membranes are also other mechanisms that saponins use for their antimicrobial effects. According to Bohm and Kocipal-Abyazan (1994), saponins extracted from *Paullinia pinnata* have shown antimicrobial and antifungal properties against multiple types of bacteria and yeast.

2.8 Terpenoids.

These are metabolite compounds exhibited by plants, terpenoids are widely known for their unregular structures and a wide range of biological activities. Terpenoids possess some antimicrobial activities through several mechanisms. The mechanisms include, the ability of terpenoids to disrupt the formation of biofilms in microorganisms' communities, the inhibition of enzyme activities, microbial cell membrane destruction and permeabilization (Moyo *et al.*, 2013). Research done by Rita *et al.* (2022), have shown the effectiveness of terpenoids extracts of *Myrmecodia pendans* against Streptococcus mutants, a bacterium that promotes tooth decay as well as oral infections. Also, terpenoids extracts from *Commiphora spp.* resin have shown some antimicrobial properties towards *Mycobacterium tuberculosis*, a bacterium that is responsible for tuberculosis in humans (Zhu & Wang, 2016). Examples of specific terpenoids include limonene and carvacrol in which both have shown promising effects as antimicrobial and antifungal agencies. Terpenoids can also have other medicinal properties that include anticancer effects, antioxidant effects as well as reducing swelling and inflammation (Moyo *et al.*, 2013).

2.9 Other Compounds.

Other plant-based metabolites that can be also classified as phytochemicals because they have shown to exhibit some antimicrobial properties that can be taken into consideration are waxes, sterols, fixed oils, essential oils, carbohydrates and mucilage (Smith & Stitt, 2007). Initially,

these metabolites were believed to be primary plant metabolites only to later discover that they exhibit secondary metabolites properties. Some plant carbohydrates such as polysaccharides, although they are primary metabolites have shown to inhibit microbial growth, and mucilage has shown to contain some biological activities such as preventing microorganisms from spreading by creating a surface barrier which reduces microbial growth. These other phytochemicals can also act as supporting agencies or work in synergy with major phytochemicals to help combat antimicrobial resistance of different microbes. Sterols can have antimicrobial effects due to their ability to interfere with microbial membrane structure and function and causing damage to the membrane (Tiwari & Rana, 2015). On average, these plant-based metabolites have a role in the plant extract effectiveness against microbes thanks to its capacity to inhibit microbial growth, modulate the immune response as well as the ability to possess a physical barrier against different microorganism (Tiwari & Rana, 2015).

2.10 Advantages of plant-based antibiotics

Medicinal plants are important for developing novel antimicrobial medicines and also for the concept of drug discovery as a whole. They pose a variety of advantages that supports their effectiveness as valuable antimicrobial resources. Effective plant-based therapies can significantly lower costs associated with antimicrobial resistance hence promoting economical budget of phytochemical derived antibiotics in contrast with synthetic antibiotics (Tiwari *et al.*, 2011). Most plants were used traditionally as medicine, so they exhibit a good foundation for the modern drug discoveries (Efferth & Koch, 2011). Medicinal plants are also readily available for use as compared to the time taken to synthesize and use of new, complex and synthetic pharmaceuticals. Plant extracts can also exhibit synergistic effects with other antibiotics, thereby enhancing the overall antimicrobial efficacy (Mthembu *et al.*, 2020). Plant extract-based antibiotics have shown to pose fewer negative side effects due to their natural origin and also their complex, natural composition of phytochemicals contributes to their sustainability in suppressing rapid bacterial resistance development (Moyo *et al.*, 2013)

2.11 Safety of Antibacterial Phytochemicals.

Although medications from medicinal plants have shown to be less associated with harmful side effects, standard safety precautions cannot be ignored. Precautions such as ensuring appropriate dosages and concentrations, attentiveness to allergies, potential interactions with other medications and proper quality controls of the plant-based medications should be appropriately monitored. Ensuring proper dosages will help to avoid toxicity and adverse effects, potential reactions for sensitive individuals should also be kept in attentiveness, caution

is also needed when using plant-based medications in pregnant women (Rita *et al.*, 2022). Also, good and accurate regulatory compliance is also necessary to ensure the safety of plant derived phytochemical medicines. Adequate guidance and consultation with trusted health professionals is also necessary for safety use of phytochemically produced medications.

2.12 Effectiveness of Phytocompounds.

Many researches confirmed the ability of phytocompounds to inhibit microbial growth through a wide range of in vitro assays. The compounds have good effectiveness as they possess antimicrobial activities, antivirulence activities and antibiofilm activities towards many pathogenic microorganisms. Mthembu *et al*. (2020), outlined that some species of the pepper plant contain phytochemicals that can exhibit antifungal and antibiofilm activities against *Candida spp*. Also, some pepper species have shown to have phytochemicals that have antimicrobial activities towards *S. aureus* and *B. subtilis* in relatively lower concentrations. Alkaloid extracts of pepper have shown to exhibit antibiofilm activities by reducing *Serratia marcescens* biofilm formation ability and making bacteria more susceptible to the treatment (Rita *et al.*, 2022). Although these compounds have shown promising results of antimicrobial activities, in vivo assessment is still limited especially in developing countries.

2.13 The current state of medicinal plant extract uses in Zimbabwe.

In Zimbabwe, medicinal plant extracts are much more used in traditional settings as compared to the scientific use. In vitro scientific researches and experiments to evaluate the efficacy of different medicinal plant extracts has been done and it is still in progress as scientists are fighting the battle of containing different diseases and infections (Maroyi, 2013). However, in vivo experiments concerning the efficacy of medicinal plants are greatly limited in Zimbabwe (Gelfand *et al.*, 1985)

Examples of some scientifically researched medicinal plants in Zimbabwe include *Aloe ferox* commonly known as Bitter Aloe in which studies have shown that it possesses anti-inflammatory, antimicrobial and antioxidant activities. It contains compounds such as anthraquinones that have therapeutic potential in wound healing and digestive health (van Wyk & Wink, 2004). *Sutherlandia frutescens* known as cancer bush has been used and incorporated to alleviate symptoms of HIV or AIDS as well as other chronic diseases (Mills *et al.*, 2005). *Sutherlandia* spp has demonstrated immunomodulatory effects and is rich in flavonoids and other phytochemicals that may help fight infections and support overall health. Research

indicates its potential in managing stress and inflammation. Leone *et al.*, (2015), also mentioned that *Moringa oleifera,* common name 'drumstick tree' is rich in vitamins, minerals, and antioxidants. Studies suggest it has anti-inflammatory, antimicrobial and cholesterol-lowering properties, making it beneficial for various health conditions.

The pharmacological involvement and application of natural plants within Zimbabwe, highlights an intersection between traditional knowledge and modern science. Many plants that have been used for generations are now the subject of scientific research, validating their efficacy and opening avenues for new therapeutic agents (Maroyi, 2013). This integration not only preserves cultural heritage but also contributes to the development of sustainable healthcare solutions in the region.

CHAPTER 3: MATERIALS AND METHODS.

3.1 Sample collection & Research sites.

This research was done at Astra Biological Sciences laboratory at Bindura University of Science Education located in Mashonaland Central, about 54.7 miles North-East of Harare, Zimbabwe. Six health *Myrothamnus flabellifolius* plants were collected from a farming rural area in Gutu, Masvingo province. The place is known for the growth of this resurrection plant and also proper identification and documentation of the plant species was confirmed.

3.2 Sample Preparation and Extraction

Fresh and health leaves of *Myrothamnus flabellifolius* were harvested from their parental plants, distilled water was used for rinsing, removing dirty and debris. Leaves were exposed to atmospheric air under a shade for direct sunlight protection.

Mortar and pestle were used as a grinding tool to crash the dried leaves into fine powder. The produced powder was then subjected to solvent extraction using ethanol, methanol and distilled water following the maceration method. Thereafter, 20g, 15g and 10g of plant powder material were soaked in ethanol, methanol and distilled water inside conical flasks. Aluminium foil paper was used to seal the conical flasks with the mixtures and left for 48 hours under room temperature; there was interval shaking to ensure even distribution and increase absorption. Whatman No. 1 filter papers and funnels were used for filtration so as to separate solid plant debris from the solvent plant extracts and obtain crude extract. The collected extracts or filtrates for each solvent were thickened under vacuum at low heat. Produced or yielded extracts were transferred from flasks to sterile vials and the vials were stored at 4˚C in a refrigerator ready for antimicrobial tests. Percentage yield was calculated with the method:

Total yield (%) = (Mean mass extraction in grams ÷ Original mass extraction in grams) ×100

3.3 Microbial Strain and Culture Conditions.

3.3.1 Bacterial Strain.

The test organism that is *Staphylococcus aureus (S. aureus)*, was obtained from a suspended laboratory culture collection and cultured in liquid broth. After culturing of the S. aureus in liquid broth, the bacteria were sub-cultured in well prepared mannitol salt agar to obtain a pure culture. Morphological and biochemical confirmation of the bacteria was done by examining the shape, size, colony nature and pigmentation of the bacterial isolate. The isolate was also

subjected to gram staining test, catalase test, and mannitol fermentation, using the following methods:

3.3.2 The Gram staining test

Gram staining is a laboratory test technique that is used for bacterial classification into two main groups using cell wall composition. With this test, strains are only grouped as Gram positive or Gram-negative bacteria. The procedure was performed by inoculating a full loop of bacteria from MSA plate, the inoculum was transferred on a microscopic viewer slide, mixed with clean water to create a smear. Air was used for drying followed by heat fixing. The sample was then stained with crystal violet dye followed by 60 seconds wait. The sample was flushed with water, gram's iodine was added and waited for 60 seconds. The sample was there-again flushed with water and of 95% ethanol was added then a wait period of 30 seconds. After that, there was addition of Safranin and waiting for 60 seconds before washing the sample with distilled water. The sample slide was blotted, dried in open air and observed using a light microscope. Gram positive *S. aureus* appeared as purple, cocci and grape like clusters under the microscope (Baron & Finegold 1990).

3.3.3 The Catalase test.

The difference between staphylococcus species and streptococcus species can be made clear by the application of a catalase test. The catalase enzyme has the ability to drive the decomposition of H_2O_2 into water and oxygen. The test can be easily applied to test for Staphylococcus species as they tend to be catalase positive while their counterparts such as Streptococcus species produce negative results in a catalase test. The procedure was done by the application of a single drop of hydrogen peroxide solution (30%) on a thin microscope slide. Using sterile loop, well isolated colony was inoculated from Mannitol Salt Agar plate and emulsified on the glass slide with hydrogen peroxide. An observation of immediate rapid bubbling was made which indicated the catalase activity hence confirming the positive test for *S. aureus* (Macfaddin, 2000).

3.3.4 Mannitol Salt Agar fermentation

MSA is a specialized agar that selects and differentiates with high salt concentration of 7.5% NaCl that allows the growth of only staphylococci and mannitol fermentation produces an acid that turns phenol red indicator yellow. The procedure was carried out by using a sterile inoculation loop to inoculate *S. aureus* onto MSA plate. Incubation of the culture plate was done for a 24hour period under 37˚C and after 24 hours, yellow colonies grew in the plate and it was confirmed that there was mannitol fermentation hence a positive MSA test for *S. aureus*.

3.4 Preparation of Inoculum.

A pure isolate of *S. aureus* colony was cultured inside a 10ml test tube of broth medium; the culture was incubated for one night at a temperature of 37˚C. There was regular shaking and a comparison was made to match the standardized bacterial suspension, 0.5 McFarland standard, equal to about 1×10^8 CFU/mL.

3.5 Antimicrobial Activity Assessment.

3.5.1 Disk Diffusion Method.

Well prepared MSA plates were inoculated with a non-contaminated swab to evenly spread the standard bacterial strain across agar surface to create a lawn of bacteria. Filter paper disks of 6mm in diameter were soaked in varying concentrations of 100, 50, 25 and 12.5 milligrams per millilitre of *Myrothamnus flabellifolius* distillates, diluted using Ringer's solution and prepared using methanol, ethanol and water as extraction solvents. The paper disks were placed onto the agar surface. Pure distilled water was used to soak disks for use as negative control. Alamycin served as a known effective control. Cultures were grown at 37˚C for 24-hour period (Das *et al.*, 2010). After incubating, the cultures were observed to see if the bacteria have grown and all zones of inhibitions were measured by a means of a ruler. A fruitful outcome that showed *S. aureus* susceptibility to *M. flabellifolius* extract was the inhibition of bacterial growth towards the paper disks.

3.5.2 Determination of the MIC of *M. flabellifolius.*

A macrodilution technique was applied to assess and find MIC values of *M. flabellifolius* extract, giving the lowest concentration value that showed complete absence of turbidity. Binary successive dilutions of plant extract were created aseptically in Tryptic Soy liquid medium (TSB) inside 5ml test tubes. All extract concentrations of 12.5, 25, 50 and 100 milligrams per millilitre were tested. A test tube with TSB only was used as a negative control and an alamycin treated test tube was the positive. Standardized inoculum of bacteria was then added to each test tube. Each test tube received a 50µl bacterial inoculum with a final concentration of approx. 1.5×10^6 CFU/ml. Cultures were grown at 37˚C for 24 hours with partial sealings to permit aeration (Eloff, 1998). After incubation, tubes were analysed for visual turbidity. A clear tube indicated no bacterial growth whereas a cloudy tube indicated some bacterial metabolism activities. MIC values were noted, indicating the lowest concentration needed for each extract to prevent visible *S. aureus* growth.

3.5.3 Determination of the MBC of *M. flabellifolius.*

A total of 10µl from each test tube, that is test tubes with the recorded MIC values was inoculated and streaked on MSA plates. Incubation occurred at 37˚C for a 24-hour period. Post incubation analysis of the culture plates was done for the lowest concentration showing ≤ 3 colonies or ≥ 99.9% killing. Presence of colonies at MIC influenced the MBC values to be recorded as 2× the MIC values.

3.6 Phytochemical screening and preliminary analysis.

Biochemical tests were used to identify presence of different bioactive compounds associated with *Myrothamnus flabellifolius* plant. Alkaloids were identified by a Mayer's test, terpenoids were identified by Salkowski test, ferric chloride was used to identify tannins, a Shinoda test was used for flavonoids and phenolic compounds were identified by ferric chloride test.

3.6.1 Alkaloids test

M. flabellifolius plant extract was mixed with potassium mercuric iodide that is Mayor's reagent, a greenish colour emerged which confirmed the presence of alkaloids.

3.6.2 Terpenoids test

Terpenoids were confirmed by a Salkowski test whereby a mixture of extract, 2ml chloroform and 3ml concentrated sulfuric acid formed a light brown colour, indicating a positive test for terpenoids (Harborne, 1998).

3.6.3 Tannins test

Tannins were confirmed by mixing the extract with 1% ferric chloride solution and a deep green precipitate confirmed the detection of tannins (Trease & Evans 2000).

3.6.4 Flavonoids test

Bioflavonoids were confirmed by mixing one ml sample extract with 1ml strong alkaline solution (10% concentrated NaOH). Observations were made under UV light whereby a yellow colour was formed, confirming the positivity of flavonoids. (Bohm & Kocipai-Abyazan ,1994).

3.6.5 Phenolic test

Phenolic compounds were confirmed by mixing 1 ml extract with 2ml of $FeCl_3$ Solution. Formation and observance of deep green dye confirmed a positive phenolic test (Singleton & Rossi, 1965).

3.7 Statistical Analysis.

Data from the antimicrobial assays that are zone of inhibition and MIC values was analyzed using a statistical software known as SPSS, R. Total results then presented with average mean ± SD. The gaps within groups were analysed using ANOVA with a threshold of significance less than 0.05.

CHAPTER 4: RESULTS.

4.1 Morphological and Biochemical identification of bacterial culture (*S. aureus*).

Post a day incubation period, colonies were observed on MSA petri dishes with golden yellow, round and convex morphology. The colonies were also able to ferment mannitol and they tested positive for catalase test and gram staining test**.**

Sample of *S. aureus* colonies.	Catalase Test	Gram Staining	MSA fermentation
	Positive	Positive	Positive

Table 1 Confirmatory biochemical tests for S. aureus colonies

4.2 Phytochemical Assessment of *M. flabellifolius*: Screening and Characterization.

Extract Phytochemical	Methanol	Ethanol	Aqueous
Alkaloids	+	+	+
Phenols	+	+	+
Tannins	+	+	+
Reducing sugars	+	+	+
Flavonoids	+	-	+

Table 2 Assessment of M. flabellifolius for the exhibition of antimicrobial responsible phytochemicals

Key

\+ = Presence

\- = Absence

Solvent	Original mass (g)	Mean mass of extract (g)	Percentage yield (%)
Methanol	20	2.800±0.3	14.0
	15	2.010±0.2	13.4
	10	1.180±0.1	11.8
Ethanol	20	1.708±0.3	8.5
	15	0.905±0.04	6.0
	10	0.116±0.4	1.16
Distilled water	20	1.008±0.003	5.0

Table 3 Extraction yields of M. flabellifolius

4.3 Effects of different solvent extraction concentrations of *M. flabellifolius* against *S. aureus* growth.

4.3.1 Disk diffusion method

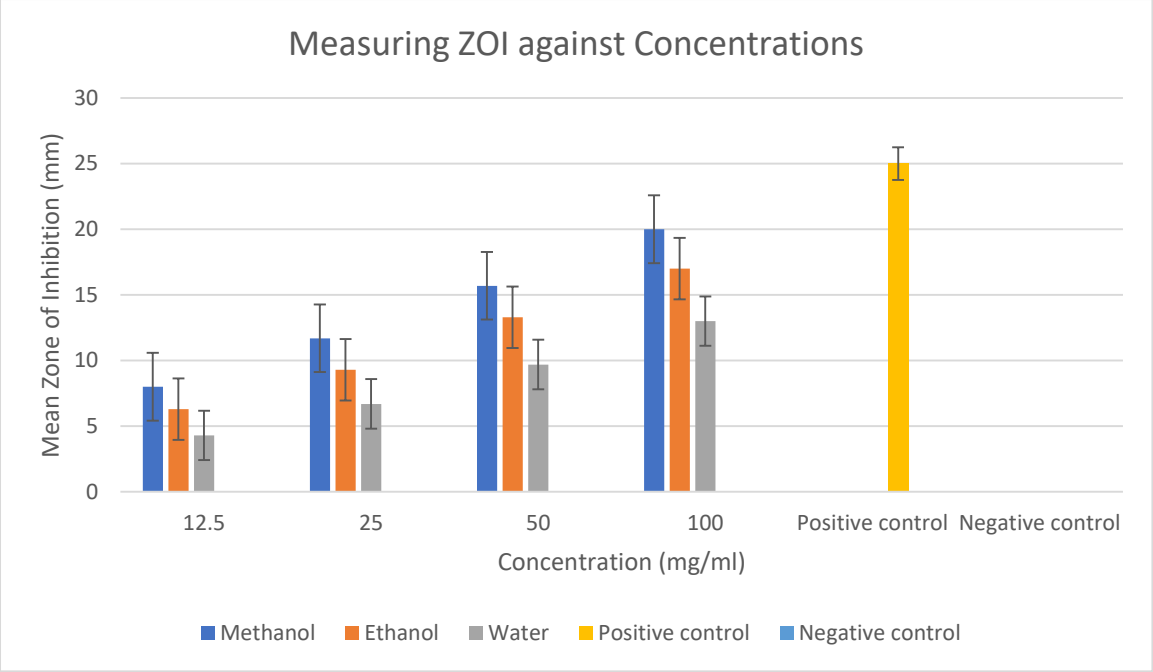

Figure 2 The mean Zone of Inhibitions of filter paper disks treated with different concentrations of M. flabellifolius plant extract

After 24 hours of incubation, the growth of *S. aureus* seized towards the filter paper disks. There was a significant difference between concentrations 12.5mg/ml, 25mg/ml, 50mg/ml and 100mg/ml.

4.3.2 Determination of MIC

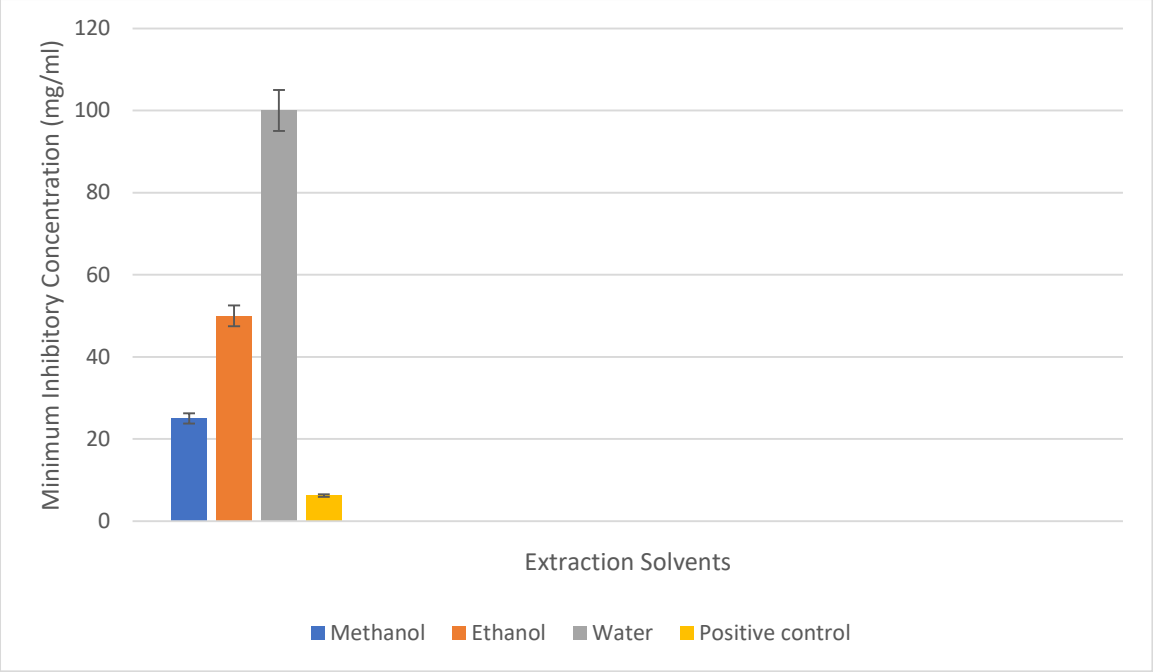

Figure 3 The Minimum Inhibitory Concentrations of different solvent M. flabellifolius plant extracts

There was a significant difference between each solvent with respect to the other, $p < 0.05$.

4.3.3 Determination of MBC

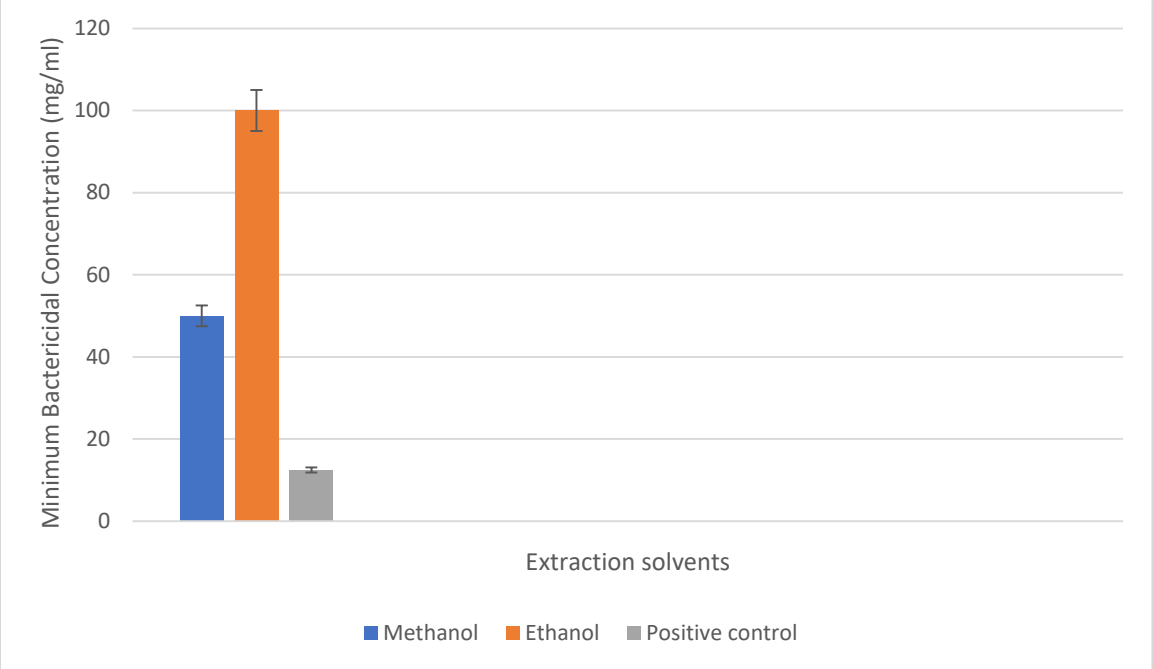

Figure 4 The Minimum Bactericidal Concentrations of different solvent M. flabellifolius plant extracts

4.4 Data Analysis

Statistical analysis was run separately for each solvent group and the results confirmed significant differences among the solvents. For MIC, methanol extract had F value 47.62 and p-0.000, ethanol extract had F value 36.84 and p-0.000. Water extract had F value 28.39 and p-0.000. For MBC, ($F = 90.00$, $p < 0.005$). Hence all groups showed statistically significant differences in bacterial growth inhibition.

CHAPTER 5: DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The characteristics used to identify the test bacteria (*S. aureus*) are in the line with standard characterization of *Staphylococcus*, therefore this indicates that the result findings of the bacterial strain were of the *Staphylococcus* species.

Pure culture of *S. aureus* exhibited typical characteristics under microscopic examination by showing some cocci shaped, clustered gram-positive bacteria and also smooth yellow-golden like colonies were observed on Mannitol Salt Agar (MSA). These features are consistent with standard microbiological identification protocols. However, upon exposure to *M. flabellifolius* extracts, significant morphological disruptions were observed.

Phytochemical analysis of *M. flabellifolius* established the existence of several bioactive metabolites found in the plant's leaves, flavonoids, tannins, alkaloids and also terpenoids were present. Flavonoids and tannins were particularly abundant, which aligns with existing literature on their antimicrobial properties. Flavonoids are known to interfere with microbial cell membranes and energy metabolism, whereas tannins can precipitate proteins in which both will result in bacterial growth inhibition.

Methanol is a polar organic solvent known for its high efficiency in extracting variety of plant bioactive compounds. Methanol extracts indicated were found to possess a high concentration of phytochemicals, (Flavonoids, phenolics, tannins and essential oils). This was supported in the fact that methanol extracts were the most effective in showing antimicrobial activities.

Ethanol, as another polar solvent has showed moderate and noteworthy antimicrobial activities. Due to its moderate polarity, ethanol extractions contained all bioactive compounds needed for antimicrobial assessment, but the bioactive compounds had less intensities compared to methanol extracts.

Water extraction was accompanied by the yield of hydrophilic compounds that were simple sugars, glycosides and proteins. These compounds do not necessarily exhibit strong antimicrobial properties. Important antimicrobial metabolites (flavonoids, alkaloids, tannins

and terpenoids) have shown limited solubility in water hence the poor performance of *M. flabellifolius* water extracts in antimicrobial testing against *S. aures*.

The disk diffusion assay displayed a clear inhibitory effect proportional to dose on *S. aureus* growth. A negative control with entirely distilled water had no zone of inhibition verifying that the antimicrobial activities shown by other extracts were results of the plant extract rather than the extraction solvent. Alamycin as the positive control, produced the highest ZOI of 25mm. Methanol extracts dominated the tests by producing highest inhibition zones across all concentrations. Ethanol extracts also showed inhibition of *S. aureus* growth, but it showed less efficacy compared to methanol extract while distilled water extracts showed minimal inhibition.

At 12.5mg/ml concentration, all the extracts showed weak zones of inhibition. Starting with methanol extract, it showed 8.0mm, followed by ethanol which showed 6.3mm and lastly distilled water extract which showed 4.3mm mean ZOI. From 25mg/ml, there was a notable increment in the ZOI of all extracts, starting with methanol at 50mg/ml, it exhibited a 15.7mm mean ZOI followed by ethanol extract which showed of 13.3mm and lastly aqueous extract with 9.7mm. All extracts at 100mg/ml concentration showed pleasant results and great improvement compared to 25mg/ml and 50mg/ml. Methanol extract showed a mean ZOI of 20mm, ethanol extract showed 17mm and aqueous extract showed 13mm, these extracts at 100mg/ml concentration had very strong inhibitory effects that at times doubled the inhibition zone sizes of 25mg/ml concentration. These findings exhibited a dose dependent antimicrobial activity that aligned to findings of Cowan (1999).

The F-values and p-values confirmed statistic differences among varying concentrations between mean zones of inhibition within each solvent extraction group. The F-value was 58.73 with statistical significance at $p = 0.000$ for methanol, F-statistic for ethanol was 42.31 with a p-value of 0.000 and for aqueous extract, the F-value was 31.76 with a p-value of 0.000. These differences were significant as they produced p values, $p < 0.05$. Post-hoc Tukey tests further validated that each concentration produced distinct inhibitory effects, reinforcing the dose-response relationship. These results suggest that *M. flabellifolius* extract concentrations could be optimized for higher efficacy in antimicrobial formulations against *S. aureus*.

The MIC results further confirmed the disk diffusion results by showing varying degrees of antimicrobial efficacy in response to the solvent used. Methanol extracts displayed the maximum potency featuring an MIC at 25mg/ml, this gave an insight that methanol extract could completely inhibit bacterial growth at this partially lower concentration. MIC values indicating a higher potency in inhibiting bacterial growth. Ethanol extract came in second potency showing MIC at 50 mg/ml, showing moderate inhibitory activity. Lastly, water extract required a higher concentration of 100mg/ml to start inhibiting bacterial growth, this suggested a comparatively weaker antimicrobial potential for aqueous extract. Controls further validated the results as positive control (alamycin) showed inhibition at 6.25mg/ml while the negative control (TSB), just as expected, it showed no inhibition of *S. aureus* growth.

This pattern emphasizes that methanol-extracted compounds inhibit bacterial metabolism or replication at much lower doses. The MIC values reinforce that the bioactive components responsible for antibacterial activity of *M. flabellifolius* are more soluble in organic solvents. These findings align with Kuete (2010), who narrated lower or minimum MICs for methanolic plant bioactive across multiple Gram-positive strains, including *S. aureus*.

The MBC results further agreed with the MIC outcomes but provided deeper insight into the bactericidal capabilities of the extracts. Highest bactericidal activity was observed in methanol extract with a complete growth inhibition of viable *S. aureus* cells at 50mg/ml. At 100mg/ml, ethanol extract showed bactericidal activity, while aqueous extract showed no bactericidal or remained inactive even at 100mg/ml (highest dose tested), this indicated that aqueous extract was only bacteriostatic and needed a far higher concentration for it to be bactericidal. Methanol and ethanol extracts showed MBC values within a twofold range of the MIC, suggesting a bactericidal rather than bacteriostatic effect. Alamycin as the positive control, was effective at 12.5mg/ml whereas TSB (negative control) showed the expected bacterial growth hence no MBC.

These results are significant because bactericidal agents are typically preferred for treating acute type of infections caused by *S. aureus*, preferably MRSA, where swift eradication of the pathogen is critical (Pankey & Sabath, 2004). The negative control showed no bactericidal effects hence confirming that the observed activities were solely due to the plant extracts.

The disk diffusion assay in this study showed that methanol-extracted samples exhibited the largest zones of inhibition, followed by ethanol and water. This finding corroborates a research by Dey *et al.*, (2012), according to whom methanolic plant extracts demonstrate potent antimicrobial efficacy towards *S. aureus* with zones of inhibition exceeding 18 mm at high concentrations. Their study attributed this activity to the presence of flavonoids and polyphenolic compounds, which are known to be efficiently extracted using methanol. Similarly, a study by Steenkamp *et al.* (2004), tested several South African medicinal plants, including *M. flabellifolius*, and methanol emerged as the most effective solvent for extracting antimicrobial agents active against Gram-positive bacteria, including *S. aureus*. The researchers noted that aqueous extracts often failed to demonstrate any measurable antimicrobial activity, which aligns with the current study's observation that water extracts yielded the weakest inhibition zones and had the highest MIC with no MBC value.

Based on considerations of MIC and MBC measurements, this current study found that methanol extracts exhibited the lowest values in both, indicating strong bactericidal activity. This is consistent with previous findings by Fyhrquist *et al.* (2002), who noted that *M. flabellifolius* methanol left extract can demonstrate MIC values as low as covering a spanning of 0.12-0.5 mg/ml on *S. aureu*s. Their study exhibited MBC values close to the MIC values, suggesting that the extract exerted a bactericidal effect rather than merely inhibiting growth.

In contrast, ethanol extracts in both this and previous studies showed moderate antibacterial activity across all concentrations. Dlamini and Masuku (2017) evaluated ethanol extracts of *M. flabellifolius* and found a moderate inhibitory effect against *S. aureus*, with inhibition zones ranging between 10 and 14 mm and MIC values averaging 1 mg/mL at higher concentration. These findings reinforce the current study's observation that while ethanol can extract some plant's bioactive compounds, it is less efficient than methanol.

The less antimicrobial efficacy of aqueous extracts observed in this study is not unusual. According to Eloff (1998), water is often a poor solvent for antimicrobial compound extraction, particularly when it comes to flavonoids, terpenoids, and alkaloids that are not water-soluble. As such, despite the widespread traditional use of aqueous preparations, their actual antimicrobial efficacy may be limited unless combined with other practices or solvents. Moreover, the work conducted by Madivoli *et al.* (2020), explored the microbe-killing properties regarding *M. flabellifolius* from Kenyan highlands and reported similar findings regarding solvent efficiency. They observed that methanolic and ethanolic extracts had

statistically significant inhibitory effects on *S. aureus*, whereas water extracts showed almost no activity, thus confirming a cross-regional consistency in plant pharmacological behavior.

The consistent observation across the literature suggests that solvent polarity plays a critical role in determining antimicrobial potency. Methanol, due to its high polarity appears to extract a wider range of bioactive secondary metabolites than water or even ethanol. This positions methanolic extracts of *M. flabellifolius* as a promising candidate for further development into phytopharmaceuticals, particularly to combat *S. aureus* related infections.

5.2 Conclusion

As of the conclusion drawn from this investigation, it was observed that *Myrothamnus flabellifolius* holds significant antimicrobial potential when extracted using appropriate solvents, especially methanol and ethanol. Methanolic extracts consistently produced the strongest antibacterial responses across all assay namely disk diffusion, MIC and MBC. Ethanol derived extracts showed moderate action, while aqueous extracts demonstrated minimal to no inhibitory effect on *S. aureus* growth. The disk diffusion results revealed a clear gradient of increasing inhibition with higher extract concentration, particularly in methanolic and ethanolic samples. This trend was also reflected in the MIC and MBC data, with the lowest values observed in methanol extracts indicating potent bacteriostatic and bactericidal properties. The water-based extracts, even at full concentration yielded weak or negligible antimicrobial effects, reinforcing the notion that traditional water extractions may not fully harness the therapeutic potential of *M. flabellifolius*. In light of the global challenge posed by antibiotic resistance, the study's outcomes have strong support to potential innovations of plant-based antimicrobial agents. Furthermore, the results lend scientific credibility to the traditional use of *M. flabellifolius*, while highlighting the need to modernize extraction and formulation processes to achieve maximum therapeutic benefits.

5.3 Recommendations

Zimbabwean academic and research institutions should expand and intensify scientific investigations into indigenous plants with medicinal potential, including *M. flabellifolius*. Government bodies such as the Research Council of Zimbabwe and universities with life sciences faculties should fund targeted phytochemical and pharmacological studies to explore the plant(s) full range of bioactivity. Given the superior performance of methanol and ethanol extracts, there might be a need to standardize these extraction solvents for both laboratory and traditional use. Though methanol extract has shown to be highly effective, its toxicity limits the suitability for direct medicinal use, therefore, future works should explore food-grade solvents or derivations of methanolic extraction methods that retain efficacy while ensuring safety. The pharmaceutical sector in Zimbabwe, including small to medium scale enterprises, should be advised to explore the formulation of topical agents, ointments or disinfectants using

M. flabellifolius extracts as these products could serve as cost-effective alternatives in rural healthcare settings where access to synthetic antibiotics is often limited.

Traditional medical practitioners remain an integral part of Zimbabwe's healthcare system, especially in rural areas, hence training programs should be organized to share scientific findings from researches such as this one so as to promote best practices in the preparation and application of plant-based remedies. Emphasis should also be placed on the risks of antimicrobial resistance and the importance of correct dosage and extraction methods. Also, before any commercial or clinical application, it is essential to undertake comprehensive in vivo studies and toxicology of the plant. These studies will provide critical data on safe dosage limits and potential side effects, particularly if the plant is to be used in therapeutic formulations.

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APPENDICES

Appendix A: *S. aureus* in MSA

Appendix B: Negative control (disks dipped in distilled water only), no zone of inhibition observed.

Appendix C: Test-tube macrodilution determining extract MICs in TSB.