A COMPARATIVE STUDY OF THE ANTIMICROBIAL EFFICACY OF A HERBAL PREPARATION TO SYNTHETIC ANTIBIOTICS FOR BOVINE MASTITIS TREATMENT IN ZAMBIA

By

JOSHUA NGWISHA, BVM

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UNIVERSITY OF ZAMBIA

DECLARATION

The investigational effort described in this thesis was conducted at The University of Zambia, School of Veterinary Medicine in the Department of Paraclinical Studies. This work has not been submitted for any other degree and therefore, I **Dr NGWISHA JOSHUA** do hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous submission for a degree, that the work of which it is a record has been done by me and that all sources of information have been explicitly recognised by means of references.

Signature……..

Date ……..

CERTIFICATE OF APPROVAL

This thesis submitted by NGWISHA JOSHUA, has been approved as fulfilling the requirements for the award of Master of Science in Microbiology by the University of Zambia.

Internal Examiner I:

Signature:_____ **Date:**_____

Internal Examiner II:

Signature:_____ **Date:**_____

External Examiner:

Signature:_____ **Date:**_____

Board of Examiners Chairperson:

Signature:_____ **Date:**_____

Supervisor:

Signature:_____ **Date:**_____

DEDICATION

This work is dedicated to my daughter Twasanta, son Benny, and my loving wife Talitha. Thanks for keeping up in all the times I kept away. You're an awesome team guys!

For my Late Mother and Father and my wonderful and supportive Mother-in-Law.

ABSTRACT

Mastitis is a universal concern that accounts for reduced milk yield, loss from milk discarding due to antibiotic residues, increased veterinary costs and early culling of cows. This study aimed to compare the antimicrobial efficacy of locally available ethno veterinary herbal (*Aloe* genus and *Curcuma longa*) fresh formulations to synthetic antimicrobials on bovine mastitis causing bacteria *in-vitro*. Herbal formulations for the treatment of mastitis may not have any residual effects in the milk and blood of the treated cows.

The plant materials of the genus *Aloe* and *Curcuma longa* were sourced within Zambia. The active compounds of these herbs were obtained as crude extracts using the water, ethanol and ethyl acetate solvents. The test microbes comprised of standard controls and field isolates. The agar dilution method was used to determine the antimicrobial activity by observing the susceptibility graded as Susceptible, intermediate or resistant. The synthetic anti-mastitis' susceptibility was compared to that of the herbal preparations. The Minimum Inhibitory Concentrations (MIC) of the herbal formulation was also determined using two-fold serial dilutions.

On average, the agar dilution trials revealed; 41.8% resistance , 13% intermediate and 45.3% susceptibility for the herbal formulation, while the synthetic antimicrobials produced 23.5% resistance, 5.2% intermediate and 71.3% susceptibility. The gram positive microbes (genera *Staphylococcus, Streptococcus, Bacillus, Lactobacillus*) when analysed alone for the agar dilution tests indicated; 18.8% resistance, 25.3% intermediate and 56% susceptibility on the herbal treatment, while the synthetic anti-mastitis formulation averaged; 16.8% resistance, 4.75% intermediate and 78.5% susceptibility. The gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were mostly resistant and were absent in the field isolates assessed and therefore did not warrant sole analysis. Mean MIC was 11.5mg/ml at the 5% serial dilution for herbal extract compared to the Cephalexin (semi-synthetic antimicrobial) MIC for *S. aureus* at 0.5mg/L or 0.0005mg/ml.

The *Aloe* genus and *Curcuma longa* crude herbal formulation from Zambia has antimicrobial efficacy on bovine mastitis causing microbes *in-vitro* and may be used as an alternative to synthetic anti-mastitis preparations.

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LIST OF ABBREVIATIONS & ACRONYMS

AMR- Antimicrobial Resistance

ANOVA- Analysis of Variance

ATCC- American Type Culture Colony

CDC- Centre for Disease Control

CLSI- Clinical Laboratory Standards Institute

DMSO- Dimethyl Sulfoxide

EVM- Ethno Veterinary Medicine

EVP- Ethno Veterinary Practices

GIZ- Deutsche Gesellschaft fur Internationale Zusammenarbeit

MHA- Mueller Hinton Agar

MIC- Minimum Inhibitory Concentration

NCCLS- National Committee for Clinical Laboratory Standards

NHREB- National Health Research Ethics Board

OD- Optical Density

TANUVAS- Tamil Nadu University of Veterinary and Animal Science

TDU- Trans-Disciplinary University

UNZABREC- University of Zambia Biomedical Research Ethics Committee

UNZA SoVM- University of Zambia School of Veterinary Medicine

WHO- World Health Organisation

CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Mastitis is a universal concern that accounts for reduced milk yield as the mammary gland is affected by pathogenic microorganisms. This condition leads to losses as a result of discarding milk due to antibiotic residues usually used to alleviate the condition. This also translates to increased veterinary costs and early culling of cows. Herbal formulations for the treatment of mastitis have been shown to have no residual effects in the milk and blood of the treated cows subsequent to extra-mammary application due to their organic nature that enables ease of metabolism (Balakrishnan et al, 2019). The slow rate at which new antimicrobials are being developed and the advent of antimicrobial resistance in recent times have led to increased interest to research on alternatives such as traditional therapeutics. Ethno medical practice stems back from many centuries ago, and the World Health Organisation (World Health Organisation, 1993) estimates that as many as 80% of the world's people depend on traditional medicine for their primary health care needs. Several researchers have noted the availability and widespread use of Ethno Veterinary Medicine in rural parts of Southern Africa and have stressed on the need to undertake further research into the available potential and for the validation of herbs in veterinary medicine to reduce disease control costs (Syakalima et al, 2018; Chinsembu et al, 2014) and curb antimicrobial resistance.

The current and expected growth of the world's population warrants an increased production of high-quality animal protein. Dairy farming is regarded as one of the important ways of satisfying this need to meet the growing demand for milk, especially in developing countries. The focus on crossbreeding and increasing the productivity of dairy cattle has, besides enhanced milk production, also resulted in an increased use of agro-chemicals, mainly antibiotics and anti-parasite drugs (Groot and Van't Hooft, 2016). The residues of these agro-chemicals, if not managed properly, could leak into the environment, affecting natural processes, biodiversity, and soil life. Public health can also be affected due to residues in milk and meat, especially in countries with insufficient food quality controls. These processes contribute to the growing global

threat to human and animal health posed by multi-resistant microbes (Groot and Van't Hooft, 2016). In their discussion of the differences and similarities of dairy farming, and the effect on public and environmental health, between the Netherlands, India, Ethiopia, and Uganda, Groot (2016) elaborated on the strategies that had been developed during the Exchange Motive (E-Motive) project to reduce the use of antibiotics and other chemicals in dairy farming. Most notable and of interest in their multi-national Natural Livestock Farming five-layer approach is the strategy of revitalizing ethno veterinary knowledge and the use of medicinal plants to counter current and future livestock production challenges.

In order to bridge the knowledge gap between the traditional undocumented ethno veterinary practices and the conventional, countries such as India and the Netherlands have taken deliberate steps to advance ethno veterinary research and practices. These steps have involved training of farmers and orienting field veterinary physicians on the principles, theory and application of ethno veterinary health sciences (Tamil Nadu University of Veterinary and Animal Sciences, 2011). Furthermore, one of the immediate demands by those working on antimicrobial resistance is to reduce the use of antibiotics in animal production. It is inarguable that the unwarranted and haphazard use of antibiotics in humans, food production and animal health care, has resulted in the establishment of antimicrobial resistance (Marshall and Levy, 2011).

Ranganathan (2017) reviews that issues related to antimicrobial resistance may be minimized by mainstreaming ethno veterinary practices in the veterinary curriculum and research as this effort might facilitate herbal research in academia with hypotheses rested with traditional healers on primary health care of livestock and poultry. Ranganathan (2017) additionally argues that this would prevent further loss of ethno veterinary knowledge through increased research within the logic of local strategies and enhance livestock production and health. The advantage of the possibility of such practices minimizing occurrence of synthetic antimicrobial residues in milk, meat and eggs can thus not be overemphasized.

The main question under experimentation in this study was on the effect of the locally available ethno veterinary herbal formulations of *Aloe vera* and *Curcuma longa* (TANUVAS formula) on the microbial activity of Bovine mastitis causing bacteria *in-*

vitro. Furthermore, comparison was made to the effect of synthetic antimicrobials currently in use in Zambia. The herbal formulation carries with it, the prospective promulgation as an alternative for treatment of bovine mastitis. We focused on assessing the in-vitro activity of the herbal remedy by determining the antimicrobial activity of the extract of the formulation. The study enriches us with knowledge on the local and cheaper possibilities of managing conditions of economic importance in livestock production using what is locally and readily available. This study therefore raises the urgent need to promote the complementary use of Ethno Veterinary Medicine (EVM) in livestock production on a wider scale than in its current form as a mere living tradition, in order to complement the fight against antimicrobial resistance. The study particularly focused on the EVM management of bovine mastitis and by so doing, it comparatively looked at reducing the use of synthetic antimicrobials.

1.2 Problem Statement

The natural diversity of the Zambian vegetation makes it possible for most herbs of ethno veterinary importance to thrive in addition to the cultivation prospect. However research into the usefulness of these herbs in Veterinary Medicine and their relevance to providing alternative, cheaper and local solutions capable of impacting the battle against antimicrobial resistance in Zambia has not been adequately studied. This study experimented and provided indications on whether or not the locally available herbs; *Aloe* genus and *Curcuma longa*, in Zambia could produce the same cure effects and results in the management of mastitis as had been revealed in other countries such as India with similar ecological diversities and mastitis challenges, with a view of comparatively providing a useful alternative to synthetic antimicrobials.

1.3 Study Justification

Milk and milk products are an important source of nutrition and income globally. Mastitis treatment can be lengthy and unrewarding using synthetic antimicrobials, thereby raising the potential risk of farmers retaining milk containing antimicrobials on the market so as to limit their losses, thus contributing to antimicrobial resistance especially in the human population. Locally available and produced ethno veterinary herbal formulations have the potential of providing alternative cheaper and local solutions adept in impacting the fight against antimicrobial resistance in Zambia hence the need to conduct this research. In India for instance, 240 bulk milk samples collected

from the farmers of different milk cooperatives from Kerala and Karnataka before and after intervention showed a reduction in antibiotic residues of 18 to 49% in the milk. This indicated that Ethno Veterinary Practice based natural products are an effective alternative to synthetic chemicals in dairy farming, hence justifying their introduction in Zambia.

The experimentally unstudied potential of our Zambian savannah in comparison to other regions of the world with similar climatic conditions poses a challenge to undertake research into local herbs as solutions to local animal and human health challenges. Preliminary findings indicated that herbs such as *Aloe vera* and *C longa* are readily available in Zambia. The question that remained to be answered is on whether or not the locally available latter mentioned herb varieties in accordance with the TANUVAS formulation could reproduce the same beneficial effects mentioned above and comparatively correlated as alternatives to synthetic anti-mastitis formulations currently in use in Zambia. Therefore, this research probed the effect of locally available ethno veterinary herbal formulations as well as the individual plant materials and made efficacy comparisons with synthetic antibiotics on the microbial activity of bovine mastitis causing bacteria *in-vitro*.

1.4 General Objectives

To assess the antimicrobial efficacy and effectiveness of an *Aloe* genus and *Curcuma longa* ethno veterinary herbal formulation on bovine mastitis causing bacteria in-vitro and compare with synthetic antimicrobials.

1.4.1 Specific objectives

1. To determine and compare the extractive values (yield of extractable matter from the solvents used in milligrams per gram of the dried material) of the herbal extracts in the aqueous, ethanol and ethyl acetate solvents.
2. To determine and compare the *in-vitro* antimicrobial activity of individual *Aloe* genus and *Curcuma longa* extracts on isolated bacteria.
3. To determine the antimicrobial activity of the combined *Aloe* genus and *Curcuma longa* herbal preparation in comparison to the other commercially available synthetic antimicrobials.

4. To determine the minimum inhibitory concentration (MIC) value of the herbal formulation relative to MIC values of common commercially available antimicrobials against the isolated mastitis causing bacteria.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Dairy Production and Mastitis

2.1.1 General Overview

Bovine mastitis is common in both developed and developing countries and is a significant disease in the dairy industry worldwide. Mastitis causes direct and indirect losses through the decrease in milk production, discarded or rejected milk and costs of disease treatment and management including the death of cows through culling (Halasa T., Huijps K., Østerås O. and Hogeveen H. Economic effects of bovine mastitis and mastitis management: A review, *Veterinary Quarterly*. 2007, 29:1, 18-31).

2.1.2 Dairy Production and Mastitis in Zambia

Smallholder dairy farming is understood to be economically viable in Zambia with a major role to play in rural development and poverty reduction (Mumba et al, 2011). However, the occurrence of diseases especially those affecting udder health, directly threaten full attainment of the productivity potential. Hofer, (2015) highlights the negative impact that udder infections pose on milk yields and small scale dairy production as a whole in Zambia.

2.2 Antimicrobial Resistance in the Dairy Industry

2.2.1 General Overview

In the current modern times, livestock production seems to go hand in hand with antibiotic medication to improve productivity. It is well-known that both therapeutic and non-therapeutic antimicrobial use in food animal production is prevalent. Nair et al (2015) indicated that the widespread use of antimicrobials and other veterinary drugs in dairy animals caused residues in animal products and also contributed to the rising number of antimicrobial resistance, both of which are directly related to consumer health hazards. The advent of antimicrobial resistance has not spared the dairy industry globally and if the problem is not tackled exhaustively, it has the potential of causing serious food safety challenges on the human populace. Reports of incidences of milk-borne multi-drug resistant bacteria in processed dairy products such as cheese, reported

in Mexico (CDC, 2018), raise a public health worry that could be addressed through alternatives such as those provided by this study.

2.2.2 Antimicrobial Resistance in the Zambian Dairy Industry

In a recent study in Lusaka Zambia, Kunda (2015) reported a higher percentage of antibiotic residues in raw milk in comparison to what has been reported in many other countries in the Sub-Saharan region. In this study, antibiotic residues from penicillin, cephalosporin, tetracycline, sulphonamide, aminoglycoside and macrolide derivatives were observed to be high (Kunda, 2015).

Furthermore, the incidence of antimicrobial resistance among the smallholder dairy farmers in Zambia (Eriksson, 2013), also poses a supplementary threat to both productivity and human health.

Such occurrences necessitate the need to take more proactive measures to adequately safeguard the health of Zambian consumers.

2.3 Use of EVM in Livestock production and Environmental protection

2.3.1 Overview of EVM in Livestock Production

Morbidity is a major constraint to livestock production and development in rural and peri-urban communities where half of the world's livestock population is found (Wanzala et al, 2005). In Zambia, such areas are not always easily accessible to current veterinary information and services and people face huge animal health challenges. The review by Wanzala et al (2005) further hypothesizes that endurance mechanisms and tactics for continued livestock production are mainly based on people's traditional knowledge from time immemorial. As such, improvement of the lives of the rural people through livestock productiveness must therefore begin by understanding and recognizing the levels of ethnoveterinary medicine in the ethnic way of life. Such an approach also has the potential of fostering environmental conservancy through management strategies for attaining sustainability, accessibility, convenience and affordability of prevailing and new herbal therapies and ethno practices. Coupled with the increased advent of antimicrobial resistance and the slow rate of discovery of new synthetic antimicrobials in recent times, the implementation and upholding of a more economical community-based ecological livestock production system is cardinal. It is equally fundamental to study, assess, promote and integrate the beneficial aspects of

traditional animal health care practices into current primary livestock health care delivery services.

In Zambia, ethno medicines could have been used for a long time, but documentation of these medicines seems problematic. The most commonly used ethnoveterinary remedies in Zambia include *Cassia abbreviata* and *Trema orientalis* which are used in several conditions such as theileriosis, foot and mouth disease, blackleg, bloody diarrhoea, lumpy skin disease, coughing, bloat and hemorrhagic septicemia, while herbs such as *Cissus quadrangularis* and *Piliostigma thonningi* are utilised for worm infestations and cobra snakebites respectively (Syakalima et al, 2018).

2.3.2 Ethno Veterinary Practices in the Dairy Industry

In a topical review, traditional health care approaches were hypothesized by Nair et al (2015) to be effective solutions for humans, animals and agriculture. The Ethnoveterinary program of the Trans-disciplinary University (TDU) of Bangalore documented local ethnoveterinary practices (EVP) in cooperation with TANUVAS. Using the rapid assessment method, 353 out of the 460 documented ethnoveterinary formulations were assessed as safe and efficacious. A total of 140 medicinal plants were used in these formulations. The use of these formulations was subsequently mainstreamed into the livestock primary health care through field veterinarians and farmers. Veterinarians were trained in EVP to manage 15 different clinical diseases in livestock. The vets were also given practical training for identification of the plants and raw drug, preparation of the formulations and the application under field conditions. Farmers were also educated in managing and preventing certain clinical diseases in dairy animals using EVP. The Field studies conducted thereafter using the fresh EVP formulation for mastitis on 314 cases in three Indian states (Kerala, Karnataka and Tamil Nadu) indicated 97% clinical healing. From this research, 240 bulk milk samples collected from the farmers of different milk cooperatives from Kerala and Karnataka before and after intervention showed a reduction in antibiotic residues of 18 to 49% residue in the milk which indicated that EVP-based natural products are an effective alternative to synthetic chemicals in dairy farming (Nair et al, 2015).

Medicinal plants and other natural products are a major resource for preventive, health promotion and curative purposes in dairy farming in developing countries (Van't Hooft et al, 2017). India has an especially rich experience and history of documenting, validating, promoting and legitimizing traditional ethno-veterinary practices and this experience is being shared across the international partners. An additional aspect of this facet is to focus on natural feeds. Natural grasslands that include herbal products are increasingly seen as an important way to reduce antibiotic use in dairy farming as well as to improve soil fertility and close nutrient cycles (Van't Hooft et al, 2017).

2.3.3 Veterinary Homoeopathy in Mastitis Management

Homoeopathy is defined by the Merriam-Webster dictionary (2021) as a system of medical practice that treats a disease especially by the administration of minute doses of a remedy that would in larger amounts produce in healthy persons or animals symptoms similar to those of the disease. In his book on Veterinary homoeopathy, Naveen (2017) provides alternative medical treatments to manage all types and stages of mastitis using homoeopathic herbal remedies from plants such as *Aconite, Byronia* or *Belladonna,* for the management of peracute or acute mastitis, *Echinacea angustifolia,* for mastitis complicated by toxaemia and septicaemia and *Conium maculatum,* for subacute mastitis.

2.4 Ethno Veterinary Medicine Foliage used for Mastitis Management

2.4.1 *Curcuma longa*

Turmeric (*Curcuma longa*) is a rhizomatous herb belonging to the ginger family Zingerberacea and requires a temperature range of between 20-35^{o}C and rainfall between 80-125cm^{3} for its growth according to the "Turmeric Cultivation Guide" (2019). "Zambia Tourism" (2018) estimates the average minimum and maximum temperatures in Zambia to be between the ranges 6.8-34.8^{o}C and rainfall in the range of 74-132cm, conditions that without a doubt enable the cultivation of the herb locally. Knowledge on the cultivation of *Curcuma longa* is necessary if the herb is to be propagated widely and at a larger scale for its medicinal uses in Zambia. Fortunately enough, "Growing Turmeric-An Overview" (2017), provides a guide on how to grow Turmeric in Zambia and to date, the prospects seem very promising.

Curcuma longa is extensively used as a spice, food preservative and colouring material in India, China and South-East Asia (Chattopadhyay et al, 2004). It has been used in traditional medicine as a household remedy for various diseases, including biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis. For the last few decades, extensive work has been done to establish the biological activities and pharmacological actions of turmeric and its extracts. Chattopadhyay et al (2004) further state that curcumin, the main yellow bioactive component of turmeric has been shown to have a wide spectrum of biological actions. These include its anti-inflammatory, antioxidant, anti-carcinogenic, anti-mutagenic, anticoagulant, antifertility, anti-diabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive and hypocholesterolemic activities (Chattopadhyay et al, 2004). Clinically, curcumin has been used to reduce post-operative inflammation. Safety evaluation studies indicate that both turmeric and curcumin are well tolerated at a very high dose without any toxic effects. Thus, both turmeric and curcumin have the potential in modern medicine for the treatment of various diseases. Chattopadhyay et al (2004) in their review also highlight that both curcumin and the oil fraction of Turmeric suppress the growth of several bacteria like *Streptococcus, Staphylococcus* and *Lactobacillus*. The aqueous extract of turmeric rhizomes is also stated to have antibacterial effects and to prevent the growth of *Helicobacter pylori* cytotoxin-associated gene A (CagA+) strains *in-vitro* (Chattopadhyay et al, 2004). Therefore, the combination of the *Aloe vera* and the *C. longa* could potentially have additional synergistic effects against the other bacterial strains where no inhibitory effects were observed from a singular plant extract.

Evaluation of *C. longa* in previous studies has produced remarkable findings. Liju et al (2011) evaluated the chemical composition, antioxidant potential *in-vitro* and *in vivo*, anti-inflammatory, and antinociceptive activity of turmeric oil and found that the main constituent of the essential oil of turmeric was ar-turmerone (61.8%), curlone (12.5%), and ar-curcumene (6.1%). Turmeric oil was found to have *in-vitro* antioxidant activity. Oral administration of turmeric oil for one month to mice was also found to significantly increase superoxide dismutase, glutathione, and glutathione reductase enzyme levels in blood and glutathione-S-transferase and superoxide dismutase enzymes in the liver. The turmeric oil was also found to show a significant

reduction in paw thickness in carrageenan, dextran-induced acute inflammation, and formalin-induced chronic inflammation. The drug was also stated to have produced significant antinociceptive activity at all doses studied. Liju et al (2011) therefore concluded that turmeric oil had potential health benefits as it could scavenge the free radicals and produce significant anti-inflammatory and antinociceptive activities.

2.4.2 *Aloe vera*

Aloe vera (L) Burm f. (synonym= *Aloe barbadensis* Miller) is widely spread and cultivated at both backyard (small-scale) and commercial levels in Zambia. Chinsembu et al (2014) furthermore underline the frequent use of Aloes and other herbs in Ethno Veterinary Medicine within Southern Africa, with most resource-poor small-scale farmers having valuable traditional knowledge of these herbs but only lacking concise data on usage. This is in most part due to scanty clinical trials, confounded by the absence of an Ethno Veterinary pharmacopoeia. Park et al (2009), experimentally revealed the anti-inflammatory activity of Aloin and Aloe-emodin, an attribute of the *Aloe vera*, which could be valuable in the treatment of the inflammatory condition, mastitis.

Studies on *Aloe vera* have shown promising results on gram-positive isolates. Alemdar and Agaoglu (2009) conducted a study to determine the antimicrobial activity of the *Aloe vera* juice against Gram-positive bacteria (*Mycobacterium smegmatis, Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus* and *Bacillus sphericus*), Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and *Salmonella typhimurium*) and *Candida albicans in-vitro*. The disc diffusion method was used to test the antimicrobial activity. Their study revealed that *Aloe vera* juice had antimicrobial activity against *M. smegmatis, K. pneumoniae, E. faecalis, M. luteus, C. albicans* and *B. sphericus,* but showed no inhibitory effect against the other bacterial strains.

2.4.3 Curcuma longa and Aloe vera

In-vitro tests of the anti-mastitis formulation have proved to be hypothetically stimulating. Punniamurthy and Ramakrishnan (2015) in their test of the antimicrobial

activity of the TANUVAS formulation stated that the anti-mastitis herbal preparation had inhibitory effects against pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus* under *in-vitro* conditions. Their study involved the extraction of the active ingredients of the *A. vera* and *C. longa* fresh herbal formulation and tested it for antimicrobial activity against field isolates of mastitis-causing pathogens. Arutselvi et al (2012) in their study phytochemically screened and compared the anti-microbial activity of leaves and rhizomes of turmeric varieties and concluded that both parts of the herb regardless of variety had resounding anti-microbial activity against pathogenic bacterial strains. The agar well diffusion method was most useful in the experiments of both Punniamurthy (2015) and Arutselvi (2012). Arutselvi (2012) also screened the turmeric extract for anti-fungal activity against fungi such as *Candida albicans*, highlighting the broad spectrum potential of the curcumin containing herb, which should stimulate more research on its usefulness.

Field trials of the combined formulation have also yielded very promising results. Balakrishnan et al (2017) in their field trials using the Tamil Nadu University of Veterinary and Animal Science (TANUVAS) ethnoveterinary anti-mastitis remedy reported that the formulated topical combination of *Aloe Vera*, *Curcuma longa* and calcium hydroxide act synergistically to provide an effective 6-7 days cure remedy in clinical mastitis. Their intervention impact analysis also revealed a remarkable reduction in antimicrobial residues in the milk over one year. The research also revealed that the ethnoveterinary remedy had no detectable residues in both milk and blood. Such findings in addition to those of Liju et al (2011) are therefore thought to be a possible game-changer in the use of antibiotics in veterinary practice.

2.4.4 Antimicrobial potential of *Aloe vera, Curcuma longa* formulation with other herbs

Aloe vera and *C. longa* have been successfully studied in combination with other herbs. Pandey et al (2010) in their study formulated and evaluated a herbal ointment containing *Aloe vera*, Neem and Turmeric for antibacterial and antifungal activity using cup plate method for the zone of inhibition and two-fold dilution method for MIC (Minimum Inhibitory Concentration). Their study showed that the Aloe ointment exhibited broad-spectrum antifungal and antibacterial activity. Comparatively, the overall experiment revealed that Aloe and Turmeric ointments showed more

antifungal activity than the Neem ointment and that the mixed ointment of *Aloe vera*, Neem and Turmeric had more prominent antifungal activity than antibacterial activity. Such scientific evidence brings about the possibility of utilization of herbal plants in the treatment of fungal and bacterial infections and the development of anti-bacterial and anti-fungal products.

2.5 Methods used for *in vitro* herbal antimicrobial efficacy tests

2.5.1 Disk diffusion Method

In the disk diffusion method, the bacterial inoculum is adjusted to a certain concentration (usually 0.5 McFarland) and inoculated onto the entire surface of the Mueller-Hinton agar (MHA) plate. The paper disks impregnated with diluted antibiotic solution are placed on the surface of each MHA plate using a sterile pair of forceps. Then the plates are incubated aerobically and the diameter of the zone of inhibition is measured by a ruler or calliper. The bigger the diameter of the inhibition zone, the more susceptible the microorganism is to the antimicrobial.

The major disadvantages of this method were hypothesized as the inability to generate the MIC value and difficulty to examine the susceptibility of fastidious and slow-growing bacteria (Wilkins and Thiel, 1973) compounded by a lack of standard Clinical Laboratory Standards Institute (CLSI) interpretive criteria of disk diffusion results to support natural antimicrobials susceptibility testing; thus, it is unable to explain the zone diameter generated by disk diffusion for natural antimicrobials. Like other agar-based methods, disk diffusion is also stated to be labour-intensive and time-consuming (Klancnik et al, 2010). Cos et al (2006) indicated the inappropriateness of the diffusion method for testing non-polar samples or samples that do not easily diffuse into the agar. They stated that generally, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar. Furthermore, agar-diffusion methods are also stated to be difficult to run on high capacity screening platforms. Due to these concerns, disk diffusion may not be a suitable one to determine the antimicrobial activity of natural compounds.

2.5.2 Agar Dilution Method

The agar dilution technique is a quantitative susceptibility testing method because MIC values can be obtained using this method (Jiang, 2011). In this method, two-fold serial dilutions of an antibiotic are made in Mueller-Hinton agar (MHA) medium and then bacterial suspensions are inoculated on the MHA. The agar dilution method has also been studied extensively as a recommended reference method for the bacteria growing aerobically (CLSI, 2009). The advantages of agar dilution are held to include the ability to simultaneously test the susceptibility of several bacteria in one plate and the ability to test the susceptibility of fastidious organisms since the agar with supplements can adequately support the bacteria growth. However, agar dilution may not be commonly used in most microbiology laboratories due to its time consuming and labour-intensiveness.

2.5.3 Broth Microdilution Method

Broth microdilution is another quantitative reference method routinely used in clinical laboratories. In this method, the susceptibility panel in 96-well microtiter plates contains various concentrations of antimicrobial agents to which, standardized numbers of bacteria are inoculated and incubated overnight at 35^{o}C. The MIC value is observed as the lowest concentration where no viability is observed in the wells of 96-microwell plates after incubation. Broth microdilution is a widely utilized method, allowing for the simultaneous testing of multiple antimicrobials with ease. Compared with the agar-based method, broth microdilution is less laborious and time-consuming. However, limitations of the method primarily are understood to be associated with the lack of or poor growth of many anaerobic microorganisms (Jiang, 2011).

2.5.4 Contrast of Disk diffusion, Agar dilution and Broth microdilution techniques

The importance of selecting the most appropriate antimicrobial susceptibility testing method for herbal assays is immense. Jiang (2011) made a comparative overview of the disk diffusion, agar dilution and broth microdilution methods. Jiang (2011) indicated that the disk diffusion method allowed for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner.

Comparatively, agar dilution and broth microdilution are found to overcome some limitations of the disk diffusion method, primarily that capability to draw a quantitative conclusion by determining the MIC value for antimicrobials (Kim et al, 2007). As such, both agar dilution and broth microdilution are conveniently used for routine antimicrobial susceptibility testing in the clinical laboratory. However, in studies examining antimicrobial susceptibility of natural antimicrobials including *Aloe vera* and *Curcuma longa*, disk diffusion seems to be the most popular method used (Punniamurthy et al, 2015; Arutselvi et al, 2012). Nevertheless, Eloff (2019) in his review highlights that a lot of energy is wasted by using techniques such as agar diffusion that does not work well with plant extracts. Eloff (2019) further states that many manuscripts are rejected before sending to reviewers because the wrong method is used and concludes that antimicrobial activity of plant extracts based on agar diffusion studies have limited value.

CHAPTER 3

3.0 MATERIALS AND METHODS

This experimental study focused on the in-vitro activity of the ethno veterinary herbal formulation containing the plant materials of the *Aloe* genus and *Curcuma longa*. Corroborated *in-vitro* methods were used to screen a combined formulated crude extract of the *Aloe* genus and *C. longa* for antimicrobial activity and the activity was compared to that of synthetic antimicrobials. This served as an indicator of the *in vivo* efficacy of the extract against microbes implicated in bovine udder inflammation.

3.1 Plant Material

To ensure the repeatability of activity, plant materials were sourced from different areas within Zambia. The plant material, *Aloe barbadensis* Miller and *Curcuma longa* used for this study was sourced locally from Luanshya (-13.045408°, 28.455703°) in the Copperbelt province and Lusaka district (-15.370980°, 28.408393°; -15.397228°,28.393323°; -15.402436°,28.389208°) in Lusaka province of Zambia respectively (Figure 3.1), following a non-participatory rapid appraisal (Kemm et al, 2004).

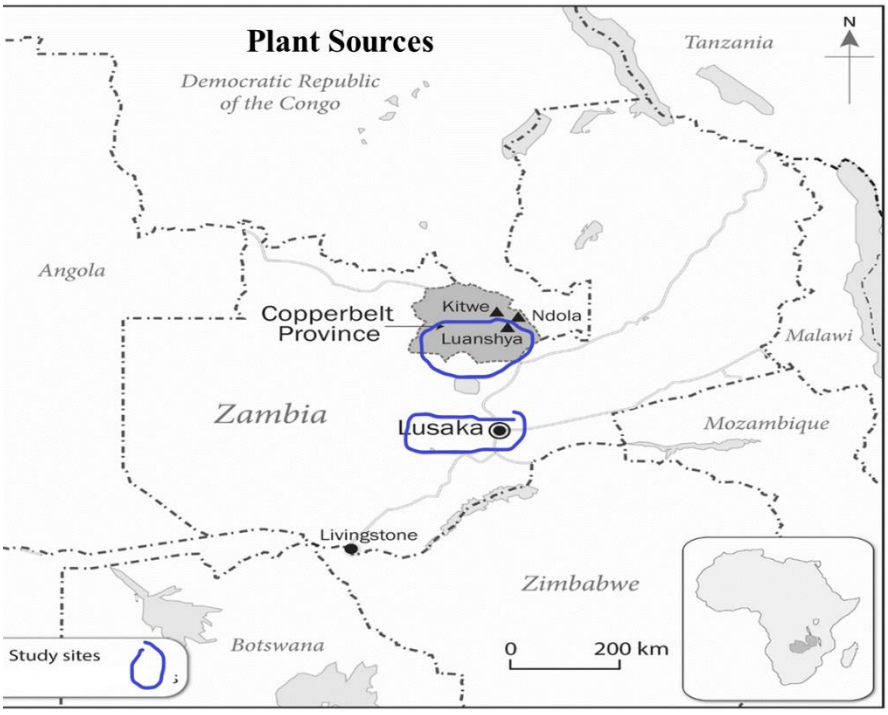


Figure 3.1 Map showing sources of plant materials

The whole plant material (Figure 3.2) was collected in pairs. A set from the paired plant sample was prepared and deposited (Figure 3.3) at the University of Zambia Biology department herbarium for accurate identification of the plants, specimen voucher numbering as well as for future taxonomic review (Mekala, 2017).

Figure 3.2: Whole Plant

Figure 3.3: Herbarium plant press preparation

The *Aloe* leaves and *C. longa* rhizomes in proportions of 300g and 60g (Figure 3.4) respectively (Punniamurthy, 2017), were dried in a drying oven (Figure 3.5) at 35ºC for Seven days. The oven was situated in a dark, warm and well-ventilated room. The herbs were crumbling dry and ready for extraction of active matter in one weeks' time.

Figure 3.4: Herb formulation drying in formulation proportions

Figure 3.5: Yamato Drying oven pre-set at 35ºC

3.2 Bacterial organisms used in the verification of Antibacterial activity

For the antibacterial screening, the National Committee for Clinical Laboratory Standards (CLSI, formerly, NCCLS) recommends gram positive *Staphylococcus aureus* (ATCC 25923) and gram negative *Escherichia coli* as well as *Pseudomonas aeruginosa* bacterial cultures (Mekala, 2017). These isolates were used in the preliminary trials. The isolates were collected from the field isolates of the University of Zambia, School of Veterinary Medicine (UNZA-SoVM). In the third trial, *Streptococcus*, *Bacillus* and *Candida* isolates were used and tested accordingly.

In the fourth and fifth trials, *Eighty Four* critical case isolates derived from *Forty Nine* clinical and subclinical mastitis milk samples for cows from Dairy Cooperatives (Magoye, -16.002262°, 27.608795°) in Mazabuka, (Kayuni, -16.341070°, 27,48669°), Monze (Monze, -16.274660°, 27.474601°), Kalomo (Kalomo, -17.031228°,26.490204°), Namwala (Niko, -16.300620°,26.900439°) and Choma (Batoka, -16.776273°,27.245060°) districts were tested as part of the Deutsche Gesellschaft fur Internationale Zusammenarbeit (GIZ) Green Innovations Centre (GIC) dairy value chain project in the Southern Province of Zambia (Figure 3.6).

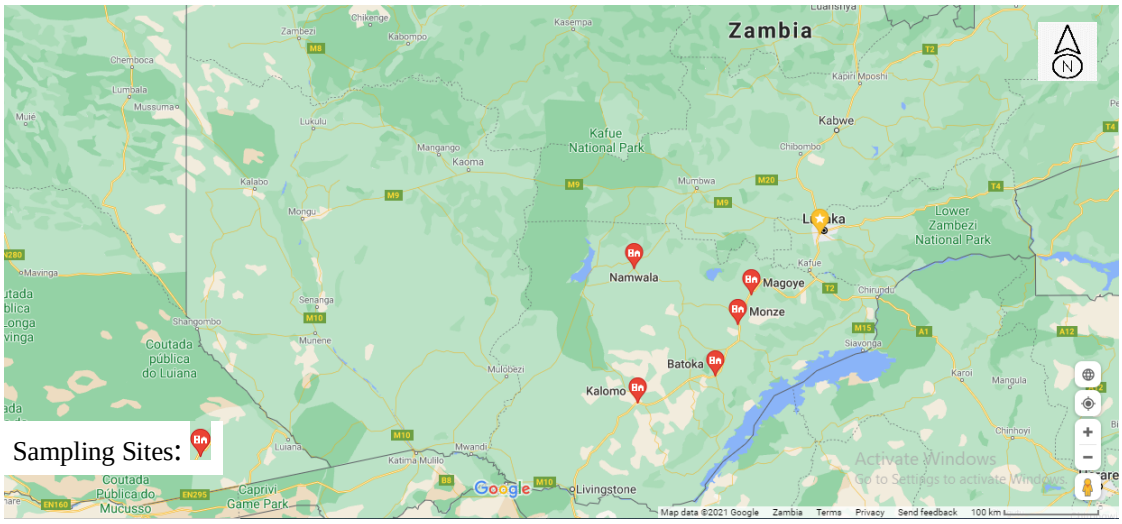


Figure 3.6: Map showing Critical Case Sampling Sites in Southern Zambia (courtesy of google maps)

The field milk samples were collected in conformity to the UNZABREC ethical clearance 1073-2020 and NHREB approval. The milk samples were aseptically collected and transported under ice in a cooler box to the laboratory.

At the laboratory, 9mL of Brain Heart Infusion (BHI, Oxoid,UK) was pipetted into sterile test tubes for first enrichment. One millilitre of thawed milk sample was added into the 9mL of BHI and vortexed for 5 to 10 seconds to mix thoroughly. The mixture was then incubated at 37°C for 20 hours.

On the second day, the tubes containing the overnight cultures for the milk samples were placed on the working bench and mixed thoroughly by gently vortexing, after which 100µL was transferred aseptically from the sample suspension of the overnight

cultures of day 1 to Blood (Oxoid, UK), MacConkey (Oxoid, UK) and Nutrient agar (Oxoid, UK) plates. Spreading of the inoculum over the surface of the agar plate was done using a sterile streaking rod. The plates were maintained in the upright position until the inoculum was absorbed by the agar. The plates were then inverted and incubated for 20 hours at 37°C.

The bacteria was identified by gram staining and biochemical reactions as described by Bisen et al, (2012). Briefly, the pure isolates were identified as Gram positive or negative, followed by biochemical reactions that included sulphur indole motility, citrate, Triple sugar iron and sugar utilization. Following phenotypic confirmation of the isolates, the culture colonies were then constituted to the 0.5 McFarland and processed thereafter as described under subheading 3.5 below.

3.3 Culture media and antibiotics

Mueller-Hinton (MH) agar media (Oxoid, UK) was used for the growth of bacterial cultures as well as for the antibacterial determination/ screening, while Mueller-Hinton broth (Oxoid, UK) was incorporated for MIC determination. Brain Heart Infusion and Blood agar were also utilised for initial culture of the fastidious bacteria. Furthermore, the Zambian market available anti-mastitis preparations Terrexin ® (Each injector of 10 gram contains: Cefalexin 200 mg and Kanamycin monosulfate 100,000 IU) and Rilexine 200 L.C® (containing Cefalexin 100mg, Neomycin Sulphate 100mg and Prednisolone 10mg, per 10ml) were utilised for comparative purposes and as experimental controls.

3.4 Preparation of herbal extract and extractive matter determination

After drying as referred to under subheading 3.1 above, the herbal extracts were prepared as described by Punniamurthy and Ramakrishnan(2015). Specifically, 300g *A. vera* and the 60g *C. longa* was coarsely powdered together (Figure 3.7). Thereafter, five grams of the dried coarsely powdered crude herbs were transferred into flasks and macerated with 100ml of solvents (Water, Ethanol, Ethyl acetate) in flasks for 36 hours. The 36hours was divided into continuously shaking of flasks on a shaker for 18 hours and then standing them for 18 hours (Figure 3.8). The flask contents were then filtered rapidly using No. 1 Whatman™ filter paper, taking precautions against loss of solvent (Figure 3.9). For the extractive matter determination, 25ml of the filtrate was

transferred and vaporized to dryness on a shallow well in a water bath (Figure 3.10) at 105 °C for 6 hours, and thereafter the extract was cooled and weighed immediately. The content of extractable matter was then calculated in duplicates for the three different solvents used in milligrams per gram of the dried material (World Health Organisation, 2011).

Figure 3.7: Coarsely powdered herbs

Figure 3.8: Maceration of herbs

Figure 3.9: Filtered Extracts in Solvents

Figure 3.10: Vaporization of Solvents in water bath at 105^{o}C

The remainder of the filtrates (not used for extractive matter determination) meant for antimicrobial activity assaying were equally vaporized in a water bath for 6 hours, leaving only the extract. 1ml of 100% DMSO was then added using a Pasteur pipette to the extracts for complete dissolution of all solid extract particles. The extracts were then transferred into air tight Eppendorf tubes in readiness for agar dilution for antimicrobial activity and minimum inhibitory activity determination (Figure 3.11) as described under subheadings 3.5 and 3.6 respectively below.

The individual plant material extraction (for the single *Aloe* and *C. longa*) was also conducted in like manner to the combined extracts mentioned above.

The optical densities (OD) of all the extracts were measured at 450nm and 492nm in duplicates in order to ensure presence of viable cell biomass. DMSO and water were used as blanks for the spectrophotometric optical density measurements (Figure 3.12).

Figure 3.11: Herbal Extract in Eppendorf Tubes

Figure 3.12: Spectrophotometer Measuring OD

3.5 Determination of antimicrobial activity

Antimicrobial activity of all extracts (combined formulation and individual plant extracts) was tested by the agar dilution method. The extract was diluted (initial volume (V_i)) (0.5ml extract into 19.5ml agar for initial trials and 1ml into 19ml for latter trials) into the media to make up to 20ml (final volume (V_f)) before agar solidification at temperatures of between 45-55^oC and petri-dish plated. Selection of the maximum concentration was largely dependent on the volume of compound extracted. After solidification and drying, the test saline dissolved bacterial isolates were spot inoculated onto the MHA at the CLSI recommended inoculum rate of 0.5 McFarland and marked for bacterial test strain identification after which the plates were incubated at 37^oC for 24hours (Figure 3.13). In similar manner, plates with dilute antibiotic (Terrexin ® and Rilexine®; 0.5ml antibiotic to 19.5ml agar) and undiluted agar control were also prepared and spot inoculated with the test bacterial strains for comparative purposes (Figure 3.14).

Comparisons were recorded based on the susceptibility analysis and graded as Susceptible (S), Intermediate (I) or Resistant (R). Susceptible was interpreted as the antimicrobial completely inhibiting growth of the bacteria; Intermediate was recorded for minor growth, while Resistant was recorded for completely uninhibited growth. The bacterial cultures were observed immediately after 24 hours post incubation and continued over a period ranging from 10 to 37 days in order to determine if the effects were bacteriostatic or bactericidal and the susceptibility observations were recorded.

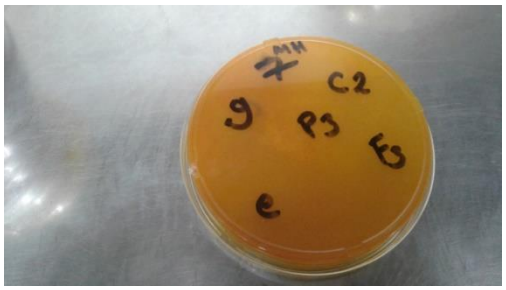


Figure 3.13: Herbal formulation extract diluted in MHA

Figure 3.14: Synthetic Antimicrobial diluted in MHA

3.6 Minimum Inhibitory Concentration (MIC) Determination

The MIC was defined as the lowest concentration that completely inhibited the growth of microorganisms for 24 hours. To determine the MIC, a serial dilution in Mueller-Hinton Broth was prepared beginning with, a 10% extract in agar initial dilution to least final dilution concentrations of 1.25% and 0.15625% for the first and second trials respectively. *Staphylococcus aureus* was utilised for the MIC determination of which a 100µl stock control was added to all the dilutions individually, vortexed and incubated for 24 hrs, and then inoculated onto Nutrient agar and thereafter observations were made and recorded. A recording was also made for the *Staphylococcus* stock solution and a plain negative control. Ethanol extracts were used for the MIC determination.

The recordings were related to the MIC values for the synthetic antimicrobials and observations made were recorded.

CHAPTER 4

4.0 RESULTS

4.1 Extractive Value Determination

Specific to the solvents used, extraction was conducted in duplicates and the mean extract weights were recorded and compared. Table 4.1 shows the recordings of the extractive values for the three solvents used for the extraction and their respective means. Figure 4.1 graphically summarizes the extractive values and their means.

Table 4.1: Results of Extractive Values (Weights) with ANOVA

	Aqueous(mg)(1)	**Ethanol(mg)(2)**	**Ethyl Acetate(mg)(3)**
Run 1	330	65	30
Run 2	168.2	53.3	25
Mean	249.1	59.15	27.5
T_{1-3}	498.2	118.3	55
n_{1-3}	2	2	2
$SumSq_{1-3}$	137191.2	7065.89	1525
T^2/n_{1-3}	124101	6997	1512
T	671.5		
n	6		$\alpha=0.05$
SumSumSq	145782.1		$df_{btn}=2$
$SumT^2/n$	132610		$df_{wtn}=3$
T^2/n	75152		$F_{(2,3)}=9.552$
SS_{wtn}	13172.13		
SS_{btn}	57458		Hypothesis
SS_{tot}	70630.13		H_o: $\mu_1=\mu_2=\mu_3=\mu$
MS_{wtn}	4390		A: $\mu_1\neq\mu_2\neq\mu_3\neq\mu$
MS_{btn}	28729		
F_{obs}	6		
			$F_{obs}< F_{(2,3)}$
			$\therefore H_o$ Not rejected

Simuunza (2019) highlights that the decision criteria for the F-test designates that the hypothesis should not be rejected if the F (observed) is less than the F (critical), therefore the hypothesis is not rejected despite the mean extractive weights being visibly different.

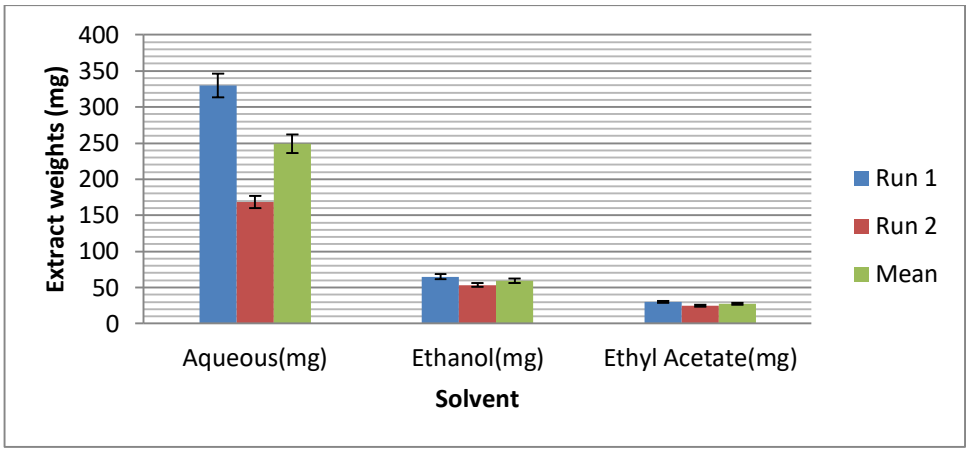


Figure 4.1: Extract Weight Comparison

The recordings indicate that the aqueous extract had the highest extract weight. The mean extract weights from the 25ml of macerated plant extract in each solvent which was vaporized yielded 250mg from the aqueous solvent which was the highest, while Ethanol yielded 59.2mg and 27.5mg from the Ethyl Acetate. The extractive values which are a percentage of the 5g dried herbs were therefore 5%, 1.2% and 0.6% respectively for the aqueous, ethanol and ethyl acetate extracts.

4.2 Minimum Inhibitory Concentration (MIC)

The results of the Minimum Inhibitory Concentration (MIC) arising from the serial dilutions made are tabulated below and the corresponding key is indicated. Table 4.2 illustrates the key for interpretation of the colony count data provided in tables 4.3 and 4.4. The coding illustrated in the table was formulated to estimate the number of colonies observed and counted on each plate. Table 4.3 shows the results from Run 1 while Table 4.4 shows the readings from Run 2.

TABLE 4.2: MIC RUN 1

	SERIAL DILUTIONS

SAMPLE ID	Initial Conc^{n} (mg/ml)	10%	5%	2.50%	1.25%
9(T2)	260	±	2+	4+	5+
7(T2)	280	±	±	±	±

TABLE 4.3: MIC RUN 2

		SERIAL DILUTIONS						
Sample ID	Initial Conc^{n} (mg/ml)	10%	5%	2.50%	1.25 %	0.63 %	0.31 %	0.16 %
7(T1)	250	±	5+	5+	5+	3+	4+	1+
18(T2)	180	1+	5+	5+	1+	5+	2+	3+
Negative Control		5+	N/A					
Staphylococcus (known bacteria control)		5+						

Table 4.4: An illustration of the key to interpretation of Data provided in Table 4.2 and Table 4.3

KEY:	**Bacterial colony growth count**
±*	less than 5 Colonies
1+	50 to 100 colonies
2+	100 to 150 colonies
3+	150 to 250 colonies
4+	250 to 400 colonies
5+	No Inhibition(full growth)

*Interpreting less than 5 colonies or no colonies observed.

The Minimum Inhibitory concentration (MIC) serial dilution assays in Run 1 indicated that the MIC of the anti-mastitis preparation was between 10% and 5% (13mg/ml) for the first sample (9T2), while results from the second extract sample in trial 1 were inconclusive, hence necessitating the conduct of a second Run.

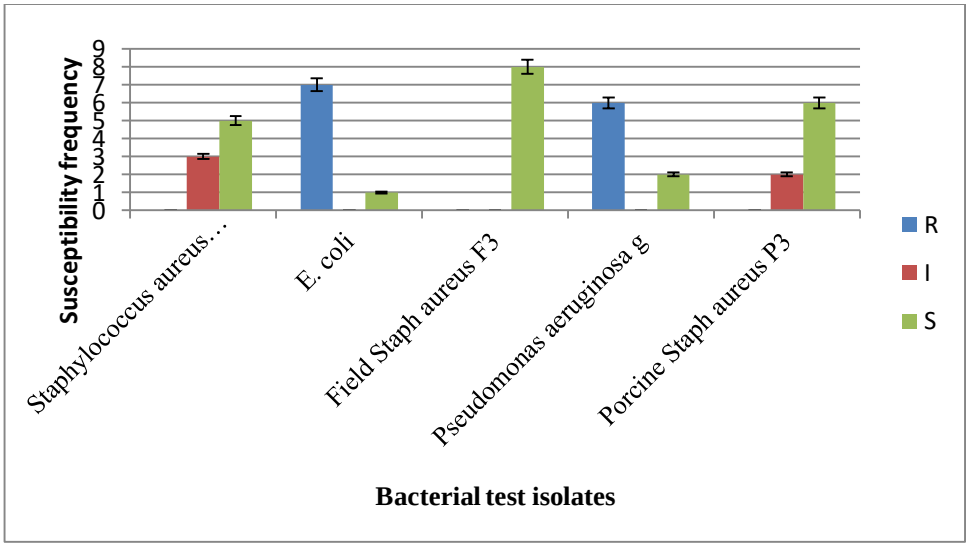
In the second Run (Run 2) both anti-mastitis ethanol extracts tested indicated an MIC between 10% and 5% (12.5mg/ml & 9mg/ml). Consequently the MIC was concluded

according to the case definition to be 5% concentration and from the three readings, the mean MIC was identified as being 11.5mg/ml.

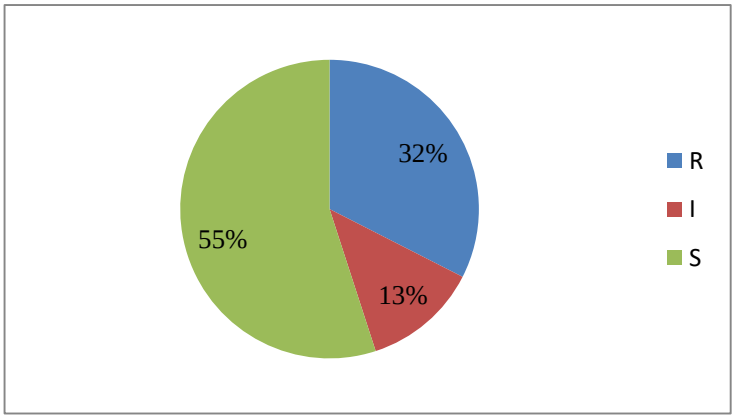
4.3 Antimicrobial activity of *Aloe* and *Curcuma longa* individually

4.3.1 *Curcuma longa* antimicrobial activity

Susceptibility results from the antimicrobial activity of *Curcuma longa* ethanol extract are summarized graphically and in the pie chart in Figure 4.2 (a) and (b) below for the individual bacterial susceptibility (See Table 4.5 for details) and cumulatively for all recorded observations respectively on all isolates at 2.5% agar dilution concentration.



(a)



(b)

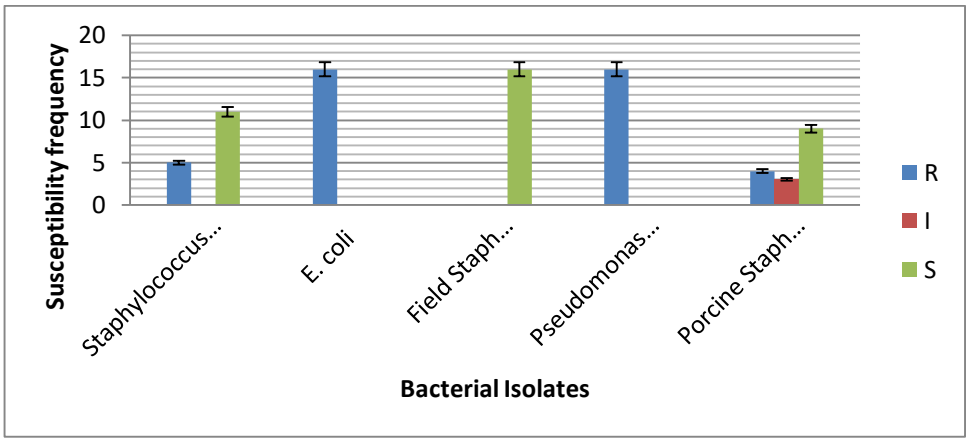
Figure 4.2 (a) & (b): *Curcuma longa* only Bacterial Susceptibility (R is resistant, I intermediate and S is Susceptible, n=40)

Gram negative *E. coli* and *Pseudomonas aeruginosa* were the most resistant and accounted for the 32% resistance recorded cumulatively. The *Staphylococcus aureus*

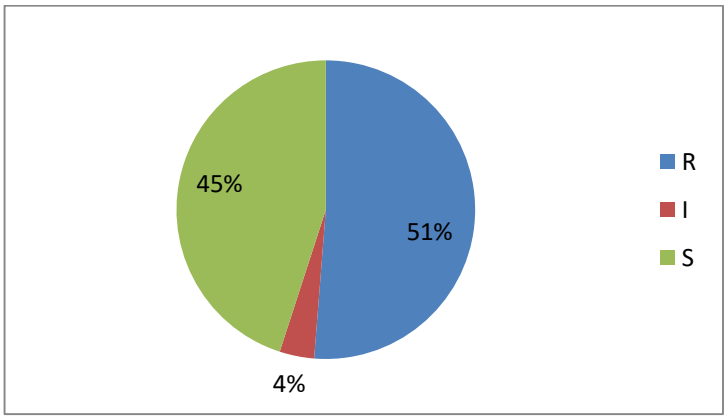
control and isolates were the most susceptible to this treatment with variations noted between intermediate susceptibility and full susceptibility with time. On the overall the treatment was Susceptible on 55% of all observations and in combination with the 13% intermediate susceptibility, yielded a total of 68% susceptibility on all observations made.

4.3.2 Plain Aloe antimicrobial activity

Susceptibility results from the antimicrobial activity of *Aloes* on their own are summarized graphically and in the pie chart in Figure 4.3 (a) and (b) below for the individual bacterial susceptibility (See Table 4.5 for details) and cumulatively (pie chart) for all recorded observations respectively.



(a)



(b)

Figure 4.3 (a) & (b): Aloe Extract only Bacterial Susceptibility (R is resistant, I intermediate and S is Susceptible, n=80)

Gram negative *E. coli* and *Pseudomonas aeruginosa* were resistant and accounted for the most part of the 51% resistance recorded cumulatively. The *Staphylococcus aureus* control and isolates were the most susceptible to this treatment with variations between intermediate susceptibility and full susceptibility noted. On the overall the treatment was Susceptible to the bacteria on 45% of all observations and in combination with the 4% intermediate susceptibility yielded a total of 49% susceptibility on all observations made.

4.4 Antimicrobial activity of the Combined *Aloe* and *Curcuma longa* formulation

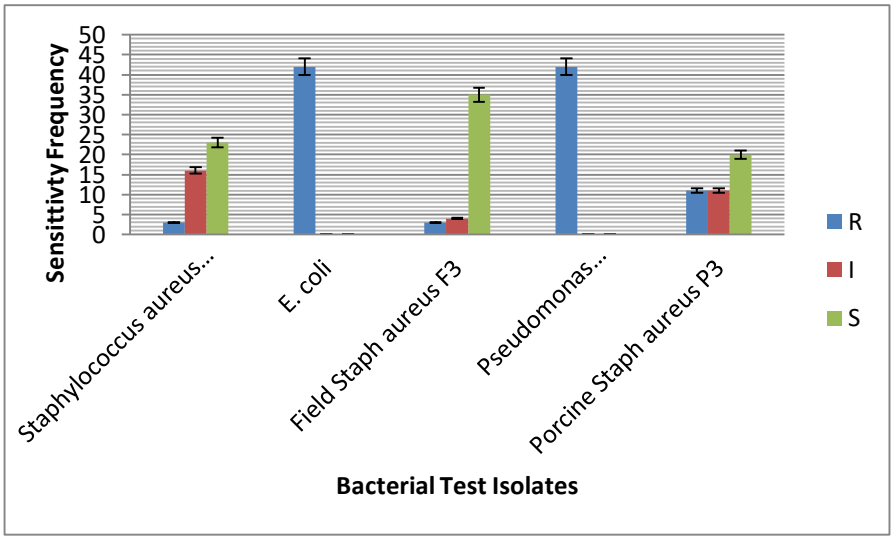
Susceptibility results from the antimicrobial activity of the combined *Aloe* genus and *C. longa* formulation are summarized below beginning with trial 1.

4.4.1 Trial 1

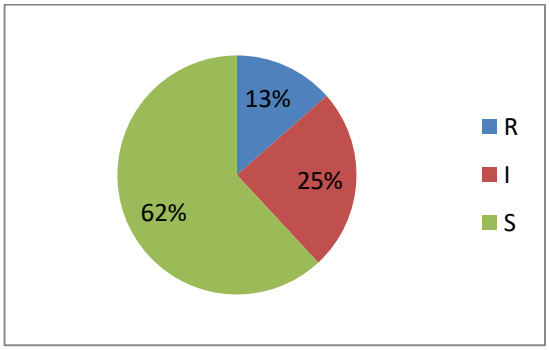
Results from trial 1 are illustrated graphically below. Figure 4.4(a) depicts individual susceptibility of the bacterial isolates (See Table 4.5 for details) to the herbal formulation. Figure 4.4(b) shows the overall performance of the herbs against all bacterial isolates, while Figure 4.4(c) describes the overall susceptibility of the herbal formulation against the gram positive isolates on their own.

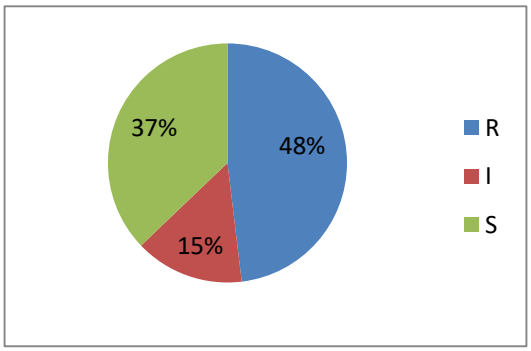
Gram negative *E. coli* and *Pseudomonas aeruginosa* were resistant and accounted for the most part of the 48% resistance recorded cumulatively. The *Staphylococcus aureus* control and isolates were the most susceptible to this treatment with variations noted between intermediate susceptibility and full susceptibility. On the overall the treatment was Susceptible to the bacteria on 37% of all observations and in combination with the 15% intermediate susceptibility yielded a total of 52% susceptibility on all observations made.

Preliminary findings indicated that the gram positive strains are the most prevalent mastitis associated aetiological agents and therefore, an analysis of their susceptibility independently was prepared and presented in Figure 4.4(c). Results from the gram positive isolates assayed alone showed 62% susceptibility, and when combined with the 25% intermediate susceptibility totalled 87% susceptibility.



(a)





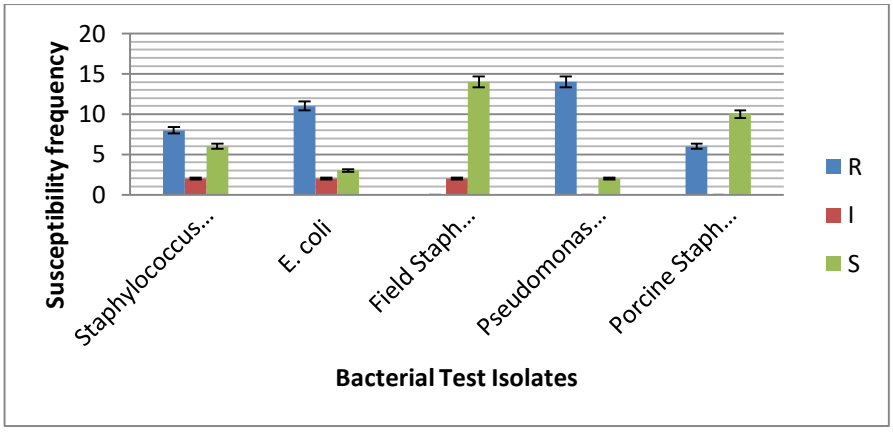
(b)(n=210) (c)(n=126)

Figure 4.4 (a)-(c): Trial 1 *Aloe* and *Curcuma longa* formulation antimicrobial susceptibility (R is resistant, I intermediate and S is Susceptible)

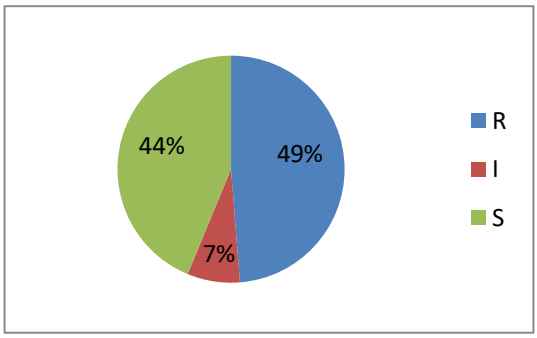
Figure 4.5(a)-(d) displays results from trial 1 of the synthetic antimicrobials against the same isolates depicted in Figure 4.4. Figure 4.5(a) depicts individual susceptibility of the bacterial isolates (See Table 4.5 for details) to the synthetic anti-mastitis drugs which also served as positive controls. Figure 4.5(b) shows the overall performance of the synthetic drugs against all bacterial isolates, while Figure 4.5(c) describes the overall susceptibility of the herbal formulation against the gram positive isolates on their own. In Figure 4.5(d), we compared the sensitivities of the two anti-mastitis preparations (Rilexine® and Terrexin®) to each other.

Gram negative *E. coli* and *Pseudomonas aeruginosa* were again the most resistant and accounted for over two thirds of the 49% resistance recorded cumulatively. The *Staphylococcus aureus,* control and the porcine nasal swab isolate also showed some

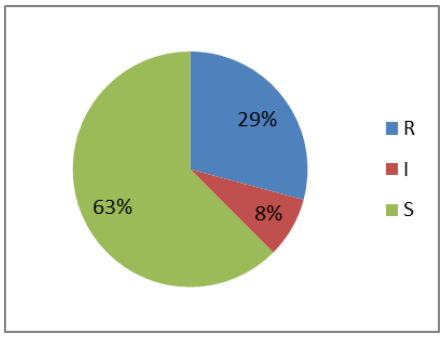
level of resistance with time to the synthetic antimicrobials during the period of observation. The field isolated *S. aureus* was the most susceptible to this treatment with variations noted between intermediate susceptibility and full susceptibility. On the overall the treatment was Susceptible to the bacteria on 44% of all observations and in combination with the 7% intermediate susceptibility yielded a total of 51% susceptibility from all observations made.



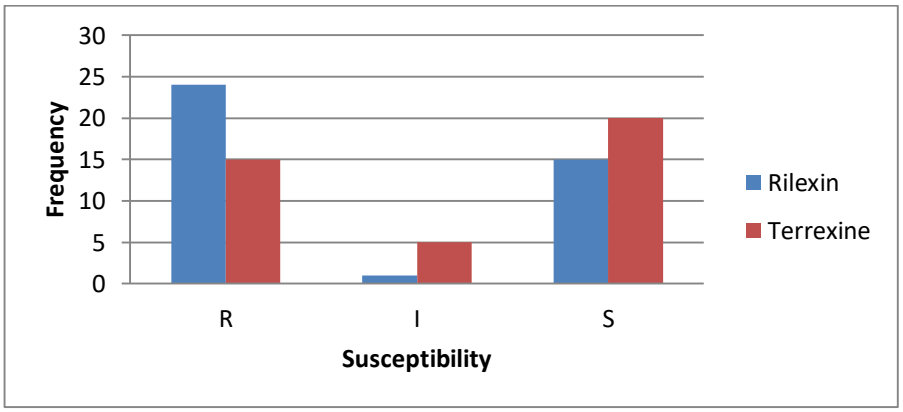
(a)



(b)(n=80)



(c)(n=48)



(d)

Figure 4.5(a)-(d): Trial 1 Synthetic Anti-mastitis preparations' Susceptibility (R is resistant, I intermediate and S is Susceptible)

Outcomes from the gram positive isolates assayed alone (Figure 4.5(c)) exhibited 63% susceptibility, and when combined with the 8% intermediate susceptibility equalled 71% susceptibility. Comparatively, the Terrexin® was 12.5% more Susceptible to the bacterial isolates than the Rilexine® at the 2.5% agar dilution concentration as depicted in Figure 4.5(d).

Figure 4.6 graphically displays results for trial 1 from the negative controls (Dimethylsulfoxide (DMSO) treatment and media without any treatment) against the bacterial isolates. As anticipated there was no inhibition to the growth of the bacterial isolates. 100% resistance was recorded overall from the negative control in this trial after the 24 hours incubation period and observation was thereafter discontinued.

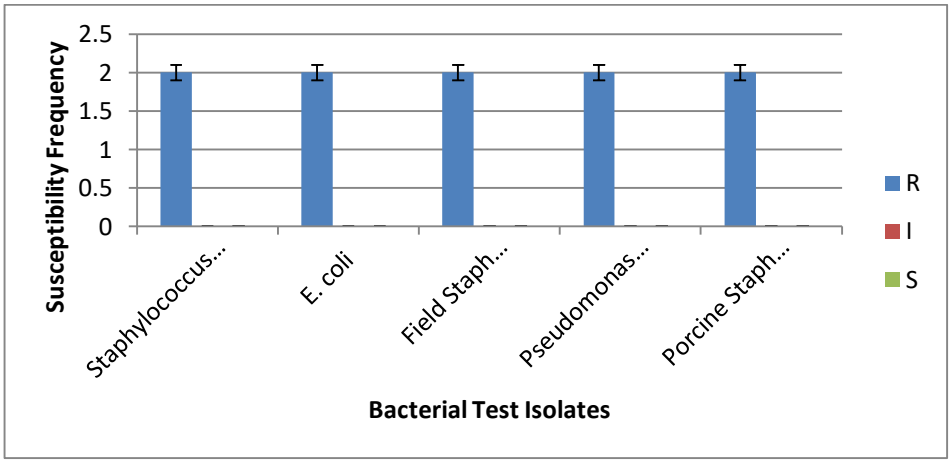


Figure 4.6: Trial 1 Negative controls

The table below portrays the bacterial isolates used for this trial, their source and coding assigned to them for the experiment.

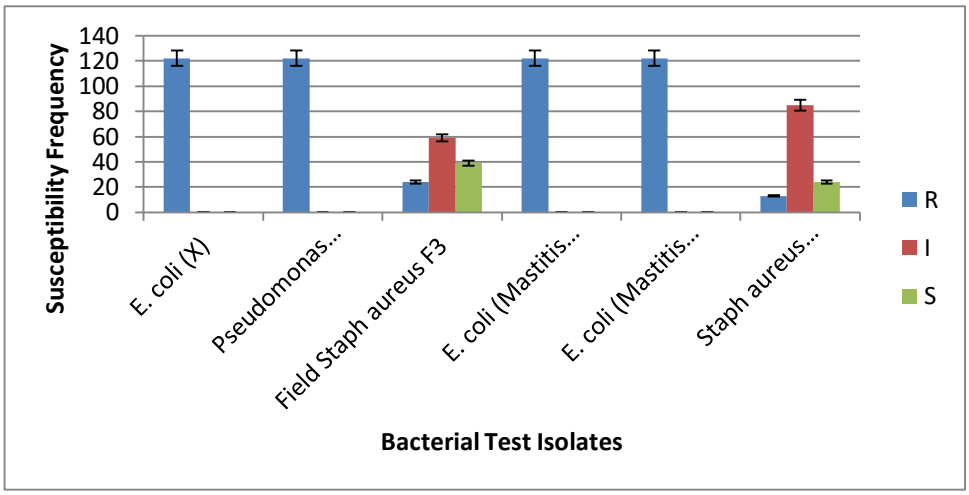
Table 4.5: Microbes and Codes Assigned For Trial 1 and Individual Herb Tests

S/N	**Code Assigned**	**Source**	**Bacteria (Graph Depiction in Brackets)**
1	e	Piglet	*Escherichia coli* (*E. coli*)
2	F_3	Field Mastitis Isolate	*Staphylococcus aureus* (Field Staph…)
3	C_2	Mastitis Control Strain (ATCC 25923)	*Staphylococcus aureus* (*Staphylococcus...*)
4	g	Borehole water Isolate	*Pseudomonas aeruginosa* (*Pseudomonas*)

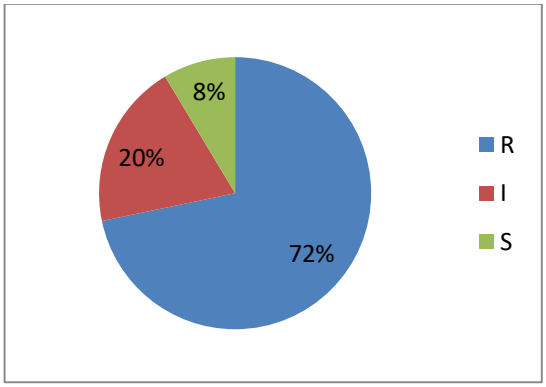
5	P_3	Field porcine nasal swab	*Staphylococcus aureus* (Porcine Staph…)

4.4.2 Trial 2

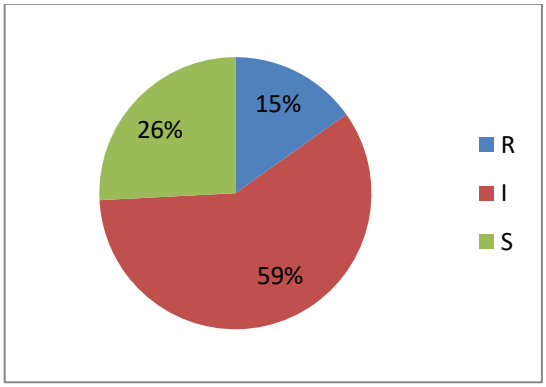
Outcomes from trial 2 are illustrated below. Figure 4.7(a) depicts individual susceptibility of the bacterial isolates to the herbal formulation. The pie chart in Figure 4.7(b) shows the overall performance of the herbs against all bacterial isolates tested, while the pie chart in Figure 4.7(c) describes the overall susceptibility of the herbal formulation against the gram positive isolates specifically.



(a)



(b)(n=732)



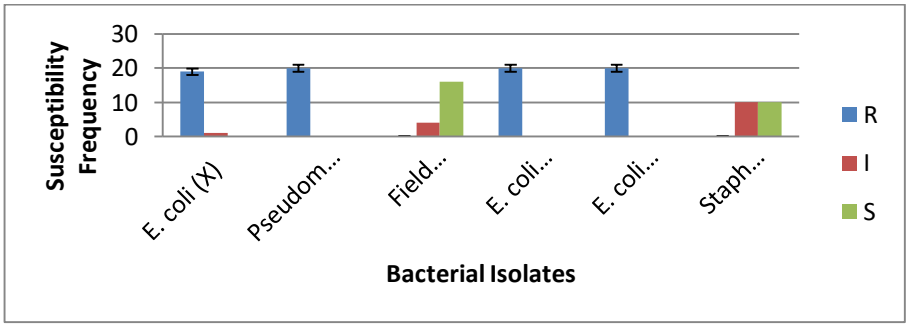
(c)(n=244)

Figure 4.7 (a)-(c): Trial 2 *Aloe* and *C. longa* formulations Susceptibility Results (R is resistant, I intermediate and S is Susceptible)

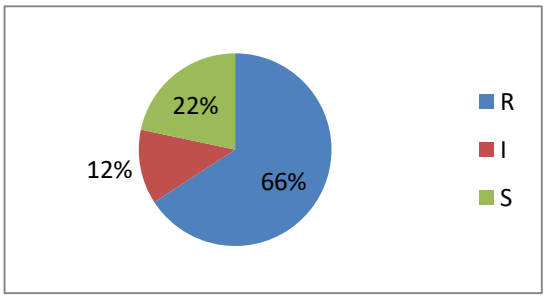
The gram negative isolates (*E. coli* and *Pseudomonas aeruginosa*) again presented the most resistance and accounted for over three quarters of the 72% resistance recorded cumulatively. The *Staphylococcus aureus*, control strain and the field mastitis isolate

accounted for less than 10% of the overall resistance. On the overall the treatment was Susceptible to the bacteria on 8% of all observations and in combination with the 20% intermediate susceptibility yielded a total of 28% susceptibility from all observations made. However, results from the gram positive isolates assayed when analysed alone (Fig 4.7(c)) exhibited 26% susceptibility, and when combined with the 59% intermediate susceptibility equalled 85% susceptibility. The intermediate resistance was the most predominant susceptibility reading during this trial for the herbal formulations.

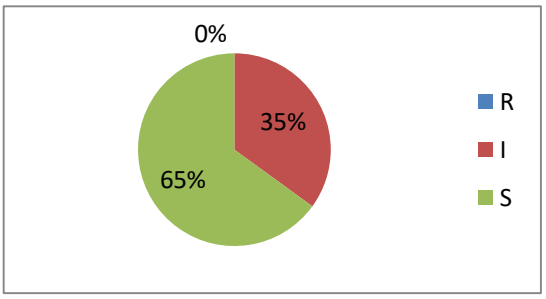
The isolates in this trial were also tested at three times the initial yields of some select extracts (1, 2 & 7) from Trial 1. The quantity of powdered pre-macerated herbs was tripled from the 5g to 15g and the quantity of solvent was also tripled from 100ml to 300ml of ethanol. The remainder of the procedure is identical to the all other trials as specified in the previous chapter. The extracts were preserved at -20^{o}C prior to use along with the extracts from the second trial. The trials and observations for these extracts were conducted together with the trial 2 extract trials. The results are presented below in Figure 4.8(a)-(c). Figure 4.8(a) graphically demonstrates the frequency of the susceptibility observations of the microbes against the select herbal treatments.



(a)



(b)(n=120)



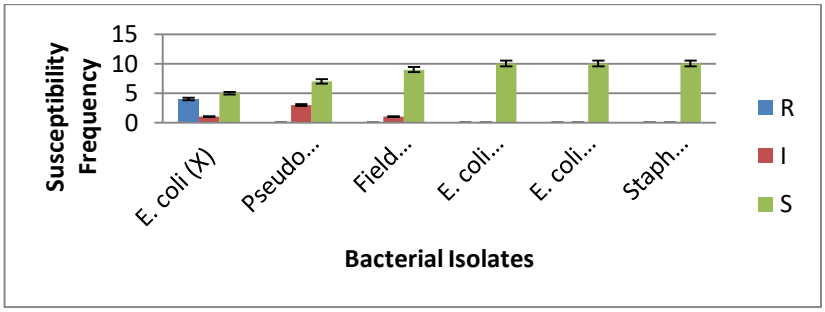
(c)(n=40)

Figure 4.8 (a)-(c): Trial 2 Select Triple yield *Aloe* and *C. longa* formulation susceptibility (R is resistant, I intermediate and S is Susceptible)

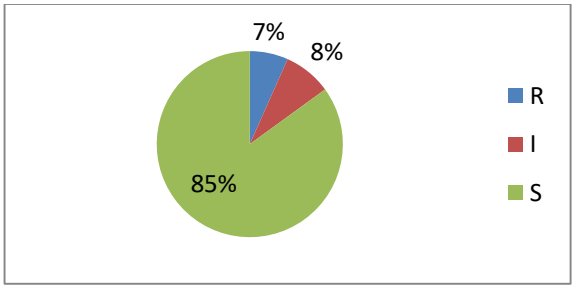
Figure 4.8(b) indicates the overall response to the select formulations while Figure 4.8(c) separately summarizes solitary results of the gram positive bacterial isolates. Based on these findings, the extract has gram positive spectrum of activity only.

The gram negative isolates *E. coli* and *Pseudomonas aeruginosa* were resistant and accounted for the whole of the 66% resistance recorded cumulatively. The *Staphylococcus aureus,* control strain and the field mastitis isolate accounted for 0% of the overall resistance on all observations made. On the overall the treatment was Susceptible to the bacteria on 22% of all observations and in combination with the 12% intermediate susceptibility yielded a total of 34% susceptibility from all observations made. However, results from the gram positive isolates assayed when analysed alone exhibited 65% susceptibility, and when combined with the 35% intermediate susceptibility equalled 100% susceptibility.

Figure 4.9(a)-(b) displays results from trial 2 of the synthetic antimicrobials against the same isolates. Figure 4.9(a) depicts individual susceptibility of the bacterial isolates to the synthetic anti-mastitis drugs which also served as positive treatment controls. Figure 4.9(b) summarizes the overall performance of the synthetic drugs against all bacterial isolates.



(a)



(b)

Figure 4.9(a) &(b): Trial 2 Synthetic anti-mastitis preparations susceptibility (R is resistant, I intermediate and S is Susceptible, n=60)

The *E. coli* was the sole contributor to the 7% resistance noted cumulatively. Overall, the treatment was Susceptible to the bacteria on 85% of all observations and in combination with the 8% intermediate susceptibility yielded a total of 93% susceptibility from all observations made.

Figure 4.10 graphically displays results for trial 2 negative controls (DMSO treatment and media without any treatment) against the bacterial isolates. As projected there was no inhibition to the growth of the bacterial isolates. 100% resistance was recorded overall from the negative control in this trial after the 24 hours incubation period and observation was thereafter dropped.

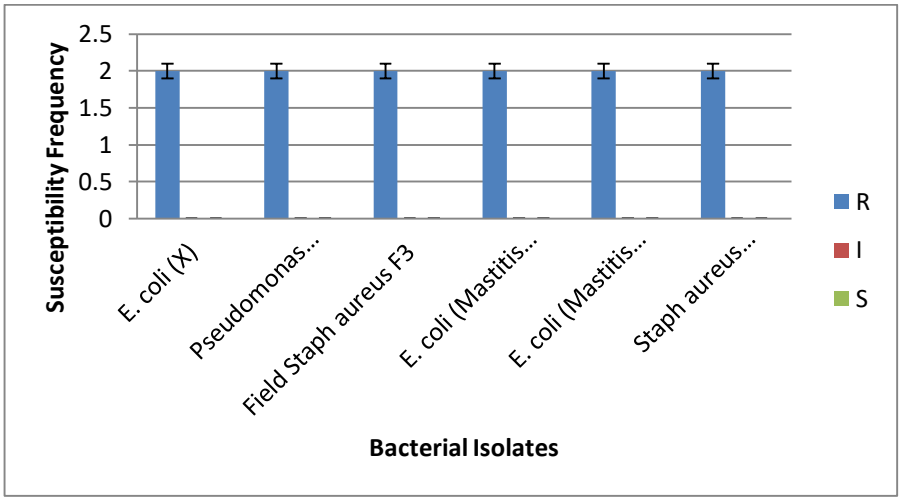


Figure 4.10: Trial 2 Negative Controls (DMSO & MHA) (R is resistant, I intermediate and S is Susceptible)

Table 4.6 below reveals the bacterial isolates used for this trial, their source and results coding assigned to them for the experiment.

Table 4.6: Microbes Used and Codes Assigned For Trial 2 Tests

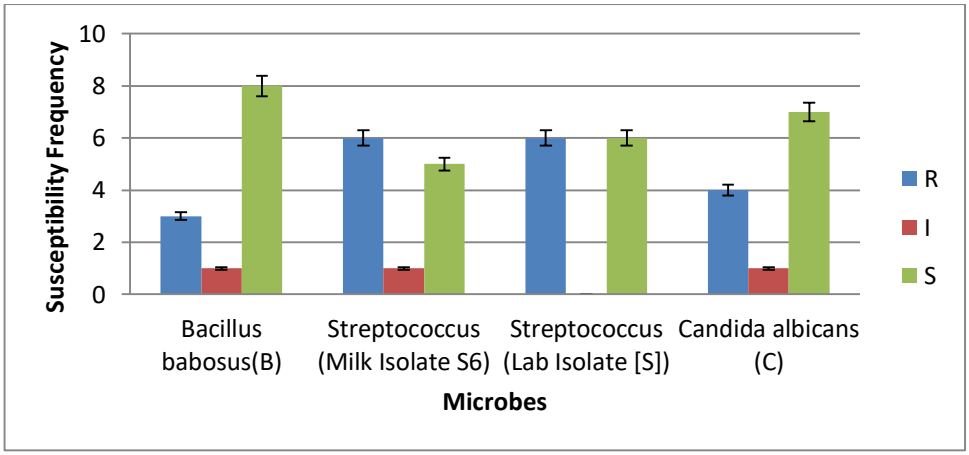
S/N	Results Code Assigned		Source	Bacteria
1	X	E.coli	Piglet	*Escherichia coli*
2	F_3	Field staph aureus	Field Mastitis Isolate	*Staphylococcus aureus*
3	C_2	Staph aureus...	Mastitis Control Strain (ATCC)	*Staphylococcus aureus*
4	P	Pseudomonas	Chick	*Pseudomonas aeruginosa*
5	M	E.coli (Mastitis)	Mastitis Isolate (mm25)	*Escherichia coli*

6	N	E. coli (Mastitis 2)	Mastitis Isolate (mm16)	*Escherichia coli*

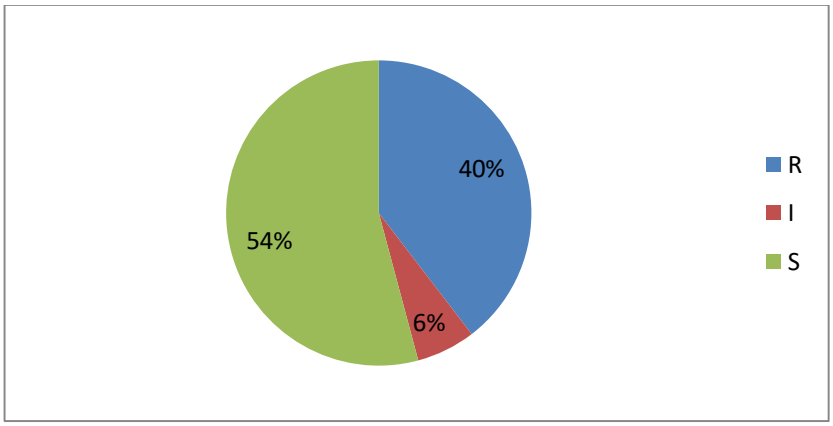
4.4.3 Trial 3

Effects of treatments from trial 3 are illustrated below. Figure 4.11(a) depicts individual susceptibility of the trial 3 microbial isolates (See Table 4.7 for details) to the herbal formulation. The pie chart in Figure 4.11(b) displays the overall performance of the herbs against all microbial isolates tested.

The stored (-20^{o}C) extracts used in trials 1 and 2 were utilized for trial 3 experimentation. All the microbes tested exhibited varying degrees of resistance which was cumulatively recorded at 40%. *Bacillus babosus*, was the most susceptible, while *Streptococcus* depicted a balance between susceptibility and resistance based on the observations made. The *Candida albicans* was also more Susceptible than resistant.



(a)



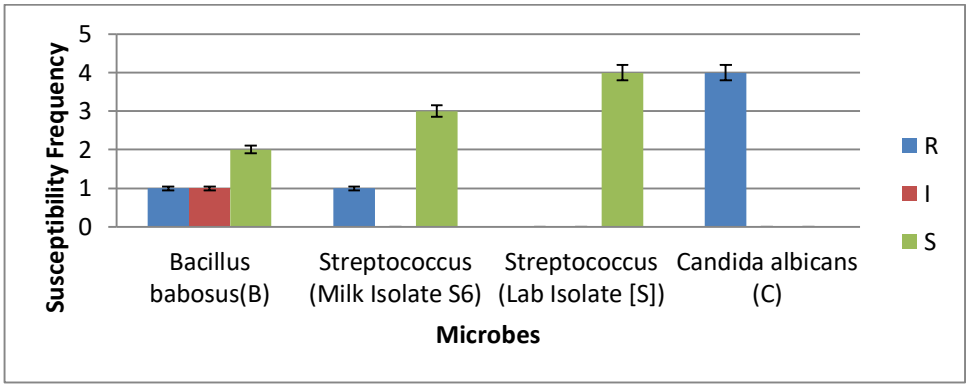
(b)

Figure 4.11 (a) & (b): Trial 3 Herbal preparations' Susceptibility (R is resistant, I intermediate and S is Susceptible, n=48)

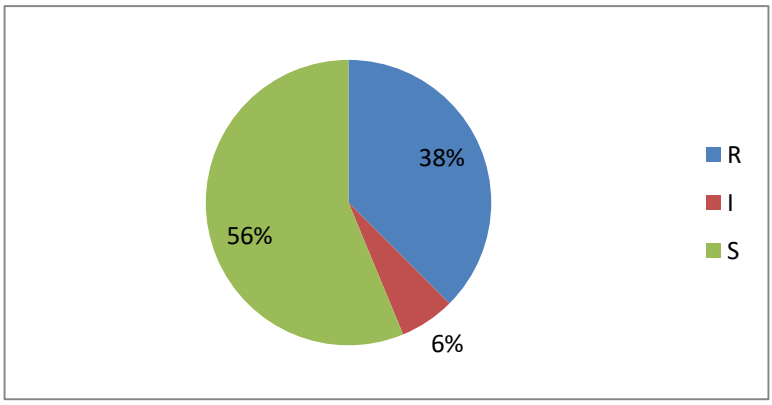
On the overall the treatment was Susceptible to the microbes on 54% of all observations and in combination with the 6% intermediate susceptibility yielded a total of 60% susceptibility from all observations made.

Figure 4.12(a)-(b) displays results from trial 3 of the synthetic antimicrobials against the trial 3 test microbes. Figure 4.12(a) depicts individual susceptibility of the microbes (See Table 4.7 for details) to the synthetic anti-mastitis drugs (positive controls), while Figure 4.12(b) summarizes the overall performance of the synthetic drugs against the entirety of test microbes.

The synthetic antibiotics in trial 3 had no inhibitory effect on the *Candida albicans* (fungal) which in itself accounted for two thirds of the overall resistance on all observations. *Streptococcus* isolates were the most susceptible to the synthetic antimicrobials while *Bacillus babosus* showed the least susceptibility of the three bacterial isolates.



(a)



(b)

Figure 4.12 (a) & (b): Trial 3 Synthetic Antibiotics Susceptibility (R is resistant, I intermediate and S is Susceptible, n=16)

Figure 4.13 explicitly parades results for trial 3 negative controls (DMSO treatment, media without any treatment and with 6% defibrinated blood) against the test microbes. There was no inhibition to the growth of the microbes. No growth inhibition was recorded overall from the negative control in this trial after the 24 hours incubation period and observation was thereafter withdrawn.

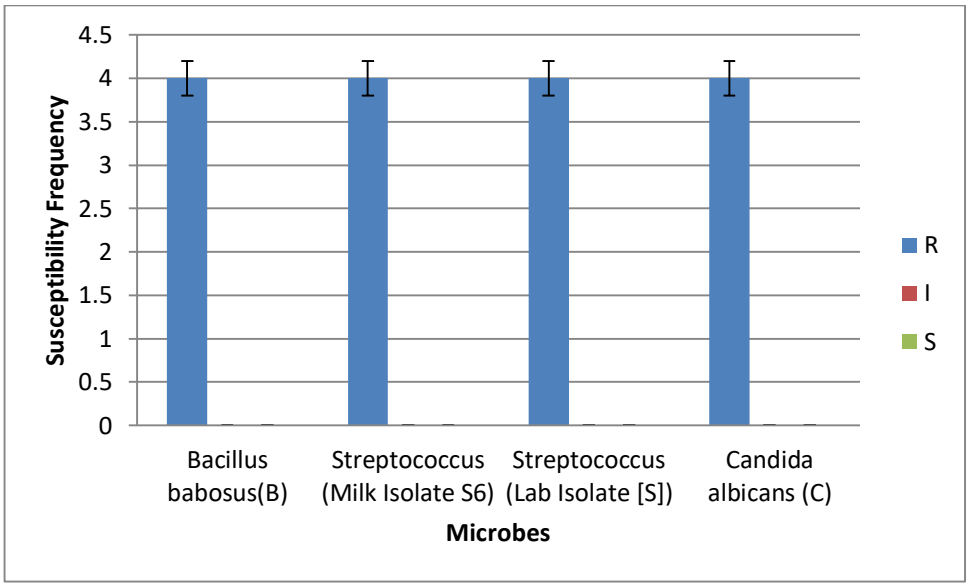


Figure 4.13: Trial 3 Negative Controls (DMSO, MHA and MH with 6% Blood) (R is resistant, I intermediate and S is Susceptible)

Table 4.7 below shows the microbial isolates used for trial 3, their source and coding assigned to them for the experiment.

Table 4.7: Microbes Used and Codes Assigned For Trial 3 Tests

S/N	Code Assigned	Source	Microbe
1	B	Lab	*Bacillus babosus*
2	S	Milk Isolate	*Streptococcus*
3	C	Lab	*Candida albicans*
4	S_6	Milk Isolate	*Streptococcus*

4.3.4 Trial 4

Table 4.8 below summarizes the agar well diffusion zones of inhibition results for field samples for Trial 4 and also displays the ANOVA results at α=0.05 analysed using

Microsoft Excel 2010. All the field mastitis samples were collected as critical case samples and all showed the presence of Gram positive bacterial species. The combined herbal formulation extracts are indicated as "Herb" in table 4.6.

The agar well diffusion test was conducted in this trial out of interest and in the significance of gaining knowledge and experimenting with the procedure for any future use and comparative purposes. 100 µL of drug/extract was added per well.

Table 4.8: Trial 4 Agar Well diffusion Susceptibility and F_{test} Results (100 µL/well)

Sample Lab ID	**Herb (cm)**	**Rilexine (cm)**	**Terrexin (cm)**	**Microscopy; Species Queried**	**Milk Collection Centre**
1	0.45	1.1	1.2	*Staphylococcus*	
2	0.6	1.3	1.5	*Staphylococcus* & *Streptococcus*	
3	1.2	1.4	1.5	*Staphylococcus*	
4	0.6	1.4	1.2	*Lactobacillus*	
5	0.6	1.7	1.2	*Staphylococcus, Streptococcus* & *Diplococci (Streptococcus pneumoniae queried)*	
6	0.9	1.3	1.2	*Staphylococcus* & *Streptococcus*	
7	0.7	1.3	1.4	*Staphylococcus*	NIKO
8	0.5	1.5	1.2	*Staphylococcus*	(NAMWALA)
9	0	0	0	*Streptococci* & *Diplococci (Strep pneumoniae)*	
10	0.5	0.8	1	*Streptococcus*	
11	0.5	1.5	1.7	*Streptococcus*	
12	0.25	0.7	0.9	*Streptococcus*	
13	0.15	1	0.8	*Streptococcus, Lactobacillus, Streptococcus pneumoniae, Bacillus spores*	
14	0.2	0.55	0.8	*Streptococcus*	
15	0.15	1.25	1.2	*Staphylococcus* & *Streptococcus*	
16	0.2	1.1	1	*Staphylococcus*	
17	0.1	1.25	1.15	*Staphylococcus* & *Streptococcus*	KALOMO
18	0.95	1.2	1.7	*Streptococcus*	(KALOMO)
19	0.35	1.25	0.85	*Streptococcus* & *Staphylococcus*	
20	0.25	1.2	1.25	*Staphylococcus, Streptococcus, Diplococci (S. pneumoniae), Spores*	KAYUNI (MONZE)

21	0.4	1	0.7	*Streptococcus*	
22	0.6	1	1.1	*Streptococcus*	
23	0.5	0.9	1.1	*Streptococcus*	
24	0.5	0.8	0.55	*Staphylococcus* & *Streptococcus*	
25	0.6	0.75	0.65	*Streptococcus*	
26	0.7	1	1	*Staphylococcus, Streptococcus and Diplococcus*	
T_{1-3}	12.45	28.25	27.85		
n_{1-3}	26	26	26		
$SumSq_{1-3}$	7.9275	33.8125	33.2125		
T^2/n_{1-3}	5	30	29		
T	68.55				
n	78			$\alpha=$	0.05
SumSumSq	74.9525			df_{btn}	2
$SumT^2/n$	64			df_{wtn}	75
T^2/n	60			$F_{(2,75)}$	3.15
SS_{wtn}	10.9525				
SS_{btn}	4				
SS_{tot}	14.9525				
MS_{wtn}	0.14603				
MS_{btn}	2				
F_{obs}	13				
			$F_{obs} > F_{(2,75)}$		
			Therefore, $H_o \neq \mu$, rejected		

Only one sample (9) revealed some resistance to all treatments and the species identified/queried in this sample were *Streptococcus* and the Diplococcal *Streptococcus pneumoniae*. Gram positive bacteria were isolated from all the samples collected giving an impression of being the most prevalent cause of mastitis. All clinical mastitis cases were treated with the synthetic anti-mastitis preparations on site, as an ethical necessity.

4.3.5 Trial 5

This trial, similar to trial 4 was the second phase that involved collection of critical case samples from selected cows of farmers who are members of Milk Collection

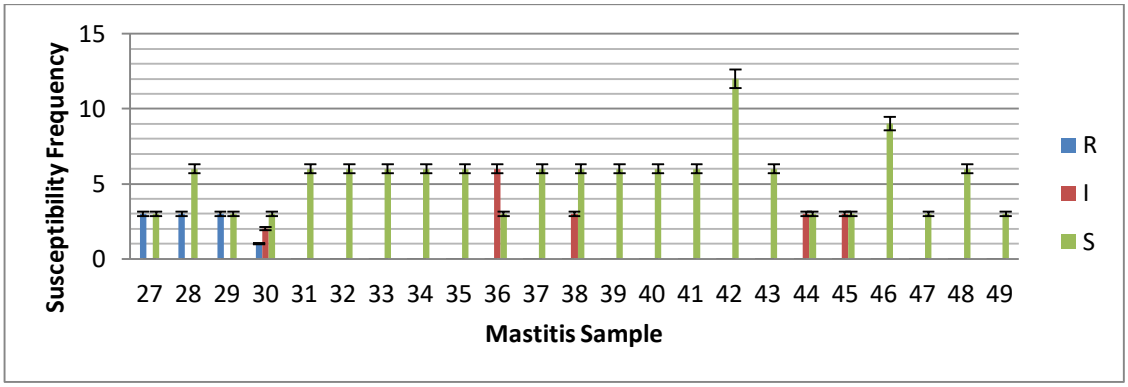
centres/ dairy cooperatives under the GIZ, Green Innovation Centres (GIC) Project in the Southern Province of Zambia. These are Batoka Dairy Cooperative in Choma, Monze Dairy Cooperative in Monze and Magoye dairy Cooperative in Mazabuka (their actual positions are as indicated in Chapter 3 above). Table 4.9 below summarizes the microscopically detected bacterial species. These susceptibility findings were communicated promptly to the farmers from whom the samples were collected as an ethical requisite and in order to render immediate benefits to the participants.

Table 4.9: Microscopy Results From Trial 5 Mastitis (Field) Samples

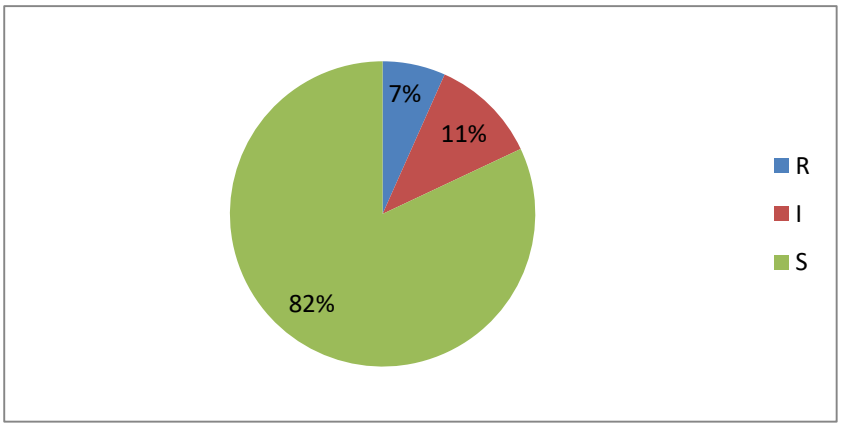
DISTRICT	**MILK COLLECTION CENTRE**	**SAMPLE ID**	**MICROSCOPY SPECIES QUERRIED**
CHOMA	BATOKA	27	*Staphylococcus* & *Bacillus*
		28	*Staphylococcus* & *Bacillus*
		29	*Staphylococcus* & *Bacillus*
		30	*Lactobacillus, Staphylococcus & Streptococcus*
		31	*Streptococcus*
		32	*Staphylococcus*
		33	*Bacillus*& *Streptococcus*
MONZE	MONZE	34	*Staphylococcus*& *Bacillus*
		35	*Staphylococcus*
MAZABUKA	MAGOYE	36	*Bacillus*& *Staphylococcus*
		37	*Staphylococcus*
		38	*Staphylococcus*
		39	*Staphylococcus*
		40	*Staphylococcus*
		41	*Staphylococcus*
		42	*Staphylococcus*
		43	*Staphylococcus*
		44	*Streptococci* & *Staphylococci*
		45	*Staphylococcus*

		46	*Staphylococcus & Coccobacilli*
		47	*Bacillus*
		48	*Bacillus & Staphylococcus*
		49	*Staphylococcus*

The *in-vitro* effects of treatments on all isolates from mastitis cases from trial 5 are illustrated below. Figure 4.14(a) categorically depicts individual susceptibility of the trial 5 microbial isolates (See Table 4.9 for details) to the herbal formulation. The pie chart in Figure 4.14(b) displays the overall performance of the herbs against all microbial isolates tested.



(a)



(b)

Figure 4.14 (a) & (b): Trial 5 Herbal Extracts Susceptibility on *thirty four* Field Mastitis Isolates (R is resistant, I intermediate and S is Susceptible, n=150)

Freshly prepared extracts were utilized for trial 5 experimentation. The Mastitis sample isolates were randomly spot inoculated on the agar containing the herbal extracts at 5% concentration. All the microbes isolated were gram positive as shown in Table 4.9

above (See Table 4.9 for details). High levels of susceptibility against the herbal extracts were noted. On the overall, 82% of the sample isolates were Susceptible to the herbal formulation and when combined with 11% intermediate susceptibility, totalled 93% susceptibility and only 7% resistance on all observations made was recorded. The results of the susceptibility of the mastitis sample isolates to the synthetic antimicrobials are depicted in Figure 4.15 below.

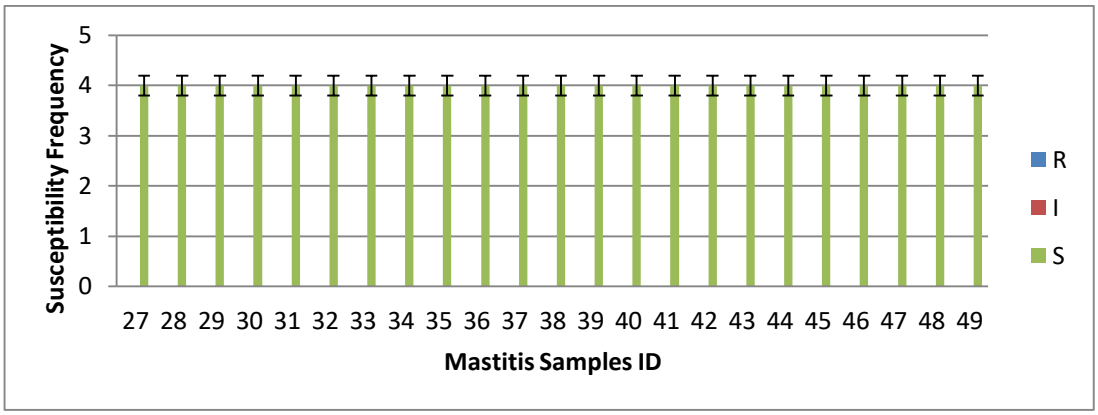


Figure 4.15: Trial 5 Synthetic Anti-mastitis' Susceptibility on all *thirty four* Field Mastitis Isolates (R is resistant, I intermediate and S is Susceptible, n=92)

All the isolates were Susceptible to the synthetic antimicrobials which were used for comparative purposes and as positive controls. 100% susceptibility was recorded at the 5% dilute agar concentration from the initial concentrations (Terrexin ® :Each injector of 10 gram : Cefalexin 200 mg and Kanamycin monosulfate 100,000 IU ; Rilexine 200 L.C®containing Cefalexin 100mg, Neomycin Sulphate 100mg and Prednisolone 10mg, per 10ml).

The negative controls were cultured on DMSO treatment and plain MHA without any treatments and showed 100% resistance.

It was observed that all sterile water extracts despite yielding the highest extractable compound, were resistant after the 20-24hrs of incubation. Therefore observation was discontinued and subsequently use of water as a solvent was disregarded after consistency in the first 2 trials.

CHAPTER 5

5.0 DISCUSSION

In this study, we established the efficacy of Zambian grown *Aloe* genus and *Curcuma longa* ethno veterinary herbal formulations' antimicrobial activity on bovine mastitis causing micro-organisms *in-vitro* and the effectiveness was compared to the locally available synthetic antimicrobials for treatment of mastitis.

The determined extractive values from the 5g of the dried herbs were 5% (250mg), 1.2% (59.15mg) and 0.6% (27.5mg) for the aqueous, ethanol and ethyl acetate solvents respectively. These values differ markedly from those of Punniamurthy and Ramakrishnan (2015) whose experiment showed an extractive value of 10.3% for the aqueous, 21.3% for the ethanol and 3.82% for the ethyl acetate. Our experiment indicated that the aqueous extract had the highest extractive value seconded by ethanol and then ethyl acetate was third. However, the Analysis of Variance (α=0.05) test whose null hypothesis was that there was no difference between the means of the three extracts was not rejected as the F observed was found to be less than the F expected (F critical). It was also noted that the extractive values had no implication on the quality of the extract as the extract from aqueous solvent was the least efficacious compared to that from the other two extracts. Therefore, this part of our experiment showed that the extractive value has no bearing in estimating the potency of the extract based on the solvent used. Other resources such as phytochemical analysis may provide answers to this variation. This in most part could be pivoted on the realization that water, only extracts water soluble compounds leaving out the lipid soluble ones. Ethanol on the contrary, extracts both water and lipid soluble compounds because of its polarity, while ethyl acetate, extracts only lipid soluble compounds, leaving out water soluble ones. However further phytochemical analysis may be required to streamline all the compounds in the crude extracts from each solvent.

The experiment determined the individual *in-vitro* antimicrobial activity of the herbal ethanol extract constituents of the herbs. The *Curcuma longa* treatment indicated that the gram negative *E. coli* and *Pseudomonas aeruginosa* were most resistant and accounted for the 32% resistance recorded cumulatively. The gram positive *Staphylococcus aureus* control and isolates were the most susceptible to this treatment

with variations noted, whereby, susceptibility observed 24hrs post incubation changed to intermediate susceptibility with the passage of time and reverted to full susceptibility again. Therefore the *C. longa* antimicrobial activity can be understood to be bacteriostatic based on variations of bacterial growth during the observation period. On the overall the treatment was Susceptible on 55% of all observations and in combination with the 13% intermediate susceptibility, yielded a total of 68% susceptibility on all observations made. These findings are concur with those of Chattopadhyay et al (2004) who in their review highlighted that both curcumin and the oil fraction of Turmeric suppressed the growth of several bacteria like *Streptococcus, Staphylococcus* and *Lactobacillus.* The *C. longa* extract can thus be stated to have efficacy against gram positive bacteria, which from our field isolates were found to be the most prevalent causes of mastitis.

Similar to the individual *C. longa* extract, the *Aloe* extract could not suppress the Gram negative *E.coli* and *Pseudomonas aeruginosa*, whose resistance accounted for the most part of the 51% resistance recorded cumulatively. The gram positive *Staphylococcus aureus* control and isolates were again the most susceptible to the treatment with variations being noted where the susceptibility observations changed from Susceptible to intermediate and vice-versa and also to resistant. On the overall the treatment was Susceptible to the bacteria on 45% of all observations and in combination with the 4% intermediate susceptibility yielded a total of 49% susceptibility on all observations made. The findings show some minor variations from those of Alemdar and Agaoglu (2009) who determined the antimicrobial activity of the *Aloe vera* juice against Gram-positive bacteria (*Mycobacterium smegmatis, Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus* and *Bacillus sphericus*), Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and *Salmonella typhimurium*) and *Candida albicans in-vitro*. Their study revealed that *Aloe vera* juice had antimicrobial activity against *M. smegmatis, K. pneumoniae, E. faecalis, M. luteus, C. albicans* and *B. sphericus*, but showed no inhibitory effect against the other bacterial strains like *Staphylococcus aureus* which was susceptible in our study. Gram negative *Pseudomonas aeruginosa* and *Escherichia coli* were resistant in their study just like in this study. Such findings prompt further investigations into the observed efficacy variations with hypothesis bent on factors such as plant growth stage, time of plant

collection and species variations in addition to other influences such as the drying conditions and extraction equipment as well as use of the *Aloe vera* juice relative to the ethanol extract. It can however be deduced that the *Aloe vera* efficacy tends more towards the gram positives.

Comparatively, the study established that the discrete *Curcuma longa* extract was 19% more Susceptible to the isolates tested than the *Aloes* (Susceptible and Intermediate combined).

Five trials were conducted for determination of the antimicrobial activity of the combined *Aloe vera* and *Curcuma longa* formulation and the findings were compared to the commercially available synthetic antimicrobials used for mastitis management.

In the first trial, gram negative *E. coli* and *P. aeruginosa* were again resistant and accounted for the most part of the 48% resistance recorded for the herbal formulation cumulatively. The *Staphylococcus aureus* control and isolates were the most susceptible to the herbal treatment with variations noted between intermediate susceptibility and full susceptibility with time. On the overall the treatment was Susceptible to the bacteria on 37% of all observations and in combination with the 15% intermediate susceptibility yielded a total of 52% susceptibility on all observations made. Results from the gram positive isolates when analysed alone showed 62% susceptibility, and when combined with the 25% intermediate susceptibility totalled 87% susceptibility.

When compared to the susceptibility results of the synthetic antimicrobials (Terrexin® and Rilexine®) in trial 1, it was noted that the gram negative *E. coli* and *P. aeruginosa* were again the most resistant and accounted for over two thirds of the 49% resistance recorded cumulatively. The *S. aureus* also showed some level of resistance with time to the synthetic antimicrobials during the period of observation. The Mastitis isolated *S. aureus* was the most susceptible to this treatment with variations noted between intermediate susceptibility and full susceptibility. On the overall the synthetic antimicrobials treatment was susceptible to the bacteria on 44% of all observations and in combination with the 7% intermediate susceptibility yielded a total of 51% susceptibility from all observations made. The gram positive isolates when analysed

alone exhibited 63% susceptibility, and when combined with the 8% intermediate susceptibility equalled 71% susceptibility.

The susceptibility on the overall for the herbal formulation in trial 1 was 52% (intermediate plus full susceptibility) while that of the synthetics was 51%, and when the gram positives were analysed alone, the herbs yielded susceptibility rates of 87% to 71% for the synthetics. The herbal formulation can therefore be said to have performed better than the synthetics during this trial. These findings are only slightly inconsistent with the findings of Punniamurthy and Ramakrishnan (2015), who in their agar well diffusion test of the antimicrobial activity of the TANUVAS formulation at 200µL per well, stated that the anti-mastitis herbal preparation had inhibitory effects against pathogenic bacteria such as *E. coli* and *S. aureus* under *in-vitro* conditions. Our agar dilution test findings at concentrations of 2.5-10% however showed *E. coli* resistance, but were consistent on the *S. aureus*.

A porcine nasal swab isolate of *Staphylococcus aureus* was also utilised for this trial and was noted to be Susceptible to the herbal formulation. This finding therefore opens up more avenues for ethno veterinary research into porcine infectious conditions as a way of fighting antimicrobial resistance and reducing use of synthetic antimicrobials in this species.

In the second trial an analysis of the herbal formulation revealed that the gram negative isolates (*E. coli* and *P. aeruginosa*) were again consistently resistant and accounted for over three quarters of the 72% resistance recorded cumulatively. The *Staphylococcus aureus*, control strain and the field mastitis isolate accounted for less than 10% of the overall resistance. On the overall the bacteria was Susceptible to the formulation on 8% of all observations and in combination with the 20% intermediate susceptibility yielded a total of 28% susceptibility from all observations made. However, when analysed separately, results from the gram positive isolates exhibited 26% susceptibility, and when combined with the 59% intermediate susceptibility equalled 85% susceptibility. The intermediate resistance was the most predominant susceptibility during this trial for the herbal formulations.

The isolates in the second trial were also tested at three times the initial applications of some select extracts from the first trial. The results revealed that the gram negative isolates *E. coli* and *P. aeruginosa* were again resistant and accounted for the whole of the 66% resistance recorded cumulatively. The *Staphylococcus aureus,* control strain and the field mastitis isolate accounted for 0% of the total resistance on all observations made. On the overall the treatment was effective on the bacteria on 22% of all observations and in combination with the 12% intermediate susceptibility yielded a total of 34% susceptibility from all observations made. However, when analysed in isolation, the gram positive isolates exhibited 65% susceptibility, and when combined with the 35% intermediate susceptibility equalled 100% susceptibility at this higher application. These findings suggest that increasing the application quantity of the formulation may increase efficacy against the gram positives, however, this may require further toxicity analysis. However, this may not be absolutely necessary as the formulation has been proven effective at the current formulation proportions (Balakrishnan et al, 2017)

As per the performance of the synthetics' in the second trial, the *E. coli* was found to be the sole contributor to the 7% resistance noted cumulatively. In this trial, the synthetics were Susceptible to the bacteria on 85% of all observations and in combination with the 8% intermediate susceptibility yielded a total of 93% susceptibility from all observations made.

The synthetic antimicrobials performed better than the herbal formulations in trial 2 where the most prevalent susceptibility reading was intermediate. The poor performance of the herbs could be attributed to the poor drying conditions of the herbs which could have caused some degradation as part of the drying was conducted in the incubator for 5 days (with some becoming mouldy) before being transferred to the drying oven (when it was available for use). Therefore drying conditions can be said to be of utmost importance in the extraction process. This assumption is in line with that of Rocha et al (2011), who in their review of the influence of the drying process on the quality of medicinal plants concluded that the drying method, velocity and temperature of drying air influence the quantity and quality of the active ingredients present in aromatic and medicinal plants.

In the third trial, when the herbal formulation was assayed, all the microbes tested (*Bacillus babosus, Candida albicans* and *Streptococcus*) exhibited varying degrees of resistance which was cumulatively recorded at 40%. *Bacillus babosus,* was the most susceptible, while *Streptococcus* displayed equality between susceptibility and resistance based on the observations made. The *Candida albicans* was noted to be more Susceptible than resistant to the herbal formulation.

On the whole, the formulation in trial 3 was Susceptible to the microbes on 54% of all observations and in combination with the 6% intermediate susceptibility yielded a total of 60% susceptibility from all observations made.

The synthetic antibiotics in trial 3 had no inhibitory effect on the *Candida albicans* (fungal) which in itself accounted for two thirds of the overall resistance on all observations. *Streptococcus* isolates were the most susceptible to the synthetic antimicrobials while *Bacillus babosus* showed the least susceptibility of the three pathogenic microbial isolates. Statistically, the combined susceptibility of the microbes to the synthetic antimicrobials was 62% (56% Susceptible and 6% Intermediate) from all observations.

Therefore the synthetic antimicrobial was 2% superior over the herbal formulation in the third trial despite not being able to suppress the growth of the *C. albicans.* It should however be noted that the commercial anti-mastitis formulations are a mixture of both gram positive and gram negative antimicrobials but have no antifungal. The herbal extract on the other hand displayed gram positive and antifungal activity.

The findings from the third trial are consistent with earlier mentioned findings of Alemdar and Agaoglu (2009), who in their study revealed that *Aloe vera* juice had antimicrobial activity against microbes such as *Candida albicans* and *Bacillus sphericus*. Therefore the antimicrobial activity of the formulation on the *C. albicans* could be attributed to the *Aloe* thereby making the formulation more advantageous for management of mastitis of fungal aetiology. This high efficacy of the herbal extract against *Candida* is an interesting finding that opens up room for further investigation. This is because epidemiologically, bacteria are the most prevalent causes of mastitis, however, prolonged use of antimicrobials potentially suppress the commensal bacteria

too, which leads to increased prevalence of fungal infections. The resultant is a chain reaction of more secondary opportunistic bacteria and inevitably higher antimicrobial use. Therefore, the herbal formulation may be more beneficial in management of persistent mastitis in which opportunistic fungal infections may arise. This finding therefore necessitates further clinical justification.

The fourth trial involved collection of twenty six (26) critical case mastitis samples. The agar well diffusion method was experimented with for this trial and the zones of inhibition recorded for the three treatments had their variances analysed (ANOVA at α=0.05) using Microsoft Excel 2010 with the hypothesis that there was no significant difference in the means of the three treatments (herbal formulation, Rilexine® and Terrexin®). This hypothesis after analysis was rejected as the F (observed) was greater than the F (critical). This therefore entails that the mean zones of inhibitions for the synthetic antimicrobials (Rilexine®= 1.09cm, Terrexin®= 1.07cm) were more significant to the mean of the herbal formulation (mean= 0.48cm).

The presence of only gram positive isolates from the field critical case samples during trial 4 provides further relevance to the use of the herbal formulation which in all trials proved to be efficacious against gram positive bacteria. Only one sample (ID 9) revealed some resistance to all treatments and the species queried in this sample were *Streptococcus* and *Streptococcus pneumoniae*. The other isolates queried are tabulated in Table 4.8 above. The presence of such isolates consolidates the findings of Eriksson (2013), who reported that the most common udder pathogens in Mapepe, Batoka and Choma areas in Zambia were the gram positives, and therefore justifies the need to adopt the use of this herbal formulation in Zambia

The herbal formulation assays in the fifth trial that used freshly prepared extracts depicted high levels of susceptibility against the cultured microbes. On the overall, 82% of the sample isolates were Susceptible to the herbal formulation and when combined with 11% intermediate susceptibility, totalled 93% susceptibility. Therefore, only 7% resistance was recorded on all observations made for this trial.

The synthetic antimicrobials suppressed the growth of all the isolates during the fifth trial thereby recording 100% susceptibility.

The synthetic antimicrobials once more proved to be marginally (7%) superior to the crude herbal extract with regards to susceptibility in trial 5.

The use of freshly prepared extracts in the susceptibility analysis though inconclusive proved to be highly valuable in the fifth trial as the extracts were immediately diluted into the agar at the end of the extraction process unlike in previous trials where the isolates had been stored at -20^{o}C for short periods of time (1day to 3 weeks). This study therefore endorses use of freshly prepared extracts for *in-vitro* ethno veterinary medicine trials.

The herbal extracts' susceptibility against mastitis causing micro-organisms was determined *in-vitro* and the formulation was found to be mostly effective against the gram positive bacteria and fungi. The herbal extract proved to possess more superior antifungal properties over the cephalexin, kanamycin and neomycin containing synthetic anti-mastitis preparations available on the Zambian market while the synthetic antimicrobials displayed their antibacterial superiority. On the overall, the average for all agar dilution trials revealed; 41.8% Resistance, 13% Intermediate and 45.2% Susceptibility for the herbal formulation, while the synthetic antimicrobial produced 23.5% Resistance, 5.3% Intermediate and 71.2% Susceptibility. The gram positive microbes when analysed alone for the agar dilution tests indicate; 18.7% Resistance, 25.3% Intermediate and 56% Susceptible for the herbal preparation, while the synthetic anti-mastitis formulation produced; 16.7% Resistance, 4.8% Intermediate and 78.5% Susceptible. The comparison can however be said to be subjective because the synthetic antimicrobials contain single useful antimicrobial elements, while the crude extracts include even those elements that may not be very beneficial, therefore, this comparison can be said to be imbalanced.

From the three categorically analyzed herbal ethanol extracts for the Minimum Inhibitory concentration (MIC) determination on *Staphylococcus aureus* the 5% dilution was inferred as the MIC, and the average concentration arising from the initial concentrations was determined to be 11.5mg/ml. This is the mean arising from the 13mg/ml for the first sample (9T2) in run 1 and from run 2, the correlated concentrations at 5% dilution which were 12.5mg/ml & 9mg/ml for extract 7(T1) and

18(T2) respectively. These findings vary slightly with those of Punniamurthy (2015) who found an ethanol extract MIC on *S. aureus* of 20mg/ml. This variation noted could be speculatively attributable to several factors including concentration of the inoculant in addition to potential human error. The Cephalexin MIC for *S. aureus* is much lower at 0.5mg/L or 0.0005mg/ml (Andrews 2001). Therefore, comparison of the herbal preparation MIC to that of the synthetic anti-mastitis preparations can be said to be only indicative based on the wide variation of the active constituents which are more purified for the synthetic preparation in comparison to the crude extract. Given that the crude extract shows good efficacy on most gram positive isolates, it is a promising candidate for further investigation and development to separate pure active compounds that can ultimately be compared on the appropriate equimolar basis.

In this study, the antimicrobial activity of individual extracts of the *Aloe* genus and *Curcuma longa* on pathogenic mastitis causing bacteria was established for the very first time in Zambia. The extracts were found to mostly suppress the growth of gram positive micro-organisms.

This study, in line with the findings of Alemdar and Agaoglu (2009) established that the combined formulation of *Aloe vera* and *Curcuma longa* possesses antifungal attributes in addition to the antimicrobial properties against gram positive microbes *in-vitro*. The abundance of gram positive mastitis field isolates in comparison to gram negative isolates potentially suggests that the anti-mastitis herbal formulation could be an ideal alternative or addition to the current management protocol for mastitis treatment in Zambia.

CHAPTER 6

6.1 CONCLUSION

This study established that obtainable Zambian *Aloe* genus and *Curcuma longa* ethno veterinary herbal preparations, possess gram positive antibacterial and antifungal efficacy on bovine mastitis causing and other pathogenic micro-organisms *in-vitro*. The combined herbal formulations' ethanol extracts, possess superior effectiveness over the singular herb extracts, at an MIC of 5%, and can be utilized as an effective alternative or adjunct to locally used synthetic anti-mastitis preparations.

6.2 RECOMMENDATIONS

- More studies are required to purify compounds in order to identify the active ingredients and their concentrations per unit crude extract, which would be useful in standardizing the therapeutic dose.
- This study recommends the conduct of clinical trials of the ethno veterinary remedy for mastitis in Zambia.
- The study also suggests the need to conduct an intervention impact analysis on the reduction of antibiotic residues in the milk when the herbal formulation are available for use.
- There is a need to extend this study into evaluation of the *in vitro* pharmacognostic understanding of the elements in the formulation based on the available literature and phytochemical assessment.
- There is also need to promote networking among institutions and individuals working in the field of ethno veterinary knowledge in Zambia in order to establish the status of ethno-veterinary knowledge in Zambia.
- There is need to develop a cadre of ethno veterinary practitioners through a deliberate and elaborate post graduate diploma in ethno veterinary practices that will facilitate good use of our local biodiversity. Ethno veterinary practice if harnessed through a one health approach can potentially provide solutions to antimicrobial resistance and foster further empirical solution based research into other beneficial herbal formulations for tackling various zoonotic

conditions affecting the livestock sector and of human medical importance in Zambia.

- The study also calls for concerted efforts in motivating environmental conservancy of ethno botanical resources and fostering agricultural diversification through medicinal herb production for wealth and employment creation.

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