Efficacy of selected South African plants against multidrug-resistant staphylococci isolated from clinical cases of bovine mastitis

Ayodele Omolade Akinboye

18374052

Submitted in fulfilment of the requirements for the degree

Magister Scientiae: Paraclinical Science

Phytomedicine Programme, Department of Paraclinical Sciences

Faculty of Veterinary Science

University of Pretoria

Supervisor: Prof. Lyndy McGaw

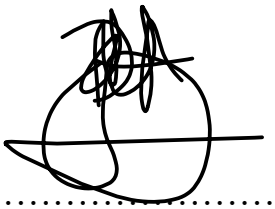
Co-supervisor: Prof. Inge-Marié Petzer

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

May 2021

Declaration

I, Ayodele Omolade Akinboye, declare that the dissertation titled 'Efficacy of selected South African plants on multidrug-resistant staphylococci isolated from clinical cases of bovine mastitis', which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



Signed ……..

.

Date**23/05/2021**……..

Dedication

To God, the Great Physician who has eternally prescribed ‘the leaves of the tree .. for the healing of the nations’ (Revelations 22:2). To Him be all the glory forever. Amen.

Acknowledgements

I would like to express my gratitude to the following contributors:

Prof. Lyndy McGaw, my supervisor, for who she is, for her kindness, depth of understanding and guidance that has made this work a success. I would like to thank her very much for all her support. I am indeed grateful.

Prof. Inge-Marié Petzer, my co-supervisor during this project, I would like to thank her for all her contributions. Her professional insight added depth to this work.

My family: Debbie, Deola, Theopistos, El-Aman, El-Checed and Abraham-El for inspiring me to succeed. My parents and siblings, for all your care and love.

My friends and mentors; Nzwakie, Tosin, and Bro. Muyiwa, they inspire me and push me hard to the finish line. I want to thank them for believing in me.

Sanah Nkadimeng for her valuable assistance in the general lab as well as during cytotoxicity testing.

Ibukun Famuyide for his ever-ready-to-help attitude whenever I needed his help.

My colleagues at the Phytomedicine lab, I am grateful to each and every one of them. The unity within the lab and the environment created by all involved make it a pleasant place to work. Their willingness to always help is a quality rarely seen and is something I am thankful to have been a part of.

The Department of Paraclinical Sciences for the use of their facilities and their helpful staff.

Abstract

Among cattle diseases, bovine mastitis remains a serious problem with financial implications to the farmer as it adversely affects milk production. *Staphylococcus aureus* and non-aureus staphylococcus (NAS) species are often isolated from milk samples in cases of bovine mastitis. Various antimicrobial agents have been used for treatment of mastitis pathogens in veterinary medicine with limited success because of increasing prevalence of resistance to commonly used antibiotics. Some bacteria causing bovine mastitis are known for their ability to form biofilms, which helps them resist antibiotic effects. The use of natural plant products is being explored as an alternative antimicrobial treatment for mastitis and a template for new mastitis drug development.

The primary aim of this study was to investigate antibacterial and antibiofilm activities of selected South African plants against drug-resistant staphylococci organism isolated from clinical cases of bovine mastitis. The study also examined the cytotoxic, anti-inflammatory and antioxidant activities of some of the plants.

Plants for this study were selected based on known antimicrobial activity, chemotaxonomic relationships to plant species with antibacterial activity, availability, and/or the existence of traditional uses against infectious diseases. The nine selected South African plants include *Antidesma venosum*, *Elaeodendron croceum*, *Erythrina caffra*, *Indigofera frutescens*, *Pleurostylia capensis*, *Searsia lancea*, *Searsia leptodictya*, *Trichilia emetica* and *Ziziphus mucronata*. Eight *S. aureus* isolates, three *Staphylococcus chromogenes* isolates, one *Staphylococcus haemolyticus* isolate and one ATCC strain of *S. aureus* were used to determine the antibacterial activity of extracts of the plants prepared with acetone and ethanol using standard methods, while one *S. aureus* isolate and one ATCC strain of *Staphylococcus epidermidis* were used in the antibiofilm assay. The leaf extracts of the plants with good antimicrobial activity were also assessed for their antibiofilm, antioxidant, anti-inflammatory and cytotoxic activities using standard methods.

The range of antibacterial minimum inhibitory concentration (MIC) values obtained in this study was relatively low and promising. All the plant extracts had good to weak antibacterial activity against all the drug resistant isolates with MIC ranging between 0.01 – 1.41 mg/ml. The lowest MIC value of 0.01 mg/ml obtained in this study was shown by the acetone extract of *S. lancea*

while the highest MIC value of 1.41 mg/ml was recorded with the acetone extract of *T. emetica*. Generally, the acetone extracts of all the plants showed better activity than their ethanol counterparts except for *E. caffra.* Ethanol is generally preferred as an extraction solvent in industrial applications, such as large-scale preparation of bioactive plant extracts, owing to its relative safety compared to acetone, which is more flammable. The *S. aureus* strains appeared to be more susceptible to the extracts than the NAS strains. An interesting finding was that the drug resistant isolates used in this study were generally more susceptible to the extracts than the ATCC strain which was in turn more susceptible to gentamicin, the positive control. All the plant extracts tested had LC_{50} values higher than the recommended cytotoxic cut-off concentration of 0.02 mg/ml. The ethanol extract of *E. caffra* had the best mean selectivity index (SI), calculated as LC_{50}/MIC against all the pathogens, of 8.30.

The antibiofilm investigation revealed that most of the plant extracts had very good inhibitory activity, inhibiting more than 50% of the test organism biofilm biomass, with *E. caffra* and *A. venosum* showing outstanding activities. The results also suggest that the *S. epidermidis* (NAS) ATCC strain was more susceptible to the antibiofilm activities of the plant extracts than the *S. aureus* isolate. The antioxidant activity investigation of the crude extracts of three of the plants showed that the ethanol extract of *S. lancea* had the best antioxidant activity against the 2, 2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals when compared to the other extracts. The ethanol extract of *S. lancea* had better anti-inflammatory activities than other extracts prepared using different solvents.

The results reported in this dissertation demonstrate the potential of the selected South African plants to manage bovine mastitis as the plant extracts showed good antibacterial activities at relatively non-cytotoxic concentrations. The results further reveal the abilities of the plants to disrupt the biofilm-forming activities of drug-resistant *Staphylococcus* spp. implicated in bovine mastitis. The anti-inflammatory and antioxidant activities demonstrated by some of the plants also showed the potential of the plants to arrest oxidative stress and anti-inflammatory activities involved in the pathophysiology of the disease. *S. lancea* and *E. caffra* appeared to show great potential in the overall management of infectious bovine mastitis.

Potential for synergistic antibacterial activity of the plant extracts needs to be examined for potential formulation of a polyherbal product. The active compounds need to be isolated and

identified from the most active plant extracts to provide possible marker compounds for quality control if an active extract or fraction is to be developed for use against mastitis pathogens. The mechanism of action of the active plant preparations (extracts, fractions or isolated compounds) needs to be elucidated to identify how they can be most useful in preventing or treating diseases caused by mastitis pathogens for possible complementary applications in the management of bovine mastitis.

Table of Contents

List of abbreviations, acronyms and symbols

%	Percentage
+	Plus
=	Equal to
±	Plus or minus
ºC	Degrees Celsius
µg	Microgram
µl	Microliter
µm	Micromolar
ABTS	2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ATCC	American type culture collection
AMR	Antimicrobial resistance
BEA	Benzene/ethanol/ammonia
BLG	β-lactoglobulin
CEF	Chloroform/ethyl acetate/formic acid
CFU	Colony forming unit
CM	Clinical mastitis
CV	Crystal violet
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl

EMW	Ethyl acetate/methanol/water
EPS	Extracellular polymeric substance
FOX	Ferric oxidation
FBS	Fetal bovine serum
g	Gram
h	Hour
INT	p-iodonitro-tetrazolium violet
LC	Lethal concentration
LOX	Lipoxygenase
m	Meter
MALDI-TOF-MS	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
MBC	Minimum bactericidal concentration
MDR	Multidrug resistance
MEM	Minimal essential medium
MFC	Minimum fungicidal concentration
MH	Mueller-Hilton
mg/ml	Milligram per millilitre
MIC	Minimal inhibitory concentration
Min	Minute
ml	Millilitre
MRSA	Methicillin resistant *Staphylococcus aureus*
MTT	3-(4.5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide
NAS	Non-aureus staphylococci

NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
OD	Optical density
PBS	Phosphate-buffered saline
PSF	Penicillin/streptomycin/fungizone
SCC	Somatic cell count
SCM	Subclinical mastitis
SI	Selectivity index
STA	*Staphylococcus aureus*
TAA	Total antibacterial activity
TSA	Tryptic Soy agar
TSB	Tryptic Soy broth
TLC	Thin layer chromatography
UV	Ultraviolet

List of Figures

List of Tables

1. General introduction

1. Introduction

1.1 Background

Dairy production systems provide milk that is rich in carbohydrates, fats, vitamins and minerals for the growing human population (Mekibib et al. 2010; Wilson et al. 2004). However, milk production does not always meet the population's demands due to several factors, common among which is mastitis (Kurjogi and Kaliwal 2014; Cervinkova et al. 2013; Islam et al. 2010). Mastitis is a serious health and economic problem in the bovine dairy industry worldwide (Ngu Ngwa et al. 2018).

Bovine mastitis is an infectious disease that occurs in dairy cattle. It is often defined as an inflammatory response of the mammary gland, which is caused by either physical or microbial factors (Petzer et al. 2009). In dairy cattle, mastitis occurs when the udder becomes inflamed because of pathogenic (mostly bacterial) invasion of the teat canal (Vasudevan et al. 2003). In the mammary gland, the organisms multiply and produce toxins, resulting in injury to the milk-secreting tissue owing to physical trauma and chemical irritation (Reshi et al. 2015), with potential to damage the milk-secreting tissues (Vasudevan et al. 2003).

Mastitis severity is divided into clinical and subclinical mastitis. Clinical mastitis is further divided into three levels of severity. Level 1 is characterized by secretion of abnormal milk (i.e. watery milk or presence of flakes in milk) while level 2 includes changes in the milk and signs of inflammation of the udder leading to redness, swelling, hardness and pain of the udder (Perini et al. 2014; Sharif and Muhammad 2009; Chebel 2007; Gruet et al. 2001). In level 3 the two latter signs are present as well as systemic signs in the animal that can in some cases be fatal. In subclinical mastitis, there is a lack of noticeable changes in the udder and the milk, and increased somatic cell count (SCC) (Petzer et al. 2009).

Common mastitis-causing organisms include bacteria, fungi and viruses (Tiwari et al. 2013). Mastitis pathogens are classified generally as contagious (host adapted) or environmental. Contagious pathogens are organisms that can adapt to surviving within the mammary gland of the host. These organisms can cause subclinical infections which show in elevation of SCC of milk as

well as clinical mastitis. They spread from one cow to another during milking. Examples of such contagious pathogens are *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* (Bradley 2002). Environmental pathogens are opportunistic invaders of the mammary gland, and most of them are not adapted to survive within the host for a long period of time as is the case with contagious pathogens. After invading the mammary gland, environmental pathogens multiply and stimulate the host response system. The environmental pathogens include mainly species belonging to the Enterobacteriaceae (particularly *Escherichia coli*), *Streptococcus uberis* and non-aureus staphylococci (NAS) (Perini et al. 2014; Petzer et al. 2009; Taemchuay et al. 2009).

Among cattle diseases, mastitis remains a serious problem with financial implications to the farmer as it adversely affects milk production (Petrovski et al. 2006). The economic impact of mastitis may be a result of two origins: control cost and losses. These losses mostly occur because of cost of treatment, decreased milk yield, decreased milk quality, and an increase in the risk of culling (Halasa et al. 2007; Petrovski et al. 2006; Bradley 2002).

Antimicrobial agents have been widely used for treatment of mastitis pathogens in veterinary medicine with limited success. Apart from pharmacodynamic and pharmacokinetic failures, the prevalence of resistance to commonly used antimicrobial agents is increasing (Du Preez 2000; Craven 1987). Meanwhile, natural products from plants are proven templates for new drug development and have shown various useful biological activities. The use of natural plant products has increasingly become a recognized treatment for several ailments, and this is a promising growing sector in the agribusiness industry (Makunga et al. 2008).

Some bacteria causing bovine mastitis have been known to exhibit biofilm formation as a virulent property (Phophi et al. 2019). This involves the use of a mechanism whereby bacterial cells group together to communicate and overcome the effects of antimicrobials (Van Vuuren and Holl 2017).

In the current study, extracts of nine South African plants were evaluated for their antibacterial, antibiofilm, antioxidant and anti-inflammatory potential as well as their cytotoxicity. These plants may be further investigated as possible complementary applications for the management of bovine mastitis.

1.2. Research hypothesis

Null hypothesis

- The selected South African plants do not possess antibacterial and anti-biofilm activities against drug-resistant *Staphylococcus* spp. isolated from clinical cases of bovine mastitis.
- The selected South African plants do not possess anti-inflammatory and antioxidant activities.

Alternative hypothesis:

- The selected South African plants possess antibacterial and anti-biofilm activities against drug-resistant *Staphylococcus* spp. isolated from clinical cases of bovine mastitis.
- The selected South African plants possess anti-inflammatory and antioxidant activities.

1.3. Justification

- Bovine mastitis is a known cause of severe losses in the dairy industry in South Africa and in other nations of the world, thereby negatively impacting all stakeholders in the industry. The economic impact runs into millions of dollars in losses worldwide annually.
- The major causative agents of mastitis are strains of staphylococcal bacteria which are rapidly evolving to be resistant to multiple antibiotics used in pharmacological management of bovine mastitis, thus making the disease increasingly difficult to manage.
- Natural products from plants are proven templates for new drug development and have shown biological activities against some mastitis-causing staphylococci that are resistant to several antibiotics commonly used in treatment of mastitis.
- Biofilm formation is one of the factors responsible for enhanced virulence and resistance to antibiotics among some mastitis-causing staphylococci. Plants with activity against biofilm formation can be useful in fighting bovine mastitis caused by drug resistant staphylococci.
- Mastitis is an inflammatory disease which is aggravated by oxidative stress induced by mastitis pathogens. Plants with anti-inflammatory and antioxidant activities may be useful in contributing to management of the disease.

1.4. Aims

The aim of this study is to investigate antibacterial and antibiofilm activities of selected South African plants against drug-resistant staphylococci isolated from clinical cases of bovine mastitis. The study will also examine the anti-inflammatory and antioxidant activities of some of the plants, as well as their cytotoxic effects.

1.5. Objectives

- To determine the antibacterial activity of selected South African plants against drug-resistant staphylococcal isolates from clinical cases of bovine mastitis.
- To determine cytotoxicity of the active plant species to evaluate selective antibacterial efficacy.
- To determine the antibiofilm activity of selected South African plants against a biofilm-forming ATCC strain of *Staphylococcus epidermidis* and a biofilm-forming *S. aureus* isolate from clinical cases of bovine mastitis.
- To determine the antioxidant and anti-inflammatory activities of selected plants.

2. Literature review

2.1. Introduction

Dairy products are consumed worldwide. As the world population grows, the demand for milk and other dairy products is expected to increase exponentially. This highlights the need to efficiently manage bovine mastitis, which is the most common cause of milk losses in the dairy industry worldwide.

2.2. Prevalence of bovine mastitis

Bovine mastitis occurs among dairy herds worldwide irrespective of the climate, however, studies have shown variability in epidemiology of mastitis pathogens based on different geographical locations (Krukowski et al. 2020). Prevalence and incidence of mastitis vary among countries and herds (Getaneh et al. 2017; Taponen et al. 2017). Risk factors such as management practices, host and dietary factors have been reported to play important roles in the epidemiology of both clinical and subclinical mastitis (Doherr et al. 2007; Biffa et al. 2005; Barkema et al. 1999).

Africa has a wide distribution of cattle, with countries such as Uganda, Kenya, Ethiopia and South Africa having the largest number of herds and dairy farms. Studies have been conducted to estimate the prevalence of bovine mastitis in approximately 30% of African countries and reported that Ethiopia has the highest prevalence of bovine mastitis (Yirga 2018; Mutaung et al. 2017).

Table 2.1 shows the prevalence of bovine mastitis according to studies that researched dairy herds in different countries of the world, publishing their findings between 1997 and 2020. The prevalence ranges from 2.1% to 85.3.% in Nigeria. The mastitic cows' populations were predominantly diagnosed using the California mastitis test (CMT) and bacteriological assay, among others.

Table 2.1: Prevalence of bovine mastitis among dairy herds according to reviewed studies around the world

Prevalence of Bovine Mastitis in Some Countries						
Continents	**Countries**	**No. of Cows**	**Type of Mastitis**	**Testing Method**	**Prevalence (%)**	**References**
Africa	Algeria	600	SCM	CMT	37.66	Zaatout et al. 2020
		140	SCM	CMT	28.57	Saidi et al. 2013
	Cameroon	240	SCM & CM	CMT & CE	68.30	Ngu Ngwa et al. 2018
		224	SCM & CM	CMT	63.39	Ngu Ngwa et al. 2020
	Ethiopia	386	SCM & CM	CMT & CE	34.70	Shiferaw et al. 2017
		302	SCM & CM	CMT & CE	41.70	Sarba and Tola 2017
		686	SCM & CM	CMT & CE	54.20	Abebe et al. 2020
		529	SCM	CMT	62.60	Abebe et al. 2016
		444	SCM & CM	CMT	68.00	Tilahun et al. 2015
		322	SCM & CM	CMT & CE	64.30	Zenebe et al. 2014
		349	SCM & CM	CMT & CE	29.50	Yohanis et al. 2013
		499	SCM & CM	CMT & CE	74.70	Zeryehun et al. 2013
		384	SCM & CM	CMT & CE	52.90	Lidet et al. 2013
		384	SCM & CM	CMT & CE	23.18	Girma et al. 2012
		107	SCM & CM	CMT	71.00	Mekibib et al. 2010
		195	SCM & CM	CMT	44.10	Delelesse 2010
		223	SCM & CM	CMT & CE	65.60	Lakew et al. 2009
		500	SCM & CM	CMT	24.10	Getahun et al. 2008
	Kenya	100	SCM & CM	CMT & CE	30.00	Ndirangu et al. 2017
	Nigeria	360	SCM & CM	CMT	26.90	Umaru et al. 2017
		339 Milk samples	SCM	CMT	30.90	Suleiman et al. 2013a
		300	SCM	CMT	85.30	Shittu et al. 2012
		360	SCM & CM	CMT	26.90	Umaru et al. 2017

		5 000	CM	PE & BE	2.10	Ameh et al. 1999
	Rwanda	123	SCM	CMT	50.40	Mpatswenumugabo et al. 2017
		195	SCM & CM	MECT	8.20	Iraguha et al. 2015
	Senegal	101	SCM	CMT & SCC	11.90	Kalandi et al. 2017
	Tanzania	416	SCM	CMT	48.80	Suleiman et al. 2018
		163	CM, SCM	CMT	31.3, 55.80	Suleiman et al. 2013b
Asia	Bangladesh	175	SCM & CM	CMT	28.60	Hoque et al. 2018
		228	SCM	CMT	64.90	Hoque et al. 2015
		330	CM, SCM	CMT	2.12, 37.58	Islam et al. 2010
		388	SCM & CM	CMT & PE	19.9 (D), 44.8 (W)	Rahman et al. 2009
	Iran	1 545	SCM & CM	CMT & CE	44.70	Hashemi et al. 2011
	India	244	SCM & CM	SFMT	72.95	Sadashiv et al. 2013
	Pakistan	500	SCM	SFMT	36.00	Bachaya et al. 2011
	Sri Lanka	152	SCM	CMT	43.00	Sanotharan et al. 2016
Europe	Croatia	61	SCM & CM	BE	22.50	Maćešić et al. 2012
	Finland	3 282	SCM & CM	CMT & CE	0.31	Krukowski et al. 2020
		9 410	SCM & CM	CMT & CE	37.80	Honkanen-Buzalski et al. 1996
North America	Mexico	259	SCM	CMT	20.50	Olivares-Pérez et al. 2016
	USA	108 312	SCM & CM	BE	48.50	Wilson et al. 1997

CMT= California mastitis test (Mastest), CE= Clinical examination, PE= Physical examination, SFMT= Surf field mastitis test, BE= Bacteriological examination, SCC= Somatic cell count, SCM= Subclinical mastitis, CM= Clinical mastitis.

Since the late 1970s, the prevalence of mastitis and the causal pathogens have been investigated in South Africa, but no official national survey has been done to determine the prevalence of mastitis, occurrence of udder pathogens and drug-resistant bacteria. Based on investigation of routine milk samples, the prevalence of mastitis increased from 8.1% in 2002 to 15.4% in 2006. In the herds selected for one such study, the overall mastitis prevalence remained stable and did not increase (Petzer et al. 2009). In 2018, Karzis et al. also reported that the overall prevalence of

mastitis did not change significantly over the study period of 2001 to 2010, although a visual decrease (non-significant) in the incidence of *S. aureus* mastitis was observed (Karzis et al. 2018).

2.3. Economic importance of bovine mastitis worldwide

Mastitis reduces dairy farm productivity and economic efficiency due to the reduction of milk yield, milk returns and increasing costs of treatment (Ibrahim and Ghanem 2019), and therefore it is still one of the major sources of economic losses for farmers. These losses are both direct and indirect (Zecconi and Cipolla 2019). Estimating the direct and indirect costs associated with bovine mastitis is extremely difficult. Direct costs to the dairy industry include the costs of treatment (veterinarian's time and drugs), discarded milk and fatalities (Petrovski et al. 2006). It is harder to quantify indirect losses that are associated with subclinical mastitis as well as longevity of cows, as they are not visible to the farmers (Petrovski et al. 2006)

Mastitis is responsible for economic losses of approximately US$35 billion worldwide (Gomes and Henriques 2016; Doss et al. 2012; Modi et al. 2012). The University of Glasgow in 2016 revealed that mastitis costs the global dairy industry €16–26 billion per annum based on a global dairy cow population of 271 million dairy cows (University of Glasgow 2016). The US alone estimates that it loses US$2 billion to the disease, and the cost of subclinical mastitis exceeds $1 billion annually in the US dairy industry (Ott et al. 1999). According to a University of Montreal study, mastitis costs the Canadian dairy industry CA$400 million annually or about CA$500 - $1000 per cow annually.

Published estimates of the economic losses of clinical mastitis range from €61 to €97 per cow on a farm, with large differences between farms, e.g., in the Netherlands, losses due to clinical and subclinical mastitis varied between €17 and €198 per cow per year (Hogeveen et al. 2011). Ruegg (2011) estimated the overall product loss of subclinical mastitis is US$110 per cow annually. A case of subclinical mastitis may cause a production loss that ranges between 10% and 20% less milk over a lactation per cow and year (Ruegg 2011).

According to a more recent study, a case of clinical mastitis may lead to an economic loss that ranges between US$128 (€ 105) and US$444 (€ 360) (Rollin et al. 2015). Some other studies have estimated the cost of mastitis per case to be €146 in the USA (Bar et al. 2008), €519 in England

(Kossaibaiti et al. 1997), €275 in Sweden (Nielson et al. 2009), €205 in Netherland (Hujips et al. 2008), €347 in Denmark (Ostegaard et al. 2005) and €69 in the Czech Republic (Wolfova et al. 2006). In the Czech Republic, financial losses due to mastitis range from €43.63 to €84.84, while in the United States losses of €134 were reported (Petrovski *et al*., 2006; Manombe, 2014). In Nigeria, the cost of a case of clinical mastitis at a base risk incidence of 35.2% was ₦5,005.85 ($15.87). The costs increased by 7.5% in a herd with 10% higher milk yield and the cost was 1.64% higher in a herd with fifty per cent of its cows in first parity than a herd with fifty per cent of its cows in third parity (Moru et al. 2018).

A deterministic partial budget model estimated the direct and indirect costs per case of clinical mastitis occurring during the first 30 days of lactation by approximate cost to be 31% milk production losses, 24% veterinarian and drugs costs, 18% discarded milk, 4% excess labour demand and 23% premature culling (Rollin et al. 2015; Cha et al. 2011; Huijps et al. 2008).

In South Africa, in the year 2015, it is estimated that an annual milk loss of 46 190 L valued at ZAR 205 544.84 occurred in a particular herd where all the lactating cows have elevated somatic cell counts (SCC) (Petzer et al. 2016). A study has also shown an estimation of the total cost per mastitic cow per year in South African dairy farms to be on average ZAR 919.96 with an average incidence of 0.9 cases/cow/year (Manombe 2014).

The total value of annual milk losses due to mastitis in South Africa was estimated at ZAR 29.68 million on farms (Giesecke et al. 1971). More recent figures are not readily available, possibly owing to the observation that in most countries, including South Africa, clinical mastitis cases are not widely and routinely recorded (Man'ombe 2014). Available estimates of the incidence and prevalence of mastitis in the population are a prerequisite for the estimation of its real cost to the dairy industry (Petrovski et al. 2006).

2.4. Prevalence of organisms isolated from cases of bovine mastitis infections

Bacteria are the most frequently encountered pathogens among a wide variety of mastitis-causing microorganisms (Getaneh 2016). *Mycoplasma* species and bacteria such as *S. aureus*, non-aureus staphylococci (NAS) (formerly known as coagulase-negative staphylococci (CNS)), *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis*, environmental

streptococci, and *Enterobacteriaceae* like *Escherichia coli* (*E. coli)* and *Serratia* spp. are among pathogens increasingly implicated in causing mastitis (Tilahun and Aylate 2015; Radostits et al. 2007; Bradley 2002). Researchers across the world have reported a wide range of bacterial species isolated from cases of bovine mastitis as shown in Tables 2.2 and 2.3.

In Algeria, Saidi et al. (2013) reported that staphylococci or streptococci accounted for almost 60% of the bacterial isolates in subclinical mastitis. A survey in Netherlands also highlighted the importance of staphylococcal and streptococcal contagious mastitis (Miltenburg et al. 2006). In Sudan both *Staphylococcus* spp. (50%) and *Streptococcus* spp. (68.7%) were more commonly isolated than other bacteria (Bagadi 1970). In Egypt, it was found that the most frequently isolated major causative agents were *S. aureus*, *Str. agalactiae* and *E. coli* with prevalence of 52.5%, 31.25% and 16.25% respectively (Abdel-Rady and Sayed 2009). Although *S. aureus* and streptococci represent more than half (52%) of pathogens involved in subclinical mastitis, other bacterial species were also reported, but in smaller proportions (Saddek et al. 1996). Endale et al. (2016) reported 57.14% *Staphylococcus* spp. and 28.57% *Streptococcus* spp. in Ethiopia. Meanwhile a more recent study in Ethiopia has identified three major pathogenic bacteria, namely *S. aureus* (59.26%); *Str. agalactiae* (38.27%) and *E. coli* (2.47%) (Gemechu et al. 2019).

Petzer et al. (2009) reported that pathogens like αβ-haemolytic *S. aureus, Enterococcus canis* and *Str. agalactiae* were the causes of many outbreaks of mastitis in the test samples. They isolated NAS most frequently in the milk samples from both dry cows and lactating, followed by *Str. agalactiae and S. aureus*. In as much as *S. aureus* remained the major mastitis pathogen in South Africa, most mastitis cases were caused by NAS. This underscores the importance of NAS significantly, as this previously minor pathogen is becoming a major one. The trend of isolated mastitogenic pathogens is shifting. Frequency of isolation of *Str. agalactiae* reached its peak between the year 2000 and 2005 and decreased in 2007. NAS isolates were on the rise from 2002 and maintained the upward turn in 2007. Recent data as at 2020 (Table 2.4), also show that NAS is still on the increase. *Str. agalactiae*, *Str. uberis* and *Enterococcus canis* were recovered more frequently from lactating cows compared with dry cows, while dry cow samples yielded more *Enterococcus faecalis*.

Table 2.2: Bacterial spectrum in bovine mastitis

Area	Species	Prevalence	Year	Reference
Algeria	*Staphylococcus aureus*	40%	2012	Saidi et al. 2013
	Streptococcus spp.	12.50%		
	Enterobacteriaceae	2.50%		
	Pseudomonas spp.	2.50%		
	Staphylococcus aureus + *Streptococcus* spp.	12.50%		
	Streptococcus spp.+ *Escherichia coli*	7.50%		
	Staphylococcus aureus + *Mycoplasma* spp.	7.50%		
	Staphylococcus aureus +*Streptococcus* spp.+ *Escherichia coli*	5%		
Sudan	*Staphylococcus aureus*	11.50%	2003 - 2004	El-Zubeir and Ahmed 2007
	Streptococcus agalactiae	0.83%		
	Escherichia coli	7%		
	Klebsiella pneumoniae	9.40%		
	Enterococcus faecium	2.50%		
	Pseudomonas aeruginosa	9.50%		

Among studies that have recorded incidence of staphylococcal mastitis with a frequency of approximately 40% are those done in Algeria (Heleili et al. 2012), Jordan (Lafi et al. 1994) and Italy (Moroni et al. 2006). Findings in Ontario, Canada, recorded isolation of *S. aureus* (40%) (Sargeant et al. 1998) while in Jordan, *Staphylococcus* spp. (30%) were the most common organisms isolated from clinical and subclinical cases (Lafi et al. 1994). Some studies reported *Staphylococcus* spp. to be 39.0% in France (Bouchot et al. 1985), 44.7% in Jordan (Azmi et al. 2008), and 29.0% in India (Harini and Sumathi 2011; Ben Hassen et al. 2003; Fallet 1999).

Laboratory results in Nigeria showed that *S. aureus* and NAS were implicated as causes of mastitis in a study where 56.7% were caused by *S. aureus* and NAS caused 12.3% of the cases examined (Umaru et al. 2017). This indicates that 69.0% of mastitis cases were caused by staphylococci, while other pathogens and agents represent the remaining 31.0% (Umaru et al. 2017). Ameh et al. (1999) also showed that NAS and *S. aureus* occurred predominantly in both clinical and sub-clinical bovine mastitis. Similarly, Mekibib et al. (2010) revealed that *S. aureus* and NAS were the

Table 2.3: **Distribution of mastitis pathogens in 38 852 milk samples from 22 587 cows with clinical and subclinical mastitis from mastitis cases in Poland (Krukowski et al. 2020)**

Group of microorganisms	Frequency	Percentage of samples (%)
Gram-positive catalase-negative cocci	**12 491**	**32.15**
Streptococcus agalactiae	1 017	2.61
Other*	11 474	29.53
***Staphylococcus* spp.**	**9 564**	**24.61**
Staphylococcus aureus	2 360	6.07
Non-aureus staphylococci (NAS)	7 204	18.54
Gram-negative bacteria	**2 485**	**6.39**
*Enterobacteriaceae***	1 703	4.38
Gram-negative non-fermentative bacilli	782	2.01
Coryneform bacteria and *Listeria monocytogenes*	**774**	**1.99**
Gram-positive bacilli	**249**	**0.64**
Yeasts	**394**	**1.01**
***Prototheca* spp.**	**483**	**1.24**
Culture-negative	**8 993**	**23.14**
Contamination	**3 419**	**8.8**
Total	**38 852**	**100**

*When identified to the species level 5751 isolates (14.8%) were *Str. uberis*, 2758 (7.1%) *Str. dysgalactiae* and 1864 (4.8%) *Str.* spp. isolates. *Enterococcus* spp. were isolated from 1101 (2.8%) samples.

** Enterobacteriaceae isolates identified to the species/genus level included – *E. coli* (1074; 2.7%), *Klebsiella* spp. (194; 0.5%), *Serratia* spp. (155; 0.4%), *Enterobacter* spp. (117; 0.3%), *Proteus* spp. (133; 0.3%) and *Citrobacter* spp. (30; 0.007%).

predominant bacteria isolated in mastitic milk in Ethiopia with 47.1% and 30.1% prevalence rates, respectively. Interestingly, a report by Suleiman et al. (2013) showed the prevalence of *S. aureus* in bovine mastitic milk was up to 98.1% in Plateau State, Nigeria while an earlier study in Egypt showed that 29.1% of mastitis was caused by *S. aureus* (Saddek et al. 1996).

In South Africa, Schmidt et al. (2015) isolated 146 *S. aureus* and 102 NAS from milk samples. They reported that 12 (15.2%) samples from human origin yielded *S. aureus* and 95 NAS. The

NAS of bovine origin were predominantly *S. chromogenes* (78.4%), and other seven *Staphylococcus* spp., while S. *epidermidis* (80%) dominated NAS species from human origin, followed by *S. chromogenes* (6.3%).

Among the contagious organisms, *S. aureus* is reported to be the most predominant cause of subclinical mastitis and is considered to be responsible for chronic and persistent infections (Gomes and Henriques 2016; Bradley 2002). Therefore, *S. aureus* remains of concern as it has developed mechanisms that allow it to survive within the host and avoid removal by regular milking, and to evade the immune system in order to cause persistent infection in mammary glands

Table 2.4: Prevalence of bacteria isolated from milk samples in the Onderstepoort milk laboratory*

Bacteria spectrum of the isolates					
Bacteria Isolated		**2008 - 2015**		**2018 - 2020**	
		Number of bacteria isolates	**% of bacteria isolated**	**Number of bacteria isolates**	**% of bacteria isolated**
Major Gram-positive bacteria	*Staphylococcus aureus* (STA)	9550	6.39	4092	4.48
	Staphylococcus aureus (STH)	3972	2.66	59	0.06
	Staphylococcus aureus (STI)	1458	0.98	1015	1.61
	Streptococcus agalactiae	4759	3.18	1340	1.47
	Streptococcus uberis	9173	6.14	7338	8.03
	Streptococcus dysgalactiae	3159	2.11	1196	1.31
	Total	**32071**	**21.46**	**15040**	**16.96**
Non-aureus staphylococcus	**Total**	**111461**	**74.59**	**59274**	**76.5**
Major Gram Negatives	*Escherichia coli*	341	0.23	231	0.25
	Klebsiella spp.	282	0.19	90	0.1
	Serratia spp.	240	0.16	41	0.04
	Total	**863**	**0.58**	**362**	**0.4**

*data provided by the Onderstepoort milk laboratory

and be prevalent in dairy herds (Bradley 2002). Most contagious mastitis pathogens are transmitted by fomites from infected cows to non-infected herd mates during the milking process. Milkers' hands, milking units and udder wash cloths are also implicated as primary fomites (Fox and Gay 1993).

The prevalence of *S. aureus* has been reported to vary with the type of sample and geographic location of the area sampled. Another study by Ateba *et al.* (2010) in South Africa reported a high prevalence of *S. aureus* in milk from communal farms compared to commercial farms. The high rate of isolation of *S. aureus* may be attributed to the fact that the principal reservoirs of *S. aureus* are the skin of the udder and milk of the infected gland. In addition, *S. aureus* has the capacity to penetrate the tissue, producing deep-seated foci protected by a tissue barrier (Ranjan et al. 2010). The high frequency of staphylococcal mastitis is considered to be due to the existence of inadequate hygiene in the dairy industry, poor animal health services and lack of proper attention to the health of the mammary gland in general (Saidi et al. 2013).

The prevalence of NAS as etiological agents of mastitis is higher in primiparous than in older cows (Krukowski et al. 2020). The occurrence of different NAS species isolated from cases of bovine mastitis varies in studies conducted in different nations of the world (Table 2.5).

Table 2.5: Predominance of NAS species in some countries

Predominant NAS species Isolated in studies			
Countries	**NAS species**	**Frequency (%)**	**Reference**
South Africa	*S. chromogenes*	78.4	Schmidt et al. 2015
Sweden	*S. chromogenes*	24	Persson-Waller et al. 2011
	S. epidermidis	22	
Switzerland	*S. xylosus*	36	Frey et al. 2013
	S. chromogenes	16.8	
United States	*S. chromogenes*	72.2	Park et al. 2011
	S. xylosus	9.1	
	S. haemolyticus	6.1	

NAS= Non-aureus staphylococci

In general, the most commonly isolated species of NAS are *S. chromogenes, S. xylosus, S. epidermidis, S. haemolyticus,* and *S. simulans* (Vanderhaeghen et al. 2015). It has been proposed

that the spread of mastitogenic NAS species is herd-specific and may be subject to particular management practices that may differ between countries (Supré et al. 2011; Thorberg et al. 2009). Epidemiological studies done at the species-level in some European dairy herds have revealed that *S. chromogenes* is a more specific udder pathogen than the other NAS species and it usually causes chronic bovine mastitis (Supré et al. 2011).

2.5. Antibacterial susceptibility profile of *Staphylococcus aureus* and non-aureus staphylococcal isolates in bovine mastitis

Antimicrobial agents have been widely used for treatment of mastitis pathogens in veterinary medicine with limited success because of the increasing prevalence of resistance to commonly used antimicrobial agents (Du Preez 2000; Craven 1987). The commonly used antibiotic classes for treatment of mastitis caused by *S. aureus* include β-lactams (e.g., penicillin G), macrolides (e.g. erythromycin) and lincosamides (e.g. pirlimycin) (Kasravi et al. 2011; Barkema et al. 2006; Du Preez 2000). However, mastitis caused by *S. aureus* is mostly difficult to treat as this bacterial strain frequently results in deep-seated abscesses that allow it to survive in intracellular compartments after phagocytosis, where antibiotic concentrations are lower. *S. aureus* has been reported to be resistant to most antibiotics used in mastitis treatment (mostly β-lactams) and it can release toxins that impair the udder's natural defenses (Gruet et al. 2001). Resistance of *S. aureus* has led to further overuse of antimicrobials which has resulted in increasing amounts of drug residues in milk, affecting its quality and additionally posing a public health concern as it increases risk of antibiotic resistant strains of bacteria entering the food chain (Gomes and Henriques 2016; Khodaei Motlagh et al. 2014; Dhanabalan et al. 2008).

In her work in 2011, Schmidt indicated a remarkably high level of resistance to the beta-lactam antibiotics: 47.8% of the isolates showed resistance to penicillin while 65.6% were resistant to ampicillin. Minimal resistance to oxacillin, cephalothin and trimethoprim-sulfamethoxazole (1.1 %) was found. Seventeen (18.9%) of the isolates tested were found to be resistant to three or more antimicrobials. The need for vigilant monitoring of bacterial resistance trends in the dairy industry is warranted as the potential public health implications are significant.

In work done by Schmidt et al. (2015), they reported 67.1% of *S. aureus* isolates of bovine origin were susceptible to all the antimicrobials used in the assay; 26.7% of the isolates showed resistance to one class of antimicrobial, and 4.8% of the isolates showed resistance to two classes of antibiotics. Only 1.4% of the *S. aureus* isolates demonstrated multidrug resistance. Both *S. aureus* isolates of bovine (28.8%) and human (75%) origins showed resistance to penicillin. A similar pattern was observed with the NAS, 89.5% of NAS isolates of human origin and 37.3% of that of the bovine origins demonstrated resistance to penicillin. The study found 39% of the NAS isolates of human origin were resistant to three or more classes of antibacterial. In the end, they concluded that the results suggest that the antimicrobial resistance among *Staphylococcus* spp. causing bovine mastitis in South Africa is not common and should not be a cause for concern (Schmidt et al. 2015). Meanwhile, they opined that the antimicrobial resistance which was frequently observed in *Staphylococcus* spp. of human origin represent a possible reservoir of resistance genes. They therefore suggested that the *Staphylococcu*s spp. isolated from cases of mastitis should be continuously monitored for changes in the pattern of antimicrobial susceptibility to different classes of antibacterial. Table 2.6 shows more recent data of the prevalence of multidrug resistant bacteria isolated from milk samples in the University of Pretoria milk laboratory.

Table 2.6: 2020 prevalence of multidrug resistant bacteria isolated from cases of bovine mastitis at Onderstepoort milk laboratory

Prevalence of multidrug resistant bacteria isolated in the milk lab	
Bacteria	**% Multidrug resistance (n)**
Staphylococcus aureus	5% (189)
Non-aureus staphylococci	2% (216)
Streptococcus agalactiae	3% (36)
Streptococcus dysgalactiae	3% (122)
Streptococcus uberis	11% (97)
Gram negative bacteria	50% (8)

Reports of studies on the antimicrobial resistance of NAS to some antibiotics commonly used in mastitis around the world are shown in Table 2.7.

Table 2.7: Prevalence of mastitis NAS antimicrobial resistance in some studies across the world

Antibiotics	Countries	Prevalence %	Reference
Penicillin	South Africa	63	Phophi et al. 2019
	Finland	32	Simojoki et al 2012
	Estonia	38.5	Pitkälä et al. 2007
	Zimbabwe	8	Kudinha and Simango 2012
Erythromycin	South Africa	49	Phophi et al. 2019
	Argentina	29	Raspanti et al. 2016
	Germany	22	Lüthje et al. 2006
	Turkey	73.2	Kenar et al. 2012
Vancomycin	South Africa	9	Phophi et al. 2019
	Turkey	58.2	Kenar et al. 2012
	Sweden	0	Bengtsson et al. 2009
Cefoxitin	South Africa	9	Phophi et al. 2019
	Tunisia	29.41	Klibi et al. 2018
	Switzerland	40	Sakwinska et al. 2011
β-lactam	South Africa	63	Phophi et al. 2019
	Argentina	100	Gentilini et al. 2010
	Finland	23	Taponen et al. 2017

In more recent research reported in South Africa by Phophi et al. (2019), 90% of NAS strains isolated from mastitic milk exhibited resistance to at least one of the antibacterial agents, namely erythromycin (64%). Consequently, Phophi et al. (2019) concluded that the majority of NAS in their study were resistant to penicillin, and the majority of the isolated NAS were β-lactam resistant and 51% of the isolates were reported to be multidrug-resistant (MDR). NAS were most resistant against ampicillin (90%) and penicillin (89%), while a few of the isolates demonstrated resistance to vancomycin and cefoxitin (9% in both cases). They similarly reported that MDR-*S. epidermidis*

(65%), MDR-*S. chromogenes* (52%) and MDR-*S. haemolyticus* (44%) mainly exhibited resistance to penicillin. The most frequent pattern of resistance observed was resistance to combinations of penicillin-ampicillin (16%) as well as penicillin-ampicillin-erythromycin (10%). A similar study in Sweden recorded 68.6% AMR resistance in clinical mastitis cases of lactating cows (Bengtsson et al. 2009). More than half (51%) of NAS were MDR, higher than 45% reported in clinical mastitis cases in India (Mahato et al. 2017). Multidrug resistant NAS were mainly resistant to penicillin (88%), ampicillin (85%) and lactam antibiotics and were MDR. The study highlighted the presence of AMR resistance of clinical concern, and urgent action is needed to address the situation.

Long term usage of antibiotics leads to the emergence of MDR strains. For example, introduction of β-lactamase-stable AMR drugs into clinical use has led to the emergence of methicillin-resistant *S. aureus* (MRSA) strains. MRSA is a zoonotic nosocomial pathogen that has been reported among veterinarians, farmers, milkers, and persons working at slaughterhouses, with evidence of cross transmission (Juhász-Kaszanyitzky et al. 2007). High rates (10-30%) of treatment failure have been reported in mastitis cases associated with *S. aureus* infection (Gomes and Henriques, 2016). A matter of concern in utilization of antibiotics is the probable influence of reverse zoonosis that may have had a major impact in South Africa due to many milkers suffering from compromised immunity due to bovine mastitis pathogens (Petzer et al. 2009). Therefore, an alternative to antibiotic treatment should be considered (Gomes and Henriques 2016).

2.6. Antibiotic resistant mastitis staphylococcal isolates and biofilm formation

Antibiofilm formation is another approach to investigating antimicrobial activity. Biofilm formation is a mechanism whereby cells group together to communicate and overcome the effects of antimicrobial drugs (Van Vuuren and Holl 2017). A biofilm is a population of cells growing on a surface and enclosed in an exopolysaccharide matrix. Biofilms are notoriously difficult to eradicate and are a source of many recalcitrant infections (Lewis 2001). Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species, as the protected environment of the biofilm enables them to interact and become increasingly resistant to antimicrobials (Jefferson 2004).

Microbial biofilms have been used by microorganisms for their survival and improved virulence. Their incidence makes it difficult to treat common infections and the possibility of recurrent infections is high. As a result of emerging resistance, conventional control methods are fast becoming ineffective (Erhabor et al. 2019), therefore, there is a need to highlight the role of biofilm formation in antimicrobial resistance.

Studies have shown that mastitic staphylococcal pathogens form biofilms. However, Phophi et al. (2019) concluded that biofilm-forming NAS isolates were not common according to their observation, and there was no significantly different incidence of MDR-NAS based on their biofilm-forming ability. They reported that 18% of the isolated mastitis NAS were biofilm-forming. Researchers such as Tremblay et al. (2012) in Canada reported 96.7%, Srednik et al. (2017) in Argentina reported 85.1% and Simojoki et al. (2012) in Finland reported 31.3% proportion of biofilm formation among NAS from dairy cattle with mastitis which is a concerning trend. Recent studies also revealed both *S. aureus* and NAS isolates of bovine mastitis that are biofilm-forming to be on the high side, as 100% *S. aureus* isolates (Zaatout et al. 2020), 83.5% NAS (Turchi et al. 2020) and 86% *S. aureus* isolates by YeHui et al. (2019) were recorded as being able to form biofilms.

Horiuk et al. (2019) reported that, on average, *S. aureus* formed biofilms 1.5 times more than *Str. agalactiae* and *Str. dysgalactiae* strains. They also noted that *S. aureus* strains isolated from cows with the subclinical form of mastitis formed biofilms 2.0 times more often than the *S. aureus* strains isolated during the clinical form of mastitis. Also, the study discovered that bacterial cells in the biofilm are more resistant to antibacterial drugs, contrary to Phophi et al. (2019), the only available work in South Africa that compared antimicrobial resistance patterns and biofilm formation in subclinical mastitis in dairy cattle. Phophi et al. (2019) concluded that there was no significant association between biofilm formation and multidrug resistance, so further research is needed in this area.

Available human medicine study reported a high prevalence of multidrug resistance in biofilm positive NAS compared to biofilm negative NAS (Shrestha et al. 2017). Also, another human study in Korea revealed that multidrug resistance among *S. aureus* from human clinical isolates was more common in biofilm-forming isolates (Kwon et al. 2008). In fact, de Oliveira et al. (2016)

reported that human bacterial isolates grown as biofilms are up to 1000 times more resistant compared to the planktonic bacteria.

2.7. South African medicinal plants and potential antibiofilm activity

Plants produce a great diversity of substances that could be useful in many fields of medicine. Approximately 3 000 species of southern African plants are therapeutically used for disease treatment (Van Wyk et al. 1997). Worldwide, several studies have been done on plants that are used in ethnoveterinary medicine for various diseases (Okem et al. 2012; Maphosa and Masika 2010; Dhanabalan et al. 2008). Some plant extracts distinguish themselves by their inhibitory power on bacteria and fungi (Kuete 2010).

Several studies (e.g. Famuyide et al. 2019; Erhabor et al. 2019), used an anti-biofilm approach to investigate the antimicrobial activity of southern African medicinal plants, but only one could be identified as using bacterial isolates of bovine mastitis (Sserunkuma et al. 2017). A study of the stem bark of *Sclerocarya birrea* using methanol as the extractant, showed that it disrupted the quorum sensing mediated production of biofilm formation and inhibited the swarming ability of *Pseudomonas aeruginosa*, thus providing some evidence for the use of this plant in the traditional setting for the management of dysentery, diarrhoea and various other infectious diseases (Sarkar et al. 2014).

Antimicrobial efficacies of medicinal plants have largely focused on planktonic bacterial growth. However, infectious bacterial populations sometimes occur as biofilms (Van Vuuren and Holl 2017). Studies undertaken on South African medicinal plants inhibiting biofilm formation have been sorely neglected. Only a handful of relevant studies have been done (Van Vuuren and Holl 2017). Some biofilm studies have investigated the effects against *Listeria monocytogenes* (Nyila et al. 2012), *Pseudomonas aeruginosa* and *Candida albicans* (Sandasi et al. 2011), as well as *Str. mutans* (Naidoo *et al*., 2012). Biofilms are known to form with motile strains and thus future medicinal plant studies should incorporate biofilm aspects on such strains as well as other species, including mixed cultures (Van Vuuren and Holl 2017).

In a recent review seeking to summarize present knowledge on South African medicinal plants with antibiofilm and quorum quenching potential, Erhabor et al. (2019) highlighted the potential of plants native to South Africa as sources of antibiofilm and quorum quenching bioactive

secondary metabolites and the role of quorum sensing and biofilm formation in antimicrobial resistance. Forty-six species of plants from 27 families as well as 31 isolated bioactive compounds were documented to have antibiofilm and quorum quenching capacity against foodborne pathogens. The review also provided support for continuing investigations on South African medicinal plants with previously reported good to excellent antimicrobial activities.

Other recent studies published in 2019 by Shirinda et al., Kharsanya et al., and Khunoana et al. have all shown very good to excellent results in the anti-biofilm and quorum quenching abilities of South African plants.

2.8. Research motivation

In the review by Van Vuuren (2008), it was recommended that when investigating antimicrobial activity, more specific attention should be given to the relevant pathogen in question. For example, if a medicinal plant is used to treat respiratory infections, the relevant respiratory pathogens should be tested. As such, a more targeted approach has arisen with better relevance to the traditional use (Van Vuuren and Holl 2017). A few different approaches have been undertaken to study the antimicrobial properties of medicinal plants. Usually, the plant is selected ethnobotanically and testing follows based on the traditional use with respect to the relevant pathogens (Van Vuuren and Holl 2017). In bovine mastitis, therapies such as ethnoveterinary medicine are worthy of further study since bacteria isolated from bovine mastitis cases are increasing in frequency and are becoming increasingly resistant to conventional antibiotic therapy. While many researchers use the validation of emerging antimicrobial resistance to justify their reasoning to undertake screening studies on medicinal plants, very few studies follow through with investigating activity against the resistant strains (Van Vuuren and Holl 2017). Nielsen et al. (2012) carried out a study on 12 South African plants on a wide but non-specific array of pathogens including resistant strains. Other studies that have included resistant strains include Heyman et al. (2009), Bisi-Johnson et al. (2011), Njume et al. (2011b), Mabona et al. (2013), Khan et al. (2014) and Nciki et al. (2016). It is well known that globally the problem of antimicrobial resistance has led to the urgent need to find newer antibiotics with more potent effects (Hwang and Gums 2016) and natural product research has become a source for investigating novel antimicrobials with high anti-infective potential (Van Vuuren and Holl 2017).

One aspect of natural product antimicrobial analysis that has been lacking is the reporting of the minimum bactericidal or fungicidal concentration (MBC or MFC). This is a simple addition to the MIC assay but yields information that demonstrates the killing effect rather than just the inhibitory effects. This information is becoming more important for the future as the impact of cidal over inhibitory activity reduces the possibility of antimicrobial resistance (Van Vuuren and Holl 2017). Suleman et al. (2015) and Kharsany et al. (2019) are examples of such studies that have demonstrated the killing effect of South African propolis, one of the most potent natural products studied to date, and MBCs were reported for all samples. Another study to report cidal activity of South African plant extracts is that by Okem et al. (2012).

2.9 Botanical descriptions and biological activity of selected plant species

The selection of plants for the current study was based on their known antimicrobial activity, chemotaxonomic relationships to plant species with antibacterial activity, availability, and/or the existence of traditional uses against infectious diseases.

2.9.1 *Antidesma venosum* E. Mey. ex. Tul.

Family: Phyllanthaceae. The common names include Tasselberry (English), Voëlsitboom, Tosselbessie (Afrikaan), isiBangamlotha, Isiqutwane, Umhlabahlungulu, Umhlalanyoni (isiZulu); modulane (Northern Sotho), Kgôbê-tsabadisana, Moingwe, Segagama (Tshwane), Mufhala-khwali (Venda), Umtyongi (isiXhosa). It is native to the Eastern Cape, KwaZulu-Natal, Limpopo, and Mpumalanga provinces in South Africa.

Antidesma venosum is an evergreen to semi-deciduous, perennial tree or shrub from 0.55 m up to 15 m tall, with a dense, roundish crown. Old stems are buff-grey in shade and pale grey in sunny habitats. Middle age branchlets are glabrous and brown to grey-brown with scattered pale grey lenticels. New twigs are very hairy, green becoming pale brown with age. The bark is channeled longitudinally, soft and very hairy, scattered with pale grey lenticels. Leaves are simple, alternate, spirally arranged, with rounded or bluntly pointed tips, about 150 mm long and 70 mm broad. The leaf blade is leathery, dark glossy green, and the lower surface is covered with rusty hairs and has

prominent venation looping along the margin. The leaf is paler green below with a raised midrib and smooth margins. The leaf stalk is hairy, and up to 6 mm long (Mnxati 2014).

Figure 2.1: *Antidesma venosum* (Photograph credit: Ayodele Akinboye (2020); @ Manie van der Schijff Botanical Garden, University of Pretoria)

Different plant parts are used medicinally; a root extract is used for the treatment of heart diseases; an infusion of roots and leaves is taken for the treatment of coughs; a leaf infusion is taken for an upset stomach (Mnxati 2014). According to the available ethnobotanical information, it is used against many diseases by the healers in the city of Lubumbashi, Democratic Republic of Congo (Shengo et al. 2013). Phytochemical screening revealed the presence of terpenoids, tannins and steroids in both the root and stem bark extracts. The results of a study into the antibacterial efficacy of the root and stem extracts showed activity against Gram-positive bacteria, thus supporting the popular use of this plant for the treatment of conditions associated with bacterial infections such as cut wounds, chest infections and some types of diarrhoea (Mwangomo et al. 2012). Both methanol and ethanol extracts of *A. venosum* leaves had antibacterial activity against *S. aureus*, *E. coli, Proteus vulgaris, Salmonella typhi, Streptococcus lactis* and *Shigella* sp. (Adegoke et al. 2013). However, the plant also caused *in vitro* mutagenicity or DNA damage (Elgorashi et al. 2002).

2.9.2 *Erythrina caffra* Thunb.

Family: Fabaceae. Common names are coast coral tree (English), kuskoraalboom (Afrikaans), umsinsi (Zulu) and umsintsi (Xhosa).

Erythrina caffra, the coast coral tree or African coral tree, is a tree native to southeastern Africa. The coast coral tree is an ideal garden plant, and because of its unique appearance, it has continued to attract the attention of botanists, horticulturists, nature lovers, and the general gardening public for many decades (Kirstenbosch 2006). The plant is an important medicinal plant native to South Africa. In 2014, Desta and Majinda isolated and identified a total of 13 compounds including 3 new isoflavonoids from the stem bark of *E. caffra*. Some isolated flavonoids from the stem bark were found to be active against human cervix carcinoma KB-3-1 cells with IC_{50} values in the micromolar range, while some showed weak to moderate antibacterial activity against some organisms (Desta et al. 2016).

Figure 2.2: *Erythrina caffra* (Photograph credit: Ayodele Akinboye (2020), @ Manie van der Schijff Botanical Garden, University of Pretoria))

The stem bark when extracted with hexane yielded two known compounds, erythrinasinate B and lupeol, which demonstrated moderate antiplasmodial activity (Chukwujekwu et al. 2016). The

stem bark acetone extract had good antimicrobial activity against some bacterial and fungal isolates with the increases in the inhibition zones in the disc diffusion assay being concentration dependent (Olajuyigbe and Afolabi 2012). Since there has been little work done on other plant parts, this work sought to examine the antimicrobial action of the leaves, which are more renewable resources than the stem bark.

2.9.3 *Pleurostylia capensis* Turcz. (Loes)

Family: Celastraceae. *Pleurostylia capensis* is commonly known as coffee-pear (English), koffiepeer (Afrikaans) and murumelela (Tshivenda) (Mabogo 1990). Its habitat is mostly along rivers and streams, and in coastal and mountain forests. It is native to the Eastern Cape, KwaZulu-Natal, Limpopo and Western Cape provinces, and can also be found in Swaziland (Schmidt and Lotter 2002). *Pleurostylia capensis* was described by Retief and Herman (1997) as a large tree that is distributed commonly in scrub, wooded ravines, along rivers and streams, and in coastal and mountain areas.

The bark is greyish-brown in colour and the leaves are shiny dark green to fresh green above, with some being paler green below. It can grow to heights of about 20 m, although it can sometimes be found as a shrub in forest. It was previously used in wagon construction (Retief and Herman 1997).

It is used medicinally for the treatment of various diseases such as colic pains in babies, epilepsy, as well as mental illness. Its bark is used for cosmetics, in steam baths and ritual body washes and as a purgative to treat symptoms of witchcraft, whereas the stem is used together with the roots or the bark in a powdered form mixed together with other semi-parasitic plants and other ingredients of either plant or animal origin to make magical mixtures (Michelle and Dold 2012; Seqaws and Kasenene 2007; Reid et al. 2006; Mabogo 1990; De Jager 1963). When extracts were evaluated for antimicrobial activity against Gram-positive bacteria such as *S. aureus*, *Bacillus cereus*, and *Mycobacterium smegmatis*, and Gram-negative bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*, and the yeast *Candida albicans*, moderate to potent inhibitory activity was observed in all extracts (Razwinani et al. 2014).

Figure 2.3: *Pleurostylia capensis* (Photograph credit: Friends of Van Staden., 2017: http://www.vanstadens.co.za/Photo/Trees?page=2)

2.9.4 *Elaeodendron croceum* (Thunb.) DC.

Elaeodendron croceum is a shrub or a small evergreen tree species which is a member of the Celastraceae family. The common names of the species include forest saffron, saffron or wood saffron. The plant is distributed in the Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape in South Africa.

In its ideal habitat *E. croceum* occurs on the margins of coastal and other moist inland forests. In wet conditions the outer portion of the bark breaks off more frequently, exposing the characteristic yellow pigments that the species shares with other Celastraceae such as *Cassine peragua* L. and *Pterocelastrus echinatus* N.E.Br. The wood is of fine quality and is used for fine furniture. It is rare to find it in trade, but the preparations of the root and bark have proven to be fatal to humans and the leaves toxic to rabbits, presumably due to the presence of alkaloids of which little is known (Kunene et al. 2003; Palmer and Pitman 1972; Watt and Breyer-Brandwijk 1962). The bark is used in dyeing and tanning (Watt and Breyer-Brandwijk 1962).

Figure 2.4: *Elaeodendron croceum* (Photography credit: H. Robertson., 2016. Cape Town: http://pza.sanbi.org)

Elaeodendron croceum is reported to have been used as an emetic and purgative (van Wyk et al. 2013; Long 2005), and as herbal medicine against human immunodeficiency virus (HIV) opportunistic infections (Mamba et al. 2016). The bark is also traditionally used to treat and manage tuberculosis and other related diseases such as blood in sputum, chest congestion, cough and sore throat (van Wyk et al. 2013; Lall and Meyer 1999). The bark (used for the treatment fever (Lall and Meyer 1999), roots and leaves of the species are employed as herbal medicine to clean the digestive tract (van Wyk et al. 2013; Schmidt et al. 2002).

Phytochemical compounds identified from the species include alkaloids, cardiac glycosides, flavonoids, phenols, proanthocyanidins, saponins, sugars, tannins and triterpenoids (Odeyemi and Afolabi 2017; Elisha et al. 2016). Pharmacological studies revealed that *E. croceum* extracts and compounds have antimycobacterial (Elisha et al. 2017a; Lall and Meyer 1999), anti-arthritic (Elisha et al. 2016), antibacterial (Kaikabo 2008; Eloff 2000), anti-HIV (Elisha et al. 2016; Mamba et al. 2016), antioxidant (Prinsloo et al. 2011), anti-inflammatory (Prinsloo et al. 2011) and antifungal activity (Mamba et al. 2016), but cytotoxicity (Elisha et al. 2016; Prinsloo et al. 2010) was also noted. Elisha et al. (2017b) reported that the acetone extract of *E. croceum* leaves showed cytotoxic activities with LC_{50} value of 5.2 µg/ml and selectivity index (SI) value ranging from 0.01 to 0.07 while doxorubicin, the positive control, had LC_{50} of 8.3 µg/ml. They therefore

concluded that the extract exhibited high cytotoxicity (Elisha et al. 2017b; Yelani et al. 2010). Future studies should therefore focus on ethnopharmacological safety and efficacy of *E. croceum* extracts and compounds isolated from the species using in vivo preclinical studies and clinical trials (Maroyi 2019).

2.9.5 *Indigofera frutescens* (L.f.) Kuntze

Indigofera frutescens belongs to the family Leguminosae, and its common names include River Indigo (English) and Rivierverfbos (Afrikaans) (Random Harvest 2021). It is a hardy, semi-deciduous, graceful, small tree with delicate compound leaves and attractive bark. It blooms at a very young age - from 2 to 3 years old (Random Harvest 2021). Elegant, upright sprays of showy light to dark pink flower spikes adorn this little tree for an exceptionally long time in summer from October to March (Random Harvest 2021). This pretty tree reaches a size of 1 to 4 m with a 2 – 3 m spread, and the tree is found mostly in the Eastern Cape and Western Cape (The Plant Library 2021). it grows in savanna-bushveld and forest, as well as rocky slopes in Eastern Cape, Gauteng and KwaZulu-Natal (The Plant Library 2021).

Figure 2.5: *Indigofera frutescens* (Photograph credit: Ayodele Akinboye (2020), @ Manie van der Schijff Botanical Garden, University of Pretoria)

Indigofera frutescens has been reported to be used traditionally in the treatment of helminth infections (Hutchings et al. 1996; Watt and Breyer-Brandwijk 1962). In one study, it was reported that the acetone extract showed antibacterial activity with MIC = 0.31, 0.16, 0.16 and 0.08 mg/ml against *P. aeruginosa*, *S. aureus*, *E. coli* and, *E. faecalis*, respectively (Adamu et al. 2014). The study also reported its antioxidant activity (Trolox equivalent antioxidant concentration of 0.5 µg/ml), cytotoxicity (0.052 mg/ml), and selectivity index values of 0.33, 0.33, 0.65, 0.17 against *S. aureus, E. coli, E. faecalis* and *P. aeruginosa*, respectively (Adamu et al. 2014).

2.9.6 *Searsia lancea* (L.f.) F.A. Barkley

Searsia lancea (previously *Rhus lancea)* belongs to the family Anacardiaceae and has the common names: karee (English); karee, rooikaree (Afrikaan); mokalabata, monhlohlo, motshakhutshakhu (Northern Sotho); inhlangutshane (Siswati); mosinabele, mosilabele (Southern Sotho); mosabele, mosilabele (Tswana); mushakaladza (Venda); umhlakotshane (Xhosa). The family Anacardiaceae is the fourth largest tree family in southern Africa and many species are used for medicinal purposes, for food (fruits) or as building material (wood) (van Wyk and van Wyk 1997).

Figure 2.6: *Searsia lancea* (Photograph credit: M. Ritter and DeLorenzo Inc. SelecTree. "Searsia lancea Tree Record." 1995-2021. Jan 26, 2021.

S. lancea is an evergreen, drought tolerant tree, most common in the Midlands of Zimbabwe and is also found in most areas of the southern African region. The leaves of *S. lancea* are used as a valuable fodder for livestock and are believed to taint the flavor of milk if eaten in large quantities by dairy cattle (Venter and Venter, 1996). The organic extracts of *S. lancea* leaves exhibited promising antibacterial activity with MIC values < 0.05 mg/ml (Mulaudzi et al. 2012). A study done to determine the major phytoconstituents as well as the antioxidant and the antimicrobial activities of *S. lancea* essential oil against eight bacterial and four fungal species, led to the discovery of three major phytoconstituents, namely pinene, benzene and delta-3-carene. The oil exhibited remarkable antioxidant as well as dose dependent antibacterial and antifungal activities with highest activities against *E. coli* (19.2 mm zone of inhibition when 100 µg/ml of oil was used compared to 20.7 mm for the positive control), *Clostridium perfringens* (15.0 mm) and *Aspergillus flavus* (74.2 mm zone of inhibition when 100 µg/ml of oil was used compared to 76.2 mm for the positive control) (Gundidza et al. 2008).

2.9.7 *Trichilia emetica* Vahl.

Trichilia emetica belongs to the family Meliacae. Common names include Natal mahogany, rooiessenhout (Afrikaans), mamba (Northern Sotho), umathunzini (isiZulu), umkuhlu (SiSwati), umkhuhlu (isiXhosa), nkulu (Xitsonga) and mutuhu (TshiVenda). It is native to Africa and is a tree or bush with many different traditional uses.

It is used frequently in traditional setting to treat various ailments such as abdominal pains, dermatitis, haemorrhoids, jaundice and chest pain (Watt and Breyer-Brandwijk 1962). It is used for its emetic, diuretic and purgative properties and for induction of labour (Watt and Breyer-Brandwijk 1962). The extensive traditional use of this species has encouraged scientists to explore several biological activities including anti-infective (Shai et al. 2008; Germano et al. 2005), anti-inflammatory (McGaw et al. 1997), antischistosomal (Sparg et al. 2000), antiplasmodial (El Tahir et al. 1999), anticonvulsant (Bah et al. 2007), antitrypanosomal (Hoet et al. 2004), antioxidant (Frum and Viljoen 2006; Germano et al. 2006), antitussive, anticancer (Traore et al. 2007) and hepatoprotective properties (Germano et al. 2005). Several limonoids have been isolated from the stem bark. Trichilia substance Tr-B and nymania-1 exhibited selective inhibitory activity towards

DNA repair-deficient yeast mutants and exerted antifungal, bactericidal, antiviral, antifeedant and growth regulating properties (Komane et al. 2011).

Figure 2.7: *Trichilia emetica* (Photograph credit: Ayodele Akinboye (2020), @ Manie van der Schijff Botanical Garden, University of Pretoria)

Polysaccharides have been isolated and the pectin rhamnogalacturonan type I with side chains of arabinogalactan type II exhibited fixation ability suggesting the possible mode of action as a wound healing remedy (Komane et al. 2011). Germano et al. (2006) have also reported the antioxidant activity of phenolic acids extracted from the root of *Trichilia emetica*.

2.9.8 *Searsia leptodictya* (Diels) T.S.Yi, A.J. Mill & J.Wen

Searsia leptodictya (previously known as *Rhus leptodictya*) is a member of the family Anacardiaceae and its common names include mountain karee and rock karee in English; bergkaree and klipkaree in Afrikaans, Mohlwehlwe in Sotho and Inhlangushane in Siswati. It is a large shrub or tree native to South Africa. The leaves are divided into three leaflets which are bright green with toothed margins and the tree can grow up to 9 m but it is usually rather a shrubby bush of about 3 – 4 m.

Figure 2.8: *Searsia leptodictya* (Photograph credit: Ayodele Akinboye (2020), @ Manie van der Schijff Botanical Garden, University of Pretoria)

Its natural distribution stretches across the four northern provinces in South Africa and includes the northern Free State It is also found in other surrounding countries such as Zimbabwe, Mozambique, Lesotho and Botswana (Aubrey 2017). The mountain karee grows naturally in a variety of habitat types including woodland, forest margins and bushveld (Mitchell 2004; Tshisikhawe and Van Rooyen 2014).

Phytochemical analysis of *S. leptodictya* extracts revealed the presence of phenols, tannins, saponins and flavonoids (Mtunzi et al. 2017). *Searsia* extracts have promising potential to provide renewable bio-products with the following excellent bioactivities: antifibrogenic, anti-inflammatory, antioxidant, antimalarial, antimicrobial, antimutagenic, antithrombin, antifungal, cytotoxic, antitumorigenic, antiviral, antileukopenia and hypoglycemic (Salimi et al. 2015; Rahideh et al. 2014; Rayne and Mazza 2007).

2.9.9 *Ziziphus mucronata* Willd.

The genus *Ziziphus* belongs to the Rhamnaceae family. The members of the taxon are drought tolerant and very resistant to heat (Paroda and Mal 1989). *Z. mucronata* is known as buffalo thorn in English, blinkblaarwag-n-bietjie in Afrikaans, mokgalo in Sepedi Tswana, umphafa in isiXhosa

and umlahlankosi in isiZulu. It is a small to medium-sized tree, with a spreading canopy. Its distribution is throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia. Its bark and roots find good use medicinally in the management of various disease conditions such as rheumatism, gastrointestinal complaints, and snake bites (Tas et al. 1991).

Warm bark infusions are used for body pains, as expectorants in cough, and to treat respiratory infections and chest problems. The root infusions are used for treating gonorrhea, diarrhoea and dysentery. In ethnomedicine, decoctions made from the leaves and roots are used externally for pain relief when applied on boils, sores and glandular swellings (Venter and Venter 2002). While several species of the genus *Ziziphus* have been scientifically investigated, there is a dearth of information on the phytochemical properties and antioxidant activities of this plant species (Olajuyigbe and Afolabi 2011).

Figure 2.9: *Ziziphus mucronata* (photograph credit: ©Top Tropicals LLC, 2003 – 2021. https://toptropicals.com/catalog/uid/ziziphus_mucronata.htm)

2.10 Conclusion

The prevalence and the economic impact of bovine mastitis is worldwide. As an inflammatory disease caused by microorganisms such as bacteria, fungi, viruses etc., management approaches include the use of conventional antibiotics. *Staphylococcus* spp. are widely reported to have been isolated from both clinical and subclinical cases of mastitis, with NAS showing a rising trend, and antibacterial resistance reportedly common among these species. Some of the isolates have been reported to be resistant to as many as six commonly used antibiotics, justifying the widespread concerns of treatment failure and subsequent increasing cost of bovine mastitis. While various studies have attempted to explain and link the resistance phenomenon to quorum sensing and biofilm forming nature of some of the mastitic staphylococci, natural sources such as South African plants have been explored to find compounds that demonstrate antibacterial and antibiofilm activities, in order to find alternative approaches to the failing commonly used antibiotic management of bovine mastitis. In line with this drive, this research seeks to investigate the above South African plants of potential relevance in bovine mastitis management and control for their antibacterial and anti-biofilm activity against drug resistant strains of *S. aureus* and NAS isolated from clinical cases of bovine mastitis.

3. *In vitro* antibacterial and cytotoxic activities of selected South African plants on multidrug-resistant staphylococci isolated from clinical cases of bovine mastitis

3.1 Background

Mastitis is a serious health and economic problem in the bovine dairy industry worldwide (Ngu Ngwa et al. 2018), as it is still one of the major sources of economic losses for farmers. Mastitis reduces the productivity and economic efficiency of dairy farms due to the reduction of milk yield, milk returns and increasing costs of treatment (Ibrahim and Ghanem 2019). Bovine mastitis has been described as an inflammatory reaction of the mammary gland that is usually caused by a microbial infection and is recognized as the costliest disease in dairy cattle (Xhao and Lacasse 2008). The severity of the disease is divided into clinical and subclinical mastitis (Perini et al. 2014). The damage to the mammary tissue reduces the number and activity of epithelial cells, and this consequently contributes to decreased milk production. Mastitis is responsible for economic losses of approximately US$35 billion worldwide (Gomes and Henriques 2016). The economic cost estimation of this disease indicated that decreased milk production accounts for approximately 70% of the total cost of mastitis (Xhao and Lacasse 2008). Other economic costs of mastitis are incurred from treatment, decreased milk quality and an increase in the risk of culling (Manombe 2014). Among cattle diseases, mastitis remains a serious problem with financial implications to the farmer as it adversely affects milk production (Petrovski et al. 2006; Bradley 2002).

Mastitis organisms include bacteria, fungi and viruses (Tiwari et al. 2013) and they are classified generally as contagious or environmental. Contagious pathogens, which include *Staphylococcus aureus*, certain non-aureus staphylococci (NAS), *Streptococcus dysgalactiae* and *Streptococcus agalactiae*, are organisms that can survive within the mammary gland of the host (Bradley 2002). Environmental pathogens including members of the Enterobacteriaceae (particularly *E. coli*), *Streptococcus uberis* and some NAS (Petzer et al. 2009), are opportunistic invaders of the mammary gland, and they are not adapted to survive within the host as is the case with contagious pathogens. They can invade and multiply within the mammary gland and stimulate the host response system (Perini et al. 2014; Taemchuay et al. 2009). In South Africa, Petzer et al. in their

2009 review suggested that *S. aureus* remains a major pathogen of bovine mastitis and the prevalence of NAS is on the increase.

Antimicrobial agents have been widely used for treatment of mastitis pathogens in veterinary medicine with limited success because of the increasing prevalence of resistance to commonly used antimicrobial agents (Du Preez 2000; Craven 1987). *S. aureus* is reported to be resistant to most antibiotics used in mastitis treatment (mostly β-lactams) and it can release toxins that impair the udder's natural defenses (Gruet et al. 2001). In more recent research reported in South Africa 90% of 142 NAS strains isolated from mastitic cases exhibited resistance to at least one antibacterial agent while 51% were multidrug resistance (Phophi et al. 2019). Natural products from plants are proven templates for new drug development and have shown various useful biological activities. The use of natural plant products has increasingly become a recognized treatment for several ailments, and this is one of the rapidly growing sectors of the agribusiness industry (Makunga et al. 2008). There is a worldwide drive to explore natural products for new and safer drugs because of the increasing incidence of *Staphylococcus* species and the increasing occurrence of their resistance to commonly used antibiotics.

Many plant metabolites are toxic both in vitro and in vivo, therefore, the general assumption that plants and their constituents are safe is wrong. It is necessary to determine their cytotoxicity to provide preliminary scientific evidence whether they are likely to be safe or not before further development and commercialization of plant derived herbal remedies or drugs (Dzoyem et al., 2016; Elisha et al. 2016; Tshikalange et al. 2005).

In the current study, extracts of nine selected South African plants were evaluated for their antibacterial activity against multidrug resistant *Staphylococcus* species isolated from clinical cases of bovine mastitis. Cytotoxic effects of extracts of three plants selected based on their unique antibacterial activities were also determined. The study also investigated the chemical composition of the extracts to detect potential active compounds suspected to be responsible for antibacterial activities in the three selected plant species.

3.2 Materials and Methods

3.2.1 Plant Collection and Identification

The leaves of the plants were harvested from the Manie van der Schijff Botanical Garden at the University of Pretoria, Onderstepoort campus of University of Pretoria and the Lowveld National Botanical Garden, Nelspruit, Mpumalanga. Herbarium voucher specimens were prepared and deposited in the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria where possible but sterile specimens (those of *Elaeodendron croceum*, *Pleurostylia capensis*, *Antidesma venosum* and *Erythrina caffra*) were not able to be deposited.

Table 3.1: Selected plants and their herbarium accession number

Plant species	**Family**	**Accession numbers**
Indigofera frutescens L.f.	Leguminosae	PRU 128111
Ziziphus mucronata Willd. ssp. *mucronata*	Rhamnaceae	PRU 128 112
Searsia lancea (L.f.) F.A.Barkley	Anacardiaceae	PRU 128113
Trichilia emetica Vahl.	Meliacae	PRU 128115
Searsia leptodictya T.S.Yi, A.J.Mill & J.Wen	Anacardiaceae	PRU 128116
Antidesma venosum E. Mey. ex. Tul.	Phyllanthaceae	
Erythrina caffra Thunb.	Fabaceae	
Pleurostylia capensis Turcz. (Loes)	Celastraceae	
Elaeodendron croceum (Thunb.) DC.	Celastraceae	

3.2.2. Storage and Grinding

The leaves were harvested and stored immediately in open mesh loosely woven bags to make sure there was adequate air flow for quick drying to minimize chemical changes due to microbial attack after collection. The leaves were dried indoors at room temperature under ventilated conditions, and when completely dried, they were ground to a fine powder using a Janke and Künkel Model A10 mill. The powder was stored in tightly closed glass containers in the dark at room temperature. Dried material was used because there are fewer problems associated with large scale extraction of dried plant material compared to fresh plant material, and dried material may retain its biological activity for many decades (Eloff and McGaw 2006).

3.2.3 Plant extraction

Acetone and ethanol were used for extraction of the plant material. Extraction with acetone is considered the best choice for preparing plant extracts because it can extract compounds of a wide range of polarities, it is non-toxic to bioassay systems and easy to remove from extracts (Eloff 1998b; Eloff 2001). Ethanol was selected based on the preference for less flammable solvents in commercial usage. Four grams of ground dry leaf samples were extracted with 40 ml each of the solvents. The mixture was sonicated for 20 min, vigorously shaken, and then poured into a 50 ml polyester centrifuge tube and centrifuged at 4000 x g for 10 min (Hettich Centrifuge, Rotofix 32 A, Labotec, Johannesburg, South Africa). The supernatant was collected and filtered through Whatman No. 1 filter paper into pre-weighed glass vials and concentrated by drying under a stream of cold air. The dried extracts were weighed, and the yields were obtained by dividing the mass extracted by the initial mass. A concentration of 10 mg/ml (stock solution) in the extracting solvent was prepared for use in the assays.

3.2.4 Analysis of extracts by thin layer chromatography (TLC)

Qualitative screening of crude acetone and ethanol extracts of *Antidesma venosum*, *Erythrina caffra* and *Searsia lancea* was performed to obtain thin layer chromatography (TLC) fingerprints on aluminum-backed silica gel plates following an established protocol (Kotzé and Eloff 2002).

Three different solvent systems of diverse polarities, namely benzene: ethanol: ammonium hydroxide (18:2:0.2, BEA, non-polar, basic), chloroform: ethyl acetate: formic acid (5:4:1, CEF, intermediate polarity, acidic) and ethyl acetate: methanol: water (40:5.4:5, EMW, polar, neutral) were used to elute 100 µg of the extract loaded in a band of 1 cm width on the TLC plates. Visible bands were marked under white light and ultraviolet light (254 nm and 360 nm wavelengths, Camac universal UV light lamp TL-600) and then sprayed with freshly prepared vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) spray reagent. The plates were then heated to 110°C until optimal colour development.

3.2.5 Bacteriological analysis

3.2.5.1 Preparation of bacterial culture

Four strains of non-aureus staphyloccous (NAS) and eight strains of *S. aureus* isolated from composite milk samples of clinical mastitis cases that were submitted to the Onderstepoort Milk Laboratory, Faculty of Veterinary Science, University of Pretoria in 2019 were used for this study. An ATCC strain of *S. aureus* was also obtained and used for some of the bacteriological assays. The Milk Laboratory receives milk samples from dairy farms across South Africa for routine diagnosis of mastitis. The bacterial isolates were retrieved from the -70°C storage prior to the antibacterial assay and then thawed at room temperature. Using a sterile loop, a small amount of the thawed culture was inoculated on Mueller-Hinton (MH) agar and incubated at 37°C for 18- 24 hours. After 24 hours incubation, a colony was used to inoculate 10 ml of MH broth and further incubated for 18- 24 hours at 37°C on a shaker.

3.2.5.2 Identification of the isolates

3.2.5.2.1 Phenotypic identification

The Milk Laboratory (Faculty of Veterinary Sciences, University of Pretoria) conducted the phenotypic assays by streaking milk samples on bovine blood tryptose agar plates (Oxoid, Quantum Biotechnologies (Pty) Ltd., South Africa). The inoculated agar plates were incubated aerobically for 24–48 h at 37°C (±1 °C). Presumptive *Staphylococcus* spp. colonies were initially

identified based on phenotypic morphology, and biochemical tests (Markey et al. 2013). The *Staphylococcus* isolates were confirmed using Staph API (Biomerieux South Africa (Pty) Ltd., South Africa).

3.2.5.2.2 Species identification using MALDI-TOF-MS

All *Staphylococcus* isolates were subjected to matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as previously described (Nonnemann et al. 2019; Fox et al. 2011). Single pure colonies were transferred onto MALDI plates (Sigma Aldrich, St. Louis, MO) in duplicate and covered with alpha-cyano-4-hydroxycinnamic acid in an organic solution (50% acetonitrile and 2.5% tri-fluoro-acetic acid). The preparation was crystallized by air drying at room temperature. Flex Control software (Bruker Daltonics) recorded spectra sets for bacterial identification. MALDI Biolayer 3.0 software (Bruker Daltonics) with an integrated pattern-matching algorithm was used to compare generated peak lists against the reference library and a score was generated based on similarity.

3.2.5.3 Antibiotic susceptibility testing

The 12 isolates and *S. aureus* ATCC 29213 (control) were subjected to antimicrobial susceptibility testing against a panel of 10 drugs using the modified disc diffusion method (Kirby-Bauer method) on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute guidelines (Clinical Laboratory Standard Institute 2017). The antimicrobials investigated included 10 µg ampicillin (AMP), 30 µg chloramphenicol (C), 30 µg tetracycline (TE), 30 µg doxycycline (DO), 30 µg amoxicillin + clavulanic acid (AMC), 30 µg amikacin (AK), 10 µg colistin sulphate (CT), 25 µg trimethoprim + sulfamethoxazole (SXT), 10 µg gentamicin (CN) and 30 µg ceftiofur (EFT). Based on the diameter of the zone of inhibition, isolates were classified as sensitive, intermediate or resistant (Clinical Laboratory Standard Institute 2017). For the purpose of analysis, the intermediate susceptibility was considered as resistant. Isolates that were resistant to at least one antimicrobial drug were defined as “resistant” while those resistant to three or more antimicrobial categories were defined as “multidrug resistant” (Margiorakos et al. 2012).

3.2.5.4 Qualitative antibacterial assay by TLC bioautography

Thin layer chromatograms of both acetone and ethanol extracts of *Antidesma venosum*, *Erythrina caffra* and *Searsia lancea* were prepared as described above (section 3.2.4) except that the plates were not sprayed with vanillin. The plates were allowed to dry overnight in a stream of cold air to remove the eluents. Each plate was then sprayed with an actively growing suspension of either a *S. aureus* isolate (STA 3), a NAS isolate (NAS D) or an ATCC (29213) strain of *S. aureus* cultured for 18-24 h at 37°C. The moist plates were then allowed to dry slightly and incubated at 37°C in a closed plastic humidified sterile container for 24 h to allow the bacteria to grow on the plates. After incubation, the plates were sprayed with 2 mg/mL of freshly prepared p-iodonitrotetrazolium (INT) violet (Sigma) in sterile distilled water and incubated further for 1-2 h for the development of clear zones against a purple-red background which indicates inhibition of bacterial growth by the compounds separated on the chromatograms (Begue and Kline 1972).

3.2.5.5 Quantitative antibacterial assay by minimum inhibitory assay

A widely accepted, simple, reproducible, low cost and sensitive serial dilution microplate method (Eloff 1998a) was used to determine the minimum inhibitory concentration (MIC) of the crude plant extracts against eight isolates of *S. aureus*, four isolates of NAS and one ATCC 29213 strain of *Staphylococcus aureus*. Bacterial cultures were grown overnight in MH broth (Sigma Aldrich, SA) and adjusted to McFarland standard 1 which is equivalent to 3.0×10^8 cfu/mL (*Staphylococcus aureus*, ATCC 29213). The dried extracts were dissolved in their extracting solvents to a concentration of 10 mg/mL. 100 µl was added to the first well of a sterile 96-well microtitre plate containing 100 µl of water and serially diluted 1:1 with sterile distilled water. One hundred microliters of appropriately adjusted bacterial cultures were then added to each well. The bacteria were subjected to final extract concentrations of 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, 0.04 and 0.02 mg/ml. Gentamicin (Virbac) at a starting concentration of 0.1 mg/ml (also serially diluted two-fold) and solvents alone served as positive and negative controls respectively while sterile distilled water served as the sterility control. The bacteria were subjected to decreasing concentrations of solvent starting with 25% in the first well with a two-fold decrease in subsequent wells. It has been established that acetone at these concentrations does not inhibit bacterial growth (Eloff et al. 2007). The microplates were covered with parafilm and incubated at 37°C for 18-24 h. After incubation,

the plates were removed from the incubator and 40 µl of 0.2 mg/mL p-iodonitrotetrazolium violet INT dissolved in hot water were added to the wells and incubated further at 37°C for 2 h. The minimum inhibitory concentration (MIC) values were determined visually as the lowest concentration that led to growth inhibition (Eloff 1998). Bacterial growth in the wells was indicated by a red colour, whereas clear wells indicated inhibition of the bacterial growth by plant extracts. Clear wells were re-cultured on MH agar to determine the bactericidal effects of the plant extracts.

Additionally, the total activity (ml/g) of the extracts which is calculated by dividing the mass in mg extracted from 1 g of plant material with the MIC in mg/ml was calculated (Eloff 2000). This value considers the mass extracted as well as the antibacterial activity and is used to compare the potential use of extracts of different plant species.

3.2.6 Cytotoxicity assay

The cytotoxicity of selected plant extracts against Vero monkey kidney cells was determined using the 3-(4,5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium-based colorimetric (MTT) assay as described by Mosmann (1983). The cells were grown in Minimal Essential Medium (MEM) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Cells of a sub-confluent culture were harvested and centrifuged at 200 x g for 5 min and re-suspended in MEM to 5 x 10^4 cells/ml. Cell suspensions (100 µl) were pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Minimal Essential Medium (200 µl) was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37ºC in a 5% CO_2 incubator, to allow the cells to attach and reach the exponential phase of growth. One hundred microliters of the extract at differing concentrations prepared in MEM was added to the plates in quadruplicate. The microtitre plates were incubated at 37°C in a 5% CO_2 incubator for 48 h with the plant samples. Untreated cells and a positive control (doxorubicin chloride, Pfizer Laboratories) were also included. After incubation, the MEM with plant extract was aspirated from the cells and washed with 150 µl phosphate buffered saline (PBS, Whitehead Scientific) and replaced with 200 µl of fresh MEM. Following the washing step, 30 µl MTT (Sigma, stock solution of 5 mg/ml in phosphate-buffered saline (PBS)) was added to all the wells and the plates were incubated for a further 4 h at 37°C. After incubation, the medium

in all wells was carefully removed, without disturbing the MTT crystals in the wells. The cells were washed with PBS and the MTT formazan crystals were then dissolved by adding 40 µl of dimethyl sulfoxide (DMSO) to all the wells. The plates were shaken gently to allow the MTT solution to dissolve. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader at a wavelength of 540 nm and a reference wavelength of 630 nm. The wells in column 1 and 12, containing medium and MTT, without cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of plant samples resulting in a 50% reduction of absorbance (correlating to killing 50% of the cells) compared to untreated cells.

3.2.7 Statistical analysis

Experimental results were expressed as mean ± standard error (SE) of at least three replicates. Data were collated and analyzed using Microsoft Excel Version 2010.

3.3 Results and discussion

3.3.1 Plant extract yield

Different yields of crude extracts were obtained with acetone and ethanol extraction of the nine plants. Among the acetone extracted plants, *Searsia lancea* had the highest yield (5.49%) followed by *Erythrina caffra* (5.35%) while *Indigofera frutescens* gave the lowest yield (1.68%). Among the ethanol extracts, *Searsia lancea* yielded the highest (10.75%), followed by *Elaeodendron croceum* (8.12%) and *Erythrina caffra* yielded the lowest (2.68%). Generally, the ethanol extracts of all the plants yielded more than the acetone extracts except in the case of *Erythrina caffra* where the acetone extract yielded more than the ethanol extract (Table 3.2). The yield of a plant extract is important in calculating the total activity to compare plant extracts for bioprospecting (Eloff, 2000). The yield also affects the total activity of the different plant species.

Table 3.2: Crude extract yield of the selected plants.

Plants	% Acetone extract yield (g/g)	% Ethanol extract yield (g/g)
Pleurostylia capensis	5.11	6.80
Antidesma venosum	1.92	3.28
Searsia leptodictya	1.98	4.00
Searsia lancea	5.49	10.75
Indigofera frutescens	1.68	6.87
Erythrina caffra	5.35	2.68
Elaeodendron croceum	4.96	8.12
Ziziphus mucronata	2.80	6.04
Trichilia emetica	2.79	6.99

3.3.2 Antibiogram results

The antibacterial susceptibility of the bacteria used is shown in Table 3.3. All the organisms were resistant to at least one of the antibiotics used. They were all resistant to colistin sulphate which is not unexpected as this antibiotic is primarily used to treat infections caused by Gram-negative bacteria. Eight of the organisms were resistant to three antibiotics, namely amikacin, colistin sulphate and gentamicin, except for NAS 2 which was resistant to chloramphenicol and amoxicillin-clavulanic acid instead of amikacin and gentamicin. Three bacteria were resistant to only two drugs while one was resistant to only one antibiotic. Ten of the organisms were resistant to either of the macrolide antibiotics (amikacin and gentamicin) included while seven were resistant to both. Worthy of note is the fact that gentamicin at concentrations ranging between 0.2 and 25.0 µg/ml was used as the positive control in the quantitative antibacterial assay and against most of the organisms, gentamicin had relatively poor activity (MIC above 9.1 µg/ml) as shown in Table 3.5 and 3.6. Since the concentration of gentamicin in the antibiogram disc is 10 µg, there is, therefore, a correlation between the resistance patterns shown by the organisms in the MIC and the antibiogram assays.

Table 3.3: Antibiogram results showing resistant strains and total number of resistances per strain (n=10 antibiotics / combinations).

Species	Strains	Resistant Pattern	No. of Resistance
S. aureus	STA 1	AK, CT, CN	3
	STA 2	AK, CT, CN	3
	STA 3	CT, CN	2
	STA 4	AK, CT	2
	STA 5	AK, CT, CN	3
	STA 6	AK, CT, CN	3
	STA 7	AK, CT, CN	3
	STA 8	AK, CT, CN	3
	ATCC (259213) (*S. aureus*)	CT	1
NAS	NAS A	CT, CN	2
	NAS B	CT, C, AMC	3
	NAS C	AK, CT, CN	3
	NAS D	CT	1

AK= Amikacin, CT= Colistin sulphate, Gentamicin, C= Chloramphenicol, AMC= Amoxicillin-Clavulanic acid

3.3.3 Identification of bacterial isolates*

The test organisms were identified as shown in Table 3.4.

To make analysis of the data generated easier in this study, NAS 1 and NAS 2 will be reported as STA 7 and STA 8, respectively as they were initially thought to be NAS but were later identified as strains of *Staphylococcus aureus,* while NAS 3 – NAS 6 will be reported as NAS A – NAS D. This will be done to clearly differentiate between the two groups of *Staphylococcus* strains used in this work.

Table 3.4: Species names of the test bacteria

Strain codes	Species names
ATCC 29213	*Staphylococcus aureus*
STA 1	*Staphylococcus aureus*
STA 2	*Staphylococcus aureus*
STA 3	*Staphylococcus aureus*
STA 4	*Staphylococcus aureus*
STA 5	*Staphylococcus aureus*
STA 6	*Staphylococcus aureus*
NAS 1	*Staphylococcus aureus*
NAS 2	*Staphylococcus aureus*
NAS 3	*Staphylococcus chromogenes*
NAS 4	*Staphylococcus haemolyticus*
NAS 5	*Staphylococcus chromogenes*
NAS 6	*Staphylococcus chromogenes*

3.3.4 Bioautography

Out of the three solvent systems used for elution of TLC plates, the non-polar solvent system BEA gave the best separation of compounds before introduction of organisms. This suggests that the active compounds are more non-polar. An ATCC strain of *S. aureus*, one *S. aureus* strain (STA 3) and one NAS strain (NAS D) were selected based on their sensitivity to the selected plant extracts that were used for the bioautography assay, based on the MIC data generated. The portion of the chromatogram that shows white zones surrounded by pinkish areas denotes where the active compound in each extract lies (Figure 3.1 – 3.6). In some of the extracts tested against the ATCC strain, extracts such as AA, EA, and RA, more than one active compound was observed, while for extracts AB, EB and RA, the active compounds did not separate clearly. Against the NAS D strain, each of the extracts showed a few separate clear zones, while against the STA 3 strain, the clear zones were mostly at the origin of the TLC plate, suggesting that the active compounds were not clearly separated by the solvent system used and were likely to be more polar. In Figure 3.7 and 3.8, the plates were sprayed with vanillin after TLC assay. This is to stain separated compounds

and differentiate different compounds that are present in the extracts. The plates showed the zones of separation of the constituent compounds of the extracts.

Figure 3.1: Acetone extracts + ATCC

Figure 3.2: Ethanol extracts + ATCC

Figure 3.3: Acetone extracts + NAS D

Figure 3.4: Ethanol extracts + NAS D

Figure 3.5: Acetone extracts + STA 4

Figure 3.6: Ethanol extracts + STA 4

Figure 3.7: Chromatogram of acetone extracts

Figure 3.8: Chromatogram of ethanol extracts

Note: AA, EA and RA stands for acetone extracts of *Antidesma venosum*, *Erythrina caffra* and *Searsia lancea* leaves, while AB, EB and RB represents their ethanol extracts, respectively.

3.3.5 Antibacterial activity (minimum inhibitory concentration) and total antibacterial activity

For this study, classification of antimicrobial activity of plant extracts described by Kuete (2010) was adopted, with good MIC< 0.1 mg/mL, moderate as 0.1 ≤ MIC ≤ 0.63 mg/mL, and weak as MIC > 0.63 mg/mL.

As shown in Table 3.5, the acetone extract of *S. lancea* had good activity against all the organisms with MIC values ranging from 0.01 – 0.07 mg/ml. The lowest MIC value of 0.01 mg/ml obtained was found with *S. lancea* against STA 3 and the highest MIC value of 1.41 mg/ml was recorded with *T. emetica* against STA 2. Apart from *S. lancea*, there were only two other plant extracts, out of the remaining 8 plants extracts, that showed MIC values which are less than 0.1 mg/ml against any of the bacterial strains. They are *I. frutescens* which showed good activities (MIC = 0.05 mg/ml and 0.09 mg/ml) against STA 4 and STA 3, respectively, and *A. venosum* which showed a good activity (MIC value = 0.09 mg/ml) against STA 3 only. *S. leptodictya* (a member of the same genus as *S. lancea*) showed moderate activities against all the organisms while *E. caffra, P. capensis, Z. mucronata* and *T. emetica* showed moderate to poor activities across all the strains of the isolates.

According to Table 3.6, the ethanol extract of *S. lancea* had moderate activities against all the organisms except NAS C, against which it showed a good activity with MIC = 0.08 mg/ml. The lowest MIC value of 0.08 mg/ml obtained was found with *S. lancea* against NAS C and *E. caffra* against STA 4, while the highest MIC value of 0.94 mg/ml was recorded with *S. leptodictya against STA 7 and T. emetica* against STA 3, STA 6 and NAS B . *E. caffra* which showed good activity (MIC value = 0.08 mg/ml) against STA 4 showed moderate activities against the rest of the isolates. Like its acetone counterpart the ethanol extract of *S. leptodictya* showed moderate activity against all the organisms except STA 7, against which it showed a poor activity (0.94 mg/ml). *A. venosum* showed good to moderate activities while *P. capensis, Z. mucronata* and *T. emetica* showed moderate to poor activities across all the strains.

Literature search did not reveal any work involving any of the plants used in this study to have been tested against *Staphyloccocus* spp. of mastitis origin. However, several studies have reported the antibacterial activities of these plants against *Staphylococcus* spp. of different origins, as well as antimicrobial activities of other plants against *Staphylococcus* spp. isolated from cases of bovine mastitis. An example of such papers is the work of Gundidza et al. (2008) which reported *S. lancea*

exhibited antibacterial activity against a strain of *S. aureus* at a MIC value 0.01 mg/ml. This is the same value obtained with acetone extract of *S. lancea* against an isolate of *S. aureus* in this work. The acetone extract of *S. leptodictya* has been shown to inhibit the same ATCC strain of *S. aureus* used in this work at the same MIC value of 0.16 mg/ml (Fanyana et al. 2017). The acetone extract of *T. emetica* was also reported to show a MIC value of 1.3 mg/ml against a strain of *S. aureus* which is the same as obtained with one of the S. aureus isolates used in this study. Generally, the acetone extracts of all the plants showed better activity than their ethanol counterparts except for *E. caffra*.

A striking observation from Table 3.5 and 3.6 is that only the acetone extract of *S. lancea* showed good activity against all the strains of NAS. Neither the ethanol extract of *S. lancea* nor both (acetone and ethanol) extracts of other plants had good activity against any strain of NAS. The results suggest that the STA strains showed a broader range of susceptibility to both extracts of all the plants and appear to be more susceptible to the extracts than the NAS strains. Further research needs to be conducted to unravel the reason for this pattern. Knowing that the NAS and the STA strains are Gram-positive organisms, it might appear that this observed pattern may not be due to the cell wall infrastructure of both groups of bacteria. A similar pattern of susceptibility was reported by Okmen et al. (2017). All extracts prepared from *Liquidambar orientalis* leaves using 3 different solvents (acetone, methanol, and ethanol) were able to show significant antibacterial properties against mastitis-causing bacteria. However, antibacterial activity of these extracts was significantly lower towards NAS species compared to *S. aureus* (Okmen et al. 2017). The highest antibacterial activity (12 mm inhibition zone diameter) was observed for *S. aureus* 17 with acetone extracts. Interesting but almost similar antibacterial properties (11 mm inhibition zone diameter) were also observed for methanol and ethanol extracts towards *S. aureus* 17. The lowest antibacterial activity (7 mm inhibition zone diameter) of acetone extracts was found towards NAS-36 (Okmen et al. 2017). The MIC (3.2 mg/ml) of different solvent extracts was determined using the broth dilution method and it was the same for all *S. aureus* and NAS. Meanwhile, Piotr et al. (2018) reported varying susceptibility patterns. Though all the reference strains of *S. aureus* were less susceptible to the reference strains of NAS, they reported that some isolates of NAS (*S. epidermidis* and *S. xylosus*) (MIC = 0.156%) appeared to be less susceptible than some isolates of *S. aureus* (MIC = 0.078%) to essential oil of *Pelargonium graveolens* while some isolates of NAS

(MIC = 0.020%) appeared to be more susceptible than some isolates of *S. aureus* (MIC = 0.156%) to essential oil of *Cinnamomum cassia.*

Isolates of both *S. aureus* and NAS were susceptible at the same concentration to essential oils of other plants such as *Juniperus virginiana* (MIC = 0.010%), *Leptospermum scoparium* (MIC = 0.005%), *Pogostemom cablin* (MIC = 0.010%) and *Thymus vulgaris* (MIC = 0.010%) (Piotr et al. 2018). In another study, Sserunkuma et al. (2017) reported that the acetone leaf extract of *Acacia nilotica* showed higher antibacterial activity against *S. epidermidis* and *S. chromogenes* (MIC = 0.156 mg/ml) than *S. aureus* (MIC = 0.625 mg/ml). The acetone leaf extract of *Aloe arborescens* showed similar MIC (>2.5 mg/ml) against *S. aureus* and *S. chromogenes,* and lower MIC (2.5mg/ml) against *S. epidermidis* (Sserunkuma et at. 2017).

According to Table 3.7 NAS strains were more susceptible to the extracts over a shorter MIC range than *S. aureus* strains which are susceptible over a wider MIC range. This is similar also to the work of Piotr et al. (2018) which reported geranium oil to have a wider MIC range (0.078 – 1.25%) for *S. aureus* and a narrower MIC range (0.156 – 1.25%) for NAS.

From another perspective when considering the average MIC of the extracts against all bacteria (Table 3.7; column "STA & NAS strains"), only the acetone extract of S. lancea showed good overall antibacterial activity (mean MIC = 0.03 mg/ml). The acetone extracts of the other plants showed only moderate activity, while the ethanol extracts of all the plants showed moderate to poor activity. Average MIC values of all the acetone extracts of each of the plants were lower than those of their ethanol counterparts except for E. caffra. The average MIC value of the ethanol extract of E. caffra (mean MIC = 0.18 mg/ml) for all bacteria appears to be less than half of the average MIC value of its acetone extract (mean MIC = 0.45 mg/ml). This is an interesting observation because, though acetone is the preferred extractant because of the observed better antibacterial activity, ethanol remains the preferred solvent because of the potential to commercialize the findings of this study, as ethanol is less flammable and dangerous to work with in large quantities compared to acetone. Ethanol is also believed to be a suitable solvent as it can efficiently penetrate cell membranes, permitting extraction of higher levels of endo-cellular components than solvents with lower polarity (Panda, 2014; Panda et al., 2016). E. caffra should therefore be further investigated. The observation of better activities with the acetone extract of the other plants is consistent with various studies which have suggested acetone to be the preferred

solvent of extraction for antimicrobial investigation of plants because acetone is able to extract compounds of wider range of polarity (Eloff and McGaw 2006; Eloff 2001: Eloff 1998b). Against the ATCC strain of *S. aureus*, only the acetone extracts of *S. lancea* had good activity (MIC = 0.07 mg/ml). Both ethanol and acetone extracts of the other plants showed moderate activities, except for the ethanol extracts of *P. capensis* (MIC = 0.94 mg/ml), *Z. mucronata* (MIC = 0.83 mg/ml) and *T. emetica* (MIC = 0.83 mg/ml) which showed poor activities. It generally appears that the isolates were more susceptible to the extracts than the ATCC strain, which is an interesting result, while the ATCC strain was more susceptible to gentamicin (positive control) than the isolates (Tables 3.5 and 3.6).

The total antibacterial activity (TAA) which is the measure of the potency of plant extracts can be measured on the basis of both the MIC in mg/ml and extract yield in mg/g (Eloff 2004). Dividing the yield of each of the extracts by the mean MIC values of each extract against the species group of the isolates, their TAA were obtained. The TAA gives the volume (ml) to which the extract obtained from one g of plant material can be diluted and still be able to inhibit the bacteria. In this study, the acetone extract of *S. lancea* had the highest mean total antibacterial activities of 2385.87 (*S. haemolyticus*), 1964.02 (*S. aureus*) and 1291.18 mL/g (*S. chromogenes*) respectively (Table 3.8), followed by its ethanol extract. TAA is useful to determine the most suitable plant extract for compound isolation and bioprospecting.

Table 3.5: Antibacterial assay results showing MIC values of the acetone extracts against different strains of bacteria.

Mean MIC (mg/ml) ± SEM													
Plants	**STA1**	**STA2**	**STA3**	**STA4**	**STA5**	**STA6**	**STA7**	**STA8**	**NAS A**	**NAS B**	**NAS C**	**NAS D**	**ATCC**
Pleurostylia capensis	0.23 ± 0.09	0.31 ± 0.00	0.31 ± 0.00	0.23 ± 0.09	0.31 ± 0.00	0.23 ± 0.09	0.18 ± 0.15	0.39 ± 0.26	0.23 ± 0.09	0.23 ± 0.09	0.23 ± 0.09	0.73 ± 0.43	0.47 ± 0.17
Antidesma venosum	0.12 ± 0.04	0.14 ± 0.03	0.09 ± 0.07	0.10 ± 0.06	0.12 ± 0.04	0.27 ± 0.30	0.20 ± 0.13	0.64 ± 0.66	0.20 ± 0.13	0.16 ± 0.00	0.10 ± 0.06	0.18 ± 0.15	0.12 ± 0.04
Searsia leptodictya	0.31 ± 0.00	0.20 ± 0.13	0.23 ± 0.09	0.12 ± 0.04	0.16 ± 0.00	0.29 ± 0.06	0.23 ± 0.09	0.31 ± 0.00	0.23 ± 0.09	0.23 ± 0.09	0.12 ± 0.04	0.29 ± 0.06	0.16 ± 0.00
Searsia lancea	0.05 ± 0.02	0.04 ± 0.01	0.01 ± 0.00	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.05 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.07 ± 0.02
Indigofera frutescens	0.16 ± 0.00	0.37 ± 0.13	0.09 ± 0.03	0.05 ± 0.03	0.42 ± 0.16	0.10 ± 0.04	0.10 ± 0.06	0.12 ± 0.04	0.10 ± 0.04	0.14 ± 0.03	0.12 ± 0.04	0.12 ± 0.04	0.16 ± 0.00
Erythrina caffra	0.47 ± 0.17	0.47 ± 0.17	0.31 ± 0.00	0.31 ± 0.00	0.63 ± 0.00	0.39 ± 0.26	0.42 ± 0.16	0.37 ± 0.13	0.63 ± 0.00	0.39 ± 0.26	0.73 ± 0.57	0.31 ± 0.00	0.16 ± 0.00
Elaeodendron croceum	0.63 ± 0.00	0.47 ± 0.17	0.31 ± 0.00	0.47 ± 0.17	0.73 ± 0.26	0.47 ± 0.17	0.31 ± 0.00	0.63 ± 0.00	0.31 ± 0.00	0.18 ± 0.06	0.12 ± 0.04	0.39 ± 0.26	0.47 ± 0.17
Ziziphus mucronata	0.78 ± 0.51	0.94 ± 0.34	0.23 ± 0.09	0.47 ± 0.17	0.47 ± 0.17	0.31 ± 0.00	0.26 ± 0.21	0.08 ± 0.00	0.47 ± 0.17	0.63 ± 0.00	0.78 ± 0.51	0.47 ± 0.17	0.63 ± 0.00
Trichilia emetica	0.47 ± 0.17	1.41 ± 1.20	1.29 ± 1.33	0.23 ± 0.09	0.23 ± 0.09	0.63 ± 0.00	0.35 ± 0.30	0.20 ± 0.13	0.21 ± 0.12	0.39 ± 0.26	0.39 ± 0.26	0.31 ± 0.00	0.31 ± 0.00
Gentamicin (µg/ml)	14.6	4.7	0.8	0.2	9.1	> 25.0	> 25.0	> 25.0	> 25.0	4.0	0.7	> 25.0	< 0.2

STA= *Staphylococcus aureus*, NAS= Non-aureus staphylococci, ATCC= American type culture collection, MIC= Minimum inhibitory concentration

SEM= Standard error of mean

Table 3.6: Antibacterial assay result showing MIC values of the ethanol extracts against different strains of bacteria

Mean MIC (mg/ml) ± SEM												
Plants	**STA1**	**STA2**	**STA3**	**STA4**	**STA5**	**STA6**	**STA7**	**STA8**	**NAS A**	**NAS B**	**NAS C**	**NAS D**	**ATCC**
Pleurostylia capensis	0.78 ± 0.51	0.63 ± 0.00	0.57 ± 0.13	0.42 ± 0.16	0.63 ± 0.00	0.63 ± 0.00	0.63 ± 0.00	0.47 ± 0.17	0.63 ± 0.00	0.57 ± 0.13	0.31 ± 0.00	0.57 ± 0.13	0.94 ± 0.34
Antidesma venosum	0.47 ± 0.17	0.16 ± 0.00	0.31 ± 0.00	0.31 ± 0.00	0.47 ± 0.17	0.31 ± 0.00	0.31 ± 0.00	0.31 ± 0.00	0.34 ± 0.15	0.31 ± 0.00	0.16 ± 0.00	0.31 ± 0.00	0.31 ± 0.00
Searsia leptodictya	0.63 ± 0.00	0.63 ± 0.00	0.57 ± 0.13	0.47 ± 0.17	0.63 ± 0.00	0.63 ± 0.00	0.94 ± 0.34	0.31 ± 0.00	0.47 ± 0.17	0.31 ± 0.00	0.31 ± 0.00	0.63 ± 0.00	0.68 ± 0.46
Searsia lancea	0.20 ± 0.13	0.13 ± 0.04	0.13 ± 0.04	0.18 ± 0.06	0.16 ± 0.12	0.14 ± 0.09	0.13 ± 0.04	0.13 ± 0.04	0.14 ± 0.03	0.14 ± 0.03	0.08 ± 0.00	0.18 ± 0.06	0.16 ± 0.00
Indigofera frutescens	0.47 ± 0.17	0.31 ± 0.00	0.52 ± 0.16	0.63 ± 0.00	0.63 ± 0.00	0.52 ± 0.16	0.47 ± 0.17	0.37 ± 0.13	0.31 ± 0.00	0.47 ± 0.17	0.26 ± 0.08	0.63 ± 0.00	0.63 ± 0.00
Erythrina caffra	0.12 ± 0.04	0.12 ± 0.04	0.12 ± 0.04	0.08 ± 0.00	0.12 ± 0.04	0.12 ± 0.04	0.12 ± 0.04	0.47 ± 0.17	0.16 ± 0.00	0.26 ± 0.08	0.31 ± 0.00	0.16 ± 0.00	0.31 ± 0.00
Elaeodendron croceum	0.44 ± 0.21	0.31 ± 0.00	0.31 ± 0.00	0.57 ± 0.13	0.31 ± 0.00	0.47 ± 0.17	0.47 ± 0.17	0.42 ± 0.16	0.37 ± 0.13	0.31 ± 0.00	0.23 ± 0.09	0.31 ± 0.00	0.31 ± 0.00
Ziziphus mucronata	0.47 ± 0.17	0.78 ± 0.51	0.42 ± 0.16	0.63 ± 0.00	0.78 ± 0.51	0.73 ± 0.26	1.15 ± 0.81	0.63 ± 0.00	0.73 ± 0.26	0.73 ± 0.26	0.63 ± 0.00	0.47 ± 0.17	0.83 ± 0.47
Trichilia emetica	0.52 ± 0.16	0.63 ± 0.00	0.94 ± 0.34	0.73 ± 0.26	0.63 ± 0.00	0.94 ± 0.34	0.63 ± 0.00	0.63 ± 0.00	0.57 ± 0.13	0.94 ± 0.34	0.47 ± 0.17	0.63 ± 0.00	0.83 ± 0.32
Gentamicin (µg/ml)	14.6	4.7	20.8	0.2	9.1	> 25.0	> 25.0	> 25.0	> 25.0	4.0	0.7	> 25.0	< 0.2

STA= *Staphylococcus aureus*, NAS= Non-aureus staphylococci, ATCC= American type culture collection, MIC= Minimum inhibitory concentration

SEM= Standard error of mean

Table 3.7: Antibacterial activity result showing the MIC range of both extracts across the groups of the bacteria strains.

MIC range and average MIC of the extracts (mg/ml) ± SEM										
Plants	**MIC Range**		**STA strains**		**NAS strains**		**STA & NAS strains***		**ATCC strain**	
Solvents	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**
Pleurostylia capensis	0.18 - 0.73	0.31 - 0.78	0.25 ± 0.06	0.59 ± 0.11	0.38 ± 0.24	0.52 ± 0.14	0.29 ± 0.15	0.57 ± 0.12	0.47 ± 0.17	0.94 ± 0.34
Antidesma venosum	0.09 - 0.64	0.16 - 0.47	0.15 ± 0.06	0.33 ± 0.22	0.14 ± 0.05	0.28 ± 0.08	**0.15 ± 0.06**	**0.31 ± 0.09**	0.12 ± 0.04	0.31 ± 0.00
Searsia leptodictya	0.12 - 0.31	0.31 - 0.94	0.22 ± 0.06	0.60 ± 0.33	0.22 ± 0.07	0.43 ± 0.15	**0.22 ± 0.06**	0.54 ± 0.18	0.16 ± 0.00	0.68 ± 0.46
Searsia lancea	0.01 - 0.05	0.08 - 0.20	0.03 ± 0.01	0.15 ± 0.44	0.04 ± 0.02	0.14 ± 0.04	**0.03 ± 0.01**	**0.15 ± 0.03**	0.07 ± 0.02	0.16 ± 0.00
Indigofera frutescens	0.05 - 0.37	0.26 - 0.63	0.17 ± 0.14	0.49 ± 0.55	0.11 ± 0.01	0.42 ± 0.16	**0.15 ± 0.12**	0.46 ± 0.13	0.16 ± 0.00	0.63 ± 0.00
Erythrina caffra	0.31 - 0.73	0.08 - 0.47	0.43 ± 0.10	0.16 ± 0.66	0.50 ± 0.21	0.22 ± 0.08	0.45 ± 0.14	**0.18 ± 0.11**	0.16 ± 0.00	0.31 ± 0.00
Elaeodendron croceum	0.12 - 0.73	0.23 - 0.47	0.46 ± 0.15	0.41 ± 0.77	0.28 ± 0.12	0.31 ± 0.05	0.40 ± 0.16	0.38 ± 0.10	0.47 ± 0.17	0.31 ± 0.00
Ziziphus mucronata	0.23 - 0.94	0.42 - 1.15	0.47 ± 0.26	0.70 ± 0.88	0.49 ± 0.22	0.64 ± 0.12	0.47 ± 0.24	0.68 ± 0.19	0.63 ± 0.00	0.83 ± 0.47
Trichilia emetica	0.21 - 1.41	0.47 - 0.94	0.56 ± 0.50	0.70 ± 0.99	0.55 ± 0.50	0.65 ± 0.20	0.56 ± 0.47	0.69 ± 0.16	0.31 ± 0.00	0.83 ± 0.32

***** bold indicating the best three average MIC against both staphylococcal groups. SEM= Standard error of mean

STA= *Staphylococcus aureus*, NAS= Non-aureus staphylococci, ATCC= American type culture collection, MIC= Minimum inhibitory concentration

Table 3.8: Yield of acetone and ethanol extracts of the plants, total activity and mean MIC values of the *S. aureus, S. chromogenes, S. haemolyticus* isolates and ATCC strain of *S. aureus.*

Plants	% Yield		*S. aureus* isolates				*S. chromogenes* isolates				*S. haemolyticus* isolates				*S. aureus* ATCC strain			
			Mean MIC (mg/ml)		Total Activity		Mean MIC (mg/ml)		Total Activity		Mean MIC (mg/ml)		Total Activity		Mean MIC (mg/ml)		Total Activity	
	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol
Pleurostylia capensis	51.10	67.98	0.25 ± 0.06	0.59 ± 0.11	204.96	114.71	0.40 ± 0.29	0.50 ± 0.17	127.93	134.96	0.23 ± 0.09	0.57 ± 0.13	217.91	118.63	0.47 ± 0.17	0.94 ± 0.34	108.96	72.51
Antidesma venosum	19.20	32.75	0.15 ± 0.06	0.33 ± 0.22	125.49	98.53	0.16 ± 0.05	0.27 ± 0.17	122.81	121.62	0.10 ± 0.06	0.31 ± 0.00	123.08	104.63	0.12 ± 0.04	0.31 ± 0.00	164.10	104.63
Searsia leptodictya	19.75	39.95	0.22 ± 0.06	0.60 ± 0.33	89.17	66.69	0.21 ± 0.09	0.47 ± 0.17	92.82	85.18	0.12 ± 0.04	0.31 ± 0.00	84.22	127.64	0.16 ± 0.00	0.68 ± 0.46	126.60	58.98
Searsia lancea	54.88	107.53	0.03 ± 0.01	0.15 ± 0.44	1,964.02	714.65	0.04 ± 0.01	0.13 ± 0.17	1,291.18	800.10	0.04 ± 0.01	0.14 ± 0.03	2,385.87	751.92	0.07 ± 0.02	0.16 ± 0.00	767.48	689.26
Indigofera frutescens	16.78	68.73	0.17 ± 0.14	0.49 ± 0.55	97.46	140.69	0.11 ± 0.01	0.40 ± 0.17	148.89	172.00	0.05 ± 0.03	0.47 ± 0.17	117.31	146.54	0.16 ± 0.00	0.63 ± 0.00	107.53	109.96
Erythrina caffra	53.48	26.83	0.43 ± 0.10	0.16 ± 0.66	125.33	171.82	0.56 ± 0.22	0.21 ± 0.17	96.23	128.76	0.31 ± 0.00	0.26 ± 0.08	136.94	102.91	0.16 ± 0.00	0.31 ± 0.00	342.79	85.70
Elaeodendron croceum	49.60	81.18	0.46 ± 0.15	0.41 ± 0.77	107.24	196.20	0.27 ± 0.14	0.30 ± 0.17	181.35	266.88	0.47 ± 0.17	0.31 ± 0.00	272.40	259.35	0.47 ± 0.17	0.31 ± 0.00	105.76	259.35
Ziziphus mucronata	27.98	60.38	0.47 ± 0.26	0.70 ± 0.88	60.07	86.65	0.57 ± 0.18	0.61 ± 0.17	48.81	99.35	0.47 ± 0.17	0.73 ± 0.26	44.76	82.80	0.63 ± 0.00	0.83 ± 0.47	44.76	72.44
Trichilia emetica	27.90	69.90	0.56 ± 0.50	0.70 ± 0.99	49.68	99.41	0.30 ± 0.09	0.56 ± 0.17	91.78	125.79	0.23 ± 0.09	0.94 ± 0.34	71.45	74.56	0.31 ± 0.00	0.83 ± 0.32	89.14	83.88

ATCC= American type culture collection, MIC= Minimum inhibitory concentration, mg/ml= milligram per millimetre

3.3.6 Cytotoxicity

According to Kuete (2010), a plant extract is considered to be cytotoxic when the LC_{50} is 0.02 mg/ml and below. However, it has been observed that *in vitro* cellular toxicity may not equate to *in vivo* toxicity due to different factors such as gut interactions and bioavailability. Therefore, acute and chronic animal toxicity studies are needed to conclusively determine the toxicity of plant extracts (Ahmed et al. 2012).

In this study, all the plant extracts had LC_{50} values higher than the recommended cytotoxic concentration of 0.02 mg/ml (Table 3.9). Both extracts of E. *caffra* had the lowest cytotoxicity (LC_{50} > 1mg/ml) followed by the ethanol extracts *S. lancea* (0.79 mg/ml), and *A. venosum* (0.43 mg/ml). The highest cytotoxicity (0.08 mg/ml) was noted with the acetone extract of *A. venosum*. Desta et al. (2016) performed cytotoxicity tests on the crude extracts of *E. caffra* and only the n-hexane and ethyl acetate extracts of the root bark were active against the human cervix carcinoma cells at concentrations of 0.11 mg/ml and 0.06 mg/ml, respectively. The toxicity test of the ethanol extracts of *A. venosum* by Mwangomo et al. (2012) showed that the stem back is more toxic (LC_{50}= 0.026 - 0.041 mg/ml) than the root (LC_{50}= 0.063 – 0.080 mg/ml). Water extract of *S. lancea* leaf has been shown by McGaw et al. (2007) to have a LC_{50} of 0.6 mg/ml against brine shrimp. This is close to the value (0.79 mg/ml) obtained in this study. The other species of Searsia genus such as *S. leptodictya*, *S. pendulina* and *S. pentheri* have shown LC_{50} value of 0.025, 0.022 and 0.051 mg/ml against Vero cells according to Ahmed et al. (2014). They concluded that the phenolic-enriched extracts of *S. pendulina*, and *S. leptodictya* need to be applied orally with caution unless the fraction(s) containing toxic component(s) is identified and removed. The LC_{50} of ethanol extracts of the plants in this study were higher than their acetone counterparts which suggests that the ethanol extracts are relatively less toxic compared to the acetone extracts. Mwangomo et al. (2012) also favours ethanol extracts as well. This is significant for potential commercialization as ethanol is preferred as a solvent for industrial use because it is less flammable and dangerous to work with in large quantities.

The cytotoxicity (mg/ml) and MIC (mg/ml) values are the two variables used to obtain the selectivity index (SI) of a plant extract, which is a measure of the safety margin of the extract (Dzoyem et al., 2016). A selectivity index (SI) value greater than 1 means that the extract is more toxic to the pathogen than to the mammalian cells used for cytotoxicity testing. The higher the SI

value, the more promising is the activity of the plant extract as it is not likely to be owing to general toxicity. Therefore, the higher the SI, the higher the potential of the plant extract to be developed as a safe herbal product.

The ethanol extract of *E. caffra* had the best mean SI against all the pathogens (Table 3.9) while the acetone extract of *A. venosum* had a poor mean SI. Both extracts of all the three plants in this study have potential to be developed into useful products in tackling microbial infections as herbal remedies, except for the acetone extract of *A. venosum*. Alternatively, isolation of active compounds can be a template for the development of new drugs.

Table 3.9: Cytotoxicity against Vero cells LC_{50} (mg/ml) and selectivity index of the six selected crude extracts.

Selectivity Index of selected plant extracts																
Plants	**Extractant**	**LC_{50} (mg/ml) ± SEM**	**Mean SI ± SEM**	**STA 1**	**STA 2**	**STA 3**	**STA 4**	**STA 5**	**STA 6**	**STA 7**	**STA 8**	**NAS A**	**NAS B**	**NAS C**	**NAS D**	**ATCC STA**
Antidesma venosum	Ace	0.08 ± 0.00	0.65 ± 0.15	0.68	0.56	0.91	0.82	0.68	0.30	0.68	0.68	0.68	0.68	0.68	0.45	0.67
Antidesma venosum	Eth	0.43 ± 0.16	1.24 ± 0.51	0.92	2.76	1.37	1.37	0.92	1.37	0.92	0.92	0.92	0.92	0.92	1.37	1.39
Searsia lancea	Ace	0.15 ± 0.02	4.69 ± 0.89	2.88	4.13	10.27	4.13	8.11	10.23	2.88	2.88	2.88	2.88	2.88	4.71	2.14
Searsia lancea	Eth	0.79 ± 0.09	4.64 ± 0.78	4.04	6.08	6.08	4.34	4.85	5.52	4.04	4.04	4.04	4.04	4.04	4.34	4.94
Erythrina caffra	Ace	> 1	2.69 ± 1.18	2.13	2.13	3.19	3.19	1.60	2.56	2.13	2.13	2.13	2.13	2.13	3.19	6.25
Erythrina caffra	Eth	> 1	8.30 ± 2.05	8.55	8.55	8.55	12.82	8.55	8.55	8.55	8.55	8.55	8.55	8.55	6.41	3.23

STA = *Staphylococcus aureus*, NAS= Non-aureus staphylococci, Ace= Acetone, Eth= Ethanol, ATCC= American type culture collection, LC= Lethal concentration, SEM= Standard error of mean

3.4 Conclusion

Little is known about the antimicrobial activities of the selected plants in this study against *Staphylococcus aureus* and NAS isolated from clinical cases of bovine mastitis, though most of the plants in this study are known for their antibacterial activities. The crude extracts of the plants had good to moderate antibacterial activity against the investigated drug-resistant staphylococcal isolates of mastitis origin. The antibacterial activity of *S. lancea* was found to be better than the rest of the plants used in this study.

The acetone extracts showed better antibacterial activity compared to the ethanol extracts of all the plants except for *E. caffra*. The ethanol extracts of the three plants selected for cytotoxicity evaluation had lower toxicity which makes them good candidates for possible development into herbal products or for isolation of novel pure compounds that can serve as templates for new antimicrobial drugs which can be useful alternatives in the management of bovine mastitis.

It is imperative to determine the antibiofilm activities of the ethanol extracts of these selected plants on biofilm-forming mastitis staphylococcal strains. This will help in further understanding if the extracts which are active on the planktonic organism can also be active on staphylococcal biofilms. Furthermore, the knowledge of their antioxidant and anti-inflammatory activities is much desired since mastitis is an inflammatory disease involving significant oxidative stress. A plant-based preparation combining promising antibacterial, antioxidant and anti-inflammatory activity coupled with low cytotoxicity would assist in developing a product capable of inhibiting or reversing the disease's pathogenic processes.

4. Antibiofilm, antioxidant and anti-inflammatory activities of three South African plants with antibacterial activity against drug resistant staphylococci isolated from clinical cases of bovine mastitis

4.1 Introduction

Mastitis, an inflammation of the mammary gland, is among most important production disease of dairy animals, severely affecting farm economics by decreasing milk yield and increasing the cost of treatment (Srivastava and Kumaresan 2015). The inflammatory process in bovine mastitis has been linked repeatedly with oxidative stress, which is a major underlying cause of many metabolic and physiological changes, including immunosuppression (Politis et al. 2012; Sordillo and Aitken 2009; Sordillo 2005). This suppression of the immune response impairs how the organism counteracts inflammation, and thus cows are more prone to mastitis (Jóźwik et al. 2012; Sordillo 2005; Cai et al. 1994). In another perspective, a study has shown that the levels of whey proteins and oxidative stress markers change depending on the bacterial strain inducing inflammation, and selected whey proteins can be a marker for the diagnosis of individual mastitis-inducing strains. The study concluded that *Staphylococcus* spp. infection increased the β-lactoglobulin (BLG) level in the milk of infected cows (Puppel et al. 2020).

It has been confirmed that most microbial infections are associated with bacterial biofilms (Jamal et al. 2015). *Staphylococcus* spp. isolated from clinical cases of bovine mastitis include strains that are biofilm formers (Phophi et al. 2019). The ability to produce enzymes such as coagulase and biofilm-producing capacity are important virulence factors of *S. aureus* (Otto 2010; Qiu et al. 2010). Apart from antimicrobial resistance, bacterial biofilm formation is the most significant of all the factors playing an important role during establishment of disease inside the body (Jamal et al. 2015).

Biofilms can be considered as complex communities of microorganisms on a living or non-living surface, living together within a matrix of amorphous extracellular polymeric substance (EPS) produced by them, which is composed of exo-polysaccharides, extracellular DNA, lipids and proteins (Hall-Stoodley et al. 2004; Jamal et al. 2015). Many species of bacteria can communicate with one another through quorum-sensing, a mechanism for co-ordination of gene expression

during biofilm formation (Jamal et al. 2015). Bacteria may remain less accessible to antibiotics and the immune system of the body inside the biofilms; therefore, biofilms have tremendous public health and clinical importance (Jamal et al. 2015). It is advantageous for bacteria to grow in a biofilm because microbial biofilms protect the bacteria in the biofilm's microenvironment as long as the conditions are favorable (Jefferson 2004). In biofilms, bacteria account for less than 10% of their dry mass, whereas the matrix can account for over 90% of their dry mass (Flemming and Wingender 2010). It is believed that this EPS helps the bacterial cells to live in close proximity and interact, which makes their behaviour profoundly different from their planktonic counterparts (Hu et al. 2012). These EPS provide an extra covering around the cells to provide a shield against various stresses. This makes the bacteria within the biofilm resistant to antibiotics and environmental stresses. This makes it easier for them to escape the host immune response while in a biofilm, therefore causing serious problems in industrial as well as in clinical settings (Alam et al. 2020; Kim and Park 2013; Hoiby et al. 2010).

Antioxidants are multifaceted molecules playing a crucial role in several cellular functions. There is by now well-established knowledge about their involvement in numerous processes associated with aging, including vascular damage, neurodegenerative diseases and cancer (Francolini and Piozzi 2020). An emerging area of application has been recently identified for these compounds in relation to findings indicating their ability to affect biofilm formation by some microbial pathogens, including *Staphylococcus aureus, Streptococcus mutans* and *Pseudomonas aeruginosa* (Francolini and Piozzi 2020). This finding of the potential benefits of the use of natural antioxidants as antimicrobial/antibiofilm compounds was confirmed by Francolini and Piozzi (2020) in a review of 65 articles. In the same paper, they also suggested that, generally, Gram-positive bacteria are more sensitive to antioxidants than Gram-negative species.

Based on the reported interrelation between mastitis, inflammatory responses, mastitic organisms and oxidative stress (Huma et al. 2020; Laliotis et al 2020; Puppel et al. 2020; Zigo et al. 2019), this study aimed to investigate the antibiofilm, antioxidant and anti-inflammatory activities of three South African plants that showed antibacterial efficacy against drug-resistant *Staphylococcus* spp. isolated from clinical cases of bovine mastitis (Chapter 3). Plant extracts prepared using ethanol, and bioactive plant compounds have been shown by researchers in recent studies to have antibiofilm, antioxidant and anti-inflammatory activities (Alam et al. 2020; Lahiri et al. 2019).

However, no information was found on work done using the selected plant species (chapter 3 of this study) and clinical isolates of bovine mastitis. Therefore, this study was initiated to investigate whether the selected plant species have antibiofilm, antioxidant and anti-inflammatory activities.

The three selected plant species are *Antidesma venosum, Erythrina caffra* and *Searsia lancea*. The plants were selected based on their average minimum inhibitory concentrations (MIC) of the ethanol extracts. The three plant species had the lowest average MIC values. Investigation of the antibiofilm activities of these extracts will additionally give more insight into the mode of action of these extracts.

4.2 Materials and Methods

4.2.1 Collection of plant material, drying, storage and preparation of the extracts.

The leaves of the plants were harvested from the Manie van der Schijff Botanical Garden at the University of Pretoria and Onderstepoort campus of University of Pretoria. Herbarium voucher specimens were prepared and deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria, South Africa (Table 3.1). Harvested leaves were processed, and the extracts were prepared as previously described in sections 3.2.2 and 3.2.3 of Chapter 3.

4.2.2 Test bacterial strains

Staphylococcus epidermidis ATCC 35984 and a clinical isolate of *S. aureus*, both of which are known biofilm formers (Phophi et al. 2019) were obtained from the collection of the Onderstepoort Milk Laboratory, Faculty of Veterinary Science, University of Pretoria and used in this study. Tryptic Soy agar (TSA) and Tryptic Soy broth (TSB) were used in the biofilm assays. The bacteria were grown on TSA at 37°C for 18-24 h. The bacterial colonies were then cultured in TSB for at least 18-24 h at 37^{o}C.

4.2.3 Antibiofilm assay

4.2.3.1 Inhibition of biofilm formation – prevention of initial bacterial cell attachment

The potential of the extracts to prevent initial cell attachment was investigated using the biofilm inhibition assay (Sandasi et al. 2008). Briefly, a 100 µl aliquot of standardized concentration of cultures with OD_{560} = 0.02 (equivalent to 1.0 x 10^6 CFU/ml) *S. aureus*, was added into individual flat-bottomed 96-well microtitre plates and incubated at 37°C for 4 h without shaking. Then the plates were removed from the incubator and 100 µl (2 mg/ml) aliquots of plant extracts were added in replicates into the wells of 96-well microtitre plates to give a final concentration of 1 mg/mL and then incubated further at 37°C for 24 h without agitation. Gentamicin (Virbac) served as positive control while the solvents and sterile distilled water served as negative controls. The biomass was quantified using the modified crystal violet staining method (Djordjevic et al. 2002). Established criteria as determined by Sandasi et al. (2008) indicate that percentage inhibition values between 0 to ≥ 100% signify inhibition of biofilm, while values below 0% signify enhancement of growth. Inhibition values above 50% are regarded as good activity, while those between 0 and 49% reflect poor activity. These criteria will be adopted in the interpretation of results obtained in this study (Tables 4.1 and 4.2).

4.2.3.2 Inhibition of development of pre-formed biofilms – assessment of destruction of biofilm mass

The ability of the extracts to prevent further biofilm development or destruction of pre-formed biofilms was investigated. A 100 µl aliquot of standardized concentration of cultures at 1.0 x 10^6 CFU/ml) *Staphylococcus aureus* was added into each well of flat-bottomed 96-well microtitre plates and incubated at 37°C for 24 h (to reach irreversible attachment phase) and 48 h (mature biofilm) without shaking for the development of a multilayer biofilm. Following respective incubation periods, 100 µl aliquots of plant extracts (at 2 mg/mL) were added into the wells of the 96-well microtitre plates to give a final concentration of 1 mg/mL and the plates were incubated at 37°C for a further 24 h. Gentamicin (Virbac) served as positive control while solvents and sterile distilled water served as negative controls. The biofilm biomass was assayed using the modified crystal violet (CV) staining assay (Djordjevic et al. 2002).

4.2.3.3 Crystal violet staining assay

The assay was done as previously described (Djordjevic et al. 2002) with some modifications (Sandasi et al. 2008). Briefly, the 96-well microtitre plates were washed five times with sterile distilled water, air dried and then oven-dried at 60°C for 45 min. The wells were then stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed thrice with sterile distilled water to remove unabsorbed stain. At this point, biofilms were observed as purple rings at the side of the wells. The semi-quantitative assessment of biofilm formation was done by adding 125 µl of ethanol to destain the wells. A 100 µl aliquot of the destaining solution was then transferred to a new sterile plate and the absorbance was measured at 590 nm using a microplate reader (BioTek Synergy). The mean absorbance of the samples was determined, and percentage inhibition of biofilm determined using the equation below (Sandasi et al. 2008):

$$\text{Percentage (\%) inhibition} = \frac{\text{(OD Negative control – OD Experimental)}}{\text{OD Negative control}} \times 100$$

4.2.4 Antioxidant activity assays

4.2.4.1 The 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The simple, quick and widely used method to measure the ABTS radical scavenging activity of the acetone crude extracts of the plants was used with slight modifications (Re *et al*., 1999). Ascorbic acid served as positive control, methanol as negative control, while extract without ABTS was the blank. Briefly, ABTS (160 µl) was mixed with extracts (40 µl) at different concentrations, incubated for 5 min in the dark, followed by measuring the absorbance at 734 nm using a microplate reader (Epoch, BioTek). The percentage of radical scavenging activity was calculated using the formula below:

Percentage (%) scavenging activity = 100 – [(absorbance of sample – absorbance of sample blank) × 100/(absorbance of control) – (absorbance of control blank)].

The 50% inhibitory concentration (IC_{50}) values of samples were obtained from the graph plotted as inhibition percentage against the concentrations. Each test was done in triplicate.

4.2.4.2 The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The ability of the acetone crude extracts to scavenge radicals was also determined using the DPPH assay as previously described (Brand-Williams et al. 1995) with slight modifications. Ascorbic acid and methanol served as positive and negative controls respectively while extract with methanol only served as blank control. Briefly, the DPPH solution (160 µL) was added to 40 µL of different crude extracts at different concentrations. The mixture was incubated in the dark room for 30 min, and the absorbance was measured at 517 nm on a microplate reader (Epoch, Biotek). The percentage of scavenging activity was calculated using the formula:

Percentage (%) scavenging activity = 100 – [(absorbance of sample – absorbance of sample blank) × 100 / (absorbance of control) – (absorbance of control blank)].

The 50% inhibitory concentration (IC_{50}) values of samples were obtained from the graph plotted as inhibition percentage against the concentrations. Each test was done in triplicate.

4.2.5 Anti-inflammatory assays

4.2.5.1 Lipoxygenase inhibition assay

The assay was done as previously described (Pinto et al. 2007). The principle of the assay is to measure the inhibition of the 15-lipoxygenase (15-LOX) enzyme activity by the plant extracts in the presence of linoleic acid based on the formation of the complex $Fe3^{+}$/xylenol orange which can be measured at an absorbance of 560 nm in a spectrophotometer. All extracts (10 mg/ml) were dissolved in DMSO and further diluted to 2 mg/ml in Tris-HCl buffer containing 20% DMSO. Briefly, 40 µl of the enzyme (15-LOX) was incubated with 20 µl of different concentrations (100 to 0.78 µg/ml) of the extracts or quercetin (positive control) at 25°C for 5 min. Then linoleic acid (40 µl of final concentration of 140 µM) was added and the mixture was incubated at 25°C for 20 min in the dark. The assay was then terminated by adding 100 µl of freshly prepared FOX reagent which consists of sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM) in methanol/water (9:1)]. The negative control consisted of only the 15-LOX solution and Tris-HCl buffer, substrate and FOX reagent while the blanks (background) contained 15-LOX and buffer, but the substrate (linoleic acid) was added after the FOX reagent. The lipoxygenase inhibitory

activity by the extracts was then determined by calculating the percentage of the inhibition of hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25°C using the formula below:

$[(A_{control} - A_{blank}) - (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$. Where, $A_{control}$ is the absorbance of control well, A_{blank} is the absorbance of blank well and A_{sample} is the absorbance of extract well.

4.2.5.2 Nitric oxide (NO) production inhibition assay

4.2.5.2.1 Cell culture and sample testing

The RAW 264.7 macrophage cell line obtained from the American Type Culture Collection (ATCC, CRL-2278™) (Rockville, MD, USA) was used to determine the ability of the crude extracts to inhibit the production of nitric oxide when the cells were stimulated with lipopolysaccharide (LPS; Sigma) by measuring the amount of nitrite produced by the cells. Macrophages were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/fungizone (PSF) at 37°C with 5% CO_2. The cells were sub-cultured twice weekly at 75-80% confluence. Cells were seeded at a concentration of 2×10^6 cells/ml in 96-well microtitre plates and incubated overnight at 37°C with 5% CO_2 to allow for attachment. Then the cells were activated by incubation in medium containing 2 µg/ml of LPS alone (negative control) and treated simultaneously with different concentrations of the extracts or quercetin (positive control) dissolved in DMSO (concentration did not exceed 0.2%). Cells were incubated for 24 h at 37°C with 5% CO_2.

4.2.5.2.2 Nitrite measurement

The release of nitrite from the RAW 264.7 macrophages was determined by measuring the concentration of nitrite in culture supernatants using the Griess reagent (Griess 1879). Briefly, after incubation for 24 h, 100 µl of cell supernatant from each well was transferred into a new 96-well microtitre plate and an equal volume of Griess reagent was added. The absorbance of the mixture was read after 15 min of incubation in the dark at room temperature at 550 nm using a microtitre plate reader (Epoch Biotek). Nitrite concentrations were calculated from a standard curve obtained from regression analysis of different concentrations of sodium nitrite. The percentage of nitric oxide inhibition was calculated based on the ability of extracts to inhibit nitric

oxide formation by macrophages compared with the control (cells treated with LPS only). All experiments were done in triplicate

4.2.6 Statistical analysis

. Data were collated and analyzed using Microsoft Excel Version 2010. The mean and standard error, or mean and standard deviations where appropriate, for the different assays, were calculated. Percentages were also calculated. The IC_{50} and LC_{50} were determined using linear and non-linear regression curves where necessary.

4.3 Results and discussion

4.3.1 Antibiofilm activities of the plant extracts

4.3.1.1 Prevention of cell attachment: antibiofilm activity / anti-adhesion

Four crude extracts were used in the antibiofilm assay (Table 4.1). The effect of the extracts of *A. venosum*, *E. caffra* and *S. lancea* on the adhesion of the bacterial cells or the inhibition of biofilm formation was investigated.

Table 4.1 Prevention of biofilm formation by selected plant extracts.

Prevention of biofilm formation (%)		
Extracts	*Staphylococcus epidermidis* (ATCC 35984)	*Staphylococcus aureus* (Clinical isolate, known biofilm former)
Ethanol extract of *A. venosum*	117.08	108.19
Ethanol extract of *E. caffra*	162.57	131.20
Ethanol extract of *S. lancea*	62.85	-100.68
Acetone extract of *S. lancea*	116.90	100.32

Against both organisms, the plant extracts had varying degrees of activity on the prevention of attachment of the bacterial cells (Table 4.1). All the four extracts had good biofilm prevention

activity (> 50%) except the ethanol extract of *S. lancea* which showed enhancement of *S. aureus* biofilm growth (-100.68%). This disparity in the activity of the two extracts of *S. lancea* follows a pattern observed in their antibacterial activities as the acetone extracts showed good activity while the ethanol extracts showed only moderate activity (Table 3.7). Compared to others, *E. caffra* had the best inhibition activities (162.57% and 131.20%) against both strains of *S. epidermidis* (ATCC) and *S. aureus*, respectively. Next in the order of activity was *A. venosum* (117.08% and 108.19%), followed by the acetone extract of *S. lancea* (116.90% and 100.32%), respectively. The ethanol extract of *S. lancea* also had reasonable anti-adhesion activity (62.85%) against *S. epidermidis* but, in sharp contrast, could not prevent the adhesion of *S. aureus* cells to form biofilm despite showing moderate antibacterial activity against all *S. aureus* isolates tested in the MIC assay. This suggests that the ethanol extracts of *S. lancea* contain compound(s) that demonstrate(s) antibacterial activity against *S. aureus* but do not contain compound(s) that interrupt the biofilm-forming mechanism of the bacteria. All other extracts contain compound(s) that both inhibit the growth of the planktonic cells of the two organisms as well as disrupt the adhesion of the bacterial cells to surfaces. However, this does not suggest that both activities are caused by the same compound(s), therefore further research should be carried out to determine the antibacterial and antibiofilm activities of the constituent compounds of the extracts since different extraction methods extract different compounds Elsewhere (Ofek et al. 2003; Sandasi et al. 2008), some extracts with ≤ 0% inhibition were interpreted as enhancing the formation of biofilm by the organisms. They attributed this to the presence of metabolites or production of conditioning films for microbial adhesion that can enhance the growth and development of biofilms.

In this study, all the extracts had greater anti-adhesion activity against *S. epidermidis* (ATCC strain) than the *S. aureus* isolate. Further research needs to be done to explain this observation. From the above observations, all the plants had good anti-attachment activity against the two bacterial strains. A literature search yielded no similar research on mastitis-causing *Staphylococcus* spp. and any of the above extracts, therefore, to the best of my knowledge this work is the first reported of anti-biofilm activity of *S. lancea*, *E. caffra* and *A. venosum* against mastitis-causing *Staphylococcus* spp.

4.3.1.2 Inhibition of development of pre-formed biofilms – assessment of destruction of biofilm mass

The ethanol extract of *A. venosum* showed good activity against preformed biofilms after 24 and 48 h incubation of both bacterial species. The highest inhibition activity (171.64%) was observed against the 48 h preformed biofilm of *S. epidermidis*, followed by 76.82% inhibition against the 24 h preformed biofilm. Similarly, the extract's inhibition (74.85%) against 48 h preformed biofilm of *S. aureus* was more (74.03%) than that of 24 h. *E. caffra*'s ethanolic extract showed a different pattern of inhibition against both organisms. It showed good activity against all the biomass except the 24 h biomass of *S. epidermidis* against which the activity was poor (30.79%). It had highest inhibition (102.63%) against the 24 h biofilm of *S. aureus* followed by the inhibition (79.11%) of 24 h biofilm of *S. epidermidis*. The same pattern was observed against 48 h preformed biofilms of both bacteria. Unlike other plants in this study, both extracts of *S. lancea* could not disrupt the 24 h biofilm of *S. epidermidis* and *S. aureus*, while the acetone extract showed the best activity against 48 h biofilm of *S. epidermidis*.

Table 4.2: Prevention or reduction of biofilm development in 24 and 48 h preformed biofilms by selected plant extracts.

Prevention of biofilm formation (%)				
	Staphyloccous epidermidis (ATCC)		*Staphylococcus aureus*	
Extracts	**24 h**	**48 h**	**24 h**	**48 h**
Ethanol extract of *Antidesma venosum*	76.82	171.64	74.03	74.85
Ethanol extract of *Erythrina caffra*	79.11	30.79	102.63	83.29
Ethanol extract of *Searsia lancea*	-143.12	112.75	72.75	110.54
Acetone extract of *Searsia lancea*	-31.15	199.41	81.57	63.62

Generally, the highest inhibition (199.41%) was noted with the acetone extract of *S. lancea* against the 48 h preformed biofilm of *S. epidermidis*. The ethanol extracts of *A. venosum* and *S. lancea* were better at disrupting the 48 h biofilms while the *E. caffra* extract was more effective against

the 24 h biofilms of both organisms. This suggests that *S. epidermidis* (a NAS strain) is more susceptible to the antibiofilm activities of the plant extracts than *S. aureus*.

Similarly, the ethanolic crude leaf extracts of *Hypericum perforatum* showed inhibition of 56.85% against *S. aureus* biofilm formation after 24 h of (Nazli et al. 2019). Extracts from *S. gerrardii* and *Syzygium aromaticum* have also been suggested to be valuable in combating *Staphyloccocus* infections associated with mastitis in the dairy industry (Budri et al. 2015). Budri et al. (2015), reported a significant inhibition of *S. aureus* biofilm production by essential oils of *Syzygium aromaticum* on polystyrene and stainless-steel surfaces (69.4 and 63.6%, respectively). However, its major component, eugenol, was less effective on polystyrene and stainless steel (52.8 and 19.6%, respectively). Both essential oil of *Cinnamomum zeylanicum* and its major component, cinnamaldehyde, significantly reduced *S. aureus* biofilm formation on polystyrene (74.7 and 69.6%, respectively) and on stainless steel surfaces (45.3 and 44.9%, respectively) (Budri et al. 2015). Of the nine extracts tested against *S. aureus* by Famuyide et al. (2019), only *Syzygium masukuense* and *Syzygium* species acetone extracts did not prevent the attachment of the bacteria although these two plants had good MICs. *Eugenia natalitia* and *E. woodii* extracts, however, had poor activity with values between 0 and 50% (Famuyide et al. 2019).

Different classes of compounds in the plant extracts may be responsible for the various antibiofilm activities observed in this study. In a recent review, natural compounds (e.g., cinnamaldehyde) from plants have been shown to have antibiofilm activity against different bacterial pathogens such as *S. aureus*, NAS, *P. aeruginosa* and MRSA (Manilal et al. 2020; Erhabor et al. 2019; Song et al. 2018; Budri et al. 2015). Further studies should be done to investigate the compounds that are responsible for the antibiofilm activities of these plants.

Some reports have suggested that it is less difficult to inhibit cell attachment than to eliminate an established biofilm (Manilal et al. 2020; Sandasi et al. 2011; Cerca et al. 2005). This seems to be the pattern observed in this study. This favours the assumption that pathogens are able to resist the action of antimicrobials more when they exist in biofilms, and the infections are able to persist on different biotic and abiotic surfaces (Famuyide et al. 2019). Some of the extracts in this study did, however, inhibit biofilms at different stages of development, thus highlighting their potential usefulness in clinical applications. Such applications may help to enhance the immunological

defense of infected hosts against bacterial cell populations, especially those in biofilms, and subsequent host clearance and reduction of disease symptoms (Sathiya Deepika et al. 2018).

4.3.2 Antioxidant activities of the plant extracts

The results of the DPPH and ABTS radical scavenging activity of the three selected plant leaf crude extracts are presented in Table 4.3. The ethanol extract of *S. lancea* had the best antioxidant activity against the ABTS and DPPH radicals with IC_{50} values of 8.03 µg/ml and 22.69 µg/ml respectively when compared to the other extracts. The controls (Vitamin C and Trolox) had IC_{50} values of 0.02 and 1.46 against ABTS, and 4.47 and 4.35 against the DPPH radical, respectively. The results obtained in the present study indicate that *A. venosum*, *S. lancea* and *E. caffra* ethanolic crude extracts demonstrate antioxidant activities.

Table 4.3: Antioxidant potential of the three selected South African medicinal plant

	ABTS	**DPPH**
Samples	**IC_{50} (µg/ml)**	**IC_{50} (µg/ml)**
Acetone extract of *Antidesma venosum*	60.93 ± 11	> 100
Ethanol extract of *Antidesma venosum*	14.61 ± 1.37	36.18 ± 0.63
Acetone extract of *Erythrina caffra*	> 100	> 100
Ethanol extract of *Erythrina caffra*	54.57 ± 0.44	> 100
Acetone extract of *Searsia lancea*	23.93 ± 0.14	48.12 ± 1.94
Ethanol extract of *Searsia lancea*	8.03 ± 1.63	22.69 ± 2.07
Ascorbic acid	0.02 ± 0.03	4.47 ± 0.47
Trolox	1.46 ± 0.13	4.35 ± 0.46

The antioxidant activity of the plants showed their potential to boost immunity by reducing oxidative stress which may occur due to generation of deleterious reactive oxygen or nitrogen species and lipid peroxidation associated with conditions such as microbial infection, cardiovascular diseases, cancer, Parkinson's disease and Alzheimer's disease (Saba et al. 2018). A comprehensive literature search did not reveal much on the antioxidant activities of the crude extracts of these plants. One of the available studies includes the work of Gundidza et al. (2008).

They reported that the essential oil from *S. lancea* showed antioxidant activity similar to that of ascorbic acid, the positive control used in their study. They suggested the antioxidant effect may be due to the presence of the monoterpene α-pinene which acts as a radical scavenging agent (Gundidza et al. 2008). It seems to be a general trend that essential oils, which contain monoterpene hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes have good antioxidative properties (Tepe et al. 2004; Burits et al. 2001; Mau et al. 2003). In a recent review of the antioxidant activities of *Erythrina* spp., *E. caffra* was not included, but it was highlighted that most species in the genus have antioxidant activities (Jiménez-Cabrera et al. 2021).

4.3.3 Anti-inflammatory activities of the plant extracts

4.3.3.1 Lipoxygenase (LOX) inhibition

The lipoxygenase pathway is responsible for the formation of inflammatory mediators such as leukotrienes and 5S-hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid (5-HETE) during arachidonic acid metabolism (Shah et al., 2011). In this study, the potential of the extracts of the plants to inhibit the activity of 15-lipoxygenase was determined using the 96-well microplate-based ferric oxidation of xylenol orange (FOX) assay. The ethanol extract of *S. lancea* had the highest activity (IC_{50} = 48.55µg/ml) which is above that of quercetin (positive control) followed by the acetone extract of the same plant (Table 4.4). Ahmed et al. (2014) reported that *Searsia leptodictya* had IC_{50} above 100 µg/ml. The lipoxygenase enzymes have been implicated in a number of pathological states, and LOX products stimulate neutrophil migration, increase adhesion of leukocytes to endothelial cells, cause smooth muscle contraction, increase vascular permeability, and increase ion and mucus secretion (Eberhart and Dubois, 1995). Thus, inhibition of LT synthesis may be useful for the treatment animal inflammatory conditions. Inhibition of soybean 15-lipoxygenase is generally regarded as predictive for inhibition of the mammalian enzyme (Gleason et al., 1995; Gundersen et al., 2003; Lapenna et al., 2003).

4.3.2.2 Nitric oxide (NO) production inhibition

In this study, the nitric oxide production inhibitory activity of plant extracts was determined using LPS-stimulated RAW 264.7 macrophages. Four different concentrations (1.6, 12.5, 50 and 100 µg/mL) of the extracts were used and their cellular viability was also determined using the MTT

assay. None of the extracts caused cellular toxicity at the highest concentration (100 µg/mL), which means that the inhibitory effects on the NO production of the macrophages were not due to toxicity. The NO inhibitory effects of the plants were concentration dependent. At a concentration of 100 µg/ml, the acetone extract of *S. lancea* had NO inhibition activity of 63.38% with cell viability of >70% and IC_{50} value of 67.21 µg/ml, while the ethanol extract of *A. venosum* had NO inhibition activity of 38.09% with cell viability of >100% and IC_{50} value of 152.89 µg/ml (Table 4.5 and 4.6). The activity displayed by these plant extracts showed their anti-inflammatory potential which may be valuable in reversing and/or preventing inflammatory activities which have been documented in mastitis pathogenesis (Huma et al. 2020; Laliotis et al 2020; Puppel et al. 2020; Zigo et al. 2019).

Table 4.4: The half maximal lipoxygenase inhibitory concentration (IC_{50}) in µg/ml

Plant species	Extracts	Mean± SD
A. venosum	Ethanol	165.33 ± 153.42
E. caffra	Ethanol	148.02 ± 161.63
S. lancea	Ethanol	48.55 ± 20.94
S. lancea	Acetone	82.05 ± 14.59
Quercetin		24.69 ± 1.43

SD= Standard deviation, µg/ml= microgram per millilitre

Table 4.5: Nitric oxide inhibitory concentration (NO IC_{50}) of the selected crude extracts in µg/ml

Plant species	Extracts	Mean ± SD
A. venosum	Ethanol	152.89 ± 0.23
S. lancea	Acetone	67.21 ± 1.07
Quercetin		24.69 ± 0.061.43

SD= Standard deviation, µg/ml= microgram per millilitre

Table 4.6: Inhibitory activities of the selected crude extracts on nitric oxide (NO) production and cell viability in LPS-activated RAW macrophages

Plant extracts	Conc. (µg/ml)	%±SD	% ± SD
Ethanol extract of *S. lancea*	1.6	17.27 ± 3.66	108.06 ± 29.97
	12.5	21.58 ± 2.58	94.24 ± 11.42
	50.0	47.14 ± 16.51	109.96 ± 2.43
	100.0	63.38 ± 14.72	73.46 ± 6.32
Ethanol extract of *A. venosum*	1.6	8.38 ± 3.72	92.06 ± 34.08
	12.5	10.81 ± 4.24	89.31 ± 12.53
	50.0	12.73 ± 7.58	104.60 ± 4.77
	100.0	38.09 ± 7.30	103.17 ± 9.86
Quercetin	1.6	53.84 ± 6.37	99.82 ± 5.42
	12.5	89.18 ± 7.32	83.35 ± 7.66
	50.0	93.46 ± 1.71	63.97 ± 3.77
	100.0	95.68 ± 2.89	39.54 ± 4.31
Doxorubicin	2.0		79.36 ± 9.34
	4.0		62.51 ± 13.87
	10.0		9.42 ± 4.95
	20.0		1.46 ± 0.80

SD= Standard deviation, µg/ml= microgram per millilitre

4.4 Conclusion

This part of the study investigated whether the selected plants have antibiofilm, antioxidant and anti-inflammatory activities. This was done to explore the existence of complementary mechanisms in the plant extracts that may contribute to the management of pathogenic processes (infection, oxidative stress and inflammation) in bovine mastitis and other infectious disease conditions. The ethanol extracts of the three plants and acetone extract of *S. lancea* were tested in the biofilm, ABTS, DPPH and LOX assays, while only the ethanol extracts of *S. lancea* and *A.*

venosum were assayed in the NO assay because they had the best antioxidant activities among all the extracts.

The extracts showed potential to act as anti-biofilm agents due to their good anti-adherence activities against one or both strains of *S. aureus* and *S. epidermidis* used in the assay. *A. venosum* and *E. caffra* showed good activities against all forms of biofilms of both organisms. This study is the first to investigate the anti-biofilm activities of these plants against biofilm-forming *S. aureus* isolated from a clinical case of bovine mastitis and the ATCC strain of *S. epidermidis*. These species are the most commonly implicated organisms in bovine mastitis. Further studies need to be conducted to investigate the chemical components of the extracts responsible for the antibiofilm activities of the plants.

The plants, especially *S. lancea* and *A. venosum,* also demonstrated good potential to prevent or reverse oxidative stress normally triggered by bacterial infections in mastitis with their good antiradical activities. *S. lancea* had the best antioxidant activity, the best 15-LOX inhibitory activity and the best nitric oxide inhibitory activity. Since overly expressed and unregulated pro-inflammatory cytokines can be damaging beyond the control of infections, the potential of the plants to reduce nitric oxide production and 15-LOX activity demonstrates useful anti-inflammatory properties which may help to contain the inflammation within physiological limits. These activities show that these plants can help to reduce the burden of oxidative stress and inflammation in cases of bovine mastitis. Therefore, they are worthy of further investigation to determine the molecular mechanisms responsible for these observations. This study suggests that the plant extracts demonstrated antibiofilm, antioxidant and anti-inflammatory activities and they therefore have potential in contributing to the management of bovine mastitis infections.

5. General conclusions and recommendations

5. General conclusions

The purpose of this study was to evaluate the antibacterial, antibiofilm, antioxidant, anti-inflammatory and cytotoxic activities of acetone and ethanol extracts of nine selected South African medicinal plants, with the intention of determining their potential usefulness in the management of bovine mastitis. Since the pathogenesis of infectious bovine mastitis includes oxidative stress and inflammation, plants which possess antibacterial, antioxidant and anti-inflammatory activities, and are non-cytotoxic, have potential for development of treatments useful in the management of the disease. Strains of the most commonly implicated bacteria in mastitis were used to determine the antibacterial and antibiofilm activities of the plant extracts. These strains include isolates from clinical cases of mastitis: eight *Staphylococcus aureus* isolates, three *Staphylococcus chromogenes* isolates, one *Staphylococcus haemolyticus* isolate, one ATCC strain of *Staphylococcus aureus* and one ATCC strain of *Staphylococcus epidermidis*. The leaf extracts of the selected plants were assessed for their antibacterial, antibiofilm, antioxidant, anti-inflammatory and cytotoxic activities. In order to achieve the aim of the study, four objectives were set and they are summarized below.

5.1. To determine the antibacterial activity of selected South African plants against multidrug-resistant staphylococcal isolates from clinical cases of bovine mastitis

The acetone and ethanol extracts of the selected plant species were subjected to antibacterial assays. The broth serial microdilution method was used to determine the MIC activity of the nine plant extracts against the selected strains. All the plant extracts demonstrated good to weak antibacterial activity with MIC values ranging between 0.01 – 1.41 mg/ml. The lowest MIC value of 0.01 mg/ml obtained in this study was recorded with the acetone extract of *Searsia lancea* while the highest MIC value of 1.41 mg/ml was recorded with the acetone extract of *Trichilia emetica*. Generally, the acetone extracts of all the plants showed better activity than their ethanol counterparts except for *Erythrina caffra.* The average MIC value of the ethanol extract of *E. caffra* against all bacteria was less than half of the average MIC value of the acetone extract.

The results suggest that the *S. aureus* strains showed a broader range of susceptibility to both extracts of all the plants and appeared to be more susceptible to the extracts than the NAS strains. Meanwhile, the NAS strains were more susceptible to the extracts over a shorter concentration range than *S. aureus* strains which were susceptible over a wider concentration range. Further research needs to be conducted to unravel the reason for this pattern. The total antibacterial activity was determined, and both extracts of *S. lancea* had the highest mean total antibacterial activity.

The drug resistant isolates used in this study were generally more susceptible to the extracts than the ATCC strain which was more susceptible to gentamicin. This raises questions about the potential mechanisms of action of the extracts. The antibacterial activity of the extracts may be due to another mode of activity different from that of gentamicin. Hence, alternative strategies that focus on pathogenicity pathways of bacteria should be investigated to further understand how the extracts effect their antibacterial activity.

5.2. To determine cytotoxicity of the active plant species to evaluate selective antibacterial efficacy

The MIC range of the plant extracts against the mastitis isolates reported in this study was relatively low and promising. This called for a need to determine how toxic the extracts are to living mammalian cells. In this study, all the plant extracts had LC_{50} values higher than the recommended cytotoxic concentration cut-off of 0.02 mg/ml. Both acetone and ethanol extracts of *E. caffra* had the lowest cytotoxicity. The LC_{50} of ethanol extracts of the plants were higher than their acetone counterparts which suggests that the ethanol extracts are relatively less toxic than the acetone extracts. This is significant for potential commercialization as ethanol is preferred as an extracting solvent because it is less flammable and dangerous to work with in large quantities than acetone.

The ethanol extract of *E. caffra* had the best mean SI against all the pathogens while the acetone extract of *A. venosum* had a poor mean SI. Both extracts of all the three plants in this study have potential to be developed into useful products in tackling antimicrobial infections as herbal remedies. Alternatively, isolation of active compounds can possibly provide templates for the development of new drugs.

5.3. To determine the antibiofilm activity of selected South African plants against a biofilm-forming ATCC strain of *Staphylococcus epidermidis* and a biofilm-forming *Staphylococcus aureus* isolate from clinical cases of bovine mastitis.

The antibiofilm investigation revealed that most of the plant extracts investigated had very good inhibitory activity and inhibited more than 50% of the test organism biofilm biomass, and both *E. caffra* and *Antidesma venosum* showed outstanding activities. The results also suggest that *S. epidermidis* (NAS) ATCC strain is more susceptible to the antibiofilm activities of the plant extracts than the *S. aureus* isolate. The antibiofilm activity of the four extracts of the leaves of the three plants is reported for the first time. Further studies need to focus on the virulence pathways of bacteria that control biofilm formation behaviour such as quorum sensing, to understand how the extracts effect their antibiofilm activity.

5.4. To determine the anti-inflammatory and antioxidant activities of selected plants.

The antioxidant activity investigation of the crude extracts of the three selected plants showed that the ethanol extract of *S. lancea* had the best antioxidant activity against the ABTS and DPPH radicals when compared to the other extracts. The ethanol extract of *S. lancea* also showed the best anti-inflammatory activity when compared to the other extracts of the plants investigated.

5.5. Recommendations for future work

Much further work is possible in this study. The MBC values for those extracts with MIC values less than 1.0 mg/ml need to be determined. Potential for synergistic antibacterial activity of the plant extracts needs to be examined to determine whether combinations of the extracts or the active compounds will offer more therapeutic value than the single extract or active compound. It is also possible that the extracts may be used in combination with synthetic antibiotics to potentiate their activity and assist in overcoming bacterial resistance as resistance modifiers. The active compounds need to be isolated and identified from the most active plant extracts to provide possible marker compounds if an active extract or fraction is to be developed for use against mastitis pathogens. The mechanism of action of the active plant preparations (extracts, fractions,

or isolated compounds) needs to be elucidated to identify how they can be most useful in preventing or treating diseases caused by mastitis pathogens.

REFERENCES

Abdel-Rady, A., Sayed, M., 2009. Epidemiological studies on subclinical mastitis in dairy cows in Assiut Governorate. *Veterinary World* 2, 373–380. http://dx.doi.org/10.5455/vetworld.2009.373-380

Abebe, R., Hatiya, H., Abera, M., Megersa, B., Asmare, K., 2016. Bovine mastitis: prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. *BMC Veterinary Research*, 12, 1-11.

Abudoleh, S. M., Mahasneh, A. M., 2017. Anti-quorum sensing activity of substances isolated from wild berry associated bacteria. *Avicenna Journal of Medical Biotechnology*, 9, 23-30.

Adamu, H. O., Hussaini, O., Obasuyi, C., Anagha, L., Okoduwa, G. O., Rahimat, H., 2020. Prevalence of mastitis in Nigerian livestock: A Review. *Open Veterinary Science*, 1, 20-29.

Adamu, M., Naidoo, V., Eloff, J. N., 2014. The antibacterial activity, antioxidant activity and selectivity index of leaf extracts of thirteen South African tree species used in ethnoveterinary medicine to treat helminth infections. *BMC Veterinary Research*, 10, 52.

Adegoke, S. A., Agada, F. D., Ogundipe, L. O., 2013. Antibacterial activity of methanol and ethanol leaf extracts of *Antidesma venosum* and *Lannea barteri*. *African Journal of Microbiology Research*, 7, 3442-3447.

Alam, K., Al Farraj, D. A., Mah-e-Fatima, S., Yameen, M. A., Elshikhb, M. S., Alkufeidy, R. M., Mustafa, A. E. M. A., Bhasme, P., Alshammari, M. K., Alkubaisi, N. A., Abbasi, A. M., Naqvi, T. A., 2020. Anti-biofilm activity of plant derived extracts against infectious pathogen-Pseudomonas aeruginosa PAO1. *Journal of Infection and Public Health*, 13, 1734 -1741.

Aliyu, A. B., Koorbanally, N. A., Moodley, B., Singh, P., Chenia, H. Y., 2016. Quorum Sensing Inhibitory potential and molecular docking studies of sesquiterpene lactones from *Vernonia blumeoides*. Phytochemistry 126, 23–33.

Ameh, J. A., Edgbe-Nwiyi, T., Zaria, l. T., 1999. Prevalence of bovine mastitis in Maiduguri Borno State, Nigeria. *Veterinary Archives*, 69, 87-95.

Ateba, C. N., Mbewe, M., Moneoang, M. S., Bezuidenhout, C. C., 2010. Antibiotic-resistant *Staphylococcus aureus* isolated from milk in the Mafikeng Area, North West province, South Africa. *South African Journal of Science*, 106, 1-6.

Awale, M. M., Dudhatra, G. B., Avinash, K., Chauhan, B. N., Kamani, D. R., Modi, C. M., 2012. Bovine mastitis: A threat to economy. *Scientific Reports*, 1, 295.

Azmi, D., Al-Dabbas, H., Al-Dabbas, F., 2008. Prevalence and distribution of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Jordan. *American Journal of Animal and Veterinary Sciences*, 3, 36–39. http://dx.doi.org/10.3844/ajavsp.2008.36.39

Bachaya, H. A., Raza M. A., Murtaza, S., Akbar, I. U. R., 2011. Subclinical bovine mastitis in Muzaffar Garh District of Punjab (Pakistan). *The Journal of Animal & Plant Sciences*, 21, 16-19.

Bagadi, H.O., 1970. The aetiology of bovine mastitis in three areas in Sudan. *Tropical Animal Health and Production*, 2, 28–34. http://dx.doi.org/10.1007/BF02359326

Bah, B., Jäger, A. K., Adsersen, A., Diallo, D., Smestad-Paulsen, B., 2007. Antiplasmodial and GABA-benzodiazepine receptor binding activities of five plants used in traditional medicine in Mali, West Africa. *Journal of Ethnopharmacology*, 110, 451-457.

Bar, D., Tauer, L. W., Bennett, G., Gonzalez, R. N., Hertl, J. A., Schukken, Y. H., Schulte, H. F., Welcome, F. L., Gröhn, Y. T., 2008. The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. *Journal of Dairy Science*, 91, 2205-2214.

Barkema, H. W., Schukken, Y. H., Zadoks, R. N., 2006. The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *Journal of dairy science*, 89, 1877–95.

Barkema, H. W., Schukken, Y. H., Lam T. J., Beiboer, M. L., Benedictus, G., Brand, A., 1999. Management practices associated with the incidence rate of clinical mastitis. *Journal of Dairy Science*, 82, 1643-1654.

Begue, W. J., Kline, R. M., 1972. The use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography*, 64, 182.

Ben Hassen, S., Messadi, L., Ben Hassen, A., 2003. Identification and characterization of Staphylococcus species isolated from cow's milk with and without mastitis. *Annales de Medecine Vétérinaire*, 147, 41–47.

Bengtsson, B., Unnerstad, H. E., Ekman, T., Artursson, K., Nilsson-Öst, M., Waller, K. P., 2009. Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows. *Veterinary Microbiology*, 136, 142–149.

Biffa D., Debela E., Beyene F., 2005. Prevalence and risk factors of mastitis in lactating dairy cows in Southern Ethiopia. *International Journal of Applied Research and Veterinary Medicine*, 3, 189-198.

Bisi-Johnson, M. A., Obi, C. L., Hattori, T., Oshima, Y., Li, S., Kambizi, L., Eloff, J. N., Vasaikar, S. D., 2011. Evaluation of the antibacterial and anticancer activities of some South African medicinal plants. *BMC Complementary Alternative Medicine*, 11, 1–5.

Bouchot, M. C., Catel, J., Chirol, C., Ganierf, J. P., Le Menec, M., 1985. Recording antimicrobial sensitivity to antibiotics in the treatment of mammary infection in cattle. *Recueil de Medecine Vétérinaire*, 161, 587–601.

Bradley, A.J., 2002. Bovine mastitis: an evolving disease. *The Veterinary Journal*, 164, 116–128.

Brand-Williams, W., Cuvelier, M.-E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. *LWT-Food science and Technology*, 28, 25-30.

Budri, P. E., Silva, N. C. C., Bonsaglia, E. C. R., Fernandes Júnior, A., Araújo Júnior, J. P, Doyama, J. T., Gonçalves, J. L., Santos, M. V., Fitzgerald-Hughes, D., Rall, V. L. M., 2015. Effect of essential oils of *Syzygium aromaticum* and *Cinnamomum zeylanicum* and their major components on biofilm production in *Staphylococcus aureus* strains isolated from milk of cows with mastitis. *Journal of Dairy Science*, 98, 5899–5904. http://dx.doi.org/10.3168/jds.2015-9442

Cai, T. Q., Weston, P. G., Lund, L. A., Brodie, B., McKenna, D. J., Wagner, W. C., 1994. Association between neutrophil functions and periparturient disorders in cows. *American Journal of Veterinary Research*, 55, 934–943.

Cerca, N., Martins, S., Pier, G. B., Oliveira, R., Azeredo, J., 2005. The relationship between inhibition of bacterial adhesion to a solid surface by sub-MICs of antibiotics and subsequent development of a biofilm. *Research in microbiology*, 156, 650-655

Cervinkova, D., Vlkova, H., Borodacova, I., Makovcova, J., Babak, V., Lorencova, A., Vrtkova, I., Marosevic, D., Jaglic, Z., 2013. Prevalence of mastitis pathogens in milk from clinically healthy cows. *Veterinarni Medicina*, 58, 567–575.

Cha, E., Bar, D., Hertl J.A., Tauer, L. W., Bennett, G., González, R. N., Schukken, Y. H., Welcome, F. L., Gröhn, Y. T., 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. *Journal of Dairy Science*, 94, 4476-4487

Chebel, R.C., 2007. Mastitis effects on reproduction. In NMC Regional Meeting Proceedings. University of California-Davis, Tulare, California, USA. 43–55.

Chukwujekwu, J. C., Kock, C. A., de Smith, P. J., Heerden, F. R., van Staden, J., 2016. Antiplasmodial Activity of compounds isolated from *Erythrina caffra*. *South African Journal of Botany*, 106, 101-103.

Clinical Laboratory Standards Institute, 2017. Laboratory standards institute antimicrobial susceptibility testing standards M02-A12, M07-A10, and M11-A8.

Collett, D. G., 1941. The South African species of Erythrina. *Bothalia*, 4, 219-228.

Craven, N., 1987. Efficacy and financial value of antibiotic treatment of bovine clinical mastitis during lactation - A review. *British Veterinary Journal*, 143, 410–422.

De Jager, 1963. Notes on the magical charms of the Cape Nguni tribes. *Fort Hare Papers*, 2, 293-309.

de Oliveira, A., Pereira, V. C., Pinheiro, L., Riboli, D. F. M., Martins, K. B., Cunha, M., 2016. Antimicrobial resistance profile of planktonic and biofilm cells of *Staphylococcus aureus* and coagulase-negative staphylococci. *International Journal of Molecular Science*, 17, 1423.

Delelesse, G. D., 2010. Study on prevalence of bovine mastitis on cross breed dairy cow around Holeta areas, West Shewa zone of Oromia, Ethiopia. *Global Veterinaria*, 5, 318-323.

Desta, Z. Y., Majinda, R. R. T., 2014. Three new isoflavonoids from *Erythrina caffra*. *Natural Product Communications*, 9, 817-820.

Desta, Z.Y., Sewald, N., Majinda, R. R. T., 2016. Cytotoxic flavonoids from *Erythrina caffra* Thunb. *Bulletin of the Chemical Society of Ethiopia*, 30, 427-435.

Djordjevic, D., Wiedmann, M., Mclandsborough, L., 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, 68, 2950-2958.

Dhanabalan, R., Doss, A., Jagadeeswari, M., Balachandar, S., Kezia, E., Parivuguna, V., Reena Josephine, C.M., Vaidheki, R., Kalamani, K., 2008. In vitro phytochemical screening and antibacterial activity of aqueous and methanolic leaf extracts of *Tridax procumbens* against bovine mastitis isolated *Staphylococcus aureus*. *Ethnobotanical Leaflets*, 144.

Diallo, D., Paulsen, B. S., Liljebäck, T. H. A., Michaelsen, T. E., 2003. The malian medicinal plant *Trichilia emetica*; studies on polysaccharides with complement fixing ability. *Journal of Ethnopharmacology*, 84, 279-287.

Doherr, M. G., Roesch, M., Schaeren, W., Schallibaum, M., Blum, J. W., 2007. Risk factors associated with subclinical mastitis in dairy cows on Swiss organic and conventional production system farms. *Veterinarni Medicina*, 52, 487-495.

Doss, A., 2012. In-vitro antibacterial activity of certain wild medicinal plants against bovine mastitis isolated contagious pathogens. *Asian Journal of Pharmaceutical and Clinical Research*, 5, 90–93.

Du Preez, J. H., 2000. Bovine mastitis therapy and why it fails: continuing education. *Journal of the South African Veterinary Association*, 71, 201–208.

Dzoyem, J. P., Aro, A. O., McGaw, L. .J., Eloff, J. N., 2016. Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal plants used in southern Africa to treat tuberculosis and respiratory ailments. *South African Journal of Botany*, 102, 70-74

Ebojei, C. O., Davou, G. C., Salman, B. A., Mosimabale, V. O., 2010. Prevalence of mastitis among dairy cattle in Kanam Local Government Area of Plateau state, Nigeria. *Human and Veterinary Medicine International Journal of Bioflux*, 2, 77-81.

Elgorashi, E. E., Taylor, J. L. S., Maes, A., Kimpe, N., de Staden, J., Verschaeve, L., 2002. The use of plants in traditional medicine: potential genotoxic risks. *South African Journal of Botany*, 68, 408-410.

El Tahir, A., Satti, G. M. H., Khalid, S. M., 1999. Antiplasmodial activity of selected Sudanese medicinal plants with emphasis on *Maytenus senegalensis* (Lam.) Exell. *Journal of Ethnopharmacology*, 64, 227-233.

El-Zubeir, I. E. M., Ahmed, M. I. A., 2007. The hygienic quality of raw milk produced by some dairy farms in Khartoum State, Sudan. *Research Journal of Microbiology*, 2, 988 – 991.

Elisha, I. L., Botha, F. S., Madikizela, B., McGaw, L. J., Eloff, J. N., 2017a. Acetone leaf extracts of some South African trees with high activity against *Escherichia coli* also have good antimycobacterial activity and selectivity index. *BMC Complementary Alternative Medicine*, 17, 327

Elisha, I. L., Botha, F. S., McGaw, L. J., Eloff, J. N., 2017b. The antibacterial activity of extracts of nine plant species with good activity against *Escherichia coli* against five other bacteria and cytotoxicity of extracts. *BMC Complementary Alternative Medicine*, 17, 133.

Elisha, I. L., Dzoyem, J-P., McGaw, L. J., Botha, F. S., Eloff, J. N., 2016. The anti-arthritic, anti-inflammatory, antioxidant activity and relationships with total phenolics and total flavonoids of nine South African plants used traditionally to treat arthritis. *BMC Complementary and Alternative Medicine*, 16, 307.

Eloff, J. N., 1998a. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta medica*, 64, 711-713.

Eloff, J. N., 1998b. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of ethnopharmacology*, 60, 1–8.

Eloff, J. N., 2000. On expressing the antibacterial activity of plant extracts: a small first step in applying scientific knowledge to rural primary health care. *South African Journal of Science*, 96, 116-118.

Eloff, J. N., 2001. Antibacterial activity of Marula (*Sclerocarya birrea* (A. rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro) (Anacardiaceae) bark and leaves. *Journal of Ethnopharmacology*, 76, 305-308.

Eloff, J. N., McGaw, L. J. 2006. Plant extracts used to manage bacterial, fungal and parasitic infections in southern Africa. *Modern phytomedicine: turning medicinal plants into drugs*, 97-121.

Endale, M., Eyob, E., Addisu, A., Naod, T., 2016. A Study on the prevalence of bovine mastitis and associated risk factors in and the surrounding areas of Sodo Town, Wolaita Zone, Ethiopia. *Global Journal of Medical Research: Global Veterinary Science and Veterinary Medicine*, 16, 16-24.

Erhabor, C. R., Erhabor, J. O., McGaw, L. J., 2019. The potential of South African medicinal plants against microbial biofilm and quorum sensing of foodborne pathogens: A review. *South African Journal of Botany*, 126, 214–231.

Fallet, D., 1999. Some aspects of the epidemiology of clinical mastitis in dairy cows. Literature review and survey results, PhD thesis, University Claude Bernard, Lyon.

Flemming, H. C., Wingender, J., 2010. The biofilm matrix. *Natural Review Microbiology*, 8, 623–633.

Fox, K., Fox, A., Rose, J., Walla, M., 2011. Speciation of coagulase negative staphylococci, isolated from indoor air, using SDS page gel bands of expressed proteins followed by MALDI TOF MS and MALDI TOF-TOF MS-MS analysis of tryptic peptides. *Journal of Microbiology Methods*, 84, 243–250.

Fox, L. K., Gay, J. M., 1993. Contagious mastitis. *The Veterinary Clinics of North America. Food Animal Practice*, 9, 475-487

Francolini, I., Piozzi, A., 2020. "Role of Antioxidant Molecules and Polymers in Prevention of Bacterial Growth and Biofilm Formation". *Current Medicinal Chemistry*, 27, 4882. https://doi-org.uplib.idm.oclc.org/10.2174/0929867326666190409120409

Frey, Y., Rodriguez, J. P., Thomann, A., Schwendener, S., Perreten, V., 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *Journal Dairy Science*, 96, 2247–2257.

Frum, Y., Viljoen, A. M., 2006. In vitro 5-lipoxygenase and antioxidant activities of South African medicinal plants commonly used topically for skin diseases. *Skin Pharmacology and Physiology*, 19, 329-335.

Gemechu, T., Yunus, H. A., Soma, M., Beyene, A., 2019. Bovine mastitis: Prevalence, Isolation, and identification of major bacterial pathogens in selected areas of Bench Maji Zone, Southwest Ethiopia. *Journal of Veterinary Medicine and Animal Health*, 11, 30-36.

Gentilini, E., Denamiel, G., Betancor, A., Rebuelto, M., Rodriguez Fermepin, M., De Torres, R. A., 2010. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. *Journal of Dairy Science*, 85, 1913–1917.

Germanò, M. P., D'Angelo, V., Biasini, T., Sanogo, R., De Pasquale, R., Catania, S., 2006. Evaluation of the antioxidant properties and bioavailability of free and bound phenolic acids from *Trichilia emetica* Vahl. *Journal of Ethnopharmacology*, 105, 368-373.

Germanò, M. P., D'Angelo, V., Sanogo, R, Catania, S., Alma, R., De Pasquale, R., Bisignano, G., 2005. Hepatoprotective and antibacterial effects of extracts from *Trichilia emetica* Vahl

Journal of Ethnopharmacology, 96, 227-232.

Getaneh, A. M., Gebremedhin, E. Z., 2017. Meta-analysis of the prevalence of mastitis and associated risk factors in dairy cattle in Ethiopia. *Tropical Animal Health Production*, 49, 697.

Getaneh, G., 2016. Prevalence of coagulase negative staphylococcus in mastitis infection in dairy cattle in and around Bahir Dar. *World Journal of Pharmaceutical and Medical Research*, 2, 17–23.

Getahun, K., Kelay, B., Bekana, M., Lobago, F., 2008. Bovine mastitis and antibiotic resistance patterns in Selalle smallholder dairy farms, central Ethiopia. *Tropical Animal Health Production*, 40, 261–268.

Giesecke, W. H., van den Heever, L. W., du Toit, I. J., 1971. Bovine mastitis in the Republic of South Africa. *Bulletin de l'Office International des Epizooties*, 76, 621.

Girma, S., Mammo, A., Bogele K., Sori, T., Tadesse, F., Jibat, T., 2012. Study on prevalence of bovine mastitis and its major causative agents in West Harerghe zone, Doba district, Ethiopia. *Journal of Veterinary Medicine and Animal Health*, 4, 116-123.

Gomes, F., Henriques, M., 2016. Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. *Current Microbiology*, 72, 377–382.

Griess, P. 1879. Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt "Ueber einige Azoverbindungen" . *Berichte der deutschen chemischen Gesellschaft*, 12, 426-428.

Gruet, P., Maincent, P., Berthelot, X., Kaltsatos, V., 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. *Advanced Drug Delivery Reviews*, 50, 245–259.

Gundidza, M., Gweru, N., Mmbengwa, V., Ramalivhana, N. J., Magwa, Z., Samie, A., 2008.

Phytoconstituents and biological activities of essential Oil from *Rhus lancea* L. F. *African Journal of Biotechnology*, 7, 2787-2789. http://www.academicjournals.org/AJB DOI: 10.5897/AJB08.136

Hagnestam-Nielson, C., Ostergaard, S., 2009. Economic impact of clinical mastitis in a dairy herd assessed by stochastic simulation using different methods to model yield losses. *Animal*, 3, 315-328.

Halasa, T., Huijps, K., Ǿsterås, O., Hoogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: A review. *Veterinary Quarterly* 29, 18-31.

Hall-Stoodley, L., Costerton, J. W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology*, 2, 95-108.

Harini, H., Sumathi, B. R., 2011. Screening of bovine milk samples for sub-clinical mastitis and antibiogram of bacterial isolates. *Veterinary World*, 4, 358–359.

Hashemi, M. Kafi, M., Safdarian, M., 2011. The prevalence of clinical and subclinical mastitis in dairy cows in the Central Region of Fars Province, South of Iran. *Iranian Journal of Veterinary Research*, Shiraz University, 12, 236 -241.

Heleili, N., Ayachi, A., Melizi, M., Kassah, A.L., Mamache, B., 2012. Prevalence of subclinical bovine mastitis and the in vitro sensitivity of bacterial isolates in Batna Governorate, East of Algeria. *Journal of Animal Science Advances*, 2, 576–582.

Heyman, H., Hussein, A., Meyer, J., Lall, N., 2009. Antibacterial activity of South African medicinal plants against methicillin resistant *Staphylococcus aureus*. *Pharmaceutical Biology*, 47, 67-71

Hoet, S., Opperdoes, F., Brun, R., Adjakidjé, V., Quetin-Leclercq, J., 2004. In vitro antitrypanosomal activity of ethno-pharmacologically selected Beninese plants. *Journal of Ethnopharmacology*, 91 (2004), pp. 37-42

Hogeveen, H., Huijps, K., Lam T. J. G. M., 2011. Economic aspects of mastitis: New developments. *New Zealand Veterinary Journal*, 59:1, 16-23, DOI: 10.1080/00480169.2011.547165

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*, 35, 322–332.

Honkanen-Buzalski, T., Myllys, V., Kulkas, L., 1996. Prevalence of bovine mastitis in Finland. *Suomen Eläinlääkärilehti*, 102, 191-197.

Hoque, M. N., Das, Z. C., Rahman, A. N. M. A., Haider, M. G., Islam, M. A., 2018. Molecular characterization of *Staphylococcus aureus* strains in bovine mastitis milk in Bangladesh. *International Journal of Veterinary Science and Medicine*, 6, 53-60.

Hoque, M. N., Das, Z. C., Talukder, A. K., Alam, M. S., Rahman, A. N. M. A., 2015. Different screening tests and milk somatic cell count for the prevalence of subclinical bovine mastitis in Bangladesh. *Tropical Animal Health Production*, 47, 79–86.

Horiuk, Y., Kukhtyn, M., Kovalenko, V., Kornienko, L., Horiuk, V., Liniichuk, N., 2019. Biofilm formation in bovine mastitis pathogens and the effect on them of antimicrobial drugs. *Independent Journal of Management and Production*, 10, 897-910.

Hu, W., Li, L., Sharma, S., Wang, J., McHardy, I., Lux, R., et al., 2012. DNA builds and strengthen the extracellular matrix in *Myxococcus xanthus* biofilms by interacting with

exopolysaccharides. *PLoS One,* 7, e51905.

Huijps, K., Lam, T. J. G. M., Hogeveen, H., 2008. Costs of mastitis: facts and perception. *Journal of Dairy Research,* 75, 113-120.

Huma, Z. I., Sharma, N., Kour, S., Tandon, S., Guttula, P. K., Kour, S., Singh, A. K., Singh, R., Gupta, M. K., 2020. Putative biomarkers for early detection of mastitis in cattle. *Animal Production Science,* 60, 1721 – 1736.

Hwang, A. Y., Gums, J. G., 2016. The emergence and evolution of antimicrobial resistance: impact on a global scale. *Bioorganic and Medicinal Chemistry*, 24, 6440–6445.

Ibrahim, S. S., Ghanem, H. M., 2019. Factors affecting mastitis incidence and its economic losses under Egyptian conditions. *Alexandria Journal of Veterinary Sciences*, 60, 17-22.

Iraguha, B., Hamudikuwanda, H., Mushonga, B., 2015. Bovine mastitis prevalence and associated risk factors in dairy cows in Nyagatare District, Rwanda. *Journal of the South African Veterinary Association*, 86, 1-6.

Islam, M. A., Rahman, A. K. M. A., Rony, S. A., Islam, M. S., 2010. Prevalence and risk factors of mastitis in lactating dairy cows at Baghabari Milk Shed Area of Sirajganj. *Bangladesh Journal of Veterinary Medicine,* 8, 157–162.

Jamal, M., Tasneem, U., Hussain, T., Andleeb, S., 2015. Bacterial biofilm: its composition, formation, and role in human infections. Research & Reviews: *Journal of Microbiology and Biotechnology*, 4, 1-14

Jefferson, K. K., 2004. What drives bacteria to produce a biofilm? *FEMS Microbiology Letter,* 236, 163–173.

Jiménez-Cabrera, T., Bautista, M., Velázquez-González, C., Jaramillo-Morales, O. A., Guerrero-Solano, J. A., Urrutia-Hernández, T. A., De la O-Arciniega, M., 2021. Promising antioxidant activity of Erythrina genus: An alternative treatment for inflammatory pain? International Journal of Molecular Sciences, 22, 248.

Jóźwik, A., Krzyżewski, J., Strzałkowska, N., Poławska, E., Bagnicka, E., Wierzbicka, A., Niemczuk, A. K., Lipińska, P., Horbańczuk, O., 2012. Relations between the oxidative status,

mastitis, milk quality, and disorders of reproductive functions in dairy cows – A review. *Animal Science Papers and Reports*, 30, 297–307

Juhász-Kaszanyitzky, É. et al., 2007. MRSA transmission between cows and humans. *Emerging Infectious Diseases*, 13, 630–632.

Junaidu, A. U., Salihu, M. D., Tambuwala, F. M., Magaji, A. A., Jaafaru, S., 2011. Prevalence of mastitis in lactating cows in some selected commercial dairy farms in Sokoto metropolis. *Advances in Applied Science Research*, 2, 290-294.

Kalandi, M., Sow, A., Millogo, V., Faye, S., Ouédraogo, A.G., Sawadogo, G. J., 2017. Prévalence et facteurs de risque des mammites subcliniques dans les élevages traditionnels de Kaolack au Sénégal. *Journal of Applied Biosciences*, 112, 10978-10984

Kaikabo, A. A., Suleiman, M. M., Samuel, B. B., Eloff, J. N., 2008. Antibacterial activity of eleven South African plants use in treatment of diarrhoea in folkloric medicine. *Journal of Traditional and Complementary Alternative Medicine*, 5, 315-316

Karzis, J., Petzer, I.-M., Donkin, E. F., Naidoo, V., 2018. Proactive udder health management in South Africa and monitoring of antibiotic resistance of *Staphylococcus aureus* in dairy herds from 2001 to 2010', *Journal of the South African Veterinary Association* 89, a1490. https://doi.org/10.4102/jsava.v89i0.1490

Kasravi, R., Bolourchi, M., Farzaneh, N., Seifi, H. A., Barin, A., Hovareshti, P., Gharagozlou, F., 2011. Conventional and extended intramammary therapy of persistent subclinical mastitis using nafcillin-penicillin-dihydrostreptomycin in lactating dairy cattle. *Iranian Journal of Veterinary Research*, 12, 113–120.

Kenar, B., Kuyucuoğlu, Y., Şeker, E., 2012. Antibiotic susceptibility of coagulase-negative staphylococci isolated from bovine subclinical mastitis in Turkey. *Pakistan Veterinary Journal*, 32, 390–393.

Khan, F., Hill, J., Kaehler, S., Allsopp, M., Vuuren, S., 2014. Antimicrobial properties and isotope investigations of South African honey. *Journal of Applied Microbiology*, 117, 366–379.

Kharsany, K., Viljoen, A., Leonard, C., van Vuuren, S., 2019. The new buzz: Investigating the antimicrobial interactions between bioactive compounds found in South African propolis. *Journal of Ethnopharmacology*, 238, 111867

Khodaei-Motlagh, M., Hazemi, M., Khaltabadi F. A. H, Yahyaei, M., Rezaei, M., De Rensis, F., Taddei, S., 2014. Antibacterial effect of medicinal plant essence (*Thymus vulgaris*) on major bacterial mastitis pathogen in vitro. *International Journal of Advanced Biological and Biomedical Research,* 2, 286–294.

Khunoana, E. T., Madikizela, B., Erhabor, J. O., Nkadimeng, S. M., Arnot, L. F., Van Wyk, I., McGaw, L. J., 2019. A survey of plants used to treat livestock diseases in the Mnisi community, Mpumalanga, South Africa, and investigation of their antimicrobial activity. *South African Journal of Botany*, 126, 21–29

Kim, H., Park, H., 2013. Ginger extract inhibits biofilm formation by *Pseudomonas aeruginosa* PA14. *PLoS One*, 8, e76106.

Kirstenbosch W. V., 2006. *Erythrina caffra* Thunb. South African National Biodiversity Institute.

Klibi, A., Maaroufi, A., Torres, C., Jouini, A., 2018. Detection and characterization of methicillin-resistant and susceptible coagulase-negative staphylococci in milk from cows with clinical mastitis in Tunisia. *International Journal of Antimicrobial Agents*, 52, 930–935.

Koh, J., Kim, H., Peck, K., Lee, M., Lee, H., Lee, S., Lee, J., 2006. MP-01.14: the analysis of autoinducer synthesis gene expression involved in quorum sensing mechanism with catheter associated urinary tract infection (CAUTI). *Urology*, 68, 52.

Komane, B. M., Olivier, E. I., Viljoen, A. M., 2011. *Trichilia emetica* (Meliaceae) – A review of traditional uses, biological activities and phytochemistry. *Phytochemistry Letters*, 4, 1-9.

Kossaibati, M. A., Esslemont, R. J., 1997. The costs of production diseases in dairy herds in England. *Veterinary Journal*, 154, 41-51.

Kotzé, M. & Eloff, J. N. 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). *South African Journal of Botany*, 68, 62-67.

Krukowski, H., Lassa H., Zastempowska, E., Smulski, S., Bis-Wencel, H., 2020. Etiological agents of bovine mastitis in Poland. *Medycyna Weterynaryjna*, 76, 221-225. DOI: dx.doi.org/10.21521/mw.6339.

Kudinha, T., Simango, C., 2012. Prevalence of coagulase-negative staphylococci in bovine mastitis in Zimbabwe. *Journal of South African Veterinary Association*, 73, 62–65.

Kuete, V., 2010. Potential of Cameroonian plants and derived products against microbial infections: A review. *Planta Medica*, 76, 1479–1491.

Kunene, N., Wilson, R. A. C., Myeni, N. P., 2003. The use of trees, shrubs and herbs in livestock production by communal farmers in northern Kwazulu-Natal, South Africa. *African Journal of Range and Forage Science*, 20, 271-274.

Kurjogi, M. M., Kaliwal, B. B., 2014. Epidemiology of Bovine Mastitis in Cows of Dharwad District. *International Scholarly Research Notices*, Available from: http://dx.doi.org/10.1155/2014/968076.

Kwon, A. S., Park, G. C., Ryu, S. Y., Lim, D. H., Lim, D. Y., Choi, C. H., Park, Y., Lim, Y., 2008. Higher biofilm formation in multidrug-resistant clinical isolates of *Staphylococcus aureus*. *International Journal of Antimicrobial Agents*, 32, 68–72.

Lafi, S. Q., Al-Rawashdeh, O. F., Ereifej, K. L., Hailat, N. Q., 1994. Incidence of clinical mastitis and prevalence of sub clinical udder infection in Jordan. *Preventive Veterinary Medicine*, 18, 89–98. http://dx.doi.org/10.1016/0167-5877(94)90067-1

Lahiri, D., Dash, S., Dutta, R., Nag, M., 2019. Elucidating the effect of anti-biofilm activity of bioactive compounds extracted from plants. *Journal of Biosciences*, 44, 52. https://doi.org/10.1007/s12038-019-9868-4

Lakew, M., Tolosa, T., Tigre, W., 2009. Prevalence and major bacterial causes of bovine mastitis in Asella, South Eastern Ethiopia. *Tropical Animal Health Production*, 41, 1525.

Laliotis, G. P., Koutsouli, P., Sotirakoglou, K., Savoini, G., Politis, I., 2020. Association of oxidative stress biomarkers and clinical mastitis incidence in dairy cows during the periparturient period. *Journal of Veterinary Research*, 64, 421 – 425.

Lall, N., Meyer, J. J. M., 1999. In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *Journal of Ethnopharmacology*, 66, 347-54.

Lewis, K., 2001. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*, 4, 999–1007.

Lidet, G. M., Deressa, B., Begna, F., Mekuria, A., 2013. Study on prevalence of bovine mastitis in lactating cows and associated risk factors in and around Areka town, Southern of Ethiopia. *African Journal of Microbiology Research*, 7, 5051-5056.

Long, C., 2005. Swaziland's flora: siSwati names and uses. Swaziland National Trust Commission, Mbambane. Available on: http://www .sntc.org.sz/index.asp, retrieved on 4 February 2018.

Lüthje, P., Schwarz, S., 2006. Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *Journal of Antimicrobial Chemotherapy*, 57, 966–969.

Mabogo, D. E. N., 1990. The ethnobotany of the Vhavenda. M. Sc. Thesis. University of Pretoria. Pretoria, Republic of South Africa.

Mabona, U., Viljoen, A., Shikanga, E., Marston, A., van Vuuren, S., 2013. Antimicrobial activity of southern African medicinal plants with dermatological relevance: from an ethnopharmacological screening approach to combination studies and the isolation of a bioactive compound. *Journal of Ethnopharmacology* 148, 45–55.

Maćešić, N., Karadjole, T., Bačić, G., Benić, M., Karadjole, M., Vince, S., Lipar, M., Cergolj, M., 2012. Aetiology and prevalence of bovine etiology and prevalence of bovine intramammary infection at drying off. Veterinarski Arhiv, 82, 125-131.

Mahato, S., Mistry, H. U., Chakraborty, S., Sharma, P., Saravanan, R., Bhandari, V., 2017. Identification of variable traits among the methicillin resistant and sensitive coagulase negative staphylococci in milk samples from mastitic cows in India. *Front Microbiology*, 8, 1446.

Makunga, N. P., Philander, L. E., Smith, M., 2008. Current preservatives on an emerging formal natural products sector in South Africa. *Journal of Ethnopharmacology*, 119, 365- 367

Mamba, P., Adebayo, S. A., Tshikalange, T. E., 2016. Anti-microbial, anti-inflammatory and HIV-1 reverse transcriptase activity of selected South African plants used to treat sexually transmitted diseases. *International Journal of Pharmacognosy and Phytochemistry Research*, 8, 1870-1876.

Man'ombe, E., 2014. Economic value and genetic prediction of clinical mastitis in South African Holstein cattle. Stellenbosch: Thesis (MSc. Agric), Stellenbosch University.

Manilal, A., Sabu, K. R., Shewangizaw, M., Aklilu, A., Seid, M, Merdekios, B., Tsegaye, B., 2020. In vitro antibacterial activity of medicinal plants against biofilm-forming methicillin-resistant *Staphylococcus aureus*: efficacy of *Moringa stenopetala* and *Rosmarinus officinalis* extracts. *Heliyon*, 6, e03303. www.cell.com/heliyon.

Maphosa, V., Masika, P. J., 2010. Ethnoveterinary uses of medicinal plants: a 23 survey of plants used in the ethnoveterinary control of gastro-intestinal parasites of goats in the Eastern Cape Province, South Africa. *Pharmaceutical Biology*, 48, 697–702.

Margiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., 2012. Multidrug-resistant, extensively drug-resistant, and pan drug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology Infections*, 18, 268–281.

Markey, B., Leonard, F., Archambault, M., Cullinane, A., Maguire, D., 2013. Clinical veterinary microbiology e-book. United States: Mosby Ltd.

Maroyi, A., 2019. Medicinal uses, phytochemistry, pharmacology and toxicological properties of *Elaeodendron croceum*. *Tropical Journal of Pharmaceutical Research*, 18, 669-676.

McGaw, L. J., Jäger, A. K., van Staden, J., 1997. Prostaglandin synthesis inhibitory activity in Zulu. Xhosa and Sotho medicinal plants. *Journal of Phytotherapy Research*, 11, 113-117.

Mekibib, B., Furgasa, M., Abunna, F., Megersa, B., Regassa, A., 2010. Bovine Mastitis: prevalence, risk factors and major pathogens in dairy farms of Holeta Town, Central Ethiopia. *Veterinary World*, 3, 397-403.

Michelle, C., Dold, T., 2012. Voice from the forest: Celebrating nature and culture in Xhosaland. Jacan Media (Ltd), pp. 209.

Mickymaray, S., 2019. Efficacy and mechanism of traditional medicinal plants and bioactive compounds against clinically important pathogens. *Antibiotics*, 8, 257.

Miltenburg, J. D., De Lange, D., Crauwels, A. P. P., Bongers, J. H., Tielen, M. J. M., Schukken, Y. H., Elbers, A. R. W., 1996, 'Incidence of clinical mastitis in a random sample of dairy herds in the southern Netherlands', *Veterinary Record*, 139, 204–207. http://dx.doi.org/10.1136/vr.139.9.204, PMid:8883335

Mitchell, J. D., 2004. Anacardiaceae. In: Smith N, Mori SA, Henderson A, Stevenson DW, Heald SV (eds.). Flowering Plants of the Neotropics., Princeton University Press, Princeton, NJ, USA, 2004, 14-17.

Mnxati, S., 2014. *Antidesma venosum*. KwaZulu-Natal Herbarium. South African National Biodiversity Institute. http://pza.sanbi.org/antidesma-venosum.

Motaung, T. E., Petrovski, K. R., Petzer, I.-M., Thekisoe, O., Tsilo, T. J., 2017. Importance of bovine mastitis in Africa. *Animal health research reviews*, 18, 58-69.

Moroni, P., Pisoni, G., Antonini, M., Villa, R., Boettcher, P., Carli, S., 2006. Antimicrobial drug susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in Italy. *Journal of Dairy Science*, 89, 2973–2976. http://dx.doi.org/10.3168/jds.S0022-0302(06)72569-3

Moru, N. H., Umoh, J. U., Maikai, B. V., Barje, P. P., Amuta, P., 2018. Milk yield losses and cost of clinical mastitis in Friesian × Bunaji crossbred dairy cows in Zaria, Nigeria. *Sokoto Journal of Veterinary Sciences*, 16, 28 - 34. http://dx.doi.org/10.4314/sokjvs.v16i2.4

Mpatswenumugabo, J. P., Bebora, L. C., Gitao, G. C., et al. 2017. Prevalence of subclinical mastitis and distribution of pathogens in dairy farms of Rubavu and Nyabihu districts, Rwanda. Journal of Veterinary Medicine Issue: 2017 Pages: Article ID 8456713

Mtunzi, F. M., Ejidike, I. P., Matamela, T., Dikio, E., Klink, M. J., 2017. Phytochemical profiling, antioxidant and antibacterial activities of leaf extracts from *Rhus leptodictya*. *International Journal of Pharmacognosy and Phytochemical Research*, 9, 1090-1099.

Mulaudzi, R. B., Ndhlala, A. R., Kulkarni, M. G., van Staden, J., R., 2012. Pharmacological properties and protein binding capacity of phenolic extracts of some Venda medicinal plants used against cough and fever. *Journal of Ethnopharmacology*, 143, 185-193.

Mwangomo, D. T., Moshi, M. J., Magadula, J. J., 2012. Antimicrobial activity and phytochemical screening of *Antidesma venosum* root and stem bark ethanolic extracts. *International Journal of Research in Phytochemistry & Pharmacology*, 2, 90-95.

Naidoo, R. Patel, M., Gulube, Z., Fenyvesi I., 2012. Inhibitory activity of *Dodonaea viscosa* var. angustifolia extract against *Streptococcus mutans* and its biofilm. Journal of ethnopharmacology, 144, 171-174.

Nciki, S., van Vuuren, S., van Eyk, A., de Wet, H., 2016. Plants used to treat skin diseases in northern Maputaland, South Africa: antimicrobial activity and in vitro permeability studies. *Pharmaceutical Biology*, 54, 1–17.

Ndirangu, P. N., Siamba, D., Wesonga, H. O., Mungube, E. O., Maichomo, M. W., Mugambi, J. M., 2017. Prevalence of bovine mastitis and multi-antibiotic resistant *Staphylococcus* and *Streptococcus species* in a research centre farm at Naivasha, Kenya. *Bulletin of Animal Health and Production in Africa*, 65, 291-303.

Ngu Ngwa, V., Cuteri, V., Awah-Ndukum, J., Tangwa, B. V., Manchang, K. T., Attili, A. R., 2020. Bacterial pathogens involved in bovine mastitis and their antibiotic resistance patterns in the Adamawa region of Cameroon. *Journal of Dairy Research and Technology*, 3, 012.

Ngu Ngwa, V., Awah-Ndukum, J., Cuteri V., Tanyi Kingsley, M., Souaibou, A., Laouane, F., Kofa, H., Attili A. R., 2018. Prevalence study on bovine mastitis in the Adamawa Region of Cameroon. *Large Animal Review*, 24, 21-29.

Nielsen, T. R. H., Kuete, V., Jager, A. K., Meyer, J. J. M., Lall, N., 2012. Antimicrobial activity of selected South African medicinal plants. *BMC Complementary and Alternative Medicine*, 12, 1-6.

Njume, C., Jide, A. A., Ndip, R. N., 2011. Aqueous and organic solvent-extracts of selected South African medicinal plants possess antimicrobial activity against drug resistant strains of

Helicobacter pylori: inhibitory and bactericidal potential. *International Journal of Molecular Science*, 12, 5652–5665.

Nyila, M. A., Leonard, C. M., Hussein, A. A., Lall, N., 2012. Activity of South African medicinal plants against *Listeria monocytogenes* biofilms, and isolation of active compounds from *Acacia karroo*. *South African Journal of Botany*, 78, 220-227.

Odeyemi, S. W., Afolayan, A. J., 2017. Biological activities and phytochemical screening of *Elaeodendron croceum* (Thunb.) DC. leaves and stem barks extracts. *International Journal of Phytomedicine*, 9, 566-575.

Ofek, I., Hasty, D. L., Sharon, N., 2003. Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunology and Medical Microbiology*, 38, 181-191.

Okem, A., Finnie, J., van Staden, J., 2012. Pharmacological, genotoxic, and phytochemical properties of selected South African medicinal plants used in treating stomach-related ailments. *Journal of Ethnopharmacology,* 139, 712–720.

Okmen, G., Cantekin, Z., Alam, M.I., Turkcan, O., Ergun, Y., 2017. Antibacterial and antioxidant activities of *Liquidambar orientalis* Mill. various extracts against bacterial pathogens causing mastitis. *Turkish Journal of Agriculture - Food Science and Technology*, 5, 883-887.

Olajuyigbe, O. O., Afolayan, A. J., 2011. Phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. mucronata Willd. *BMC Complementary Alternative Medicine*, 11, 130.

Olajuyigbe, O. O., Afolayan, A. J., 2012. In vitro pharmacological activity of the crude acetone extract of *Erythrina caffra* Thunb: antibacterial and antifungal assessment. *Journal of Medicinal Plants Research*, 6, 1713-1720.

Olivares-Pérez, J., Kholif, A. E., Rojas-Hernández, S., Elghandour, M. M. M. Y., Salem, A. Z. M., Bastida, A. Z., Velázquez-Reynoso, D., Cipriano-Salazar, M., Camacho-Díaz, L. M., Alonso-Fresán, M. U., DiLorenzo, N., 2015. Prevalence of bovine subclinical mastitis, its etiology and diagnosis of antibiotic resistance of dairy farms in four municipalities of a tropical region of Mexico. *Tropical Animal Health Production*, 47, 1497–1504.

Ondiek, J. O., Ogore, P. B., Shakala, E. K., Kaburu, G. M., 2013. Prevalence of bovine mastitis, its therapeutics and control in Tatton Agriculture Park, Egerton University, Njoro District of Kenya. *Basic Research Journal of Agricultural Science and Review*, 2, 15-20.

Ostegaard, O., Chagunda, M. G. G., Friggens, N. C., Bennedsgaard, T. W., Klaas, I. C., 2005. A stochastic model simulating pathogen-specific mastitis control in a dairy herd. *Journal of Dairy Science,* 88, 4243-4257.

Otto, M., 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annual review of microbiology,* 64, 143-162.

Ott, S., 1999. Costs of herd-level production losses associated with subclinical mastitis in US Dairy Cows. PP 152-156 in Proceedings of the 38th annual meeting of National Mastitis Council, Arlington VA. Natl. Mast Coun. Madison WI.

Palmer, E., Pitman, N., 1972. Trees of Southern Africa: covering all known indigenous species in the Republic of South Africa, South-West Africa, Botswana, Lesotho and Swaziland. Volumes 1 & 2.

Panda, S.K., 2014. Ethno-medicinal uses and screening of plants for antibacterial activity from Similipal biosphere reserve, Odisha. *Indian Journal of Ethnopharmacology* 151, 158–175. https://doi.org/10.1016/j.jep.2013.10.004.

Panda, S.K., Mohanta, Y.K., Padhi, L., Park, Y.-.H., Mohanta, T.K., Bae, H., 2016. Large scale screening of ethnomedicinal plants for identification of potential antibacterial compounds. *Molecules* 21. https://doi.org/10.3390/molecules21030293.

Park, J. Y., Fox, L. K., Seo, K. S., McGuire, M. A., Park, Y. H., Rurangirwa, F. R., Sischo, W. M., Bohach, G. A., 2011. Detection of classical and newly described staphylococcal superantigen genes in coagulase-negative staphylococci isolated from bovine-intramammary infections. *Veterinary Microbiology,* 147, 149-154.

Paroda, R. S., Mal, B., 1989. “New plant sources for food and industry in India,” in New Crops for Food and Industry, G. E. Wickens, N. Haq, and P. Day, Eds., pp. 135–149, Chapman & Hall, London, UK.

Perini S., Piccoli R. H., Nunes C. A., Bruhn F. R. P., Custódio D. A. C., Costa G. M., 2014. Antimicrobial activity of essential oils against pathogens isolated from bovine mastitis. *Journal of Natural Products Plant Resources*, 4, 6-15.

Persson-Waller, K., Aspán, A., Nyman, A., Persson, Y., Andersson, U. G., 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Veterinary Microbiology*. 152:112–116.

Petrovski, K. R., Trajcev, M., Buneski, G., 2006. A review of the factors affecting the costs of bovine mastitis. *Journal of South African Veterinary Association*, 77, 52–60.

Petzer, I.-M., Karzis, J., Watermeyer, J. C., Van der Schans, T. J., Van Reenen, R., 2009. Present trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association,* 80, 17–22

Petzer, I.-M., Karzis, J., Donkin, E. F., Webb, E. C., 2016. A pathogen-specific approach towards udder health management in dairy herds: Using culture and somatic cell counts from routine herd investigations. *Onderstepoort Journal of Veterinary Research*, 83, 1–12.

Phophi, L., Petzer, I.-M., Qekwana, D.N., 2019. Antimicrobial resistance patterns and biofilm formation of coagulase-negative Staphylococcus species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. *BMC Veterinary Research,* 15,420. https://doi.org/10.1186/s12917-019-2175-3

Pinto, M. D. C., Tejeda, A., Duque, A. L., Macias, P., 2007. Determination of lipoxygenase activity in plant extracts using a modified ferrous oxidation−xylenol orange assay. *Journal of Agricultural and Food Chemistry*, 55, 5956-5959.

Piotr, S., Magdalena, Z., Joanna, P., Barbara, K. and Sławomir, M., 2018. Essential oils as potential anti-staphylococcal agents. *Acta Veterinaria-Beograd*, 68, 95-107.

Pitkälä, A., Salmikivi, L., Bredbacka, P., Myllyniemi, AL., Koskinen, M.T., 2007. Comparison of tests for detection of β-lactamase-producing staphylococci. *Journal of Clinical Microbiology*, 45, 2031–2033.

Politis, I., Theodorou, G., Lampidonis, A. D., Kominakis, A., Baldi, A., 2012. Oxidative status and incidence of mastitis relative to blood α-tocopherol concentrations in the postpartum period in dairy cows. Short communication. *Journal of Dairy Science*, 95, 7331–7335.

Prinsloo, G., Meyer, J. J. M., Hussein, A. A., 2011. Anti-HIV activity of a cardiac glycoside isolated from *Elaeodendron croceum*. *South African Journal of Botany*, 111, 308.

Prinsloo, G., Meyer, J. J. M., Hussein, A. A., Munoz, E., Sanchez, R., 2010. A cardiac glucoside with in vitro anti-HIV activity isolated from *Elaeodendron croceum*. *Natural Products Research*, 24, 1743-1746.

Puppel, K., Kali´nska, A., Kot, M., Slósarz, J., Kunowska-Slósarz, M., Grodkowski, G., Kuczynska, B., Solarczyk, P., Przysucha, T., Gołebiewski, M., 2020. The Effect of *Staphylococcus* spp., *Streptococcus* spp. and Enterobacteriaceae on the development of whey protein levels and oxidative stress markers in cows with diagnosed mastitis. *Animals*, 10, 1591.

Qiu, J., Wang, D., Xiang, H., Feng, H., Jiang, Y., Xia, L., Dong, J., Lu, J., Yu, L., Deng, X., 2010. Subinhibitory concentrations of thymol reduce enterotoxins A and B and α-hemolysin production in *Staphylococcus aureus* isolates. *Plosone*, 5, 9736.

Radostits, O. M., Gay, C. C., Hinchcliff, K. W., Constable, P. D., 2007. Mastitis. In: Veterinary Medicine: a textbook of disease of cattle, sheep, pigs, goats, and horses. 10th ed. Bailliere Tindall, London. pp. 674-762.

Rahideh, S. T., Shidfar, F., Khandozi, N., Rajab, A., Hosseini, S. P., Mirtaher, S. M., 2014. The effect of sumac (*Rhus coriaria* L.) powder on insulin resistance, malondialdehyde, high sensitive C-reactive protein and paraoxonase-1 activity in type 2 diabetic patients. *Journal of Research in Medical Sciences*, 19, 933-938.

Rahman, M. A., Bhuiyan, M. M. U., Kama, M. M., Shamsuddin, M., 2009. Prevalence and risk factors of mastitis in dairy cows. *The Bangladesh Veterinarian*, 26, 54–60.

Random Harvest, 2021. *Indigofera frutescens.* https://www.randomharvest.co.za/South-African-Indigenous-Plants/Show-Plant/PlantId/66/Plant/Indigofera-frutescens. Retrieved on 29th January 2021.

Ranjan, R., Gupta, M. K., Singh, S., Kumar, S., 2010. Current trend of drug sensitivity in bovine mastitis. *Veterinary World*, 3, 17–20.

Raspanti, C. G., Bonetto, C. C., Vissio, C., Pellegrino, M. S., Reinoso, E. B., Dieser, S. A., Bogni, C. I., Larriestra, A. J., Odiernoa. L. M., 2016. Prevalence and antibiotic susceptibility of coagulase-negative Staphylococcus species from bovine subclinical mastitis in dairy herds in the central region of Argentina. *Review of Argentina Microbiology*, 48, 50–56.

Rayne, S., Mazza, G., 2007. Biological activities of extracts from sumac (*Rhus* spp.): a review. *Plant Foods for Human Nutrition*, 62, 165-175.

Razwinani, M., Tshikalange, T. E., Motaung, S. C. K. M., 2014. Antimicrobial and anti-inflammatory activities of *Pleurostylia capensis* Turcz (Loes) (celastraceae). *African Journal of Traditional, Complementary and Alternative Medicines : AJTCAM / African Networks on Ethnomedicines*, 11, 452–457.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26, 1231-1237.

Reid, K. A., Maesa, A., Van Staden, J., Dekimpec, N., Mulholland, D. A., Verschaevea, L., 2006. Evaluation of the mutagenic and antimutagenic effects of South African plants. *Journal of Ethnopharmacology*, 106, 44-50.

Retief, E., Herman, P. P. J., 1997. Plants of the northern provinces of South Africa: Keys and diagnostic characters. National Botanical Institute. pp 369.

Rollin E., Dhuyvetter K.C., Overton M.W., 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. *Preventive Veterinary Medicine*, 122, 257–264. https://doi.org/10.1016/j.prevetmed.2015. 11.006 PMID: 26596651

Ruegg, P., 2011. Premiums, production and pails of discarded milk: how much money does mastitis cost you? University of Wisconsin, Madison. https://milkquality.wisc.edu/wp-content/uploads/sites/212/2011/09/how-much-money-does-mastitis-cost.pdf

Ryu, J. H., Ahn, H., Kim, J. Y., Kim, Y. K., 2003. Inhibitory activity of plant extracts on nitric oxide synthesis in LPS-activated macrophages. *Phytotherapy Research*, 17, 485-489.

Saba, E., Lee, Y. Y., Kim, M., Kim, S.-H., Hong, S.-B. & Rhee, M. H. 2018. A comparative study on immune-stimulatory and antioxidant activities of various types of ginseng extracts in murine and rodent models. *Journal of Ginseng Research*, 42, 577-584.

Sadashiv, S. O., Kaliwal, B. B., 2013. Prevalence of bovine mastitis in North Karnataka, India. *International Journal of Pharmaceuticals and Health care Research*, 1, 169-177.

Saddek, S. R., Abd-Elkader, H. A., Abd-Elhaffez, M. M., 1996. Bacteriological studies of subclinical mastitis in Friesian cattle in Assiut Governorate. *Assiut Veterinary Medicine Journal*, 42, 77–88.

Sakwinska, O., Morisset, D., Madec, J. Y., Waldvogel, A., Moreillon, P., Haenni, M., 2011. Link between genotype and antimicrobial resistance in bovine mastitis related Staphylococcus aureus strains, determined by comparing swiss and French isolates from the Rh??Ne valley. *Applied Environmental Microbiology*, 77, 3428–3432.

Saidi, R., Khelef, D., Kaidi, R., 2013. Subclinical mastitis in cattle in Algeria: Frequency of occurrence and bacteriological isolates. *Journal of the South African Veterinary Association* 84, Art. #929, 5 pages.

Salimi, Z., Eskandary, A., Headari, R., Nejati, V., Moradi, M., Kalhori, Z., 2015. Antioxidant effect of aqueous extract of sumac (*Rhus coriaria* L.) in the alloxan-induced diabetic rats. *Indian Journal of Physiology and Pharmacology*, 59, 87-93.

Sandasi, M., Leonard, C., Van Vuuren, S., Viljoen, A., 2011. Peppermint (*Mentha piperita*) inhibits microbial biofilms in vitro. *South African journal of botany*, 77, 80-85.

Sanotharan, N., Pagthinathan, M., Nafees, M. S. M., 2016. Prevalence of bovine subclinical mastitis and its association with bacteria and risk factors in milking cows of Batticaloa District in Sri Lanka. *International Journal of Scientific Research and Innovative Technology*, 3, 137-150.

Sarba E. J., Tola G. K., 2017. Cross-sectional study on bovine mastitis and its associated risk factors in Ambo district of West Shewa zone, Oromia, Ethiopia. *Veterinary World*, 10, 398-402.

Sargeant, J. M., Morgan-Scott, H., Leslie, K. E., Ireland, M. J., Bashiri, A., 1998. Clinical mastitis in dairy cattle in Ontario: frequency of occurrence and bacteriological isolates. *Canadian Veterinary Journal*, 39, 33–38. PMid:9442950, PMCid:1539829.

Sarkar, R., Chaudhary, S. K., Sharma, A., Yadav, K. K., Nema, N. K., Sekhoacha, M., Karmakar, S., Braga, F. C., Matsabisa, M. G., Mukherjee, P. K., Sen, T., 2014. Anti-biofilm activity of Marula - a study with the standardized bark extract. *Journal of Ethnopharmacology*, 154, 170-175.

Sathiya Deepika, M., Thangam, R., Sakthidhasan, P., Arun, S., Sivasubramanian, S., Thirumurugan, R., 2018. Combined effect of a natural flavonoid rutin from *Citrus sinensis* and conventional antibiotic gentamicin on *Pseudomonas aeruginosa* biofilm formation. *Food Control*, 90, 282-294.

Schmidt, T., 2011. In vitro antimicrobial susceptibility of Staphylococcus aureus strains from dairy herds in Kwazulu-Natal. *Journal of the South African Veterinary Association*, 82, a38.

Schmidt, T., Kock, M. M., Ehlers, M. M., 2015. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *Journal Dairy Science*, 98, 6256–6269.

Schmidt, E., Lotter, M., 2002. Trees and shrubs of Mpumalanga and Kruger National Park. *Jacana Media*, 358-359.

Schmidt, E., Lotter, M., McCleland, W., 2002. Trees and shrubs of Mpumalanga and Kruger National Park. Jacana Media, Johannesburg. 702.

Seqaws, P., Kasenene, J. M., 2007. Medicinal plants diversity and uses in Sango bay area, Southern Uganda. *Journal of Ethnopharmacology*, 113, 521-548.

Sgorbini M., Bonelli F., Fratini F., Sbrana A., Brombin M., Meucci V., Corazza M., Ebani V., Bertelloni F., Turchi B., Gatta D., Cerri D., 2014. Diagnosi delle mastiti bovine: test di screening e batteriologia a confronto e loro indice predittivo. *Large Animal Review*, 20, 9-16.

Shah, M. A., Bosco, S. J. D., Mir, S. A., 2014. Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, 98, 21-33.

Shai, L. J., McGaw, L. J., Masoko, P., Ellof, J. N., 2008. Antifungal and antibacterial activity of seven traditionally used South African plant species active against *Candida albicans. South African Journal of Botany*, 74, 677-684

Sharif, A., Muhammad, G., 2009. Mastitis control in dairy animals. *Pakistan Veterinary Journal* 29, 145–148.

Shengo, L. M., Mundongo, T. H., Kasamba, I. E., Malonga, K. F., Kapend, A. K. L., 2013. A survey of the antibacterial activity of three plants used in the Congolese herbal medicine practiced by the healers in the city of Lubumbashi. *African Journal of Pharmacy and Pharmacology*, 7, 1870-1875

Shiferaw, J., Telila, I., 2017. Prevalence of Bovine Mastitis and Assessment of Risk Factors in and Around WolaytaSodo, Ethiopia. *International Journal of Homeopathy & Natural Medicines*, 2, 1-7.

Shirinda, H., Leonard, C., Candy, G., Van Vuuren, S., 2019. Antimicrobial activity and toxicity profile of selected southern African medicinal plants against neglected gut pathogens. *South African Journal of Science*, 115 (11/12), Art. #6199, 10 pages. https://doi.org/10.17159/sajs.2019/6199.

Shittu, A., Abdullahi, J., Jibril, A., Mohammed, A. A., Fasina, F. O., 2012. Sub-clinical mastitis and associated risk factors on lactating cows in the Savannah Region of Nigeria. *BMC Veterinary Research*, 8, 134-142.

Shoukat, S., Wani, H., Ali, U., Ali, M., Para, P., Singh, C., Ganguly, S., 2018. Mastitis and its diagnosis: A Review. 10.22271/ed.book01.a06.

Shrestha, L. B., Bhattarai, N. R., Khanal, B., 2017. Antibiotic resistance and biofilm formation among coagulase-negative staphylococci isolated from clinical samples at a tertiary care hospital of eastern Nepal. *Antimicrobial Resistance and Infection Control*, 6, 89.

Simojoki, H., Hyvönen, P., Plumed Ferrer, C., Taponen, S., Pyörälä, S., 2012. Is the biofilm formation and slime producing ability of coagulase-negative staphylococci associated with the persistence and severity of intramammary infection? *Veterinary Microbiology*. 158, 344–52.

Sordillo, L. M., 2005. Factors affecting mammary gland immunity and mastitis susceptibility. *Livestock Production Science*, 98, 89–99.

Sordillo, L. M., Aitken, S. L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. *Veterinary Immunology and Immunopathology*, 128, 104–109

Sparg, S. G., van Staden, J., Jäger, A. K., 2000. Efficiency of traditionally used South African plants against schistosomiasis. *Journal of Ethnopharmacology*, 73, 209-214.

Srednik, M. E., Tremblay, Y. D. N., Labrie, J., Archambault, M., Jacques, M., Cirelli, A. F., 2017. Biofilm formation and antimicrobial resistance genes of coagulase negative staphylococci isolated from cows with mastitis in Argentina. *FEMS Microbiology Letter*. 2017; 364: fnx001.

Srivastava, A. K., Kumaresan, A., 2015. Mastitis in dairy animal: current concepts and future concerns. In 'Mastitis in dairy animals: an update'. (Eds AK Srivastava, A Kumaresan, A Manimaran, S Prasad) pp. 25–39. (Satish Serial Publishing House: New Delhi, India).

Sserunkuma. P., McGaw, L. J., Nsahlai, I. V, Van Staden, J., 2017. Selected southern African medicinal plants with low cytotoxicity and good activity against bovine mastitis pathogens. *South African Journal of Botany*, 111, 242–247.

Suleiman, T. S., Karimuribo, E. D., Mdegela, R. H., 2018. Prevalence of bovine subclinical mastitis and antibiotic susceptibility patterns of major mastitis pathogens isolated in Unguja island of Zanzibar, Tanzania. *Tropical Animal Health Production*, 50, 259–266.

Suleiman, A, B., Umoh, V. J., Kwaga, J. K. P., Shaibu, S. J., 2013a. Enterotoxigenic and antibiotic resistance of Staphylococcus aureus isolated from sub-clinical bovine mastitis in Plateau State, Nigeria. *Research Journal of Microbiology*, 8, 101-107.

Suleiman, T. S., Karimuribo, E. D., Mdegela, R. H., 2013b. Prevalence of mastitis in smallholder dairy cattle in Pemba Island, Tanzania. *Tanzania Veterinary Journal*, 28, 70-81.

Supré, K., Haesebrouck, F., Zadoks, R. N., Vaneechoutte, M., Piepers, S., De Vliegher, S., 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. *Journal of Dairy Science*. 94, 2329-2340.

Taemchuay, D., Rukkwamsuk, T., Sakpuaram, T., Ruangwises, N., 2009. Antibacterial activity of crude extracts of *Centella asiatica* against *Staphylococcus aureus* in bovine mastitis. *Kasetsart Veterinarians*, 19, 119–128.

Taponen S., Liski E., Heikkilä A. M., Pyörälä S., 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis*, or *Escherichia coli*. *Journal of Dairy Science*, 100, 493-503.

Tas, P., Stopper, H., Koschel, K., Schiffmann, D., 1991. Influence of carcinogenic oestrogen diethylstilboestrol on the intracellular calcium level in C6 rat glioma cells. *Toxicology In Vitro*, 5, 463-465. 10.1016/0887-2333(91)90073-M.

The Plant Library, 2021. *Indigofera frutescens*. https://www.theplantlibrary.co.za/plants/Indigofera-frutescens-#:~:text=Size%3A%202%20%E2%80%93%204%20m%20with,moderate%20frost%3B%20easy%20to%20prune. Retrieved on the 29th of January 2021.

Tilahun, A., Aylate, A., 2015. Prevalence of bovine mastitis in lactating cows and its public health implications in selected commercial dairy farms of Addis Ababa. *Global Journal of Medical Research: (G) Veterinary Science and Veterinary Medicine*, 15, 17–24.

Tiwari, J. G., Babra, C., Tiwari, H. K., Williams, V., DeWet, S., Gibson, J., Paxman, A., Morgan, E., Costantino, P., Sunagar, R., Isloor, S., Mukkur, T., 2013. Trends in therapeutic and prevention strategies for management of bovine mastitis: an overview. *Journal of Vaccines and Vaccinations* 4, 1000176.1–1000176.11.

Traore, M., Zhai, L., Chen, M., Olsen, C. E., Odile, N., Pierre, G. I., Bosco, J. O., Guigemde, T., Robert, T. G., Christensen, S. B., 2007. Cytotoxic kurubasch aldehyde from *Trichilia emetica*. *Natural Products Research*, 21, 13-17.

Tremblay, Y. D. N., Lamarche, D., Chever, P, Haine, D., Messier, S., Jacques, M., 2012. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *Journal of Dairy Science*. 96, 234–246.

Tshisikhawe, M.P., Van Rooyen, M.W., 2012. Population biology of *Brackenridgea zanguebarica* in the presence of harvesting. *Journal of Medicinal Plants Research*, 6, 5748-5756.

Turchi, B., Bertelloni, F., Marzoli, F., Cerri, D., Tola, S., Azara, E., Longheu, C. M., Tassi, R., Schiavo, M., Cilia, G., Fratini, F., 2020. Coagulase negative staphylococci from ovine milk: genotypic and phenotypic characterization of susceptibility to antibiotics, disinfectants, and biofilm production. *Small Ruminant Research*, 183, 106030.

Umaru, G. A., Kwaga, J. K. P., Bello, M., Raji, M. A., Maitala, Y. S., 2017. Occurrence of bovine mastitis and isolation of *Staphyloccocus* species from fresh cow milk in settled Fulani herds in Kaduna State, Nigeria. *Bayero Journal of Pure and Applied Sciences*, 10, 259-263.

University of Glasgow, 2016. Potential biomarkers of mastitis in dairy cattle milk identified (2016, July 18). Retrieved on 30 May 2020 from https://phys.org/news/2016-07-potential-biomarkers-mastitis-dairy-cattle.html.

Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F., De Vliegher, S., 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. *The Veterinary Journal*, 203, 44–51. http://dx.doi.org/10.1016/j.tvjl.2014.11.001.

van Wyk, B.-E., Van Oudtshoorn, B., Gericke, N., 2013. Medicinal Plants of South Africa, Second Edition. Briza Publications, Pretoria; pp. 336. South Africa

van Wyk, B., van Wyk, P., 1997. Field Guide to Trees of Southern Africa. Cape Town: Struik Nature.

Vasudevan, P., Nair, M. K. M., Annamalai, T., Venkitanarayanan, K. S., 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. *Veterinary Microbiology*, 92, 179–185.

Van Vuuren, S., Holl, D., 2017. Antimicrobial natural product research: A review from a South African perspective for the years 2009–2016. *Journal of Ethnopharmacology*, 208, 236–252

Venter, F., Venter, J. A., 2002. Making the most of indigenous trees. Briza Publications, Pretoria, South Africa, 28 (rev. edition).

Watt, J. M., Breyer-Brandwijk, M. G., 1962. The medicinal and poisonous plants of southern and eastern Africa. 2nd Edition, E. and S. Livingstone Ltd., Edinburgh.

Wilson, D. J., Gonzalez, R. N., Hert, J. A., Schulte, H., Bennett, G., Schukken, Y., Groohn, Y., 2004. Effect of clinical mastitis on the lactation curve: a mixed model estimation using daily milk weight. *Journal of Dairy Science*, 87, 2073-2084.

Wilson, D. J., Gonzalez, R. N., Das, H. H., 1997. Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production. *Journal of Dairy Science*, 80, 2592-2598.

Wolfova, M., Stipkova, M., Wolf, J., 2006. Incidence and economics of clinical mastitis in five Holstein herds in the Czech Republic. *Preventive Veterinary Medicine*, 77, 48-64.

YeHui, W., QingLing, M., Jun, Q., Jing, L., KuoJun, C., DengFeng, W., XuePeng, C., 2019. Analysis of biofilm formation, related genes distribution and transcription level in Xinjiang isolates of Staphylococcus aureus from cows. *Journal of Southern Agriculture*, 50, 1829-1835.

Yelani, T., Hussein, A. A., Meyer, J. J. M., 2010. Isolation and identification of poisonous triterpenoids from *Elaeodendron croceum*. *Natural Products Research*, 24, 1418- 1425.

Yirga, M., 2018. An updated review on prevalence and major pathogens of bovine mastitis in Ethiopia. *Bulletin of Animal Health and Production in Africa*, 66, 275-279.

Yohannis, M., Molla, W., 2013. Prevalence, risk factors and major bacterial causes of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia. *African Journal of Microbiology Research*, 7, 5400-5405.

Zaatout, N., Ayachi, A., Kecha, M., 2020. Epidemiological investigation of subclinical bovine mastitis in Algeria and molecular characterization of biofilm-forming Staphylococcus aureus. *Tropical Animal Health Production*, 52, 283–292.

Zecconi, A., Cipolla, M., 2019. Mastitis costs are not imputable to milk losses only. *Summa, Animali da Reddito*, 14, 5-13.

Zenebe, N., Habtamu, T., Endale, B., 2014. Study on bovine mastitis and associated risk factors in Adigrat, Northern Ethiopia. *African Journal of Microbiology Research*, 8, 327-331.

Zeryehun, T., Aya, T., Bayecha, R., 2013. Study on prevalence, bacterial pathogens, and associated risk factors of bovine mastitis in small holder dairy farms in and around Addis Ababa, Ethiopia. *The Journal of Animal & Plant Sciences*, 23, 50-55.

Zigo, F., Elecko, J., Vasil, M., et al., 2019. The occurrence of mastitis and its effect on the milk malondialdehyde concentrations and blood enzymatic antioxidants in dairy cows. *Veterinarni Medicina*, 64, 423 – 432.

Appendix: Ethics committee approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 15 April 2020

LETTER OF APPROVAL

Ethics Reference No	**REC236-19**
Protocol Title	**Anti-Biofilm, Bactericidal and Quorum Sensing Inhibition Activities of Selected South African Plants on Multidrug Resistant Staphylococci Isolated from Clinical Cases of Bovine Mastitis.**
Principal Investigator	**Mr AO Akinboye**
Supervisors	**Prof LJ McGaw**

Dear Mr AO Akinboye,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC236-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

Room 6-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel +27 (0)12 529 8390
Email marie.watson-kriek@up.ac.za
www.up.ac.za