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Pharmacological activity of selected medicinal plants with potential for preventing and treating infectious bovine mastitis, and identification of compounds from active fractions of *Kalanchoe gunniae*

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Thesis submitted in fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR (PhD)

Phytomedicine Programme, Department of Paraclinical Sciences

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DEDICATION

This work is dedicated to the Holy Trinity; God the Father, God the Son Jesus Christ and God the Holy Spirit, the covenant keeping God, my all in all, who kept me going and who provided me with the strength to do this work.

DECLARATION

I, the undersigned, declare that this thesis is a presentation of my original research work done at the University of Pretoria while in candidature for a Doctor of Philosophy (Veterinary Science) degree. Neither the entire thesis nor parts of it have/shall be submitted to any institution for examination for a degree or any other professional qualification. Where the contributions of others are included, I have acknowledged clearly and consistent with the plagiarism policy guidelines of the University of Pretoria.

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Student name:	Signature:	Date:

ETHICAL STATEMENT

I, Eucharia Chika Ogbuadike (student number 18378928) declare that ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy Guidelines for Responsible Research were followed accordingly.

The Research Ethics Committee of the Faculty of Veterinary Science (University of Pretoria) granted the research protocol of the present study a clearance with number: REC124-19. All experimental procedures were carried out in the Department of Paraclinical Sciences, located within the Faculty of Veterinary Sciences, University of Pretoria.

The author further declares that the ethical standards required in terms of the University of Pretoria's Code of Ethics for researchers and the policy guidelines for responsible research were observed.

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Student name:	Signature:	

ETHICS CERTIFICATE

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Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Anti-infective agents from selected medicinal plants with activity against mastitis and skin ulcer; and their mechanisms of action.**
Project Number	**REC124-19**
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Supervisor	**Prof LJ McGaw**

APPROVED	Date: **2020-01-06**
CHAIRMAN: UP Research Ethics Committee	Signature:

Applications using animals requires AEC approval before commencing with the project.

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LIST OF ABBREVIATIONS

ABTS	2,2-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
Avg	Average
BD	Bovine dermal
BSA	Bovine serum albumin
^{o}C	Degrees Celsius
CFU	Colony forming units
CLSI	Clinical Laboratory Standards Institute
$CHCl_3$	Chloroform
COX	Cyclooxygenase
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2,-Diphenyl-1-picrylhydrazyl
ETOAC	Ethyl acetate
FADD	Fas associated death domain
FASL	Fas ligand
FBS	Foetal bovine serum
FOX	Ferrous oxidation-xylenol orange

GC-MS	Gas chromatography-mass spectrometry
h	hour
H_2O	Water
IC_{50}	Inhibitory concentration 50%
IL1 β	Interleukin 1 beta
IL10	Interleukin 10
INOS	Inducible nitric oxide synthase
INT	p-Iodonitro-tetrazolium violet
LC_{50}	Lethal concentration 50%
LOX	Lipoxygenase
LPS	Lipopolysaccharides
LTB4	Leukotriene B4
MEOH	Methanol
MH	Mueller-Hinton
MIC	Minimal inhibitory concentration
min	Minute
MQSIC	Quorum sensing inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MTT	3-(4,5-dimethyl tetrazolium bromide)
NA	Not applicable
NAS	Non-aureus staphylococci
N-BUOH	N-butanol
NF-ϰB	Nuclear factor kappa B

NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OD	Optical density
PBS	Phosphate buffer saline
PGE2	Prostaglandin endoperoxide synthase 2
PGG2	Prostaglandin G2
PSF	Penicillin/streptomycin/fungizone
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SANBI	South African National Botanical Institute
SCC	Somatic cell count
SI	Selectivity index
TNFR1	Tumour necrosis factor receptor 1
TNF-α	Tumour necrosis factor alpha
TRADD	TNF receptor associated death domain
Trolox	6-hydroxy-2, 5, 7, 8-tetrahydroxy-chroman-2-carboxylic acid
UPLC-MS	Ultra performance liquid chromatography-mass spectrometry
WHO	World Health Organization

LIST OF SYMBOLS AND UNITS

±	Plus, or minus
µg	Microgram
µl	Microliter
µM	Micromolar
%	Percentage
+	Plus
=	Equal to
g	Gram
m	Meter
mg/ml	Milligram per millilitre

CONFERENCE PRESENTATIONS

1. Ogbuadike, E.C., Nkadimeng, S.M., Makhubu, F.N., Igwe, C.C., Dzoyem, J.P., Qekwana, D.N., Petzer, I.M., McGaw, L.J. (2022). Anti-quorum sensing, anti-biofilm activity, antibacterial activity, total phenolic and flavonoid contents of *Kalanchoe gunniae* and other selected plants with potential for managing mastitis *Faculty of Veterinary Sciences, University of Pretoria - Faculty Day. Poster presentation*

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MANUSCRIPTS UNDER REVIEW AND IN PREPARATION

1. Ogbuadike, E.C., Nkadimeng, S.M., Khunoana, E.T., Igwe, C.C., Qekwana, D.N., Petzer, I.M., McGaw, L.J. Mechanisms of anti-inflammatory and anti-oxidant activity of extracts of selected South African Celastraceae and Crassulaceae plant species with potential use against bovine mastitis.

2. Ogbuadike, E.C., Nkadimeng, S.M., Makhubu, F.N., Igwe, C.C., Dzoyem, J.P., Ndivhuwo, K.K., Qekwana, D.N., Petzer, I.M., McGaw, L.J. Identification of compounds and bioactivity of crude extract and fractions of *Kalanchoe gunniae* against *S. aureus* reference strain and isolates from bovine mastitis**.**

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ABSTRACT

Infectious mastitis is an inflammation of the udder usually associated with increased somatic cells and presence of microorganisms. It is challenging to the health of cattle due to antibiotics resistance resulting in huge financial losses. Inflammation is a complex reaction of living tissues to microbial infection or injury. Normally it promotes healing, but when uncontrolled it can result in cell damage or death. In cattle and humans, inflammation is a major feature of infectious mastitis which has significant health and financial challenges. Excess reactive oxygen species accompanying uncontrolled inflammation are also harmful. *Staphylococcus aureus* is increasingly becoming resistant to antibiotics for the management of infectious bovine mastitis. This is often coupled with significant financial losses for the farmers. Plant secondary metabolites like flavonoids, phenolic compounds and their synergetic activity have been reported to provide alternative treatment due to their antibacterial, antioxidant and anti-inflammatory activities.

In this study, the antimicrobial activity of 15 plants selected on the basis of preliminary activity of related plant species in the same family (chemotaxonomic selection), or relevant traditional uses of the species for treatment of infections in humans (ethnopharmacological selection) was initially investigated. A broth microdilution assay using acetone and ethanol extracts of these plants was used to determine activity against an ATCC strain of *Staphylococcus aureus (S. aureus* ATCC 29213). Among these 15 plants, extracts of two plants from chemotaxonomy selection and two plants from ethnopharmacological selection, namely *Maytenus undata* and *Maurocenia frangula*, and *Kalanchoe gunniae* and *Bryophyllum pinnatum* (synonym *Kalanchoe pinnata*), respectively, with promising minimum inhibitory concentration (MIC) values ranging from 0.02 to 0.31 mg/ml were selected for further assay. These extracts were then tested against six multiple-drug resistant *S. aureus* isolates from clinical bovine mastitis cases. The four plant extracts were further analysed for cytotoxicity and anti-quorum sensing activities. Two of the plants with good results were assessed for anti-biofilm activity. The anti-inflammatory and antioxidant activities of acetone and ethanol leaf extracts of *Maurocenia frangula*, *Maytenus undata*, *Kalanchoe gunniae* and *Bryophyllum pinnatum* were further investigated. Antioxidant activity was evaluated using radical scavenging 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and electron reducing 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. Anti-inflammatory activity was determined via inhibition of 15-lipoxygenase (15-LOX) and cyclooxygenase (COX) enzymes, as well as inhibition of nitric oxide (NO) production by lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. Anti-inflammatory activity through regulating cytokine production was further investigated using ELISA kits. *Kalanchoe gunniae* consistently gave good results for all the above-mentioned

assays so *K. gunniae* 80% methanol crude extract was fractionated with hexane, dichloromethane, ethyl acetate, butanol and water. Antibacterial activity of crude extracts and fractions of *K. gunniae* were investigated against *S. aureus* (ATCC 29213) and six *S. aureus* bovine mastitis isolates using a broth microdilution assay**.** Cytotoxicity of the crude extract and fractions were further investigated against Vero cells and their selective indices were determined. The total flavonoid and total phenolic contents of the fractions were determined using the aluminium chloride spectrometric method and the Folin-Ciocalteu method respectively. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analysis of the crude extract and active fractions were performed.

Minimum Inhibitory Concentration (MIC) values of the extracts against *S. aureus* isolates ranged from 0.02 to 0.63 mg/ml*. Kalanchoe gunniae* extracts were the least cytotoxic with selectivity index (SI) ranging from 12 to 25. Significant biofilm inhibition against *S. aureus* mastitis isolates was observed at time zero (0 h), however there was no eradication of developed biofilm. *Kalanchoe gunniae* extracts had the best anti-quorum sensing activity with minimum quorum sensing inhibition concentration (MQSIC) and MIC values of 0.04 mg/ml and 0.63 mg/ml respectively on *Chromobacterium violaceum* ATCC 12472. From the results of antioxidant and anti-inflammatory assays, *K. gunniae* extracts had the best antioxidant potency with IC_{50} values ranging from 0.06 to 0.42 µg/ml. *K. gunniae* extracts also had the best 15-LOX inhibitory activity with IC_{50} values of 1.25 and 2.03 µg/ml for acetone and ethanol extracts respectively. At the highest concentration (100 µg/ml), the acetone extract of *B. pinnatum* had the best NO inhibition of 80.48% and cell viability of 96.75%. From COX-2 assay it was observed that *K. gunniae* extracts and *B. pinnatum* extracts were able to significantly inhibit COX-2. *B. pinnatum* extracts had the best cytokine activity. MIC values of the 80% methanol crude extract and fractions of *K. gunniae* against *S. aureus* ATCC 29213 ranged from 0.03 mg/ml to 0.63 mg/ml. MIC values against the six *S. aureus* bovine mastitis clinical isolates ranged from 0.02 mg/ml to 2.50 mg/ml. Total flavonoid content of fractions and crude extract ranged from 2.12 mg QE/g to 140.30 mg QE/g. The total phenolic content of fractions and crude extract ranged from 252.75 mg GAE/g to 943.09 mg GAE/g. From the crude extract, the major compounds 1-docosene, 1-nonadecene and benzenedicarboxylic acid and butyl 2-ethylhexyl ester were identified from GCMS. The compounds genistin, myristicin, apigenin-7-o-α-l-rhamnose (1→4)-6” o-acetyl-β-d-glycoside and mudanpioside H, were identified from UPLC-MS analyses. From active fractions, the major compounds 1-docosene, butane, 2,2,3,3-tetramethyl, cetene, tetradecane, nonadecane and heptadecane, 2-methyl-nonadecane were identified from GCMS-MS and the compounds genistin, mudanpioside h, apigenin-7-o-α-l-rhamnose (1→4)-6” o-acetyl-β-d-glycoside, myristicin, kaempferol-3-o-rhamnoside and safflor yellow A were identified from UPLC-MS analyses.

These results demonstrate that *K. gunniae* extracts were not cytotoxic and had good antimicrobial, anti-quorum and anti-biofilm activities as well as good antioxidant and anti-inflammatory activities. The presence of flavonoids and phenolic compounds in the active fractions of *K. gunniae,* especially high phenolic content in the active fractions as well as the identified compounds in the active samples was significant. These compounds have been reported to have antibacterial, antioxidant and anti-inflammatory activities, supporting the premise that active extracts and fractions of *K. gunniae* are potential alternative antibacterial and anti-inflammatory agents for the treatment and management of mastitis.

Results of this research also demonstrate that active extracts of *K. gunniae and B. pinnatum* (which is also non-cytotoxic) have potential for utilization for the management of other inflammatory and oxidative degenerative diseases. Further work is recommended to investigate the bioactive principles of these active extracts and fractions in herbal therapeutic formulations.

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CHAPTER 1 Introduction

1.1 Introduction

Infectious mastitis is an inflammation of the mammary parenchyma caused by microbes (Sserunkuma et al., 2017). For infection to take place, microorganisms typically infiltrate underlying tissues, colonise the tissues, replicate, and as acute colonisation starts, pain and inflammation ensues, leading to inflammatory reactions. With the multiplication of microorganisms, the immune response, which brings about inflammatory reactions, is initiated and infection is thus established (Negut et al., 2018). In bovines, infectious mastitis and the subsequent inflammatory reaction is brought about by entry of pathogens into the udder.

Microbial infections in general cause high morbidity and mortality to the extent that 50 000 people die every day worldwide as a result of infections (Boakye et al., 2016; Pauw & Eloff, 2014). The serious challenge of microbial multi-drug resistance makes it even more complex and pathogenic organisms causing mastitis are no exception to multi-drug resistance (Bérdy, 2012; Eloff, 2000; MILK, 2014). Mastitis is an epidemic and infectious disease of mammals that is very difficult to treat or manage (Goni et al., 2004). According to Sharma et al. (2011), bovine mastitis is both an animal welfare problem and a food safety problem for humans and as such a significant economic and public health challenge.

In South Africa, there are reports of increasing prevalence of clinical mastitis and even 100% prevalence of subclinical mastitis (Ateba et al., 2010; Petzer et al., 2009). Mastitis causes great losses to the dairy industry in South Africa and the world at large (Fernández et al., 2014; MILK, 2014; Sserunkuma et al., 2017). Some strains of *S. aureus* can possibly produce staphylococcal enterotoxins (SEs) in milk, resulting in food poisoning (Pexara et al., 2013). In the world ranking of food-borne diseases, *S. aureus* food poisoning is ranked third (Boerema et al., 2006).

The disease of mastitis is challenging to the health of both animals and humans (dairy cattle producers, veterinary and health professionals) due to the complex resistance patterns of *S. aureus,* the major causative organism of mastitis (Ateba et al., 2010). The dangerous adverse effects of synthetic drugs like antibiotics also contribute to the problem (Panichayupakaranant & Kaewsuwan, 2004; Petrovska, 2012). If not taken care of, methicillin resistant *S. aureus* (MRSA) from animals and even its resistant genes can spread to humans through direct contact with animals or by consuming infected animal food products (Kluytmans et al., 2002).

Anti-infective agents from medicinal plants have the possibility of serving three strong purposes needed for eradication of mastitis: (i) They can act as antimicrobials by directly killing the microorganism (bacteriocidal) or indirectly slowing the growth of the microorganism (bacteriostatic) (Leekha et al., 2011). (ii) They may reduce side effects accompanying administration of synthetic antimicrobial drugs (Madhuri et al., 2012) . (iii) Some plant constituents prevent microorganisms from gaining entrance into cells due to their membrane stabilizing properties. Thus, they show promising potential to be used for preventative therapy (Iwalewa et al., 2007).

1.2 Research question

Multidrug resistance of causative agents of mastitis currently present a major challenge to the management of mastitis with synthetic antibiotics. Medicinal plants have been used for ages for the treatment of diseases due to the presence of active natural product constituents in plants. These medicinal plants can be utilized for the management of mastitis and its symptoms. However, availability together with dose related issues make the use of medicinal plants unreliable at the actual times of need. The question now is, can natural active plant constituents for alternative/complementary management of mastitis be identified and developed into readily available standardized therapeutic herbal product formulations for medication and easy application?

1.3 Hypothesis

Active substances that have the ability to treat or prevent microbial infections caused by antimicrobial resistant etiological agents of mastitis can be obtained from some medicinal plants of chemotaxonomical and ethnopharmacological importance.

1.4 Aim

The aim of this study is to identify biological activity and possible chemical compounds responsible for activity from selected medicinal plants with potential application in treating mastitis, as well as clinical signs and inflammation associated with the disease.

1.5 Objectives

1. To determine the antibacterial activity of plant extracts prepared from selected South African species of Celastraceae and Crassulaceae families, for activity against

reference and drug-resistant clinical isolates of major causative organism of bovine mastitis.

2. To determine cytotoxicity, and mechanisms of inhibiting bacterial infection through anti-quorum sensing and anti-biofilm activities of most active extracts.
3. To characterize anti-inflammatory and antioxidant extracts of active plant species with potential for standardization and use against bovine mastitis**.**
4. To identify compounds from bioactive crude extract and fractions of most active plant species with potential for standardized preparation and use against bovine mastitis.

CHAPTER 2 Literature review

2.1 Mastitis

Infectious diseases caused by microorganisms are a major public health problem worldwide (Cos et al., 2006; Jiménez-González et al., 2013). According to Boakye et al. (2016) Boakye et al. (2016), infectious diseases are ranked among wars and famine as one of the most serious factors that negatively influence the survival of man worldwide. Pauw and Eloff (2014) stated that infections are the world's leading cause of premature death, killing almost 50 000 people every day. One such challenging infectious disease is mastitis.

Mastitis is an inflammation of the mammary parenchyma or breast caused mainly by microbes and physical factors. It affects both animals (cattle) and humans. It is characterized, depending on its severity, by tenderness, redness of the skin, heat, and fever (Semba, 2000; Sharma et al., 2011; Sserunkuma et al., 2017). According to Viguier et al. (2009), in the (Latin) word mastitis, *mast* stands for breast and *itis* stands for inflammation. Viguier et al. (2009) went further to state that mastitis can be clinical, subclinical or chronic. The clinical stage is sometimes accompanied by abscesses or ulcers. Chronic mastitis is a situation where there is continuous inflammation of the mammary gland. Clinical mastitis can be visually detected by changes in the milk (which may be watery or show the presence of floccules or blood), the udder (pain, heat, oedema) or systemic changes in the animal in severe cases. Chronic mastitis persists and changes can often be palpated in the udder parenchyma (fibrosis, nodules and atrophy). The subclinical stage can only be detected in a laboratory by identifying a somatic cell count (SCC) in excess of 200 000 cells/ml with presence of microbial pathogens in milk (Sargeant et al., 2001). Subclinical mastitis is not visible nor is it easily detected. Mastitis usually starts with subclinical symptoms. Undetected and untreated subclinical mastitis may progress to clinical mastitis which is more dangerous if the body cannot cope with it. Early detection of subclinical mastitis is therefore very important and SCC plays a very important part in early detection of mastitis.

2.2 Prevalence of mastitis

It has been reported that the prevalence of bovine mastitis in South Africa is increasing. For example, there was an increase in teat canal infection (subclinical mastitis) from 8.1% in 2002 to 15.4% in 2006 and visibly observable mastitis (clinical mastitis) from 24.1% to 30.0% in South Africa (Petzer et al., 2009). Studies carried out by Ateba et al. (2010) on 28 milk samples from communal farms and commercial farms in the Mafikeng area of South Africa

showed that all the milk samples had bacterial contamination, indicating 100% prevalence. These prevalence rates observed by Ateba et al. (2010), support the above reports of Petzer et al. (2009) of increasing prevalence rates of mastitis over the years in South Africa. The challenge of mastitis is not limited to South Africa. Junaidu et al. (2011) found 52% prevalence of mastitis in milk samples from lactating cows in commercial dairy farms in Sokoto, Nigeria with *S. aureus* being the most prevalent causative organism. In research carried out on small holder dairy farms in Zimbabwe, Katsande et al. (2013) reported 21.1% prevalence of mastitis with subclinical mastitis being 16.3% and clinical mastitis 4.8% on cows and prevalence of 49.3% mastitis at herd-level.

Of more concern is resistant mastitis pathogens. There are reports of their existence and prevalence in South Africa and other parts of the world. Scientific research showing prevalence of mastitis resistant pathogens includes the work of Ateba et al. (2010), which further reported MRSA prevalence of 6.35% in commercial farms and 87.2% in communal farms in the Mafikeng area of South Africa. Most of the isolates were resistant to at least three groups of antibiotics including methicillin and this is very worrisome. Methicillin Resistant *Staphylococcus aureus* prevalence has also been reported in other countries in Africa. For example, in Nigeria, MRSA prevalence of 7.6% from cow milk was reported (Suleiman et al., 2012). In Ethiopia, Daka et al. (2012) reported MRSA prevalence of 60.3% in cow milk samples. The problem of mastitis and especially resistant *S. aureus* is also not limited to Africa. Across the world, prevalence of MRSA has been reported. In Brazil, 25% MRSA prevalence was reported in cow milk (Coelho et al., 2009). In USA, 4% prevalence of MRSA in the herd and 1.3% in milk was reported (Haran et al., 2012). In the Czech Republic, MRSA prevalence of 14.7% was reported in milk samples (Stastkova et al., 2009). In Germany 4.4% occurrence of MRSA in milk was reported (Kreausukon et al., 2012) . MRSA prevalence has also been reported in milk samples in the following countries, India 13.1% (Kumar et al., 2011), Thailand 19.7% (Intrakamhaeng et al., 2012), Pakistan 10.4% (Farzana et al., 2004) and Iran 28.3% (Alian et al., 2012) .

Management practices can help to reduce the prevalence of subclinical mastitis caused by strains of bacteria that are not resistant to antibiotics (Karzis et al., 2018). However, Karzis et al. (2018) also reported that they observed decreased prevalence of mastitis in the selected herds with good management that was only numerical. Their report went further to state that overall prevalence of mastitis did not decrease significantly. If nothing is done to eradicate resistant mastitis pathogens, as long as they are still in existence in farms, animals and humans, the problem persists because they will continue to spread in one way or the other. One way in which they spread is by transferring resistance to sensitive bacteria through conjugation, referred to as R-plasmid mediated antibiotic resistance (MILK, 2014). Resistant

strains can also develop by gradual mutation of a formerly non-resistant strain (susceptible strain) to resistance strain. Alternatively, an already resistant strain can simply infect an animal/human.

2.3 Economic effect and consequences of mastitis

Mastitis pathogens can contaminate dairy milk and human breast milk and render it unfit for consumption even at the subclinical stage if not pasteurised. It is a very costly disease that causes great losses to dairy industries in South Africa and the world over (Fernández et al., 2014; MILK, 2014; Sserunkuma et al., 2017). In humans, it increases mother to child transmission of human immunodeficiency virus (HIV) via breast milk (Semba, 2000).

In South Africa, a 1% increase in somatic cell count of individual milk bulk-tank leads to a profit loss of between R491.48 and R1 795.57 per cow per year (Banga et al., 2014). In the United States and UK, producers lose about $2 billion and £300 million respectively as a result of reduced milk synthesis due to mastitis in dairy herds. This figure excludes the loss that occurs after the milk has left the farm e.g. losses due to changed milk composition, low milk quality and low quality of other dairy products (Viguier et al., 2009).

Mastitis affects both the quantity and quality of milk and milk products produced. On the part of quantity, the volume of milk produced is reduced due to reduced milk synthesis and discarding of milk when mastitis is detected on time or is easily visible (Halasa et al., 2007). On the part of quality, there is reduced quality owing to changes in milk consistency and milk constituents. There is low casein part of the milk protein, low fat, decrease in lactose and α-lactalbumin in the milk due to lowered synthetic activity of the mammary gland. Increased somatic cells in the milk also leads to reduced shelf life of the milk and may lead to undesirable taste due to increased enzymatic activity of somatic cells and free fatty acids leading to rancid flavour. Dairy products made from mastitis milk are inferior. For example, the fermentation of yogurt may be incomplete or even stopped. Increased fatty acid content in the yogurt leads to reduced shelf life of the yogurt. With high somatic cell counts in milk, there is also reduced cheese production due to the increase of whey protein and the decrease of casein. Mastitis also reduces the hygiene standard of the milk, thus drinking of raw milk with high somatic cells increase the risk of ingestion of pathogens, their toxins and cattle neutrophils by humans. Mastitis makes the process of pasteurisation of milk more difficult and even when the milk is pasteurised, microorganisms may be removed but toxins will still be present (Harmon, 1994; Janzen, 1970; Sharma et al., 2011). On the part of the animal itself, mastitis reduces the productive life of the animal and causes premature culling. In severe cases the culled cattle meat has reduced value because of low quality and yield or even no value in the case of disposal (Halasa et al., 2007; Viguier et al., 2009).

The total cost and consequences of mastitis are actually more than what is usually indicated. This is because it is very difficult to quantify the exact cost of subclinical mastitis since the signs are not visible to the farmer (Petrovski et al., 2006). Also, mastitis, especially clinical mastitis, causes severe pain to the animal (Leslie & Petersson-Wolfe, 2012) and this pain cannot easily be measured. The true cost and consequences of mastitis should therefore be seen as being more than what is possibly calculated.

2.4 Causative organisms of mastitis

Most causative organisms of mastitis are bacteria and fungi, with bacteria being the most common. However, there are sometimes cases of algal and viral pathogenic causes of mastitis (Viguier et al., 2009)**.** The infection may be caused by a single microbial species or a combination of different microorganisms. For example, a combination of different bacteria (Waage et al., 1999) and in some cases both bacteria and fungi are found together in individual milk samples) (Bourtzi-Hatzopoulou et al., 2003). Bacterial causative organisms of mastitis are grouped into major or minor causative organisms. The bacteria can also be contagious or environmental.

Major causative organisms of mastitis include the contagious bacteria *Staphylococcus aureus (S. aureus)* (coagulase positive staphylococcus), *Streptococcus agalactiae* and environmental bacteria e.g. coliforms, streptococci, and enterococci. The coliforms include *Escherichia coli, Klebsiella* spp., *Enterobacter* spp*.,* and *Citrobacter* spp. Major causative organisms within the streptococci include *Streptococcus dysgalactiae, Streptococcus uberis* and *Streptococcus bovis.* For enterococci, major causative organisms are *Enterococcus faecium and Enterococcus faecalis.* With contagious major pathogens, infection is serious and can easily progress to clinical mastitis. With environmental major pathogens, although the infection is also serious, the infection usually exists for less than 30 days (Harmon, 1994).

Minor causative organisms of mastitis include non-aureus staphylococci (NAS) (previously known as coagulase-negative staphylococci) and *Corynebacterium bovis*. Infections by minor causative organisms are usually not considered serious because they hardly lead to clinical mastitis. They also neither lead to serious decreases in milk production, nor remarkable changes in milk composition nor serious/prolonged inflammation. With minor causative organisms the increase in somatic cell count can only be 2- to 3-fold. Other very uncommon pathogens that can cause periodic cases of mastitis include *Pseudomonas* spp*.*, *Actinomyces pyogenes* and *Serratia* spp*.* (Harmon, 1994).

Although *S. aureus* with its chronic and destructive nature is still the major causative organism of mastitis, NAS are increasing in importance in South Africa. Studies carried out by Petzer et al. (2009) showed NAS to be the most common causative organisms of mastitis between the

years 1996–2007 in South Africa. The authors also reported that for herds that have controlled major mastitis pathogens, NAS are the most frequently isolated mastitis pathogen. Non-aureus staphylococci causative organisms of mastitis include *Staphylococcus simulans, S. chromogenes, S. hyicus, S. xylosus, S. haemolyticus, S. capitis, S. warneri, S. sciuri* and *S. auricularis*. Other organisms detected as etiological pathogens of mastitis include *Arcanobacterium pyogenes*, *Bacillus* spp., for example *Bacillus cereus*, and *Pasteurella haemolytica* (Waage et al., 1999) (Waage et al., 1999).

Staphylococcus aureus is the major and most serious causative organism of mastitis (Harmon, 1994; Islam et al., 2012; Waage et al., 1999). It has also been reported as the predominant causative organism of mastitis in South Africa (Swartz & Novello, 1984).

For fungi, species of the genus *Candida* are the major causative organisms of mastitis. Pachauri et al. (2013), reported *Candida albicans* as the most common isolated fungal causative organism of mastitis. Other *Candida* species implicated in causing mastitis include *C. tropicalis, C. krusei, C. pseudotropicalis, C. parapsilosis* and *C. rugosa*. *Aspergillus fumigatus, A. niger, Geotrichum candidum* and *Rhodotorula* spp. are also other fungal causative organisms of mastitis (Bourtzi-Hatzopoulou et al., 2003; Pachauri et al., 2013).

2.5 Clinical and subclinical signs of mastitis

2.5.1 Skin ulceration accompanying mastitis

Ulceration, as one of the clinical signs of mastitis, is most predominantly associated with MRSA infection, although other organisms implicated in causing mastitis have also been isolated from such ulcers (Baba et al., 2015; Salavastru et al., 2012). Ulcers do not as yet have a clear, readily available conventional or standard drug for (Krishnamoorthy et al., 2012; Moffatt et al., 1992) . Skin ulcers are wounds resulting from a breakdown of the skin or mucous membrane, resulting in a very difficult to heal sore that erodes away tissue and may be accompanied by abscesses and pus. As previously mentioned, skin ulcers mostly result from microbial infection but can also be caused by injury or oxidative damage in the body, accompanied by inflammation, forming a wound with a burning sensation. Wounds lead to a loss of continuity of epithelium in the skin with or without the loss of underlying connective tissue (Krishnamoorthy et al., 2012). Wound healing is a complex process of restoring impaired cells and tissues back to their normal state. It occurs as a cellular response to injury and involves activation of fibroblast, endothelial cells, and macrophages (Muhammad et al., 2013). Sometimes an infection is treated but the wound or ulcer that has already been created still remains. Therefore, completely treating ulcers associated with mastitis involves treating both the infection and the wound. The complicated process of wound healing depends on cell

migration and proliferation. There is a shortage of scientific reports on agents that can facilitate the process of wound healing at cellular level (Krishnamoorthy et al., 2012). According to Negut et al. (2018), natural products are a promising group for new antimicrobial agents against resistant organisms in wound management. Consequently, researching and finding skin ulcer/wound healing bioactive agents is of extreme importance.

2.5.2 Somatic cell count (SCC)

Somatic cells are protective cells of the animal body and they fight pathogenic organisms. Somatic cells are made up of leukocytes (white blood cells) and epithelial cells. The leukocytes comprise lymphocytes, macrophages, erythrocytes and neutrophils which are mainly polymorphonuclear neutrophils (PMNs) (Harmon, 1994). The leucocytes enter the mammary gland in response to injury. Epithelial cells are milk secreting cells shed from the mammary gland. The leukocyte content of somatic cells is about 75% and the epithelial cells are about 25% (Sharma et al., 2011).

Keratin lining the sphincter muscles of the teat canal usually prevents the entry of bacteria through the teat canal into the udder (Viguier et al., 2009). This keratin contains long chain antimicrobial fatty acids that help in fighting invading pathogens. However, as the time of giving birth comes closer, there is an increase in fluid within the mammary gland. This leads to increased intramammary pressure and dilation of the teat canal which may be followed by leakage of mammary secretions. The dilation of the teat canal permits entry of pathogens through the teat to the mammary gland. During milking, about 30% of the keratin is milked out and it usually takes at least 2 hours for the sphincter to return to its tight and firm position. During this interval of loose sphincter, microorganisms can invade the mammary gland. Inside the mammary gland, bacteria multiply and release toxins which induce epithelial cells to release inflammatory mediators like cytokines, chemokines and defence peptides (Sharma et al., 2011; Viguier et al., 2009).

Bacterial toxins also induce leukocytes to stimulate rapid influx of polymorphonuclear neutrophils (PMNs) (Schukken et al., 2003). PMNs have granules that store bactericidal agents like enzymes, peptides and proteins. Once inside the mammary gland, PMNs engulf and destroy bacterial cells through oxygen-dependent and oxygen-independent systems. However, although PMNs are major defence mechanism of the udder, their activity against bacteria is reduced because they also engulf fat globules and casein particles. Reduced casein leads to lower quality of milk. The influx of PMNs in milk constitute the major increase in SCC. Macrophages from leukocytes help in phagocytosing bacteria and destroying them with proteases and oxidants released by the macrophages. Released proteases and oxidants

from macrophages also destroy milk producing cells, the epithelia cells. Toxins produced by bacteria likewise contribute in destroying epithelial cells.

Destruction of the epithelial cells leads to reduced milk production and release of lactate dehydrogenase (LDH) and N-acetyl-b-D-glucosaminidase (NAGase) enzymes into the milk. When the activity of PMNs is finished, they are either destroyed by apoptosis or engulfed and ingested by macrophages. Shed and dead epithelial cells as well as dead leucocytes are secreted into the milk. All these add to the high somatic cell levels in milk (Harmon, 1994; Viguier et al., 2009)**.** The determination of the total sum of somatic cells in milk is known as somatic cell count (SCC) (Sharma et al., 2011). During the above explained stage of infection leading to somatic cell increase with no visible effect on the udder, the animal is undergoing sub-clinical mastitis. If the immune system is not able to eliminate the pathogen, the infection persists and inflammation response goes on and on leading to visible mastitis symptoms which is the clinical mastitis (Schukken et al., 2003).

Apart from udder infection which is the major factor for increased SCC in milk, other minor factors like stage of lactation, diurnal variation, milk transportation and or management, age of the animal, breed of the animal, season, stress, can also affect somatic cell count in milk (Harmon, 1994). Because of these, following a high SCC, subclinical mastitis is confirmed by microbial culture test (Sargeant et al., 2001).

2.5.3 Inflammation

Inflammation often accompanies mastitis and skin ulcers. It is a complex reaction of living tissues to microbial infection, chemical/physical injury, environmental pollution or irritation that can result in cell damage or death. It is usually associated with painful sensation, heat, redness and swelling, and loss of function (Jalil et al., 2003). Inflammation can be acute or chronic. Acute inflammation is short term inflammation characterised by rapid onset and short duration. It is a defence and healing response which serves to protect the body by killing microbes and facilitating wound repair. The killing of microbes and wound healing is brought about by migration of leukocytes to the site of infection or injury, and normal short term/moderate activities of phagocytes like neutrophils. Through exudation of fluids and plasma proteins, digested or ingested microbes and foreign particles are removed from the body by the activities of phagocytes (Iwalewa et al., 2007).

Chronic inflammation occurs when the inflammatory process does not shut off. It has a prolonged duration in which the activities of the immune system continue and do not bring about the desired effect. Instead of healing, more damage is done to the body (Iwalewa et al., 2007). The complex process of inflammation that leads to a chronic state is such that upon

microbial infection or presence of foreign particles, phagocytes like neutrophils and macrophages are activated to carry out phagocytosis to cause excess activation of those phagocytes (Latha et al., 2011). This excess activation of phagocytes leads to increased oxygen consumption by the neutrophils (Colin & Monteil, 2003). This in turn leads to oxidative stress/burst at the site of microbial invasion (Jalil et al., 2003). Then reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot}$), hydroxyl (OH) and peroxyl (OOH, $ROO^{\cdot}$) – free radicals and hydrogen peroxide (H_2O_2) - non-free radical species are generated (Sakat et al., 2010). Excess production of these free radicals and non-free radicals harms the surrounding tissue either by powerful direct oxidizing action with O_2, or indirectly with H_2O_2 and $^{\cdot}OH$ radicals formed from $O_2^{\cdot}$ (Latha et al., 2011). The complex process is such that there is also an increase in the following enzymes; phospholipase A_2 (PLA_2), 5-lipoxy-genase (5-LOX), cyclooxygenases e.g., cyclooxygenase-2 (COX -2) and inducible nitric oxide synthase (iNOS). Increased iNOS then brings about the enzymatic oxidation of L – arginine to citrulline thereby forming nitric oxide ($^{\cdot}NO$). The $O_2^{\cdot}$ reacts with $^{\cdot}NO$ to form peroxynitrite ($ONOO^-$) which is a strong oxidant. As a strong oxidant, $ONOO^-$ initiates lipid peroxidation (Adibhatla and Hatcher, 2006). An inflammatory response is triggered (Latha et al., 2011) leading to production of more ROS generating enzymes and activation of the transcription factor, nuclear factor kappa B (NFkB) (Iwalewa et al., 2007), pro-inflammatory cytokines and other inflammatory mediators like tumor necrosis factor (TNF-α) (Paterson et al., 2003). More membrane destruction and tissue damage take place, leading to increased and persistent inflammation. At this stage there are degenerative or pathological processes of various diseases, and inflammation itself is one of them (Jalil et al., 2003).

Identification of naturally occurring agents from plants (bioactive compounds), which could decrease the neutrophil accumulation in inflamed areas, thus scavenging these reactive oxygen species, may be beneficial in the treatment of inflammatory disorders. In addition, lipoxygenase (LOX) activity mediates leukotriene production from arachidonic acid. (Hu & Ma, 2018), and leukotriene is likewise a mediator of inflammation. Another property of lipoxygenase enzymes is that they are able to oxidise fatty acids to their corresponding hydroperoxide. This property can be very useful in determination of lipoxygenase enzyme inhibition in anti-inflammatory assay. Thus extracts/compounds that are able to inhibit lipoxygenase activity will be good anti-inflammatory agent. Also plant bioactive principles which can inhibit or reduce any of the other molecular targets involved in chronic inflammation can as well be beneficial in inhibiting or reducing inflammatory process, thereby treating inflammatory disorders.

2.5.4 Microbial resistance to antibiotics

Despite the breakthrough made in discovering the therapeutic efficacy of antibiotics in the 20th century (Rangasamy et al., 2007), and the tremendous advances made in the understanding of microorganisms and their control, the incidence of epidemics due to drug resistant microorganisms together with emergence of new disease–causing microorganisms presently poses a huge public health concern (Boakye et al., 2016). Due to the frightening increase in the resistance of microorganisms to antibiotics, some scientists have declared that a post antibiotic era is imminent (Eloff, 2000). These resistant microorganisms may be multi-drug resistant. This issue of drug resistance is also noted amongst mastitis-causing microorganisms.

The challenges posed by infectious diseases are not only those related to decreasing efficacy (due to microbial resistance) of antibiotics but there are also increasing contraindications of their usage (Petrovska, 2012). For example, synthetic drugs can be carcinogenic (Panichayupakaranant & Kaewsuwan, 2004). There is a need to look for antimicrobial active substances from other sources because: (i) Antibiotic resistant strains of clinically important pathogens are spreading at an alarming rate leading to the emergence of new bacterial strains that are multidrug-resistant (WHO, 2001 [http/www.who.int/mediacentrefactsheets/fs/134]) (Bagozzi; Gerits et al., 2016). (ii) There is non-availability and high cost of new generation (synthetic) antibiotics (that are without dangerous side effect) (Akinsulire et al., 2007; Jiménez-González et al., 2013).

Among microbial infections, bacterial and fungal infections as reported by Boakye et al. (2016) are significant causes of morbidity and mortality despite advances in medicine. On the part of fungi, discovery of new antifungal agents is increasingly becoming scarce (Boakye et al., 2016). Major pathogenic organisms of mastitis and skin ulcer are bacteria and fungi. Among all the pathogenic organisms of mastitis and skin ulcer, *S. aureus* is implicated as the major causative organism.

2.6 Resistance of the major mastitis causal organism, *Staphylococcus aureus*

2.6.1 Antibiotic resistance

Control of mastitis with antibiotics is very difficult because of the development of antibiotic resistant, including multi-drug resistant, strains. Resistant traits are carried by certain genes on bacterial plasmids, chromosomes, transposons and gene cassettes (Ateba et al., 2010). The major causative organism of mastitis, *S. aureus,* has developed resistance to antibiotics which is of great concern. The resistance of *S. aureus* like with other resistant bacteria

developed as a result of widespread and indiscriminate use of antibiotics e.g. for management of mastitis, antibiotics are used for therapy, prevention and as growth-promoting agents in animal feed (Goni et al., 2004).

A remarkable type of *S. aureus* resistance is methicillin-resistance, and such *S. aureus* strains are referred to as methicillin-resistant *S. aureus* (MRSA). Following the licensing of methicillin in 1961, MRSA was isolated in the same year. MRSA refers to all *S. aureus* strains that are resistant to the antibiotic methicillin and all other semi-synthetic, penicillinase-resistant, β-lactams like cloxacillin and oxacillin. MRSA are also resistant to cephems and cephalosporins and all other β-lactam antibiotics like ampicillin, amoxicillin and ticarcillin. Resistance of MRSA also extends to aminoglycosides, tetracycline, chloramphenicol, fluoroquinolones and macrolides. Vancomycin resistant strains of MRSA has also been reported (Lee, 2003; Pexara et al., 2013).

On susceptible strains of *S. aureus,* β-lactam antibiotics work by inactivating penicillin-binding proteins found in *S. aureus*. The penicillin-binding proteins are enzymes that are important in the assembly of the bacterial cell wall. By inactivating penicillin-binding proteins, the cell wall becomes weak and lyses, and bacteria are destroyed. The resistance of MRSA is brought about by the presence of the *mec*A gene or its homologue*, mec*C, in the organism. The MRSA *mec*A gene encodes the penicillin-binding protein PBP2' (also referred to as PBP2a) which confers resistance to all β-lactam antibiotics, including the semi-synthetic penicillins. PBP2' has a very low binding affinity for β-lactam antibiotics, thus it is not inactivated and helps in cell wall assembly when ordinary penicillin-binding proteins are rendered non-active. Some strains of MRSA have another mechanism of resistance brought about by their hyper-production of the *S. aureus* β-lactamase enzyme. The over produced β-lactamase enzyme inactivates β-lactam antibiotics by hydrolysing the β-lactam ring (Pexara et al., 2013).

There are other *S. aureus* resistant strains e.g., the aminoglycoside-modifying strains. These *S. aureus* resistant strains confer their resistance by producing aminoglycoside-modifying enzymes (AGMEs). AGMEs modify the antibiotic and renders them inactive. Production of the aminoglycoside-modifying enzymes N-acetyltransferase [AAC (6')] and 2''-O-phosphotransferase [APH (2'')] enzymes can mediate resistance to the following antibiotics kanamycin, tobramycin, gentamicin, streptomycin, tetracycline, chloramphenicol, ampicillin, and neomycin. Production of the enzyme 3'-O-phosphotransferase [APH(3')I] can mediate resistance to methicillin, neomycin, gentamicin, streptomycin, apramycin, cefepime, tetracycline, ceftazidime, chloramphenicol and cefotaxime (Goni et al., 2004).

2.6.2 Biofilm formation and quorum sensing as mechanisms of resistance

One of the mechanisms that bacteria use to become highly resistant to antibiotics is by being able to adhere to both biotic and abiotic surfaces to form biofilms. A biofilm is a community of complex matrices of sessile micro-organisms attached to a surface surrounded by polysaccharides called glycocalyx. The glycocalyx protects the biofilm cells from external and deleterious agents (Sandasi et al., 2008).

According to Sandasi et al. (2010), biofilm accounts for 60% of infections. In the biofilm system, bacteria exert more resistant to antibiotics and immune system defence than bacterial in planktonic forms (Mohsenipour & Hassanshahian, 2015). Usually, bacteria condition a suitable surface for cell attachment by adsorbing nutrients and substances necessary for cell growth and easy adhesion. Following the surface conditioning, the next stage in biofilm formation is cell attachment. The ability of plant extracts to inhibit cell attachment will help to reduce microbial colonization and control infection (Sandasi et al., 2008).

Another mechanism by which micro-organisms exert resistance to antibiotics and the immune system is by quorum sensing. Quorum sensing (QS) is a process of communication by microorganisms using a signalling mechanism of molecules called autoinducers (AI) which are dependent on microbial density and result in simultaneous action of the microbial group in the activation or repression of important genes once a threshold density is perceived (Padder et al., 2018). The threshold density is referred to as a quorum (Nazzaro et al., 2013). A wide spectrum of physiological and cellular processes is controlled by the QS system. These include regulation of virulence factor expression, pigment production, adhesion, biofilm formation, bioluminescence, exopolysaccharide production, and sporulation in different micro-organisms (Chenia, 2013). Micro-organisms use QS to regulate virulence gene expression and with this ability, microbial pathogens have been able to develop multiple drug resistance (Padder et al., 2018).

Apart from autoinducers, other elements used by quorum sensing systems include signal receptors, signal response regulator, signal synthase and regulated genes (QS regulon). In bacteria autoinducers are also called bacterial pheromones. Depending on the bacteria, autoinducers can differ. Autoinducers are classified into 3 sub-groups. Autoinducers produced by Gram-negative bacteria are acylated homoserine lactone (AHL), which belongs to the first sub group. In Gram-positive bacteria, autoinducers produced are secreted peptides, which belong to the second sub group (Nazzaro et al., 2013).

The autoinducer produced by *Staphylococcus aureus*, a Gram-positive bacterial species, is a peptide AI called AIP (autoinducing peptide). *Staphylococcus aureus* uses the AIP and the *Agr* QS to control pathogenesis (Eickhoff & Bassler, 2018). The *Staphylococcus* QS system controls protein expression. It is responsible for the production of staphylococcal virulence

factors such as α-, β-, and δ- hemolysin. In the QS system the *agr* locus of *S. aureus* QS is a quorum-sensing gene cluster of five genes (*hld, agrA, agrB, agrC*, and *agrD*). The *agr* locus, is responsible for the production of δ-hemolysin a translational protein product of RNAIII. Expression of *hld* gene leads to transcription of RNAIII which in turn mediates translations into δ-hemolysin. Δ-hemolysin is also called δ-toxin (Quave et al., 2011). Anti-infective agents that have activity against *Staphylococcus aureus* QS can stop or inhibit the production of δ-hemolysin.

For intra- and interspecies communication, both Gram-positive and Gram-negative bacteria use a furanosyl boronated diester molecule called AI-2 and a non-boronated diester molecule, termed vA1-2. These belong to the third sub-group of autoinducing (Nazzaro et al., 2013). Although Gram-negative and Gram-positive bacterial species use acylated homoserine AHL and AIP as autoinducers respectively, both Gram-positive and Gram-negative bacteria use the diester molecules, AI-2 and vA1-2 for quorum sensing communication, (Nazzaro et al., 2013). Once these diester molecules are activated, autoinducers are also activated and autoinducers mediate pigment production (Padder et al., 2018). Thus, activation of an autoinducer, is an indication that the diester molecules are activated and that quorum sensing communication has taken place.

Quantification of δ-hemolysin produced by *S. aureus* could be used to check the mechanism of action of an anti-infective agent against *S. aureus*. Nevertheless, this is usually not easy since δ-hemolysin protein is not pigmented and obtaining a standard pure reference of δ-hemolysin protein to use as a control in a quantification assay is also not easy. However, since diester molecules, the third sub-group of autoinducing molecules is present in both Gram-positive and Gram-negative bacteria and these diester molecules control both the first subgroup of autoinducers (which belong to Gram-negative bacteria) and the second subgroup of autoinducers (which belong to Gram-positive bacteria), regulation of the activation of any autoinducer in any bacteria (be it Gram-positive or Gram-negative bacteria) by an antimicrobial agent (e.g. a plant extract) is an indication that the antimicrobial agent is capable of regulating the activation of the diester molecules present in bacteria. Thus, *Chromobacterium violaceum* with its pigment production, which shows that the diester molecules are activated is usually used as standard organism for QS assay since violacein production or inhibition which can easily be observed in culture media due to the violet colour of violacein can be measured in the QS assay against *Chromobacterium violaceum* (Vattem et al., 2007).

Since biofilms are mediated by quorum sensing, targeting QS with phytochemicals can be used to fight biofilm infections. Non bactericidal but antipathogenic agents from plants are also important. This is because finding nontoxic inhibitors i.e., antipathogenic agents that are QS inhibitors from plants can help to control infection without promoting the occurrence of resistant

bacterial strains. The anti-quorum sensing of phytochemicals is still poorly understood (Nazzaro et al., 2013). Research into the QS mechanism of action of plant derived anti-infective agents is therefore crucial.

Considering these challenges in the use of antibiotics for the management of mastitis, there is an urgent need to research and find new non-synthetic therapeutic agents. One such alternative may be including the use of plant extracts since plant extracts have been reported in several instances to have promising deleterious effects on biofilm formation as well as quorum sensing Erhabor et al. (2019).

2.7 Approaches for the management of mastitis in South Africa

2.7.1 Use of antibiotics in South African dairy farms.

In South Africa, antibiotics use in general which includes antibiotics use for bovine mastitis is controlled by two separate Acts. The first is Acts 101 of 1965, Medicine and Related Substance Act as amended. With this act drugs are only available on prescription (Burgos et al., 2005). Following this Act, only six antibiotics (four for lactating cows and two for dry cows) are available for use in South Africa for intramammary remedies which mastitis is part of it (Karzis et al., 2021). The second Acts is Acts 36 of 1947, the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act. With this second Act, antibiotics can be bought over the counter without prescription (Burgos et al., 2005). The free use of different kinds of antibiotics which the second Acts allows, has apparently led to dairy farmers using antibiotics without confirmation but with symptoms only. This second act also allows for use of antibiotics as growth promoters. These indiscriminate and apparent overuses of antibiotics may not only promote the ongoing antibiotics resistance but may as well lead to emergence of new resistance to more antibiotics (Burgos et al., 2005; Karzis et al., 2021).

It has become obvious that drug resistance which has resulted as a result of misuse and overuse of antibiotics has threatened the efficacy of the drugs (Department of Health and Department of Agriculture Forestry and Fisheries [DAFF] 2018) (Health, 2014). Steps being taken presently to curb the present situation to some extent, is the development and implementation of a National Antimicrobial Resistance Strategy Framework (NARSF), (DAFF 2018) (Health, 2014). The mission of NARSF include improved surveillance and early detection of antimicrobial resistance as well as enhanced infection prevention and control. With the challenges presented by antibiotics resistance, use of plant extracts may be an alternative remedy to antibiotics.

2.7.2 Plants used in traditional medicine for the treatment of bovine mastitis in South Africa.

There are not many reports on plants used in ethnoveterinary medicine for the treatment of bovine mastitis in South Africa. This could be as a result of non-literature documentation of plants in traditional medicine for the treatment of bovine mastitis or as a result very limited use of plants for the treatment of mastitis in south Africa. However, Chitura et al. (2018) reported the traditional use of *Ziziphus mucronata* Willd specifically for the treatment of mastitis in Vhembe district of South Africa. The common name of the plant is Buffalo thorn tree whereas the local name in Vhembe is Mutshetshete. The traditional use of the plant involves the topical application of the plant leaf paste.

In vitro scientific studies have been done on *Ziziphus mucronate,* which to some extent validate its use in traditional medicine. McGaw et al. (2007) reported that the minimum inhibitory concentration (MIC) activity of hexane, methanol and water extracts of the leaf of *Z. mucronate* ranged from 0.2 mg/ml to 12.5 mg/ml against *S.aureus* ATCC strain with hexane extract being non-toxic and methanol extract as well as water extract having no lethal effect on brine shrimp. Also Nemudzivhadi and Masoko (2015) reported that MIC activity of *Z. mucronate* extracts (ethyl acetate, hexane, chloroform, dichloromethane, acetone, ethanol and methanol) ranged from 0.27 mg/ml to 1.05 mg/ml against *S. aureus* ATCC strain. Nemudzivhadi and Masoko (2015) further stated that *Z. mucronate* extracts had LC_{50} value of 105.5 µg/ml (0.11 mg/ml) against Vero kidney cells (monkey). Although this LC_{50} is above 0.02 mg/ml (a concentration below which a plant extract can be considered toxic) (Kuete, 2010), it is not very safe because even with the lowest MIC (0.27 mg/ml) they obtained, the selectivity index will be 0.41 which is very low as it is below 1 indicating that the observed activity is more of toxicity (Sserunkuma et al., 2017). Report on activity of *Z. mucronate* against *S. aureus* isolate from clinical cases of mastitis is lacking

Finding plant extract/s with very good MIC activity less than 1 mg/ml against *S. aureus* ATCC and *S. aureus* isolates from clinical cases of mastitis that also has/have very good LC_{50} that will result in selectivity index not just slightly above one nor four digits above 1 but clearly high (from five upwards) will be more promising for the management of bovine mastitis.

2.7.3 Use of plant extracts in South African against mastitis symptoms and pathogens implicated in causing mastitis.

As ideal plant/s (with very good activity and safe) used traditionally for the treatment/management of mastitis in South Africa, was not found, searches were made on plants used against mastitis related symptoms and against microbial related diseases as mastitis is an example of microbial related diseases. Table 2.1 presents a list of South African

plants used in ethnomedicine in South Africa for treatment against mastitis related symptoms and diseases caused by microbes implicated in causing mastitis.

2.7.4 Activity and cytotoxicity of bio-actives (extracts/compounds) from South African plants against pathogens implicated in causing mastitis

Literature on South African plant bio-actives clearly specified to be tested particularly against clinical isolates from bovine mastitis cases were lacking. Therefore, searches were also made on plant extracts that have been tested against ATCC strains, clinical isolate strains and field isolate strains of organisms implicated in causing mastitis. Literature on both the activity and cytotoxicity of South African plant bio-actives tested against ATCC, clinical isolates and field isolates strains of organisms implicated in causing mastitis were made. These isolates were not necessarily from mastitis cases but the organisms are organisms known to possible cause bovine mastitis upon infection of bovine udder. The activities and cytotoxicity of these plants are summarised in Table 2.2. Table 2.3 presents the activity and cytotoxicity of two compounds isolated from one of the plants.

It was observed that most of the plant extracts that were active following MIC test (broth microdilution) were toxic with the exception of acetone extracts of four plants (*Acacia nilotica:* bark, *Tetradenia riparia:* flowers, *Aloe arborescens*: leaves and *Crassula multicava*: whole plant), which were active with mild safety having SI ranging from 1.0551 to 5.2308. However, the highest SI index obtained for activity against an *S. aureus* strain (field strain) was 2.6070 from acetone extract of whole plant of *Crassula multicava.* Although this SI value is above 1, it only indicates mild safety. The higher the SI value of an extract the safer the plant extract (Dzoyem et al., 2014). It will thus, be interesting to find very active plant extracts with high SI value which will evidently indicate safety.

Significant activities were also observed from some plant extracts following MIC (agar disk diffusion), however their safely is unknown as cytotoxicity of these plant extracts were not tested and thus their selective indices were not determined.

Two compounds (Quercetin 3-*O*-β-galactoside-6-gallate and Kaempferol 3-*O*-α-arabinopyranoside) isolated from one of the active plants (*Melianthus major)* were tested for activity against both *S. aureus* Methicillin sensitive strain and *S. aureus* Methicillin-resistant strain. The cytotoxicity of the compounds was also tested. Although the crude extract of *Melianthus major* was active but toxic, the authors wanted to know whether after isolation, the isolated compounds could be active as well as safe. Unfortunately, the isolated compounds were not active and had moderate toxicity. Thus, the observed activity in the crude extract

could most likely be as a result of synergistic effect of the constituents of the crude extract or as a result of the activity from another compound/s not isolated.

Finding ideal plant bio-active/s (extracts/fractions/compounds) that have strong activity against the predominant causative organism of mastitis (*S. aureus*) with high selective index value which will clearly indicate safety will be very interesting. It will be even much more interesting if the ideal plant extract with high SI has the strong activity against both *S. aureus* ATCC strain and *S. aureus* clinical isolates from bovine mastitis cases.

Table 2. 1 South African plants used in ethnomedicine in South Africa for treatment against symptoms related to *S. aureus* infections and other microbial related diseases.

Plants species/ Family	Common name/ Local name	South African province where plant grows/ is used in ethomedicine	Diseases treated against in ethnomedicine	Reference
Melianthus major L / Melianthaceae	Giant honey flower/ touch-me-not/ ubutyayi/ ibonya	Gauteng, Xhosa, Zulu	*S. aureus* infections	(Heyman et al., 2009). (SANBI, Feb 2023, Melianthus major) https://pza.sanbi.org/melianthus-major
Melianthus comosus Vahl / Melianthaceae	Honey flower	Gauteng, North-West, Free State, North cape, Mpumalanga, Eastern Cape, Western Cape	*S. aureus* infections. Skin rashes	(Heyman et al., 2009). (SANBI, Feb 2023, Melianthus comosus) https://pza.sanbi.org/melianthus-comosus
Dodonaea viscosa Jacq. var. *angustifolia* (L.f.) Benth / Sapindaceae	Sand olive/ Mutepipuma/ Mutata-vhana	Gauteng, Mpumalanga, KwaZulu-Natal, Eastern Cape, Western Cape, Limpopo, North West.	*S. aureus* infections. Itchy skin	(Heyman et al., 2009). (SANBI, Feb 2023, Dodonaea viscosa) https://pza.sanbi.org/dodonaea-viscosa-var-angustifolia
Withania somnifera L. Dunal / Zygophyllaceae	Winter cherry/ Ubuvimbha/ Ubuvuma/ Bofepha	Across south Africa	*S. aureus* infections. Inflammation, Abscesses, Sores	(Heyman et al., 2009). (SANBI, Feb 2023, Withania somnifera) http://pza.sanbi.org/withania-somnifera
Albizia harveyi E. Fourn / Leguminosae	Common false thorn/ Umbola/Mmola/ Molalakgaka/Muvhola	Gauteng, Mpumalanga, Limpopo	*S. aureus* infections	(Heyman et al., 2009). (SANBI, Feb 2023, Albizia harveyi) http://redlist.sanbi.org/species.php?species=278-12

Piper capensis (L.f) var. *capense* / Zygophyllaceae	Wild pepper/ Uluphokwane/ Ihlolane	Gauteng, Mpumalanga, Limpopo, KwaZulu-Natal, Eastern Cape, Western Cape	*S. aureus* infections	(Heyman et al., 2009). (SANBI, Feb 2023, Piper capensis) http://redlist.sanbi.org/species.php?species=4109-1
*Plantago longissima*Decne / Plantaginaceae	Fleaworts/ Plantain (non-cooking)	Gauteng, Mpumalanga, Limpopo, KwaZulu-Natal, Eastern Cape, North West	*S. aureus* infections	(Heyman et al., 2009). (SANBI, Feb 2023, Plantago longissima) http://redlist.sanbi.org/species.php?species=3482-13
Boophone disticha. (L.f) / Amaryllidaceae	Soer-eye flower/Century plant/poison bulb/ Incwadi/Ibhade/ Motlastsisa	Across South Africa.	Boils. Abscesses. Wounds. Pains. *S. aureus* infections.	(Heyman et al., 2009). (Plantzafrica, Feb 2023, *Boophone disticha*). https://www.plantzafrica.com/plantab/boophdist.html . (SANBI, Feb 2023, *Boophone disticha*). https://pza.sanbi.org/boophone-disticha
Drimia altissima (L.F) Ker Gawl / Hyancinthaceae	Tall white squill/ Umgulube/ Isiklenama/ Umahlokolosi/ Lukhovu	Across South Africa.	*S. aureus* infections	(Heyman et al., 2009). (SANBI, Feb 2023, Drimia altissima) http://pza.sanbi.org/drimia-altissima
Euphorbia damarana L.C.Leach / Euphorbiaceae	NA (Not available)	Pretoria	*S. aureus* infections	(Heyman et al., 2009).
Combretum caffrum Kuntze / Combretaceae	Cape Bushwillow/ Umdubu/ Umdubi	Eastern Cape	Conjuctivitis. Redwater	(Masika & Afolayan, 2002, 2003). (SANBI, Feb 2023, Combretum caffrum) http://redlist.sanbi.org/species.p

				hp?species=2039-8
Salix capensis Thunb(Synonym: *Salix mucronate*) / Salicaceae	Willow/ Cape Willow/ Safsaf Willow/ Umzekana/ Umngcunube/Umnyezane/Mogokare/Munengeledzi	Eastern Cape, Western Cape, Gauteng, Limpopo, Free State, KwaZulu-Natal, Mpumalanga, North West	Gallsickness, Redwater, fever. Retained placenta, Pain. Burns, Rheumatism	(Masika & Afolayan, 2002, 2003). (SANBI, Feb 2023, *Salix capensis*) https://pza.sanbi.org/salix-mucronata . (Treesa, Feb 2023, *Salix capensis*) https://treesa.org/salix-mucronata
Schotia latifolia Jacq / Caesalpinioideae	Bush boer-bean/ Forest boer-bean/ Umgxam/ Umgxamu, Umxamo	Eastern Cape, Limpopo, Mpumalanga	Diarrhoea. Redwater. Fever	(Masika & Afolayan, 2002, 2003). SANBI, Feb 2023, Schotia latifolia) https://pza.sanbi.org/schotia-latifolia
Solanum tomentosum L / Solanaceae	Snake apple	Eastern Cape. (Bantu tribe), Northern cape, Western Cape, Free State	Boils. Syphilis. Sore throat, and toothache.	(Aliero & Afolayan, 2006). (SANBI, Feb 2023, Solanum tomentosum). http://redlist.sanbi.org/species.php?species=2853-88
Acacia nilotica, (L.) Delile. (Synonym: *Vachellia nilotica*) / Fabaceae	Scented-pod acacia/ Gum Arabic tree/ Babul/ Umnqawe/Mogohlo, Motsha, Umncawe	KwaZulu-Natal, Gauteng, Mpumalanga, North West, Limpopo	Ulcer. Sores. Used as antiseptic for wounds healing. Colds. Bronchitis, Diarrhoea. Bleeding. Leukoderma. Cough. Tuberculosis. Diarrhoea	(Bansal & Goel, 2012; Mattana et al., 2012; Singh & Arora, 2009; Sserunkuma et al., 2017). (SANBI, Feb 2023, Acacia nilotica). http://pza.sanbi.org/vachellia-nilotica-subsp-kraussiana
Tetradenia riparia (Hochst.) Codd/ Lamiaceae	Ginger bush/ Misty plume bush/ Ibozane/ Iboza	Mpumalanga, KwaZulu-Natal, Tswana	Gall sickness (in cattle). Pains. Fever.	(Sserunkuma et al., 2017). (SANBI, Feb 2023, Tetradenia riparia) https://pza.sanb

				i.org/tetradenia-riparia
Aloe arborescens Mill/ Asphodelaceae.	Bitter aloe/ Krantz aloe/ Khala elincinci/ Umhlabana/ Inkalane/ Ikalene	Eastern Cape, Mpumalanga, Limpopo, KwaZulu-Natal	Ulcer. Burns. Pains. Diarrhoea, Septic wounds. Anthelmintic (in goats),	(Maphosa & Masika, 2010; Sserunkuma et al., 2017). (SANBI, Feb 2023, Aloe arborescens) https://pza.sanbi.org/aloe-arborescens
Crassula multicava Lem / Crassulaceae.	Fairy crassula/ Pitted crassula/ Umadinsane	Across South Africa	Stomach related ailments. Antibacterial. Anthelmintic	(Okem et al., 2012; Sserunkuma et al., 2017). (SANBI, Feb 2023). (Okem et al., 2012). (SANBI, Feb 2023, Crassula multicava) https://pza.sanbi.org/crassula-multicava

Table 2. 2 From literature: Tested Antibacterial activity (MIC) of plant extracts against organisms implicated in causing mastitis, their cytotoxicity and selective index.

S/N	Plants and parts used / Plant part extracted	Extractants/Solvents	Antibacterial activity (mg/ml) against mastitis causative organisms - wild type and their selectivity indices (SI)	Antibacterial activity (mg/ml) against drug-resistant /clinical strain/field strain mastitis causative organisms and their selectivity indices (SI)	Cytotoxicity IC_{50} (µg/ml) / LC_{50} (mg/ml) IC_{50} (µg/ml)	Reference
1.	*Melianthus major* L/ Leaves	Ethanol	*S. aureus* / MIC (Microtitre) / 0.558 mg/ml (Active)	Methicillin-resistant *S. aureus* (MRSA) / MIC (Microtitre) / 0.781mg/ml (Active)	IC_{50} (µg/ml) / 52.76 ± 7.1875 / (toxic)	(Heyman et al., 2009)
2.	*Melianthus comosus* Vahl / Leaves	Ethanol	*S. aureus* / MIC (Microtitre) / 0.500 mg/ml (Active)	Methicillin-resistant *S. aureus* (MRSA) / MIC (Microtitre) / 0.391mg/ml (Active)	IC_{50} (µg/ml) / 51.41 ± 8.9475 / (toxic)	(Heyman et al., 2009)
3.	*Dodonaea viscosa* / Leaves	Ethanol	*S. aureus* / MIC (Microtitre) / 0.500 mg/ml (Active)	Methicillin-resistant *S. aureus* (MRSA) / MIC (Microtitre) / 0.586mg/ml (Active)	IC_{50} (µg/ml) / 42.11 ± 9.805 / (toxic)	(Heyman et al., 2009)
4.	Withania somnifera L. Dunal / Leaves	Ethanol	*S. aureus* / MIC (Microtitre) / 0.500 mg/ml (Active)	Methicillin-resistant *S. aureus* (MRSA) / MIC (Microtitre) / 1.561mg/ml (Active)	IC_{50} (µg/ml) / 20.18 ± 4.630 / (toxic)	(Heyman et al., 2009)
5.	*Albizia harveii* E. Fourn / Bark	"	*S. aureus* / MIC (Microtitre)/ 2.00 mg/ml / (Not active)	Not tested	Not tested	(Heyman et al., 2009)
6	*Piper capensis* (L.f) var. *capense* / Root.	"	*S. aureus* / MIC (Microtitre) / 3.125 mg/ml / (Not active)	Not tested	Not tested	(Heyman et al., 2009)

7	*Plantago longissimi* Decne / *Root*	Ethanol	*S. aureus* / MIC (Microtitre) / *12.5 mg/ml* / (Not active)	Not tested	Not tested	(Heyman et al., 2009)
8	*Boophane disticha* (L.f)	Ethanol	*S. aureus* / MIC (Microtitre) / *7.25 mg/ml* / (Not active)	Not tested	Not tested	(Heyman et al., 2009)
9	*Drimia altissima* / *Scales (bulb)*		*S. aureus* / MIC (Microtitre) / *> 12.5 mg/ml* / (Not active)	Not tested	Not tested	(Heyman et al., 2009)
10	*Euphorbia damarana* / *Stem*	Ethanol	*S. aureus* / MIC (Microtitre) / *6.25 mg/ml* / (Not active)	Not tested	Not tested	(Heyman et al., 2009)
11	*Combretum caffrum* / Bark		*Staphylococcus aureus*			(Masika & Afolayan, 2002).
		Water	MIC (on agar plates) / 0.5 mg/ml / (Significant activity)	Not tested	Not tested	
		Methanol,	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone,	MIC (on agar plates) / 0.5 mg/ml / (Significant activity)	Not tested	Not tested	
		Decoction	MIC (on agar plates) / 0.5 mg/ml / (Significant activity)	Not tested	Not tested	
			Klebsiella pneumonia			
		Water	Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Decoction	MIC (on agar plates) / Not active	Not tested	Not tested	

			Escherichia coli			
		Water	MIC (on agar plates). / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Decoction	MIC (on agar plates) / Not active	Not tested	Not tested	
			Pseudomonas aeruginosa			
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Decoction	MIC (on agar plates) /Not active	Not tested	Not tested	
12	*Salix capensis* Thunb / Bark		*Staphylococcus aureus*			(Masika & Afolayan, 2002)
		Water	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / 0.5 mg/ml / (Significant activity)	Not tested	Not tested	
		Decoction	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested	Not tested	
			Klebsiella pneumonia			
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	

		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Decoction	MIC (on agar plates) / Not active	Not tested	Not tested	
			Escherichia coli			
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml/ (Significant activity)	Not tested		
		Acetone	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested		
		Decoction	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
			Pseudomonas aeruginosa			
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Decoction	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
13	*Schotia latifolia*. Jacq / Bark		*Staphylococcus aureus*			(Masika & Afolayan, 2002)
		Water	MIC (on agar plates) / 0.5 mg/ml /	Not tested	Not tested	

			(Significant activity)			
		Methanol	MIC (on agar plates) / 0.1 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates) / 0.5 mg/ml / (Significant activity)	Not tested	Not tested	
		Decoction	MIC (on agar plates) / 1.0 mg/ml / (Significant activity)	Not tested	Not tested	
			Klebsiella pneumonia			
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / Not active	Not tested	Not tested	
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Decoction	MIC (on agar plates)/ Not active	Not tested	Not tested	
			Escherichia coli			
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / Not active	Not tested	Not tested	
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Decoction	MIC (on agar plates) / Not active	Not tested	Not tested	
			Pseudomonas aeruginosa		Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Acetone	MIC (on agar plates)/ Not active	Not tested	Not tested	

		Decoction	MIC (on agar plates) / Not active	Not tested	Not tested	
14	*Solanum tomentosum* L / Leaves		*Staphylococcus aureus*			(Aliero & Afolayan, 2006)
		Acetone	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
			Staphylococcus epidermidus		Not tested	
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / Not active	Not tested	Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
			Streptococcus pyrogens		Not tested	
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
			Klebsiella pneumonia			
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	
		Methanol	MIC (on agar plates) / Not active	Not tested	Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
			Escherichia coli			
		Acetone	MIC (on agar plates) / Not active	Not tested	Not tested	

		Methanol	MIC (on agar plates) / Not active	Not tested	Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
			P. aeruginosa			
		Acetone	MIC (on agar plates) / 5.0 mg/ml / (Significant activity)	Not tested	Not tested	
		Methanol	MIC (on agar plates) / Not active	Not tested	Not tested	
		Water	MIC (on agar plates) / Not active	Not tested	Not tested	
15.	*Acacia nilotica* / Bark		*S. aureus*	*S. aureus* (field strain)	**LC_{50} (**mg/ml**)**	(Sserunkuma et al., 2017)
		Acetone	MIC (Microtitre) / 0.0390 mg/ml / SI = 0.8513	MIC (Microtitre) / 0.0390 mg/ml / SI = 0.8513	0.0332	
		Water	MIC (Microtitre) / 0.1560 mg/ml / SI = 0.1782	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.0888	0.0278	
			S. agalactiae	*S. agalactiae*		
		Acetone	MIC (Microtitre) / 0.0703 mg/ml / SI = 0.4723	*Not tested*	0.0332	
		Water	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.0888	*Not tested*	0.0278	
			S. uberis	*S. uberis* (field strain)		
		Acetone	MIC (Microtitre) / 0.0390 mg/ml / SI = 0.8513	MIC (Microtitre) / 0.0780 mg/ml / SI = 0.4256	0.0332	
		Water	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.0445	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.0888	0.0278	
			E. coli	*E. coli* field strain)		
		Acetone	MIC (Microtitre) / 0.1560	Not tested	0.0332	

			mg/ml / SI = 0.2128			
		Water	MIC (Microtitre) >2.500 mg/ml	Not tested	0.0278	
			K. pneumoniae	*K. pneumoniae*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.1061	Not tested	0.0332	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.0222	Not tested	0.0278	
			P. aeruginosa	*P. aeruginosa*		
		Acetone	MIC (Microtitre) / 0.0780 mg/ml / SI = 0.4256	Not tested	0.0332	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.0278	
			S. epidermidis	*S. epidermidis* (clinical isolate)		
		Acetone	Not tested	MIC (Microtitre) / 0.0097 mg/ml / SI = 3.4227	0.0332	
		Water	Not tested	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.0888	0.0278	
			S. chromogenes	*S. chromogenes* (field strain)		
		Acetone	Not tested	MIC (Microtitre) / 0.0390 mg/ml / SI = 0.8513	0.0332	
		Water	Not tested	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.0445	0.0278	
			S. aureus	*S. aureus* (field strain)		
		Acetone	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.3499	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.3499	0.2187	

		Water	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.1101	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.1101	0.0688	
			S. agalactiae	*S. agalactiae*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.6987	Not tested	0.2187	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.0550	Not tested	0.0688	
			S. uberis	*S. uberis* (field strain)		
		Acetone	MIC (Microtitre) / 0.1822 mg/ml / SI = 1.2003	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.6987	0.2187	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.0550	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.2198	0.0688	
			E. coli			
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.6987	Not tested	0.2187	
		Water	MIC (Microtitre) / >2.500	Not tested	0.0688	
			K. pneumoniae	*K. pneumoniae*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.6987	Not tested	0.2187	
		Water	MIC (Microtitre)/ 1.2500 mg/ml / SI = 0.0550	Not tested	0.0688	
			P. aeruginosa	*P. aeruginosa*		
		Acetone	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.2187	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.0688	

			S. epidermidis	*S. epidermidis* (clinical isolate)		
		Acetone	Not tested	MIC (Microtitre) / 0.6250 mg/ml / SI = 1.4019	0.2187	
		Water	Not tested	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1101	0.0688	
			S. chromogenes	*S. chromogenes* (field strain)		
		Acetone	Not tested	MIC (Microtitre) / 0.1560 mg/ml SI = 1.4019	0.2187	
		Water	Not tested	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.0550	0.0688	
16.	*Tetradenia riparia* / Flowers		*S. aureus*	*S. aureus* (field strain)		(Sserunkuma et al., 2017)
		Acetone	MIC (Microtitre) / 0.0780 mg/ml / SI = 1.0551	MIC (Microtitre) / 0.0728 mg/ml / SI = 1.1305	0.0823	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.1427	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.0714	0.1784	
			S. agalactiae	*S. agalactiae*		
		Acetone	MIC (Microtitre) / 0.0390 mg/ml / SI = 2.1103	Not tested	0.0823	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.1427	Not tested	0.1784	
			S. uberis	*S. uberis* (field strain)		
		Acetone	MIC (Microtitre) / 0.0780 mg/ml / SI = 1.0551	MIC (Microtitre) / 0.0195 mg/ml / SI = 4.2205	0.0823	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.1427	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.0714	0.1784	
			E. coli	*E. coli*		

	Leaves	Acetone	MIC (Microtitre) / 0.1560 mg/ml / SI = 0.5276	Not tested	0.0823	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.1784	
			K. pneumoniae	*K. pneumoniae*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.2629	Not tested	0.0823	
		Water	MIC (Microtitre) / 0.6250 mg/ml / SI = 0.2854	Not tested	0.1784	
			P. aeruginosa	*P. aeruginosa*		
		Acetone	MIC (Microtitre) / 0.2607 mg/ml / SI = 0.3157	Not tested	0.0823	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.1427	Not tested	0.1784	
			S. epidermidis	*S. epidermidis* (clinical isolate)		
		Acetone	Not tested	MIC (Microtitre) / 0.0390 mg/ml / SI = 2.1103	0.0823	
		Water	Not tested	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.0714	0.1784	
			S. chromogenes	*S. chromogenes* (field strain)		
		Acetone	Not tested	MIC (Microtitre) / 0.0780 mg/ml / SI = 1.0551	0.0823	
		Water	Not tested	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.1427	0.1784	
			S. aureus	*S. aureus* (field strain)		
		Acetone	MIC (Microtitre) /	MIC (Microtitre) /	0.0513	

			0.2345 mg/ml / SI = 0.2188	.1560 mg/ml / SI = 0.0444		
		Water	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1095	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1095	0.2738	
			S. agalactiae	*S. agalactiae*		
		Acetone	MIC (Microtitre) / 0.1170 mg/ml / SI = 0.4385	Not tested	0.0513	
		Water	MIC (Microtitre)/ 2.5000 mg/ml / SI = 0.1095	Not tested	0.2738	
			S. uberis	*S. uberis* (field strain)		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.1639	MIC (Microtitre) / 0.1170 mg/ml / SI = 0.4385	0.0513	
		Water	MIC (Microtitre) / 2.500 mg/ml / SI = 0.1095	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1095	0.2738	
			E. coli	*E. coli*		
		Acetone	MIC (Microtitre) / 0.1560 mg/ml / SI = 0.3288	Not tested	0.0513	
		Water	MIC (Microtitre) L / >2.500 mg/ml	Not tested	0.2738	
			K. pneumoniae	*K. pneumoniae*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.1639	Not tested	0.0513	
		Water	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.2190	Not tested	0.2738	
			P. aeruginosa	*P. aeruginosa*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 0.1639	Not tested	0.0513	

		Water	MIC (Microtitre) / 1.2500 mg/ml LSI = 0.2190	Not tested	0.2738	
			S. epidermidis	*S. epidermidis* (clinical isolate)		
			Not tested	MIC (Microtitre) / 0.1560 mg/ml / SI = 0.3288	0.0513	
			Not tested	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1095	0.2738	
			S. chromogenes	*S. chromogenes* (field strain)		
		Acetone	Not tested	MIC (Microtitre) / 0.1560 mg/ml / SI = 0.3288	0.0513	
		Water	Not tested	MIC (Microtitre)>2.500 mg/ml / MIC (Microtitre)	0.2738	
17.	*Aloe arborescens* / Leaves.		*S. aureus*	*S. aureus* (field strain)		(Sserunkuma et al., 2017)
		Acetone	MIC (Microtitre) / >2.500 mg/ml	MIC (Microtitre) / >2.500 mg/ml	0.4825	
		Water	MIC (Microtitre) / >2.500 mg/ml	MIC (Microtitre) / >2.500 mg/ml	>1	
			S. agalactiae	*S. agalactiae*		
		Acetone	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.4825	
		Water	MIC (Microtitre) / >2.500	Not tested	>1	
			S. uberis	*S. uberis* (field strain)		
		Acetone	MIC (Microtitre) / >2.500 mg/ml	MIC (Microtitre) / 0.3130 mg/ml / SI = 1.5415	0.4825	
		Water	MIC (Microtitre) / >2.500 mg/ml	MIC (Microtitre) / >2.500 mg/ml	>1	
			E. coli	*E. coli*		
		Acetone	MIC (Microtitre) /	Not tested	0.4825	

			0.6250 mg/ml / SI = 0.7720			
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	>1	
			K. pneumoniae	*K. pneumoniae*		
		Acetone	MIC (Microtitre) / 0.6250 mg/ml/ SI = 0.7720	Not tested	0.4825	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	>1	
			P. aeruginosa	*P. aeruginosa*		
		Acetone	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.4825	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	>1	
			S. epidermidis	*S. epidermidis* (clinical isolate)		
		Acetone	Not tested	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1930	0.4825	
		Water	Not tested	MIC (Microtitre)/ 2.5000 mg/ml / SI = 0.4000	>1	
			S. chromogenes	*S. chromogenes* (field strain)		
		Acetone	Not tested	MIC (Microtitre) / >2.500 mg/ml	0.4825	
		Water	Not tested	MIC (Microtitre) / >2.500 mg/ml	>1	
18.	*Crassula multicava* / whole plant.		*S. aureus*	*S. aureus* (field strain)		(Sserunkuma et al., 2017)
		Acetone	MIC (Microtitre) / 1.8750 mg/ml / SI = 0.4352	MIC (Microtitre) / 0.3130 mg/ml / SI = 2.6070	0.8160	
		Water	MIC (Microtitre) / >2.500 mg/ml	MIC (Microtitre) / >2.500 mg/ml	0.4879	

			S. agalactiae	*S. agalactiae*		
		Acetone	MIC (Microtitre) / 0.7292 mg/ml / SI = 1.1190	Not tested	0.8160	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.4879	
			S. uberis	*S. uberis* (field strain)		
		Acetone	MIC (Microtitre). / 0.3130 mg/ml / SI = 2.6070	MIC (Microtitre) / 0.3130 mg/ml / SI = 2.6070	0.8160	
		Water	MIC (Microtitre) / >2.500 mg/ml	MIC (Microtitre) / 1.2500 mg/ml / SI = 0.3903	0.4879	
			E. coli	*E. coli*		
		Acetone	MIC (Microtitre) / 0.3130 mg/ml / SI = 2.6070	Not tested	0.8160	
		Water	MIC (Microtitre) / >2.500 mg/ml	Not tested	0.4879	
			K. pneumoniae	*K. pneumoniae*		
		Acetone	MIC (Microtitre) / 0.1560 mg/ml / SI = 5.2308	Not tested	0.8160	
		Water	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1952	Not tested	0.4879	
			P. aeruginosa	*P. aeruginosa*		
		Acetone	MIC (Microtitre) / 0.1560 mg/ml / SI = 5.2308	Not tested	0.8160	
		Water	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1952	Not tested	0.4879	
			S. epidermidis	*S. epidermidis* (clinical isolate)		

		Acetone	Not tested	MIC (Microtitre) / 0.6250 mg/ml / SI = 1.3056	0.8160	
		Water	Not tested	MIC (Microtitre) / 2.5000 mg/ml / SI = 0.1952	0.4879	
			S. chromogenes	*S. chromogenes* (field strain)		
		Acetone	Not tested	MIC (Microtitre) / 0.9375 mg/ml / SI = 0.8704	0.8160	
		Water	Not tested	MIC (Microtitre) / >2.500 mg/ml	0.4879	

Table 2. 3 From Literature: Tested antibacterial activity (MIC) of compounds against mastitis causative organisms, their cytotoxicity and selective index.

S/N	Compound/ From plant	Activity against Mastitis causative organism - wild type) MIC (mg/ml) (Antibacteria)	Activity against drug-resistant mastitis organism/clinical strain/field strain MIC (mg/ml)	Cytotoxicity / IC_{50} (µg/ml)	References
1.	Quercetin 3-*O*-β-galactoside-6-gallate / *Melianthus major* / (Leaves)	*S. aureus* (Methicillin sensitive) / MIC (Microtitre) / Not active	Methicillin-resistant *S. aureus* (MRSA) / MIC (Microtitre) / Not active	64.27 ± 1.973 / Moderate toxicity / (0.064 mg/ml)	(Heyman et al., 2009)
2.	Kaempferol 3-*O*-*α*-arabinopyranoside / *Melianthus major* / (Leaves)	*S. aureus* (Methicillin sensitive) / MIC (Microtitre) / Not active	Methicillin-resistant *S. aureus* (MRSA) / MIC (Microtitre)/ Not active	IC_{50} (µg/ml) / 160.7 ± 1.441 / Moderate toxicity / (0.1607 mg/ml)	(Heyman et al., 2009)

2.8 Model organism for this research

The Gram-positive bacterium, *S. aureus*, which has been discussed above as the major causative organism of mastitis of increasing importance in South Africa, were used as the model organism in the current research project. ATCC reference strains as well as drug-resistant strains isolated from clinical cases of mastitis were used. *Staphylococcus aureus* is highly contagious, causing both sub-clinical and clinical mastitis, and is extremely difficult to manage with currently used antibiotics owing to the high incidence of drug-resistance. Alternatives to antibiotics need to be sought and it is proposed that plant extracts comprising a mixture of chemical substances with different mechanisms of action may be useful in preventing and treating mastitis.

2.9 Medicinal plants as alternatives to antibiotics

Medicinal plants have been used and valued all over the world for ages (Sharma et al., 2011) and can be sources of newer, more cost-effective drugs (Muhammad et al., 2013). It has also been reported that phytochemicals, apart from maintaining the plant's physiological activities, also protect the plant against foreign agents such as bacteria and fungi (Jiménez-González et al., 2013), indicating the anti-infective properties of plants. Natural substances in plants have antimicrobial activities (Boakye et al., 2016; Rangasamy et al., 2007). Also, naturally occurring bioactive substances with antioxidant properties e.g. plant phenols, vitamins, carotenoids, phytoestrogens and terpenoids have been shown to have anti-inflammatory activity (Iwalewa et al., 2007).

2.9.1 Plant species selected from the Celastraceae family

Plant species from the Celastraceae family were chosen from the Phytomedicine Database (PMDB), Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria based on the studies of Pauw and Eloff (2014). They investigated the correlation between taxonomy and antimicrobial activity which led to the finding that the order Celastrales (which includes the Celastraceae family) has promising antimicrobial activity against bacteria and fungi. The Celastraceae family comprises about 1 350 species belonging to 96 genera. The species are made up of herbs, vines, shrubs and trees. In this study, species from the Celastraceae family were collected based on availability for in-depth studies of their antibacterial and anti-inflammatory efficacy and related mechanisms of action.

2.9.2 Plant species selected from the Crassulaceae family

Two other plant species from South Africa belonging to the Crassulaceae family were also included with the aim of elucidating anti-infective agents that have activity against mastitis organisms and inflammation. These species are *Bryophyllum pinnatum* and *Kalanchoe x gunniae* (in future referred to as *Kalanchoe gunniae*). These species were selected based on their relevant ethnopharmacological use and highly promising (unpublished) preliminary antimicrobial activities respectively.

2.9.2.1 Bryophyllum pinnatum

Bryophyllum pinnatum (Lam.) Oken*,* synonyms *Kalanchoe pinnata, Bryophyllum calcinum* (Chibli et al., 2014; Ebere Okwu & Uchenna Nnamdi, 2011; Okwu & Nnamdi, 2011), is an erect, succulent perennial shrub. It grows to about 1.5 m in height and reproduces from seeds as well as vegetatively through leaf bulbils (Nwali et al., 2012). It can also be propagated through stems or leaf cuttings. The stems are hollow, and the leaves are dark green and fleshy. The flowers are red and have a bell-like pendulous form (Okwu & Nnamdi, 2011)with the plant flowering around November to December (Mahmood et al., 2011) . Common names of the plant include; life plant, miracle plant, air plant and resurrection plant (Afzal et al., 2012; Egharevba & Ikhatua, 2008).

Bryophyllum pinnatum is ethnopharmacologically used to treat abscesses, ulcers, burns, ear-ache, diarrhea, gonorrhea, skin infections, inflammation and cough (Chibli et al., 2014; Ebere Okwu & Uchenna Nnamdi, 2011; Okwu & Nnamdi, 2011) and wounds (Egharevba & Ikhatua, 2008). The plant is also used ethnomedicinally as a sedative (Akinsulire et al., 2007), diuretic (Olowokudejo et al., 2008), astringent and analgesic (Afzal et al., 2012). Its ethnopharmacological uses also include facilitating the dropping of remains of umbilical cord

from newborn babies as well as prevention of naval infection and facilitating naval healing of babies (Okwu & Uchenna, 2009). In its traditional use, leaf extract is mainly obtained by exposing the leaves mildly to heat and squeezing off the juice. The extracted juice is then used for medication (Nwali et al., 2012).

Antimicrobial activity, including activity against *S. aureus* has also been reported with polar crude extracts of *B. pinnatum* (Akinsulire et al., 2007) as well as with three compounds purified from *B. pinnatum*. The three compounds are 1-ethanamino 7 hex-1-yne-5^1-one phenanthrene, 5^1 methyl 4^1, 5, 7 trihydroxyl flavone 1 and 4^1,4 3, 5, 7 tetrahydroxy 5-methyl 5^1-propenamine anthocyanidine 2. The first compound, 1-ethanamino 7 hex-1-yne-5^1-one phenanthrene belongs to the alkaloid phytochemical class whereas the second and third compounds, 5^1 methyl 4^1, 5, 7 trihydroxyl flavone 1 and 4^1,4, 3, 5, 7 tetrahydroxy 5-methyl 5^1-propenamine anthocyanidines 2, belong to the flavonoid phytochemical class. The above three compounds were also isolated from polar crude extracts (Ebere Okwu & Uchenna Nnamdi, 2011; Okwu & Nnamdi, 2011).

Other compounds reported to be present in *B. pinnatum* include quercetin, kaempferol and rutin belonging to flavonoid glycosides, bryophenol and bryophollon belonging to triterpenoids, bryophyllol and bryophyllol acetate belonging to steroids, decenylphenanthrene and undecenylphananthrene belonging to aromatic hydrocarbons, bryophollenone and ψ-taraxasterol belonging to terpenoids. Further phytochemicals reported to be present in *B. pinnatum* include saponins, terpenes, lipids, glycosides, bufadienolides, tannins and carotenoids (Afzal et al., 2012; Chibli et al., 2014; Okwu & Uchenna, 2009).

Akinsulire et al. (2007) suggested that extracts of *B. pinnatum* have broad spectrum antimicrobial activity. This can equally be seen from the wide ethnomedicinal application of the plant. The suggestion that *B. pinnatum* has a broad spectrum of biological activity is supported by the works of Chibli et al. (2014) and Afzal et al. (2012), who further reported that polar crude extracts of *B. pinnatum* had anti-inflammatory activities on acute and chronic inflammation, anti-ulcer and wound healing activities. Actual compound/s responsible for each of these activities are yet to be isolated.

Researchers who reported antimicrobial activity of *B. pinnatum* carried out extraction with only polar solvents. Reports on tested antimicrobial activity on non-polar extracts or compounds originating from non-polar extracts of this plant are lacking. This research therefore included use of various extracting solvent/s with the ability to extract a wide spectrum of non-polar and polar compounds. Considering that squeezing out the juice as is done in ethnomedicinal

practice releases both polar and non-polar constituents from the plant, using broad spectrum extractants as will be done in this research will be very useful.

2.9.2.2 Kalanchoe gunniae

Kalanchoe ×*gunniae* Gideon F.Sm & Figueiredo (Crassulaceae) is a new South African nothospecies derived from *Kalanchoe paniculata* Harv. × *Kalanchoe sexangularis* N.E.Br. (Smith et al., 2019). This species has only recently been described as occurring in Pretoria, South Africa, and has not been reported to occur elsewhere. When included in a preliminary screening procedure (unpublished), extracts of the aerial parts displayed notable antimicrobial activity, and hence it was included in the current research project for further investigation into the potential of the extracts to have anti-mastitis efficacy. Sparse information is available on recorded medicinal uses of the parent plants of this nothospecies. The roots of *Kalanchoe paniculata* are chewed by Southern Sotho people to treat colds, or the powdered root is used as snuff for the same purpose (Watt & Breyer-Brandwijk, 1962).

There is a promising possibility to identify useful biological activities, including inhibition of bacterial growth as well as quorum sensing and biofilm development, and also anti-inflammatory and antioxidant properties from the selected plant species. Bioactive compounds from the most active plants may be elucidated using various techniques, and compounds, active fractions or extracts may be identified for future development into readily available, standardised forms for the prevention and treatment of mastitis, and associated wounds and inflammation.

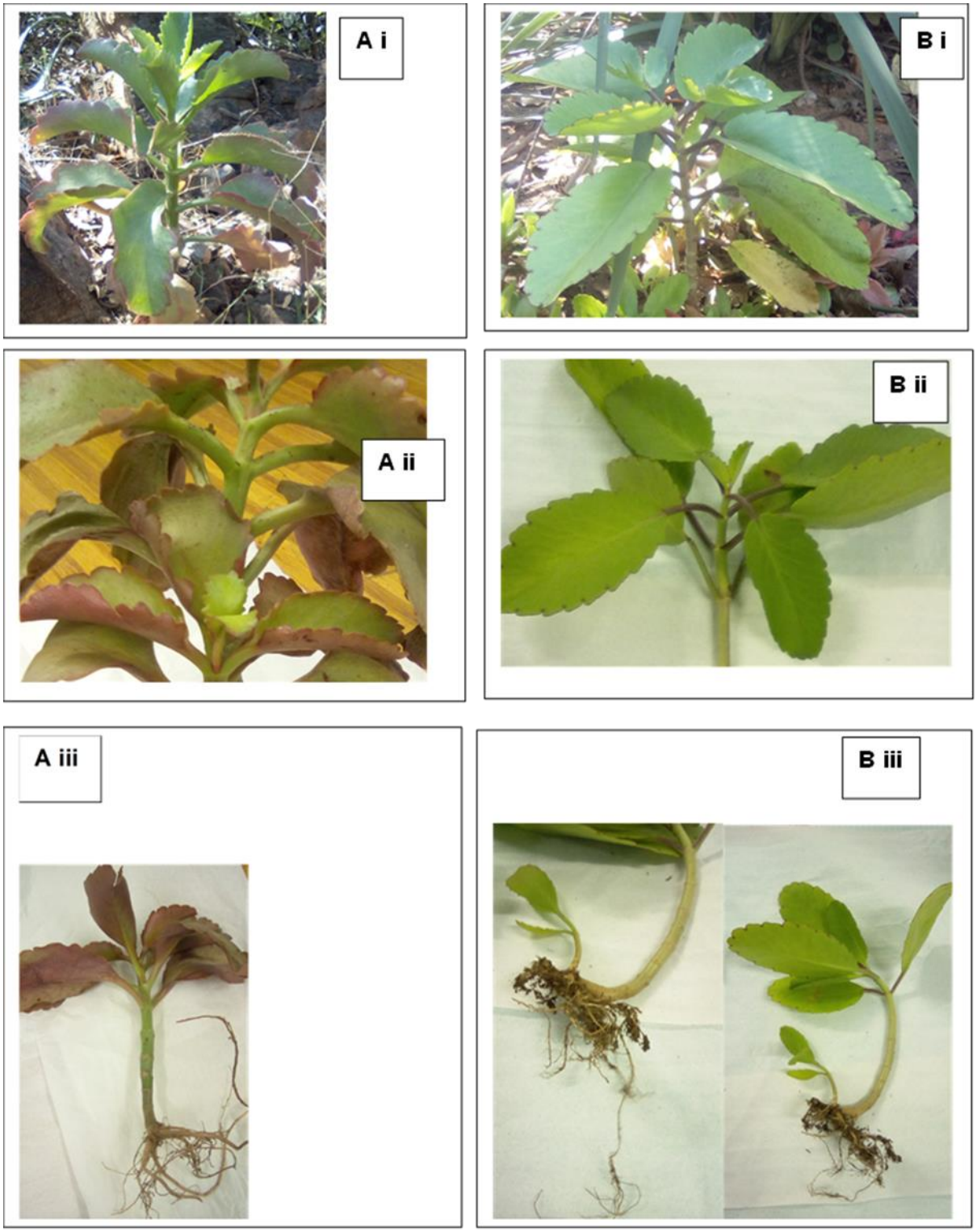

Figure 2. 1 A i = *Kalanchoe gunniae*, whole plant, A ii = *Kalanchoe gunniae* shoot, A iii = *Kalanchoe gunnia*e seedling, B i = *Bryophyllum pinnatum* whole plant B ii = *Bryophyllum pinnatum* shoot, B iii = *Bryophyllum pinnatum* seedling.

CHAPTER 3 Efficacy of South African plant extracts against wild type and drug-resistant *Staphylococcus aureus* isolated from clinical bovine mastitis cases

3.1 Preface

The first step in this study was to prepare plant extracts from the selected plants and to test them for efficacy against the reference American Type Culture Collection strain of *S. aureus* (ATCC 29213). This was then followed by testing the most active extracts against clinical strains isolated from clinical cases of mastitis in dairy cows. Additionally, the active extracts were evaluated for their cytotoxicity and ability to inhibit quorum sensing in test bacteria. Two of the plants with promising activity were also tested for their capacity to prevent or disrupt biofilm formation by the bacteria.

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3.2 Introduction

Infectious diseases caused by microorganisms are a global public health problem (Cos et al., 2006; Jiménez-González et al., 2013). One such infectious disease is mastitis, which occurs in humans and all other milk producing animals. In cattle the disease is characterized by swelling, tenderness, redness of the skin, as well as warm and painful udders (Semba, 2000; Sharma et al., 2011; Sserunkuma et al., 2017). It leads to a decrease in milk production as well as low quality of milk, resulting in huge financial losses (Banga et al., 2014). Mastitis can be subclinical or clinical, with clinical cases being characterised by the presence of clinical signs. Subclinical cases are associated with somatic cell counts of over 200 000 cells/ml and microbial organisms in milk (Sargeant et al., 2001; Viguier et al., 2009). The disease has been reported globally including in South Africa (Petzer et al., 2009), Nigeria (Salih et al., 2011) and Zimbabwe (Katsande et al., 2013). Most cases of mastitis are caused by bacterial infections, followed by fungal and in very rare cases algae and viruses have been reported as causative organisms (Bourtzi-Hatzopoulou et al., 2003; Viguier et al., 2009). Among the bacterial agents, *S. aureus* is predominant (Harmon, 1994; Swartz & Novello, 1984; Waage et al., 1999), mainly due to its intracellular nature and a high prevalence of resistance to antimicrobials (Goni et al., 2004).

Initially, penicillin and methicillin were used effectively for the treatment of *S. aureus* infections; however, resistance to these antimicrobials has emerged (Parker & Jevons, 1964; Saga & Yamaguchi, 2009; Smith et al., 1999). In recent years, resistance to other antimicrobials, including those belonging to the β-lactam and aminoglycoside groups has emerged in *S. aureus* (Lee, 2003; Pexara et al., 2013; Saga & Yamaguchi, 2009). Of great concern is the presence of mastitis caused by methicillin-resistant *S. aureus,* or MRSA, which is not responsive to readily available intramammary antimicrobials (Ateba et al., 2010; Pexara et al., 2013). Gomes et al. (2019) demonstrated that some *S. aureus* strains have strong biofilm forming properties, aiding in their ability to resist antimicrobial therapy. Together with the inherent and acquired mechanism of resistance, bacteria have the ability to adhere to both biotic and abiotic surfaces to form protective biofilms (Sandasi et al., 2008). According to Sandasi et al. (2010), biofilm forming organisms are present in 60% of bacterial infections.

Plant extracts have been reported to inhibit bacterial cell attachment, therefore limiting the ability of the organism to form biofilms. This is likely to reduce microbial colonization and control infection (Sandasi et al., 2010). Another mechanism of antimicrobial resistance relates to quorum sensing (QS) which regulates expression of virulence factors, adhesion, biofilm formation, exopolysaccharide production, and sporulation in different microorganisms (Chenia, 2013). The QS mechanism has also been linked to the ability of the organism to develop multiple drug resistance (Padder et al., 2018). This further limits prescription choice and impacts on patient prognosis (Rasko & Sperandio, 2010).

In view of limited prescription choices, medicinal plants can serve as novel natural alternative or complementary sources of antimicrobial agents (Leekha et al., 2011). Medicinal plants for ages have been used and valued all over the world (Sharma et al., 2008). It has been reported that plants act by (i) killing or slowing the growth of microorganisms (Leekha et al., 2011), (ii) reducing side effects accompanied with synthetic antibiotics (Madhuri et al., 2012) and (iii) preventing microorganisms from entering cells (Iwalewa et al., 2007). Furthermore, medicinal plants inhibit biofilm formation, and quorum sensing activity (Adnan et al., 2020). Therefore, finding safe and effective plant extracts against *S. aureus* can be important in the prevention, treatment and management of mastitis.

In this study, antimicrobial efficacy of selected plant species against a reference ATCC strain of *S. aureus* (ATCC 29213) and *S. aureus* isolates from clinical mastitis cases were investigated. Furthermore, their cellular safety was investigated against two mammalian cell lines. Their ability to inhibit bacterial quorum sensing was also investigated. Plant extracts that were active in all the above-mentioned bioassays as well as non-cytotoxic were further investigated for their effect on biofilm kinetics. It is useful to investigate potential novel

formulations with complementary mechanisms of action to current antibiotics to enhance the ability to prevent and treat mastitis.

Plant species for the study were selected based on chemotaxonomy and ethnopharmacology. In the study of Pauw and Eloff (2014), it was reported that species belonging to the order Celastrales had promising antimicrobial activity against bacteria and fungi. Twelve species from the Celastraceae family were thus chosen for this study. A further three plant species were selected based on ethnopharmacological use. From the Aquifoliaceae family, *Ilex mitis* (stem bark) is used traditionally in South Africa to treat skin infections/rashes, rheumatism, sores and fever (Mabona et al., 2013; Sagbo & Mbeng, 2018). From the Crassulaceae family *Bryophyllum pinnatum* (also known as *Kalanchoe pinnata)* (Chibli et al., 2014; Okwu & Nnamdi, 2011; Okwu & Uchenna, 2009) and *Kalanchoe gunniae* were selected. *Bryophyllum pinnatum* is used ethnopharmacologically in Nigeria, southern India, and Bengal to treat abscesses, skin ulcer, burns, earache, gonorrhoea, skin infections, inflammation, fever and cough (Nagaratna & Hegde, 2015; Okwu & Nnamdi, 2011). In Southeast Nigeria in particular, juice from the leaves of *B. pinnatum* is used traditionally to facilitate the dropping of remains of umbilical cord from newborn babies as well as for prevention of naval infection and facilitation of naval healing of babies (Okwu & Uchenna, 2009). *Kalanchoe gunniae,* a hybrid plant growing in South Africa, was only described in 2019 (Smith et al., 2019) Since it is related to *Bryophyllum pinnatum,* it is hypothesised that *Kalanchoe gunniae* could possibly also have antimicrobial activity, therefore it was included in this study.

3.3 Materials and methods

3.3.1 Plant collection and processing

Leaves of *Catha edulis* and leaves and stem bark of *Ilex mitis* and *Kalanchoe gunniae* were collected from the South African National Biodiversity Institute (SANBI), Pretoria Botanical Garden, South Africa. Leaves of *Maytenus undata, Mysroxylon aethiopicum* and *Gymnosporia buxifolia* were collected from SANBI, Walter Sisulu Botanical Garden, South Africa. Leaves and stem bark of *Bryophyllum pinnatum* were collected from the Federal Institute of Industrial Research Oshodi, Lagos Nigeria, Medicinal Plant Garden. Leaves of *Gymnosporia heterophylla, Elaeodendron transvaalense, Gymnosporia senegalensis, Elaeodendron croceum, Maurocenia frangula, Maytenus peduncularis, Robsonodendron eucleiforme* and *Pseudosalacia streyi* were collected from SANBI, National Botanical Garden, Nelspruit, Mpumalanga, South Africa.

Herbarium specimens were prepared and deposited at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (PRU) and the National Herbarium (SANBI) Pretoria (PRE), South Africa (Table 3.1). Using the method described by Eloff and McGaw (2006), harvested plant materials were cleaned, and hung inside open mesh loose woven bags and dried indoors at room temperature. The succulent plant material of *Bryophyllum pinnatum* and *K. gunniae* were dried by cutting into pieces and placing inside a constant air flow dehumidifying oven dryer at 33^{o}C. When dried, plant material was ground to fine powder using a Merck MF 10 Basic plant miller, and stored in closed glass jars at room temperature in the dark as recommended by Eloff and McGaw (2006). The ground plant materials were stored overnight and used the following day for extraction.

Table 3.1 Plants selected for the study and their voucher numbers

Plant Family	Plant species	Voucher number
Celastraceae	*Maytenus undata* (Thunb.) Blakelock	PRU 125486
	Mysroxylon aethiopicum (Thunb.) Loes.	PRU 125488
	Catha edulis (Vahl) Endl.	PRU 125485
	Gymnosporia buxifolia (Eckl. and Zeyh.) Loe.	PRU 125487
	Gymnosporia heterophylla (Eckl. and Zeyh.) Loes.	PRE1004259
	Elaeodendron transvaalense (Burtt Davy) R.H.Archer	PRE 1004258
	Gymnosporia senegalensis (Lam.) Loes.	PRE 1004261
	Elaeodendron croceum (Thunb.) DC.	PRE 1004265
	Maurocenia frangula Mill.	PRE 1004262
	Maytenus peduncularis (Sond.) Loes	PRE 1004264
	Robsonodendron eucleiforme (Eckl. and Zeyh.) R.H.Archer	PRE 1004268
	Pseudosalacia streyi Codd	PRE 1004260
Aquifoliaceae	*Ilex mitis* (L.) Radlk.	PRU 125483
Crassulaceae	*Kalanchoe gunniae* Gideon F.Sm. and Figueiredo	PRE 1004266
	Kalanchoe pinnata (Lam.) Pers. Synonym: *Bryophyllum pinnatum* (Lam.) Oken (Chibli et al., 2014; Okwu & Nnamdi, 2011; Okwu & Uchenna, 2009)	PRE 1004263

3.3.2 Plant material extraction

Acetone and ethanol were used for extraction of plant material. Acetone is able to extract a broad range of polar and non-polar compounds, and has been recommended as the best extractant to use in preparing plant extracts for antibacterial testing (J. Eloff, 1998). Ethanol is able to extract more polar compounds from plant material, and is often preferred for use in preparing samples for formulation and product development as it is less flammable than acetone (Gupta et al., 2012). For the plants selected based on ethnopharmacological uses,

extraction was done on both the leaf and the stem-bark as leaves as well as stem-bark are used in traditional medicine, although use of leaves is dominant (Iyamah & Idu, 2015). For the Celastraceae species, extraction was done on only the leaves as the initial research indicating a correlation between taxonomy and antimicrobial activity was done on the leaves (Pauw & Eloff, 2014).

For extraction, separate aliquots (4 g of ground plant material) were weighed into 50 ml Falcon tubes. Using a plant material to solvent ratio of 1:10, 40 ml of either acetone (≥ 99%, from Minema Chemicals (Pty)Ltd, South Africa) or ethanol (99.9%, from Minema Chemicals (Pty)Ltd, South Africa) was added into each tube. The tubes containing the plant samples were shaken vigorously for 25 min with a mechanical shaker and sonicated for 15 min. Tubes containing the samples were shaken vigorously again for two min and vortexed for one min. Ultrasonication of samples were again done for another five min. The supernatants containing the extracts were collected into a glass beaker by filtering the extracts through Whatman no 1 filter paper. The process was repeated two times with the plant material residue (marc) using half the volume of starting solvent. For each sample, the supernatants containing the extracts were combined and concentrated to dryness by evaporating the solvent with continuous flow of air at 30^{o}C. When the samples were completely dried, the extract yield and percentage extract yield were calculated.

3.3.3 Bacterial strains and culturing

Staphylococcus aureus (ATCC 29213) and *Chromobacterium violaceum* (ATCC 12472) were obtained from the culture collection of the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria. *Staphylococcus epidermidis* (ATCC 35984) and *S. aureus* mastitis clinical isolates; STA 1, STA 2, STA 3, STA 4, STA 5, and STA 6 were provided by the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Sciences, University of Pretoria. *Staphylococcus* species were maintained on Mueller-Hinton (MH) agar plates and prior to the bioassays were grown in MH liquid medium overnight at 37^{o}C with shaking. *C. violaceum* was maintained on Luria–Bertani (LB) agar at room temperature of 25^{o}C (±2^{o}C). When needed for experiments, single colonies were picked from the agar plates and cultured in LB broth overnight at 30^{o}C with shaking.

3.3.4 Antimicrobial susceptibility testing

Antibiogram assays to determine the susceptibility of the bacterial isolates were performed using the disc diffusion method (Bauer et al., 1966). Eight antibiotics belonging to six classes of antibiotics were used at the indicated concentrations (Table 3.2). Antibiotics were chosen in such a way that narrow spectrum and broad spectrum antibiotics will be included. Standardized bacterial suspensions of the micro-organisms were made in normal saline. This

was done by picking a single colony from bacterial strains cultured overnight on MH agar at 37ºC and suspending it in ±5 ml normal saline in tubes and measuring the optical density to obtain an inoculum equivalent to 0.5 McFarland standard. Each tube was gently vortexed to obtain a homogeneous suspension. Sterile cotton swabs were used to streak the suspensions of the microorganisms on agar plates (each comprising 25 ml MH agar). Using sterile forceps, antibiotic discs (Oxoid) were dispensed on inoculated agar plates which were incubated overnight at 37ºC. Bacterial strains tested include *S. aureus* ATCC 29213and the six *S. aureus* isolates from clinical mastitis (STA 1 to STA 6). After incubation, the zones of inhibition were measured using veneer clipper and the results were Mcinterpreted according to the performance standard of the Clinical and Laboratory Standards Institute for antimicrobial disc susceptibility tests for bacteria isolated from animals (Institute, 2015, 2020).

Table 3.2 Antibiotics used in the antimicrobial susceptibility experiment

	Class of antibiotics used	Antibiotics used	Concentration (µg/disc)
1	Penicillins	Ampicillin	10
		Amoxicillin/Clavulanic acid	30
2	Cephalosporins (3rd generation)	Ceftiofur	30
3	Tetracyclines	Doxycycline (broad)	30
		Tetracycline	30
4	Aminoglycosides	Amikacin (Broad)	30
5	Sulfonamides	Sulphamethoxazole /Trimethoprim	25
6	Amphenicols	Chloramphenicol	30

3.3.5 Antibacterial screening

Antibacterial activity of each of the plant extracts was first assayed against *S. aureus* (ATCC 29213). After this initial screening of all the plant extracts, the two most active plants from the chemotaxonomy selection and the two most active plants from ethnopharmacological selection were further assayed against six mastitis clinical isolates of *S. aureus*. The broth microdilution method (J. N. Eloff, 1998) was used and the minimum inhibitory concentration (MIC) of the extracts was determined. From MH agar plates containing the bacterial cultures, a single colony of each bacterial strain was inoculated into MH broth and grown at 37ºC for 18 h with shaking (MRC orbital shaker, 150 rpm). Overnight cultures were diluted with MH broth and the absorbance was measured with a microplate reader (Epoch Biotek), at a wavelength of 560 nm to correspond to McFarland standard No 1 (equivalent to 3×10^8 colony forming units (CFU)/mL).

Stock solutions of extracts (10 mg/ml) were prepared in 50% acetone and 50% ethanol for acetone and ethanol extracts respectively. One hundred µl of 10 mg/ml samples were added

to the first wells of each column of a 96-well microtitre plate. Gentamicin was used as a positive control and negative solvent controls were also included. Then the wells were serially diluted two-fold (1:1 with sterile deionised water) downwards until the last well. The last 100 µl of the serial dilution from the last well was discarded. Following the serial dilutions, 100 µl of standardized bacterial culture (3×10^8 CFU/mL) were added to all wells before overnight incubation at 37ºC. After incubation, 40 µl of freshly prepared 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT, Sigma) was added to all wells before further incubation for 40 min. The assays were done in duplicate, each with triplicate samples. The total activity in ml/g was determined by dividing the extract yield of 1 g of plant material in mg by MIC in mg/ml (Eloff, 2000).

3.3.6 Cytotoxicity

The 3-(4,5-dimethyl thiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay method of Mosmann (1983) was used to investigated the cytotoxicity of the eight most active plant extracts on Vero monkey kidney cells and bovine dermis cells. Minimal essential medium (MEM, Highveld Biological, South Africa), supplemented with 5% foetal bovine serum (FBS, Highveld Biological) and 0.1% gentamicin (Virbac, South Africa) was used to grow the cells in a 5% CO_2 incubator (Hera Cell 150, ThermoScientific Germany), at 37ºC. When the cells reached sub-confluency, they were harvested and centrifuged (Universal 320R, Labotec, South Africa) for five min at 200 x g. Then the cells were re-suspended in MEM to a density of 5×10^4 cells/mL and 100 µl of each cell line was seeded into wells of columns 2 to 12 of sterile 96-well microtitre plates. The wells of column 1 served as blank and received 100 µl of MEM. Plates were incubated overnight at 37ºC in a 5% CO_2 incubator. After incubation, cells were treated with plant extracts.

Plant extracts used to treat the cells were first dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100 mg/ml. From this concentration, different concentrations of the plant extracts ranging from 1 mg/ml to 0.0075 mg/ml were prepared using MEM. Each of the different concentrations (100 µl) was added to their corresponding designated wells in triplicate. Doxorubicin hydrochloride (Pfizer Laboratories, South Africa) was used as positive control. Two wells served as negative controls, thus received no treatment. The blank wells also received no treatment. After treatment, the plates were incubated for 48 h at 37ºC in a 5% CO_2 incubator. After incubation, the MEM was gently aspirated and 200 µl of phosphate buffered saline (PBS, Whitehead Scientific), was then used to wash the cells. Following the washing, 200 µl of fresh MEM was added to all the wells. Then, 30 µl of 5 mg/ml MTT (Sigma) dissolved in PBS, was added to all the wells. The plates were further incubated for 4 h at 37ºC in a 5% CO_2 incubator. Then the medium was again gently aspirated and 50 µl DMSO was added to dissolve the MTT formazan crystals. The plates were shaken gently for 2 – 3 min on

a plate shaker (QG - 9001 microporous Quick shaker, Hinotek) to facilitate dissolving of the formazan crystal. A microplate reader (BioTek Synergy) was used to measure the absorbance immediately at 570 nm wavelength. The effect of the samples on the cells (Vero and bovine dermis cells) were determined by calculating the percentage cell viability of cells. The percentage cell viability was calculated as:

% Cell viability = (Mean absorbance of sample / Mean absorbance of control) * 100

Then using the percentage cell viability of the different concentrations tested, the lethal concentration (LC_{50}) was determined. The LC_{50} of test samples, the concentration that can cause 50% reduction of absorbance, compared to the untreated cells was interpreted as the concentration that can be lethal to 50% of the cells. The selectivity index values were calculated by dividing the cytotoxicity, LC_{50} (in mg/ml), by the MIC values (in mg/ml).

3.3.7 Anti-quorum sensing assay

3.3.7.1 Bacterial culture for anti-quorum sensing assay

The anti-quorum sensing activity of eight selected plant extracts with significant to moderate activity against *S. aureus* reference and mastitis clinical isolates was performed by the method described by Ahmad et al. (2015), with modification by miniaturizing into 48-well microplate. Briefly, LB agar (Miller) and LB broth (Miller) were prepared by dissolving 20 g of LB agar in 500 ml of distilled water and 12.5 g of LB broth in 500 ml distilled water respectively and autoclaving at 121^{o}C for 15 min. *Chromobacterium violaceum* was maintained on LB agar and incubated in LB broth overnight for 18 h at 30^{o}C in an orbital shaker (140 rpm) prior to the assay. The overnight bacterial suspension was diluted with sterile distilled water to achieve an absorbance of 0.1±0.02 OD at a wavelength of 590 nm equivalent to McFarland standard No 0.5. This correlated to approximately 1.5 x 10^8 CFU/ml which was diluted in LB broth to a final working inoculum of 1.5 x 10^6 CFU/ml.

3.3.7.2 Inhibition of violacein production using broth microdilution

Plant extract stock solutions in DMSO (50 mg/ml) were first prepared for each extract. From the 50 mg/ml plant extract stock solution, 50 µl was diluted with 950 µl of LB broth to obtain 2.5 mg/ml solutions. The positive control, gentamicin, was prepared in LB broth to a concentration of 500 µg/ml. *Chromobacterium violaceum* culture (500 µl) was added to wells of 48 well plates except for those wells designated for blank (sample blank and positive control blank). From 2.5 mg/ml samples of plant extracts, 500 µl was added to wells of the wells designated for extract samples. Also 500 µl was taken from the 500 µg/ml concentration of gentamicin and added to the first well of wells designated for the positive control. Extract

sample wells and positive control wells were then serially diluted by pipetting 500 µl from the first well, diluting down to the last well and discarding the last 500 µl from the last wells. After the serial dilutions the well concentrations of the samples ranged from 1.25 mg/ml to 0.009 mg/ml whereas the well concentrations of the positive control ranged from 250 µg/ml to 1.95 µg/ml. Then 500 µl of LB broth was added to all wells. The blank wells contained 1000 µl of media alone. All wells had a total volume of 1000 µl. The plates were covered with parafilm and incubated for 24 h in an orbital shaker (140 rpm) at 30^{o}C.

After incubation, the antiquorum sensing activity was determined based on growth (turbidity) and pigmentation (purple colour). Thus, the minimum inhibitory concentration (MIC) value was defined as the lowest concentration of a sample that inhibited the visible growth of *C. violaceum*, while the minimum quorum sensing inhibitory concentration (MQSIC) values was defined as the lowest concentration of a sample that inhibited the visible pigmentation.

3.3.7.3 Violacein quantification

After 24 h of incubation of the treated *C. violaceum* culture, 1 ml from each well was transferred to new replica 48 well plates. The plates were centrifuged at 1800 x g (EBA Hettich centrifuge, Labotec) for 20 min to pellet the bacteria containing violacein. The resulting supernatant was discarded and 1 ml of 100% DMSO was added to the bacterial pellet remaining at the bottom of each well. The plates were vortexed until the pellet solubilised. Then the plates were further centrifuged at 1800 x g for 20 min to separate bacterial cells from the solution. Supernatant from each well (200 µl) was transferred to wells of 96-well microtitre plates in duplicate. The absorbance was measured at a wavelength of 595 nm using a spectrophotometer (Epoch microplate reader: BioTek, United States). The percentage violacein inhibition was determined using the formula:

$$\text{Percentage violacein inhibition} = \left(\frac{\text{meanOD}_{\text{control}} - \text{meanOD}_{\text{test}}}{\text{MeanOD}_{\text{control}}}\right) \times 100$$

The concentration of each sample inhibiting 50% of violacein production was determined as the $MQSIC_{50.}$

3.3.8 Bacterial culture preparation and biofilm kinetics assay

Staphylococcus aureus ATCC 29213, *S. epidermidis* ATCC 35984 and two *S. aureus* isolates from clinical mastitis cases (samples STA 4 and STA 6) were used for the biofilm kinetics bioassay. Tryptone Soy Agar (TSA) plates were prepared and used for overnight culture of strains at 37^{o}C without shaking.

The method of O'Toole and Kolter (1998) modified by Sandasi et al. (2010) was used for the biofilm kinetics bioassay. Freshly prepared Tryptone Soy broth (TSB) broth (10 ml) in 50 ml sterile tubes were inoculated with bacteria from the TSA plates and incubated overnight for 18 h at 37^{o}C with shaking. The overnight cultures were standardized by diluting the cultures with TSB to optical density of 0.02 at 590 nm wavelength using a spectrophotometer (Epoch microplate reader: BioTek, United States) to obtain 1.0 x 10^6 CFU/ml (OD_{590} = 0.02 or 1.0 x 10^6 CFU/ml). One hundred µl of the standardised bacterial cultures were added to wells of individually labelled flat bottomed 96 well plates (Lasec, South Africa) except wells allocated for blanks and positive control. Three biofilm stages were investigated. Plates for biofilm stage 1, i.e. time zero (0 h, effect on bacterial adhesion) were treated immediately. Plates for biofilm stage 2, i.e., that were to be treated at 24 h (effect on pre-formed biofilm), and plates for biofilm stage 3, i.e., 48 h (effect on matured biofilm), were incubated until their respective time of treatment.

Plant extracts used for the treatments were selected based on their MIC activity, level of cytotoxicity as well as their anti-quorum sensing activity. Stock solutions (2 mg/ml) of the plant extracts were prepared in 50% acetone. Treatment was done by adding 100 µl of 2 mg/ml plant extract in twelve replicates to the corresponding labelled well, giving a final concentration of 1 mg/ml in the well. Gentamicin (Virbac, South Africa) was used as positive control, and 100 µl of 2 mg/ml stock solution was added to the corresponding wells to give a final concentration of 1 mg/ml. Acetone in sterile deionised water (50%) was used as negative control. Each well had a final volume of 200 µl. The above biofilm kinetic experiments were repeated three times. After treatment, plates were sealed with parafilm and incubated for another 24 h at 37^{o}C without shaking.

3.3.8.1 Assessment of biofilm kinetics with crystal violet (CV) staining assay.

The modified method described by Sandasi et al. (2010) was used for the crystal violet (CV) staining assay. After 24 h incubation, plates were washed four times by gently pouring sterile distilled water from the edges to remove unattached cells. The plates were air dried by gently turning them over clean tissue paper, followed by oven drying at 60^{o}C for 45 min after drying, 100 µl of 0.1% crystal violet was added to all wells to stain adherent cells and the plates were covered and incubated for 15 min in the dark at room temperature. Then the plates were washed again 4 times with sterile deionised water to remove unabsorbed stain. Purple rings indicating biofilms could be observed at this stage at the sides of the wells. Ethanol (150 µl of 100%) was added to all the wells to de-stain the wells and resolubilise the crystal violet bound to the adherent cells. The de-stained solution (100 µl) was transferred into new sterile plates

and the absorbance was measured at 590 nm using a spectrophotometer (Epoch microplate reader: BioTek, United States). The mean optical density (OD) of each sample was obtained and the percentage inhibition was determined using the equation below:

$$\text{Percentage inhibition} = \frac{\text{meanOD}_{\text{untreatedl}} - \text{meanOD}_{\text{treated}}}{\text{MeanOD}_{\text{untreated}}} \times 100$$

3.3.9 Statistical analysis

Data obtained were analysed statistically with Analysis of Variance (ANOVA) in Microsoft Excel (Professional Plus 2019).

3.4 Results and Discussion

3.4.1 Antimicrobial susceptibility

Three of the isolates, STA 1, STA 2 and STA 6 were each resistant to two antibiotics (Table 3.3). The reference *S. aureus* ATCC 29213 strain was only resistant to one antibiotic (ampicillin). The antibiotics to which these organisms were resistant to fell into two classes of antibiotics, namely the penicillins and the aminoglycosides. All the tested *S. aureus* strains were resistant to ampicillin, suggesting that they could have harboured the *mecA* gene or its orthologue, the *mecC* gene. The *mecA* gene encodes for the penicillin-binding protein (PBP2') which has a very low binding affinity for β-lactam antibiotics (Saga & Yamaguchi, 2009). Alternatively, the resistant strains may have the ability to produce the β-lactamase enzyme that inactivates β-lactam antibiotics by hydrolysing the β-lactam ring (Pexara et al., 2013). In addition to being resistant to ampicillin, STA 1, STA 2, and STA 6 were also resistant to amikacin. Amikacin binds to the 30s ribosomal subunit to exert activity. However, it has been found that some *S. aureus* develop resistance to amikacin via an adaptive mechanism through cell wall thickening (Yuan et al., 2013). Thus, the strains STA 1, STA 2, and STA 6 may have developed cell wall thickening and subsequent resistance to amikacin.

Table 3.3 Susceptibility of *Staphylococcus aureus* strains to commercially available antibiotics (Kirby-Bauer Disc method with measured zones)

	***S. aureus* ATCC 29213**		**STA 1**		**STA 2**		**STA 3**		**STA 4**		**STA 5**		**STA 6**	
	Diameter of inhibition (mm) and interpretation (R, S, I)													
Antibiotics														
Ampicillin (10 µg)	25.60	**R**	23.00	**R**	24.24	**R**	22.68	**R**	25.44	**R**	25.40	**R**	22.20	**R**
Amoxicillin/Clavulanic acid (30 µg)	26.60	S	25.76	S	28.12	S	25.20	S	24.80	S	27.20	S	27.00	S
Ceftiofur (30 µg)	25.05	S	25.40	S	24.04	S	26.20	S	27.20	S	24.12	S	27.20	S
Doxycycline (30 µg)	38.00	S	28.04	S	31.40	S	34.06	S	26.56	S	28.56	S	35.48	S
Sulphamethoxazole /Trimethoprim (25 µg)	30.32	S	26.08	S	27.40	S	29.06	S	27.60	S	26.60	S	26.20	S
Tetracycline (30 µg)	34.92	S	25.00	S	28.44	S	33.02	S	28.40	S	28.00	S	35.64	S
Chloramphenicol (30 µg)	23.76	S	18.04	S	25.00	S	26.08	S	23.80	S	24.44	S	25.60	S
Amikacin (30 µg)	20.72	S	12.80	**R**	14.60	**R**	18.34	S	16.08	I	16.80	I	10.00	**R**

ATCC = *S. aureus* ATCC 29213, STA 1 to 6 = = *S. aureus* mastitis clinical isolates, **R** = Resistant, S = Susceptible, I = Intermediate.

3.4.2 Plant extract yield, minimum inhibitory concentration and total antibacterial activity

For all the plants, ethanol extracts had the highest extract yield compared to acetone extracts (Table 3.4). *Elaeodendron croceum* ethanol extract had the highest extract yield of 271.05 mg (27.11%), followed by *K. gunniae* leaf ethanol extract with extract yield of 260 mg (26%). *Maurocenia frangula* acetone extract, *M. undata* acetone extract and *K. gunniae* leaf extracts had promising activity against *S. aureus* ATCC strain with MIC values ranging from 0.02 to 0.08 mg/ml (Table 3.4). MIC values above 1 mg/ml are considered inactive (Holetz et al., 2002), MIC values between 0.6 mg/ml and 1 mg/ml are considered to be weak activity (Aligiannis et al., 2001; Holetz et al., 2002), MIC values between 0.1 mg/ml and 0.5 mg/ml were regarded as moderate activity (Holetz et al., 2002), while MIC values less than 0.1 mg/ml are regarded as having very good activity (Kuete, 2010). Several plant extracts had moderate activity against *S. aureus* ATCC strain with MIC values ranging from 0.12 mg/ml to 0.47 mg/ml. Six plant extracts had weak activity against *S. aureus* ATCC strain with MIC of 0.63 mg/ml and six other plant extracts were inactive with MIC values ranging from 1.25 mg/ml to > 2.5 mg/ml.

The total antibacterial activity of all the plant extracts against *S. aureus* ATCC 29213 ranged from 6.12 ml/g to 3250.00 ml/g (Table 3.4). *K. gunniae* leaf acetone extract had the highest total antibacterial activity (3250.00 ml/g) against *S. aureus* followed by *M. frangula acetone* extract with total antibacterial activity (2292.50 ml/g). The total antibacterial activity (TAA) indicates the volume to which an extract prepared from 1 g of a plant material can be diluted and still have activity, thus the higher the TAA the better (Dzoyem et al., 2014; Eloff, 2004).

Maurocenia frangula, Maytenus undata, *Kalanchoe gunniae* and *Bryophyllum pinnatum* had the best activities against *S. aureus* ATCC 29213. Therefore, the acetone and ethanol extracts of these species were further assayed for antimicrobial activity against the six *S. aureus* mastitis clinical isolates.

Table 3.4 Minimum inhibitory concentration (MIC, mg/ml) and total antibacterial activity (TAA, ml/g) of plant extracts against *S. aureus* (ATCC 29213)

Plant extract/sample	Solvent	Extract yield (mg)	Extract yield (%)	*S. aureus* ATCC 2913 MIC	TAA
G. heterophylla	Acetone	45.45	4.545	0.63	72.14
	Ethanol	96.95	9.695	0.63	153.89
E. transvaalense	Acetone	37.85	3.785	0.16	236.56
	Ethanol	53.925	5.3925	0.31	173.95
G. senegalensis	Acetone	36.875	3.6875	0.63	58.53
	Ethanol	102.45	10.25	0.16	640.31
E. croceum	Acetone	63.775	6.3775	0.16	398.59
	Ethanol	271.05	27.11	0.16	1694.06
Ilex mitis leaf	Acetone	25	2.5	0.63	39.68
	Ethanol	137.5	13.75	0.63	218.25
Ilex mitis stem-bark	Acetone	11.625	1.1625	1.50	7.75
	Ethanol	32.925	3.2925	2.50	13.17
M. frangula	Acetone	45.85	4.585	**0.02**	2292.5
	Ethanol	68.975	6.8975	0.31	222.5
M. peduncularis	Acetone	51.95	5.195	0.63	82.46
	Ethanol	70.2	7.02	0.31	226.45
R. eucleiforme	Acetone	51.125	5.1125	0.63	81.15
	Ethanol	83.825	8.3825	0.31	270.40
P. streyi	Acetone	18.425	1.8425	0.31	59.44
	Ethanol	38.3	3.83	0.16	239.38
C. edulis	Acetone	27.5	2.75	1.25	22.0
	Ethanol	130	13	0.16	812.5
G. buxifolia	Acetone	30	3	0.47	63.83
	Ethanol	92.5	9.25	1.25	74.0
M. aethiopicum	Acetone	37.5	3.75	0.63	59.52
	Ethanol	85	8.5	1.25	68.0
M. undata	Acetone	77.5	7.75	**0.08**	968.75
	Ethanol	110	11	0.12	916.67
K. gunniae leaf	Acetone	162.5	16.25	**0.08**	2031.25
	Ethanol	260	26	**0.08**	3250.00
B. pinnatum leaf	Acetone	30	3	0.31	96.77
	Ethanol	45	4.5	0.31	145.16
K. gunniae stem-bark	Acetone	62.5	6.25	0.16	390.63
	Ethanol	170	17	0.16	1062.50
B. pinnatum stem-bark	Acetone	15.3	1.53	> 2.5	6.12
	Ethanol	22.5	2.25	1.25	18.0
Gentamicin		NA		0.0002	NA

Values in bold indicate very promising antibacterial activity

M. frangula (MIC = 0.02 mg/ml) and *K. gunniae* (MIC = 0.04 mg/ml) acetone extracts had significant activities against all the *S. aureus* mastitis clinical isolates (Table 3.5). *Maytenus undata* acetone extract had significant activity with MIC ranging from 0.04 to 0.08 mg/ml against all the *S. aureus* isolates, with the exception of STA 3, against which it exhibited moderate activity (MIC = 0.11 mg/ml). In terms of the ethanol extracts, both *M. frangula* (MIC = 0.02-0.04 mg/ml) and *K. gunniae* (MIC = 0.04-0.08 mg/ml) had significant activity against all the *S. aureus* isolates from clinical mastitis. In general, acetone extracts of *M. frangula* and *K. gunniae* had significantly better activity than ethanol extracts against all the *S. aureus* clinical isolates. This suggests that acetone extracts should be considered for further research on antimicrobial activities of the two plants.

Worth noting is that this is the first study to report on the promising antibacterial activity of *Kalanchoe gunniae,* a newly described nothospecies in South Africa (Smith et al., 2019).

Similar to the acetone extract, *M. undata* ethanol extract had significant activity with MIC ranging from 0.04 mg/ml to 0.08 mg/ml against five of the *S. aureus* isolates from clinical mastitis (STA 1, STA 2, STA 4, STA 5, and STA 6) and moderate activity of 0.11 mg/ml against one of the clinical isolates (STA 3). *Bryophyllum pinnatum* acetone extract had significant activity with MIC of 0.08 mg/ml against four of the *S. aureus* clinical isolates (STA 1, STA 2, STA 3, STA 4), moderate activity of 0.16 mg/ml against "STA 5", and weak activity of 0.63 mg/ml against "STA 6". The ethanol extract of *B*. *pinnatum* had moderate activity (MIC = 0.16 mg/ml -0.32 mg/ml) against all the *S. aureus* clinical isolates. The results also suggest that the acetone extract of *B. pinnatum* was more active than the ethanol extract against the *S. aureus* clinical isolates. The findings of this study are similar to the findings by Akinsulire et al. (2007) which reported that the methanol extract of *B. pinnatum* had good antimicrobial activity against *S. aureus*.

The mean MIC of the eight plant extracts against all seven strains ranged from 0.02 mg/ml to 0.26 mg/ml (Table 3.5). Among acetone extracts, *M. frangula* acetone extract had the most significant mean MIC activity (0.02 mg/ml) against all seven bacterial strains followed by *K. gunniae* acetone extract (0.05 mg/ml). Among the ethanol extracts, *K. gunniae* extract had the most significant mean MIC activity (0.06 mg/ml) against all seven bacterial strains followed by *M. frangula* (0.064 mg/ml).

Of the acetone extracts, *K. gunniae* leaf acetone extract had the highest total antibacterial activity (4062.50 ml/g) against all *S. aureus* mastitis clinical isolates followed by *M. frangula* acetone extract (2292.50 ml/g), also against all *S. aureus* mastitis clinical isolates. Total antibacterial activity of the extracts against *S. aureus* mastitis clinical isolates ranged from 47.62 ml/g to 6500.0 ml/g (Table 3.4). From the ethanol extracts, *K. gunniae* leaf ethanol extract had the highest total antibacterial activity against *S. aureus* mastitis clinical isolates ranging from 3250.00 ml/g to 6500.00, followed by *M. frangula* acetone extract with total antibacterial activity ranging from 1724.38 ml/g to 3448.75 ml/g. The ethanol extract of *K. gunniae* which had the highest total activity is a promising candidate for isolation, bioprospecting as well as for development into a herbal formulation.

Table 3.5 Minimum inhibitory concentration (MIC, mg/ml) and total antibacterial activity (TAA, ml/g) of acetone and ethanol plant extracts against *Staphylococcus aureus* clinical isolates

			ATCC 29213		STA 1		STA 2		STA 3		STA 4		STA 5		STA 6	
Plant extracts	**Extract yield (mg)**	**% Extract yield**	**MIC**	**TAA**	**MIC**	**TAA**	**MIC**	**TAA**	**MIC**	**TAA**	**MIC**	**TAA**	**MIC**	**TAA**	**MIC**	**TAA**
Acetone																
MF	45.85	4.585	0.02	2292.5	0.02	2292.5	0.02	2292.5	0.02	2292.5	0.02	2292.5	0.02	2292.5	0.02	2292.5
MU	77.5	7.75	0.08	968.75	0.08	968.75	0.08	968.75	0.11	704.55	0.08	3448.75	0.08	968.75	0.04	1937.5
KP	162.5	16.25	0.08	2031.25	0.04	4062.5	0.04	4062.5	0.04	4062.5	0.04	4062.5	0.04	4062.5	0.04	4062.5
BP	30	3	0.32	93.75	0.08	375	0.08	375	0.08	375	0.08	375	0.16	187.5	0.63	47.62
PC	NA		0.0002	NA	0.0006	NA	0.0002	NA	0.0006	NA	0.0002	NA	0.0002	NA	0.0008	NA
Ethanol																
MF	68.975	6.90	0.31	222.5	0.02	3448.75	0.04	1724.38	0.02	3448.75	0.02	3448.75	0.02	3448.75	0.02	3448.75
MU	110.0	11.0	0.12	916.67	0.08	1375.0	0.08	1375	0.11	1000.0	0.08	968.75	0.08	1375.0	0.04	2750.0
KP	260.0	26.0	0.08	3250	0.08	3250.0	0.04	6500.0	0.08	3250.0	0.08	3250.0	0.04	6500.0	0.04	6500.0
BP	45.0	4.5	0.32	140.63	0.16	281.25	0.27	166.67	0.32	140.63	0.27	166.67	0.16	281.25	0.32	140.63
PC	NA		0.0002	NA	0.0006	NA	0.0002	NA	0.0006	NA	0.0002	NA	0.0002	NA	0.0008	NA

Staphylococcus aureus isolates: STA 1, STA 2, STA 3, STA 4, STA 5 and STA 6. MF = *Maurocenia frangula*, MU = *Maytenus undata*, KP = *Kalanchoe gunniae*, BP = *Bryophyllum pinnatum*, PC = (Positive control) Gentamicin, N.A = Not applicable.

3.4.3 Cytotoxicity

Extracts of *K. gunniae* and *B. pinnatum* were least cytotoxic to Vero and bovine dermis cells, while extracts of *M. undata* were most toxic (Table 3.6). Cytotoxicity investigation helps to ascertain whether the activity observed with a plant extract is as a result of toxicity (Elisha et al., 2017). According to Kuete (2010), a plant extract should be considered toxic when the lethal concentration killing 50% of cells (LC_{50}) is 0.02 mg/ml and below. None of the plant extracts tested had LC_{50} value below 0.02 mg/ml (Table 3.6).

K. gunniae extracts had the best selective index (SI) values for both Vero cells and for bovine dermis cells for all tested organisms, followed by *B. pinnatum* (Table 3.6). Acetone and ethanol extracts of *M. frangula* in all cases had SI values greater than one for Vero cells. However, the ethanol extract had SI values greater than one for bovine dermis cells with the clinical isolates and less than one with the ATCC strain. Of interest is that, although *M. frangula* extracts have low LC_{50} values, the much lower MIC values obtained for its acetone extract (0.02 mg/ml) against all strains and its ethanol extract against the clinical isolates, increased the corresponding SI values.

Extracts of *K. gunniae*, *M. frangula* and *B. pinnatum* had average SI values >1 for Vero and bovine dermis cells for all seven strains (Table 3.7). *K. gunniae* extracts had the highest average SI values followed by *M. frangula* extracts. The average SI of acetone extracts of *K. gunniae* were 20 and 31.80 for Vero and bovine dermis cells respectively, which is highly promising. *K. gunniae* ethanol extract also had a high average SI of 16.00 and 16.67 for Vero and bovine dermis cells respectively. The average SI of *M. frangula* acetone extract was 13.5 (Vero) and 7.5 (bovine dermis) cells and the ethanol extract had average SI values of 7.85 (Vero) and 3.75 (bovine dermis) cells.

SI values above 1 indicate greater activity against bacteria than toxicity to mammalian cells (Sserunkuma et al., 2017). The higher the SI value of a plant extract, the better and the safer it is to develop herbal therapeutic formulations from such plant extracts (Dzoyem et al., 2014). Thus, *K. gunniae* extracts which had the highest average SI values hold much value for the future development of herbal therapeutic formulations for the treatment of clinical mastitis associated with *S. aureus.*

Table 3.6 LC_{50} of most active extracts on Vero and bovine dermis (BD) cells and their selective index values on *S. aureus* isolates from clinical mastitis

Plant extracts	LC_{50} (mg/mL)		Selectivity index						
			ATCC	STA 1	STA 2	STA 3	STA 4	STA 5	STA 6
MF. AC	Vero	0.27±0.05	13.5	13.5	13.5	13.5	13.5	13.5	13.5
	BD	0.15±0.02	7.5	7.5	7.5	7.5	7.5	7.5	7.5
MF. ET	Vero	0.50±0.06	1.61	25	12.5	25	25	25	25
	BD	0.24±0.02	0.77	12	6	12	12	12	12
MU. AC	Vero	0.05±0.007	0.63	0.63	0.63	0.46	0.63	0.63	1.25
	BD	0.12±0.005	1.50	1.50	1.50	1.09	1.50	1.50	3.0
MU. ET	Vero	0.06±0.009	0.5	0.75	0.75	0.55	0.75	0.75	1.5
	BD	0.23±0.04	1.92	2.88	2.88	0.55	2.88	2.88	5.75
KP. AC	Vero	>1.0±0.10	**12.50**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**
	BD	>1.0±0.09	**12.50**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**
KP. ET	Vero	0.96±0.05	**12**	**12**	**24**	**12**	**12**	**24**	**24**
	BD	>1.0±0.12	**12.50**	**12.50**	**25.0**	**12.50**	**12.50**	**25.0**	**25.0**
BP. AC	Vero	0.86±0.01	2.69	10.75	10.75	10.75	10.75	5.38	1.37
	BD	0.62±0.05	1.94	7.75	7.75	7.75	7.75	3.88	1.94
BP. ET	Vero	>1.0±0.14	3.13	6.25	3.70	3.13	3.70	6.25	3.13
	BD	0.46±0.06	1.44	2.88	1.70	1.44	1.70	2.88	1.44
Doxorubicin	Vero	0.0099 ± 0.001327							
	BD	0.0087 ± 0.0008							

MF.AC = *Maurocenia frangula* acetone extract, MF. ET= *Maurocenia frangula* ethanol extract, MU. AC = *Maytenus undata* acetone extract, MU.ET = *Maytenus undata* ethanol extract. KP. AC = *Kalanchoe gunniae* acetone extract, KP. ET = *Kalanchoe gunniae* ethanol extract, BP. AC = *Bryophyllum pinnatum* acetone extract, BP. ET = *Bryophyllum pinnatum* ethanol extract. ATCC = *S. aureus* ATCC 29213. STA 1, STA 2, STA 3, STA 4, STA 5 and STA 6 are mastitis clinical isolates

Table 3.7 Mean minimum inhibitory concentration (MIC) and average selective index values of active acetone plant extracts on all seven strains (mastitis clinical isolates and *S. aureus* ATCC 29213)

Samples	ATCC	STA 1	STA 2	STA 3	STA 4	STA 5	STA 6	Mean MIC (mg/ml)	Vero Cells		Bovine Dermis Cells	
									LC_{50} (mg/ml)	Average SI	LC_{50} (mg/ml)	Average SI
Acetone extracts												
MF	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.27	13.5	0.15	7.5
MU	0.08	0.08	0.08	0.11	0.08	0.08	0.04	0.08	0.05	0.63	0.12	1.5
KP	0.08	0.04	0.04	0.04	0.04	0.04	0.04	0.05	>1.0	20.0	>1.0	20.0
BP	0.32	0.08	0.08	0.08	0.08	0.16	0.63	0.20	0.86	4.3	0.62	3.1
PC	0.0002	0.0006	0.0002	0.0006	0.0002	0.0002	0.0008	0.0004	NA	NA	NA	NA
Ethanol extracts												
MF	0.31	0.02	0.04	0.02	0.02	0.02	0.02	0.064	0.50	7.81	0.24	3.75
MU	0.12	0.08	0.08	0.11	0.08	0.08	0.04	0.08	0.06	0.75	0.23	2.88
KP	0.08	0.08	0.04	0.08	0.08	0.04	0.04	0.06	0.96	16.0	>1.0	16.67
BP	0.32	0.16	0.27	0.32	0.27	0.16	0.32	0.26	>1.0	3.85	0.46	1.77
PC	0.0002	0.0006	0.0002	0.0006	0.0002	0.0002	0.0008	0,0004	NA	NA	NA	NA

MF = *Maurocenia frangula*, MU = *Maytenus undata*, KP = *Kalanchoe gunniae*, BP = *Bryophyllum pinnatum*, PC = (Positive control) Gentamicin, NA = Not applicable. ATCC = *S. aureus* ATCC 29213. STA 1, STA 2, STA 3, STA 4, STA 5 and STA 6 are mastitis clinical isolates

3.4.4 Quorum sensing

Minimum quorum sensing inhibition concentration (MQSIC) of the tested extracts ranged from 0.04 to 0.63 mg/ml and their MIC values ranged from 0.63 to 1.25 mg/ml (Table 3.8). *Kalanchoe gunniae* extracts had the best anti-quorum sensing activity with strong MQSIC of 0.04 mg/ml and weak MIC value of 0.63 mg/ml indicating that the MQSIC concentration was not toxic to the bacteria. Both extracts of *B. pinnatum*, the ethanol extract of *M. frangula* and ethanol extract of *M. undata* had anti-quorum sensing activity with moderate MQSIC of 0.16 mg/ml and inactive MIC value of either 1.25 or > 1.25 indicating that the MQSIC concentration did not prevent bacterial growth. *Maytenus undata* acetone extract had poor anti-quorum sensing activity with weak MQSIC activity of 0.63 mg/ml and inactive MIC of > 1.25 mg/ml indicating that the extract neither inhibited quorum sensing nor killed or reduced growth of bacterial cells.

Table 3.8 Minimum quorum sensing inhibition concentration (MQSIC), minimum inhibitory concentration (MIC) of plant samples against *C. violaceum* and their $MQSIC_{50}$ (50% inhibitory concentration)

Plant/Samples	Solvent (extract)	MQSIC (mg/ml)	MIC (mg/ml)	$MQSIC_{50}$ (mg/ml)
M. frangula	Acetone	0.31	> 1.25	0.95
	Ethanol	0.16	1.25	0.87
M. undata	Acetone	0.63	> 1.25	0.79
	Ethanol	0.16	1.25	0.63
K. gunniae	Acetone	0.04	0.63	0.08
	Ethanol	0.04	0.63	0.07
B. pinnatum	Acetone	0.16	1.25	0.67
	Ethanol	0.16	1.25	0.57
Gentamicin		0.008	0.008	0.99

In *C. violaceum*, violacein pigment production is mediated by acylated homoserine lactone (AHL). Quorum sensing mechanism of action can be quantified by targeting the autoinducer, AHL mediated functions. Therefore, violacein production or inhibition of *C. violaceum* in culture media can be used to measure the QS mechanism (Vattem et al., 2007). The minimum quorum sensing inhibition concentration that inhibited 50% of violacein production ($MQSIC_{50}$) of the extracts against *C. violaceum* ranged from 0.07 mg/ml to 0.95 mg/ml (Table 3.8). *Kalanchoe gunniae* extracts had the best $MQSIC_{50}$ with values for ethanol and acetone

extracts of 0.07 mg/ml and 0.08 mg/ml respectively. These results indicate that both extracts greatly inhibited quorum sensing, without being toxic to the bacteria.

The quorum sensing selectivity index (quorum sensing SI) of the plant extracts against Vero and bovine dermis cells was also calculated as it is desirable to highlight extracts with good anti-quorum sensing activity without being toxic to the bacteria or mammalian cells. Extracts of *K. gunniae* and *B. pinnatum* had the highest quorum sensing SI with values greater than 1 with all the extracts (Table 3.9). The quorum sensing SI of *K. gunniae* extracts ranged from 24.0 to 25.0 for both Vero and bovine dermis cells with mean quorum sensing SI values of 25.0 (Vero and BD cells) and 24.5 (Vero and BD cells) for the acetone and ethanol extracts respectively. For *B. pinnatum* extracts, quorum sensing SI ranged from 2.88 to 6.25 for both Vero and bovine dermis cells with mean quorum sensing SI values of 4.63 (Vero and BD cells) and 4.57 (Vero and BD cells) for acetone and ethanol extracts respectively.

Vasavi et al. (2014) reported that a plant extract fraction containing flavonoids was able to result in significant anti-QS activity. The extracts of *K. gunniae* and *B. pinnatum,* which had significant to good anti-QS activity respectively, most likely have varying degrees of flavonoids or other particular plant secondary metabolite(s) that have activity against bacterial quorum sensing. Further work is necessary to isolate and characterize the active compounds.

Table 3.9 Selectivity index values of the acetone extracts of selected plants against Vero and bovine dermis (BD) cells using their MQSIC

Samples		LC_{50} (mg/mL)	Quorum SSI	Mean quorum SSI	MQSIC (mg/ml)
Acetone extracts					
MF	Vero cells	0.27±0.05	0.87	0.68	0.31
	BD cells	0.15±0.02	0.48		
MU	Vero cells	0.05±0.007	0.08	0.14	0.63
	BD cells	0.12±0.005	0.19		
KP	Vero cells	>1.0±0.10	25.0	25.0	0.04
	BD cells	>1.0±0.09	25.0		
BP	Vero cells	0.86±0.01	5.38	4.63	0.16
	BD cells	0.62±0.05	3.88		
Ethanol extracts					
MF	Vero cells	0.50±0.06	3.13	2.32	0.16
	BD cells	0.24±0.02	1.5		
MU	Vero cells	0.06±0.009	0.38	0.91	0.16
	BD cells	0.23±0.04	1.44		
KP	Vero cells	0.96±0.05	24.0	24.5	0.04
	BD cells	>1.0±0.12	25.0		
BP	Vero cells	>1.0±0.14	6.25	4.57	0.16
	BD cells	0.46±0.06	2.88		

MF = *Maurocenia frangula*, MU = *Maytenus undata*, KP = *Kalanchoe gunniae*, BP = *Bryophyllum pinnatum*. Quorum SSI = Quorum sensing selectivity index, Mean quorum SSI = Mean quorum sensing selectivity index, MQSIC = Minimum quorum sensing inhibition concentration.

3.4.5 Biofilm kinetics

Considering results of the antibacterial activity, cytotoxicity and anti-quorum sensing assays, extracts of *K. gunniae* and *B. pinnatum* were selected for investigation of their biofilm inhibition ability. The anti-biofilm assay was performed against four strains (*S. aureus* ATTC 29213, *S. epidermidis* ATCC 35984*,* and two of the *S. aureus* isolates, namely STA 4 and STA 6). *Staphylococcus aureus* ATTC 29213 was chosen to represent wild type *S. aureus*, while *S. epidermidis* ATCC 35984 was chosen because it is known to be biofilm producer. Based on the results of the antibiogram assay, STA 4 and STA 6 were chosen to represent *S. aureus* mastitis clinical isolates. The biofilm assay was done to check the effect of the extracts on prevention of cell attachment: (anti-adhesion) – 0 h, inhibition of development of pre-formed biofilms – 24 h and evaluation of destruction of matured biofilm –48 h.

3.4.5.1 Effect on bacterial adhesion

At time zero (0 h), all the extracts had good biofilm inhibition above 50% against all the tested organisms (Figs 1 to 4). *B. pinnatum* ethanol extract had the best biofilm inhibition ranging

from 69.95% to 95.55% at time zero. The acetone extract of *B. pinnatum* had the second-best biofilm inhibition activity ranging from 67.00% to 93.89% at time zero. Biofilm inhibition activities of *K. gunniae* extracts ranged from 51.53% to 83.73%.

Phytochemicals can interfere with the activities of bacterial receptors which are important in the attachment stage of the biofilm formation. This can happen by either directly binding to the bacterial receptors or by downregulating the expression and activities of binding factors in the bacterial cells (Hidalgo et al., 2011). For example, plant secondary metabolites can bind to bacterial cell walls and inhibit enzyme activity, thus preventing the initial cell attachment and biofilm formation (Tiwari et al., 2011). Therefore, it is possible that the above plant extracts contain phytochemicals with anti-biofilm properties and or that they may interfere with the functioning of bacterial signaling receptors.

3.4.5.2 Effect on pre-formed biofilm

On 24 h-old pre-formed biofilm, *K. gunniae* acetone and ethanol extracts and *B. pinnatum* acetone extracts had weak antibiofilm activity (1.34% to 15.19%) against *S. epidermidis,* whereas *B. pinnatum* ethanol extract enhanced biofilm development of *S. epidermidis.* All the extracts enhanced biofilm development of *S. aureus* and STA 4 on 24 h pre-formed biofilm (Figs 1 to 4). For STA 6, *B. pinnatum* acetone and ethanol extracts had weak antibiofilm activity (31.35% and 6.95% respectively) on 24 h pre-formed biofilm, whereas *K. gunniae* acetone and ethanol extracts enhanced the growth of pre-formed biofilm.

3.4.5.3 Effect on matured biofilm

On 48 h matured biofilm none of the extracts had good antibiofilm demolition activity against any of the tested organisms (Figs 1 to 4). However, *K. gunniae* acetone and ethanol extracts and *B. pinnatum* ethanol extracts had weak biofilm destruction activity (0.25% to 4.36%) on *S. epidermidis. Bryophyllum pinnatum* acetone extract enhanced the growth of matured biofilm on *S. epidermidis.* None of the extracts had biofilm inhibition activity on the 48h matured biofilm of *S. aureus and* STA 4. The extracts instead enhanced the growth of these organisms. For STA 6 with 48 h matured biofilm, only the *B. pinnatum* acetone extract was able to have at least a weak biofilm inhibition activity (33.74%). All the other extracts enhanced growth of STA 6 in the 48 h matured biofilm.

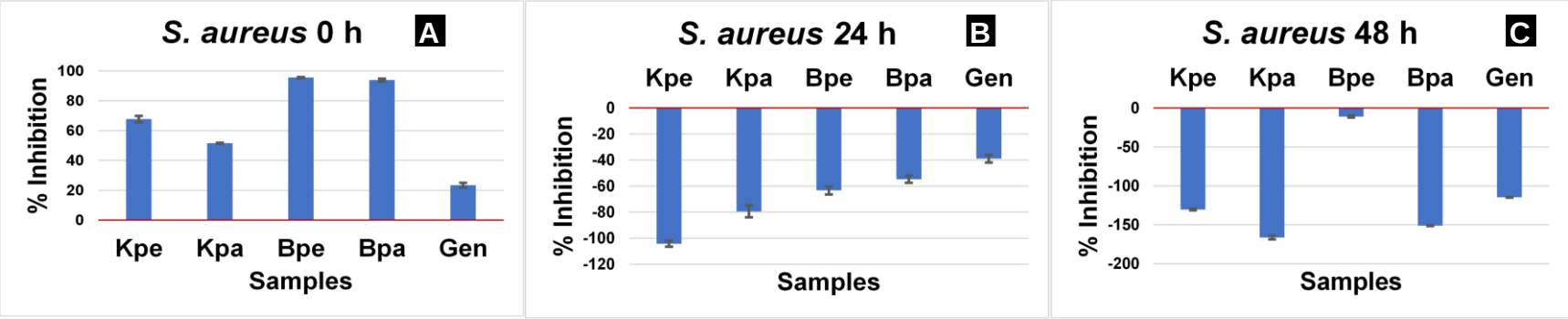


Figure 3.1. Biofilm inhibition of plant extracts on *S. aureus* ATCC 29213. A = Treatment at 0 h (bacterial adhesion stage), B = Treatment after 24 h (pre-formed biofilm stage), C = Treatment after 48 h (matured biofilm stage). Kpa = *Kalanchoe gunniae* acetone extract, Kpe = *Kalanchoe gunniae* ethanol extract, Bpa = *Bryophyllum pinnatum* acetone extract, Bpe = *Bryophyllum pinnatum* ethanol extract, Gen = Gentamicin.

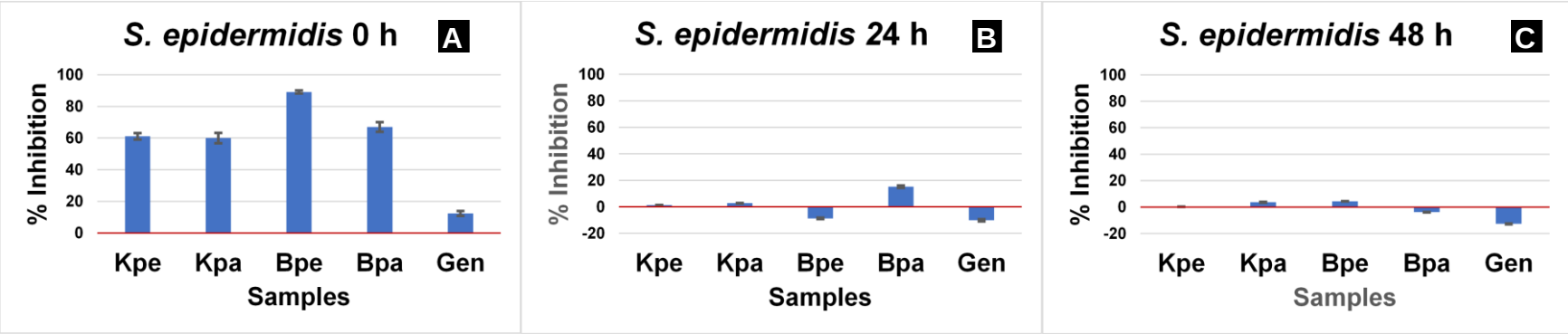


Figure 3.2 Biofilm inhibition of plant extracts on *S. epidermidis* ATCC 35984. A = Treatment at 0 h (bacterial adhesion stage), B = Treatment after 24 h (pre-formed biofilm stage), C = Treatment after 48 h (matured biofilm stage). Kpa = *Kalanchoe gunniae* acetone extract, Kpe = *Kalanchoe gunniae* ethanol extract, Bpa = *Bryophyllum pinnatum* acetone extract, Bpe = *Bryophyllum pinnatum* ethanol extract, Gen = Gentamicin.

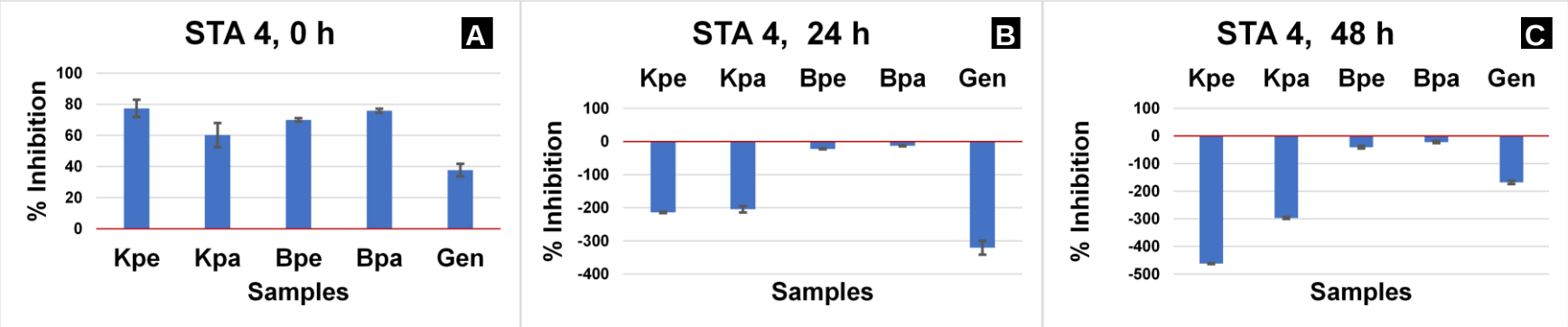


Figure 3.3 Biofilm inhibition of plant extracts on STA 4. A = Treatment at 0 h (bacterial adhesion stage), B = Treatment after 24 h (pre-formed biofilm stage), C = Treatment after 48 h (matured biofilm stage). Kpa = *Kalanchoe gunniae* acetone extract, Kpe = *Kalanchoe gunniae* ethanol extract, Bpa = *Bryophyllum pinnatum* acetone extract, Bpe = *Bryophyllum pinnatum* ethanol extract, Gen = Gentamicin.

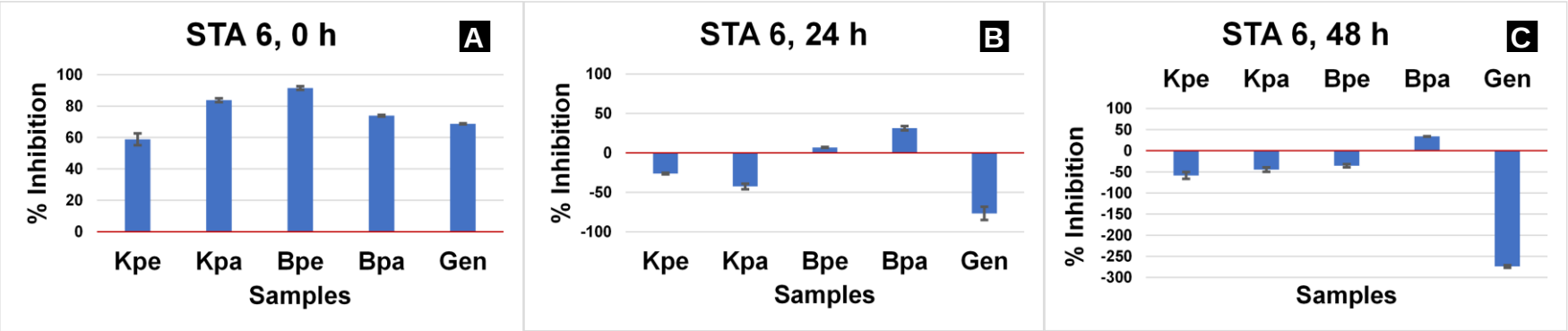


Figure 3.4 Biofilm inhibition of plant extracts on STA 6. A = Treatment at 0 h (bacterial adhesion stage), B = Treatment after 24 h (pre-formed biofilm stage), C = Treatment after 48 h (matured biofilm stage). Kpa = *Kalanchoe gunniae* acetone extract, Kpe = *Kalanchoe gunniae* ethanol extract, Bpa = *Bryophyllum pinnatum* acetone extract, Bpe = *Bryophyllum pinnatum* ethanol extract, Gen = Gentamicin

3.5 Conclusions

Bovine mastitis is an economically important disease, with the emergence of drug-resistant strains of bacteria complicating treatment and reducing productivity of dairy cows. In this study, extracts of *Kalanchoe gunniae* and *Bryophyllum pinnatum* were non-toxic to mammalian cells, with very good to moderate activity against both *S. aureus* ATCC 29213 and drug-resistant *S. aureus* isolates from clinical cases of mastitis. These plant extracts, especially the extracts of *Kalanchoe gunniae*, had excellent activity against resistant clinical isolates from mastitis cases not only directly as an antibacterial agent, but also in terms of activity via other mechanisms of inhibiting bacterial infection through quorum sensing and biofilm inhibition. In the anti-biofilm assay, although they were strongly effective in preventing biofilm formation, the extracts were unable to eradicate pre-formed and matured biofilm.

To the best of our knowledge, this is the first report of research on the selected plants as potential alternatives or complementary treatments for the management of mastitis through their activity against mastitis caused by drug-resistant *S. aureus* strains and their mechanisms of action. Extracts of *K. gunniae* have potential for development as formulations for the prevention and possible treatment of mastitis. Further work is ongoing to explore the possibilities of *in vivo* studies of herbal therapeutic mastitis spray formulations for both prophylaxis and treatment of bovine mastitis.

CHAPTER 4 Mechanisms of anti-inflammatory and antioxidant activity of extracts of selected South African Celastraceae and Crassulaceae plant species with potential use against bovine mastitis

4.1 Introduction

Infectious mastitis is caused by various microbes, and is characterised by inflammation of the mammary gland and udder tissues which develops following activation of the immune response (Sserunkuma et al., 2017). Acute inflammation is necessary to control infection as it assists in removing bacteria and cellular debris following infection. However, if the inflammatory process is not well-regulated, leading to prolonged chronic inflammation, damage is done to the body in place of healing (Iwalewa et al., 2007).

Excess activation of phagocytes during inflammation results in increased oxygen consumption by the neutrophils (Colin & Monteil, 2003). This then leads to oxidative stress (oxidative burst) burst at the site of microbial invasion (Jalil et al., 2003). Following this, reactive oxygen species (ROS) such as superoxide ($O_{2^{\cdot}}$), hydroxyl ($^{\cdot}OH$) and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) – free radicals and hydrogen peroxide (H_2O_2) - non-free radical species are generated (Sakat et al., 2010). Production of these free radicals and non-free radicals in excess harms the surrounding tissue either by powerful direct oxidizing action with O_2, or indirectly with H_2O_2 and OH radicals formed from $O_{2^{\cdot}}$(Latha et al., 2011). This complex process also results in an increase in several inflammation-related enzymes including phospholipase A_2 (PLA_2), 5-lipoxy-genase (5-LOX), cyclooxygenases and inducible nitric oxide synthase (iNOS) (Adibhatla & Hatcher, 2006). Increased iNOS then enzymatically oxidises L–arginine to citrulline, forming nitric oxide ($^{\cdot}NO$). The $O_{2^{\cdot}}$reacts with $^{\cdot}NO$ to form peroxynitrite ($ONOO^{-}$), which is a strong oxidant able to initiate lipid peroxidation (Adibhatla & Hatcher, 2006). An inflammatory response is triggered (Latha et al., 2011) leading to production of more ROS generating enzymes and activation of the transcription factor, nuclear factor kappa B (NFkB) (Iwalewa et al., 2007), pro-inflammatory cytokines and other inflammatory mediators such as tumour necrosis factor (TNF-α) (Paterson et al., 2003). More membrane destruction and tissue damage takes place, leading to increased and persistent inflammation (Jalil et al., 2003).

In bovines, following chronic inflammation when the immune system is not able to eliminate the pathogen, the infection does not clear and the inflammatory response persists, leading to visible mastitis symptoms, i.e. clinical mastitis (Schukken et al., 2003). Mastitis is a significant problem in the dairy industry, causing vast financial losses as a result of a decrease in milk production and low quality of milk (Banga et al., 2014). Clinical cases of mastitis are characterised by clinical signs while subclinical cases are detected by a SCC of over 200 000 cells/ml as well as the presence of microbial organisms in milk (Sargeant et al., 2001; Viguier et al., 2009). The majority of mastitis cases are caused by bacteria, with *S. aureus* one of the predominant species (Harmon, 1994; Swartz & Novello, 1984; Waage et al., 1999).

In the previous chapter, it was reported that *Maytenus undata* and *Maurocenia frangula* from the Celastraceae family, and *Kalanchoe gunniae* and *Bryophyllum pinnatum* (Crassulaceae) were reported to have good activities against various *Staphylococcus aureus* strains. These test strains included reference ATCC strains and isolates from clinical cases of mastitis in dairy cows. With the aim of targeting plant species for development into complementary medications, further research on these species is warranted to determine their efficacy in preventing and treating bovine mastitis. In the present study, *in vitro* antioxidant and anti-inflammatory efficacy of the selected plant extracts was tested using various bioassays.

4.2 Materials and methods

4.2.1 Collection of plants, drying and extraction

Maytenus undata was collected from SANBI, Walter Sisulu Botanical Garden South Africa. *Maurocenia frangula was* collected from SANBI, National Botanical Garden, Nelspruit, Mpumalanga, South Africa. *Kalanchoe gunniae* was collected from the South African National Biodiversity Institute (SANBI), Pretoria Botanical Garden, South Africa. *Bryophyllum pinnatum* was collected from the Federal Institute of Industrial Research Oshodi, Lagos Nigeria, Medicinal Plant Garden. Herbarium sample of *Maytenus undata was* prepared, identified and deposited at the H.G.W.J. (Herold Georg Wilhelm Johannes) Schweickerdt Herbarium, University of Pretoria and the voucher specimen number were obtained. Herbarium samples of *Bryophyllum pinnatum and Kalanchoe gunniae* were prepared, identified, and deposited at the SANBI Herbarium, Pretoria Botanical Garden, South Africa and their voucher specimen numbers were also obtained. The voucher numbers are shown in Table 4.1.

Collected plant materials of *M. undata* and *M. frangula* were dried indoors at room temperature in open mesh loose woven bags. *Bryophyllum pinnatum* and *K. gunniae* were cut into pieces

and dried with sample dehumidifying oven drier at 33°C. Plant materials were extracted with acetone (≥ 99% from Minema Chemicals (Pty)Ltd, South Africa) and ethanol (99.9% from Minema Chemicals (Pty)Ltd, South Africa) using the utrasonication method of Sserunkuma et al. (2017) with modification. Briefly, 4 g of ground plant material was extracted with 40 ml of either acetone or ethanol by shaking the samples vigorously for 25 minutes with a mechanical shaker and then sonicating in an utrasonicator for 15 minutes. Each of the extracts were filtered through Whatman no 1 filter paper in to a glass vial. The plant material residues were re-extracted with 20 ml of extracting solvent and the filtrates from the three extractions were combined. The extracts were dried by evaporating the solvents under a stream of air at 30°C.

Table 4.1 Voucher numbers of plant species investigated

Plant family	Plant species	Voucher number
Celastraceae	*Maytenus undata* (Thunb.) Blakelock	PRU 125486
	Maurocenia frangula Mill.	PRE 1004262
Crassulaceae	*Kalanchoe gunniae* Gideon F.Sm. & Figueiredo	PRE 1004266
	Kalanchoe pinnata (Lam.) Pers. Synonym: *Bryophyllum pinnatum* (Lam.) Oken	PRE 1004263

4.2.2 Antioxidant activity

4.2.2.1 DPPH assay

The antioxidant assay was performed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay according to the method described by Re et al. (1999) with some modifications (Nkadimeng et al., 2020). Briefly 40 µl of methanol (Minema) was added into all the wells of a U-bottom 96 well microtitre plate (Lasec). Thereafter 40 µl of the extracts (1 mg/mL in methanol) were added each into 4 wells in the second row of the microtiter plate and serially diluted from 100 to 1.5625 µg/mL concentrations. Freshly prepared DPPH with an optical density of 0.91 to 0.99 at 517 nm was added (160 µl) into the two wells of each sample while 160 µl of methanol was added into the other two wells to be used as blank. The plate was allowed to stand at room temperature for 30 min in the dark and absorbance measured at 517 nm using a microplate reader (Biotek, Synergy HT). The positive controls used were trolox, which is a water-soluble analogue of vitamin E, and ascorbic acid (vitamin C. The experiments were repeated three times with three replicates in each experiment. The percentage of inhibition of DPPH oxidation was calculated according to the formula:

% DPPH inhibition = ((Abs control – Abs sample)/Abs control) x 100

where Abs = absorbance. The antioxidant ability was expressed in IC_{50} values (the concentration of sample necessary to scavenge DPPH by 50%).

For plant extracts it has been established that IC_{50} < 10 µg/mL are regarded as strong potent/significant (strong radical scavenging activity), IC_{50} from 10 µg/mL to 50 µg/mL are regarded as good antioxidant activity, IC_{50} > 50 and ≤ 100 µg/ml are regarded as moderate activity and IC_{50} < 100 and ≤ 250 µg/ml are regarded as weak activity (Bakasatae et al., 2018; Omisore et al., 2005; Phongpaichit et al., 2007). Thus, IC_{50} less than one as observed in the results of some of the plant extract in this study will be regarded as potent significant activity.

4.2.2.2 ABTS assay

The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity of the extracts was assessed using the method of Brand-Williams et al. (1995) with modifications. Ascorbic acid and trolox were used as positive controls. Briefly, the ABTS solution was prepared with potassium persulphate (Sigma-Aldrich) in equal amounts and allowed to react for 16 h at room temperature in the dark before use. Then 40 µl of methanol was added into all the wells of a U-bottom 96 well microtitre plate. Thereafter 40 µl of extracts (1 mg/mL in methanol) were added into each of 4 wells in the second row of the microtitre plate and serially diluted in the same manner as above with DPPH. Freshly prepared ABTS with an optical density of 0.71 to 0.79 at 734 nm was added (160 µl) into two wells of each sample while methanol was added into the other two wells used as blank. The ABTS scavenging ability of the extracts were also measured in terms of a colour change after 5 min incubation in the dark. The experiments were repeated three times and IC_{50} values were calculated according to the formula:

% ABTS inhibition = (Abs control – Abs sample)/Abs control) x 100

where Abs = absorbance. Antioxidant activity was expressed in IC_{50} values (concentration of the sample necessary to scavenge ABTS by 50%.

4.2.3 Anti-inflammatory activity

4.2.3.1 Inhibition of 15-lipoxygenase (15-LOX)

The anti-inflammatory activity of extracts was evaluated *in vitro* using the soybean 15-lipoxygenase (15-LOX) inhibitory assay according to the method of (Ondua et al., 2019). Briefly, the extracts were diluted to 10 mg/mL in DMSO and then reconstituted to 2 mg/mL in buffer (Tris-HCL buffer (50Mm), pH 7.4). Quercetin was used as a positive control also prepared 10 mg/mL in DMSO and reconstituted to 1 mg/mL in buffer. Then 20 µL of the buffer was added to all wells and 20 µL of the extracts were added into the first raw and serial diluted.

Thereafter 40 µL of the 15-lipoxygenase enzyme (Sigma-Aldrich) prepared in buffer to a concentration of 0.1 mg/mL was added into all the wells and incubated for 5 min. Approximately 40 µL of the substrate, linoleic acid (140 µM) (Sigma-Aldrich), was added to all the wells except in the blank and incubated for 20 min. Thereafter, 100 µL of freshly prepared FOX reagent (prepared with xylenol orange and ferrous sulphate) was added and incubated for 30 min. Then 40 µL of the substrate was added into the blank wells just before reading the absorbance at 560 nm. The experiments were done in triplicate and repeated twice. The percentage of inhibition was calculated using the formula (% enzyme inhibition= [(Absorbance (sample)-Absorbance (blank))/ ((Absorbance (negative control)-Absorbance (blank)) X 100] and IC_{50} determined using a linear regression from the graphs plot of % enzyme inhibition against the concentrations of the extracts and quercetin**.**

4.2.3.2 Inhibition of nitric oxide (NO) production

RAW 264.7 macrophage cells purchased from the American Type Culture Collection (ATCC ® TIB-71™, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Pan Biotech, Separations) supplemented with 10% foetal bovine serum (Gibco, Sigma) and 1% penicillin (100 units/mL) and streptomycin (100 ug/mL) (Celtic Molecular Diagnostics) at 37^{o}C in a 5% CO_2 atmosphere (HERAcell 150, Thermo Electron Corp., USA). RAW 264.7 cells were seeded at a density of 4 x 10^4 cells/well into each well of column 2 to 11 of sterile tissue culture treated 96-well plates (NEST, Whitehead Scientific) and incubated for 24 h at 37^{o}C in 5% CO_2. Then media was aspirated from all the wells and replaced with fresh medium. The cells were treated with lipopolysaccharide (LPS) (Sigma-Aldrich) at a concentration of 1 µg/mL in the presence of different concentrations of the extracts (1.6 to 100 µg/mL) and incubated for 24 h.

The amount of nitrite, a stable metabolite of nitric oxide (NO) was measured using Griess reagent (Sigma) and the concentration of nitrite in the culture media was used as an indicator of NO production. Briefly 100 µl of cultured media after 24 h incubations was transferred into a new plate and 100 µl of Griess reagent (Sigma-Aldrich) was added. The mixture was incubated for 15 min in the dark at room temperature. Then absorbance was measured at 540 nm using a microplate reader (Biotek, Synergy HT). The wells in column 1 and 12 containing only media with Griess reagent were used as a blank in every experiment. The amount of nitrite in the media was calculated from the sodium nitrite ($NaNO_2$) standard curve.

Cytotoxicity on the remaining RAW 264.7 cells following removal of the supernatant for use in the NO assay was measured using the MTT assay. Viability of cells was determined using the

tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) with modifications. Briefly after 24 h incubation with 1 µg/mL LPS in the presence of different concentrations of the extracts, medium was removed in all the wells. RAW 264.7 cells were washed with 200 uL pre-warmed PBS and then 100 µL of fresh media were added. MTT (40 µL of a stock solution of 5 mg/mL dissolved in PBS) was added to each well and the cells were incubated for 4 h at 37^{o}C in a 5% CO_2. Medium was aspirated from all the cells and DMSO was added to solubilise the formazan salt precipitate, and absorbance measured at a wavelength of 540 nm and a reference wavelength of 630 nm using a microplate reader. The percentage of dead cells was calculated relative to the untreated cells. Viability of cells in percentages was calculated using the formula: % Viability= ((Sample Absorbance/control Absorbance) x 100). The experiments were repeated two times on different occasions and each sample was tested in quadruplicate.

4.2.3.3 Inhibition of cyclooxygenase-2 (COX-2) and cytokines

The effects of the extracts on cycloxygenase-2 (COX-2) were measured using the methods of Noreen et al. (1998) modified by du Toit et al. (2005) and Nkadimeng et al. (2020). Briefly, when confluent, the RAW 264.7 macrophages were plated into several 25 cm^2 tissue culture flasks (NEST, Whitehead Scientific) at a density of 1 x 10^6 cells per flask and incubated for 24 h. The medium was removed, fresh medium was added and the cells were stimulated with LPS (1 µg/mL) and treated with different concentrations of the extracts. Quercetin, a well-known antioxidant and a flavonol found in many fruits and plants, and N-Nitro-L-Arginine Methyl Ester (-NAME), a nitric oxide synthase (NOS) inhibitor, were used as positive controls. Control cells were stimulated with LPS but not treated while the normal cells were cells which were neither stimulated with LPS nor treated with extracts. After 24 hours medium was removed and stored in a -80°C freezer until day of analysis.

4.2.3.3.1 Cyclooxygenase-2 enzyme measurements

The effects of the extracts on COX-2 were determined using the mouse PTGS2/COX-2 Prostaglandin endoperoxide synthase 2 (PGE_2) ELISA kit (Elabscience, Biocom Africa) according to the manufacture manual protocol. Concentrations of mouse COX-2 in the cell culture media samples were calculated from the standard curve. The absorbance was directly proportional to the concentrations of PGE_2 in the sample medium.

4.2.3.3.2 Cytokine measurements

The effects of the extracts on levels of pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) and anti-inflammatory cytokine Interleukin 10 (IL-10) were determined and quantified using the mouse ELISA kits with catalogue numbers E-EL-M0049, E-EL-M0037 and E-EL-M0046, (Elabscience, Biocom Africa) respectively following manufacturer's instructions.

4.2.4 Statistical analysis

Processing and analyses of obtained data were done with Analyses of Variance (ANOVA) in Microsoft Excel, Sigmastat and SPSS. Pairwise multiple comparison was done using Holm-Sidak method. Results were stated as mean ± standard deviations. Statistically significant values were compared and p-value of ≤ 0.050 was considered as statistically significant.

4.3 Results

4.3.1 Antioxidant activity

Of the eight extracts prepared from four plants, *Kalanchoe gunniae* extracts had the best scavenging activity for both ABTS and DPPH assays (Table 4.1). *Kalanchoe gunniae* extracts had potent scavenging activity with IC_{50} below 1 µg/ml for both ABTS and DPPH assays. *Kalanchoe gunniae* ethanol extract was the most active for ABTS (IC_{50} = 0.06 ± 0.007 µg/ml) and DPPH (IC_{50} = 0.13 ± 0.007 µg/ml). Similar to the ethanol extract, the acetone extract of *Kalanchoe gunniae* also had very strong activity in the ABTS assay (IC_{50} = 0.30 ± 0.01 µg/ml) and DPPH (IC_{50} = 0.42 ± 0.034 µg/ml) (Table 4.1). The ethanol extracts of *B. pinnatum, M. frangula* and *M. undata* had strong activity in both ABTS and DPPH assays. However, there was no significant difference ($p \leq 0.05$) between the ethanol extracts of *B. pinnatum, M. frangula, M. undata, Kalanchoe gunniae* extracts compared to the positive controls (Table 4.2) Regarding the acetone extract of *B. pinnatum, M. frangula* and *M. undata*, *M. frangula* acetone extract had strong potent activity for DPPH assay (9.37 ± 0.43 µg/ml) and good activity in the ABTS assay (16.68 ± 0.65 µg/ml). There was also no statistically significant difference between the activities of the *M. frangula* acetone extract when compared to the activities of the positive controls. Whereas *M. undata* acetone extract and *B. pinnatum* acetone extracts had good to moderate activity with IC_{50} ranging from 47.51 ± 6.99 µg/ml to 118.50 ± 11.81 µg/ml for both ABTS and DPPH and these acetone extracts of these plants had significant difference when compared to the positive controls.

Table 4.2 Antioxidant assays of selected plant extracts with DPPH and ABTS

Plant extracts	Extractant	DPPH - IC_{50} (µg/ml)	ABTS - IC_{50} (µg/ml)
M. undata	ethanol extract	**9.43 ± 0.17**	**4.41 ± 0.23**
	acetone extract	118.50 ± 11.81 *	47.51 ± 6.99 *
M. frangula	ethanol extract	**4.92 ± 0.77**	**6.79 ± 1.21**
	acetone extract	**9.37 ± 0.43**	16.68 ± 0.65
K. gunniae	ethanol extract	**0.13 ± 0.007**	**0.06 ± 0.007**
	acetone extract	**0.42 ± 0.034**	**0.30 ± 0.01**
B. pinnatum	ethanol extract	**6.78 ± 0.55**	**7.04 ± 0.38**
	acetone extract	38.14 ± 5.97 *	47.38 ± 8.16 *
Trolox		**0.58 ± 0.02**	**0.46 ± 0.03**
Ascorbic acid		**0.19 ± 0.004**	**0.22 ± 0.01**

* = indicates statistically significant difference (P ≤ 0.05) when compared with controls (Trolox and Ascorbic acid). DPPH = 2,2 diphenyl -1- picrylhydrazyl and ABTS = 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). Bolded = strong activity, IC_{50} below 10µg/ml.

4.3.2 Anti-inflammatory activity

4.3.2.1 Inhibition of 15-lipoxygenase

The 15-LOX inhibitory activities of the plant extracts are presented in Table 4.3. The IC_{50} of the plant extracts ranged from 1.25 ± 0.02 µg/ml (strong activity) to 119.18 ± 0.99 µg/ml (weak activity). The lower the IC_{50}, the higher the anti-inflammatory activity. As with antioxidant activity, *K. gunniae* extracts also had the best 15-LOX inhibitory activity with IC_{50} values of 1.25 µg/ml and 2.03 µg/ml for the acetone and ethanol extracts respectively. Extracts of *K. gunniae* were more potent than the positive control quercetin (IC_{50} = 4.93 µg/ml), however, there was no statistically significant difference (P ≤ 0.05) between them.

All the ethanol extracts of the other three plants (*B. pinnatum, M. frangula* and *M. undata*) had good inhibitory activity with IC_{50} values ranging from 13.26 µg/ml to 18.99 µg/ml. The acetone extracts had good to weak enzyme inhibitory activity ranging from 27.50 µg/ml to 119.18 µg/ml. With IC_{50} values of 48.51 and 119.18 for acetone extracts of *B. pinnatum* and *M. undata* respectively, these extracts had statistically significant difference (P ≤ 0.05) when compared with the positive control quercetin.

Table 4.3 Anti-anti-inflammatory assays of selected plant extracts with lipoxygenase (LOX)

Plant	Extractant	LOX - IC_{50} (in µg/ml)
K. gunniae	**ethanol extract**	**2.03 ± 0.13**
	acetone extract	**1.25 ± 0.02**
B. pinnatum	ethanol extract	18.99 ± 0.57
	acetone extract	48.51 ± 5.12 *
M. frangula	ethanol extract	17.26 ± 0.42
	acetone extract	27.50 ± 0.47
M. undata	ethanol extract	13.26 ± 0.29
	acetone extract	119.18 ± 0.99 *
Quercetin		**4.93 ± 0.28**

* = indicates statistically significant difference (P ≤ 0.05) when compared with the control (Quercetin). LOX = 15-lipoxygenase. Bold = strong activity, IC_{50} below 10µg/ml.

4.3.2.2 Inhibition of nitic oxide (NO) production and effect on macrophage viability

In this study the effects of plant extracts on NO production in LPS-activated RAW 264.7 macrophages, and viability of cells following treatment were determined. The LPS induced the production of nitric oxide (NO) in the activated RAW 264.7 macrophages. Measurement of nitrite, which is a stable oxidising product of NO, correlates to the amount of NO produced. The inhibition of nitric oxide (NO) by the plant extracts and the effect of the plant extracts on cell viability of LPS-activated RAW 264.7 macrophages showed concentration dependent effects (Table 4.4, Figs 4.1 and 4.2).

At the highest concentration tested (100 µg/ml), extracts of *B. pinnatum* had the best NO inhibition activity ranging from 78.93% to 80.48% and the % cell viability ranged from 92.19% to 96.75% on the LPS-activated macrophages**.** *Kalanchoe gunniae* extracts were the second best for NO inhibition activity. At the highest concentration tested, *K. gunniae* extracts showed

Table 4.4 Anti-inflammatory assays of selected plant extracts with nitric oxide (NO), and viability of treated RAW 264.7 macrophages

Extracts	Conc. µg/ml	IC_{50} (µg/ml)	% NO Inhibition	% macrophage cell viability
Maurocenia frangula ethanol	1.6	1.43±0.20	53.77±1.68	115.52 ± 0.37
	12.5		75.72±4.88	96.82 ± 0.69
	50		116.88±2.09	30.44 ± 1.18
	100		109.81±13.07	5.10 ± 0.007
Maurocenia frangula acetone	1.6	1.62±0.08	55.89±8.47	104.14 ± 6.45
	12.5		69.03±1.88	85.63 ± 2.80
	50		118.0±1.68	26.35 ± 1.32
	100		118.17±1.08	2.88 ± 0.004
Maytenus undata ethanol	1.6	0.91±0.15	57.19±4.45	81.71 ± 2.14
	12.5		68.35±4.26	68.56 ± 5.60
	50		77.28±4.91	62.45 ± 5.24
	100		94.96±4.39	61.35 ± 0.18
Maytenus undata acetone	1.6	1.25±0.14	59.55±3.30	82.58 ± 4.99
	12.5		66.66±4.05	68.66 ± 1.65
	50		94.08±1.07	60.89 ± 0.30
	100		112.55±0.11	57.62 ± 0.76
Kalanchoe gunniae ethanol	1.6	49.62±7.86	39.95±0.43	104.20 ± 5.14
	12.5		48.90±4.06	101.78 ± 1.34
	50		**53.64±9.47**	**98.65 ± 1.40**
	100		**56.73±7.03**	**95.75 ± 3.50**
Kalanchoe gunniae acetone	1.6	76.25±7.37	32.50±4.04	121.58 ± 3.22
	12.5		36.49±3.04	111.75 ± 2.40
	50		45.90±5.0	99.38 ± 8.39
	100		**54.71±5.14**	**96.88 ± 3.99**
Bryophyllum pinnatum ethanol	1.6	19.23±2.64	41.69±4.40	115.64 ± 3.78
	12.5		45.66±1.74	105.16 ± 3.53
	50		**67.58±3.24**	**99.99 ± 5.68**
	100		**78.93±0.32**	**92.19 ± 0.41**
Bryophyllum pinnatum acetone	1.6	23.51±3.97	37.57±0.30	115.16 ± 11.86
	12.5		45.70±0.47	106.41 ± 10.29
	50		**66.48±8.99**	**101.15 ± 9.35**
	100		**80.48±1.87**	**96.75 ± 3.94**
Quercetin	1.6	NA	39.54 ± 4.31	95.68 ± 2.89
	12.5		63.97 ± 3.77	93.70 ± 1.48
	50		83.35 ± 7.66	89.18 ± 7.32
	100		99.82 ± 5.42	53.84 ± 6.37
Doxorubicin	2	NA	NA	79.36 ± 9.34
	4		NA	60.76 ± 12.09
	10		NA	16.92 ± 0.88
	20		NA	2.14 ± 0.28

NA = not applicable

Bold = extracts that gave NO inhibition above 50% with cell viability above 90%

good inhibition activity ranging from 54.71% to 56.73% with cell viability ranging from 95.75% to 96.88%.

For *M. undata* extracts, at the highest concentration, *M. undata* ethanol extract showed NO inhibition activity of 94.96% and low cell viability of 61.35%. Also, at the highest concentration, *M. undata* acetone extract, *showed activity* above 100% with low cell viability of 57.62%. Similar to the activities of *M. undata* extracts, *M. frangula* extracts showed activity above 100% with low cell viability. The cell viability observed with *M. frangula* extracts at the highest concentration tested were extremely low and ranged from 2.88% to 5.10%, similar to the effect of the negative control doxorubicin which showed cell viability of 2.14% at the highest concentration tested.

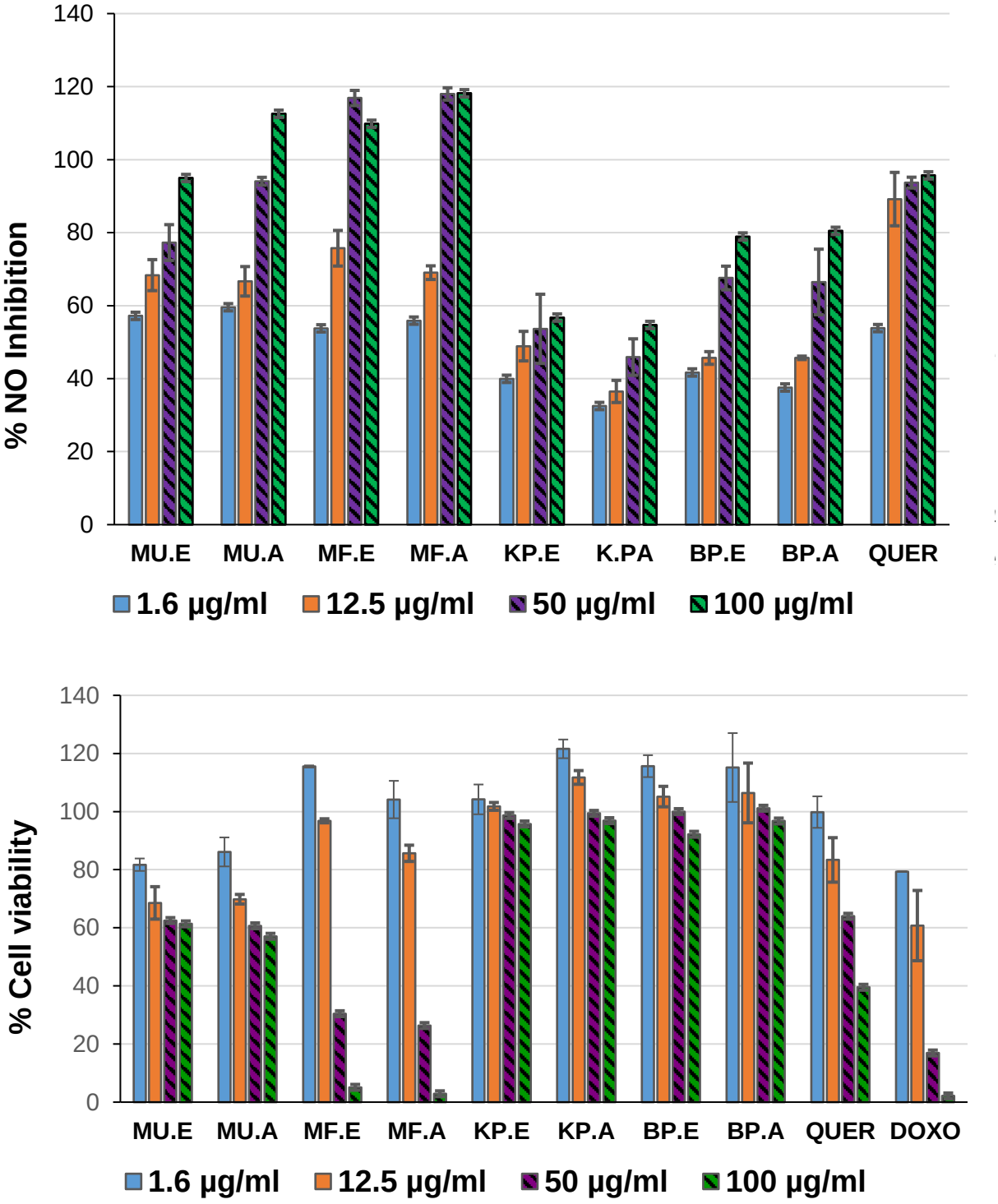


Figure 4 1. Effect of the selected plant extracts on the cell viability of treated Raw macrophage cells, following nitric oxide inhibition

KP. A = *K. gunniae* acetone extract, KP. E = *K. gunniae* ethanol extract, BP. A= *B. pinnatum* acetone extract, BP. E = *B. pinnatum* ethanol extract, M. A = *M. frangula*

acetone extract, MF. E= M. *frangula* ethanol extract, MU. A = *M. undata* acetone extract, MU. E = *M. undata* ethanol extract, Quer = = Quercetin, Doxo = Doxorubicin

4.3.2.3 Inhibition of COX-2 enzyme

The results of the COX-2 inhibition in the LPS activated RAW 264.7 macrophage cells are presented in Fig 4.3**.** Compared to the control normal cells (inactivated and untreated cells), the LPS-activated macrophages were significantly induced to increase the concentration of COX-2 ($p < 0.001$). At both concentrations used for the treatment, all the plant extracts showed very good activity and significantly inhibited the production of COX-2 ($p < 0.001$) compared to the LPS activated cells. The plant extracts had similar effects as the positive control quercetin (significant at $p < 0.001$). Extracts of *K. gunniae* (100 µg/ml), were the most active with more than 80% inhibition of COX-2 production. These extracts at 100 µg/ml concentration, even reduced the level of COX-2 more than the positive control, quercetin.

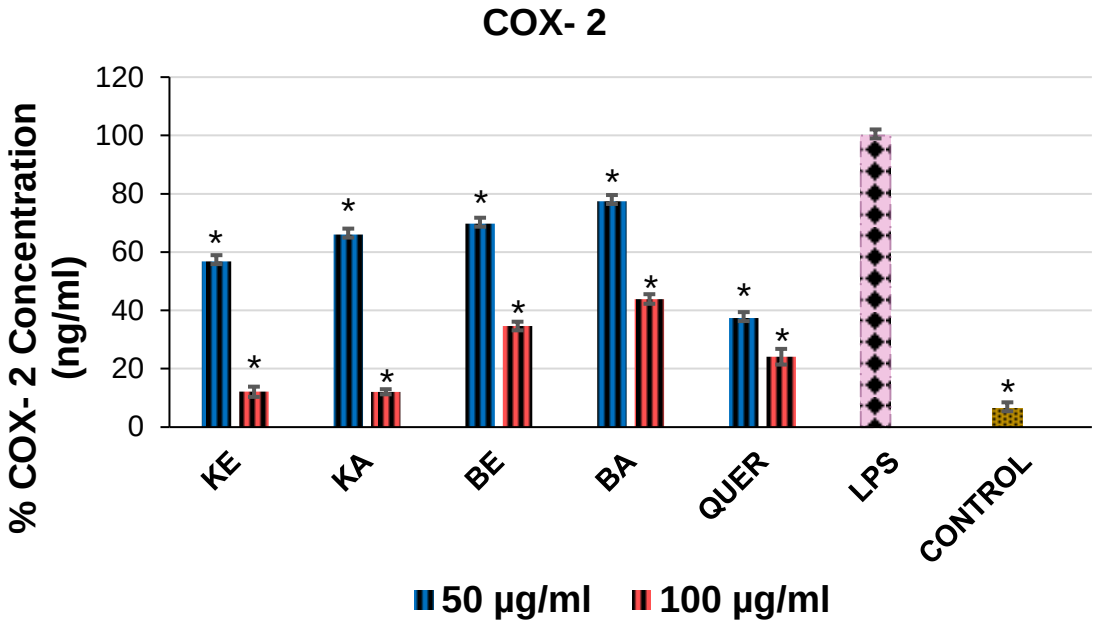


Figure 4.3 Effect of the selected plant extracts on LPS-induced COX-2 concentration for a period of 24 h

KE = *K. gunniae* ethanol extract, KA = *K. gunniae* acetone extract, BE = *B. pinnatum* ethanol extract, BA = *B. pinnatum* acetone extract, Quer = Quercetin (positive control), LPS = Differentiated and LPS-induced COX-2, Control = Differentiated cells that were neither induced with LPS nor treated (*Statistically significant).

4.3.2.4 Effect of extracts on cytokine production

4.3.2.4.1 Inhibitory effects of the extracts on pro-inflammatory cytokine TNF-α

The LPS activated RAW 254.7 macrophage significantly increased production of TNF-α compared to the control normal cells ($p < 0.001$). The positive control quercetin (100 µg/ml) reversed this effect significantly ($p < 0.001$). *B. pinnatum* ethanol extract (100 µg/ml) and *B. pinnatum* acetone extract (50 µg/ml) also reversed the effect of LPS inhibiting the production of TNF-α significantly ($p < 0.038$) and ($p < 0,036$) respectively. Extracts of *K. gunniae* were not able to significantly reverse the activated production of TNF-α. The results of the LPS activation and the effect of the plant extracts are presented in Fig 4.4.

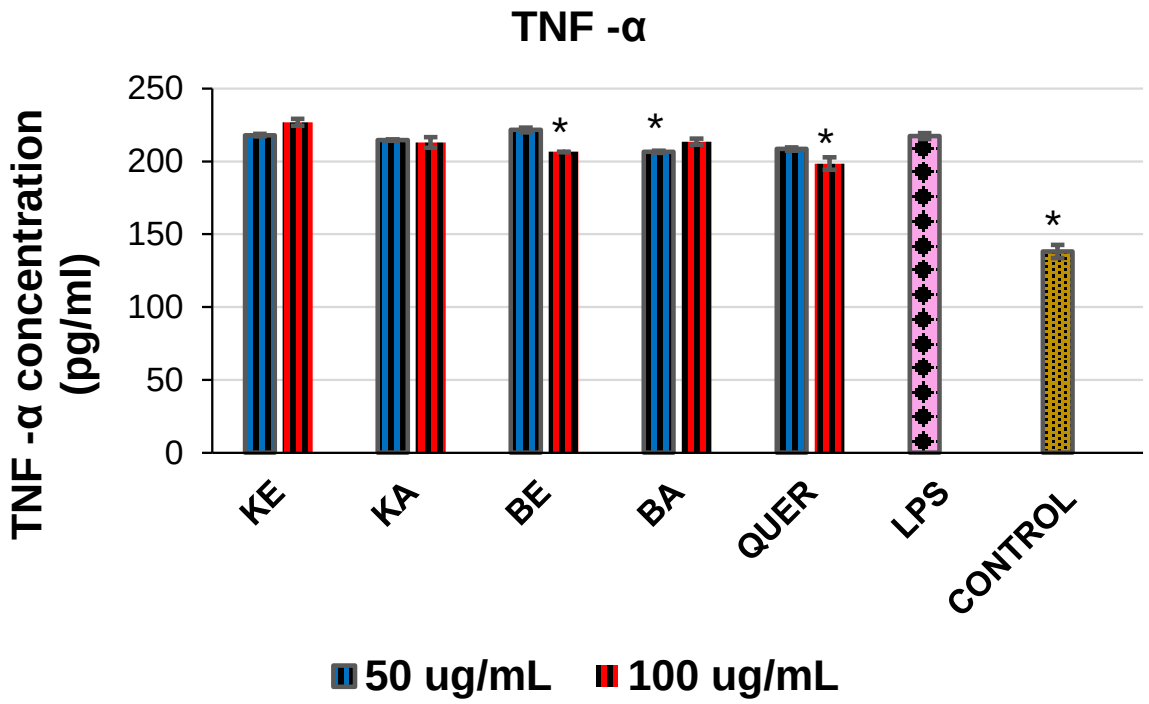


Figure 4.4 Effect of the selected plant extracts on LPS-induced TNF-α, pro-inflammatory cytokine production in RAW macrophages for a period of 24 h

KE = *K. gunniae* ethanol extract, KA = *K. gunniae* acetone extract, BE = *B. pinnatum* ethanol extract, BA = *B. pinnatum* acetone extract, Quer = Quercetin (positive control), LPS = Differentiated and LPS-induced COX-2, Control = Differentiated cells that were neither induced with LPS nor treated (*Statistically significant).

4.3.2.4.2 The effects of the extracts on pro-inflammatory cytokine IL-1β

Compared to the control normal cells, the LPS-activated macrophages increased the secretion of IL-1β non-significantly Fig 4.5. All the extracts decreased the secretion of IL-1β non-significantly. Thus, all the extracts only had slight reversal effect to the LPS activated cells.

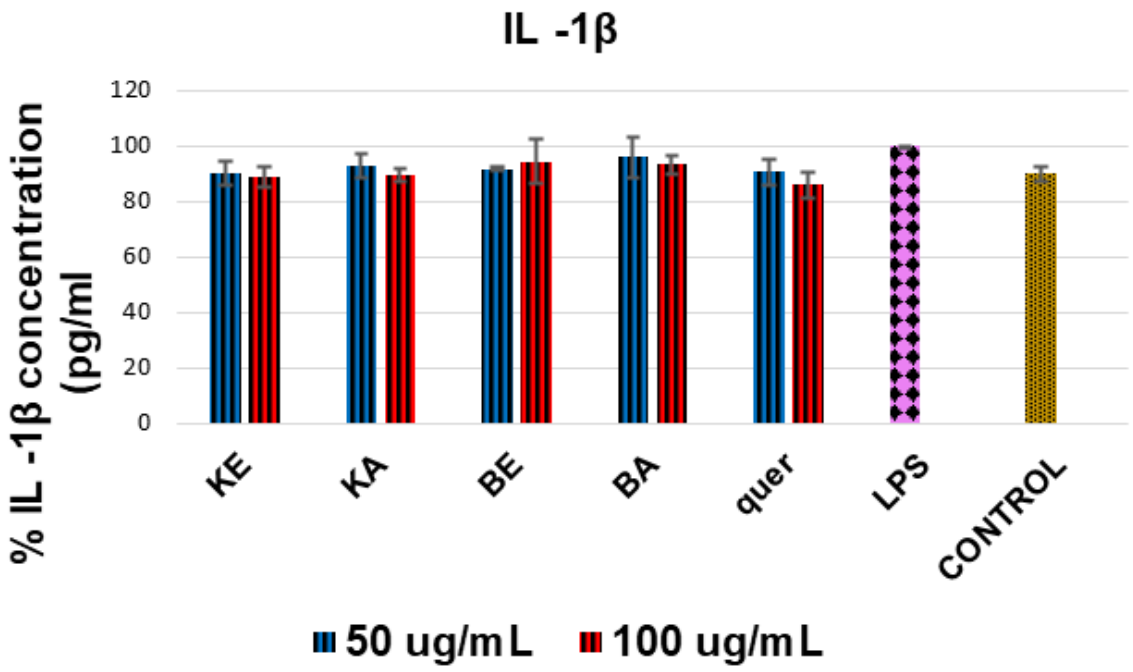


Figure 4.5 Effect of the selected plant extracts on LPS-induced IL-1β pro-inflammatory cytokine production in RAW macrophages for a period of 24 h

KE = *K. gunniae* ethanol extract, KA = *K. gunniae* acetone extract, BE = *B. pinnatum* ethanol extract, BA = *B. pinnatum* acetone extract, Quer = Quercetin (positive control), LPS = Differentiated and LPS-induced COX-2, Control = Differentiated cells that were neither induced with LPS nor treated (*Statistically significant).

4.3.2.4.3 The effects of the extracts on anti-inflammatory cytokine IL-10

In comparison to the control normal cells, the LPS activated RAW 254.7 macrophage significantly increased the production of IL-10 ($p > 0.001$) Fig 4.6. *Bryophyllum pinnatum* acetone extract (100 µg/ml and 50 µg/ml) had the best activity and increased the production of IL-10 significantly ($p < 0{,}001$) in comparism to the LPS activated cells. These were followed by *B. pinnatum* ethanol extract (100 µg/ml and 50 µg/ml) which maintained the production of IL-10 almost at the level of LPS activated cells. Extracts of *K. gunniae* and the positive control quercetin significantly ($p < 0.001$) decreased the production of IL-10 compared to the LPS activated cells, however they will still higher than the control normal cells level.

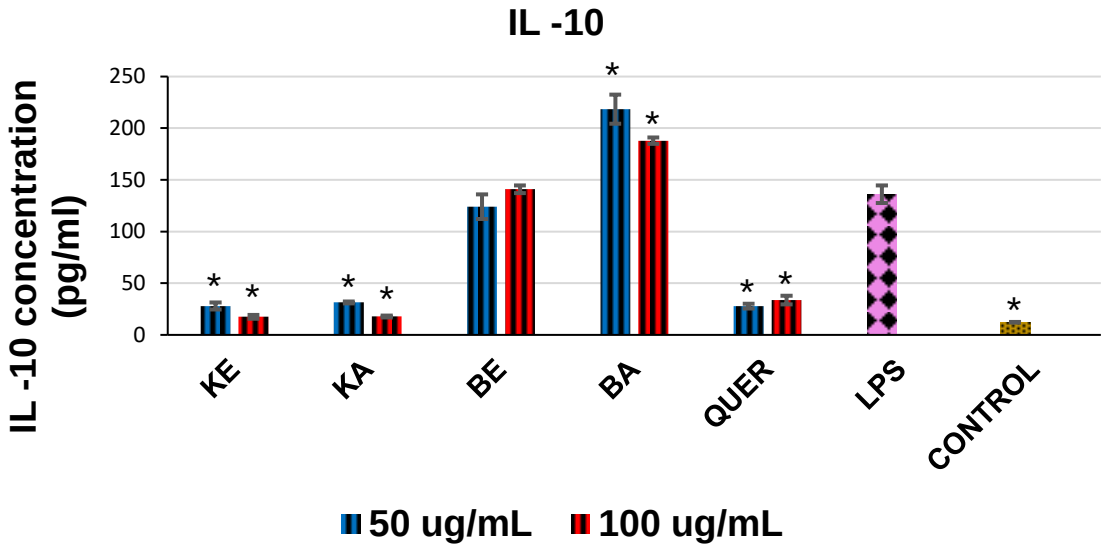


Figure 4.6 Effect of the selected plant extracts on LPS-induced IL-10 anti-inflammatory cytokine concentration in RAW macrophages for a period of 24 h

KE = *K. gunniae* ethanol extract, KA = *K. gunniae* acetone extract, BE = *B. pinnatum* ethanol extract, BA = *B. pinnatum* acetone extract, Quer = Quercetin (positive control), LPS = Differentiated and LPS-induced COX-2, Control = Differentiated cells that were neither induced with LPS nor treated (*Statistically significant).

4.4 Discussion

4.4.1 Antioxidant activity

Oxidative stress and low concentrations of antioxidants in cells promote pathological inflammation (Nkadimeng et al., 2020), and inflammation is a major feature of mastitis disease (Sserunkuma et al., 2017). Substances that have scavenging properties will be very useful in controlling and inhibiting pathological , thereby aiding healing in inflammation related diseases (Nkadimeng et al., 2020). In this study plant extracts were tested to determine their ability to scavenge free radicals like DPPH and ABTS. Lower concentrations of extracts that can cause 50% inhibition/reduction of the radicals means higher scavenging activity of that extract (Ahmed et al., 2014).

Extracts of *K. gunniae* had the best scavenging activity (IC_{50} <1 µg/ml) (Table 4.1). These were followed by ethanol extracts of *B. pinnatum, M. frangula* and *M. undata (*IC_{50} <10 µg/ml) whereas their acetone extracts had much lower activity, indicating that the ethanol and acetone extracts of each of these plants most likely contain very different phytochemicals. The

antioxidant activity of the active extracts imply that the active extracts contain active phytochemicals that exert their activity through the reactive oxygen sub-pathway of the arachidonic acid pathway and are capable of stopping the generation of free radicals (reactive oxygen species), non-free radicals and products of lipid oxidation, which are mediators of uncontrolled inflammation (Nkadimeng et al., 2021; Ondua et al., 2019)

Bhatti et al. (2012) reported IC_{50} values of 94 µg/ml and 120 µg/ml for ethanol and acetone extracts of *B. pinnatum* using the DPPH antioxidant assay method. Their reported activity is a moderate and weak activity for *B. pinnatum* ethanol and acetone whereas in this study, *B. pinnatum* ethanol and acetone extracts had strong potent activity and good activity for DPPH antioxidant assay method. The different range of activity observed in their study could be as a result of their use of the Soxhlet extraction method used previously (Bhatti et al., 2012). Due to high temperatures applied in Soxhlet extraction, the method is not suitable for any thermolabile phytochemical present in the plant material (Abubakar & Haque, 2020). Thus, bioactive compounds/molecules present in the plant material may break down, decompose, disintegrate or change into non-active or less active forms. In this study, extraction was done with ultrasonication method at room temperature of 25^{o}C. However, similar to the observation in this study that for antioxidant assays, ethanol extracts were generally more active than acetone extracts, the ethanol extracts were also more active compared to the acetone extracts.

Ahmed et al. (2013) reported IC_{50} values of 3.48 ± 0.07 µg/ml and 7.89 ± 0.31 µg/ml for DPPH and ABTS assays respectively for the crude extract of *M. undata* extracted with a mixture of acidified 70% acetone and n-hexane. With the presence of water in the extractant mixture they used, their extractant is more polar than pure acetone. They reported strong activities in both the DPPH and ABTS assays, similar to the potent activities obtained in this study with the ethanol (polar) extract of *M. undata* in both DPPH and ABTS assays.

Reports on antioxidant activity of *K. gunniae* are lacking. This is most likely because the plant is still not widely known as it was only recorded for the first time as a nothospecies in South Africa in 2019 (Smith et al., 2019), and extensive bioactivity research on the plant is yet to take place. Also, no available reports were found relating to the antioxidant activity of *M. frangula* extracts.

4.4.2 Anti-inflammatory activity

Through the LOX inflammatory pathway, the 15-lipoxygenase (15-LOX) enzyme mediates the synthesis of the inflammation mediator, leukotriene from arachidonic acid (Hu and Ma, 2018). The LOX assay was done to determine the ability of the extracts to inhibit lipoxygenase

enzyme activity. Extracts that are able to inhibit lipoxygenase activity will be good anti-inflammatory agents (Dzoyem et al., 2017).

Extracts of *K. gunniae* extracts had excellent 15-LOX inhibitory activity (IC_{50} <10µg/ml) comparable to that of the positive control. The ethanol extracts of the other plants (*B. pinnatum, M. frangula* and *M. undata*) had good anti 15-LOX activity (IC_{50} >10µg/ml to <20µg/ml). Similar to the results obtained in the antioxidant assay, the acetone extracts also had much lower activity, further affirming that the ethanol and acetone extracts of each of these plants most likely contain very different plant constituents (Table 4.2). The observed 15-LOX inhibition of the active extracts indicate that these extracts could be acting against inflammatory mediators in the arachidonic acid pathological inflammatory pathway which controls the secretion of 15-lipoxygenase or directly against 15-lipoxygenase enzyme secretion in the 15-LOX pathway, which is a sub pathway of the arachidonic acid pathway (Ondua et al., 2019).

Previous work by Ahmed et al. (2013) reported moderate anti-15-LOX activity of the acidified 70% acetone extract of *M. undata*, which confirms that different solvents extract different phytochemicals with varying activity. Reports are lacking on the anti-inflammatory activity of *M. frangula*. Thus, to the best of our knowledge, the work in this study is the first report of the anti-inflammatory activity of *M. frangula*. Previous work on the inhibitory activity of *K. gunniae* against 15-LOX or on any other anti-inflammatory activity are also lacking. The fact that *K. gunniae* was recently identified in 2019 (Smith et al., 2019) still explains lack of efficacy and biological assay research on *K. gunniae*. Also, reports are lacking on *in vitro* anti-inflammatory activity of *B. pinnatum* in general. However, there are reports of *in vivo* anti-inflammatory activity of *B. pinnatum.* Ojewole (2005) reported significant in vivo activity of aqueous extract of *B. pinnatum* which inhibited fresh egg albumin-induced acute inflammation in rats.

4.4.2.1 Anti-inflammatory assay through inhibition of NO production and effect on cell viability

Following the stimulation of macrophage cells with inflammatory mediators like LPS, nitric oxide synthase (iNOS) is expressed (Salvemini et al., 1993). Increased iNOS leads to enzymatic oxidation of l – arginine to citrulline thereby forming nitric oxide (NO). Reactive oxygen species (ROS) like superoxide (O_2), react with NO to form peroxynitrite (ONOO) which is a strong oxidant and, as a strong oxidant, ONOO initiates lipid peroxidation (Adibhatla & Hatcher, 2006). An inflammatory response is triggered (Latha et al., 2011) leading to production of more ROS generating enzymes (Iwalewa et al., 2007), pro-inflammatory cytokines like tumour necrosis factor (TNF-α) (Paterson et al., 2003) . Thus, NO production in

the cells leads to more oxidation or production of pro-inflammatory cytokines or a combination of both.

Substances which can inhibit NO production and yet are not toxic to the RAW 264.7 macrophage cells are considered good anti-inflammatory agents (Dzoyem et al., 2017). For plant extracts, effects on RAW macrophages with cell viability of ≥ 80% are considered safe (Nkadimeng et al., 2020) and nitric oxide inhibition, below 50% = weak activity (Ana et al., 2007), 50% to 69% = good activity (Ana et al., 2007; Yang et al., 2009) and above 70% = strong activity (Yang et al., 2009).

Extracts of *B. pinnatum* had the strongest NO inhibition activity which were not due to toxicity as their % cell viability ranged from 92.19% to 96.75% (Table 4.3). Extracts of *K. gunniae* were also safe (cell viability ranging from 95.75% to 96.88%) at the highest concentration tested with good NO inhibition activity.

With cell viability of 61.35% at the highest concentration tested, the *M. undata* ethanol extract showed cell toxicity indicating that the observed 94.96% NO inhibition may have been influenced by toxicity of the extract to the RAW 264.7 macrophages. Similarly, at the highest concentration tested, *M. undata* acetone extract and *M. frangula* extracts showed activity above 100%, with low cell viability indicating toxicity rather than NO inhibition.

The toxic effects observed with *M. undata* and *M. frangula* extracts on RAW 264.7 macrophage cells indicate that the antioxidant activities and the 15 LOX inhibitory activities of these extracts were also most likely as a result of their toxicity. Therefore, *K. gunniae* and *B. pinnatum* extracts which have shown either strong or good activity for all the antioxidant assays, anti 15-LOX assay and nitric oxide inhibition assay and were safe (with cell viability above 90%), were selected for the COX -2 and cytokine regulation assays.

4.4.2.2 Anti-inflammatory assay through inhibition of COX -2 enzyme

Cyclooxygenase enzymes (COX-1 and COX-2), also known as prostaglandin synthases, catalyse the production of prostaglandins from arachidonic acid. Prostaglandins are one of the mediators of chronic inflammation (Sales & Jabbour, 2003). In this study COX-2 inhibition was investigated to determine whether the plant extracts are able to inhibit the activities of the cyclooxygenase enzyme.

All the extracts had very good anti-inflammatory activity against COX-2 as they all significantly inhibited the production of COX-2. However, extracts of *K. gunniae* were more active than extracts of *B. pinnatum* as the level of COX-2 production was lower in cells treated with *K. gunniae* extracts, compared with cells treated with *B. pinnatum* extracts. This indicates that

although *K. gunniae* and *B. pinnatum* are from the same family, extracts of *K. gunniae* extracts and extracts of *B. pinnatum* extracts most likely have different active plant constituents/compounds and that the plant constituents in *K. gunniae* extracts have stronger inhibitory activity against COX-2 compared with *B. pinnatum* extracts.

From the results of antioxidant assays and anti-inflammatory assays (15-LOX and NO) above it was observed that *K. gunniae* extracts had strong inhibitory activity against COX-2, and 15 LOX as well as strong antioxidant activity. *B. pinnatum* extracts had strong inhibitory activity against COX-2 and NO. This indicates that the active compounds in *K. gunniae* extracts, and those in *B. pinnatum* extracts exert their anti-inflammatory activity through slightly different anti-inflammatory pathways.

The COX 2 pathway is one of the pathways that can be activated through either arachidonic acid pathway or through nitric oxide pathway. Through the arachidonic acid pathway, phospholipase A2 is stimulated by factors like leukotrienes/nitrogen species/reactive oxygen species (ROS)/excess prostaglandins/pro-inflammatory cytokines to cleave arachidonic acid from membrane phospholipids for metabolism, leading to the activation of the arachidonic acid inflammatory pathways of which COX -2 is one (Ondua et al., 2019). Through the nitric oxide pathway, inducible nitric oxide synthase (iNOS) is expressed after stimulation of cells with inflammatory mediators like LPS (Salvemini et al., 1993). The iNOS then synthesises NO and NO in turn modulates the activity of COX-2 (Nkadimeng et al., 2020; Salvemini et al., 1993).

Thus, it seems that the active constituents in *K. gunniae* extracts exert their COX-2 inhibition activity mainly through the arachidonic acid pathway, whereas the active constituents in *B. pinnatum* extracts exert their COX-2 inhibition activity mainly through the nitric oxide pathway. This then implies that for maximum efficacy, *K. gunniae* extracts will be more useful in treating chronic inflammation that is initiated mainly through the arachidonic acid pathway whereas *B. pinnatum* extracts will be more useful in treating chronic inflammation that is initiated mainly through nitric oxide pathway. Fractionation of *K. gunniae* extracts and *B. pinnatum* extracts as well as isolation of compounds from these plants and testing of the antioxidant and anti-inflammatory activities of the fractions and compounds from these two plants, could help to understand the type of active plant secondary metabolites and or compounds present in these plants as well as understand whether the active constituents are acting in synergy.

4.4.2.3 Inhibitory effects of extracts on pro-inflammatory cytokine TNF-α

Presence of pathogens in animal body (cells) can lead to upregulation of inflammatory reactions. The upregulation of inflammatory reactions then leads to production of proinflammatory cytokines, and the produced proinflammatory cytokines in turn promotes

further upregulation of inflammatory reactions which again leads to more production of inflammatory cytokines leading to a stage known as cytokines storm (Pasare & Medzhitov, 2005; Tang et al., 2021). TNF-α is one of the proinflammatory cytokines and uncontrolled production of TNF -α is unhealthy (Dinarello, 1991). In this study, extracts of *B. pinnatum* were able to significantly inhibit the production of TNF-α whereas extracts of *K. gunniae* had no significant effect on the inhibition of production of TNF-α. As observed with the COX-2 inhibition assay, the TNF-α inhibition assay further shows that the extracts of *B. pinnatum* and *K. gunniae* act through different inflammatory pathways.

TNF-α is one of the inflammatory mediators that can stimulate the expression of inducible nitric oxide synthase (iNOS) which in turn synthesises NO that can possibly modulate the activity of COX-2 (Nkadimeng et al., 2020; Salvemini et al., 1993). Therefore, following this pathway, any anti-inflammatory agent that will be able to inhibit the production of TNF-α, will automatically inhibit the production of NO and COX-2. The results of the anti-inflammatory activity of *B. pinnatum* extracts correlates very well with the statement above. The results indicate that as *B. pinnatum* extracts were able to inhibit the production of TNF-α, the extracts consequently inhibited the production of NO and COX-2 as observed in this study thus supporting that the anti-inflammatory activity of *B. pinnatum* is through cytokine activated nitric oxide pathway.

4.4.2.4 Effects of extracts on pro-inflammatory cytokine IL-1β

Inhibition of cytokine IL-1β, a proinflammatory cytokine (Dinarello, 2000) could be one of the ways that anti-inflammatory agents exert their anti-inflammatory activity on cells with pathological inflammation. In this study we determined whether our extracts are able to inhibit the production of IL-1β. Our results show that although the extracts slightly decreased the level of production of IL-1β (Fig 4.5), the effect was statistically non-significant, indicating that the mechanism of anti-inflammatory activity of extracts of *B. pinnatum* and *K. gunniae* is not through the inhibition of IL-1β.

4.4.2.5 Effects of extracts on anti-inflammatory cytokine IL-10

The uncontrolled production of inflammatory cytokines aids the development of pathogenic inflammatory diseases (Tang et al., 2021). A common feature of cytokines is that an activated cytokine can affect the production and activity of other cytokines in a synergistic, additive or antagonistic way (Turrin & Plata-Salamán, 2000). If only pro-inflammatory cytokines are secreted and they function together in synergistic or additive manner, this will obviously be unhealthy because inflammation will increase. A balance is needed in the secretion of proinflammatory cytokines and anti-inflammatory cytokines to maintain good health. A balance

resulting in controlled inflammation can be achieved in secreted cytokines when there is an antagonistic effect of anti-inflammatory cytokines towards pro-inflammatory cytokines (Chaudhry et al., 2013).

The anti-inflammatory cytokine IL-10 controls inflammation through an immunosuppressive effect, which includes inhibition of macrophage activation as well as having an antagonistic effect to pro-inflammatory cytokines by attenuating their expression (Steen et al., 2020). An anti-inflammatory agent that is able to increase the production of cytokine IL-10 will help in establishing antagonistic effects towards pro-inflammatory cytokines, thereby discontinuing ongoing pathological inflammation. In this study we determined the effect of the plant extracts on cytokine IL-10. Our study showed that extracts of *B. pinnatum* had very good positive activity on the production of the anti-inflammatory cytokine IL-10 (Fig 4.6). The acetone extract of *B. pinnatum* significantly increased the production of IL-10 and the ethanol extract of *B. pinnatum* maintained the production of IL-10 at almost the same level of production with LPS activated cells. This indicates that the acetone and ethanol extracts of *B. pinnatum* most likely contain different plant active constituents and thus further supports the same observation seen with the effects of these extracts on anti-oxidant assays and 15-LOX assay. These results further strongly affirm that extracts of *B. pinnatum* exert their anti-inflammatory activity mainly through the cytokine activated nitric oxide pathway. The results demonstrate that through this pathway, extracts of *B. pinnatum,* while inhibiting the production of the pro-inflammatory cytokine TNF-α, at the same time increase the production of the anti-inflammatory cytokine IL-10 and these activities consequently trigger the inhibition of the production of NO (Table 4.3) and the pro-inflammatory enzyme COX-2 (Fig 4.3). However, for *B. pinnatum* ethanol extracts, the moderate increase production of IL-10, the potent antioxidant activity (Table 4.1) and the potent 15-LOX activity (Table 4.2), further indicate that the plant constituents of *B. pinnatum* ethanol extracts in particular act through an additional anti-inflammatory pathway which is the arachidonic acid pathway.

For *K. gunniae* extracts the observation that they decreased the production of IL-10 (although not up to the low level of normal control cells), further shows that their anti-inflammatory activity is neither through inhibition of pro-inflammatory cytokines nor promotion of anti-inflammatory cytokines. The effect of *K. gunniae* extracts on IL-10, their strong antioxidant activity and strong 15-LOX activity further confirms that their anti-inflammatory efficacy is mainly through the arachidonic acid pathway. However, although *K. gunniae* extracts do not increase the production of the anti-inflammatory cytokines (Fig 4.6) and do not inhibit the production of the proinflammatory cytokines TNF-α and IL-1β (Fig 4.4 and 4.5), their moderate inhibition of NO shows that they have slight activity through the NO pathway. It is possible that *K. gunniae*

extracts may have some positive activities towards other pro-inflammatory and anti-inflammatory cytokines not tested in this study. Testing of the effect of *K. gunniae* extracts on other pro-inflammatory and anti-inflammatory cytokines not covered in this study is recommended.

4.5 Conclusions

During chronic inflammation, the anti-inflammatory activity of phytochemicals can be through their free radical scavenging activity or through inhibition of pro-inflammatory enzymes like 15-LOX and COX-2, or through inhibition of inducible nitric oxide (Ondua et al., 2019) or through the inhibition of pro-inflammatory cytokines or through up-regulation of anti-inflammatory cytokines (Nkadimeng et al., 2020; Nkadimeng et al., 2021).

This study demonstrates that *K. gunniae* extracts have strong radical scavenging activity, strong inhibitory activity against pro-inflammatory enzymes 15-LOX and COX-2, good inhibitory activity against nitric oxide and are not cytotoxic. The antioxidant and anti-inflammatory activity of *K. gunniae* extracts is reported here for the first time. Extracts of *B. pinnatum* on the other hand have strong to moderate radical scavenging activity, good and strong inhibitory activity against pro-inflammatory enzymes 15-LOX and COX-2 respectively. Additionally, they have strong inhibitory activity against nitric oxide and are not cytotoxic.

The pro-inflammatory cytokines promote inflammation whereas the anti-inflammatory cytokines fight against inflammation (Dinarello, 2000). The severity of a pathological disease can be reduced by reducing the activity of pro-inflammatory cytokines e.g., by reducing their expression and increasing the activity of anti-inflammatory cytokines(Cen et al., 2016).

Bryophyllum pinnatum extracts also had strong inhibitory activity against pro-inflammatory cytokine TNF-α, strong up regulatory activity towards anti-inflammatory cytokine IL-10. Biological therapeutic anti-inflammatory agents act by neutralising inflammatory cytokines or their receptors (Kalpachidou et al., 2022). It is thus possible that *B. pinnatum extracts* are either neutralising the inflammatory cytokines or their receptors. This study is the first report on the in vitro activity of *B. pinnatum* extracts on cytokines.

This study thus has established that *K. gunniae* extracts and *B. pinnatum* extracts have significant antioxidant and anti-inflammatory activities. However, the extracts of the two plants act through somewhat different anti-inflammatory mechanisms. *Kalanchoe gunniae* extracts exert their activity mainly through the arachidonic acid pathway and slightly through the nitric oxide pathway whereas *B. pinnatum* extracts exert their activity mainly through the nitric oxide pathway and slightly through the arachidonic acid pathway.

In view of the cellular safety and significant antioxidant and anti-inflammatory activities of *K. gunniae* extracts and *B. pinnatum* extracts, these plant extracts stand as potential therapeutic agent for the treatment and prevention of infectious mastitis disease, a disease with profound inflammation. Earlier studies Ogbuadike *et al* (submitted article) also showed that these plant extracts have antimicrobial activity against major causative organism of bovine infectious mastitis.

Further work is needed to identify the active compounds responsible for the antioxidant and anti-inflammatory activities of *K. gunniae* extracts and *B. pinnatum* extracts and to ascertain whether the compounds in the extracts have activity as single compounds or if work in synergy.

CHAPTER 5 Identification of compounds and bioactivity of crude extract and fractions of *Kalanchoe gunniae* against *S. aureus* reference strain and isolates from bovine mastitis cases

5.1 Introduction

Bovine mastitis is an inflammatory response of the mammary gland caused by either physical or microbial factors (Petzer et al., 2009). It is classified into clinical or sub-clinical, with clinical mastitis often associated with abnormal milk, redness, swelling and hardness of the udder (Viguier et al., 2009). With subclinical mastitis there are no apparent clinical signs, however, there is increased somatic cell count (SCC) above the 200 000 cells/ml threshold (Petzer et al., 2009). It causes severe economic losses to dairy industries in South Africa and worldwide (Fernández et al., 2014; MILK, 2014; Sserunkuma et al., 2017).

Infectious mastitis is associated with presence of microbial pathogens (Sargeant et al., 2001) including bacteria, fungi and viruses (Viguier et al., 2009). However, the majority of infectious mastitis cases are associated with bacterial pathogens such as *Staphylococcus aureus* (Karzis et al., 2021) and non-aureus staphylococci, for example *Staphylococcus simulans* (Labuschagne et al., 2022)

Although the prevalence of mastitis in South Africa is reducing, there is concern of increasing prevalence of antibiotic resistant mastitis pathogens in South Africa (Etter et al., 2019). As at 2019 prevalence of *S. aureus* in mastitis cases was reducing but prevalence of multidrug resistance (MDR) *S. aureus* was highly increasing (Mphahlele et al., 2020). There is also increase in the prevalence of resistant coagulase negative *Staphylococcus* (Phophi et al., 2019).

Antibiotics have been easily used for both treatment and control of mastitis as well as in feeds (Goñi *et al.,* 2004). Use of antimicrobials in communal cattle with a knowledge gap in prudent use leads to increasing resistance of antibiotics (Mupfunya et al., 2021). In South Africa antibiotics are commonly used as stock remedies, i.e. over the counter medicine and indiscriminate use by farmers has led to increasing resistance (Henton et al., 2011). Use of

antibiotics enhances the development and spread of antimicrobial resistant (AMR) *Staphylococcus* strains (Rodríguez & Gomez, 2022) and as mentioned above, *Staphylococcus* strains are one of the major bacteria types that cause mastitis.

Moreover, the common and continuous use of antibiotics in food producing animals is not only adding to the increase of antibiotic resistance to pathogens but is also imposing consumer risk on human (JN et al., 2013). Although farmers contribute immensely to the spread of antibiotics, once pathogens develop antibiotic resistance to any antibiotic, these resistant pathogens may continue to spread in nature through water, wind, manure, animals and crops (Van den Honert et al., 2018). Van Boeckel et al. (2015) projected that antibiotics consumption in humans through animal food products may rise by 67% globally by 2030 and may even double in some countries if the free, routine and indiscriminate use of antibiotics is continued. There is therefore a need to discover new natural antimicrobials to address the crucial problem of increasing microbial resistance against current antibiotics, and plant chemical biodiversity can be a valuable potential resource for new antimicrobials (Larayetan et al., 2019).

Plants, as the natural source of medication for humankind against several ailments have been used for many centuries. They contain hidden substances such as metabolites which hinder the development and movement of pathogenic microbes (Larayetan et al., 2019). These metabolites can be classified into four major classes: terpenoids, phenolic compounds, alkaloids and sulphur-containing compounds (Cruz et al., 1996). Phenolic compounds are among the most important groups of plant secondary metabolites and they often serve as defence against pathogens (Özeker, 1999). Among phenolic compounds, flavonoids constitute one of the most ubiquitous groups (Tapas et al., 2008). Flavonoids are structurally diverse, with a multitude of functions. They have anti-inflammatory, anti-microbial, anti-oxidant, anti-hepatotoxic, and anti-tumour biological activities depending upon the type of flavonoid (Meserole, 2002). In addition, synergistic activities of plant phytochemicals among crude extracts and fractions can lead to very significant antibacterial activity (Ahmad & Aqil, 2007).

In Chapter 3 the activity of selected plant species against a reference ATCC strain and drug-resistant *S. aureus* isolated from clinical mastitis show that extracts of *Kalanchoe gunniae* had excellent antibacterial efficacy, coupled with low cytotoxicity, and had promising biofilm inhibitory activity. This is not surprising as *Kalanchoe* species are known to have interesting biological activities, including anti-inflammatory (Agarwal & Shanmugam, 2019), antibacterial (Khoa et al., 2017), and wound healing (Mekonnen et al., 2013) efficacy.

The nothospecies *Kalanchoe gunniae* (Crassulaceae), a hybrid between *Kalanchoe paniculate* and *Kalanchoe sexangularis*, was recently described by Smith et al. (2019). Further research on this new hybrid is warranted as a result of its promising activity against mastitis causing bacterial isolates. In this research, antimicrobial activity of *K. gunniae* crude extract and fractions against a reference ATCC strain of *S. aureus* (ATCC 29213) and *S. aureus* isolates from clinical mastitis cases was studied. Cytotoxicity of the crude extract and fractions were also investigated. Total phenolic and total flavonoid contents of the crude extract and fractions were also determined. Finally, both volatile and non-volatile compounds present in active samples were identified using LCMS-MS and UPLC-MS respectively.

5.2 Materials and methods

5.2.1 Collection of plant material and processing

Kalanchoe gunniae fresh leaves were collected from the South African National Biodiversity Institute (SANBI), Pretoria Botanical Garden, South Africa. A herbarium sample was prepared and deposited at the SANBI Herbarium, Pretoria Botanical Garden, South Africa with voucher number PRE 1004266. The plant material was cut into pieces, spread on a table and dried indoors at room temperature. Dried plant materials were ground into fine power with a Merck MF 10 Basic mill.

5.2.2 Bulk extraction of plant material and fractionation

Ground plant material (900 g) was extracted with 80% methanol using plant material to solvent ratio of 1:2. The maceration method at room temperature (25^{o}C) and continuous vigorous shaking with a mechanical shaker for 24 h was used. The extract was filtered into a glass beaker through Whatman No 1 filter paper. The extraction process was repeated three times and the filtrate was combined and evaporated using a Büchi rotary evaporator (R-114, Labotec).

Fractionation was done by first redissolving the 80% methanol crude extract with distilled water inside a separatory funnel using extract to water ratio of 1: 4. An equal volume of *n*-hexane was added to the extract dissolved in water and separated to obtain the hexane fraction. Fractionation with *n*-hexane was repeated twice. Then the water layer was mixed with an equal volume of dichloromethane (DCM) and extracted twice to obtain the DCM fraction. This was followed by extracting the water layer with an equal volume of ethyl acetate twice to obtain the ethyl acetate fraction. The remaining water layer was further extracted twice with *n*-butanol and separated to give the butanol fraction and water fraction. The fractions were evaporated and dried to give five fractions, namely the hexane, DCM, ethyl acetate, butanol and water fractions.

5.2.3 Culture of bacterial strains

Bacterial cultures (*S. aureus* ATCC 29213 and *S. aureus* mastitis clinical isolates; STA 1, STA 2, STA 3, STA 4, STA 5, and STA 6) were maintained on Mueller-Hinton (MH) agar plates and subsequently grown overnight in MH liquid media 37^{o}C with shaking before using them for antimicrobial assay. The *S. aureus* ATCC strain was obtained from the culture collection of the Phytomedicine laboratory, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria. The *S. aureus* mastitis clinical isolates; STA 1, STA 2, STA

3, STA 4, STA 5, and STA 6) were obtained from the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Sciences, University of Pretoria.

5.2.4 Minimum inhibitory concentration (MIC) assay of crude extract and fractions against *S. aureus* mastitis clinical isolates

The Minimum Inhibitory Concentration (MIC) assay which involves broth microdilution was determined using the method described by J. N. Eloff (1998). Using this method, for each bacterial strain, a single colony was picked from an agar plate containing the bacteria and then used to inoculate sterile MH broth. The liquid culture was grown overnight with shaking at 37°C. Overnight cultures were diluted to McFarland standard No 1 (equivalent to 3×10^8 colony forming units (CFU)/mL) with sterile MH broth. One hundred µl of sterile water was added to each well of 96 sterile microplates. The 80% methanol crude extract and extract fractions of *K. gunniae* were re-suspended in 50% acetone (10 mg/ml). The resuspended crude extract and fractions (100 µl) were added to the first well of a corresponding labelled 96-well microtitre plate and the wells were serially diluted to the last well. The last 100 µl from the serial dilution was discarded. Gentamicin was used as positive control and 50% acetone was used as negative control. From the already standardised bacterial cultures, 100 µl of bacterial cultures were added to each of the wells. The microplates were incubated overnight (18 h) at 37°C. Following the overnight incubation, 40 µl of freshly prepared 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT, Sigma) was added to all wells and the cultures were incubated for 40 min in the dark. The INT helps to indicate the minimum inhibitory concentration (i.e., the lowest concentration of samples that can inhibit bacterial growth) through its colour change. The assays were done in duplicate with triplicate samples.

Results of the MIC assays can be interpreted as very good activity (significant activity), when the MIC value is less than 0.1 mg/ml (Kuete, 2010), moderate activity when the MIC value is between 0.1 mg/ml and 0.5 mg/ml (Holetz et al., 2002), or weak activity when MIC value is within 0.6 mg/ml and 1 mg/ml (Aligiannis et al., 2001; Holetz et al., 2002) and not active when the MIC value is more than 1 mg/ml (Holetz et al., 2002),

5.2.5 Cytotoxicity of crude extract and fractions

Cytotoxicity of the crude extracts and fractions of *K. gunniae* was determined using the tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) on Vero monkey kidney cells. The Vero cells were grown in Minimal Essential Medium (MEM, Highveld Biological, South Africa), supplemented with 5% foetal calf serum (Highveld Biological) and 0.1% gentamicin (Virbac, South Africa) in a 5% CO_2 incubator (Hera Cell 150, Thermo

Scientific Germany), at 37ºC. Cells were harvested and centrifuged (Universal 320R centrifuge, Labotec, South Africa) for five min at 200 x g and resuspended in MEM to 5 x 10^4 cells/ml. Cells (100 µl) were added to wells of columns 2 to 12 of the 96-well microplates. MEM alone was added to wells of column 1 which was used as a blank. Cells were incubated at 37ºC in a 5% CO_2 incubator. Following this, MEM was aspirated from the cells and the cells were treated with 200 µl of serial prepared concentration range of crude extract and fractions prepared in MEM. The plates were incubated at 37ºC in a 5% CO_2 incubator for 48 h. Doxorubicin was used as positive control while untreated cells served as negative control.

After incubation, the MEM containing plant extracts was aspirated and cells were washed with 200 µl of phosphate buffered saline (PBS, Whitehead Scientific). Then 200 µl of fresh MEM was added to the wells. Thirty µl of 5 mg/ml MTT (Sigma) dissolved in PBS, was added to all the wells and the plates were incubated again at 37ºC in a 5% CO_2 incubator for 4 h. The media was again carefully removed by aspiration and 50 µl DMSO was added to dissolve the MTT crystals. Plates were shaken for two to three min using a plate shaker (QG - 9001 microporous Quickshaker, Hinotek) and the absorbance was measured at 570 nm wavelength using a microplate reader (BioTek Synergy). To determine the effect of the crude extract and fractions on the cells, the percentage cell viability was calculated using the formula:

% Cell viability = (Mean absorbance of sample / Mean absorbance of control) *100.

The lethal concentration (LC_{50}), i.e., the concentration that can be lethal to 50% of the cells was determined and the selectivity indices were calculated by dividing the cytotoxicity, LC_{50} (in mg/ml) by the MIC values (in mg/ml).

5.2.6 Total phenolic content and total flavonoid content of crude extract and fractions

5.2.6.1 Total phenolic content

The Folin-Ciocalteu method (Folin, 1927) was used with modifications (Ondua et al. (2019). The experiment was done using a 96-well plate. Twenty µl of crude extract and fractions of *K. gunniae* (1 mg/ml dissolved in methanol) was loaded to corresponding labelled wells. Then 100 µl of Folin-Ciocalteu reagent (10% in deionised water) was added, mixed well and left for five min after which 80 µl of 7.5% Na_2CO_3 solution in deionised water was added. The solution mixture was placed in the dark for 2 h and the absorbance was measured at 750 nm wavelength in a microplate reader (Epoch, BioTek). Gallic acid was used as positive control. Blank wells received 180 µl of distilled water in place of Folin-Ciocalteu reagent and Na_2CO_3

solution. Negative control wells received 20 µl of distilled water in place of crude extract/sample. A calibration curve prepared with a standard solution of gallic acid was used to calculate the total phenolic concentration. The experiment was repeated three times and data was expressed as gallic acid equivalent (GAE)/g.

5.2.6.2 Total flavonoid content

Total flavonoid content of crude extract and fractions of *K. gunniae* was determined using the aluminium chloride spectrophotometric method (Chang, 2002.) with modifications (Ondua et al. (2019). One hundred µl (1 mg/ml in methanol) of crude extract or fraction was added to the corresponding labelled well of a 96-well plate. Then 100 µl of 2% $AlCl_3$ was added to the wells except for the negative control well (blank). The mixture was gently shaken and incubated at room temperature (25^{o}C) for 15 min and then the absorbance was read at 430 nm wavelength in a microplate reader (Epoch, BioTeK). Quercetin was used as positive control. Blank wells received 100 µl of deionised water in place of $AlCl_3$ whereas wells that received 100 µl of distilled in place of crude extract/fraction served as the negative control. Three separate experiments were done. A calibration curve was prepared with a standard solution of quercetin and the results were expressed as quercetin equivalent per gram of extract or fraction.

5.2.7 Gas chromatography-mass spectrometry (GCMS-MS) analysis of crude extract and active fractions of *K. gunniae*

Fractions that had excellent antimicrobial activity as well as high contents of total phenolics and total flavonoids were subjected to GCMS-MS analysis. The crude extract was also included for comparison. Dr Yvette Naude of the Chemistry Department, University of Pretoria assisted with analyses of the samples with the GCMS-MS equipment. For GC-MS, a stock solution of 1 mg/ml of each of the samples was prepared. The crude extract was dissolved in methanol, ethyl acetate sample was dissolved in ethyl acetate and butanol fraction was dissolved in ethanol. Blank ethyl acetate, ethanol and methanol served as solvent controls. The injection volume was 1 µl, inlet temperature of 250^{o}C, oven temperature of 40^{o}C (hold for 3 min) at 10^{o}C/min to 300^{o}C (hold for 5 min), UHP Helium carrier gas at 1 ml/mi set at constant flow mode, 5 min solvent delay and detector voltage of 1750 V were used.

Data from the resulting chromatograms were interpreted in terms of compounds corresponding to major peaks with high similarity above 80% with 1.5% and above area % were recorded.

5.2.8 Liquid chromatography-mass spectrometry (LC-MS) analysis of crude and active fractions of *K. gunniae*

Fractions that had excellent antimicrobial activity as well as high content of total phenolics and total flavonoids were also subjected to LC-MS analysis. The LC-MS Synapt Facilicity (Synapt Waters, Johannesburg, South Africa) of Department of Chemistry, University of Pretoria was used with the help of Dr Madelien Wooding to analyse the crude extract and active fractions of *K. gunniae.* One mg/ml of ethyl acetate fraction (in ethyl acetate), butanol fraction (in ethanol) and crude extract (in ethanol was prepared). The samples were sonicated for 5 min and centrifuged. An aliquot of 500 µl of each of the samples were gently transferred into new vials. To each vial, 250 µl of acetonitrile and 250 µl of water was added. The samples were sonicated and centrifuged. Then 900 µl of each of the samples was transferred into a corresponding labeled injection vial. Blank acetonitrile and water 1:1 was used as control solvent. The injection volume was 5 µl, and a flow rate of 400 µl/min was used. The column for separation was Waters BEH C18, (2.1 mm × 100 mm, 1.7 µm column). Mobile phase was solvent A, consisting of 0.1% formic acid in purified water and solvent B, consisting of acetonitrile mixed with 0.1% formic acid

Chromatograms and mass spectra were produced and the Waters UINIFI® Scientific Information System (version 1.9.2 - accessing the Chinese Natural Products database software) Facility of Department of Chemistry, University of Pretoria was used for total data analyses, processing, interpretation and identification of compounds from the chromatograms and mass spectra.

5.2.9 Statistical analysis

Analysis of Variance (ANOVA) in Microsoft Excel was used to analyse the data statistically.

5.3 Results

5.3.1 MIC assay of crude extracts and fractions against *S. aureus* ATCC 29213 and *S. aureus* mastitis clinical isolates

The ethyl acetate and butanol fractions of *K. gunniae* had significant activity against *S. aureus* ATCC 29213 (MIC = 0.03 mg/ml) and *S. aureus* mastitis clinical isolates (MIC = 0.02 to 0.04 mg/ml) with average MIC of 0.03 mg/ml (Table 5.1). These were followed by the crude extract which also had very good activity against *S. aureus* ATCC 29213 (MIC = 0.04 mg/ml) and *S. aureus* mastitis clinical isolates (MIC = 0.03 to 0.08 mg/ml) with average MIC of 0.05 mg/ml.

The water and hexane fractions had moderate activity against *S. aureus* ATCC 29213 and their activity to *S. aureus* mastitis clinical isolates ranged from weak activity to no activity with average MIC of 0.88 mg/ml and 1.48 mg/ml respectively. Activity of the DCM fraction ranged from weak to no activity for all the strains with average MIC of 0.90 mg/ml.

Table 5.1 MIC of crude and fractions of *K. gunniae* against *S. aureus* ATCC 29213 and *S. aureus* isolates from clinical mastitis cases (mg/ml)

Samples	ATCC	STA 1	STA 2	STA 3	STA 4	STA 5	STA 6	Avg. MIC
Crude	**0.04**	**0.08**	**0.04**	**0.06**	**0.03**	**0.04**	**0.08**	**0.05**
Hexane fraction	0.32	2.5	1.25	2.5	0.63	2.5	1.25	1.48
DCM fraction	0.63	1.25	0.63	1.25	0.63	0.63	1.25	0.90
Ethyl acetate fraction	**0.03**	**0.03**	**0.02**	**0.03**	**0.03**	**0.03**	**0.03**	**0,03**
Butanol fraction	**0.03**	**0.03**	**0.04**	**0.04**	**0.03**	**0.04**	**0.03**	**0.03**
Water fraction	0.32	1.25	0.63	1.25	0.63	1.04	1.04	0.88
Gentamicin	0.0002	0.0007	0.0002	0.0005	0.0002	0.0002	0.0008	0.0004

Avg MIC = average MIC, bold = excellent activity

5.3.2 Cytotoxicity of crude extracts and fractions against Vero cells and selective indices

The water fraction had the best LC_{50} (1 mg/ml), however, the selectivity index was above one for only three bacterial strains out of the seven strains tested, and the average selectivity index (SI) was 1.14 (Table 5.2). The butanol and ethyl acetate fractions had the second best and third best LC_{50} of 0.55 mg/ml and 0.42 mg/ml respectively and high selective index values ranging from 13.75 to 18.33. The butanol extract had the best average SI of 18.33 followed by the ethyl acetate fraction with average SI of 14. The crude extract had an LC_{50} of 0.36 mg/ml and good selective index above one for all the tested seven bacterial strains with average SI of 7.2.

The hexane DCM fractions had moderate to toxic LC_{50} values (0.21 and 0.01 mg/ml) respectively and their SI values were below one for all the tested seven bacterial strains.

Table 5.2 LC_{50} of crude extracts and fractions of *K. gunniae* on Vero cells and their selective index values on *S. aureus* ATCC 29213 and *S. aureus* isolates from clinical mastitis

Samples	LC_{50} (mg/mL)	Selectivity index (SI)							Avg. SI
		ATCC	STA 1	STA 2	STA 3	STA 4	STA 5	STA 6	
Crude extract	0.36±0.03	**9.00**	**4.50**	**9.00**	**6**	**12**	**9**	**4.5**	**7.2**
Hexane fraction	0.21±0.007	0.66	0.08	0.17	0.08	0.33	0.08	0.17	0.14
DCM fraction	0.01±0,002	0.02	0.01	0.02	0.01	0.02	0.02	0.01	0.01
Ethyl acetate fraction	0.42±0.02	**14**	**14**	**21**	**14**	**14**	**14**	**14**	**14**
Butanol fraction	0.55±0.01	**18.33**	**18.33**	**13.75**	**13.75**	**18.33**	**13.75**	**18.33**	**18.33**
Water fraction	1.0±0.02	**3.13**	0.8	**1.59**	0.8	**1.59**	0.96	0.96	**1.14**

SI = Selective index, Avg. SI = average selectivity index, Bold = good selectivity index i.e., Selectivity index above 1

5.3.3 Total phenolic content and total flavonoid content of crude extract and fractions

5.3.3.1 Total phenolic content

Results show that the *K. gunniae* ethyl acetate, butanol and water fractions and the crude extract were very rich in phenolics. The ethyl acetate fraction had the highest total phenolic content (943.09 mgGAE/g) followed by the butanol fraction (922.63 mgGAE/g) (Table 5.3). These were followed by the water fraction and crude extract which had similar high total phenolic content of 880.91 mgGAE/g and 880.73 mgGAE/g respectively. The remaining samples – the DCM and hexane fractions - also contained good total phenolic contents (299.99 mgGAE/g and 252.75 mgGAE/g respectively).

5.3.3.2 Total flavonoid content

The ethyl acetate fraction had the highest total flavonoid content (140.30 mgQE/g) (Table 5.3). This was followed by the butanol fraction and crude extract which had similar total flavonoid contents of 62.80 mgQE/g and 60.85 mgQE/g respectively. The hexane and water fractions contained quite low amounts of total flavonoids, while the DCM fraction contained no detectable flavonoids.

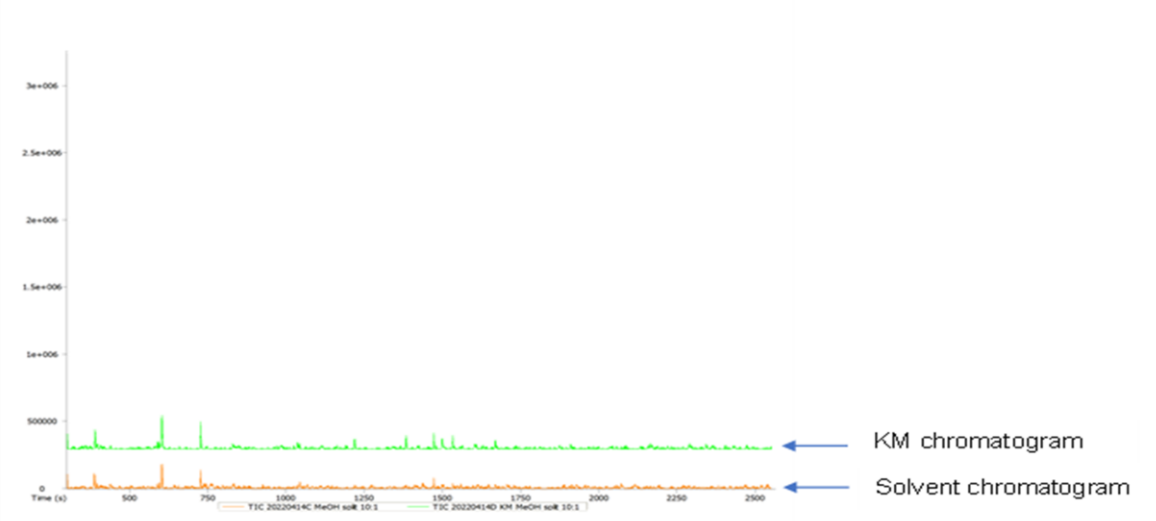
Table 5.3 Total flavonoid and total phenolic contents of crude extract and fractions of *K. gunniae*

Samples	TFC (mgQE/g)	TPC (mgGAE/g)
Ethyl acetate fraction	140.30±11.36	943.09±19.75
Butanol fraction	62.80±3.07	922.63±15.75
Crude	60.85±4.21	880.73±24.15
DCM fraction	0±0.0	299.99±53.25
Hexane fraction	18.12±2.4	252.75±19.88
Water fraction	2.12±0.33	880.91±13.94

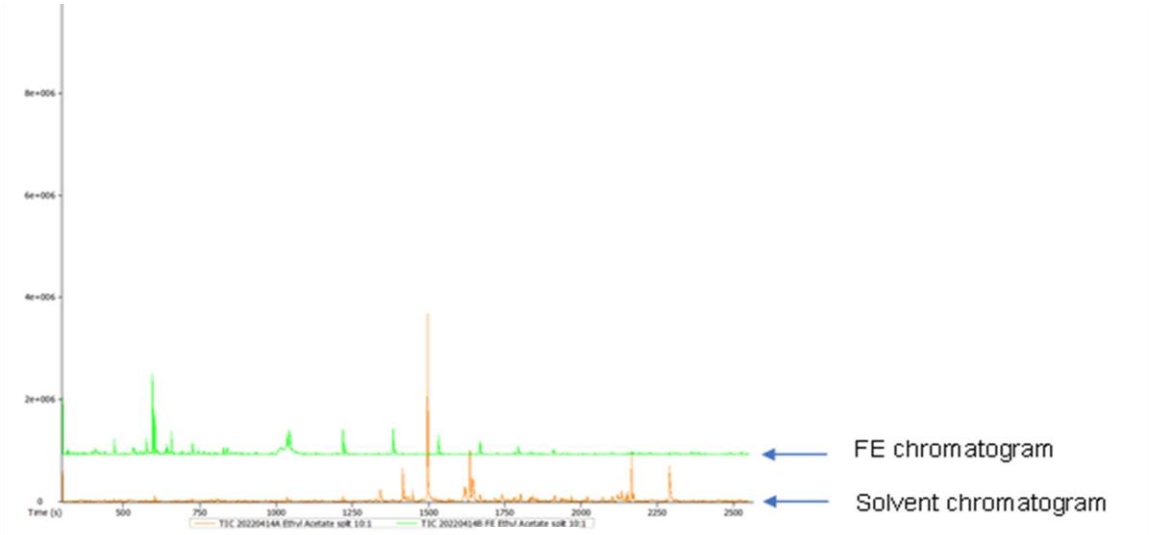
mgQE/g = milligram per quercetin equivalent in one gram of sample, mgGAE/g = milligram per garlic acid equivalent in one gram of sample. DCM = dichloromethane fraction.

5.3.4 Identification of compounds in active fractions of *K. gunniae* using GCMS

In the ethyl acetate fraction, the three most abundant volatile compounds were butane, 2,2,3,3-tetramethyl-, cetene and 1-docosene (Table 5.4). Figure 5.1 shows the chromatogram of the samples compared with the solvent chromatogram. GC-MS analysis showed that the butanol fraction contained tetradecane, nonadecane and heptadecane, 2-methyl-nonadecane as the major compounds present in the sample. Three major volatile constituents of the crude extract were 1-nonadecene, benzenedicarboxylic acid, butyl 2-ethylhexyl, and 1-docosene. The compounds 2,2,4,4-tetramethyloctane and tetradecane are present in two of the fractions (ethyl acetate fraction and butanol fraction) analysed, although these compounds were not among three most abundant compounds in any of the fractions.



(A)



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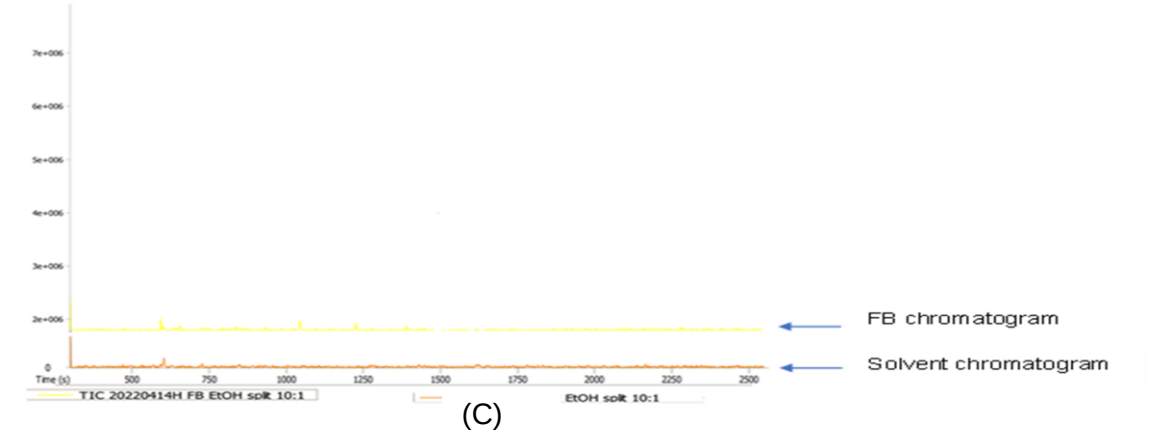

(C)

Figure 5.1 Gas chromatography-mass spectrometry (GCMS-MS) chromatogram of crude extract (A), ethyl acetate fraction (B) and Butanol fraction (C) of *K. gunniae.* FE = ethyl acetate fraction, FB = butanol fraction, KM = crude extract.

Table 5.4 GCMS profile of *K. gunniae* fractions and crude extract

Sample	Compound name	Molecular weight	Formula	CAS	% Similarity	Retention time (s)	Area %
FE – fraction	p- xylene	106	C8H10	106-42-3	94.6	408.1	2.79
		106	C8H10	106-42-3	96.2	438.2	(2.1496 +0.63673)
	Nonane	128	C9H20	111-84-2	86.6	470.4	3.28
	Benzene, 1-ethyl-2-methyl-	120	C9H12	611-14-3	93.7	533,5	1.97
		120	C9H12	611-14-3	92.0	555,8	(1.017 + 0.95164)
	Butane, 2,2,3,3-tetramethyl-	114	C8H18	594-82-1	89.4	595.3	**12.05**
	2,2,4,4-Tetramethyl octane	170	C12H26	62183-79-3	90.3	641	1.78
	Undecane	156	C11H24	1120-21-4	88.7	726.9	3.87
		156	C11H24	1120-21-4	90.0	841	(2.1798 + 1.6887)
	1,2,3-Benzenetriol	126	C6H6O3	87-66-1	80.2	1015.4	4.61
	Cetene	224	C16H32	629-73-2	91.8	1036.3	**14.08**
		224	C16H32	629-73-2	93.8	1219.5	(4.4718 + 4.5953 +
		224	C16H32	629-73-2	92.6	1384.1	5.0117)
	Tetradecane	198	C14H30	629-59-4	90.8	1046.6	3.17
	Eicosane (antifungal)	282	C20H42	112-95-8	91.9	1228.5	2.02
	1-Docosene	308	C22H44	1599-67-3	95.3	1533.2	**7.57**
		308	C22H44	1599-67-3	94.4	1669.3	(3.2721 + 2.7447 +
		308	C22H44	1599-67-3	95.5	1794.3	1.5485)
	2,2,4,4-Tetramethyl octane	170	C12H26	62183-79-3	92.5	640.2	2.70
	Propane, 1-(1,1-dimethylethoxy)-2-methyl-	130	C8H18O	33021-02-2	83.8	643.8	2.34
FB – fraction	**Tetradecane**	198	C14H30	629-59-4	909	1046.2	**17.17**
	Nonadecane	268	C19H40	629-92-5	921	1228.3	**8.96**
	Heptadecane, 2-methyl-	254	C18H38	1560-89-0	91.1	1391.6	**5.95**
	Butane, 2-bromo-	136	C4H9Br	78-76-2	80.0	658	1.612
	1-Nonadecene	266	C19H38	18435-45-5	91.6	1384.1	**7.72**
KM – crude	**1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester**	334	C20H30O4	85-69-8	90.7	1480.7	**1.78**
	1-Docosene	308	C22H44	1599-67-3	91.4	1533.2	**8.46**

FE = ethyl acetate fraction, FB = butanol fraction, KM = crude extract. In bold are the three most abundant compounds in the samples.

5.3.5 Identification of compounds in active fractions of *K. gunniae* using LC-MS

Four compounds were identified from the positive mode UPLC-MS chemical profile of crude extract of *K. gunniae.* These compounds were fenamiphos deisopropyl, genistin, 1,3,6-trihydroxy-2-methylanthraquinone-3-O-(O-6-acetyl) neohesperidin and 4α,6α-dihyroxyeud-esman-8β,12-olide. From the negative mode UPLC-MS chemical profile of crude extract of *K. gunniae* four compounds were also identified, namely myristicin, (E)-3-tridecene-5,7,9,11-tetryne-1,2-diol, apigenin-7-O-α-L-rhamnose (1→4)-6" O-acetyl-β-D-glycoside and mudanpioside H. Figures 5.2 and 5.3 show the positive mode and negative mode chromatograms and peaks respectively of these compounds and the details of these compounds are presented in Table 5.5.

From the positive mode UPLC-MS chemical profile of ethyl acetate fraction of *K. gunniae,* genistin (which is also present in the crude extract) and kaempferol-3-O-rhamnoside were identified. From the negative mode chemical profile of the ethyl acetate fraction, mudanpioside H (also present in the crude extract) and three other compounds, namely epimedin C, thiazopyr, and apigenin-7-O-α-L-rhamnose (1→4)-6" O-acetyl-β-D-glycoside, were identified. The positive mode and negative mode chromatograms and peaks of these compounds identified from ethyl acetate fraction, are given in Figures 5.4 and 5.5 and their details are presented in Table 5.6.

Analysis of the positive mode UPLC-MS chemical profile of ethyl butanol fraction of *K. gunniae* identified two compounds, namely genistin (also present in the crude extract and ethyl acetate fraction) and fenamiphos deisopropyl. From the negative mode chemical profile of the butanol fraction, four compounds were identified, namely myristicin (also present in the crude extract), Safflor yellow A, patuletin-7-O-(6''-isobutyryl)-glycoside and apigenin-7-O-α-L-rhamnose (1→4)-6"O-acetyl-β-D-glycoside (which was also present in the crude extract and ethyl acetate fraction). Chromatograms and peaks of the compounds in the positive mode and negative mode of butanol fraction sample are shown in Figures 5.6 and 5.7 with Table 5.8 showing more detail.

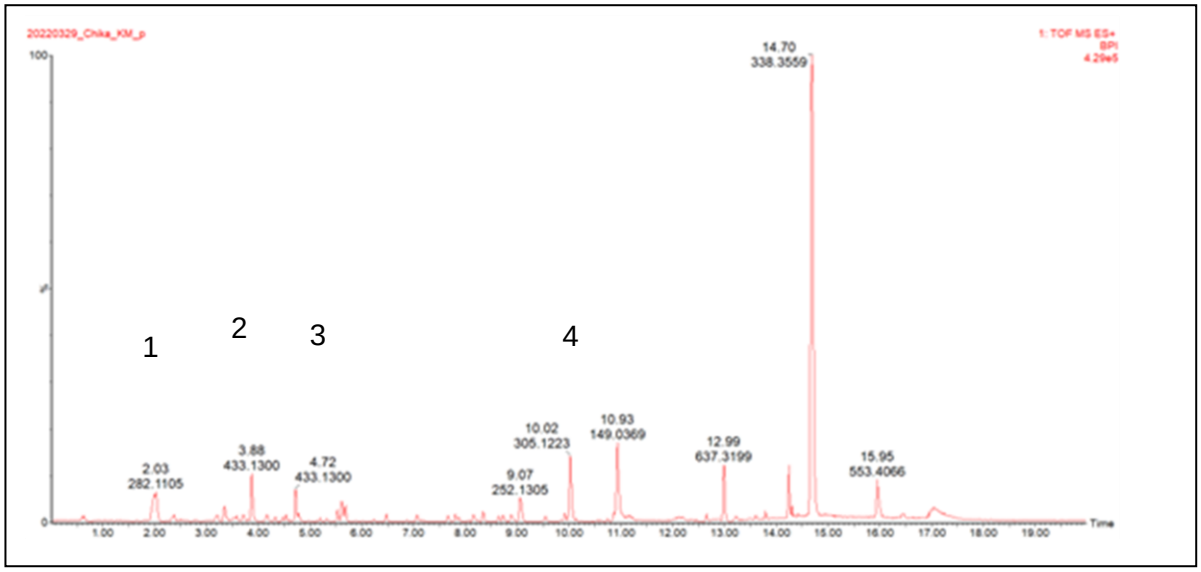

Figure 5.2 UPLC-MS chromatogram of *K. gunniae* crude extract, ESI positive mode.

1, 2, 3, 4 = peaks of identified compounds, listed in Table 5.5 under positive mode

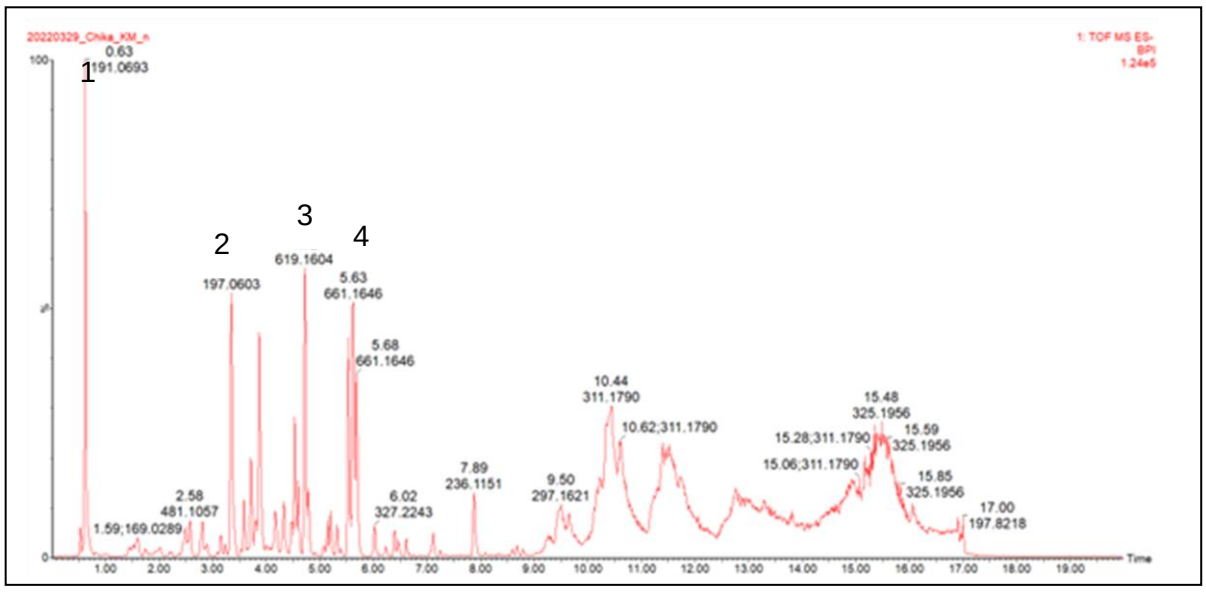

Figure 5.3 UPLC-MS chromatogram of *K. gunniae* crude extract, ESI negative mode

1, 2, 3, 4 = peaks of identified compounds, listed in Table 5.5 under negative mode

Table 5.5 UPLC-MS compound profile of *K. gunniae* crude extract, from positive mode and negative mode chromatograms

Positive mode	Peak	Retention time	Observed	Exact mass (m/z)	Formula	MS/MS (Mass fragmentation)	Compound name	Reference
	1	2.03	282.1105 $[M+NH_4]^{+}$	262.0575	$C_{10}H_{16}NO_3PS$	98.0701, 201.0184, 282.1032	Fenamiphos deisopropyl	Waters UINIFI®
	2	3.88	433.1300 $[M+H]^{+}$	432.1156	$C_{21}H_{20}O_{10}$	287.0698	Genistin	Waters UINIFI®
	3	4.72	433.1300 $[M+H]^{+}$	620.1792	$C_{29}H_{32}O_{15}$	433.1162, 287.0599, 229.1473	1,3,6-Trihydroxy-2-methylanthraquinone-3-O-(O-6-acetyl) neohesperidin	Waters UINIFI®
	4	10.02	306.1223 $[M+K]^{+}$	266.0129	$C_{15}H_{21}O_4K$	169.0910	4α,6α-Dihyroxyeud-esman-8β,12-olide	Waters UINIFI®
Negative mode	1	0.63	191.0704 $[M+H]^{+}$	192.0704	$C_{11}H_{12}O_3$	-	Myristicin	Waters UINIFI®
	2	3.35	197.0603 $[M+H]^{+}$	198.0603	$C_{13}H_{10}O_2$	121.0443, 153.0710	(E)-3-Tridecene-5,7,9,11-tetryne-1,2-diol	Waters UINIFI®
	3	4.72	619.1619 $[M+H]^{+}$	620.1619	$C_{15}H_{10}O_5$	255.0425, 283.0343, 285.0526, 431.1035	Apigenin -7-O-α-L-rhamnose (1→4)-6" O-acetyl-β-D-glycoside	Waters UINIFI®
	4	5.63	661.1716 $[M+H]^{+}$	618.171	$C_{30}H_{32}O_{14}$	255.0425, 283.0343, 430.0968	Mudanpioside H	Waters UINIFI®

- The mass fragmentation was not identified, m/z = mass per charge

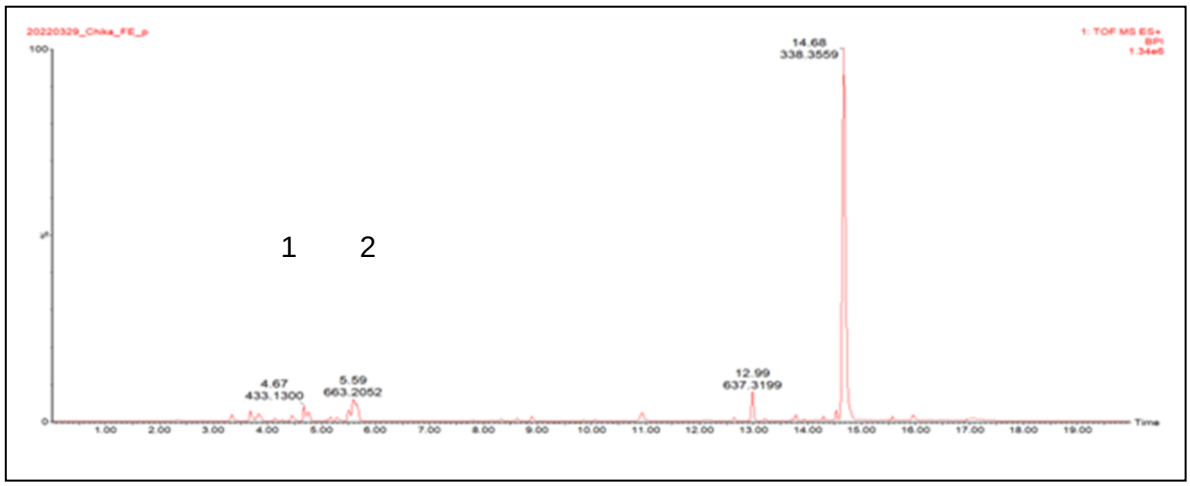

Figure 5.4 UPLC-MS chromatogram of *K. gunniae* ethyl acetate fraction, ESI positive mode

1, 2 = peaks of identified compounds, listed in Table 5.6 under positive mode

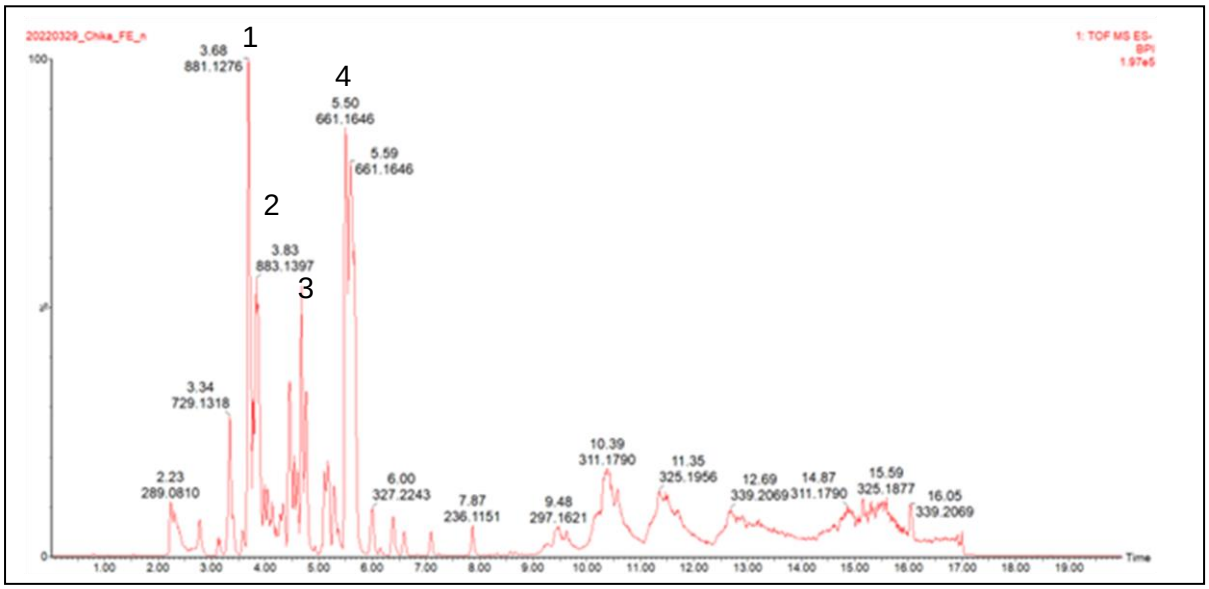

Figure 5.5 UPLC-MS chromatogram of *K. gunniae* ethyl acetate fraction, ESI negative mode

1, 2, 3, 4 = peaks of identified compounds, listed in Table 5.6 under negative mode

Table 5.6 UPLC-MS compound profile of the ethyl acetate fraction of *K. gunniae*, from positive mode and negative mode chromatograms

Positive mode	Peak	Retention time	Observed	Exact mass (m/z)	Formula	MS/MS (Mass fragmentation)	Compound name	Reference
	1	4.67	433.1300 $[M+H]^+$	433.11611	$C_{21}H_{20}O_{10}$	287.30698	Genistin	Waters UINIFI®
	2	5.59	663.2052 $[M+H]^+$	663.1884	$C_{21}H_{20}O_{10}$	287.0698, 153.0307, 111.0525	Kaempferol-3-O-rhamnoside	Waters UINIFI®
Negative mode	1	3.68	881.1276 $[M+CH_3COO]^+$	940.1409	$C_{39}H_{50}O_{19}$	289.0809, 407.0736, 559.1158	Epimedin C	Waters UINIFI®
	2	3.83	883.1397 $[M+HCOO]^+$	441,0907/838.14205	$C_{16}H_{17}F_5N_2O_2S$	169.0289, 289.0809	Thiazopyr	Waters UINIFI®
	3	4.68	619.1604 $[M-H]^-$	620.1684	$C_{15}H_{10}O_5$	255.0425, 283.0343, 285.0526, 431.0944	Apigenin -7-O-α-L-rhamnose (1→4)-6"O-acetyl-β-D-glycoside	Waters UINIFI®
	4	5.59	661.1646 $[M+HCOO]^+$	616.1669	$C_{30}H_{32}O_{14}$	255.0425, 283.0343, 430.0877	Mudanpioside H	Waters UINIFI®

- The mass fragmentation was not identified, m/z = mass per charge

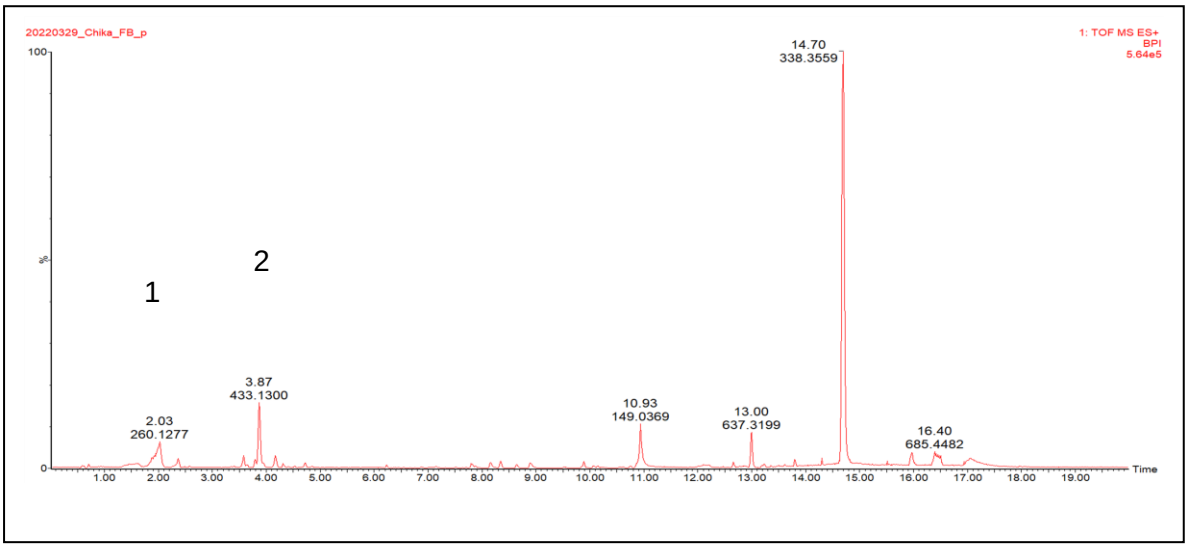

Figure 5.6 UPLC-MS chromatogram of *K. gunniae* butanol fraction, ESI positive mode

1, 2 = peaks of identified compounds, listed in Table5.7 under positive mode

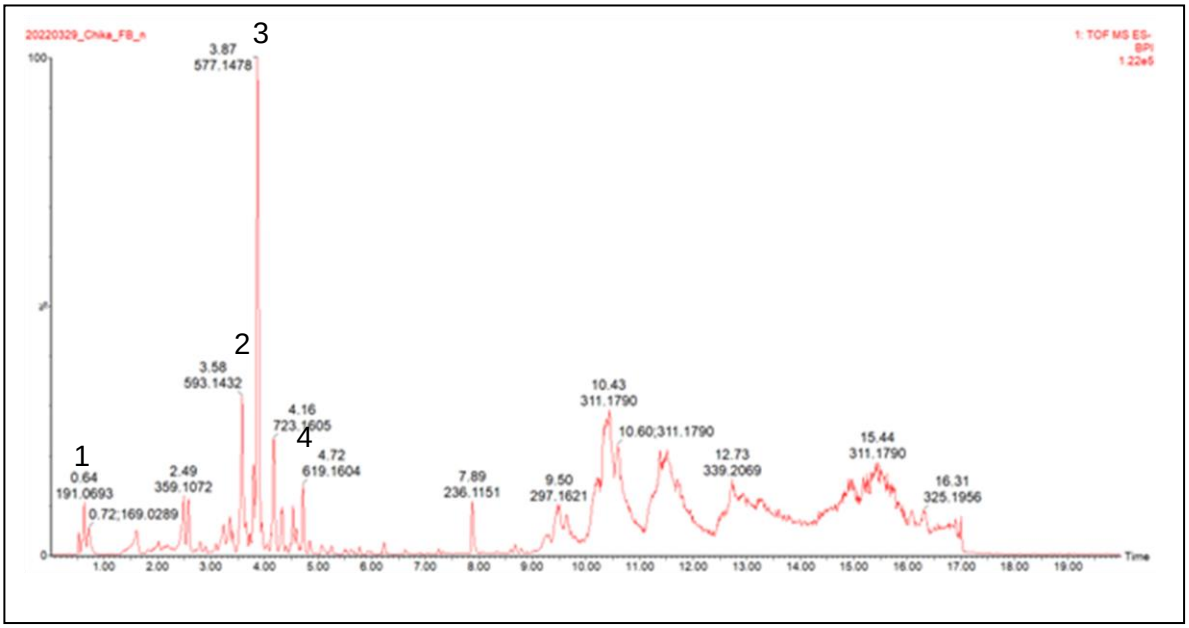

Figure 5.7 UPLC-MS chromatogram of *K. gunniae* butanol fraction, ESI negative mode

1, 2, 3, 4 = peaks of identified compounds, listed in Table 5.7 under negative mode.

Table 5.7 UPLC-MS compound profile of the butanol fraction of *K. gunniae*, from positive mode and negative mode chromatogram

Positive mode	Peak	Retention time	Observed	Exact mass (m/z)	Formula	MS/MS (Mass fragmentation)	Compound name	Reference
	1	2.03	260.1277 [M+Na]	261.0918	$C_{10}H_{16}NO_3PS$	201.0184, 110.9835, 242 1123, 98.0701	Fenamiphos deisopropyl	Waters UINIFI®
	2	3.87	433.1147 $[M+H]^+$	432.1147	$C_{21}H_{20}O_{10}$	287.0698	Genistin	Waters UINIFI®
Negative mode	1	0.64	191.0704 $[M+H]^+$	192.0704	$C_{11}H_{12}O_3$	-	Myristicin	Waters UINIFI®
	2	3.58	593.1472 $[M+H]^+$	594.1492	$C_{27}H_{30}O_{15}$	271.0320, 299.0335	Safflor yellow A	Waters UINIFI®
	3	3.87	577.1478 $[M+H]^+$	578.1547	$C_{26}H_{28}O_{14}$	255.0425, 283.0343, 285.0526	Patuletin-7-O-(6''-isobutyryl)-glycoside	Waters UINIFI®
	6	4.72	619.1604 $[M+H]^+$	620.1618	$C_{15}H_{10}O_5$	255.0425, 283.0343, 285.0526, 431.0944	Apigenin -7-O-α-L-rhamnose (1→4)-6" O-acetyl-β-D-glycoside	Waters UINIFI®

- The mass fragmentation was not identified, m/z = mass per charge

5.4 Discussion

5.4.1 MIC assay of crude extracts and fractions against *S. aureus* ATCC 29213 and *S. aureus* mastitis clinical isolates

The crude extract and two fractions (ethyl acetate and butanol fractions) had excellent activity against *S. aureus* ATCC 29213 and *S. aureus* clinical isolates from bovine mastitis cases. For the active fractions, activity was improved after fractionation as these fractions were more active than the crude extract. The average MIC of the crude extract against the seven bacterial strains was 0.05 mg/ml whereas the average MIC activity of each of the active fractions was 0.03 mg/ml.

This is the first time that antibacterial activity was investigated on fractions of *K. gunniae* as this plant was only described in 2019 and has not previously undergone any efficacy, biological activity or pharmacological activity studies.

5.4.2 Cytotoxicity of crude extracts and fractions against Vero cells and selective indices

Activity of a plant extract could be as a result of toxicity (general toxicity to both animals and micro-organisms) or as a result of selective activity against microbes alone (Elisha et al., 2017). The cytotoxicity assay was therefore performed to determine whether the crude extract and fractions of *K*. *gunniae* are toxic or safe *in vitro*. For plant extracts, an LC_{50} of 0.02 mg/ml and below is regarded as toxic (Kuete, 2010).

When the SI value is 1 and above, it demonstrates that the plant extract/fraction has more activity against bacteria than to mammalian cells (Sserunkuma et al., 2017). This implies that, the higher the SI value of a plant extract or fraction, the better and more selective antibacterial activity of the extract/fraction. Therefore, higher SI values for plant are preferable because the higher the SI value the safer the plant extracts/fractions and thus more suitable for further investigation for potential use in therapeutic preparations (Dzoyem et al., 2014).

In this study, the butanol and ethyl acetate fractions had extremely promising activity with average selective indices of 18.33 and 14.00 respectively. These excellent selective indices indicate that these samples are safe *in vitro* and thus can be recommended for further development of therapeutic applications in the treatment and management of mastitis. The crude extract with average selective index of 7.2 also indicated relative safety and good activity.

5.4.3 Total phenolic content and total flavonoid content of crude extract and fractions

Plant-derived antimicrobial compounds belong to a broad range of classes, including phenolics, alkaloids, terpenoids and peptides (Kuete et al., 2011). Antioxidant substances include polyphenols and flavonoids, which are able to scavenge free radicals like hydroxyl, hydroperoxide, and lipid peroxyl radicals as well as quench hydrogen peroxide and superoxide anion, averting oxidative damage associated with disease states (Nasab et al., 2020; Sevgi et al., 2015). It is also known that many flavonoids, which are a sub-class of polyphenols, have anti-inflammatory activities (Bernstein et al., 2018). Since phenolic compounds and flavonoids can act as antimicrobial, antioxidant and anti-inflammatory agents, tests for the presence and quantity of phenolic compounds and flavonoids were done on the three active samples (crude extract, ethyl acetate fraction and butanol fraction).

The lower quantity of total flavonoid content observed in the samples compared to the very high quantity of total phenolic content is understandable since flavonoids are one group of

phenolic compounds (Panche et al., 2016; Yuan et al., 2009). Several groups of secondary metabolites are phenolic compounds, thus in measuring for total phenolic content, quantity of flavonoids alone was measured, whereas in measuring for phenolic compounds, quantity of all secondary metabolites that are phenolics (which includes flavonoids as well) are measured.

5.4.4 Identification of compounds in active fractions of *K. gunniae* using GCMS-MS

1-Docosene, one of the major compounds identified by GC-MS, was present in the crude extract and ethyl acetate fraction, but reports on the bioactivity of 1-docosene are lacking. Cetene, also known as 1-hexadecene, an alkene (long-chain hydrocarbon) which was identified from one of the active fractions (ethyl acetate fraction) in this research has been reported to stimulate the production of antimicrobial and antioxidant substances when added in microbial culture media. Mou et al. (2013) reported the enhanced production of palmarumycin C_3 and palmarumycin C_2 which are both antimicrobial and antioxidant) in the fungus *Berkleasmium* sp. Dzf12. Perhaps the presence of cetene in the plant extract /fraction added to bacterial culture may have also stimulated the same effect producing palmarumycin C_3 and palmarumycin C_2 or other types of antibacterial and antioxidant substances. Addition of cetene into bacterial cultures, especially into *S. aureus* cultures, and identifying the subsequent compounds in the culture media before and after the addition of cetene is recommended to ascertain whether the activity of cetene observed with fungal culture has a similar effect in bacterial culture.

Tetradecane, one of the major compounds identified in the butanol fraction, has been reported to have strong antibacterial activity against *S. aureus* (Sallam & Abed, 2022). Other plant extracts containing tetradecane have also been reported to have antioxidant and anti-inflammatory activity (Nkadimeng et al., 2020).

1-Nonadecene, a long chain fatty acid identified in the crude extract, has been reported to have antibacterial, antifungal, antioxidant, antituberculosis and anticancer activities (Amudha et al., 2018; Premathilaka & Silva, 2016; Sreenivasan et al., 2022).

5.4.5 Identification of compounds in active fractions of *K. gunniae* using LC-MS

Genistin, a compound identified through LC-MS in all three of the samples tested (crude extract, ethyl acetate fraction and butanol fraction) of *K. gunniae* is an isoflavone glycoside. It has antimicrobial, antioxidant and anti-inflammatory activity (Islam et al., 2020; Panche et al., 2016). Genistein has also been found to have very significant antibacterial activity against *S.*

aureus particularly (Hong et al., 2006). Other known bioactivities of genistin include anti-cancer, anti-apoptotic, cardioprotective, hepatoprotective and neuroprotective (Islam et al., 2020). Genistin is also a phytoestrogen and thus also helps to reducing the risk of osteoporosis and post-menopausal symptoms (Zaheer & Humayoun Akhtar, 2017)

Genistin is an isoflavone and isoflavones comprise a large subclass of flavonoids (Islam et al., 2020). Thus, genistin, being a flavonoid as well as a phenolic compound (since flavonoids are members of phenolics), supports the presence of flavonoids and especially the very high quantity of total phenolics in the two active fractions (ethyl acetate and butanol fractions) and the crude extract of *K. gunniae*. This finding also indicates that genistin is most likely one of the active compounds responsible for the excellent activity of the ethyl acetate fraction, butanol fraction and crude extract of *K. gunniae* against *S. aureus* ATCC strain (MIC = 0.03 to 0.04 mg/ml) and *S. aureus* mastitis clinical isolates (MIC = 0.02 to 0.08 mg/ml). Interestingly, the crude acetone and ethanol extracts of *K. gunniae* had excellent anti-oxidant activity in research reported in a previous chapter, and genistin which was present in *K. gunniae* is a known antioxidant substance.

Apigenin-7-O-α-L-rhamnose (1→4)-6” O-acetyl-β-D-glycoside, a compound belonging to the apigenin group of flavonoids (Salehi et al., 2019) was identified in all three of the active samples. Reports on bioactivity of apigenin-7-O-α-L-rhamnose (1→4)-6” O-acetyl-β-D-glycoside, is lacking, however another form of an apigenin flavonoid derivative, namely apigenin-7-O-β-D-glucuronide has been reported to have antioxidant activity as well as *in vitro* and *in vivo* anti-inflammatory activity (Hu et al., 2016). Isolation of apigenin-7-O-α-L-rhamnose (1→4)-6” O-acetyl-β-D-glycoside, and testing it for bioactivities like antimicrobial activity, antioxidant and anti-inflammatory activity is recommended.

Myristicin and mudanpioside H. were identified in two of the samples. Myristicin was identified in the crude extract and butanol fraction whereas mudanpioside H was identified in the crude extract and ethyl acetate fraction. Myristicin has been reported to have anti-oxidant and anti-inflammatory activities (Seneme et al., 2021). Antibacterial activity of myristicin against *S. aureus* has also been reported (Lee et al., 2020).

5.5 Conclusions

Two fractions, namely the ethyl acetate and butanol fractions, and the crude extract of *K. gunniae*, had excellent antimicrobial activity and were safe when tested against mammalian cell cultures. These two fractions and the crude extract also had high total phenolic contents

as well as good total flavonoid contents, indicating that the active compounds in these fractions and crude most likely include flavonoids or other phenolics. Chromatographic analysis using UPLC-MS confirmed that these fractions and crude extract contain flavonoids and phenolics, as the same flavonoid compounds were identified in the fractions and crude extract. Moreover, in all the active samples (ethyl acetate fraction, butanol fraction and the crude extract) there were at least two identified compounds (through GC-MS and UPLC-MS) that have previously been reported to have bioactivity.

The synergistic activity of identified compounds with reported bioactivity, especially the major compounds present in at least two of the active samples are likely to be responsible for the activities of the active samples of *K. gunniae*. Plants produce an extraordinary diversity of chemicals that may have interesting and useful biological activities. Several such compounds are components of many herbal remedies, nutraceuticals, pharmaceuticals and functional foods (Susanna et al., 2022). As genistin has been found to have beneficial bioactivities like antioxidant and antimicrobial, further research is warranted into isolating or synthesising the compound and investigating it for development of pharmaceutical therapeutic medicine for the treatment and management of mastitis. Since genistin has also been found to be present in food crops (Zaheer & Humayoun Akhtar, 2017), genistin products could also be developed as nutraceutical products.

This research has demonstrated that the active fractions and crude extracts of *K. gunniae*, contain known active compounds and these compounds may have synergistic effects. Isolation of the identified compounds in this research and testing them for antimicrobial activity singly and in combination to determine whether they only act in synergy or whether they act both in synergy and in isolation is recommended. Molecular docking studies of the identified compounds in the active fractions is also recommended to see if these compounds can have activity or improved activity in modified forms as well as to detect other possible biological activities of the compounds. Molecular docking of the identified compounds could also help to identify the most active chemical derivative form of the identified compounds so that the best derivative forms of these compounds could be used in the development of therapeutic products.

CHAPTER 6 General conclusions and recommendations

Mastitis is an extremely important disease in dairy production, resulting in vast economic losses worldwide. The emergence of drug-resistant bacterial strains from clinical cases of mastitis has necessitated the search for alternative remedies to prevent or treat mastitis. Plants are a valuable source of phytochemicals with interesting biological activities, including antibacterial, antioxidant and anti-inflammatory. Various plant extracts and bioactive compounds have also been shown to have the ability to inhibit quorum sensing and the formation of bacterial biofilms, features commonly associated with antibiotic resistance.

The aim of this study was to identify biological activity and chemical compounds likely to be responsible for activity from selected medicinal plants with potential application in treating mastitis, as well as clinical signs and inflammation associated with the disease. The overall discussion of the results presented in the thesis will be provided in terms of the initial objectives of the study.

6.1.1 To determine the antibacterial activity of selected South African plant extracts against *S. aureus* reference strain and drug-resistant *S. aureus* clinical isolates from bovine mastitis cases

The first objective was to test selected South African species of Celastraceae, Aquifoliaceae and Crassulaceae families for activity against reference strain and drug-resistant strains of major mastitis causative pathogen. Activity against the reference strain (*S. aureus* ATCC 29213) was determined first and two most active plant species chosen from chemotaxonomic selection and another two most active plants from ethnopharmacological selection were selected for further study. The first plant family was chosen based on a previous study that showed that species of the Celastraceae family have antimicrobial activity. The second and third plant families were chosen based on the use of species of the families in traditional medicine. As *S. aureus* is the major causative organism of mastitis, plant extracts were tested against *S. aureus* reference strain (ATCC 29213) and drug-resistant *S. aureus* clinical isolates from bovine mastitis cases after antibiotic susceptibility test on all the *S. aureus* strains. Total antibacterial activity of the plant extracts was also evaluated. From the Celastraceae family, extracts of *Maurocenia frangula* and *Maytenus undata* plants had the best activity and from the Crassulaceae family, extracts of *Kalanchoe gunniae* and *Bryophyllum pinnatum* (also known as *Kalanchoe pinnata*) had the best activity. The extracts of these four plants (acetone and ethanol extracts) were selected for further studies. Notably, of these selected four plants,

K. gunniae extracts had the best antibacterial activity. The acetone crude extract and ethanol crude extract of *K. gunniae* had average MIC of 0.05 mg/ml and 0.06 mg/ml respectively against all the seven *S. aureus* strains tested.

6.1.2 To determine cytotoxicity, quorum sensing inhibition and anti-biofilm activity of most active extracts

The second objective was to determine the cytotoxicity of the selected plant extracts with the best antibacterial activity and to also evaluate their mechanisms of inhibiting antibacterial infection through anti-quorum sensing and anti-biofilm activities. *In vitro* cytotoxicity assay showed that *K. gunniae* and *B. pinnatum* extracts were not cytotoxic against mammalian cells (Vero and bovine dermis cells) and they also had good selectivity indices further indicating safety. *Kalanchoe gunniae* extracts had the best selectivity indices. The acetone crude extract of *K. gunniae* had average selectivity index of 20 with both Vero cells and bovine dermis cells against all the seven *S. aureus* strains tested in the first objective. The ethanol crude extract of *K. gunniae* had average selectivity indices of 16.0 and 16.67 with Vero cells and bovine dermis cells respectively against all the seven *S. aureus* strains tested in the first objective. For *M. frangula and M. undata,* although their LC_{50} values were low, indicating cytotoxicity, their average selectivity indices with bovine dermis cells against all the seven *S. aureus* strains tested (reference and clinical isolates) were above 1, indicating mild safety.

Thus, the anti-quorum sensing activities of extracts of *K. gunniae*, *B. pinnatum, M. frangula* and *M. undata,* were evaluated. *Kalanchoe gunniae* and *B. pinnatum* had the best quorum sensing inhibition activity as well as the best quorum sensing selectivity indices. Considering that *K. gunniae* and *B. pinnatum* extracts had good antibacterial activity against *S. aureus* reference strain and clinical isolates from mastitis cases, were not cytotoxic and also had good quorum sensing inhibition activity, they were selected for anti-biofilm activity investigation. Extracts of *K. gunniae* and *B. pinnatum* had good activity (above 50%) against biofilm formation, however they were not able to disrupt preformed and matured biofilm.

6.1.3 To characterize anti-inflammatory and antioxidant extracts of active plant species with potential for standardization and use against bovine mastitis.

A major feature of infectious mastitis is inflammation. Uncontrolled inflammation results in cell damage and also leads to oxidation which is also harmful to cells and surrounding tissues. Thus, the third objective of this study was to evaluate and characterise anti-inflammatory and antioxidant activities of extracts of antibacterial active plant species.

Extracts of *M. frangula* and *M. undata* plants from the Celastraceae family, and extracts of *K. gunniae* and *B. pinnatum* from the Crassulaceae family, which in earlier tests had the best

activity against *S. aureus* reference strain and *S. aureus* clinical isolates from mastitis cases, were evaluated for anti-inflammatory and antioxidant activities *in vitro*. Antioxidant assays were DPPH and ABTS assays. Inflammatory evaluations included tests on effects of the plant extracts on inhibition of 15-LOX activity, inhibition of nitric oxide production, inhibition of COX-2 production, inhibition of pro-inflammatory cytokines (TNF-α and IL-1β) and increase in the production of the anti-inflammatory cytokine, IL-10. For antioxidant activity with DPPH and ABTS, *K. gunniae* extracts had the best scavenging activity. The activity of both acetone and ethanol extracts of *K. gunniae* were excellent (IC_{50} < 1 µg/ml). The ethanol extracts of all the other plants (*B. pinnatum, M. frangula* and *M. undata*) also had potent activity, with IC_{50} < 10µg/ml for both DPPH and ABTS assays. For the anti-inflammatory assay with 15-LOX, *K. gunniae* extracts again had the best potent activity (IC_{50} < 10µg/ml).

For nitric oxide (NO) and cell viability evaluation, extracts of the four plants were tested against activated RAW 264.7 macrophage cells. Plant extracts which are able to inhibit nitric oxide production without being toxic to RAW 264.7 macrophage cells are considered to have good nitric oxide inhibition, and thus anti-inflammatory activity. Extracts of *B. pinnatum* had the best nitric oxide inhibition (at test concentrations of 50 and 100 µg/ml) which were not due to toxicity to RAW 264.7 macrophage cells (cell viability above 92%). At the highest concentration tested of 100 µg/ml, extracts of *K. gunniae* also had good nitric oxide inhibition and were safe (cell viability above 95%). Nitric oxide activities observed with *M. undata* and *M. frangula* extracts, apparently were as a result of their toxicity to RAW 264.7 macrophage cells. The extracts of these plants (*M. undata* and *M. frangula*), at the highest concentration tested, exhibited activity above 100% with low cell viability, indicating that they did not have nitric oxide inhibition but rather that the observed activity was as a result of their toxicity to RAW 264.7 macrophages. Similarly, the activity observed with *M. undata* ethanol extract at test concentration of 50 µg/ml had low cell viability of 61.35% (below the acceptable safe cell viability of 80%), again indicating toxicity rather than NO inhibition.

Toxicity of the extracts of *M. frangula* and *M. undata* to RAW 264.7 macrophage cells as well as the toxicity earlier observed with these plant extracts against mammalian cells (Vero and bovine dermis cells), confirmed that all biological activities observed with these plant extracts were most likely as a result of their toxicity. Therefore, only extracts of *K. gunniae* and *B. pinnatum* were used for the remaining anti-inflammatory assays.

Very good COX-2 inhibition activity was obtained with all extracts of *K. gunniae* and *B. pinnatum,* with the extracts of *K. gunniae* having the best activity. For inflammatory assays with cytokines, extracts of *B. pinnatum* inhibited the production of the pro-inflammatory

cytokine TNF-α, whereas extracts of *K. gunniae* were not able to inhibit the production of TNF -α. For the pro-inflammatory cytokine IL-1β, extracts of *K. gunniae,* and *B. pinnatum* where not able to significantly decrease its production. Effect of the plant extracts on anti-inflammatory cytokines IL-10 differed. Extracts of *B. pinnatum* had very good positive activity on IL-10 production. The acetone extract of *B. pinnatum* increased the production of IL-10 and the ethanol extract maintained the production of IL-10 at the level of LPS activated cell. However, extracts of *K. gunniae* decreased the production of IL-10, though not below the level of normal control cells.

The overall results of antioxidant and anti-inflammatory activities of the extracts show that extracts of *K. gunniae,* and *B. pinnatum* are safe *in vitro* and that they have antioxidant and anti-inflammatory activities but act through different pathways.

6.1.4 To identify compounds from bioactive crude extract and fractions of most active plant species (*Kalanchoe gunniae*) with potential for standardized preparation and use against bovine mastitis

The fourth objective of this study was to analyse the crude extract and fractions of the most active plant for bioactivity and important secondary metabolites, and to select active fractions and identify compounds in these fractions. This objective was carried out in search of suitably bioactive and safe crude extract and fractions that have the potential to be used against infectious mastitis. To achieve this objective, *Kalanchoe gunniae* was used as it was the most bioactive and safe plant species from all the plant species evaluated for different bioactivities and safety thus far.

The 80% methanol crude extract of *K. gunniae* was fractionated into five fractions (hexane, dichloromethane, ethyl acetate, butanol and water fractions). The crude extract and fractions were tested against *S. aureus* reference strain and *S. aureus* clinical isolates from bovine mastitis. The cytotoxicity of the crude and fractions was evaluated and their selective indices were also determined. Further the crude extract and fractions were analysed for total phenolic content (TPC) and total flavonoid content (TFC). This is because flavonoids and other phenolics are known to have antimicrobial, anti-inflammatory and antioxidant activities, and these activities are important for combating infectious mastitis. Then the crude extract and active fractions were subjected to chromatographic analysis using GC-MS and UPLC-MS in order to identify the compounds present in the active samples which may be responsible for the different bioactivities of the active samples.

Ethyl acetate and butanol fractions had the best antimicrobial activity against *S. aureus* ATCC 29213 and *S. aureus* mastitis clinical isolates, with each having average MIC of 0.03 mg/ml

against the seven *S. aureus* strains tested. These were followed by the crude extracts which had average MIC of 0.05 mg/ml against the seven strains. The butanol fraction, ethyl acetate fraction and the crude extract were also non-cytotoxic and had average selectivity indices of 18.33, 14.0 and 7.2 respectively for all the seven strains tested.

For TPC and TFC, the ethyl acetate fraction had the highest amount of these secondary metabolites followed by the butanol fraction and then the crude extract. The antibacterial results of these active samples (ethyl acetate fraction, butanol fraction and crude extract) correlated very well with the results of the TPC and TFC in these active samples indicating that there are active compounds in these fractions and crude extract that most likely fell into the phenolics and flavonoids class of plant secondary metabolites.

From chromatographic analysis with GCMS and UPLC-MS, at least two compounds with known bioactivity were identified from each of the active samples. Genistin, a phenolic compound and a flavonoid particularly, was identified in all three of the active samples (ethyl acetate fraction, butanol fraction and crude extract). Presence of this flavonoid phenolic compound, supports the high content of phenolic and flavonoids found in these samples from TPC and TFC assays. The compound genistin is also known to have antimicrobial, antioxidant activity and anti-inflammatory activities. Its activity against *S. aureus* particularly is known to be very strong. Other known bioactivities of genistin include anti-cancer, anti-apoptotic, cardioprotective, hepatoprotective, neuroprotective as well as anti-osteoporosis.

Another compound identified in all the three active samples was also a flavonoid, the apigenin flavonoid called apigenin-7-O-α-L-rhamnose (1→4)-6” O-acetyl-β-D-glycoside. Although the activity of this compound particularly seems not yet known, another apigenin flavonoid derivative (apigenin-7-O-β-D-glucuronide) is known to have antioxidant and anti-inflammatory activities. From two of the active samples, namely the butanol fraction and crude extract, myristicin was identified. Myristicin is known to have antioxidant and anti-inflammatory activities as well as antibacterial activity against *S. aureus.*

Other major compounds were also found to be present in each of the active samples alone. From the ethyl acetate fraction, cetene was identified as one of the major compounds and cetene is known to stimulate the production of antimicrobial and antioxidant substances when added to microbial liquid media. From the butanol fraction, tetradecane was identified as one of the major compounds and tetradecane is known to have strong antibacterial activity against *S. aureus.* Tetradecane is also reported to have antioxidant and anti-inflammatory activities. From the crude extract 1-nonadecene was identified as one of the major compounds. 1-

Nonadecene is known to have antibacterial, antifungal, antioxidant as well as antituberculosis and anticancer activities.

These identified compounds known to have antibacterial, antioxidant and anti-inflammatory activities apparently are responsible for the excellent pharmacological activities observed with crude and fractions of *K. gunniae* and these active compounds most likely work synergistically. The *in vitro* bioactivities and safety observed with crude and active fractions of *Kalanchoe gunniae* present these samples as potential active constituents to be used in standardized herbal therapeutic preparations for treatment and prevention of infectious mastitis.

6.2 Conclusions

This study has established that *K. gunniae* and *B. pinnatum* crude extracts have antibacterial activity against both *S. aureus* ATCC 29213 and drug resistant *S. aureus* isolates from clinical cases of mastitis and are not cytotoxic to mammalian cells, namely Vero and bovine dermis cells. While the antibacterial activity of *B. pinnatum* extract was moderate, *K. gunniae* extracts had strong antibacterial activity. Crude extracts of these two plants also had other mechanisms of inhibiting bacterial infection which were through anti-quorum sensing and antibiofilm. For quorum sensing, *K. gunniae* extracts again had strong inhibition activity while *B. pinnatum* had moderate activity. For biofilm inhibition, extracts of the two plants were strongly effective in preventing biofilm formation, though they were not able to disrupt preformed and matured biofilm.

This study also established that *K. gunniae* and *B. pinnatum* have different degrees and broad ranges of antioxidant and ant-inflammatory activities and were not cytotoxic to RAW macrophage cells. *Kalanchoe gunniae* extracts had strong radical scavenging activity, strong inhibitory activity against pro-inflammatory enzymes 15-LOX and COX-2, good inhibitory activity against nitric oxide, poor inhibitory activity against the pro-inflammatory cytokine TNF-α, and poor up-regulatory activity towards the anti-inflammatory cytokine IL-10.

Bryophyllum pinnatum extracts had strong to moderate radical scavenging activity, and good to strong inhibitory activity against pro-inflammatory enzymes 15-LOX and COX-2 respectively, strong inhibitory activity against nitric oxide, strong inhibitory activity against pro-inflammatory cytokine TNF-α, and strong up-regulatory activity towards the anti-inflammatory cytokine IL-10.

The results of the antioxidant and anti-inflammatory activities of *K. gunniae* and *B. pinnatum* crude extracts demonstrated that extracts of these plants act through somewhat different anti-inflammatory mechanisms. *Kalanchoe gunniae* extracts act mainly through the arachidonic

acid pathway and slightly through the nitric oxide pathway, whereas *B. pinnatum* extracts act mainly through the nitric oxide pathway and slightly through the arachidonic acid pathway.

Furthermore, this study also showed that the butanol and ethyl acetate fractions of *K. gunniae* have strong antibacterial activity and are not cytotoxic. Fractionation increased the activity slightly, however compared with the acetone crude extract, the selectivity index of the fractions was reduced. Butanol and ethyl acetate fractions of *K. gunniae* each had average MIC of 0.03 mg/ml against the seven *S. aureus* strains tested and average selectivity indices of 18.33 and 14.0 respectively with Vero cells. The acetone crude extract of *K. gunniae* had average MIC of 0.05 mg/ml against all the seven tested *S. aureus* strains and average selectivity index of 20 with Vero cells.

In all the active samples of *K. gunniae* (ethyl acetate fraction, butanol fraction and the crude extract) there were at least two identified compounds with known bioactivity. Genistin, one of the major compounds identified in the ethyl acetate fraction, butanol fraction and crude extract of *K. gunniae,* is a flavonoid with known antimicrobial, antioxidant and anti-inflammatory activity.

Through this study, it has been demonstrated that, in view of the low cytotoxicity, significant antioxidant activity, anti-inflammatory activity and antibacterial activity of *B. pinnatum* extracts and *K. gunniae* extracts and active fractions, these plant extracts and active fractions are potential therapeutic agents for development of standardized herbal formulations for the treatment and prevention of infectious mastitis disease. To the best of our knowledge, this is the first report of study on the selected plants as potential alternatives for the management of mastitis.

6.3 Recommendations for further study

1. Combinations of extracts of *B. pinnatum and K. gunniae* and testing the combinations for antibacterial, antioxidant and anti-inflammatory activities to detect possible stronger activity and broad-spectrum anti mastitis activity is recommended.

2. Since the bioactivity of apigenin -7-O-α-L-rhamnose (1→4)-6” O-acetyl-β-D-glycoside (one of the compounds identified in this study) is not yet known and it was present in all the active samples, isolation of this compound and testing it for bioactivities like antimicrobial activity, antioxidant and anti-inflammatory activity is recommended.

3. Isolation of other identified compounds with known activity and testing them for antimicrobial, antioxidant and anti-inflammatory activities singly and in combination to

see whether they only act in synergy or whether they act both in synergy and as a single compound is recommended.

4. *In vivo* antibacterial and toxicity tests of the active crude extracts and fractions are recommended.

5. Molecular docking of the identified compounds in active fractions is also recommended to see if these compounds can have activity or improved activity in modified forms as well as to investigate other possible biological activities of the identified compounds. Molecular docking of the identified compounds could also help to detect the most active chemical derivative form of the identified compounds so that the best derivative forms of these compounds may be searched for in plant extracts or synthesized and used in the development of therapeutic products.

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