ANTIMICROBIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST PATHOGENS CAUSING CLINICAL MASTITIS IN DAIRY COWS IN GURSUM DISTRICT, OROMIA, ETHIOPIA

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I certify that I have read and evaluated this thesis entitled **Antimicrobial Activities of Selected Medicinal Plants against Pathogens Causing Clinical Mastitis in Dairy Cows in Gursum District, Oromia, Ethiopia** prepared under my guidance by Abdinur Mohammed. I recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

This Thesis is dedicated to my wife Mrs. Iftuu Gaaromsaa and my children for their unwavering love, support and encouragement throughout my academic journey even in challenging times.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this Thesis is my own work. I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis and compilation of this Thesis. Any scholarly matter that is included in the Thesis has been given recognition through citation.

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BIOGRAPHY

Abdinur Mohammed Ali was born on September 20, 1970, in Gursum district, East Hararghe Zone, Oromia Regional State, Ethiopia. He completed his primary education at Lafto Primary School and his secondary education at Gursum Secondary School. Abdinur earned a diploma in Animal Health from Addis Ababa University and began his career with the Ministry of Agriculture as an Animal Health Assistant, serving in various capacities.

He later returned to Addis Ababa University to obtain a BSc degree in Veterinary Laboratory Technology, after which he continued his work at the Gursum District Agriculture Office. Driven by his passion for advancing veterinary science, Abdinur pursued postgraduate studies in Veterinary Microbiology at Haramaya University, further solidifying his expertise in the field.

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LIST OF ABREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CD-RW	Rewritable Compact Disc
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase Negative Staphylococci
CSA	Central Statistical Agency
IMViC	Indole, Methyl Red, Voges Proskaur and Citrate Test
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibition Concentration
NCEs	New Chemical Entities
ND	Newcastle Disease
PAs	Peasant Associations
SD	Standard Deviation
SEM	Standard Error of the Mean
SPSS	Statistical Package for Social Science
TSI	Triple Sugar Iron

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ABSTRACT

The main objective of this study was to evaluate the antimicrobial activities of selected medicinal plants against bacterial and fungal pathogens isolated from clinical mastitis in dairy cows in Gursum District, Oromia, Ethiopia. Antimicrobial activities of *Dolichos oliveri (D. oliveri)*, *Mirabilis jalapa* L. (*M. jalapa* L.), *Euphorbia schimperiana (E. shimperiana)*, and *Cyphostemma adenocaule (C. adenocaule)* on *Staphylococcus aureus, Enterobacter aerogenes*, *Candida albicans*, and *Aspergillus fumigatus* was conducted by Methanol extraction and disc diffusion method measuring the mean zone of inhibition. The Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC)was determined by serial two-fold dilution using Tube Broth Dilution (broth macrodilution). Data analysis involved one-way ANOVA and Tukey post hoc tests using SPSS version 20. Phytochemical analysis identified active compounds, including alkaloids, phenols, saponins, and terpenoids, responsible for the antimicrobial properties. *Dolichos oliveri* exhibited the highest inhibition zone (13.33 ± 0.1 mm) against *S. aureus* standard strains at 125 mg/ml. *D. oliveri* and *E. shimperiana* showed significantly higher ($P<0.05$) activity against standard strains of *S. aureus*, *E. aerogenes*, *C. albicans* and *A. fumigatus* at 125 mg/ml than *M. jalapa* L. On the other hand, *M. jalapa* L.. had significantly higher activity against *C. albicans* ($P<0.05$) at the concentration of 125 mg/ml than all the tested plants. *E. shimperiana* and *C. adenocaule* showed significantly higher ($p<0.05$) activity against the clinical isolate of *A. fumigatus* at 125 mg/ml. Minimum Inhibitory Concentration (MIC) values ranged from 37.5 mg/ml (*E. shimperiana*, *C. adenocaule*, and *M. jalapa* L*)* against *S. aureus* and *E. aerogenes* to 125 mg/ml (*D. oliveri) against S. aureus and C. albicans*. Minimum Bactericidal Concentration (MBC) ranged from 100 mg/ml (D. oliveri) against *S. aureus* to 400 mg/ml (C. adenocaule and *M. jalapa* L.) against *S. aureus* and *E. aerogenes*. Minimum Fungicidal Concentration (MFC) was exhibited only at 200 mg/ml by *M. jalapa* L.. against *A. fumigatus*. *M. jalapa* was the only plant showing anti-fungal activity at MIC value (37.5 mg/ml) against *A. fumigatus*. It showed selective bacteriostatic and fungistatic effects on *E. aerogenes* and *A. fumigatus*. These findings justify the traditional use of the plants for the treatment of mastitis in dairy cows underscoring their promising potential as alternative antimicrobial agents for managing mastitis and other livestock diseases. Therefore, phytochemical isolation and analysis of individual bioactive compounds of plant extracts, their in vivo antimicrobial activities with different extraction solvents and their toxicity levels should be further studied to use them for the development of alternative sources of drugs.

Keywords: *Antibacterial, Antifungal, Bovine mastitis, Medicinal plants, Phytochemicals*.

1. INTRODUCTION

1. 1. Background Information

Animal diseases remain a major obstacle to livestock production in Ethiopia, causing economic losses due to their impact on productivity (Sori et al., 2004). Among these, mastitis is one of the most common and widespread diseases affecting dairy cows, leading to significant economic losses by decreasing milk production, increasing treatment costs, and raising culling rates (Bradley, 2002; Halasa, 2007; Zaragoza et al., 2011; Abebe et al., 2016). It is ranked as the most costly disease in dairy cows worldwide (Zafalon et al., 2007).

The conventional drugs used for the treatment of mastitis are limited in developing countries in general and in Ethiopia in particular (Asaye et al., 2015). The causative agents of mastitis were also reported to have developed resistance to the commonly used antimicrobial agents (Dego and Tareke, 2003). Additionally, biofilm-related infections caused by bacteria such as *Staphylococcus epidermidis*, *S. aureus*, *Streptococcus* species, and *E. coli* had been recognized in human medicine many of which are also major pathogens involved in bovine mastitis making the treatment of recurrent infections difficult (Melchior *et al.*, 2006). The problem of antifungal drug resistance of *Aspergillus* and *Candida* species has also been reported by researchers as one of the possible reasons for treatment failure in livestock diseases including mastitis (Bakr *et al.,* 2015).

Due to the challenges of antimicrobial resistance, high costs and shortage of conventional drugs and social acceptability of traditional medicine among local communities in different parts of Ethiopia, there should be strategies to improve the current situation which may include a research to find new and innovative anti-microbial drugs from plants.

1.2. Problem Statement

Mastitis significantly affects dairy farming in Ethiopia and increases economic burdens on farmers (Bradley, 2002; Halasa et al., 2007; Zaragoza et al., 2011; Abebe et al., 2016). The effectiveness of conventional antimicrobial drugs is diminishing due to increasing drug

resistance among bacterial and fungal pathogens (Dego & Tareke, 2003; Melchior et al., 2006; Bakr et al., 2015). Furthermore, the availability of conventional drugs is limited (Asaye et al., 2015), and their cost is often expensive for many farmers (Shewit Kalayou et al., 2012; Habtamu Kinde et al., 2015). As a result, alternative treatments such as medicinal plants have become essential. However, little is known about the efficacy of locally available medicinal plants in treating bovine mastitis in Ethiopia, and research on their antimicrobial properties is scarce (Miruts & Teklehaymanot, 2013).

1.3. Justification

Medicinal plants have been used for the treatment and prevention of human and livestock diseases since the ancient times (Teklehaymanot and Giday, 2007; Lulekal *et al.*, 2008; Devi *et al.*, 2009). Plants can produce varieties of bioactive molecules (phytochemicals), most of which probably evolved as chemical defense against predation or infection (Okwu, 2004; Samie *et al.*, 2010). For instance, high concentrations of phytochemicals accumulated in fruits and vegetables may protect against free radical damage acting as natural antioxidants (Suffredini *et al.*, 2004) and also having antimicrobial activities (Boots *et al.*, 2008; Ammar *et al*., 2017).

Plant-based traditional medicine is used throughout the world because of local availability of different plant species, affordability, ease of administration and cumulative knowledge and wisdom acquired by local people over years of practicing (Awas and Demissew, 2009; Kibebew *et al.*, 2001; Teklehaymanot and Giday, 2007).

Approximately 20% of known plants have been used in pharmaceutical studies, impacting the healthcare system in positive ways such as treating cancer and harmful diseases (Naczk and Shahidi, 2006). Over 120 active compounds isolated from plants are widely used in modern medicine and 80% of this show a positive correlation between modern and traditional therapeutic use of the plants they are derived from (Fabricant and Farnsworth, 2001)**.** Emetine**,** quinine and taxol are some of the drugs derived from plants that were used against amoeba, malaria and cancers respectively.

Medicinal plants are still regarded as the most important source and sometimes the only alternative source for 80% of human population and more than 90% of livestock diseases in

Ethiopia (Shewit Kalayou *et al*., 2012; Habtamu Kinde *et al.*, 2015). Ethiopia is home to approximately 6,500 to 7,000 species of higher plants, of which 12% are endemic and have medicinal value (Tadeg et al., 2005; Miruts et al., 2009). The use of ethnomedicinal plants to treat bovine clinical mastitis is widely practiced in Ethiopia for years. Researchers have reported that different ethnic populations in different parts of Ethiopia use varieties of medicinal plants to treat bovine mastitis in their respective areas and most of them reported promising results (Assaye *et al*., 2015; Sahlu 2013; Shewit *et al*., 2012; Mengistu, 2004; Taddese *et al*., 2009; Mohamedamin, 2011; and Regassa and Araya, 2012).

Despite its paramount role, ethnoveterinary practice in Ethiopia is being affected by many factors. Some of the factors include: depletion of plants as a result of population pressure, drought, environmental degradation, deforestation, agricultural expansion, cultivation of marginal lands, urbanization and over exploitation of the medicinal plants (Berhan and Dessie, 2002; Teklehaymanot and Giday, 2007; Lulekal *et al.*, 2008; Miruts *et al.*, 2009; Gessese, 2010).

So far, there is limited studies conducted on the ethnoveterinary medicine in Ethiopia as compared to diverse cultural practices of traditional healing and floral diversity in the country. There is also lack of proper documentation and analysis of ethnoveterinary knowledge of Ethiopian farmers and pastoralists (Miruts and Gobena, 2003; Yineger and Yewhalaw, 2007; Miruts and Teklehaymanot, 2013). Since the traditional knowledge on ethnomedicinal plants and its associated practices in Ethiopia is conveyed verbally from generation to generation without documentation, the valuable information on the medicinal plants and its associated traditional knowledge could be lost irreversibly when a traditional healer passes away without conveying the knowledge to others (Mesfin *et al.*, 2009; Tolossa *et al.*, 2013).

Mastitis remains one of the most economically and clinically significant diseases in dairy cows, especially in developing countries (Radostits et al., 2007). Conventional antibiotic therapies, though widely used, are increasingly challenged by the global surge in antimicrobial resistance (AMR), particularly among mastitis-causing pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Ruegg, 2017; Bradley et al., 2015). This rise in resistance not only compromises treatment outcomes but also threatens public health through the potential spread of resistant bacteria and antibiotic residues in milk (Oliver et al., 2011). Furthermore, limited access to veterinary drugs in rural areas—due to cost, supply issues, and misuse—worsens the

situation (FAO, 2016). In this context, medicinal plants used in traditional veterinary medicine offer a promising and sustainable alternative. These plants harbor diverse phytochemicals with demonstrated antimicrobial properties, and their use aligns with community-based knowledge and accessible healthcare practices (Cowan, 1999; Yineger et al., 2007). Scientific validation of these plant-based treatments is thus essential, not only to ensure efficacy and safety but also to support integrated, eco-friendly approaches to mastitis control in the era of rising AMR.

To the best of our knowledge, there are no ethnomedicinal researches conducted in the current study area to investigate the use of medicinal plants for the treatment of clinical mastitis in dairy cows. Therefore, due to the increasing drug resistance of disease causing microorganisms against the existing conventional drugs, the need for affordable alternative sources of drugs from plants and its validation and integration into conventional veterinary medicine, this study was conducted to assess the antimicrobial activities of selected medicinal plants against common pathogens causing clinical mastitis in dairy cows.

1.4. Research Question

i) Do the extracts from *Dolichos oliveri*, *Mirabilis jalapa* L., *Euphorbia schimperiana*, and *Cyphostemma adenocaule* have any antimicrobial activity against the bacterial and fungal pathogens?

ii) what are the phytochemicals present in the extracts of *Dolichos oliveri*, *Mirabilis jalapa* L., *Euphorbia schimperiana*, and *Cyphostemma adenocaule*?

1.5. Hypothesis

i) Extracts from *Dolichos oliveri*, *Mirabilis jalapa* L., *Euphorbia schimperiana*, and *Cyphostemma adenocaule* do not have a bactericidal effect on bacterial pathogens and fungicidal effect on fungal pathogens.

ii) Phytochemicals present in the crude extracts from *Dolichos oliveri*, *Mirabilis jalapa* L., *Euphorbia schimperiana*, and *Cyphostemma adenocaule* are not known.

1.6. Objectives of the Study

1.6.1 General Objective

To evaluate the antimicrobial activities of selected medicinal plants against common pathogens causing clinical mastitis in dairy cows in Gursum District, Oromia, Ethiopia.

1.6.2. Specific Objectives

- To conduct methanol extraction of selected medicinal Plants and determine their phytochemical composition.
- To test the in vitro antimicrobial activity of selected medicinal plants against clinical isolates and standard strains of bacterial and fungal pathogens responsible for clinical mastitis in dairy cows
- To determine minimum inhibitory, bactericidal and fungicidal concentration of medicinal plants

1.7. Significance of the Study

The main aim of the study was to determine if the plant extracts from *Dolichos oliveri*, *Mirabilis jalapa* L., *Euphorbia schimperiana*, and *Cyphostemma adenocaule* have antimicrobial effects against the bacterial and fungal pathogens causing mastitis in dairy cows.

The study also focused on analysis of phytochemical constituents of plant extracts. The determination of phytochemicals present in the extracts was to propose the use of plant extracts as the primary material in the development of antimicrobial agents. This study may potentially provide an alternative to the conventional drugs currently in use; provide scientific validation of medicinal plants used in the treatment of bovine mastitis and other livestock disease and will contribute to preserving indigenous knowledge on traditional medicine and fostering its integration into modern veterinary practices.

2. LITERATURE REVIEW

2.1. Medicinal Plants

A medicinal plant is any plant which is in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs (Edwards, 2004). Local communities of different countries across the globe have indigenous experience in various medicinal plants whereby they use their perceptions and experience to categorize plants and plant parts to be used when dealing with different ailments (Omoruyi *et al.*, 2012). The use of medicinal plants to treat diseases is almost universal among non-industrialized societies and is often more affordable than purchasing expensive conventional drugs (Fabricant and Farnsworth, 2001).

Medicinal plants provide a rich source of raw materials for primary health care in Africa and other parts of the developing world. Ethnomedicinal studies play a major role in discovering new drugs from indigenous medicinal plants and green pharmaceuticals are getting popularity and extraordinary importance (Yaseen *et al.*, 2015). There are vast chances for new drug discoveries provided by the unrivaled availability of chemical diversity and natural products either as pure compounds or as homogenous plant extracts (Jigna and Sumitra, 2007). Previously, the synthetic drugs were approved as safe and effective because of unanticipated side effects but later had to be recalled and relabeled. The herbal medicines have no such adverse effects and because of combinations of medicinal constituents coupled with minerals and vitamins have benefits over synthetic ones (Hussain, 2007). Currently, the attention of scientists has been diverted towards ethno medicines due to the revival of knowledge in customary health practices throughout the world (Mustafa *et al.*, 2017).

Phytochemical studies based on ethnomedicinal practices are considered effective in discovering new bioactive compounds and antimicrobial agents, playing a crucial role in combating emerging and re-emerging pathogens (Savithramma et al., 2012; Duraipandiyan et al., 2006; Kordali et al., 2005). Notably, approximately 35% of the annual global market for medicines is derived from natural products or related drugs, with about 25% originating from plants (Atanasov et al., 2021).

Over the past few decades, there has been a growing interest in plants as sources of medicinal compounds. This resurgence is largely due to the successful derivation of several pivotal drugs from plants, such as digoxin, reserpine, and tubocurarine. These compounds have played significant roles in treating various ailments, underscoring the importance of plant-derived substances in modern medicine (Rates, 2001; Newman & Cragg, 2020). The isolation of active compounds from medicinal plants began with the extraction of morphine (table 1) from opium in the early 19th century, and has continued to the present day with ongoing discoveries of bioactive plant constituents (Samuelsson, 2004; Kinghorn, 2001).

Plants can synthesize and accumulate a great variety of Secondary metabolites called phytochemicals in their cells including saponins, tannins, flavonoids, cyanogenic, phenolic compounds, lignins, lignans, alkaloids and glycosides which may have probably evolved as chemical defense against predation or infection and have antimicrobial properties and can serve as an alternative source of antimicrobials (Okwu, 2004; Samie *et al.*, 2010).

Over 120 active compounds isolated from the higher plants are widely used in modern medicine and 80% of these show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived (Fabricant and Farnsworth, 2001**.** In this regard, emetine, obtained from the underground part of *Cephaelis ipecuanha and* related species have been used as amoebicidal drug for the treatment of *Amoeba histolytica* infections for many years. Quinine, which naturally occurs in the bark of the *Cinchona* tree, is another important drug of plant origin with a long history of usage against malaria. The drug taxol, (paclitaxel) is one of the most powerful anticancer drug known which was first isolated from the bark of the yew tree. *Terminalia arjuna* bark has long been used in cardiovascular therapy and continues to be studied for its cardioprotective effects (Dwivedi & Chopra, 2014), while *Andrographis paniculata* (a close relative of *Andrographis lineata*) has demonstrated antivenom properties and is traditionally used to treat snakebites (Gupta et al., 2019). The drug derived from *Taxus brevifolia* has yielded two approved treatments—paclitaxel and docetaxel—for breast and ovarian cancer (Jagessar et al., 2008). Populations consuming high amounts of plant-based foods such as vegetables, fruits, and soybeans tend to have a lower cancer risk due to the presence of antioxidants and phytochemicals (Aune et al., 2017). In poultry diseases, the stem parts of *Euphorbia candelabrum* have been used against Newcastle Disease (ND), while the

leaves of *Tetradenia riparia* (synonym of *Iboza multiflora*) combined with *Capsicum annuum* fruits have also shown effectiveness against ND (Gueye et al., 2009). Recent studies have demonstrated that herbal extracts, such as those from *Phyllanthus polygonoides* and *Carica papaya* leaves, possess therapeutic efficacy against ND in backyard poultry (Jayalakshmi et al., 2024). The extracts of several medicinal plants are also very effective against microbial and parasitic infections (Haider, 2001).

In traditional Chinese veterinary medicine, herbs have been used for centuries to treat a wide range of animal ailments, often following diagnostic principles similar to human Traditional Chinese Medicine (TCM) (Zhao et al., 2020). In India, Ayurvedic practices have long included plant-based remedies to manage livestock diseases, with formulations targeting digestive, respiratory, and infectious disorders (Patil et al., 2021). Similarly, African ethnoveterinary medicine has preserved indigenous knowledge, where local healers use plants to treat livestock ailments such as mastitis, parasitic infections, and wounds (Wanzala et al., 2012). In Latin America, especially among Andean communities, medicinal plants like Mirabilis jalapa are traditionally used in both human and animal health (Gómez-Beloz, 2002). These historical practices form the basis of modern ethnoveterinary research and underscore the importance of validating traditional knowledge through scientific methods.

In Ethiopia, the use of medicinal plants for veterinary purposes is widespread, particularly in rural and pastoral areas where access to modern veterinary services is limited (Giday et al., 2003; Lulekal et al., 2011; Tolossa et al., 2013). Several ethnobotanical studies have documented various plant species traditionally used for treating livestock ailments such as gastrointestinal problems, wounds, mastitis, and parasitic infections (Gebre et al., 2023; Regassa, 2000; Yineger et al., 2007). Conservationists and researchers have also raised concerns about the sustainability of these practices due to overharvesting and the erosion of indigenous knowledge systems (Lulekal et al., 2014).

2.2. Overview of Study Plants

2.2.1. *Dolichos oliveri*

The genus *Dolichos*, belonging to the Fabaceae family, includes herbaceous or climbing legumes predominantly found in tropical and subtropical regions *Dolichos oliveri*, in particular, is a perennial climber distributed widely in West, Central, and East Africa, including Ethiopia, Nigeria, Ghana, and Sudan (Burkill, 1985). It thrives in woodland environments and along riverbanks where the soil is fertile and well-drained. This species typically grows as a climbing vine with trifoliate leaves and produces purplish flowers in axillary racemes. The pods contain several seeds and are characteristic of legumes (Akinnibosun & Oboh, 2013).

Biologically, *D. oliveri* is notable for its nitrogen-fixing ability, contributing to soil enrichment in its natural habitat. Ethnobotanically, it has been employed in traditional African medicine for treating infections and inflammatory conditions due to its bioactive compounds such as flavonoids, alkaloids, and saponins, which contribute to its antimicrobial and anti-inflammatory potential (Ogunleye et al., 2021).

It has been employed in folk medicine to treat gastrointestinal and infectious conditions in both humans and animals (Burkill, 1985). Recent pharmacological studies have identified that its leaves and roots contain bioactive compounds such as alkaloids, saponins, and flavonoids with antimicrobial and antioxidant properties (Ayoola et al., 2018). Although it is not among the most extensively studied plants, preliminary investigations suggest potential antibacterial activity, particularly against Gram-positive organisms (Ogundare & Onifade, 2019).

2.2.2. *Mirabilis jalapa* L.

The genus *Mirabilis* is a member of the family Nyctaginaceae and includes ornamental and medicinal plants. *Mirabilis jalapa L.*, commonly referred to as the “Four o’clock flower,” is native to tropical South America but has been widely naturalized across tropical and subtropical regions worldwide, including parts of Africa (Kirtikar & Basu, 1991). It is commonly cultivated or found along roadsides, in gardens, and at forest edges. It is annual herbaceous plant up to 1m tall. It has ovate leaves and colorful, trumpet-shaped flowers that open in the late afternoon.

The plant contains a diverse range of phytochemicals, including triterpenoids, flavonoids, and alkaloids, which have been reported to possess significant antibacterial, antifungal, and antiviral activities (Saha & Verma, 2011). Studies have confirmed its antimicrobial properties against a variety of bacterial and fungal pathogens, attributing its activity to phytochemicals such as rotenoids, flavonoids, and phenolic acids (Sarma et al., 2021). The presence of various bioactive compounds validates the whole plant for different medicinal practitioners. It has been extensively used in almost all folklore remedies worldwide for treating a variety of conditions (Liya et al., 2021).

Mirabilis jalapa Linn. (Nyctaginaceae) is one of the plants that are used for health care and medicinal purposes for several thousands of years (Liya et al., 2021). Traditionally, its tuber and leaf extracts have been used in Latin America and Asia for anti-inflammatory, antimicrobial, and wound healing applications (Gómez-Beloz, 2002; Basu et al., 2020).

Mirabilis jalapa L. is also used for various human and animal ailments in Ethiopia. Bogale, Meskerem et al. (2023), in their ethnomedicinal study in tulo district, west hararghe zone, oromia region, Ethiopia, reported the use of Mirabilis jalapa L. for breast cancer by local people. In his study on in vitro antimicrobial assay of selected medicinal plants against medically important plant and food-borne pathogens, Sissay Bekele Mekbib (2016), also reported the use of Mirabilis jalapa L. for different bacterial infections.

2.2.3. *Euphorbia schimperiana*

The genus *Euphorbia*, under the family Euphorbiaceae, comprises a vast array of species ranging from herbs to shrubs and trees, many of which produce a characteristic milky latex. *E. schimperiana* is a shrubby herb with succulent stems and small, alternate leaves. It is usually found on rocky slopes, forest clearings, and grasslands with well-drained soils. The plant produces inconspicuous flowers arranged in cyathia and exudes a toxic latex upon injury.

Despite its toxicity, the latex and other plant parts are traditionally used for treating skin infections, wounds, and microbial diseases (Beentje, 1994). Pharmacologically, *Euphorbia* species are known to contain diterpenoids and phenolic compounds with strong antimicrobial properties (Chitnis et al., 2005).

Euphorbia schimperiana is indigenous to the highlands of Ethiopia and neighboring East African countries such as Kenya, and typically grows in highland and submontane environments at elevations of 1,500 to 3,000 meters above sea level (Gilbert, 1993). It is frequently used in Ethiopian ethnoveterinary medicine to treat skin infections, wounds, and respiratory ailments (Abebe & Ayehu, 1993). Its latex and leaves contain diterpenoids, triterpenoids, and tannins, which contribute to its antimicrobial and cytotoxic effects (Teklehaymanot & Giday, 2007). Recent laboratory analyses have confirmed its inhibitory effects against Staphylococcus aureus and Candida albicans (Gebrelibanos et al., 2022).

2.2.4. *Cyphostemma adenocaule*

The genus *Cyphostemma*, in the family Vitaceae, includes succulent vines and climbers widely distributed across tropical Africa. Cyphostemma adenocaule is a climbing, scrambling, or trailing herb that belongs to the Vitaceae family (Bello et al., 2019; Wickens & Burkill, 1986) and is native to tropical Africa. It is found predominantly in dry savanna, bushland, and rocky outcrop environments of countries such as Ethiopia, Nigeria, Tanzania, and Kenya (Bosch, 2006). The plant is a popular, non-cultivated vegetable eaten in many parts of Africa i.e., Nigeria, Ghana, Congo, Uganda, Ethiopia, and Eritrea (Bello et al., 2019). The plant had been documented for its ethnomedicinal value, with a comprehensive review given by Bello and colleagues (2019). The effect of Plant bioactive compounds against oxidative stress-related diseases and as an anti-infective had been well explored (Yakubu et al. 2021).

Traditionally, it is used for treating abscesses, wounds, and gastrointestinal issues in livestock (Nworu et al., 2014). Phytochemical screenings have revealed the presence of alkaloids, anthraquinones, and tannins. Research indicates that its extracts possess moderate antimicrobial and wound-healing properties, although more targeted studies are needed to confirm its efficacy against specific veterinary pathogens (Okoli et al., 2008).

2.3. Phytochemicals in Medicinal Plants

The pharmacological and medicinal properties of medicinal plants are often attributed to the presence of secondary plant metabolites (Heinrich *et al.*, 2004). Unlike the presence of universal macromolecules of primary metabolism such as monosaccharides, polysaccharides, proteins,

nucleic acids, lipids and amino acids in all plants, the secondary metabolites which have medicinal properties are observed only in a few species of plants (Kayani *et al.*, 2015).

Secondary metabolites play a role as defensive compounds against herbivores and pathogens. Others function in attracting pollinators and fruit dispersers, in reducing the growth of nearby competing plants, in mechanical support for plants or in absorbing harmful ultraviolet radiation (Ahmed *et al.*, 2015). Polysaccharides, waxes and fatty acids, terpenoids, phenolics (simple phenolics and flavonoids), alkaloids and glycosides and their derivatives are plant secondary metabolites which have medicinal properties but not limited to these compounds only.

Scientists began to isolate, purify and identify active constituents from medicinal plant extracts during the late nineteenth century and led them to find some vital drugs from medicinal plants that are still used in the field of modern medicine (Gupta, 2005).

Table 1: Discovery of some medicinal plants.

Drug	**Plant**	**Activity**
Morphine	*Papaver somniferum*	Powerful pain reliever and narcotic
Quinine	*Cinchona spp.*	Antimalarial
Taxol	*Taxus brevifollis*	Anticancerous
Vincristine	*Catharanthus rosesus*	Anticancerous
Serpentine	*Rauwolfia serpentia*	Hypertension

Source: Mustafa *et al.*, 2017.

Compounds for drug discovery have been obtained by using various methods including isolation and purification of active compounds from medicinal plants and other natural sources, combinatorial chemistry, synthetic chemistry and bioinformatics approaches such as molecular modeling (Lombardino *et al.*, 2004). Although the pharmaceutical companies and funding organizations are getting interested towards combinatorial chemistry, molecular modeling and other synthetic chemistry techniques, natural products, particularly medicinal plants remain an important source of new drugs, new chemical entities (NCEs) and new drug leads (Butler, 2004).

In Most cases, plant medicines have been used in their crude forms before nineteenth century and administered as infusions (herbal teas), decoctions (boiled extracts of bark and root),

tinctures (alcoholic extracts) and syrups (Griggs, 1981). Plants have also been applied externally as herbal washes and ointments (essential oils, poultices and balms) (Gurib-Fakim, 2005).

The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003). Numerous studies have identified compounds within herbal plants that are effective antibiotics (Afolayan, 2003).

The impact of different types of solvents, such as methanol, hexane, and ethyl alcohol have been studied and analyzed for the purpose of antioxidant extraction from various plant parts, such as leaves and seeds. In order to extract different phenolic compounds from plants with a high degree of accuracy, various solvents of differing polarities must be used (Yaseen *et al*., 2015). Moreover, highly polar solvents, such as methanol, have a high effectiveness as antioxidants and a dried powder of plants have been used to extract bioactive compounds and eliminate the interference of water at the same time. Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest (Ammar *et al*., 2017). A solvent of similar polarity to the solute will properly dissolve the solute. In this study, methanol is used as solvent to extract bioactive molecules from plants used in the study.

2.3.1. Carbohydrates and Related Compounds

Fibre, cellulose, starch and gums are carbohydrates and related compounds derived from plants (Bruneton, 1999). Carbohydrates and related compounds in addition to their use in pharmaceutical industry as bulking agents have also been shown to have immune-modulatory, hypoglycaemic, anticoagulant (e.g. heparin), antitumor and antiviral activities (Gurib-Fakim, 2005).

2.3.2. Alkaloids

The alkaloids contain nitrogen in a heterocyclic ring and are organic bases and many of them have pronounced pharmacological activities (Mc Naught, 1997; van Wyk *et al.*, 2000). On the basis their basic ring system (e.g. atropine, quinoline, indole, isoquinoline, piperdine alkaloids or imidazole), plant sources (e.g. opium, vinca, belladonna, ergot alkaloids or cinchona) or pharmacological properties (e.g. analgesic, anti-malarial alkaloids or stimulant), alkaloids can

be classified into several groups (Heinrich *et al.*, 2004). They may also sometimes contain oxygen, Sulphur, more rarely other elements such as chlorine, bromine, and phosphorus (Schardl *et al.*, 2007).

A variety of organisms including bacteria, fungi, and animals can also produce alkaloids (Kittakoop *et al*., 2014). Alkaloids are used by plants to defend against herbivores, microbial pathogens and invertebrate pests attacks because mostly they are strongly bitter in taste and are very toxic (Bruneton, 1999). They have a wide range of pharmacological activities such as antiasthma, antimalarial, anticancer, cholinomimetic, vasodilatory, antiamyhyrithic, analgesic, antibacterial and antihyperglycemic activities (Cushnie and Lamb, 2014). Alkaloids have great antimicrobial activity against bacterial pathogens such as *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aureginosa* (Maatalah *et al*., 2012). Bioactive components of alkaloids such as morphine and cordine have been found to be active not only against bacterial and fungal pathogens but also trypanosomes and plasmodia (Freiburghaus *et al*., 1996; Omulokoli *et al*., 1997).

Heinrich *et al.* 2004 and Blankenship *et al*. 2005 reported that several medicinal plants which contain alkaloids have been used by the early man as pain relievers and as recreational purposes or in some religious ceremonies to achieve a psychological state in which they could communicate with their ancestors or god.

Some of the Alkaloids found in dietary food materials have also been found to contain microbiocidal and antidiarrheal effect in the small intestines where they show the ability to intercalate with the microbial genetic material (Ghoshal *et al.*, 1996). Reserpine was derived from Vinca rosea and Rauwolfia serpentine and it reduces the production of neurotransmitters which causes hypotension and sedation by interfering membrane of synaptic vesicles (Kumar and Talapatra, 2015).

2.3.3. Phenolics

Phenolics are a class of plant secondary metabolites characterized by the presence of one or more hydroxyl (-OH) groups attached to an aromatic ring, such as benzene or other complex aromatic structures (Zhuang et al., 2023). These compounds can be broadly classified into

flavonoid and non-flavonoid phenolic compounds. Flavonoids, in particular, are responsible for various plant colors, play roles in pollination, and protect plants from UV radiation and pathogens (Kuljarusnont et al., 2024).

Structurally, flavonoids possess a 15-carbon skeleton comprising two phenyl rings and a heterocyclic ring, specifically a benzo-γ-pyrone structure (Zhuang et al., 2023). They are known to exhibit inhibitory activities against plant pathogens, such as *Fusarium oxysporum*, and possess antimicrobial properties effective against bacterial, fungal, viral, and insect pests (Cushnie & Lamb, 2005; Cushnie & Lamb, 2011). The antimicrobial mechanisms of flavonoids include inhibiting nucleic acid synthesis, disrupting cytoplasmic membrane integrity, and interfering with energy metabolism (Cushnie & Lamb, 2005). Additionally, flavonoids found in garlic have been effective in reducing atherosclerosis, coronary thrombosis, cholesterol levels, and other serious ailments (Kumar & Talapatra, 2015).

Studies have shown that flavonoids, when combined with antibiotics, exhibit synergistic effects, enhancing the suppression of various pathogenic microorganisms in both in vitro and in vivo settings (Cushnie & Lamb, 2011; Manner et al., 2013). Furthermore, in vivo studies suggest that flavonoids can be utilized as pharmaceutical agents for bacterial infections or through dietary intake to offer protection against infections (Zamora et al., 2012).

2.3.4. Terpenoids

The largest group of plant secondary metabolites is terpenoids which are also known as isoprenoids (Bruneton, 1999). They play different roles in plants such as in defense, thermotolerance, wound scaling and pollination of seed crops. Terpenoids also give flavors to fruits, fragrance to flowers and also responsible for the quality of agricultural products (Heinrich *et al.*, 2004). Structure of bisabolol is given in fig 3. Bisabolol is used as an anti-bacterial, antifungal, antimalarial and mulluscicidal drug (Heinrich *et al.*, 2006) and isolated from different plant sources such as Salvia stenophylla (Musarurwa *et al.*, 2010) and Plinia cerrocampanensis (Vila *et al.*, 2010).

2.3.5. Glycosides

Glycosides are plant secondary metabolites which are made up of two components including glycone (a carbohydrate component) and aglycone (a non-carbohydrate component).The glycone component usually consists of one or more glucose units and the aglycone component may be any one of the plant secondary metabolites from alkaloids, phenolics or terpenoids (Gurib-Fakim, 2005; Heinrich *et al.*, 2004). Anthraquinone glycosides, steroidal glycosides and coumarin glycosides are medically important glycosides but the medicinal importance is not limited to these glycosides only. Aloesin has been isolated from Aloe vera and reported for antioxidant activity, free radical scavenging and anti-inflammatory effects (Yagi *et al.*, 2002).

2.3.6. Tannins

Tannin is astringent vegetable product found in a wide range of plants parts ranging from the barks, roots, seeds, fruits, leaves, galls and roots (Ramakrishnan, 2006). They are water soluble phenolic compounds of the higher molecular weight that cross-link with proteins and other macromolecules (Ramakrishnan, 2006).

Generally, tannins are found in plants and are thought to function as chemical defenses against pathogens and herbivores (Gedir *et al*., 2005). They have been used in the preservation of leather, making glue stains and mordant (Kanth *et al*., 2009). It has also been used in the vegetable industry in different concentration in pickling process to provide protection against bacteria, mold, and yeasts (Andrade *et al*., 2005). Antimicrobial activity of tannins so far tested in various fields of medicine provided positive results such as antioxidant activities, anticarcinogenic activities, cytotoxic activities and antimutagenic properties (Lopes *et al*., 1999; Joshi *et al*., 2013). They have also been used in inhibiting the growth of many fungi, yeasts, bacteria and viruses (Chung *et al*., 1998; Akiyama *et al*., 2001). Some of the bioactive compounds of tannins such as catechin and pyrogallol found in vegetable tannins have been found to be toxic to microorganisms (Cowan, 1999).

2.3.7. Saponins

In plants, saponins are known to provide protection against microbes and fungi (Riguera, 1997). Saponins are also considered as one of the natural antimicrobial products that make up the

defense system of the plants and some can be beneficial rather than harmful to animals (Rupasighe *et al*., 2003). There has been evidence of the presence of saponins in traditional medicine preparations where the oral means of administration is expected to lead to the hydrolysis of glycosides from terpenoids (Asl and Hosseinzadeh, 2008)**.**

Studies carried out on medicinal plant extracts have shown saponins are effective against microorganisms such as *Escherichia coli*, *Salmonella typhi*, *Aeromonas hydrophilia* and other fungal pathogens such as *Candida albicans* (Deshpande *et al*., 2013). Saponins antimicrobial activity is attributed mainly to its capability of lysing microorganism's membranes rather than the surface tension of the extracellular medium (Asl and Hosseinzadeh, 2008). Apart from antimicrobial activity, saponins have shown other biological properties with its cytotoxic activity on cancer or tumor cells being considered the most important one (Yokosuka and Mimaki, 2009). Other plants such as cholestane glycosides which are known to have a broad spectrum of biological activity such as cytotoxic activity, antifungal, antibacterial and in vivo antitumor activities are known to produce steroidal saponins (Li *et al*., 2012).

2.4. Bovine Clinical Mastitis

Mastitis is a multi-factorial disease and generally occurs as a result of the interaction of various factors associated with the host, pathogen(s), environment and management factors (Kinfe, 2017). According to the National Mastitis Council's current concepts of bovine mastitis 1996, mastitis is an inflammation of the mammary gland characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues in response to injury for the purpose of destroying and neutralizing the infectious agents and to prepare the way for healing and return to normal function. Mastitis has been ranked as number one expensive disease of dairy animals all over the world (Zafalon *et al.*, 2007)**.** Because of its high incidence, mastitis is one of the most common and a very significant devastating dairy disease occurring in all dairy herds worldwide (Ojo *et al*., 2009), and has a consequence of transmitting certain diseases such as tuberculosis, brucellosis, leptospirosis etc., to consumers through milk (Nibret *et al*., 2011). It causes huge economic loses and damages to the dairy industry by decreasing milk production through increasing costs of treatment and culling rates

(Zaragoza *et al.*, 2011) and also represents a considerable welfare issue to the affected cows (Bradley, 2002; Halasa, 2007).

Mastitis encompasses both clinical and subclinical infections that adversely affect the health and welfare of dairy cows. Losses due to clinical mastitis are generally apparent and include discarded milk, transient reductions in milk yield, and premature culling (Asaye et al., 2015). Clinical mastitis is characterized by a sudden onset of udder swelling, pain, and a decline in milk quantity and quality. Affected animals may exhibit fever, depression, and anorexia, accompanied by altered milk appearance, such as the presence of flakes, clots, or a watery consistency (AHDB, 2025; Phibro, 2025).

Sometimes clinical mastitis may lead to abnormality in mammary gland which may include abnormality in teat size and consistency. But in case of sub-clinical mastitis, there are no visible signs but decrease in milk production and increased somatic cell count are evident. Abnormal quarters contain somatic cell count above 300,000 somatic cells/ml of milk while uninfected quarters contain below 200,000 somatic cells/ml of milk (Jones, 2006).

More than 150 different species of pathogenic microorganisms have been identified as causative agents of mastitis in dairy cow. Bacteria, fungi and yeasts may all play a role; but of these, bacteria have by far the largest part (Quinn *et al.,* 2002).

Different researchers have worked on isolation and identification of causative agents of bovine mastitis on different occasions. According to Watts 2002, bacterial isolates in the milk of dairy herds in and around Gondar, Ethiopia, were *Staphylococcus aureus*, S*treptcoccus agalactiae*, *Streptcoccus dysgalactiae*, *Streptcoccus uberis*, *Escherichia coli*, Coagulase Negative Staphylococci (CNS), *Micrococcus* species, *Bacillus cereus*, *Corynebacterium bovis* and *Actinomyces pyogens.*

Recent studies have also identified several bacterial species as etiological agents of bovine mastitis, including *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus epidermidis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, and *Corynebacterium bovis*. For instance, a study conducted in Asella Town, Ethiopia, reported the isolation of *Staphylococcus aureus* (38.6%), *Staphylococcus hyicus* (7.2%), and *Streptococcus*

species (2.4%) from clinical mastitic cows (Kasa et al., 2020). Additionally, a systematic review and meta-analysis focusing on Ethiopia identified *Staphylococcus aureus* as the major isolate, accounting for 13.4% and 16.5% of clinical and subclinical mastitis cases, respectively, with other significant isolates including *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Corynebacterium* species (Birhanu et al., 2022).

Getahun *et al.* 2008 also reported that the common isolates of bacteria from the clinical mastitic quarters were *St. agalactiae* (30%) and *St. dysgalactiae* (30%), while from sub-clinical cases were *S. aureus* (42.6%), *S. epidermidis* (22.1%), *St. agalactiae* (12.8%) and *St. uberis* (10.3%).But according to Kinfe 2017, in general, *Staphylococcus aureus* is the most predominant organism among all the pathogens of bovine mastitis.

Mostly, mastitis is caused by two types of pathogens: contagious and environmental pathogens. *Staphylococcus aureus*, *Streptococcus agalactiae, Corynebacterium bovis* and *Mycoplasma bovis* are major contagious pathogens which are transmitted from diseased to healthy animals during milking process while *Streptococcus disagalactiae*, *Streptococcus uberis, Klebsiella* spp, *Pseudomonas* spp and *Escherichia coli* are the major environmental pathogens responsible for mastitis and are present in animal beddings and surroundings (Eriksson, 2005; Mekonnen and Tesfaye, 2010).

Contagious mastitis pathogens, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* species, are primarily transmitted between cows during milking through contaminated milking equipment, milkers' hands, or shared udder washing cloths. Poor milking hygiene practices, such as inadequate handwashing, failure to disinfect teat cups, and improper sanitation of milking equipment, significantly increase the risk of spreading these pathogens. Implementing strict milking time hygiene—including proper handwashing before milking, disinfection of equipment, and use of post-milking teat dips—is essential in preventing the transmission of contagious pathogens from infected to healthy cows (Kasa et al., 2020; Birhanu et al., 2022).

Apart from infection of bacterial origin, the incidence of mycotic mastitis is also on rise and the most frequently isolated fungi from milk are *Cryptococcus neoformans* and *Candida albicans* (Rayaz and *Darand* 2013). Bovine mycotic mastitis can be usually caused by yeasts, but mastitis

due to filamentous fungi mostly *Aspergillus fumigatous* has been reported. It occurs as sporadic cases affecting a small percentage of cows or as outbreaks affecting the majority of animals (Abdel-Razik *et al*. 2011). Bakr *et al.* 2015 conducted therapeutic and diagnostic studies on mycotic mastitis in cattle in Egypt and identified *that C. albicans* and *Aspergillus* spp. were the main etiological agents of mycotic mastitis.

The development of antimicrobial resistance among pathogens Causing Bovine Mastitis is an emerging threat to animal health and the dairy industry worldwide. *Staphylococcus aureus* is one of the most prevalent pathogens causing both clinical and subclinical mastitis in dairy cows (Kinfe, 2017; Getahun et al., 2008). Recent studies have highlighted a concerning increase in AMR among *S. aureus* isolates: a systematic review reported that approximately 45.1% of *S. aureus* isolates from bovine mastitis cases exhibited resistance to penicillin (Naranjo-Lucena & Slowey, 2023); resistance to clindamycin and erythromycin was observed in 14.9% and 8.5% of isolates, respectively (Naranjo-Lucena & Slowey, 2023) and the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in dairy cattle is a growing concern, with implications for both animal and human health.

Antimicrobial resistance in *E. coli* isolates from bovine mastitis cases has been extensively studied. A study in Canada found that 32 out of 139 *E. coli* isolates were resistant to one or more antibiotics, with resistance observed against tetracycline, streptomycin, and sulfisoxazole (Majumder et al., 2021). The production of Extended-Spectrum β-Lactamases (ESBLs) which confer resistance to β-lactam antibiotics, has been reported in *E. coli* isolates, posing significant treatment challenges (Naranjo-Lucena & Slowey, 2023).

In Ethiopia, significant antimicrobial resistance has been observed in Streptococcus agalactiae. Approximately 20% of *S. agalactiae* isolates showed resistance to penicillin (Tessema et al., 2025) and around 40% of isolates were resistant to ampicillin (Tessema et al., 2025). Recent studies have reported resistance in *Streptococcus uberis* which causes both clinical and subclinical mastitis. A study in Thailand found that 82.02% of *S. uberis* strains were resistant to tetracycline, with the tet(M) gene being the most commonly detected resistance determinant (Zhang et al., 2021). Resistance to erythromycin was also observed in 8.33% of isolates (Zhang et al., 2021). High levels of resistance to penicillin have been reported in Coagulase-Negative

Staphylococci (CNS), which are opportunistic pathogens often isolated from subclinical mastitis cases, as reported in *S. aureus*. Resistance to other antibiotics, including macrolides and aminoglycosides, varies among CNS species and geographic regions (Naranjo-Lucena, A., & Slowey, R. (2023).

Visualization and palpation of the udder tissue and visualization of changes in milk color can be used as the common techniques for detection of clinical mastitis. Thicker or watery consistency, flakes or clots in the milk, reddening and hardening of the udder are the common signs in clinical mastitis. Flakes or clots in the milk are detected by stripping the first few squirts of milk from each quarter into a strip cup at the beginning of milking. Strip Cup or Plate Test is a test that can be used for determining the presence of clinical mastitis through detection of visible particles of milk. It is a practical and effective method of identifying cows with clinical mastitis. In strip cup test, an enamel plate divided in four strip cups is used and the bottom of the plate is black in color so that the milk flakes are easily observed by tilting the cups at an angle. Abnormal milk is usually discolored, watery or contains flakes, shreds or clot (Radostits *et al*., 2007).Isolation and identification of any pathogenic microorganisms present in the milk can also be achieved by cultural methods.

Researchers have reported that various ethnic communities across Ethiopia utilize a diverse range of medicinal plants to treat bovine mastitis. In the Tigray region, for instance, Shewit et al. (2012) reported the traditional use of plants such as *Achyranthes aspera*, *Ficus carica*, *Malva parviflora*, *Vernonia* species, *Solanum hastifolium*, *Calpurnia aurea*, *Nicotiana tabacum*, *Ziziphus spina-christi*, and *Croton macrostachyus* to manage mastitis, wounds, and gastrointestinal disorders caused by both Gram-positive and Gram-negative bacteria. Similarly, Assaye et al. (2015) evaluated the antibacterial properties of the stem bark of *Combretum molle*, the leaves of *Xanthium strumarium*, and an isolate labeled FR1 against *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from clinical cases of bovine mastitis. The in-vitro results demonstrated promising antimicrobial activity, supporting the potential use of these plants in alternative mastitis treatment strategies.

Regassa and Araya 2012 screened some herbal preparation against mastitis causing pathogens and got promising results. Mengistu 2004 screened *Brucea antidysentrica, Combertum molle,*

Cyphostema adenacuale , *Persicaria senegalensis* , *Plantago lanceolata* and *Zahneria scabra* on major isolates of bovine mastitis. Taddese *et al*. 2009 conducted in-vitro antimicrobial effects of some selected plants on *Staphylococcus aureus* isolated from bovine clinical mastitis and Mohamedamin 2011 also conducted an invitro test of *Laggera alata* and *Xanthium strumarium* on *Staphylococcus aureus* isolate and observed encouraging result. Haile 2012 conducted in-vitro sensitivity to determine and compare the in-vitro antimicrobial effects of *Xanthium strumarium* and *Grewia bicolor* juice on *Staphylococcus aureus* isolated from bovine clinical mastitis case and found a result which encourages further study. Furthermore, Sahlu 2013 conducted study on antibacterial activities and preliminary phytochemical investigation of leaves, stem bark and seeds of *Combretum molle*, stem bark of *Bereza* and leaves of *Xanthium strumarium* and *Laggora arota* against *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* and got the result which supports the previous studies that these plants can serve as famous drugs for modern use.

The traditional remedies are socially acceptable, inexpensive, and locally available and can easily be administered by traditional healers or ordinary members of local community (Kibebew *et al.*, 2001; Teklehaymanot and Giday, 2007).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted in Gursum district of Oromia Regional State in eastern Ethiopia. Geographically, the district lies between 9^0 07′ and 9^0 32′ North latitudes and 42^0 17′ and 42^0 38′E longitudes (GALRO, 2015). The total area coverage of the district is estimated at 76 261 hectares of land. The altitude of the district ranges from 1200-2938 m.a.s.l. with the annual rain fall of 650-750 mm and the mean annual minimum and maximum temperature of 18^0C and 25^0C respectively (GALRO, 2015). It is inhabited by a human population of about 168476 people (*CSA,* 2013). The district is divided into 3 agro-ecological zones: highland (5 %), midland (45 %), and lowland (50 %). The area has short rainy season (March to April) and long rainy season (June to August) (GALRO, 2015).

3.2. Study Design

This study employed a mixed-method approach combining exploratory experimental designs to comprehensively investigate the antimicrobial activities of selected medicinal plants against pathogens isolated from bovine clinical mastitis. The study was conducted from January 2024 to December 2024 in Gursum district, Eastern Ethiopia.

Data were collected through semi-structured interviews, focus group discussions, and field observations. The selection of informants was based on purposive and snowball sampling techniques to ensure knowledgeable participants were included. The purpose of this phase was to explore the ethnoveterinary knowledge and practices prevalent in the study area, and to select the most commonly cited plants for further investigation.

Secondly, a case study approach was applied to isolate the causative pathogens of bovine clinical mastitis. Milk samples were collected aseptically from clinically mastitic dairy cows brought to veterinary clinics in Gursum. The samples were subjected to bacteriological and mycological analyses in the laboratory for the isolation and identification of the mastitis-causing

microorganisms using standard microbiological procedures. This step was critical in determining the specific microbial agents targeted for antimicrobial efficacy testing.

Lastly, an in-vitro exploratory experimental study design was conducted to evaluate the antimicrobial activities of the selected medicinal plant extracts against the isolated bacterial and fungal pathogens. The experimental phase involved the preparation of crude plant extracts using methanol, followed by antimicrobial susceptibility testing through disc diffusion method and determination of minimum inhibitory concentrations (MICs). The inhibitory effects of each extract were assessed by measuring zones of inhibition and comparing them with standard antimicrobial agents.

This integrative study design allowed for a holistic understanding of both the traditional knowledge base and the scientific validation of the selected medicinal plants, thereby bridging the gap between ethnoveterinary practices and modern microbiological research.

3.3. Screening of Clinical Mastitis and Milk Sample Collection

Milk samples were collected and processed following the guidelines of the National Mastitis Council (NMC, 2021). The animals were first screened for clinical mastitis based on both physical examination of the udder and teats and visual inspection of milk.

3.3.1 Clinical Observation and Selection of Animals

Lactating cows brought to the Veterinary Clinics in the study area for examination were observed for clinical mastitis. Each quarter was physically examined for swelling, heat, redness, pain on palpation, fibrosis or hard lumps, visible injuries, lesions, or wounds, teat canal abnormalities such as blind teats, and atrophy or abnormal shape of the mammary gland. Milk from each quarter was manually stripped and observed for changes in color (e.g., yellow, brown, red), abnormal consistency (e.g., watery, thickened), Presence of flakes, clots, pus, or blood and Foul odor. All animals showing one or more of the above signs were considered clinically mastitic and selected for sampling.

3.3.2. Sample Size Description

A total of 20 lactating dairy cows with suspected clinical or subclinical mastitis were purposively selected for this study from three rural veterinary clinics: Abaadir (serving ~992 dairy cows), Gaarawadaajaa (322 dairy cows), and Qabsoo (202 dairy cows). The selection was based on the presence of clinical signs of mastitis or a history of abnormal milk production as judged by veterinary personnel. This non-randomized sampling approach aimed to maximize the likelihood of isolating and identifying the most common bacterial and fungal pathogens associated with bovine mastitis in the study area.

3.3.3. Milk Sample Collection Procedure

Milk samples were collected under strict aseptic conditions. The cows were restrained properly to avoid movement during sampling.Teats were washed with clean water and dried using a disposable paper towel. Each teat end was disinfected with 70% ethanol, using separate sterile cotton swabs for each teat. Swabbing was performed starting from the teat closest to the operator (the front right), followed by the front left, and then the rear teats (right rear and left rear) to minimize cross-contamination. After disinfection, alcohol was allowed to evaporate completely (~30 seconds) before sampling. The first 3-4 streams of milk from each teat were discarded to remove contaminants from the teat canal. Approximately 10 mL of milk was collected aseptically from each suspect quarter into a sterile, screw-capped, wide-mouth universal bottle (15 mL capacity). Gloves were worn throughout the process and changed between animals. Each sample bottle was labeled with the cow ID, quarter sampled (e.g., FR = front right), date, and clinic name. Samples were immediately placed in an icebox at 4°C and transported to the Veterinary Microbiology Laboratory, Haramaya University. Upon arrival, samples were stored in a refrigerator at +4°C and processed within 24–48 hours.

3.3.4. Laboratory Processing of Milk Samples

Within 48 hours of collection, milk samples were inoculated onto Blood Agar (5% sheep blood), MacConkey Agar (Oxoid Ltd, UK), and Potato Dextrose Agar using a sterile loop (0.01 mL). Plates were incubated at 37°C for 24-48 hours under aerobic conditions. Reagents and materials used include 70% Ethanol (analytical grade, Sigma-Aldrich, Germany), Universal sampling

bottles (sterile, polypropylene, 15 mL, Fisher Scientific), Blood agar base, MacConkey agar (Oxoid, UK), Potato Dextrose Agar and Sterile inoculation loops (1 µL and 10 µL sizes). Blood agar base, MacConkey agar and Potato Dextrose Agar were prepared according to manufacturer's instructions, using distilled water and sterilized in an autoclave at 121°C for 15 minutes. Milk samples were retained at 4°C for 3–4 days after culture inoculation in case re-culturing was needed, after which they were discarded.

3.4. Isolation and Identification of Bacterial and Fungal Test Organisms

Isolation and identification of bacterial and fungal pathogens were carried out using standard bacteriological and mycological techniques as described by Quinn et al. (2011). The procedure followed a systematic flow from sample collection**,** primary and secondary culturing**, to** biochemical characterization for precise identification of clinical isolates.

3.4.1. Primary Isolation on General and Selective Media

For initial screening, each milk sample was inoculated by streaking on three types of culture media simultaneously: Blood Agar, MacConkey Agar, and Potato Dextrose Agar (PDA). Blood agar, an enriched medium containing 5% sheep blood, was used to support the growth of fastidious bacteria and to observe hemolytic reactions indicative of organisms such as *Staphylococcus* and *Streptococcus* species. On Blood Agar, *Staphylococcus aureus* typically produces beta-hemolysis, characterized by a clear, transparent zone around colonies due to complete lysis of red blood cells. *Streptococcus* spp. exhibit variable hemolysis patterns: *Streptococcus pyogenes* (Group A) shows beta-hemolysis (clear zone), *Streptococcus pneumoniae* often shows alpha-hemolysis (greenish discoloration due to partial hemolysis), and *Streptococcus agalactiae* (Group B) may show weak beta-hemolysis or be non-hemolytic.

MacConkey Agar, a selective and differential medium, was employed to isolate Gram-negative enteric bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*. On MacConkey agar, lactose fermenters appear as pink colonies (e.g., *E. coli*, *K. pneumoniae, E. aerogenes*) but non-fermenters remain colorless or pale (e.g., *Salmonella*, *Proteus* spp.).

For fungal isolation, PDA was utilized to promote the growth of yeasts and molds. The morphological features observed on PDA helped to distinguish fungal species based on colony color, texture, margin, and pigmentation. *Candida albicans* shows round, white, moist, unpigmented colonies with smooth margins and shiny texture, while *Aspergillus fumigatus* exhibits dry, granular, pigmented colonies with green or blue-green coloration due to spore formation.

3.4.2. Secondary Isolation and Colony Purification

To ensure purity and enable accurate species-level identification, well-isolated colonies obtained from primary culture plates were sub-cultured onto various selective and differential media. This secondary isolation step helped to eliminate mixed growth and allowed better visualization of colony morphology, pigmentation, and biochemical reactions.

For the differentiation of *Staphylococcus aureus* from other *Staphylococcus* species, Mannitol Salt Agar (MSA) and purple agar base supplemented with 1% maltose were used. MSA is both selective and differential; the high salt concentration (7.5% NaCl) inhibits the growth of non-halophilic organisms while allowing *Staphylococcus* spp. to grow. The mannitol in the medium is fermented by *S. aureus*, producing acid which changes the phenol red indicator to yellow, resulting in golden-yellow colonies. Other *Staphylococcus* species that do not ferment mannitol grow as pink colonies without color change in the medium. On purple agar-maltose, *S. aureus* forms yellow colonies due to acid production from maltose fermentation, while non-fermenters retain purple coloration.

In the case of Gram-negative bacteria, well-isolated colonies from MacConkey agar were further streaked onto Eosin Methylene Blue (EMB) Agar, a selective and differential medium designed specifically for isolating fecal coliforms. EMB contains eosin Y and methylene blue dyes that inhibit the growth of most Gram-positive organisms while allowing Gram-negative enteric bacteria to grow. It also differentiates between lactose fermenters and non-fermenters. Lactose fermentation leads to acid production, which interacts with the dyes to produce characteristic colony appearances.

Escherichia coli typically produces small, dark colonies with a distinctive metallic green sheen on EMB due to vigorous lactose fermentation and strong acid production. In contrast, *Enterobacter aerogenes* shows larger, mucoid pink to purple colonies with a less intense or absent sheen, as it ferments lactose more slowly and with less acid production. Similarly, *Klebsiella pneumoniae* grows as large, moist, mucoid colonies with pink to purple coloration, also showing a moderate sheen due to its ability to ferment lactose, although less vigorously than *E. coli*. Non-lactose fermenters such as *Salmonella* or *Proteus* spp. form colorless or pale colonies with no sheen, allowing for easy preliminary differentiation.

After incubation, *Enterobacter aerogenes* produces large, mucoid, and pink to light-purple colonies with a faint sheen, indicative of slow lactose fermentation and low acid production. The pink coloration and mucoid appearance are indicative of moderate lactose fermentation, while the faint sheen results from limited acid production compared to *E. coli* (Quinn et al., 2011).

These secondary isolations provided clean, pure cultures that were subsequently subjected to microscopic and biochemical testing for further confirmation of species identity.

3.4.3. Microscopic and Biochemical Identification Tests

In addition to colony morphology, several confirmatory biochemical and microscopic tests were performed to characterize the isolates.

3.4.3.1. Microscopic Identification tests

Gram Staining was used as an initial test to classify organisms as Gram-positive or Gram-negative based on cell wall composition. *S. aureus* appears as Gram-positive cocci in clusters, while members of the Enterobacteriaceae family are observed as Gram-negative rods.

3.4.3.2. Biochemical Identification Tests

Catalase Test: The catalase test is used to detect the presence of the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). This reaction helps bacteria to protect themselves from oxidative damage caused by hydrogen peroxide, a toxic by-

product of aerobic metabolism.The test was performed by placing a drop of 3% hydrogen peroxide onto a glass slide or culture medium and then adding a small amount of the bacterial colony. Upon the addition of hydrogen peroxide to a colony, the release of bubbles (oxygen gas) indicates a positive result. Most members of the family Enterobacteriaceae, including Enterobacter aerogenes, *E. coli* and *K. pneumoniae* and *S. aureus* are catalase positive, and it helps to distinguish them from catalase-negative bacteria like Streptococcus species.

IMViC Tests: To differentiate between Gram-negative enteric bacilli, especially members of the family Enterobacteriaceae, the IMViC series of biochemical tests—Indole, Methyl Red, Voges-Proskauer, and Citrate—were conducted.

Indole Test: Detects the ability to produce indole from tryptophan via the enzyme tryptophanase. A red ring at the top of the broth after the addition of Kovac's reagent indicates a positive result. *E. coli* typically gives a positive result, while *E. aerogenes* and K.pneumoniae are negative.

Methyl Red (MR) Test: Detects stable acid end products from glucose fermentation. A red color upon the addition of methyl red indicator denotes a positive test. *E. coli* is MR positive; *E. aerogenes* and K.pneumoniae are MR negative.

Voges-Proskauer (VP) Test: Detects acetoin production from glucose fermentation via the butylene glycol pathway. Development of a pink to red color upon addition of alpha-naphthol and KOH indicates a positive test. *E. aerogenes* and K.pneumoniae are VP positive; *E. coli* is negative.

Citrate Utilization Test: Determines the ability of an organism to use citrate as its sole carbon source. A color change from green to blue in Simmons Citrate Agar signifies a positive result. *E. aerogenes* is citrate positive, while *E. coli* is citrate negative.

The Triple Sugar Iron (TSI) Agar Test: It was used to differentiate enteric bacteria based on their ability to ferment glucose, lactose, and/or sucrose and to produce hydrogen sulfide (H_2S) or gas. In the case of *Enterobacter aerogenes*, yellow coloration of both slant and butt indicates acid production, along with gas formation but no H_2S (absence of black precipitate).

The Urease Test: It was performed to identify organisms capable of hydrolyzing urea to ammonia and carbon dioxide via the enzyme urease. A negative result for *E. aerogenes* is indicated by no color change (remains light yellow).

For confirmation and standardization, the laboratory results of clinical isolates were compared with reference strains of both bacterial and fungal pathogens obtained from the Ethiopian Public Health Institute (formerly Pasteur Institute). These standard strains served as controls to validate colony morphology, staining reactions, and biochemical profiles of the test organisms.

3.5. Collection of Plant Materials

Medicinal plants investigated in this study were collected, in January 2024, from Gursum district of Oromia Regional State in Eastern Ethiopia. The area is known for its rich indigenous knowledge of traditional plant-based remedies used to treat livestock diseases, particularly bovine mastitis. The local names of some of the widely used medicinal plants include *Oggol*, *Harmal*, *Burii*, *Muka Jigoo*, *Biribiixii*, *Hamarmaddoo*, *Jifo*, *Qoramichoo*, *Qullubbii Waraabeeysaa*, *Habakat*, *Mixaaxis Shimbirroo*, and *Muka Adii*, among others.

Although these and other plant species are employed in ethnoveterinary practices across the region, only a selected few were prioritized for this study. For manageability and optimal utilization of available resources, four plants—*Oggol* (*Dolichos oliveri*), *Harmal* (*Mirabilis jalapa L.*), *Burii* (*Euphorbia schimperiana*), and *Muka Jigoo* (*Cyphostemma adenocaule*)—were chosen for investigation based on their prominence in traditional use, ease of access, and informant consensus.

The selection of these plants was underpinned by a scientifically structured ethnobotanical survey using a mixed-method approach that integrated both qualitative and quantitative data collection techniques. Data were collected through semi-structured interviews, guided field walks, and participatory ranking exercises. A purposive sampling technique was applied to identify Peasant Associations (PAs) and traditional healers or religious leaders within the Gursum district. The selection criteria for informants included agro-ecological diversity, known

availability of medicinal plants in the area, and the local credibility of the informants as knowledge bearers in ethnoveterinary medicine (Martin, 1995; Cotton, 1996).

Facilitation and coordination of the survey were supported by Animal Health Assistants stationed at veterinary clinics in the respective PAs. These professionals also played a key role in introducing researchers to the informants and ensuring culturally sensitive engagement. During the interviews, informants provided detailed information on each plant's local name, the specific part(s) used, methods of preparation and administration, dosage, duration of treatment, and the livestock diseases treated. Interviews were conducted in the local language and data were documented using pre-tested questionnaires adapted from standard ethnobotanical protocols (Alexiades, 1996) (table 2).

Field collection of plant specimens was carried out concurrently with the interviews. During the guided walks, key informants helped researchers locate and collect plant materials. All plant parts necessary for identification and herbal use—including leaves, stems, roots, tubers, and reproductive structures—were harvested and preserved for taxonomic authentication following standard herbarium procedures (Jain and Rao, 1977).

To determine the most relevant plants for antimicrobial analysis, several ethnobotanical data analysis tools were employed. These include: (i) Preference Ranking: it was conducted with informants to assess the perceived effectiveness of the plants in treating bovine mastitis (Martin, 1995); (ii) Informant Consensus Factor (ICF): it was calculated to measure the degree of agreement among informants regarding the use of plants for specific disease mastitis. High ICF values indicate strong consensus, which suggests a potentially effective remedy (Heinrich et al., 1998); (iii) Fidelity Level (FL): it was used to determine the percentage of informants claiming the use of individual plant species for the same therapeutic purpose, which reflects its healing reliability (Friedman et al., 1986); and (iv) Rank Order Priority (ROP): it was determined by integrating FL values with the relative popularity level of each species, which helps to identify plants that are both culturally and therapeutically significant (Teklehaymanot and Giday, 2007).

Based on the integration of these ethnobotanical tools and informant knowledge, *Dolichos oliveri*, *Mirabilis jalapa L.*, *Euphorbia schimperiana*, and *Cyphostemma adenocaule* were identified as the most important medicinal plants for further investigation of antimicrobial

activity against mastitis-causing pathogens. These species had high fidelity levels, strong consensus among traditional healers, and were repeatedly cited for their use in treating livestock infections.

3.6. Preparation of Plant Materials

For the purpose of crude extraction and phytochemical screening, different plant parts were selectively processed based on traditional ethnomedicinal practices and existing literature on extraction methods. In this study, the tubers of *Dolichos oliveri*, *Mirabilis jalapa L.*, and *Euphorbia schimperiana* were used, whereas the root was used for *Cyphostemma adenocaule* (Alexiades, 1996; Sofowora, 1993). Bulk samples of each plant were thoroughly washed with clean water to remove soil and contaminants, and the plant parts were chopped into smaller pieces before being air-dried under shade at room temperature for approximately three weeks to prevent photodegradation and preserve phytochemicals (Trease & Evans, 2002).

After drying, the plant materials were first ground using a sterile wooden mortar and pestle and subsequently pulverized using an electric blender to obtain a fine powder. The powdered plant parts were stored in labeled polyethylene bags and kept in a cool, dry place until extraction. All plant powders were later transported to the Microbiology Laboratory at Haramaya University for further phytochemical and antimicrobial studies.

Voucher specimens of the investigated plants were also collected, labeled, and prepared for taxonomic authentication. These specimens were submitted to the Herbarium Laboratory of Haramaya University, where they were identified by experienced taxonomists and archived for future reference in accordance with standard herbarium protocols (Jain & Rao, 1977).

3.7. Preparation of Voucher Specimen for Botanical Identification

For botanical identification, representative specimens of each medicinal plant were prepared following standard herbarium preservation techniques (Bridson & Forman, 1992; Jain & Rao, 1977). Fresh plant materials, including leaves, stems, roots, and reproductive parts, were collected in the field and pressed flat between newspaper sheets to retain their morphological

features. Each specimen was cut to fit standard herbarium sheet size and layered between newspaper sheets with interspersed cardboard to maintain structural integrity.

The layered specimens were then sandwiched between two wooden plant pressers and tightly bound using straps to maintain even pressure and prevent leaf curling. To avoid microbial decomposition due to the high moisture content of some plant parts, the press was periodically opened to check for dampness. Wet newspapers were replaced regularly to promote adequate drying and prevent fungal contamination, a common problem in tropical environments (Bridson & Forman, 1992).

After the drying process was complete, the specimens were labeled with their local names, collection date, and collector's information, and transported to the Herbarium Laboratory at Haramaya University. There, they were authenticated taxonomically and preserved in the university's botanical reference collection for future study.

3.8. Extraction of Plant Materials

The extraction of bioactive compounds from the selected medicinal plants was performed using the cold maceration technique. For each plant species, 100 grams of the powdered sample were immersed in 500 milliliters of 99.8% methanol, establishing a solute-to-solvent ratio of 1:5 (w/v). This ratio aligns with standard protocols in phytochemical research, ensuring efficient extraction of secondary metabolites while minimizing solvent usage (Sultana et al., 2009).

Methanol was chosen as the extraction solvent due to its high polarity, which enables it to dissolve a broad spectrum of phytochemicals, including phenolics, flavonoids, alkaloids, and saponins. Its ability to penetrate plant cell walls effectively facilitates the release of intracellular compounds, often resulting in higher yields of bioactive constituents compared to other solvents (Landerlee, 2024). Additionally, methanol's relatively low boiling point (64.7°C) allows for easier removal post-extraction, reducing the risk of thermal degradation of sensitive compounds (Rajak, 2014). While methanol is toxic and flammable, its efficacy in extracting a wide range of phytochemicals makes it a preferred solvent in laboratory settings, provided that appropriate safety measures are implemented.

The maceration process involved continuous agitation using an orbital shaker set at 100 rpm, with manual stirring every four hours to enhance solvent penetration. After 72 hours, the mixtures were filtered through Whatman No. 1 qualitative filter paper. The residues were re-macerated twice more with fresh methanol for 48 and 24 hours, respectively, to ensure exhaustive extraction. The combined filtrates were concentrated using a rotary evaporator set at 40°C and 60 rpm, effectively removing the methanol under reduced pressure. The resulting semi-solid extracts were transferred to evaporating dishes and dried in an oven at 40°C for 3–4 days until a constant weight was achieved. The percentage yield of each plant extract was calculated using the formula:

Percentage Yield (%) =Weight of Dried Extract (g)/Initial Weight of Plant Material (g) ×100. This calculation provides insight into the efficiency of the extraction process and the abundance of extractable compounds within each plant species. The dried extracts were stored in labeled bijou bottles with airtight caps and refrigerated at 4°C until further analysis for antimicrobial activity.

3.9. Qualitative Determination of Phytochemicals

Phytochemical screening was carried out at Haramaya University Microbiology Post-graduate Laboratory to assess the qualitative chemical composition of crude extracts using commonly employed precipitation and coloration reaction to identify the major natural chemical groups and secondary metabolites present in the plants. All the chemicals and reagents used for the testing were obtained from this laboratory and all the tests conducted under supervision of laboratory technitian in charge of laboratory tests. A combination of several methods was used to identify the phytochemicals of the medicinal plants. Standard screening tests using conventional protocol, procedure and reagents were conducted on the methanolic extracts of plants as described by WHO 1978, Trease and Evans 1989 and Sofowara 1993.The screening was done to detect the presence of bioactive principle believed to have antimicrobial activities: tannins, flavonoids, alkaloids, saponins, terpenoids, steroids, phlobatannins, glycosides and phenols.

3.9.1. Tannins

Two ml of each plant extract was mixed with 30ml distilled water in separate test tubes. Then 1ml of lead acetate was mixed with 2ml of the extracts. The formation of yellow precipitate in the tubes was an indication of positive result for tannins in the plants (Savithramma *et al*., 2011).

3.9.2. Test for flavonoids (Alkaline reagent test)

Two ml of each plant extract was mixed with 2 ml of ammonia in separate test tubes. Then 1 ml of con. H_2S0_4 added to each test tubes. The development of yellow color in the tubes was considered as an indication of the presence of flavonoids in the plants (Ayoola *et al*., 2008).

3.9.3. Test for alkaloids (Mayer's test)

Two ml of 1% HCl was added to 2 ml of each plant extract and heated for 5 minutes in water bath. The mixture was then cooled and filtered. O.4 ml of Mayer's Reagent was added to the filtrate. The formation of cream yellow precipitate was an indication for the presence of alkaloids in the extracts (Obadoni *et al.,* 2002).

3.9.4. Saponin Test (Foam test)

Two ml of each plant extract was mixed with 5 ml of distilled water. The mixture was shaken vigorously. The formation of stable foam in the tubes showed strong presence of saponins in the plants (Abba *et al*., 2009).

3.9.5. Test for Terpenoids (Salkowski test)

Two ml of each extract was mixed with 2 ml of chloroform and 3 ml of con.H2S04 was carefully added to form a layer. Formation of redish brown coloration at the interface indicated the presence of terpenoids in the plants (Edeoga *et al.,* 2005)**.**

3.9.6. Test for Steroids (Lieberman-Burchard's Test)

One ml of plant extract was added to each 4 test tubes. Then 2 ml of chloroform, 10 ml acetic acid and 2 ml of H_2S0_4 was added to each test tube. The change of red color through blue to green color of the solution indicated the presence of steroids (Edeoga and Kiruba, 2014)

3.9.7. Phlobatannin Test

Two ml plant extract was added to 4 separate test tubes and mixed with 10 ml distilled water and boiled for 10 minutes in water bath. After cooling, the mixture was filtered separately by Whatman no.1 filter paper. Then 2 ml of 1% aqueous hydrochloric acid was added to each mixture and shaken. Lack of development of red precipitate in the tubes indicated the absence of phlobatannins in the plants (Ajayi *et al*., 2011).

3.9.8. Glycosides (Keller-Kiliani Test)

In two ml plant extract, glacial acetic acid, one drop of 5% $FeCl_3$ and concentrated H_2SO_4 were added. Reddish brown color appears at junction of the two liquid layers and upper layer appears bluish green, confirming the presence of glycosides.

3.9.9. Phenols (Ellagic Acid Test)

When 5% glacial acetic acid and 5% sodium nitrite were added to each plant extract solutions, an appearance of a muddy niger brown color is a positive result for phenols.

3.10. Quantitative Determination of Phytochemicals

Determination of Phytochemical contents of medicinal plant extracts was carried out at Haramaya University Microbiology Post-Graduate Laboratory. All the chemicals, equipment, and reagents used for the testing were obtained from this laboratory and all the tests were conducted under supervision of laboratory technitian in charge of laboratory tests.

3.10.1. Total Alkaloid Determination

The determination of alkaloids was conducted using Harborne's method (1973). About 3 gm of powdered plant material was added into a 50 ml Erlenmeyer flask. Then 20 ml of 10% acetic acid in ethanol was added into the flask and the solution was allowed to stand for 4 hrs. The mixture was then filtered to remove insoluble particles. To precipitate the alkaloids, ammonium hydroxide was added to the filtrate. The resultant precipitate was collected carefully, washed with diluted ammonium hydroxide, dried completely, and weighed. The total alkaloid content was expressed as milligrams per gram (mg/g) of the plant sample.

3.10.2. Saponin Determination

Saponin content was quantified following the protocols established by Obadoni and Ochuko (2002) and Edeoga *et al*. (2005). Twenty grams (20 g) of the powdered plant sample was mixed with 100 ml of 20% ethanol and heated in a water bath at a controlled temperature. The mixture was filtered, and the residue was re-extracted with another 10 ml of 20% ethanol for completeness. The combined extracts were concentrated to 40 ml by evaporating the solvent over water bath, then transferred to a 250-ml separator funnel, and 20 ml diethyl ether added to separate the aqueous. Then, the aqueous layer was collected repeatedly until no more layer formation observed. After that ether layer was discarded and aqueous layer was collected and 60 ml n-butanol added and washed twice with 10 ml of 5% aqueoussodium chloride. At the end, the remaining solution was evaporated in a water bath, dried in an oven, weighed and the saponin content was calculated.

3.10.3. Total Terpenoid Determination

To quantify terpenoids, two grams (2 g) of the plant powder were soaked in 50 ml of 97% ethanol for 24 hours to facilitate extraction. After filtration by Whatman No 1 filter paper, the filtrate was added in to separating funnel and 50 ml of petroleum ether added. The resulting mixture was shaken and allowed to stay for 5 minutes for layer formation. Then, the bottom layer was drained and discharged while top petroleum ether was collected and concentrated to dryness using rotary evaporator at 40 ºC. The dried extract was considered as crude terpenoids, and expressed as mg per the sample powder used (Furguson, 1956).

3.10.4. Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the plant extract was determined using the Folin–Ciocalteu colorimetric method, with gallic acid as a standard reference compound, following a modified protocol described by Ainsworth and Gillespie (2007).

3.9.4.1 Preparation of standard solutions

A gallic acid stock solution (1 mg/mL or 1000 µg/mL) was prepared and serially diluted to obtain a series of standard solutions. Aliquots ranging from 0.1 to 1.0 mL of the stock solution were transferred into 10 mL volumetric flasks and diluted to volume with distilled water to yield final concentrations suitable for calibration.

3.9.4.2. Extraction of phenolics

A total of 1 g of dried plant powder was extracted with 50 mL of 80% methanol. The mixture was subjected to ultrasonic extraction for 30 minutes and then filtered through Whatman No. 1 filter paper. The clear supernatant was collected and used for TPC determination.

3.9.4.3. Reaction with Folin–Ciocalteu reagent

For each standard and sample extract, 0.5 mL was mixed with 2.5 mL of 1:10 diluted Folin–Ciocalteu reagent. After 5 minutes of incubation, 2.0 mL of 7.5% (w/v) sodium carbonate solution was added. The mixture was incubated in the dark at room temperature for 30 minutes to allow color development (Ainsworth & Gillespie, 2007).

3.9.4.4. Measurement of Absorbance

Absorbance was measured at 560 nm using a UV-Visible spectrophotometer. The absorbance of each plant extract was recorded using water as a blank.

3.9.4.5. Standard Curve and Sample phenol Concentration Determination

A standard calibration curve was plotted with absorbance on the Y-axis and concentration of gallic acid (µg/mL) on the X-axis. The regression equation derived from the curve was:

$Y=0.013X+0.01$. To calculate the phenolic concentration in the sample: $X=Y-b/m$. Thus, the phenolic content in the sample extract is expressed in gallic acid equivalents (GAE).

3.9.4.6. Calculation of Total Phenolic Content (TPC)

The TPC was expressed as mg of gallic acid equivalents per gram of dry sample(mg GAE/g dry sample) using the formula:

$TPC=C\times V/m$

Where:

- C=phenolic concentration in extract (mg/ml)
- V= volume of extract (ml)
- m=weight of dried plant sample used for the extraction (g)

3.11. Preparation of Antimicrobial Discs from Plant Extracts

The preparation of antimicrobial discs from plant extracts was conducted following standardized protocols to ensure consistency and reliability in subsequent antimicrobial susceptibility testing. Discs measuring 6 mm in diameter were punched from Whatman No. 1 filter paper using a sterile paper punch.The discs were placed into McCartney bottles and sterilized by autoclaving at 121°C for 15 minutes. Post-autoclaving, the discs were dried in a hot air oven at 50°C to eliminate residual moisture, as moisture can affect the absorption of plant extracts and the diffusion of active compounds during antimicrobial testing (Arunkumar et al., 2009).

For each plant extract, 2 grams of the dried extract were dissolved in 10 mL of DMSO, resulting in a stock solution with a concentration of 200 mg/mL. DMSO was selected as the solvent due to its ability to dissolve both polar and non-polar compounds and its minimal interference with microbial growth at low concentrations (Quinn et al., 1999).

Three working solutions with concentrations of 75 mg/mL, 100 mg/mL, and 125 mg/mL were prepared from the stock solution using the dilution formula:

$C_1V_1=C_2V_2$, Where C_1= concentration of the stock solution (200 mg/mL), V_1= volume of the stock solution required, C_2= desired concentration of the working solution and V_2= final volume

of the working solution. The mixtures were homogenized using a vortex mixer to ensure uniform distribution of the extract.

Sterile discs were impregnated with 10 µL of each working solution using a micropipette. The impregnated discs were placed in sterile Petri dishes and allowed to dry at 37°C overnight to facilitate solvent evaporation and ensure adherence of the extract to the disc surface. Dried discs were stored in sterile McCartney bottles, sealed to prevent contamination. The bottles were refrigerated at 4–8°C until use in antimicrobial susceptibility testing.

3.12. Antimicrobial Sensitivity Testing

3.12.1. Antibacterial Evaluation of Plant Extracts

The antimicrobial test was conducted using the disk diffusion method as described by Quinn et al. (1994) and in accordance with the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS, 1997; now CLSI). Muller-Hinton agar (MHA) medium was employed for the antimicrobial sensitivity test using the spread plate technique. Clinical isolates were spread on the agar surface using sterile cotton wool swabs. The MHA was prepared according to the manufacturer's instructions and poured into sterile Petri dishes. The plates were incubated at 37°C for 24 hours to ensure sterility prior to use (CLSI, 2002).

After confirming sterility, four to five well-isolated colonies of similar morphology were collected from nutrient agar using a sterile wire loop. These were suspended in sterile normal saline and vortexed to homogenize the suspension. The turbidity of the bacterial suspension was standardized by comparing it with the 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/ml), which was prepared according to the procedure described by Jain et al. (2010) and Andrews (2001). The turbidity was adjusted by visual comparison against a white background with contrasting black lines.

A sterile swab was dipped into the standardized bacterial suspension, and excess fluid was removed by pressing and rotating the swab against the inside wall of the tube. The swab was then used to evenly streak the entire surface of the MHA plate in three directions to ensure

uniform bacterial growth. A final sweep was made around the rim of the plate. The plates were left to stand for no more than 15 minutes to allow drying before placing the discs (CLSI, 2002).

Each plate was divided into four quadrants: three quadrants for three concentrations (75 mg/ml, 100 mg/ml, and 125 mg/ml) of each plant extract, and the fourth quadrant for the negative control (DMSO-impregnated disc). A positive control disc (Ciprofloxacin for bacteria or Ketoconazole for fungi) was placed at the center of the plate. Impregnated discs were positioned on the agar surface using sterile forceps, maintaining a distance of 24 mm center-to-center and 15 mm from the edge of the plate (Balouiri et al., 2016). Each disc was gently pressed to ensure complete contact with the agar surface. Plates were then inverted and incubated at 37°C for 24 hours.

Following incubation, the antimicrobial activity was determined by measuring the diameter of the zones of inhibition in millimeters using a transparent ruler (CLSI, 2002). The effectiveness of each plant extract was compared to the standard antibiotic (Ciprofloxacin), while DMSO served as the negative control.

3.12.2. Antifungal Evaluation of Plant Extracts

Antifungal evaluation of plant extracts was conducted by the technique recommended by CLSI 2008 for antifungal drug susceptibility testing. Briefly**,** the required amounts of fungal isolates were suspended in 2ml of potato dextrose broth and adjusted with 0.5 McFarland standards. The suspension was uniformly spread on Petri-plates containing potato dextrose agar media using sterile swabs. After applying the plant extracts onto the inoculums (as described for bacterial testing), the plates were incubated at 30 ºC for 24-48hrs and examined for the presence of zones of inhibition. The effectiveness of each plant extract was compared to the standard antifungal agent (ketoconazole), while DMSO served as the negative control.

3.12.3. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the plant extracts was determined using the broth tube dilution method, following established protocols with slight modifications (Wiegand et al., 2008; Balouiri et al., 2016; CLSI, 2020). The highest working concentration was selected

for each plant extract based on antimicrobial activities exhibited against test organiams by disc diffusion method.

Then, the highest working concentrations selected for each plant extract were subjected to serial two-fold dilutions (i.e., 50%, 25%, 12.5%, 6.25%, and 3.125%) by subsequently transferring 1 ml from each test tube into the next test tube by halving the concentration of each preceding test tube that already contained 1 ml of sterile Muller Hinton broth or Potato Dextrose Broth.

Inocula of clinical bacterial and fungal isolates were standardized to a 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/ml for bacteria) in physiological saline water, as per CLSI guidelines (CLSI, 2020). Each test tube received 1 ml of standardized inoculum mixed with 1 ml of the corresponding serially diluted extract solution in growth media, Muller Hinton Broth (MHB) for bacteria and Potato Dextrose Broth (PDB) for fungi, and then incubated at 37°C for 24 hours for bacteria and at 25–30°C for 24–48 hours for fungi (Clinical and Laboratory Standards Institute, 2020; Okmen et al., 2021).

Growth controls consisted of medium and inoculum without plant extract to confirm normal microbial growth. Sterility controls included medium and extract without inoculum to ensure absence of contamination.

After incubation, the MIC was defined as the lowest concentration of plant extract showing no visible microbial growth in the broth, as evidenced by absence of turbidity (Wiegand et al., 2008; Balouiri et al., 2016).

3.12.4. Determination of Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) was determined following the broth dilution assay used for MIC determination, in accordance with standardized protocols (Wiegand et al., 2008; CLSI, 2020; Balouiri et al., 2016). The MBC represents the lowest concentration of the plant extract that results in microbial death, defined by the absence of visible colony growth on an antimicrobial-free agar medium after subculturing.

Following incubation during the MIC test, aliquots (10μL) from the tubes corresponding to concentrations at least two steps higher than the MIC, the MIC tube itself, and one or two

concentrations below the MIC, were aseptically withdrawn. These samples were streaked onto sterile nutrient agar plates (free of plant extracts) using a sterile inoculating loop.

The inoculated plates were then incubated aerobically at 37°C for 24 hours for bacterial isolates. After incubation, each plate was examined for visible colony growth. The lowest concentration of the plant extract that produced no visible bacterial colonies was recorded as the MBC (CLSI, 2020; Andrews, 2001). All tests were performed in triplicate to ensure accuracy: Negative controls (media only) and positive growth controls (bacteria without extract) were also plated. Plates with growth were interpreted as indicating bacteriostatic activity, while those without growth were indicative of bactericidal activity at the tested concentration (Pankey & Sabath, 2004). It is generally accepted that if the MBC is ≤4 times the MIC, the agent is considered bactericidal; otherwise, it is considered bacteriostatic if MBC > 4 times MIC (Clinical and Laboratory Standards Institute, 2020).

3.12.5. Determination of Minimum Fungicidal Concentration (MFC)

The Minimum Fungicidal Concentration (MFC) was determined to identify the lowest concentration of plant extracts capable of killing fungal pathogens associated with mastitis in dairy cows. The procedure followed standard protocols previously described in the literature (CLSI, 2008; Espinel-Ingroff et al., 2002; Balouiri et al., 2016).

After the Minimum Inhibitory Concentration (MIC) was established, aliquots (approximately 10 µL) from the tubes that showed no visible fungal growth were aseptically subcultured onto sterile Potato Dextrose Agar (PDA) plates, which did not contain any plant extract. Each aliquot was streaked onto the agar surface in triplicate to ensure the reproducibility of results. The inoculated plates were then incubated at 28–30 °C for 24–48 hours, depending on the growth characteristics of the fungal species.

Following incubation, the plates were examined for any fungal colony formation. The MFC was recorded as the lowest concentration of the extract at which no visible fungal growth was observed, indicating fungicidal activity, defined as a 99.9% reduction in the initial fungal inoculum (Espinel-Ingroff et al., 2002; CLSI, 2008).

The MFC/MIC ratio was calculated to determine the nature of antifungal action. A ratio ≤4 was considered indicative of fungicidal activity, whereas a ratio >4 was interpreted as suggestive of fungistatic effects (Pankey & Sabath, 2004; Odds, 2003).

3.13. Data Analysis

The data obtained from antimicrobial activity tests—including zone of inhibition (ZOI), minimum inhibitory concentration (MIC), and minimum bactericidal/fungicidal concentration (MBC/MFC)—were initially entered into Microsoft Excel 2016 for organization and then exported to the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp., Armonk, NY) for statistical analysis.

Descriptive statistics, including the mean and standard error of the mean (SEM), were calculated to summarize the central tendency and variability of the antimicrobial activity of each plant extract. These measures are appropriate for continuous, quantitative data such as the diameter of inhibition zones and concentration values, allowing for concise comparisons between treatment groups.

To evaluate differences in the antimicrobial efficacy of plant extracts against the tested microbial strains, one-way Analysis of Variance (ANOVA) was employed. This test is suitable when comparing the means of more than two independent groups (in this case, different plant extracts), under the assumption that the dependent variable (e.g., ZOI) is approximately normally distributed within each group and that the variances across groups are homogenous. The rationale for using one-way ANOVA lies in its ability to assess whether observed differences in means are statistically significant beyond what would be expected by random variation alone.

Following ANOVA, Tukey's Post Hoc Multiple Comparison Test was applied to determine which specific group means differed significantly from one another. Tukey's test is particularly suitable when all pairwise comparisons are of interest, as it controls the family-wise error rate and is robust under the assumption of equal group sizes and homogenous variances.

Prior to conducting ANOVA, assumptions of normality and homogeneity of variances were assessed. Normality of residuals was evaluated using the Shapiro-Wilk test, and visual inspection of Q-Q plots and histograms. Homogeneity of variances was tested using Levene's test. ANOVA was only performed if these assumptions were met; if violations were observed, appropriate transformations or non-parametric alternatives (e.g., Kruskal-Wallis test) were considered.

The level of statistical significance for all analyses was set at $P < 0.05$, indicating that there is less than a 5% probability that the observed differences occurred by chance alone.

4. RESULTS

The selected medicinal plants *(Dolichos oliveri*, *Mirabilis jalapa* L. *Euphorbia schimperiana and Cyphostemma adenocaule)* collected from Gursum district in East Hararghe Zone of Oromia Regional State were investigated for their antimicrobial activity against mastitis causing bacteria and fungi in dairy cows. The local communities in the study area used these medicinal plants and herbs to treat different livestock and human aliments/diseases by following the prescription given by the traditional healers. The ethnomedicinal information of the medicinal plants collected for this study was summarized as described in Table 2 below.

Table 2: Ethnobotanical data of selected medicinal plants.

Scientific Name	Family Name	Local Name	GF	PU	MOP	DOT	ROA
Dolichos oliveri	Papilionoideae	Oggol	Shrub	Tuber	Crushed and mixed with water	BID for 3 days	Oral
Mirabilis jalapa L.	Nyctaginaceae	Harmal	Herb	Tuber	Crushed and mixed with wheat bran	BID for 3 days	Oral
Euphorbia schimperiana	Euphorbiaceae	Burii	Climber	Tuber	Crushed and mixed with wheat bran	BID for 3 days	Oral
Cyphostemma adenocaule	Vitaceae	Ataree	Creeper	Root	Crushed and mixed with wheat bran	BID for 3 days	Oral

Note: GF = Growth Form; PPU = Plant Part Used; MOP = Method of Preparation; DOT = Duration of Treatment; ROA = Route of Administration.

Milk samples were collected and processed following the guidelines of the National Mastitis Council. The procedure followed a systematic flow from **sample collection** to **biochemical characterization** for precise identification of clinical isolates. Each milk sample was simultaneously inoculated on Blood Agar, MacConkey Agar, and Potato Dextrose Agar (PDA) by streak plate method. The result of isolation and identification of test organisms is shown in Annex IV. From 60 milk samples collected from 25 dairy cows, *S. aureus* (Gram positive), E. aerogenes (Gram negative), *C. albicans* and *A. fumigatus* were isolated by using standard laboratory procedures. On Blood Agar, *Staphylococcus aureus* typically showed a clear,

transparent zone around colonies due to complete lysis of red blood cells. Then well-isolated colonies were transferred onto Mannitol Salt Agar (MSA) as a secondary isolation resulting in golden-yellow colonies with yellow surrounding medium. As a further confirmatory test, pure colonies were subcultured onto Purple Agar Base with 1% Maltose. On purple agar-maltose media, *S. aureus* formed yellow colonies due to acid production from maltose fermentation, while non-fermenters retained purple coloration. Catalase test was also conducted for *S. aureus* along with Gram staining test. Buble formation upon addition of a drop of 3% hydrogen peroxide on slides with colonies and purple-colored cocci in clusters during Gram staining test confirmed the bacteria tested to be *S. aureus*.

In the case of Gram-negative bacteria, well-isolated colonies from MacConkey agar were further streaked onto Eosin Methylene Blue (EMB) Agar, and *Enterobacter aerogenes* was identified as producing large, mucoid, and pink to light-purple colonies with a faint sheen**,** indicative of slow lactose fermentation and low acid production. Further biochemical tests were conducted for confirmation of E. aerogenes including IMViC, catalase, urease, TSI and Gram staining tests. In IMViC test, *E. aerogenes* was Indole and Methyl Red negative while it showed positive result for VP and Citrate utilization test. *E. aerogenes* was also identified as positive for catalase test but tested negative for urease test. In TSI test, E. aerogenes was confirmed with the formation of Yellow slant/butt and Gas formation but with absence of H_2S (no blackening), which is indicatie of positive result for E. aerogenes.

For fungal isolation, PDA was utilized to promote the growth of yeasts and molds. The morphological features observed on PDA helped to distinguish fungal species based on colony color, texture, margin, and pigmentation.*Candida albicans* was identified by its round, white, moist, unpigmented colonies with smooth margins and shiny texture, while *Aspergillus fumigatus* exhibited dry, granular, pigmented colonies with green or blue-green coloration due to spore formation.

4.3. Extraction of Medicinal Plants

The extraction of bioactive compounds from selected medicinal plants was performed using methanol as an extraction solvent. The percentage yield of methanol crude extracts varied across

the four medicinal plant species tested in this study (Table 3). The extraction was carried out using 100 grams of dried plant material, and the weight of the obtained extract was recorded after complete solvent evaporation. The percentage yield (w/w) was then calculated using the formula:

Percentage Yield= (Weight of dried extract/Weight of dried plant material) $\times$100

The highest yield was obtained from *Euphorbia schimperiana* (30.4%) (figure1), followed by *Cyphostemma adenocaule* (25.9%), *Dolichos oliveri* (24.04%), and the lowest yield was observed in *Mirabilis jalapa L.* (23.2%). All extracts were semisolid in consistency and varied in color, ranging from white to reddish-brown (Table 3).

The percentage yield results show a moderate to high extractive efficiency across all plant species, indicating the presence of significant quantities of methanol-soluble phytochemicals. The highest yield in *Euphorbia schimperiana* suggests it may contain a richer matrix of secondary metabolites, whereas the lower yield in *Mirabilis jalapa L.* could be due to higher fiber content or the predominance of compounds less soluble in methanol. All extracts displayed semisolid consistency, which is typical of crude extracts rich in mixed phytochemical constituents such as alkaloids, flavonoids, tannins, and terpenoids.

Table 3: Methanol extraction of medicinal plants.

Plant Species	**Part Used**	**Weight of Dried Powder (g)**	**Weight of Dried Extract (g)**	**Color**	**Consistency**	**% Yield (w/w)**
Dolichos oliveri	Tuber	100	24	White	Semisolid	24.04%
Mirabilis jalapa L.	Tuber	100	23.2	Reddish-brown	Semisolid	23.2%
Euphorbia schimperiana	Tuber	100	30.4	Pink	Semisolid	30.4%
Cyphostemma adenocaule	Root	100	25.9	Creamy	Semisolid	25.9%

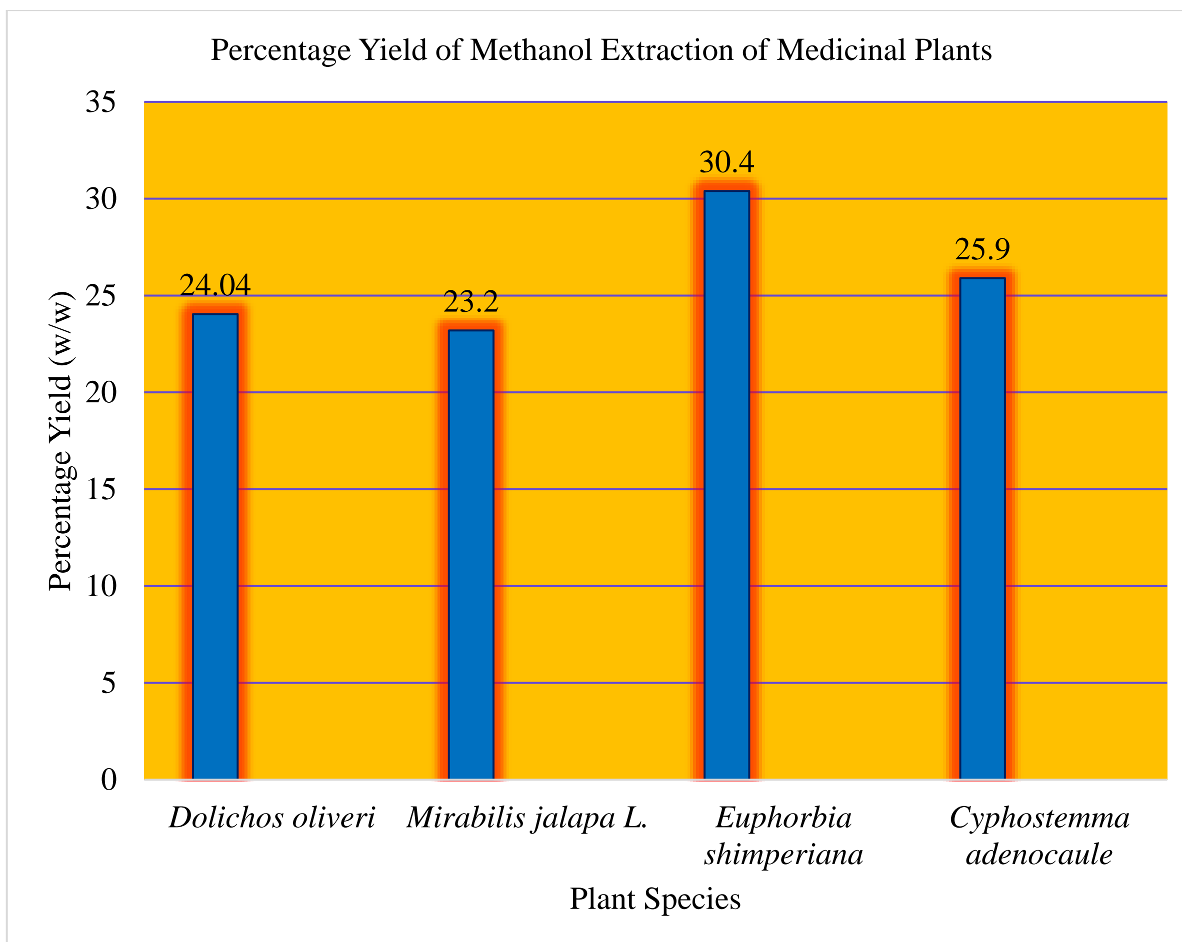

Figure 1: Graphic ilustration of percentage yield of methanol extraction of medicinal plants.

4.4. Qualitative Determination of Plant Extracts

The result of qualitative determination of plant extracts showed that there was variation among the plants (Table 4). Saponin was found in all tested plants, except *Cyphostemma adenocaule*. Tannin was identified only in *Cyphostemma adenocaule*. Terpenoid was found in all except *Dolichos oliveri*. Phenols were identified in all except *Dolichos oliveri*. Phlobatanin was not identified in any of the plants tested. From all the tested plants, *Dolichos oliveri* was found with high presence of saponins and alkaloid.

Table 4: Qualitaive phytochemical determination of plant extracts.

Name of crude extract	Phytochemicals								
	Tannins	Phlabotanins	Saponins	Flavanoid	Steroid	Terpenoid	Alkaloid	Phenols	Glycosides
Dolichos oliveri	-	-	+++	-	-	-	+++	-	+
Mirabilis jalapa L.	-	-	++	-	+	++	-	+	-
Euphorbia schimperiana	-	-	+	-	+	+	-	+	-
Cyphostemma adenocaule	+	-	-	+	-	++	++	+	+

- = absent; += present; ++= moderately present; +++ = highly present.

4.5. Quantitative Phytochemical Determination of Plant Extracts

Quantitative analysis of phenols, alkaloids, saponins, and terpenoids revealed significant variation ($p < 0.05$) among the plant extracts analyzed (Table 7).

Among the tested samples, *Dolichos oliveri* exhibited the highest alkaloid content (2.35 ± 0.01 mg/ml), significantly higher ($p < 0.05$) than other plant species. *Euphorbia schimperiana* and *Cyphostemma adenocaule* followed with values of 1.54 ± 0.02 mg/ml and 1.42 ± 1.11 mg/ml respectively, while *Mirabilis jalapa L.* had the lowest alkaloid content (0.82 ± 1.31 mg/ml). This suggests that *Dolichos oliveri* may have higher pharmacological potential related to alkaloid-associated bioactivity.

The highest saponin concentration was found in *Euphorbia schimperiana* (3.11 ± 0.00 mg/ml), showing a statistically significant difference ($p < 0.05$) from the other species. *Dolichos oliveri* had a moderate saponin content (1.43 ± 2.00 mg/ml), followed by *Mirabilis jalapa L.* (0.49 ± 1.10 mg/ml) and *Cyphostemma adenocaule* (0.47 ± 1.06 mg/ml). The high saponin level in *E. schimperiana* suggests its potential for anti-inflammatory, antioxidant, or antimicrobial applications.

Euphorbia schimperiana showed the highest terpenoid content (0.78 ± 3.11 mg/ml), significantly higher than others ($p < 0.05$). *Dolichos oliveri* followed with 0.46 ± 0.00 mg/ml, while *Cyphostemma adenocaule* and *Mirabilis jalapa L.* had lower terpenoid values of 0.23 ± 0.00 mg/ml and 0.21 ± 3.14 mg/ml respectively. These results indicate that *E. schimperiana* may possess strong pharmacological activity attributed to its terpenoid content.

Cyphostemma adenocaule exhibited the highest phenolic content (0.500 ± 1.18 mg/ml), significantly surpassing the other plant samples. *Dolichos oliveri* showed the highest alkaloid content (2.35 ± 0.01 mg/ml), while *Euphorbia schimperiana* contained the highest saponin (3.11 ± 0.00 mg/ml) and terpenoid (0.78 ± 3.11 mg/ml) concentrations.

Using the gallic acid standard solutions (Table 6), a calibration curve was constructed (Figure 2). The regression equation derived from this curve was:

$Y=1.032X+0.025(R2=0.991)$

Where:

- Y = absorbance at 560 nm,
- X = concentration of gallic acid in mg/ml.

Using the regression equation, phenolic concentrations of the samples were determined by substituting their absorbance values into the calibration equation ($Y=1.032X+0.025$). For Dolichos oliveri (Abs = 0.068) $0.068=1.032X+0.025$, $X=0.068-0.025/1.032\approx0.042$ mg GAE/g sample; for Mirabilis jalapa L. (Abs = 0.055), $0.055=1.032X+0.025$, $X=0.055-0.025/1.032\approx0.029$ mg GAE/g sample; for Euphorbia schimperiana (Abs=0.284), $0.284=1.032X+0.025$, $X=0.284-0.025/1.032\approx0.251$mg GAE/g sample; for Cyphostemma adenocaule (Abs = 0.541), $0.541=1.032X+0.025\Rightarrow X=0.541-0.025/1.032\approx0.500$ mg GAE/g sample.

Total phenolic content (TPC) was calculated using the formula: TPC = (C × V) / M, where C is phenol concentration (mg/ml), V is total extract volume (10 ml), and M is weight of the dried sample (100 g).

For Dolichos oliveri (phenol concentration=0.042), TPC=0.042 mg GAE/g x10ml/100g, TPC=0.0042 mg GAE/g sample. Similarly the TPC for Mirabilis jalapa L., Euphorbia

shimperiana and Cyphostemma adenocaule was calculated using the results of phenol concentration, total extract volume and dried sample used giving 0.0029, 0.0251 and 0.0500 mg GAE/g sample. The results confirmed that *Cyphostemma adenocaule* exhibited the highest total phenolic content among the plants tested (table 5).

Table 5: Absorbance, phenol concentration and total phenolic content of plant extracts.

Plant Extract	Weight of Dried Powder (g)	Weight of Dried Extract (g)	Absorbance at 560 nm	Phenol Concentration (mg/ml)	Total Phenolic Content (mg GAE/g sample)
Dolichos oliveri	100	24.0	0.068	0.042	0.0042
Mirabilis jalapa L.	100	23.2	0.055	0.029	0.0029
Euphorbia schimperiana	100	30.4	0.284	0.251	0.0251
Cyphostemma adenocaule	100	25.9	0.541	0.500	0.0500

Phenol concentration of each plant extract was obtained by substituiting their absorbance in the regression equation(Y=1.032X+0.025); total phenol content was calculated by using 100 gm of dried powder of sample and 10 ml of extract volume used for absorbance reading at 560 nm of spectrophotometer.

Table 6: Standard calibration data for gallic acid.

Standard Sample	Concentration (mg/ml)	Absorbance at 560 nm
Gallic Acid Standard	0.1	0.106
Gallic Acid Standard	0.2	0.124
Gallic Acid Standard	0.3	0.202
Gallic Acid Standard	0.4	0.359
Blank (Water)	0.0	0.540

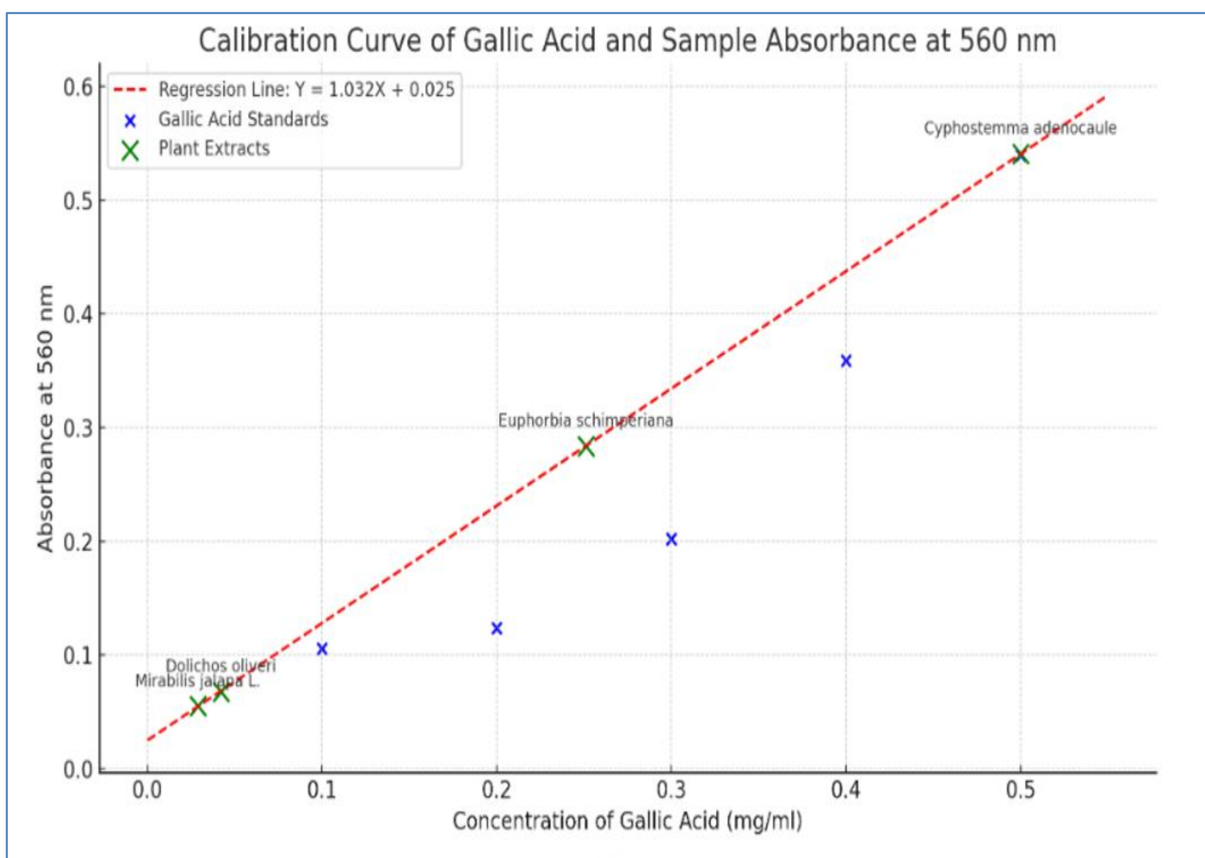

Figure 2: Calibration curve of gallic acid and absorbance of plant extracts at 560 nm

Table 7: Quantitative phytochemical determination of plant extracts.

Plant name	Phenol (mg/ml)	Alkaloid (mg/ml)	Saponin (mg/ml)	Terpenoid (mg/ml)
Dolichos oliveri	0.0042 ± 1.08^{bD}	2.35 ± 0.01^{fD}	1.43 ± 2.00^{ab}	0.46 ± 0.00^{cb}
Mirabilis jalapa L.	0.0029 ± 0.07^{bd}	0.82 ± 1.31^{Ba}	0.49 ± 1.10^{cb}	0.21 ± 3.14^{gf}
Euphorbia schimperiana	0.0251 ± 1.05^{gf}	1.54 ± 0.02^{gb}	3.11 ± 0.00^{Ac}	0.78 ± 3.11^{bd}
Cyphostemma adenocaule	0.500 ± 1.18^{df}	1.42 ± 1.11^{gd}	0.47 ± 1.06^{bc}	0.23 ± 0.00^{dg}

Column values with different superscript letters differ significantly at $p < 0.05$.

4.6. Antimicrobial Activity of Plant Extracts

4.6.1. Antibacterial Activities of Plant Extracts

The extract from *Dolichos oliveri* had increased antimicrobial activities with increased concentrations for all test organisms and standard strains indicating its broad-spectrum antimicrobial activities at high concentrations (125 mg/ml) (Table 8). The Extract from *Mirabilis jalapa L.* had only antimicrobial activities at 125 mg/ml against standard strain of *S. aureus* with 10.67 mm zone of inhibition, while it had an increased zone of inhibition from 6.92 mm to 8.83 mm on clinical isolates of *E. aerogenes* and 8.53 mm to 12.76 mm on standard isolates (Table 8). *Euphorbia schimperiana* also had no antimicrobial activity against clinical isolates of *S. aureus* at the concentration of 75 mg/ml and 100 mg/ml, while it exhibited 7 mm to 11.08 and 8.10 mm to 13.50 mm inhibition zone on clinical and standard strains *of E. aerogenes,* respectively. *Cyphostemma adenocaule* had no antimicrobial activities on both clinical and standard strains of *E. aerogenes* but showed 7.33 mm to 9.75 mm and 11 mm to 13.93 mm on clinical and standard strains of *S. aureus* respectively.

Based on the post-hoc test (Table 8), at all the tested concentrations (75, 100, and 125 mg/ml), there was no significant variation ($P>0.05$) between the activity of *Dolichos oliveri* and *Cyphostemma adenocaule* against *S. aureus*. Concerning their activity against clinical isolate of *E. aerogenes*, *Dolichos oliveri, Mirabilis jalapa L., Euphorbia schimperiana* had insignificant difference ($P>0.05$) at 75 mg/ml, while *Dolichos oliveri* had significantly ($P<0.05$) higher activity at 100 mg/ml. Moreover, at 125 mg/ml, *Mirabilis jalapa* L. had significantly ($P<0.05$) lower activity than *Dolichos oliveri,* and *Euphorbia schimperiana.* Overall, the positive control (Ciprofloxacin) had significantly higher ($P<0.05$) antimicrobial activity against *S. aureus* and *E. aerogenes* than tested plants.

Table 8: Mean zone of inhibition of plant extracts against bacterial pathogens (in mm).

Plants	Concentrations (mg/ml	*S. aureus*		*E. aerogenes*	
		Clinical	Standard	Clinical	Standard
Dolichos oliveri	75	7.17±0.2^{b}	10.19±0.2^{b}	8.17±0.7^{c}	9.23±0.1^{b}
	100	8.1667±0.2^{g}	11.78±0.1^{f}	10.83±0.4^{fh}	10.50±.1^{f}
	125	10.83±0.2^{kl}	13.33±0.1^{k}	11.9167±0.3^{l}	13.30±0.3^{m}
Mirabilis jalapa L.	75	0.0±0.0^{c}	0.0±0.0^{c}	6.92±0.2^{c}	8.53±0.3^{c}
	100	0.0±0.0^{f}	0.0±0.0^{g}	7.7500 ±.1^{fg}	10.83±.2^{f}
	125	0.00±0.0^{l}	10.67±0.3^{l}	8.8333 ±0.4^{m}	12.76±0.2^{k}
Euphorbia schimperiana	75	0.0±0.0^{c}	9.60±0.1^{b}	7.00±0.3^{c}	8.10±0.1^{c}
	100	0.0±0.0^{f}	11.50±.3^{f}	9.33±.7^{fgh}	10.33±.3^{f}
	125	8.58±0.4^{l}	13.50±0.1^{k}	11.0833±0.8^{l}	12.30±0.3^{k}
Cyphostemm adenocaule	75	7.33±0.4^{b}	11.00±0.3^{c}	0.0±0.0^{b}	0.0±0.0^{d}
	100	8.3333 ±0.3^{g}	12.73±0.1^{h}	0.0±0.0^{i}	0.0±0.0^{g}
	125	9.75±0.4^{kl}	13.93±0.1^{k}	0.00±0.0^{k}	0.00±0.0^{l}
Ciprofloxacin	10µl	18.75±0.0^{a,e,j}	19.50±0.0^{a,e,j}	19.00±0.0^{a,e,j}	18.70±0.0^{a,e,j}

The values are Mean ± S.E.M (n=3); significant at P<0.05; $^{(a-d)}$ comparison among tests in 75 mg/ml, $^{(e-i)}$ comparison among tests in 100 mg/ml, and $^{(j-n)}$ comparison among 125 mg/ml; mean comparison is among each plant's concentrations and with Ciprofloxacin. The negative control has shown no antibacterial activity.

4.6.2. Antifungal Activities of Plant Extracts

The antifungal activities of plant extract is summarized in Table 9. *Euphorbia schimperiana* exhibited 7.83 mm to 10.66 mm inhibition on clinical isolates of *C. albicans* and 9 mm to 11.90 on standard strains with increase of concentration from 75 mg/ml to 125 mg/ml. It also demonstrated 12 mm to 14.33 mm and 12.40 mm to 13.93 mm inhibition on *A. fumigatus* clinical and standard strains respectively. *Cyphostemma adenocaule* demonstrated 7.17 mm to 9.50 mm and 8.33 mm to 11.50 mm inhibition on *C. albicans* clinical and standard isolates and 9.25 mm to 13.41 mm and 7.67 mm to 10.33 mm inhibition on *A. fumigatus* clinical and standard strains. Of all plant extracts, *Euphorbia schimperiana* exhibited higher inhibition zone (11.5 mm to 14.5

mm) on *A. fumigatus* as concentration increased from 75 mg/ml to 125 mg/ml indicating its efficacy against this organism even at lower concentration.

Based on the post-hoc test (Table 9), *Mirabilis jalapa L.* had significantly higher activity against clinical isolates of *C. albicans* (P<0.05) at the concentration of 125 mg/ml than all the tested plants, while at 100 mg/ml, it only showed significant (P<0.05) variation with *Dolichos oliveri*. *Euphorbia schimperiana* and *Cyphostemma adenocaule* showed significantly higher (p<0.05) activity against the clinical isolate of *A. fumigatus* at 125 mg/ml. Overall, the positive control (Ketoconazole) had significantly higher (P<0.05) antifungal activity than tested plants.

Table 9: Mean zone of inhibition of plant extracts against fungal pathogens (in mm).

Plants	Concentrations (mg/ml)	*C. albicans*		*A. fumigatus*	
		Clinical	Standard	Clinical	Standard
Dolichos oliveri	75	4.33 ± 2.2^{b}	9.93 ± 0.1^{b}	4.33 ± 2.2^{b}	9.0 ± 0.0^{b}
	100	7.50 ± 0.3^{f}	12.46 ± 0.3^{h}	9.1667 ± 0.6^{f}	10.16 ± 0.2^{f}
	125	9.16 ± 0.2^{kh}	13.53 ± 0.0^{m}	11.83 ± 0.4^{k}	12.33 ± 0.3^{k}
Mirabilis jalapa L.	75	9.00 ± 0.6^{b}	8.20 ± 0.2^{c}	7.42 ± 0.3^{c}	9.33 ± 0.3^{c}
	100	10.16 ± 0.7^{g}	9.50 ± 0.3^{f}	9.00 ± 0.3^{f}	11.33 ± 0.3^{g}
	125	11.83 ± 0.8^{kg}	10.50 ± 0.3^{l}	11.83 ± 0.4^{k}	12.83 ± 0.2^{k}
Euphorbia schimperiana	75	7.83 ± 0.4^{b}	9.0 ± 0.0^{c}	12.0 ± 0.3^{b}	12.40 ± 0.2^{b}
	100	9.41 ± 0.7^{fg}	10.86 ± 0.1^{g}	13.58 ± 0.22^{g}	13.33 ± 0.2^{h}
	125	10.66 ± 2.0^{k}	11.90 ± 0.1^{k}	14.33 ± 0.2^{l}	13.93 ± 0.1^{l}
Cyphostemma adenocaule	75	7.17 ± 0.2^{b}	8.33 ± 0.3^{c}	9.25 ± 1.3^{c}	7.67 ± 0.3^{bc}
	100	8.08 ± 0.2^{fg}	9.53 ± 0.3^{f}	12.16 ± 0.4^{g}	9.00 ± 0.0^{i}
	125	9.50 ± 0.5^{k}	11.50 ± 0.3^{k}	13.41 ± 0.3^{l}	10.33 ± 0.3^{m}
Ketoconazole	10µ	$18.00 \pm 0.0^{a,e,j}$	$20.00 \pm 0.0^{a,e,j}$	$17.50 \pm 0.0^{a,e,j}$	$18.00 \pm 0.0^{a,e,j}$

The values are Mean ± S.E.M (n=3); significant at P<0.05; $^{(a-d)}$ comparison among tests in 75 mg/ml, $^{(e-i)}$ comparison among tests in 100 mg/ml, and $^{(j-n)}$ comparison among 125 mg/ml; mean comparison is among each plant's concentrations and with Ketoconazole.

4.7. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) exhibited by plant extracts on the growth of clinical isolates of bacteria and fungi are shown in Table 10. The extract of *Dolichos oliveri* inhibited

the growth of *S. aureus* and *C. albicans* at 125 mg/ml. But it showed MIC on *E. aerogenes* and *A. fumigatus* at 75 mg/ml. The extract of *Mirabilis jalapa* L. showed MIC at 37.5 mg/ml on *E. aerogenes* and *A. fumigatus* but didn't show MIC on *S. aureus* and *C. albicans* at all concentrations tested. *Euphorbia schimperiana* demonstrated the MIC at 37.5 mg/ml for *S. aureus*; the MIC for *E. aerogenes* and *C. albicans* was at 75 mg/ml; but it did not show MIC for *A. fumigatus* at all concentrations. The extract of *Cyphostemma adenocaule* showed MIC for *S. aureus* at a concentration of 37.5 mg/ml and for *C. albicans* and *A. fumigatus* at 75 mg/ml but didn't show MIC on *E. aerogenes* at all concentrations.

4.8. Minimum Bactericidal and Fungicidal Concentration

Based on the previous results of MIC exhibited by plant extracts (table 10), *Dolichos oliveri* had MBC on *S. aureus* at 100 mg/ml and on *E. aerogenes* at 200 mg/ml but had no MFC on *A. fumigatus* and *C. albicans* at all concentrations (Table 10) (figures 5 and 6). *Mirabilis jalapa* L. had no MBC on *S. aureus* and MFC on *C. albicans* but showed MBC and MFC at 400 mg/ml and 200 mg/ml on *E. aerogenes* and *A. fumigatus* respectively (figures 4 and 6). The extract of *Euphorbia schimperiana* had no MBC and MFC on *S. aureus*, *C. albicans* and *A. fumigatus* (figures 3, 5 and 6) but showed MBC on *E. aerogenes* at 200 mg/ml. Cyphostemma adenocaule had no MBC and MFC on *E. aerogenes*, *C. albicans* and *A. fumigatus* but showed MBC on *S. aureus* at 400 mg/ml. The growth of bacteria or fungi on the plates inoculated with the concentrations at and above MIC values showed the plant extracts were bacteriostatic (MBC: MIC ratio > 4 x MIC) for that bacteria and fungistatic (MFC: MIC ratio > 4 x MIC) for that fungus. But absence of growth on the plates at MIC value indicated the plant extracts were bactericidal or fungicidal to the organisms tested. Based on MBC: MIC and MFC: MIC ratio calculations (table 10), it was concluded that *Mirabilis jalapa* L. is not effective for *S. aureus* and *C. albicans* but bacteriostatic and fungistatic for *E. aerogenes* and *A. fumigatus* respectively. *Dolichos oliveri* is bactericidal against *S. aureus* and E, aerogenes and fungistatic against *C. albicans* and *A. fumigatus*. *Euphorbia schimperiana* is bacteriostatic against *S. aureus* and fungistatic against *C. albicans*, but not effective against *A. fumigatus*. But it is bactericidal against *E. aerogenes*. *Cyphostemma adenocaule* is not effective against *E. aerogenes* but bacteristatic against *S. aureus* and fungistatic against *C. albicans* and *A. fumigatus*.

Table 10: MIC, MBC and MFC of plant extracts against test organisms.

Plant Extract	Bacteria				Fungi			
	S. aureus		*E. aerogenes*		*C. albicans*		*A. fumigatus*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
Dolichos oliveri	125	100	75	200	125	---	75	---
Mirabilis jalapa L.	---/---	---/---/---	37.5	400	---/---	---/---/---	37.5	200
Euphorbia schimperiana	37.5	---	75	200	75	---	---/---	---/---/---
Cyphostemma adenocaule	37.5	400	---/---	---/---/---	75	---	75	---

(---) =Growth of bacteria/fungi at MIC value, (---/---) = absence of MIC due to zero inhibition zone, (---/---/---) =absence of MBC and MFC due to zero inhibition zone and MIC.

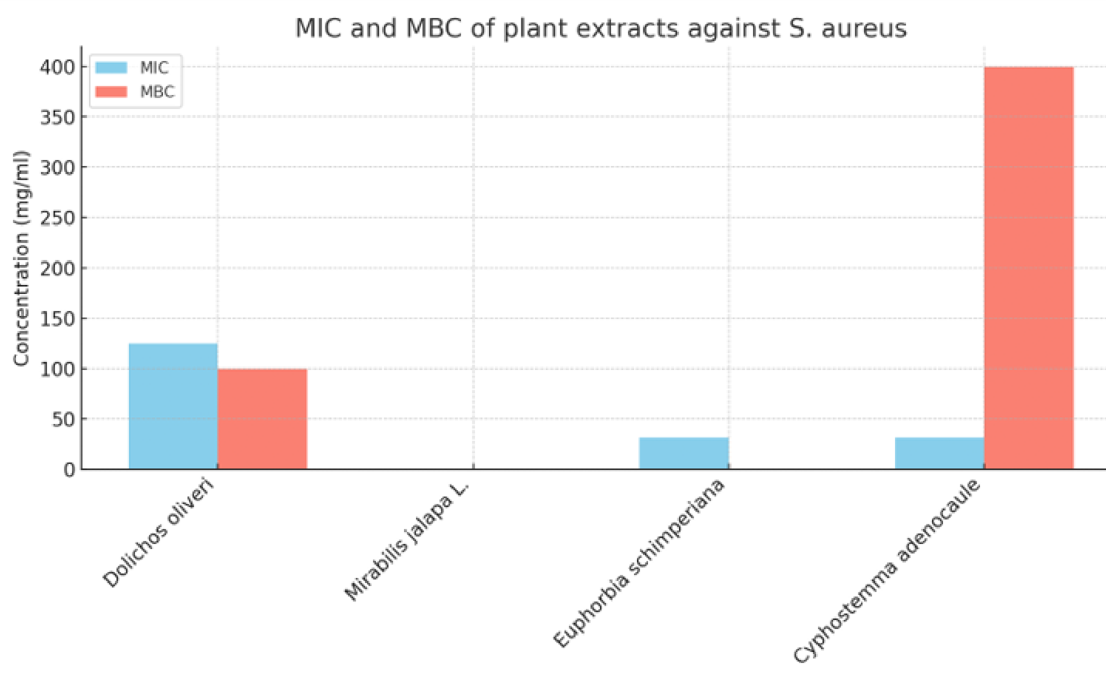

Figure 3: MIC and MBC of plant extracts against *S. aureus*.

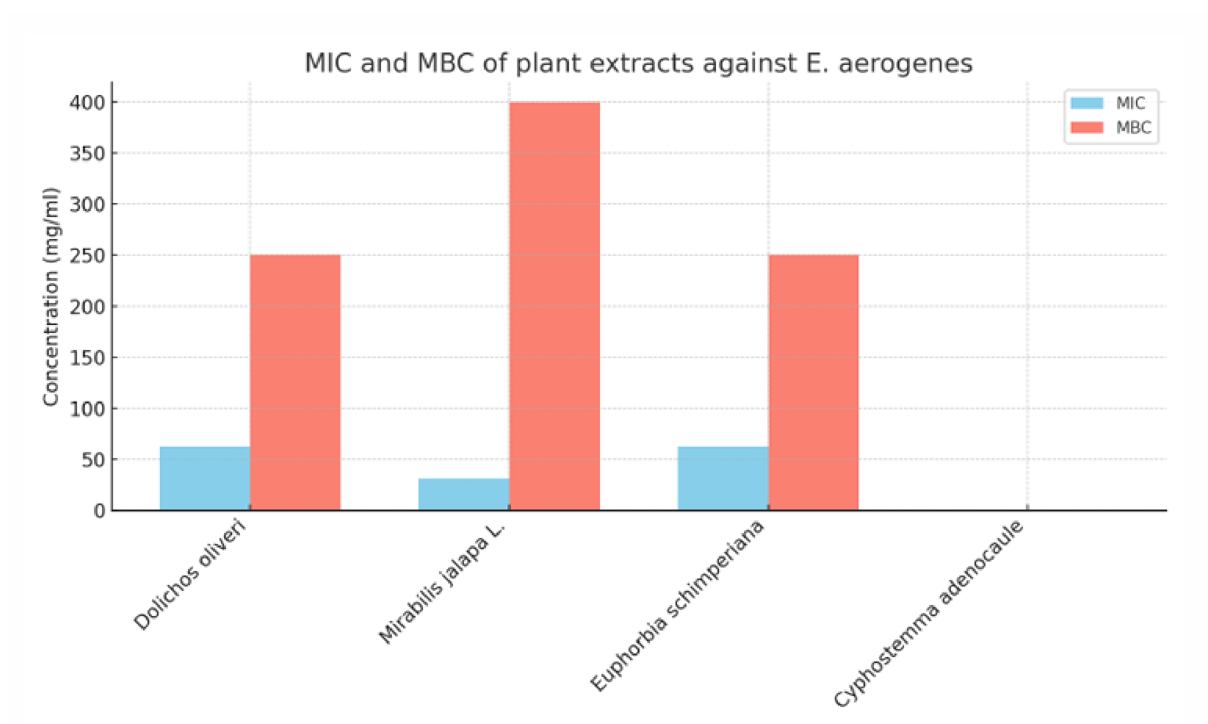

Figure 4: MIC and MBC of plant extracts against *E. aerogens*.

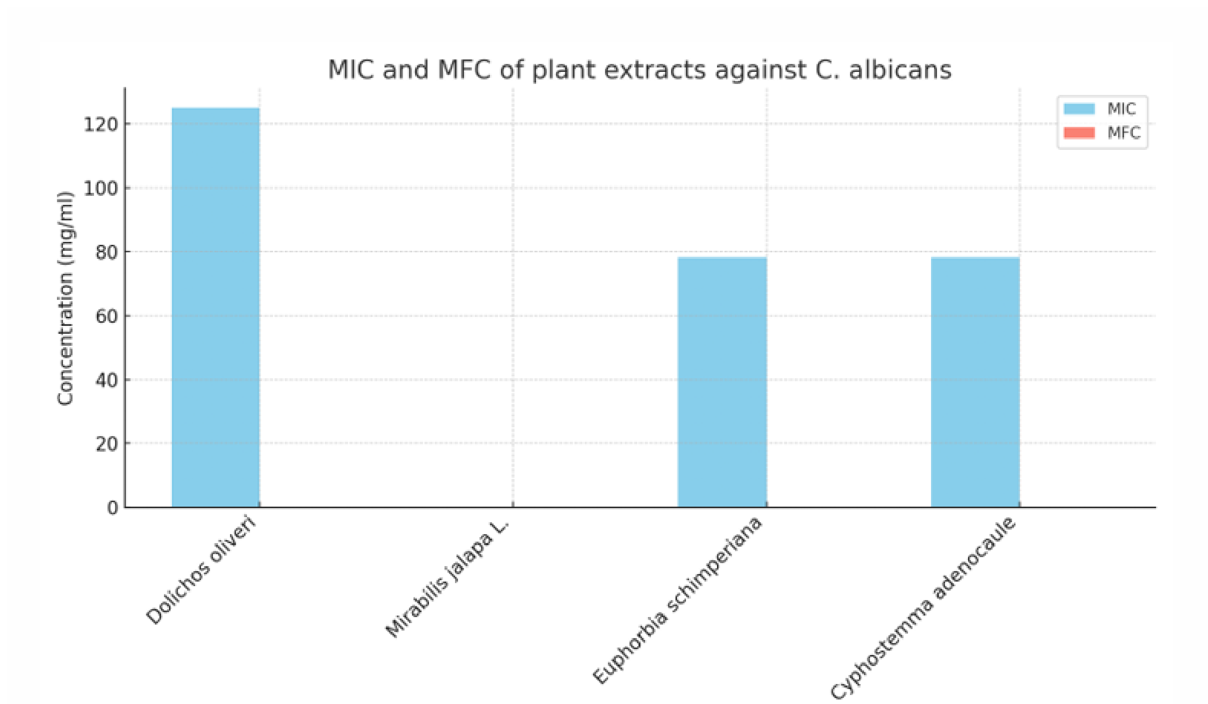

Figure 5: MIC and MFC of plant extracts against *C. albicans***.**

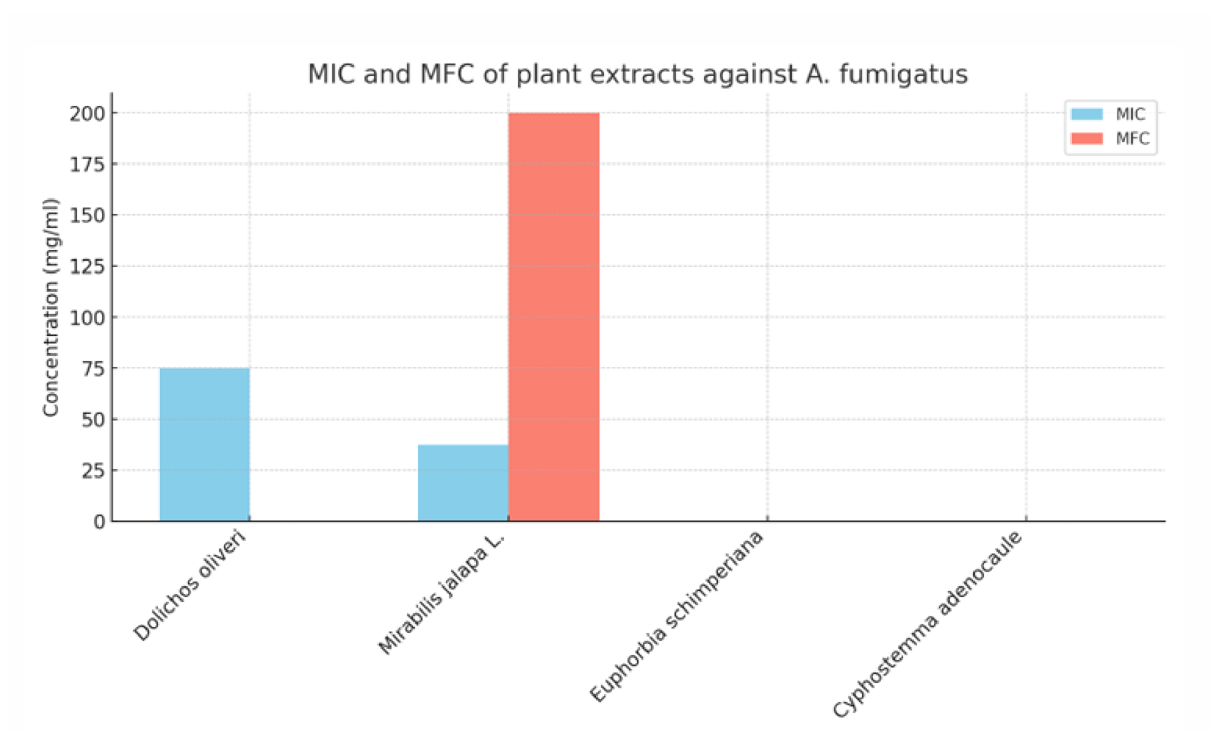

Figure 6: MIC and MFC of plant extracts against *A. fumigatus.*

5. DISCUSSION

5.1. Medicinal Plants

The increasing prevalence of antibiotic-resistant mastitis-causing pathogens has prompted global interest in plant-derived alternatives, particularly in developing regions where access to conventional drugs is limited (WHO, 2021). The current study investigated the traditional use and antimicrobial potential of four medicinal plants—*Dolichos oliveri*, *Mirabilis jalapa L.*, *Euphorbia schimperiana*, and *Cyphostemma adenocaule*—against bacterial and fungal pathogens isolated from mastitic dairy cows in Gursum district, Oromia, Ethiopia.

The use of *Dolichos oliveri* and *Euphorbia schimperiana* aligns with ethnomedicinal practices reported in southern Ethiopia by Yineger et al. (2007), who documented their local use in treating various livestock infections. Additionally, the use of *Mirabilis jalapa L.* is notable, as its antibacterial and antifungal properties have been documented in studies conducted in Nigeria and India, showing activity against *E. coli*, *S. aureus*, and *Candida* species (Adedapo et al., 2005; Karthikeyan et al., 2009). This cross-regional similarity in use suggests a common ethnopharmacological knowledge base and supports the credibility of traditional claims.

Comparative antimicrobial screenings conducted in Kenya and Uganda have demonstrated that tuber- and root-based preparations from plant species are more effective against Gram-positive bacteria such as *S. aureus* than Gram-negative ones due to the permeability barrier conferred by the outer membrane of Gram-negative bacteria (Kareru et al., 2007; Tabuti et al., 2010). Moreover, the oral administration of crushed plant material mixed with wheat bran or water, as practiced in Gursum, is mirrored in studies from Bale and Sidama zones (Lulekal et al., 2014; Regassa, 2013), highlighting widespread consistency in preparation and administration methods across diverse Ethiopian agro-ecological zones. This suggests a shared cultural pharmacology that merits preservation and scientific validation.

Although traditional remedies offer promise, challenges remain regarding standardization, dosage, and safety. The lack of toxicity data, pharmacokinetic studies, and standardized extraction methods limits the wider adoption of these ethnomedicinal plants in formal veterinary

use. Nevertheless, given the global push for reduced antibiotic use in livestock and the WHO's call for integrated approaches to antimicrobial resistance, the current findings provide a foundation for further pharmacological research and bioprospecting.

5.2. Isolation and Identification of Test Organisms

The isolation and identification of bacterial and fungal pathogens from bovine milk samples revealed the presence of multiple mastitis-causing microorganisms. Following the guidelines set by the National Mastitis Council (NMC, 2017), standard bacteriological and mycological procedures were employed to ensure accurate identification of the clinical isolates.

From a total of 60 milk samples collected from 25 dairy cows, four predominant microbial species were isolated: *Staphylococcus aureus*, *Enterobacter aerogenes*, *Candida albicans*, and *Aspergillus fumigatus*. The identification of *S. aureus*, a major causative agent of bovine mastitis, was supported by its characteristic β-hemolysis on Blood Agar, followed by the production of golden-yellow colonies with surrounding yellow zones on Mannitol Salt Agar (MSA), indicative of mannitol fermentation. Furthermore, on Purple Agar Base supplemented with 1% maltose, the formation of yellow colonies due to acid production confirmed the organism's ability to ferment maltose. The catalase test, which yielded positive bubble formation upon the addition of 3% hydrogen peroxide, and the Gram staining result showing purple cocci in clusters, further validated the identification (Cheesbrough, 2006; Quinn et al., 2011).

For Gram-negative bacteria, colonies isolated on MacConkey Agar were subcultured onto Eosin Methylene Blue (EMB) Agar, where *E. aerogenes* was identified based on the appearance of large, mucoid, pink to light-purple colonies with a faint sheen—characteristic of slow lactose fermenters with minimal acid production. Biochemical profiling through IMViC tests demonstrated that *E. aerogenes* was Indole- and Methyl Red-negative but positive for Voges-Proskauer and Citrate utilization, which is consistent with its metabolic profile. Additional confirmatory tests showed catalase positivity and urease negativity. In the Triple Sugar Iron (TSI) test, the organism produced a yellow slant and butt with gas formation and no hydrogen sulfide (H_2S), further supporting its identification (Cheesbrough, 2006; Quinn et al., 2011).

Fungal isolates of *Candida albicans* and *Aspergillus fumigatus* were identified through their growth on Potato Dextrose Agar (PDA) and subsequent microscopic examination using lactophenol cotton blue staining. The visible colony characteristics of *C. albicans* and *A. fumigatus* identified on PDA in this study align well with prior morphological descriptions by Kumar et al. (2017) and the fungal identification literature (Larone, 2011), validating the reliability of conventional identification techniques.

The results of currently isolated pathogens are consistent with previous reports identifying these pathogens as major contributors to clinical and subclinical bovine mastitis (Bradley, 2002; Krömker & Leimbach, 2017). For instance, a study by Mulugeta and Wassie (2013) in northern Ethiopia reported *S. aureus* as the most frequently isolated pathogen from mastitic milk samples, confirming its ubiquitous presence and high pathogenicity.

Similarly, *C. albicans* and *A. fumigatus* were previously identified in mastitic samples from dairy herds in Debre Zeit, suggesting the role of fungi as emerging mastitis pathogens (Bedada & Hiko, 2011). The visible colony characteristics of *C. albicans* and *A. fumigatus* identified on PDA in this study align well with prior morphological descriptions (Kumar et al., 2017), validating the reliability of conventional identification techniques. The presence of *A. fumigatus* and *C. albicans* in mastitic samples underscores the importance of including antifungal agents in mastitis treatment regimens. Previous work by Asres et al. (2014) demonstrated the antifungal activity of several Ethiopian medicinal plants, supporting the hypothesis that ethnobotanical remedies may have dual antibacterial and antifungal potential.

These findings highlight the polymicrobial nature of bovine mastitis, with both bacterial and fungal agents contributing to its etiology. The presence of *S. aureus* and *E. aerogenes*, in particular, underscores the need for accurate diagnostic techniques to inform effective antimicrobial therapy. Moreover, the detection of fungal pathogens such as *C. albicans* and *A. fumigatus* suggests that environmental and hygienic conditions may play a significant role in the occurrence of mixed infections in dairy herds.

5.3. Percentage Yield of Plant Extraction

The methanol extraction of the selected medicinal plants revealed variations in yield percentages, color, and consistency of the crude extracts. These differences can be attributed to the chemical composition, moisture content, and solvent-solubility of bioactive compounds within each plant species.

As shown in Table 3, *Euphorbia schimperiana* yielded the highest amount of extract (30.4%), indicating a relatively higher concentration of methanol-soluble phytochemicals in its tuber. In contrast, *Mirabilis jalapa L.* yielded the lowest extract amount (23.2%), suggesting it contains fewer compounds that are extractable using methanol or a higher proportion of non-soluble components.

The extract from *Dolichos oliveri* produced a 24.04% yield, and *Cyphostemma adenocaule* yielded 25.9%, both indicating moderate extractive values. The color of the extracts ranged from white (Dolichos oliveri) to reddish-brown (Mirabilis jalapa L.), while all extracts were semisolid in consistency, which is typical for methanol crude extracts rich in mixed phytochemicals such as alkaloids, flavonoids, and terpenoids.

These findings are consistent with existing literature on ethnomedicinal plant extractions, which highlights that extraction yield is influenced by plant species, part used, and solvent polarity. For instance, Njeru et al. (2015) reported a 31% methanol extract yield for *Euphorbia* species, which closely aligns with the 30.4% yield obtained for *Euphorbia schimperiana* in this study. Similarly, Mohammed et al. (2014) documented extraction yields ranging from 18.5% to 28.3% among medicinal plants used by the Borana community in southern Ethiopia, which is comparable to the the mid-range yields obtained from *Dolichos oliveri* and *Cyphostemma adenocaule* in the current study. Lower yields such as that recorded for *Mirabilis jalapa L.* are also consistent with the work of Akinmoladun et al. (2007), who reported minimal yields for some tuberous plants due to low metabolite density or higher fiber content

Yineger et al. (2008) also reported extraction yield of **15% to 30%** in their study on medicinal plants in southwestern Ethiopia, indicating that extraction efficiency is largely influenced by plant species, part used, and solvent type. The extraction results are within expected ranges

reported in ethnopharmacological literature. The high yield of *Euphorbia schimperiana* suggests it may be particularly rich in methanol-soluble bioactive compounds and is a promising candidate for further antimicrobial testing.

5.4. Phytochemical Properties of Plant Extracts

In this study, *Dolichos oliveri* contained alkaloids, saponnins and glycosides. Different studies also reported similar results. De *et al*. (2017) confirmed the presence of alkaloids in *Dolichos oliveri*, aligning with this study. The presence of saponins and glycosides was also reported by Goretti *et al*. (2015) which is also in line with the result of this study. Furthermore, Ibrahim *et al*. (2020), also reported similar phytochemical profiles for *Dolichos oliveri*, specifically noting the presence of alkaloids and saponins as key bioactive compounds in *Dolichos oliveri*. But they reported absence of terpenoids, flavonoids, tannins, and phlobatanins in this plant.

The presence of terpenoids was identified in *Mirabilis jalapa* L. in our study which was also confirmed by other research findings. Mafoki *et al*. (2021) also found terpenoids in *Mirabilis jalapa* aligning with the result of this study. Flavonoids was not found in the root extract of *M. jalapa* in our study. But Gandhi *et al*. (2018) identified flavonoids in *Mirabilis jalapa*. This variation may be due to plant parts used or extraction methods. In other studies, Chetty *et al*., (2008), Kiran *et al*. (2010), Devi *et al* (2011), Mohammed *et al*. (2012), Selvakumar *et al*. (2012), Kamel etal. (2015), Salman *et al*, (2015), Shil *et al*. (2017), Salman *et al*. (2015), Kumar *et al*. (2017), and Ali Esmail *et al*., (2023) all reported the presence of different phytochemicals in *Mirabilis jalapa* including terpenoids and flavonoids. In this study, it was generally observed that *Mirabilis jalapa* was noted for containing saponins, phenols, and terpenoids, but no alkaloids. This result corroborates findings by Kumar *et al*. (2019), who conducted similar phytochemical screenings and found that the plant exhibited a rich presence of phenolic compounds and terpenoids, which are believed to contribute to its medicinal properties, while alkaloids were absent in their study as well.

Saponins and terpenoids were identified in *Euphorbia schimperiana* in this study. The result of this study is in line with the studies of Siddiqui *et al*. (2014) which identified the presence of saponins and terpenoids in *Euphorbia schimperiana*. The presence of terpenoids in this plant is

well-documented in the literature. Qualitative determination of this study indicated the presence of saponins, phenols, and terpenoids, while alkaloids, tannins and phlobatanins were not detected. Tadesse and Ibrahim (2021) also observed similar presence of these phytochemicals in *Euphorbia schimperiana*, emphasizing the plant's potential for antimicrobial and antioxidant activity due to its rich phenolic and saponin content.

Cyphostemma adenocaule was noted to contain flavonoids, phenols, tannins and terpenoids in this study which is consistent with the studies of Molefe *et al*. (2019) which reported the presence of flavonoids and that of Pillay *et al.* (2016) which reported the presence of phenolic compounds and terpenoids in *Cyphostemma adenocaule*. For *Cyphostemma adenocaule*, the qualitative tests identified flavonoids, phenols, saponins, and terpenoids, while alkaloids, tannins and phlobatanins were absent. Liu *et al.* (2018) similarly found these phytochemicals in *Cyphostemma species*, specifically highlighting the plant's richness in flavonoids and terpenoids, which have been linked to its therapeutic and antimicrobial potential.

The alkaloid content (2.35 ± 0.01) was significantly higher in *Dolichos oliveri* compared to other plants in our study. The result of this study is in line with the report of Mafoki *et al.* (2021) which supports the finding of alkaloid content in Dolichos species although it focuses on *Mirabilis jalapa*. The saponin content in *Dolichos oliveri* was moderate in this study which is in agreement with the studies of Goretti *et al.* (2015) which also reported moderate saponin levels in *Dolichos oliveri*, aligning with our quantitative findings of 1.43 ± 2.00. The results of this study was also consistent with the findings of Olayemi *et al.* (2018), who conducted a detailed quantification of alkaloids and saponins in *Dolichos oliveri*, revealing high alkaloid concentrations as a distinguishing feature of this plant. The moderate presence of saponins and terpenoids further supports the plant's broad pharmacological applications.

In this study, it was observed that *Mirabilis jalapa L.* had the lowest terpenoid content (0.21 ± 3.14). The result of this study is in agreement with the studies of Mafoki *et al*. (2021) which found terpenoid levels that might be consistent with the lower values reported in this study. The saponin level found in Mirabilis jalapa was 0.49 ±1.10 which was supported by the study of Goretti *et al*. (2015) which reported Saponin contents in similar plant species showed moderate to low levels. Quantitative analysis of *Mirabilis jalapa* showed low alkaloid (0.82 ± 1.31 Ba),

saponin (0.49 ± 1.10 cb), and terpenoid (0.21 ± 3.14 gf) content, indicating that while the plant contains these bioactive compounds, they are present in lower concentrations compared to other plants in the study. This finding is similar to the finding reported by Kumar *et al*. (2019), who noted that although *Mirabilis jalapa* is rich in phenolic compounds, its levels of alkaloids and other phytochemicals are relatively low.

Euphorbia schimperiana had significantly the highest saponin content (3.11 ± 0.00) surpassing all other plants in this study, which is consistent with the study of Siddiqui *et al*. (2014) which reported high saponin levels. The result of this study indicated a high terpenoid level (0.78 ± 3.11) in *Euphorbia schimperiana*. This result was also in line with the result of Siddiqui *et al*. (2014) who reported the same level of terpenoid content in *Euphorbia schimperiana*. *Euphorbia schimperiana* exhibited the highest saponin content (3.11 ± 0.00 Ac) in the study, followed by moderate alkaloid levels (1.54 ± 0.02 gb) and significant terpenoid levels (0.78 ± 3.11 bd). These results also mirrored those found by Tadesse and Ibrahim (2021), who also observed a high concentration of saponins in this plant, linking it to the plant's notable antimicrobial and anti-inflammatory properties. The high saponin content is particularly noteworthy, as saponins are known for their broad-spectrum activity.

In this study, Cyphostemma adenocaule was noted for having significantly the highest phenolic content (0.0500 ± 1.18) compared to other plants, indicating a strong potential for antimicrobial activity. This finding was in agreement with the finding of Molefe *et al*. (2019) who identified the phenolic content in this plant species to be high. Cyphostemma adenocaule showed the lowest terpenoid content (0.23 ± 0.00), which is consistent with the result of Pillay *et al*. (2016) which indicated relatively low levels of terpenoids corroborating the finding of this study. The quantitative determination of Cyphostemma adenocaule generally revealed significant phenolic content (0.0500 ± 1.18 dF), moderate alkaloid (1.42 ± 1.11 gd), and low terpenoid (0.23 ± 0.00 dg) concentrations. Liu *et al*. (2018) similarly reported significant phenolic content in *Cyphostemma species*, which they linked to the plant’s strong antioxidant and antimicrobial potential. The lower levels of terpenoids and alkaloids in this species were also consistent with their earlier findings.

Qualitative tests of phytochemicals in medicinal plants may miss low-concentration compounds, which may be detected using advanced techniques such as UV-Vis spectroscopy and HPLC, as described by Egbuna et al. (2019). Some phytochemicals may be weakly detected in screening tests but have significant concentrations in quantitative analysis.

Sensitivity of qualitative tests varies, and they may fail to detect low concentrations that are measurable through more precise techniques like UV-Vis spectroscopy, HPLC, or GC-MS.

Some screening methods may yield false positives (due to interference from other compounds) or false negatives (due to low solubility or reactivity).

False positives and negatives are challenges in phytochemical screening. Roghini and Vijayalakshmi (2018) noted that qualitative tests can be influenced by the presence of interfering compounds, necessitating confirmation through precise quantitative methods. Similarly, Egbuna et al. (2019) highlighted the limitations of qualitative tests and the need for validation through quantitative assays.

If a compound is detected qualitatively, its precise concentration must be measured to assess its pharmacological relevance (Egbuna et al., 2019). Conversely, if it is absent, further advanced techniques such as HPLC or GC-MS can verify whether it is below the detection limit (Senarathna & Fernando, 2022). If a phytochemical is absent qualitatively → either it is truly absent, or its concentration is below the detection limit.

Discrepancies arise from sensitivity differences, solubility issues, or subjective interpretation of color changes in screening tests. For instance, *Euphorbia schimperiana* had the highest saponin content despite a weak (+) qualitative presence.

5.5. Antimicrobial Activities of Plant Extracts

Zone of inhibition demonstrated by plant extracts on the growth of bacteria and fungus is shown in tables 8 and 9. The presence of secondary metabolites like alkaloids, phenolics, and saponins correlates strongly with antimicrobial efficacy. Researches have reported that the antimicrobial activity of plant extracts depends on the type and amount of secondary metabolites present in

the plant tissue (Regnier and Macheix, 1996; Stankovi´c *et al*., 2015), and the pathogen's inherent resistance (Ozkan *et al*., 2004). The extract from *Dolichos oliveri* had increased antimicrobial activities with increased concentrations for all test organisms and standard strains indicating its broad spectrum antimicrobial activities at high concentrations (125 mg/ml) and above (Table 9). In their study of in vitro antimicrobial assay of selected medicinal plants against medically important plant and food-borne pathogens, Sissay Bekele *et al*., (2016) reported leaf extracts of *D. oliveri* demonstrated antimicrobial activity on both fungal and bacterial pathogens but reported the root extracts of *D. oliveri* exhibited selective antifungal activity.

Alkaloids in *Dolichos oliveri* (2.35 ± 0.01 mg/g) accounted for significant activity against Enterobacter aerogenes, as supported by Cushnie and Lamb (2011), who emphasized alkaloids' role in disrupting microbial membranes.

The Extract from *Mirabilis jalapa* L. had no any antimicrobial activities on both clinical and standard isolates of *S. aureus* at 75 mg/ml and 100 mg/ml concentrations but exhibited 10.67 mm zone of inhibition on standard strain at 125 mg/ml. It had increased zone of inhibition from 6.92 mm to 8.83 mm on clinical isolates of *E. aerogenes* and 8.53 mm to 12.76mm on standard isolates.

Sissay Bekele *et al*., (2016) reported that root extracts of *Mirabilis jalapa* showed broad spectrum antimicrobial activity to S. epidermidis (18±0.2). Aumeeruddy-Elalfi *et al*., (2015) also reported maximum inhibition was detected with *M. jalapa* against S. epidermidis, which showed better efficacy compared to other plant materials in their study. It had 9 mm to 11.83 mm zone of inhibition on clinical isolates of *C. albicans* and 8.20 mm to 10.50 mm zone of inhibition on standard isolates. It also exhibited 7.42 mm to 11.83 mm inhibition on clinical isolates of *A. fumigatus* and 9.33 mm to 12.83 mm inhibition zone on standard isolates with an increase of concentration from 75 mg/ml to 125 mg/ml.

Euphorbia schimperiana had no antimicrobial activity against clinical isolates of *S. aureus* at the concentration of 75 mg/ml and 100 mg/ml, but exhibited inhibition zone of 8.58 mm on clinical isolates at 125 mg/ml and 9.6 mm to 13.50 mm on standard strains of *S. aureus*. It exhibited 7 mm to 11.08 and 8.10 mm to 12.30 mm inhibition zone on clinical and standard strains of *E. aerogenes* respectively. It also exhibited 7.83 mm to 10.66 mm inhibition on clinical

isolates of *C. albicans* and 9 mm to 11.90 on standard strains with increase of concentration from 75 mg/ml to 125 mg/ml. Furthermore, it also demonstrated 12 mm to 14.33 mm and 12.40 mm to 13.93 mm inhibition on *A. fumigatus* clinical and standard strains respectively. Euphorbiaceae family is very diverse in range, composed of a wide variety of plants ranging from large woody trees to simple weeds that grow prostrate to the ground. The family is composed of over 315 genera and nearly 8,000 species.

The major genus is *Euphorbia L.* with over 2000 species found in the tropical and subtropical regions as well as in the temperate zones worldwide (Jassbi, 2006 and Evans *et al*., 2002). In the previous studies, a wide range of secondary metabolites, which are responsible for antimicrobial activities of the extracts, such as terpenoids, phenolic derivatives and alkaloid compounds, were reported by Shi *et al*. (2011). In the previous antimicrobial susceptibility testing, *Euphorbia schimperiana* exhibited the highest antimicrobial activity with a yield of 30.4% in methanolic extraction and significant zones of inhibition against *Staphylococcus aureus* and *Candida albicans.* This aligns with studies by Sahlu (2013) and Taddese *et al*. (2009), which also identified potent activity of phytochemical-rich plants against similar pathogens.

Cyphostemma adenocaule had no antimicrobial activities on both clinical and standard strains of *E. aerogenes* but showed 7.33 mm to 9.75 mm and 11 mm to 13.93 mm on clinical and standard strains of *S. aureus* respectively. *Cyphostemma adenocaule* demonstrated 7.17 mm to 9.50 mm and 8.33 mm to 11.50 mm inhibition on *C. albicans* clinical and standard strains respectively and 9.25 mm to 13.41 mm and 7.67 mm to 10.33 mm inhibition on *A. fumigatus* clinical and standard strains respectively. Of all plant extracts, *Euphorbia schimperiana* exhibited higher inhibition zone of inhibition (11.5 mm to 14.5 mm) on *A. fumigatus* as concentration increased from 75 mg/ml to 125 mg/ml indicating its efficacy against this organism even at lower concentration. *Cyphostemma adenocaule* (Steud. ex. Rich.) Wild and R.B.Drumm is a climbing, scrambling, or trailing herb that belongs to the Vitaceae family (Wickens and Burkill, 1986; Bello *et al*., 2019). The antimicrobial activities of this plant have been well explored by researchers. Abdulbasit *et al*., (2021), in their Phytochemical Screening, Antioxidant and Antibacterial Activities of the Root Extract of *Cyphostemma adenocaule* by methanol, n-hexane and chloroform extraction, revealed the presence of flavonoids, alkaloids,

carbohydrates and glycoside, saponins, and tannins but anthraquinone was absent. In their antibacterial assay, they revealed the methanol extract showed poor activity against the test organisms. Phenolic compounds, highest in *Cyphostemma adenocaule* (0.0500 ± 1.18 mg/g), are well-documented for their antioxidant and antimicrobial properties (Ammar *et al*., 2017; Cowan, 1999).

The extract from *Dolichos oliveri* had antimicrobial activities on all test organisms including control strains of both bacteria and fungus at lower concentrations and higher concentrations with increased inhibition zone with increasing concentrations. Gram-positive bacteria, particularly *Staphylococcus aureus*, showed higher susceptibility compared to Gram-negative bacteria and fungi. This is consistent with reports by Sahlu (2013) and Bakr *et al*. (2015), which noted the robustness of Gram-negative cell walls against plant-derived antimicrobials. Fungal isolates like *Aspergillus fumigatus* were less sensitive, indicating the need for higher concentrations or combined therapies for effective control.

The comparative analysis of plant extracts with synthetic antibiotics like ciprofloxacin and ketoconazole revealed that while plant extracts are effective, they do not yet surpass synthetic antibiotics in potency. However, the study highlighted the affordability, local availability and reduced resistance risks of plant-based treatments, which are valuable in ethnoveterinary medicine, particularly in rural areas.

These findings underscore the potential of integrating traditional knowledge with scientific validation, especially in resource-limited settings. The use of local plants like *Euphorbia schimperiana* offers a sustainable alternative for managing bovine mastitis, reducing reliance on expensive and scarce antibiotics.

While promising, the study's in-vitro nature warrants further in-vivo trials to validate efficacy under field conditions. Additionally, exploring synergistic combinations of plant extracts with synthetic drugs could enhance therapeutic outcomes.

5.6. Inhibitory, Bactericidal, and Fungicidal Activities of Plant Extracts

The antimicrobial activities of *Dolichos oliveri*, *Mirabilis jalapa* L., *Euphorbia schimperiana*, and *Cyphostemma adenocaule* against bacteria (*S. aureus*, *E. aerogenes*) and fungi (*C. albicans*, *A. fumigatus*) highlighted the diverse bioactive potential of plant-derived compounds. The findings align with several previous studies on the antimicrobial properties of medicinal plants. The extract of *Dolichos oliveri* showed significant bactericidal effects against *S. aureus* and *E. aerogenes* (MBC: 200 mg/ml) and fungistatic activity against *C. albicans* and *A. fumigatus* (MIC: 125 mg/ml and 75 mg/ml, respectively). Similar findings were reported in a study by Ahmed *et al*. (2014), where legumes exhibited antibacterial activity because of bioactive compounds they contain such as alkaloids. The antimicrobial activities of this plant extract could be attributed to its phytochemical constituents as identified in the result of this study (table 4). Bioactive components of alkaloids such as morphine and cordine have been found to be active not only against bacterial and fungal pathogens but also trypanosomes and plasmodia (Freiburghaus *et al*., 1996; Omulokoli *et al*., 1997).

The extracts from *Mirabilis jalapa* L. demonstrated bactericidal activity on *E. aerogenes* (MBC: 400 mg/ml) and fungicidal activity on *A. fumigatus* (MFC: 200 mg/ml), but no effect on *S. aureus* or *C. albicans*. The work of Sharma *et al*. (2019) found that *Mirabilis jalapa* extracts possess antifungal activity, particularly against filamentous fungi like Aspergillus spp., due to their high content of betalains and alkaloids. However, the lack of antibacterial effects against Gram-positive bacteria (*S. aureus*) aligns with studies by Singh *et al*. (2020), which noted selective activity against Gram-negative bacteria. The antimicrobial activities of *Mirabilis jalapa* could be attributed to its phytochemical constituents of saponins, steroids, terpenoids and phenols which were identified in this study. Different researchers have reported that phytochemical constituents of medicinal plants have antimicrobial activities against bacterial and fungal pathogens (Cowan, 1999; Heinrich *et al.*, 2006; Abdel *et al*., 2013; and Deshpande *et al*., 2013).

Euphorbia schimperiana exhibited Bactericidal effect on *E. aerogenes* (MBC: 200 mg/ml) and bacteriostatic effect on *S. aureus*; fungistatic on *C. albicans* but was ineffective against *A. fumigatus*. Previous research by Abebe *et al*. (2015) demonstrated that Euphorbia species

possess antimicrobial properties due to diterpenoids and triterpenoids, which disrupt bacterial protein synthesis. However, their limited fungicidal activity aligns with findings by Ogueke *et al*. (2019), who observed only fungistatic effects in similar Euphorbia extracts. The selective activity against *E. aerogenes* suggests that *Euphorbia schimperiana* contains compounds like terpenoids, as was also identified in this study, which are effective against Gram-negative bacteria, consistent with the membrane-disrupting properties of diterpenoids.

Cyphostemma adenocaule was effective as a bactericidal agent against *S. aureus* (MBC: 400 mg/ml) and fungistatic for *C. albicans* and *A. fumigatus* (MIC: 75 mg/ml). Studies by Getachew *et al*. (2018) on *Cyphostemma* species reported the presence of flavonoids and polyphenols, which inhibit bacterial growth, particularly Gram-positive bacteria like *S. aureus*. However, limited activity against Gram-negative bacteria (*E. aerogenes*) has also been noted due to differences in bacterial membrane composition. This indicates that *Cyphostemma adenocaule* primarily targets Gram-positive bacteria through mechanisms such as inhibition of peptidoglycan synthesis.

It is possible to define whether an antibacterial agent is bactericidal or bacteriostatic in vitro by using the MBC/MIC ratio. If the MBC/MIC ratio is ≤4, the effect is considered bactericidal, and if the MBC/MIC ratio is >4, the effect is defined as bacteriostatic (Angela *et al*., (2024). The MIC and MBC of antimicrobials constitute measures of in vitro efficacy against bacteria, whereas in vivo efficacy and, correspondingly, the optimal antimicrobial dose depend on pharmacokinetic and pharmacodynamics (PK/PD) indices

Cheta *et at.* (2024) also evaluated the MIC, MBC, and MFC of four herbal extracts (lemon grass oil, basil oil, peppermint oil, and Obicure tea extract) against eight common endodontic pathogens along with the MIC: MBC/MFC ratio. They evaluated antimicrobial activity of the extracts by detecting the MIC of the extracts by broth dilution method. They reported all the herbal extracts were proved to be effective antimicrobials against tested endodontic pathogens. Prasetyoputri *et al*. (2019) also reported MIC, MBC and MFC are essential for determining the antimicrobial concentration of herbal extracts.

The antimicrobial activities of plant extracts on test organisms depend on their outer membrane. The outer membrane of Gram-negative bacteria often confers resistance to plant-derived

antimicrobials (Getachew *et al*., (2018). Their study on *Cyphostemma* species confirmed the presence of flavonoids and polyphenols, which selectively target Gram-positive bacteria like *S. aureus* due to differences in bacterial membrane composition. This was evidenced by limited activity of *Cyphostemma adenocaule* against *E. aerogenes* (Gram-negative) but active against *S. aureus* (Gram-positive) in this study (table 9).

Plant extracts in this study, exhibited fungistatic effects rather than fungicidal effects. This aligns with the findings of (Ogueke *et al*. (2019) which showed plant-derived compounds inhibit fungal growth without necessarily killing fungal spores directly. Ogueke *et al*. (2019) found that Euphorbia-derived compounds had fungistatic effects against Candida species, similar to the fungistatic activity observed in this study against *C. albicans*. Sharma *et al*. (2019) also reported the antifungal activity of *Mirabilis jalapa* against Aspergillus species, which also aligns with this study's findings of fungistatic effects on *A. fumigatus*.

Certain plants, such as *Mirabilis jalapa* L., demonstrated specificity in targeting fungi (*A. fumigatus*) but not bacteria (*S. aureus*), showing selective activity against particular pathogens. In line with these findings, Singh *et al*. (2020) highlighted the selective antibacterial activity of *Mirabilis jalapa* against Gram-negative bacteria, consistent with its effectiveness against *E. aerogenes* in this study. The findings support the potential use of these plants as alternative antimicrobial agents, particularly against specific pathogens. However, their efficacy varies significantly depending on the organism and the extract, necessitating further phytochemical analysis and isolation.

6. SUMMARY AND CONCLUSION

6.1. Summary

This study evaluated the antimicrobial activities of selected medicinal plants against pathogens commonly associated with clinical mastitis in dairy cows. The pathogens isolated include *Staphylococcus aureus, Enterobacter aerogenes*, *Candida albicans,* and *Aspergillus fumigatus*. Methanol extracts from *Dolichos oliveri*, *Mirabilis jalapa L.*, *Euphorbia schimperiana*, and *Cyphostemma adenocaule* were prepared and tested for their antimicrobial properties through minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC).

The phytochemical analysis confirmed the presence of active compounds such as alkaloids, saponins, phenols, and terpenoids in varying concentrations across the plants, which are responsible for their antimicrobial activities.

The extract from *Dolichos oliveri* had antimicrobial activities on all test organisms including control strains of both bacteria and fungus at lower concentrations and higher concentrations with increased inhibition zone with increasing concentrations.

Euphorbia schimperiana had no antimicrobial activity against clinical isolates of *S. aureus* at the concentration of 75 mg/ml and 100 mg/ml. The Extract from *Mirabilis jalapa L.* had antimicrobial activities only at higher concentration (125 mg/ml) against standard strain of *S. aureus*. *Cyphostemma adenocaule* had no antimicrobial activities on both clinical and standard strains of *E. aerogenes*. At 125 mg/ml, *Mirabilis jalapa* L. had significantly ($P<0.05$) lower activity than *Dolichos oliveri,* and *Euphorbia schimperiana.*

Dolichos oliveri had significantly ($P<0.05$) higher activity at 100 mg/ml than *Mirabilis jalapa L.* and *Euphorbia schimperiana against E. aerogenes*.

Mirabilis jalapa L. had significantly higher activity against *C. albicans* ($P<0.05$) at the concentration of 125 mg/ml than all the tested plants. *Euphorbia schimperiana* and *Cyphostemma adenocaule* showed significantly higher ($p<0.05$) activity against the clinical isolate of *A. fumigatus* at 125 mg/ml

Dolichos oliveri showed broad-spectrum activity, being bactericidal against *S. aureus* and *E. aerogenes* and fungistatic against *C. albicans* and *A. fumigatus*. *Mirabilis jalapa* L. exhibited selective bacteriostatic and fungistatic activities against *E. aerogenes* and *A. fumigatus* respectively but had no effects on *S. aureus* and *C. albicans*. *Euphorbia schimperiana* demonstrated bacteriostatic and bactericidal activities against *S. aureus* and *E. aerogenes*, respectively and fungistatic effects against *C. albicans* but with no effects on *A. fumigatus*. *Cyphostemma adenocaule* demonstrated bacteriostatic effects on *S. aureus* and fungistatic effects on *C. albicans* and *A. fumigatus* but had no effects on *E. aerogenes*.

6.2. Conclusion

This study underscores the potential of selected medicinal plants in addressing the growing challenges of antimicrobial resistance in veterinary medicine. The plants tested exhibited varied antimicrobial properties, attributed to their distinct phytochemical compositions. The efficacy of these plants is linked to their rich phytochemical content, with variations in alkaloids, saponins, phenols, and terpenoids determining their specific antimicrobial properties. The plant extracts demonstrated selective activity based on pathogen type, with some showing broad-spectrum potential (e.g., *Dolichos oliveri*) and others exhibiting organism-specific activity. These findings support the integration of traditional medicinal knowledge with scientific approaches to address veterinary challenges, especially in rural and resource-constrained areas. The comparative analysis with synthetic antibiotics revealed that while plant extracts were less potent, their affordability, availability, and reduced resistance risks make them valuable in ethnoveterinary practices. From these concluding remarks, the following are forwarded as recommendations:

- Awareness should be raised among local communities by researchers and policymakers on the conservation, Cultivation, and harvesting of medicinal plants to maintain their long-term availability.
- Policy makers should consider validation and integration of ethnoveterinary practices with conventional veterinary medicine to reduce dependency on synthetic drugs and to counter the challenges posed by drug resistant pathogens in treating livestock diseases.

- ☞ Phytochemical isolation and analysis of individual bioactive compounds from the plants should be carried out to better understand their mechanisms of action and potential synergistic effects.
- ☞ Further in-vivo studies should be conducted by researchers in the field to confirm the efficacy of these plant extracts in treating bovine mastitis and other livestock diseases.
- ☞ Additional tests should be conducted to investigate the toxicity of active constituents, their side effects and pharmacokinetic effects.

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8. APPENDIX

Annex I: Format for collection of Ethno-veterinary data on traditional medicinal plants used for bovine mastitis

Name of traditional healer______________________________

Name of PA____________________

Altitude of the PA____________________

S/No.	Local name of plant	Part used	Mode of preparation	Route of administration	Duration of treatment

Annex II: Primary isolation and identification recording format for microbial isolates from milk samples

Sample code	Media used	Colony characterization			Gram-stain result	Identified MOs
		Color of colony	shape	arrangement		

Annex III: Biochemical tests of isolated pathogens

Genus of Test organism	Sample code	Type Of test	Type of chemical used			Test results		
			media	reagent	PH indicator	Color Change Of media	Gas produ-ction	Spp identifiedd

Annex IV: Isolation and identification of bacterial and fungal pathogens from milk samples

Test Organism	Cultural Characteristics				Gram Staining				Biochemical Tests			
	Media used	Colony characterization			Gram Reaction	Colony Characterization			Catalase	Citrate	Urease	TSI Agar (S/B/G/H)
		color	Shape	Appearance		color	Shape	Arrangement				
S. aureus	Blood Agar	yellow	round	smooth	+	purple	cocci	clusters	+	n/a	-	n/a
	MSA	Golden yellow	round	smooth								
	Purple-maltose	yellow	round	mucoid								
E. aerogenes	Blood Agar	pale	irregular	shiny	-	pink	rod	pairs	+	+	-	y/y/+/-
	MacConkey	pink	irregular	smooth								
	EMB	pink	round	mucoid								
C. albicans	PDA	white	round	smooth		white	round	single	n/a	n/a	n/a	n/a
A. fumigatus	PDA	Blue-green	round	powdery								

+=positive, - = negative, n/a = not applicable, y = yellow, S/B/G/H =Slant/Butt/Gas/Hydrogen sulfide

Annex V: Zone of inhibition (mm) exhibited by each plant extracts against different test organisms

Name of isolate	Zone of inhibition					Mean zone Of inhibition
	10%	5%	2.5%	1.25%	0.625%	

Annex VI: Mean zone of inhibition (mm) exhibited by plant extracts against isolates in comparison with controls

Plant extracts/controls	Test organisms					
Plant extracts						
Conventional antibiotics						
%4 DMSO						

Annex VII: Minimum bactericidal and fungicidal concentration in mgs/ml of plant extracts against bacterial and fungal pathogens

Plant extracts	Test organisms					

Annex VIII: Minimum inhibitory concentration in mgs/ml of plant extracts against bacterial and fungal pathogens

Plant extracts/antibiotics name	Test organisms					

Annex IX: Qualitative Phytochemical screening of plant extracts

Plant extracts	Screening tests						
	Flavonoid test	Saponin test	Alkaloid test	Tannin test	Steroid test	Glycoside test	Terpenoid test