South African *Searsia* and *Bauhinia* species with activity against diarrhoeagenic pathogens and isolation of compounds from *S. batophylla*

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Declaration

I, **Rasheed Omotayo Adeyemo**, declare that this thesis, which I hereby submit to the University of Pretoria for the **Doctor of Philosophy (PhD),** is my original work except where the input of others has been acknowledged. This work has never been submitted for any academic award to any other institution of higher learning.

7 February 2023

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Dedication

This work is dedicated to **Almighty God**, the creator of the entire universe who saw me through the period of my study, my wife **Hafsat Omolola Adeyemo,** who took good care of my home while I was several miles away, my sons **Ashrof Afolarin Adeyemo** and **Ahmad Afolabi Adeyemo**, my parents and my siblings.

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List of Abbreviations

ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90:10:1 v/v/v)
CEF	Chloroform/Ethyl acetate/Formic acid (5:4:1 v/v/v)
CFU	Colony forming unit
DPPH	1, 1 Diphenyl-2-picryl hydrazyl
DMSO	Dimethylsulphoxide
EMW	Ethyl acetate/Methanol/Water (40:5.4:4 v/v/v)
ESBL	Extended-spectrum-β-lactamase
Fig	Figure
FOX	Ferrous oxidation-xylenol
IC_{50}	Half maximal inhibitory concentration
LC	Liquid chromatography
LC_{50}	Lethal concentration for 50% of the cells
LOX	Lipoxygenase
LPS	Lipopolysaccharide
INT	Iodonitrotetrazolium chloride
MDR	Multi-drug resistant
MEM	Minimal Essential Medium
MH	Mueller Hinton
mL	Millilitre
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS	Mass spectrometry
N/A	Not applicable
ND	Not determined
NO	Nitric oxide
NMR (^{13}C and 1H)	Nuclear magnetic resonance (carbon 13 and proton)
OD	Optical density
PBS	Phosphate buffered saline
PRU	Pretoria University

REC	Research Ethics Committee
Rf	Retardation factor
ROS	Reactive oxygen species
SI	Selectivity index
Sp	Species
TA	Total Activity
TAA	Total Antibacterial Activity
TFC	Total flavonoid content
TLC	Thin layer chromatography
TPC	Total phenolic content
TSB	Tryptic soy broth
WHO	World Health Organisation
w/v	Weight per volume
v/v	Volume per volume

Abstract

Diarrhoea is the incessant movement of the bowel due to an alteration of the gastrointestinal tract's normal physiological and morphological functioning. Diarrhoea is a symptom of an infection in the bowel of man and animals, which can be caused by several species of bacteria, viruses, and parasites. The emergence of antimicrobial resistance has reduced the effectiveness of conventional antibiotics, leading to difficulty treating infections, and even death, especially in young animals and children under five. It is, therefore, necessary to search for alternative diarrhoeal remedies and determine their mode of activity. This study evaluated the antibacterial activity, cytotoxicity, antioxidant activity, anti-inflammatory activity, antibiofilm and quorum sensing inhibition of some selected South African medicinal plant species used to treat infectious diarrhoea.

Extracts of the leaves of *Bauhinia bowkeri, B. galpinii, B. variegata, Searsia batophylla, S. lancea, S. leptodictya, S. pendulina* and *S. guienzi,* as well as *Brachylaena transvaalensis* were screened for their antibacterial potency against a panel of bacteria using standard methods. Six plant acetone extracts of *B. bowkeri, B. galpinii, S. batophylla, S. lancea, S. leptodictya* and *S. pendulina* had good to outstanding antibacterial activity against the tested bacterial strains. *B. bowkeri* had the best minimum inhibitory concentration (MIC) of 0.01 mg/mL against *Salmonella* Enteritidis. Similar antibacterial activity was obtained against *Escherichia coli* isolates by *B. bowkeri*. The six selected plant acetone extracts exhibited notably less cytotoxicity in terms of higher LC_{50} values against Vero kidney cells compared to doxorubicin, the positive control, with values ranging from 0.03 to >1 mg/mL. The selectivity index (SI) values against the pathogens tested ranged from 0.09 to 50.75. The SI values greater than 10 are promising because the ratio of therapeutic concentration to toxic concentration is highly favourable.

Phytochemical profiling of acetone leaf extracts was evaluated using thin layer chromatography eluted with different solvent systems of varying polarity. Total polyphenol and total flavonoid contents, antioxidant and anti-inflammatory activities were determined on the antibacterially active extracts of the plant extracts a using microtube dilution method. The phytochemical study revealed that the plant extracts contain a broad spectrum of compounds. *S. lancea* and *S. batophylla* had the highest level of flavonoids (270.55 ± 30.80 mg/QE/g) and phenolic content (443.36± 24.65 gallic acid equivalent/g), respectively. In addition, the extract of *S. batophylla* exhibited the most significant free radical scavenging activity in the ABTS

assay (IC_{50} = 0.19 ± 0.02 µg/mL), while *B. bowkeri* had the best activity in the DPPH assay (IC_{50} = 14.31 ± 0.92 µg/mL). In the anti-inflammatory assays, *S. batophylla* acetone leaf extract was the most active in terms of inhibiting lipoxygenase as well as nitric oxide production.

Against at least one tested bacterial species, all the plant extracts reduced the formation of bacterial biofilms by more than 50%. Furthermore, only the fully-grown biofilm of *B. cereus* was sensitive to the tested acetone extracts, with *S. pendulina* acetone leaf extract at 1 mg/mL eradicating 98.22% of preformed biofilm. *S. lancea* exhibited the highest efficacy, with the extracts' minimal quorum sensing inhibitory concentrations ranging from 0.08 to 0.32 mg/mL. The optimum half maximum violacein production inhibitory value was 0.17 mg/mL for the *S. lancea* extract indicating promising ability to inhibit quorum sensing.

Bioassay-guided fractionation of the chloroform fraction of the acetone leaf extract of *S. batophylla* yielded three compounds, namely dodecanamide, 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione and 3-oxo-olean-18-en-28-oic. This is the first report of compounds isolated from the plant. However, individual compounds had poor antibacterial activity compared to the fraction from which they were isolated, which implies that their antimicrobial activity may be synergistic. All the compounds had low cytotoxicity to mammalian Vero cells.

This study reveals that the selected indigenous plant species based on ethnobotanical use and preliminary antibacterial efficacy are worth considering in developing alternative antimicrobial therapy in prevention and management of diarrhoea.

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Chapter 1: INTRODUCTION

1.1 Diarrhoea

Diarrhoea occurs when the gastrointestinal tract (GIT) is disrupted from its normal physiological and morphological functions, resulting in frequent bowel movements. It manifests by the rise in volume, frequency and fluidity of stools to more than three times within 24 hours (Noubissi *et al.*, 2019). According to Kotloff (2017), after pneumonia, diarrhoea is the leading cause of infant death, particularly in developing nations, accounting for approximately 10% of all deaths in children. In the animal health sector, diarrhoea is notorious for inflicting severe physiological and morphological impairments such as dehydration and weight loss, sometimes resulting in the death of animals if not treated promptly and appropriately (Arnaiz *et al.*, 2021). Diarrhoea causes a major setback in profitable animal production and thus reduces the quality of dairy and meat products (Chi *et al.*, 2002). The young of animals such as goats, sheep, cattle and other ruminants are mostly affected by this condition (Foster and Smith, 2009; Sweeny *et al.*, 2012; Cheng *et al.*, 2021).

Diarrhoea could result from parasite or microbe infections, interrupted homeostasis (in terms of secretion and absorption) of the gastrointestinal tract, stress, a dysfunctional immune system or inflammation of the intestine (Kelly *et al.*, 2018). It could be a symptom of other diseases like HIV and AIDS, cholera, irritable bowel syndrome and, more recently, severe cases of Covid-19 (Sévère *et al.*, 2016; Semá Baltazar *et al.*, 2017; Chan *et al.*, 2020). It may also be caused by secretory hypo-motility, which is a consequence of reduced reabsorption of intestinal fluid, or hyper-motility, which results in increased intestinal motility (Frampton, 2013; Kelly *et al.*, 2018).

Agents of infectious diarrhoea include viruses, parasites and bacteria. Infectious diarrhoea is mainly regarded as gastroenteritis (Bányai *et al.*, 2018; Kelly *et al.*, 2018). Viruses such as norovirus, adenovirus, rotavirus, and astroviruses cause many infections (Bányai *et al.*, 2018). Parasitic infectious diarrhoea is mostly caused by protozoans like *Entamoeba histolytica* and *Cryptosporidium* species to mention a few (Rossignol *et al.*, 2012).

Bacterial infectious diarrhoea is responsible for 10-55% of diarrhoeal episodes, especially in the developing world (Fhogartaigh and Edgeworth, 2009). Gram-negative opportunistic bacteria belonging to the Enterobacteriaceae family, which cause numerous infections, are largely responsible for this diarrhoea. Many of these species are found in the normal flora of

the intestine. The most frequently isolated bacterial species causing enteric diseases is *Escherichia coli* (Li *et al.*, 2022). There are five pathotypes of *E. coli,* namely enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and enteropathogenic *E. coli* (EPEC). These are all referred to as diarrhoeagenic *E. coli* (DEC) (Snehaa *et al.*, 2021). Apart from diarrhoea, *Escherichia coli* is also a major cause of nosocomial infections such as surgical site infections, urinary tract infection, pneumonia and bacteraemia (Yang *et al.*, 2020). Other diarrhoeic agents of the family Enterobacteriaceae include *Salmonella* spp*., Shigella* spp., *Klebsiella* spp. and *Enterobacter* spp. Non-Enterobacteriaceae species of bacteria causing diarrhoea comprise species such as *Staphylococcus aureus, Vibrio cholerae, Clostridium perfringens, Bacillus cereus, Listeria monocytogenes* and *Pseudomonas aeruginosa* (Okomo, 2018).

Conversely, diarrhoea can be considered physiologically beneficial as a mechanism of expunging harmful luminal substances out of the GIT. It becomes a medical condition when the loss of body electrolytes and fluids exceeds the replacement mechanism by the body (Kelly *et al.*, 2018).

1.2 Management of diarrhoea

Several variables come into play in the incidence and occurrence of diarrhoea, such as biological, socio-cultural and environmental factors. Since about four decades ago, the scientific research world has established efficacious measures to decrease diarrhoeal disease incidence and its deleterious effect. Some measures were targeted to lower the incidence by improving environmental conditions such as food safety, effective waste treatment, public awareness and good water supply (Brandt *et al.*, 2015).

Vaccines have been developed, especially against viral-mediated diarrhoeal disease, to mitigate the mortality rate in infants. In 2007, two oral vaccines, RotaTeq (a five-valent human-bovine reassortant vaccine) and Rotarix (a monovalent human vaccine) were developed against rotaviruses which are the most prevalent cause of acute diarrhoea, especially in children (De Francesco *et al.*, 2019). Oral rehydration therapy (ORT) and intravenous rehydration therapy (IRT) have been significant milestone solutions in medicine in the treatment of diarrhoeal disease since the twentieth century (Noubissi *et al.,* 2019).

A balanced diet is often recommended as diarrhoea is widespread in malnourished children in developing countries (WHO, 2009). Food supplements such as Vitamin A and adequate zinc

intake result in a relatively significant reduction in diarrhoea incidence. In addition, exclusive breastfeeding for the first six months of life and supplemental breastfeeding for up to two years was recommended (WHO, 2009).

Many drugs with different mechanisms of action have been deployed in the treatment of diarrhoea. Antiemetic drugs such as promethazine, which blocks the H1-histamine receptor are used when diarrhoea is associated with intense vomiting. Antidiarrhoeal and antisecretory drugs such as loperamide and other adsorbents have been used in the past. However, their usage was faced with challenges varying from toxicity to adverse effects, especially on the nervous system (Brandt *et al.*, 2015).

Antibiotics are one of the most significant discoveries in the medical sector. Prior to antibiotics, common infections were a major cause of death. However, antibiotic usage is marred with limitations. Antibiotics may sometimes trigger haemolytic uremic syndrome, most commonly in *E. coli* 0157:H7 mediated infections (Singh *et al.*, 2020). Another problem with antibiotics is that they alter the microbiota of the gastrointestinal tract (Kakoullis *et al.*, 2019). Despite the progression of antibiotics as a major therapy, morbidity and mortality rates resulting from infectious agents continue to rise.

1.3 Antimicrobial resistance

Antimicrobial resistance is a serious concern as it poses more of a threat to humanity than global warming (Davies *et al.*, 2013). Resistance of *Escherichia coli* and other Enterobacteriaceae family members to antibiotics is rapidly on the increase, especially to fluoroquinolones, cephalosporins and many others. Other diarrhoeagenic agents such as *Staphylococcus aureus, Vibrio cholerae, Clostridium perfringens* and *Listeria monocytogenes* have shown significant resistance to conventional antibiotics. In South Africa, antimicrobial resistance to fluoroquinolones is increasing among *E. coli* isolates in dogs, especially those co-presenting with urinary tract infections (Qekwana *et al.*, 2018). Drug resistance in microorganisms is mainly caused by extensive use of antibiotics as prophylactics in animal feed and poor antimicrobial stewardship, (O'Neill, 2016). The menace of resistance to antibiotics in human and animal infectious diseases will not end anytime soon, hence the need for alternative or complementary therapy that is affordable, efficacious, safe and widely available, especially to commercial farmers. Plants serve as dietary sources and therefore could be valuable in taking the place of antibiotic feed additives.

1.4 Importance and general use of medicinal plants

Medicinal plants (also known as herbal medicines, phytomedicines or pharmacologically active plants) have been used for many years as therapy by different cultures or as precursors in the manufacturing of drugs (Sofowora *et al.*, 2013). Cultures around the globe have different local techniques and common practices used in the treatment of both human and animal medical challenges. This treatment information is usually verbally transmitted from one generation to the other (Grenier, 1998). The World Health Organisation (WHO) encourages governments to allow indigenous medicine to thrive by supporting medicinal plant research that will be useful in managing various ailments in man and animals (WHO, 2013a).

South Africa houses diverse species of plants with therapeutic value, with up to 60% of the populace patronising traditional healers as substitute or complementary approaches to conventional medical services (Van Vuuren, 2008). Medicinal plants possess therapeutic attributes due to the presence of biosynthesis of secondary metabolites such as flavonoids, saponins, terpenoids, phenols and alkaloids. These phytochemicals naturally found in plants exhibit various medicinal properties, and thus are valued assets in developing new products, especially in the pharmaceutical industry (Crozier *et al.*, 2006). However, scientific advancement requires more than long-term traditional use of herbal medicines; thus, the need for empirical and scientific information expressing their potency, safety and quality assurance. Therefore, different bioassays must be carried out on the plants species to establish their bioactive capabilities and safety.

This study seeks to contribute to existing information on plants with antibacterial, antioxidant, anti-inflammatory, antibiofilm and anti-quorum sensing properties that may be useful in managing diarrhoea. Preliminary antibacterial activity studies have been conducted on different species of plants in the Phytomedicine Programme of the Department of Paraclinical Sciences, University of Pretoria (Pauw and Eloff, 2014). Plants with antimicrobial activity against microorganisms related to etiological agents of various diseases have been identified in the Phytomedicine database. This is a valuable resource for selecting plants for further investigation.

In this study, plant species were selected for scientific evaluation based on the following criteria:

1. Preliminary antibacterial assessment of the plants and their related species in the Phytomedicine Programme of the Department of Paraclinical Sciences, University of Pretoria.
2. Chemotaxonomic relatedness to plant species recorded as having ethnopharmacological use in treating diarrhoea because of the possibility of producing similar potentially bioactive chemical compounds.
3. Availability of plants for collection in botanical gardens.

Based on the listed criteria, leaves of ten different plant species from the families of Anacardiaceae, Fabaceae and Asteraceae were selected for targeted assessment of their biological activities. The selected plant species were: *Searsia pendulina* (Jacq.) Moffett, *Searsia leptodictya* (Diels) T.S.Yi, A.J.Mill. & J.Wen, *Searsia gueinzii* (Sond.) F. A Barkley, *Searsia lancea* L.f. F.A.Barkley, *Searsia batophylla* (Codd) Moffet, *Bauhinia galpinii* N.E.Br. (Syn *Bauhinia galpinii* var. *ungulata* L.), *Bauhinia bowkeri* (Harv.) A.Schmitz, *Bauhinia variegata* L. and *Brachylaena transvaalensis* E. Philips and Schweick.

1.5 Study Hypothesis

Extracts, fractions and isolated bioactive compounds from the selected plant species have antimicrobial efficacy, good antioxidant and anti-inflammatory effects; they are non-cytotoxic, with good antibiofilm and quorum sensing inhibition abilities, and thus have potential for being developed into preventative agents or treatments for diarrhoea.

1.6 Problem statement

The management of diarrhoea in humans and farm animals poses a challenge, especially in developing countries marred with poverty, non-availability of potable water and inadequate medical facilities, consequently leading to substantial economic loss and sometimes loss of lives. Bacteria belonging to the Enterobacteriaceae family are commonly responsible for bacterial infectious diarrhoea. The Enterobacteriaceae is a sizeable family of Gram-negative, facultative anaerobic, rod-shaped bacteria including the genera *Salmonella, Klebsiella, Shigella, Escherichia, Enterobacter* and *Proteus*. They are environmental bacteria capable of producing diseases by toxin production and invasive action (Li *et al.*, 2022). Various antibiotics with important therapeutic features are used to treat microbial infections. However, notable resistance has been linked to Enterobacteriaceae species. The resistance of infectious agents to many pharmacological drugs has grown due to the extensive and indiscriminate use of these

antimicrobial agents (Mladenovic-Antic *et al.*, 2016). Since 1992, resistance to virtually all therapeutically useful antibiotics has been documented (Sengupta and Chattopadhyay, 2012). Treatment of these infectious agents is even more difficult in immunocompromised patients. Hence, the need for an alternative to antibiotics (Eloff and McGaw, 2014).

1.7 Justification of the study

Plant extracts are good sources of antimicrobial compounds with a higher degree of molecular diversity when compared to chemically synthesised compounds (Surendra and Roopan, 2016). Antiviral, anti-inflammatory, and anticancer plant-based regimens have been developed. No antibacterial antibiotics from plant sources have been developed due to inherent challenges such as unwillingness of pharmaceutical industries to support "once-off" drugs (Vaou *et al.*, 2021). However, medicinal plants may have potential in the development of preparations with multiple activities that could be beneficial in helping the host to overcome the infectious agent causing diarrhoea. One of the merits of medicinal plants is that they are typically available with generally low adverse effects compared to some synthetic agents (Wadkar *et al.*, 2008). The rate at which bacteria become resistant is faster than the discovery rate of new compounds, thus leading to public health risk (Ling *et al.*, 2015). This project serves to complement and build on previous work done in the Phytomedicine Programme on pharmacological studies of medicinal plants, providing leads for development of new plant-based products in treating diarrhoeagenic pathogens. Many plants have been discovered to possess bioactive compounds, but the mechanisms of action of these bioactive chemical compounds remain largely unexamined.

1.7 Research aims and objectives

The aim of this study was to investigate the biological activity of plant extracts, fractions and active compounds from selected plant species against diarrhoeagenic pathogens. Other complementary biological activities such as anti-inflammatory and antioxidant efficacy were also investigated. This will contribute to the development of new herbal products for therapeutic purposes in both humans and animals.

The specific objectives of this study are to:

1. select plant species with known preliminary activity against Enterobacteriaceae species and those with ethnopharmacological use in treating diarrhoea in South Africa.

2. determine the antimicrobial potency of the prepared plant extracts against selected diarrhoeagenic bacterial pathogens.
3. evaluate cytotoxicity, anti-inflammatory and antioxidant activities of the most active plant extracts.
4. select a plant species with a high selectivity index and conduct bioassay-guided fractionation to isolate and characterize the active component(s).
5. evaluate the anti-quorum sensing properties of the plant extracts and active compound(s).
6. evaluate inhibition of biofilm formation of the plant extracts and their active compound(s).

CHAPTER 2: LITERATURE REVIEW

2.1 Diarrhoea and its effect on man and animals

Diarrhoea is the abnormal and rapid passage of stomach contents through the intestine. It is caused by a disruption in the gut's dynamic and delicately balanced absorption and secretion of water and electrolytes. Diarrhoea comes from the Greek words that mean "to flow through" (Kelly *et al.*, 2018)**.** The frequency of defecation in humans varies between individuals and over time within the same individual, but one movement per day is generally regarded as the median. According to the World Health Organization, diarrhoea is defined as three or more loose or watery daily stools. In animals, it is the most common symptom of illnesses resulting in substantial morbidity, mortality and loss of productivity (Santin, 2020). Diarrhoea has three clinical episodes: acute watery diarrhoea, which lasts a few hours or days, acute bloody diarrhoea, often known as dysentery, and chronic diarrhoea, which lasts 14 days or more (WHO, 2020). Diarrhoea may be classified according to pathogenesis or stool characteristics as secretory, osmotic or inflammatory diarrhoea.

2.1.1 Secretory diarrhoea

Secretory diarrhoea is usually caused by increased secretion or reduced absorption of body metabolites. This is characterised by stimulation of chloride ion secretion (Cl^-). To maintain charge balance, sodium ion (Na^+) is released along with water, leading to increased mucosal permeability. There is little to no structural damage and it may persist even with ingestion of food (Moon *et al.*, 2015).

2.1.2 Osmotic diarrhoea

Osmotic diarrhoea occurs when too much water is drawn into the bowel. Drinking solutions high in sugar or salt can cause osmotic diarrhoea by drawing water from the body into the colon (Kelly *et al.*, 2018). It can also be caused by maldigestion when nutrients are left in the lumen for extended periods, and thus pull water (coeliac disease). In addition, osmotic diarrhoea could be triggered when healthy individuals consume too much lactose, vitamin C, fructose, sugar alcohols (e.g., sorbitol) or magnesium. In most cases, osmotic diarrhoea resolves when the offending agent or inducer is stopped (Baldi *et al.*, 2009).

2.1.3 Inflammatory diarrhoea

Inflammatory diarrhoea is epitomised by blood, mucus and leukocytes in the stool. Typically, the mucosal lining is damaged, and features of other types of diarrhoea could also be present. This may be caused by autoimmune disorders such as inflammatory bowel disease, microbial infections or their products such as toxins, and other diseases like colon cancer and tuberculosis (Moon *et al.*, 2015).

2.2 Agents of diarrhoea caused by pathogens

2.2.1 Protozoa induced diarrhoea

2.2.1.1 *Giardia duodenalis*

G. duodenalis (syn *Giardia intestinalis, Giardia lamblia*) is a flagellated parasitic microbe of the genus *Giardia* that colonises the small intestine and causes giardiasis, which is characterised by diarrhoea, abdominal pain and loss of weight (Simner, 2017). Sometimes, dysentery and vomiting are accompanying symptoms (Minetti *et al.*, 2016). *Giardia* has two stages in its life cycle: cysts and trophozoites. The cyst form is the infectious stage, and this metamorphoses into the trophozoite form in a living host. This trophozoite attaches itself to the intestinal wall and reproduces within it. As trophozoites progress through the gastrointestinal tract, they revert to the cyst form, which is expelled with faeces. Only a few of these cysts need to be ingested to cause infection in another host (Coelho *et al.*, 2017). Giardiasis causes reduced expression of brush border enzymes, physical alterations to the microvillus, increased intestinal permeability, and apoptosis of small intestinal epithelial cells (Buret, 2008). Manifestation of symptoms usually begins within 1-3 weeks of exposure.

One of the most frequent causes of waterborne illness is the parasite *Giardia duodenalis*, which has a widespread distribution worldwide. Although outbreaks sometimes occur in developed nations as well, they are more likely in poorer nations where access to clean water and sanitary facilities is poor (Einarsson *et al.*, 2016). Both people and animals are susceptible to the parasite, and animal-to-human transmission is also possible. This condition can be resolved without treatment, however, metronidazole can be used as a treatment if symptoms persist (Barr and Smith, 2014).

2.2.1.2 *Cryptosporidium parvum*

Cryptosporidiosis is a parasite illness of the mammalian digestive tract caused by *C. parvum*. Acute, watery and non-bloody diarrhoea are the most common signs of *C. parvum* infection (O'Hara and Chen, 2011). Infection is caused by ingestion of the oocyst. In immunocompromised individuals, diarrhoeal episodes might occur 10–15 times per day. Other symptoms of *C. parvum* infection include anorexia, nausea/vomiting, and stomach discomfort. The lungs, liver and gall bladder are among the extra-intestinal locations where it causes respiratory cryptosporidiosis, hepatitis and cholecystitis, respectively (O'Hara and Chen, 2011).

2.2.1.3 *Entamoeba histolytica*

Entamoeba histolytica causes amoebiasis, a parasitic infectious disease. About 55 000 people are estimated to die of *E. histolytica* infection annually (Rawat *et al.*, 2020). The organism causes direct tissue damage to the host cells by parasite invasion and inflammation. The parasite possesses various enzymes, such as lipases, cysteine proteases and pore-forming proteins, typically used to break down bacteria in feeding vacuoles. However, these enzymes can also cause epithelial cell lysis by inducing cellular necrosis and programmed cell death when the trophozoite comes across the epithelial cells and attach via the lectin. In rare cases, the enzymes produced enable invasion of the intestinal epithelium, blood arteries, liver and other organs. The trophozoites consume these defunct cells. The trophozoite attracts immune cells to the damaged epithelial cell layer, which can then lyse them, releasing the lytic enzymes of the immune cells into the surrounding tissue, causing tissue destruction in a cascade reaction. Blood vessels can also be damaged, resulting in bloody diarrhoea and amoebic dysentery (Kantor *et al.*, 2018). Antiparasitic drugs, including metronidazole, tinidazole, and iodoquinol are frequently used to treat amoebiasis (Hashmey *et al.*, 1997). These medications function by preventing the parasite's development and reproduction. To remove the affected tissue in serious situations, surgery may be necessary. The parasite can also be prevented from spreading by practising good hygiene and sanitation.

2.2.1.4 *Cyclospora cayetanensis*

In humans and other primates, *Cyclospora cayetanensis* is a coccidian parasite that causes cyclosporiasis, a diarrhoeal illness. *Cyclospora cayetanensis* has become an endemic cause of

diarrhoea in tropical countries and a source of traveller's diarrhoea and food-borne diseases in developed countries (Ortega and Sanchez, 2010). This infection is self-limited, however, in immunocompromised individuals, the illness may present as severe or persistent diarrhoea. The primary mode of transmission is through faecal-oral transmission by ingesting sporulated oocyte in contaminated fresh food (Ortega and Sanchez, 2010). Direct individual transmission is not common due to the need for external sporulation of the oocyte, usually in the environment between 7-14 days (Almeria *et al.*, 2019). Despite being widespread across the globe, cyclosporiasis is mostly detected in tropical and subtropical regions. This parasite is widespread in some several Middle Eastern nations, Central and South America and the Indian subcontinent (Marques *et al.*, 2017). Antiparasitic drugs like trimethoprim-sulfamethoxazole or macrolides are used to treat cyclosporiasis (Rossignol *et al.*, 2006). These medications function by preventing the parasite's development and reproduction. Other drugs can be needed in extreme circumstances to treat symptoms and avoid consequences. The parasite can also be stopped from spreading by following good hygiene and sanitation practices, choosing and preparing food carefully, and other measures (Rossignol *et al.*, 2006).

2.2.2 Virus-induced diarrhoea

2.2.2.1 Rotavirus

Rotavirus is one of the leading causes of gastroenteritis in children. Globally, more than 100 million cases of rotavirus induced gastroenteritis cases are reported and over 200 000 deaths annually especially in children less than age of 5 years (Tate *et al.*, 2016). About 90% of the incidences occur in low-income nations, where the availability of rehydration treatment is less than in developed countries (Bányai *et al.*, 2018). Diarrhoea caused by rotavirus infection is non-inflammatory and includes malabsorptive and secretory processes. A rotavirus secretory component associated diarrhoea causes an increase in intracellular Ca2+, which is necessary for Cl^- secretion activation. In addition, diarrhoea results from the alteration of plasma-protein function leading to the reduction in absorption of Na+ by suppression of sodium-hydrogen activity (Zachos *et al.*, 2014). Supportive care, such as oral rehydration therapy, is the mainstay of rotavirus treatment to control symptoms and avoid dehydration. Since rotavirus infections cannot be treated with antiviral drugs, oral or intravenous solutions can replenish lost fluids and avoid dehydration (Hartman *et al.*, 2019).

2.2.2.2 Norovirus

Norovirus is a significant cause of gastroenteritis globally, and it affects people of all ages (Bányai *et al.*, 2018). The virus infects the small intestine and induces gastrointestinal tract dysfunction by causing malabsorption due to the surface area reduction of the villus (Troeger *et al.*, 2009). These highly contagious viruses are released with faeces of infected patients. Contamination is through direct or indirect ingestion of food or water polluted with faecal matter. Characteristically, the duration of sickness is shorter in norovirus infection compared to rotavirus infection (Teunis *et al.*, 2008).

The best treatment approach for preventing norovirus outbreaks is prevention. To stop the virus from spreading, it is crucial to maintain good hygiene habits, including routine hand washing, avoiding tainted food and water, and thoroughly cleaning and disinfecting infected surfaces (Guix *et al.*, 2019).

2.2.2.3 Human immunodeficiency virus (HIV)

HIV has been studied for over three decades as a direct cause of gastrointestinal sickness associated with diarrhoea and weight loss (Ramos *et al.*, 2012). Diarrhoea is caused by cytokines released by the immune cells infected with HIV or the alteration of mucosal structural arrangement when infected with the virus. It is also an important secondary enteric condition due to suppressed immune cells (Lucas and Nelson, 2015).

The underlying cause determines how to manage diarrhoea in HIV-positive individuals. Antibiotics or antifungal drugs are typically used as part of the treatment to combat infections, or the medication regimen may be changed to control adverse effects. Probiotics, dietary adjustments, and other strategies may occasionally be suggested to treat symptoms and avoid consequences (Misau *et al.*, 2018).

2.2.2.4 Coronavirus

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the coronavirus disease 2019 (COVID-19). Covid-19 has led to a worldwide pandemic which has caused enormous morbidity and mortality and a negative impact on the global economy. Diarrhoea is one of the typical symptoms of Covid-19 illness (Huang *et al.*, 2020). SARS-CoV-2 gains entrance to intestinal epithelial cells via the angiotensin conversion enzyme 2 (ACE2) receptor (Hoffmann *et al.*, 2020). Covid-19 patients have higher leukocyte and plasma pro-

inflammatory cytokine numbers, thus mediating systemic and local inflammation (Lin *et al.*, 2020). The inflammatory response thus leads to mucosal damage and loss in enterocyte absorption ability (Gu *et al.*, 2020).

Diarrhoea caused by Covid-19 is currently without a particular therapy (Ye *et al.*, 2020). Supportive and symptom-management-oriented treatment is usually the practice. Hydration, electrolyte replenishment, and over-the-counter or prescription drugs to treat diarrhoea may all be necessary in this situation (McIntosh *et al.*, 2020). Hospitalization could be necessary in serious situations.

2.2.2.5 Other viruses

Apart from noroviruses and rotaviruses, adenoviruses, saproviruses and astroviruses are part of medically important viruses causing gastroenteritis. The use of broad base PCR, unbiased-viral metagenomics and pan-viral microarrays for diagnosis due to the shortcoming of laboratory diagnosis to identifying pathogens showed the presence of some Picornaviridae, Parvoviridae and Astroviridae families in gastroenteritis patients (Oude Munnink and Van der Hoek, 2016). However, it is somewhat difficult to distinguish between the gastroenteritis caused by these viruses and that of enteric bacteria in terms of clinical presentations. The main symptoms include vomiting and non-bloody diarrhoea. In some cases, abdominal cramps, nausea and fever may accompany the main symptoms (Bányai *et al.*, 2018).

2.2.3 Bacterial causes of diarrhoea

2.2.3.1 *Shigella* spp.

Shigella species including *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* are Gram-negative rod-shaped, facultative anaerobic and non-motile bacteria. They belong to the Enterobacteriaceae family, and cause acute gastroenteritis, which is one of the leading causes of illness and fatalities among children in underdeveloped countries (Malani, 2010). They generally elicit symptoms such as diarrhoea, fever, abdominal pain and chills (Sethuvel *et al.*, 2017). *Shigella dysenteriae* type 1 produces shigatoxin which has entero-invasive properties, causing diarrhoea and or dysentery with frequent mucoid bloody stools and stomach pains in a severe infection that can lead to death. *Shigella* bacteria proliferate within colonic epithelial cells, kill them, and subsequently move laterally to infect and kill neighbouring epithelial cells, resulting in mucosal ulceration, inflammation and bleeding (Livio *et al.*, 2014). Antibiotics,

such as ciprofloxacin, azithromycin, or ceftriaxone, are frequently used to treat shigellosis. By eliminating the bacteria, these medications shorten the length of symptoms (Bruzzese *et al.*, 2018).

2.2.3.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium occurring normally in both human and animal microflora. It is a common opportunistic pathogen (Lowy, 1998). Under favourable conditions, pathogenic *S. aureus* strains produce toxic shock syndrome toxins and heat-stable staphylococcal enterotoxins (Tong *et al.*, 2015). These toxins are known to induce diarrhoea. Also, depending on the site of infection and strains involved, *S. aureus* can cause pulmonary, skin and soft tissue, urinary tract, and food-borne infections as well as meningitis in humans and farm animals (Tong *et al.*, 2015). There have been outbreaks of *Staphylococcus* species worldwide, a frequent source of foodborne disease. Consumption of contaminated foods, such as dairy products, pork, poultry, and shellfish, is frequently linked to outbreaks (Todd and Notermans, 2011). People with compromised immune systems, such as small children, the elderly and those with underlying medical issues, are more likely to get staphylococcal diarrhoea (Pinchuk *et al.*, 2010).

Antibiotics like penicillin or cephalosporins are frequently used to treat staphylococcal diarrhoea (Gourama, 2020). Antibiotics prevent the bacteria's development and multiplication, which enables the body to get rid of the disease. In extreme circumstances, fluid replacement treatment may be necessary to control symptoms and prevent dehydration (Bartlett, 2002).

2.2.3.3 *Campylobacter jejuni*

Globally, *Campylobacter* species are one of the most prevalent causes of bacterial gastroenteritis. *Campylobacter* spp. are Gram-negative bacteria with a spirally curled structure that colonises the mucosal surfaces of most warm-blooded animals' digestive systems, mouth cavities and urogenital tracts (Humphrey *et al.*, 2007). Most cells have a polar flagellum on either one or both ends making *Campylobacter* species mobile (Facciolà *et al.*, 2017). Several virulence factors are required to start *Campylobacter* infection, including motility, chemotaxis, adhesion and penetration of the host cell, toxin generation, cell envelope structures, iron absorption system, multidrug resistance and bile tolerance, and stress response mechanisms (Johnson, 2018). Additionally, it may produce a biofilm, which is a polymeric matrix made up

of a group of microbial cells from the same or different microorganisms that are adherent to various surfaces (Teh *et al.*, 2014). Apart from virulence factors, earlier research has shown that antibiotic resistance is increasingly being detected in *Campylobacter* spp., notably in food-borne strains (Silva *et al.*, 2018). The World Health Organization reports that campylobacteriosis represents one of every four causes of gastrointestinal sickness in developed and developing countries (WHO, 2020). Poultry is one of the major reservoirs of *Campylobacter* species and comprises a major vehicle for human transmission (Hwang and Singer, 2020). A brief overview of human campylobacteriosis is represented in **Fig 2.1.**

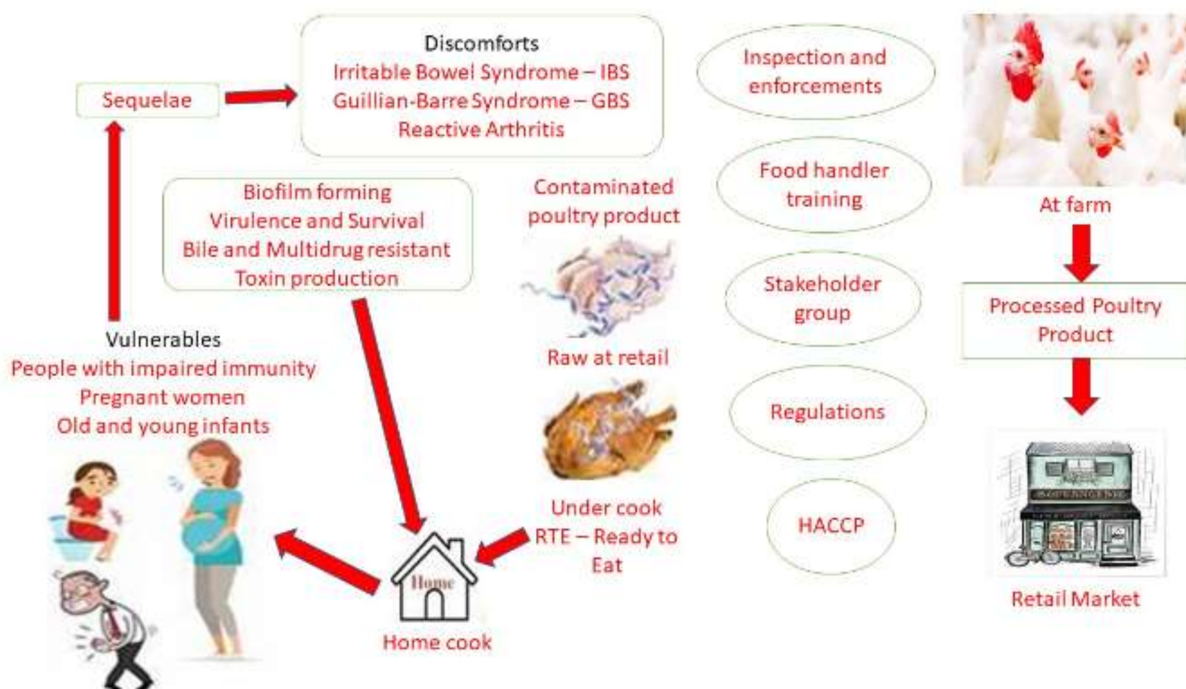

Fig 2.1: Campylobacteriosis transmission cycle (adapted from (Myintzaw *et al.*, 2022))

2.2.3.4 Vibrio cholerae

Vibrio cholerae is a significant bacterial species that causes cholera, a life-threatening form of severe diarrhoea that mostly affects third-world populations. *Vibrio cholerae* belongs to the Vibrionaceae family of Gram-negative bacteria. Despite rehydration with uncontaminated water and electrolytes, *V. cholerae* infection continues to cause substantial morbidity and death (Broeck *et al.*, 2007). *Vibrio cholerae* colonises the small intestine using toxin-co-regulated pili and engages with receptors on the intestinal epithelium. The bacteria then secrete the toxin, followed by the release of hemagglutinin/protease (HA/protease). This results in massive

secretion of water and electrolytes into the intestinal tract, followed by bacterial excretion. The patient's faeces resembles rice water and can amount to over 10 litres daily (Sánchez and Holmgren, 2005). The pathogen's major transmission mechanism is faeco-oral transmission, frequently resulting in violent outbursts (Broeck *et al.*, 2007).

Oral rehydration treatment, which replaces fluids and electrolytes lost due to diarrhoea, can be used to manage *Vibrio cholerae* infection (LaRocque and Harris, 2018). Antibiotics can also be used to treat the infection, lower the severity of the symptoms, and stop the bacteria from spreading (LaRocque and Harris, 2018).

2.2.3.5 *Bacillus cereus*

Bacillus cereus, B. mycoides, B. anthracis and *B. thuringiensis* are ubiquitous spore-forming rod-shaped bacteria that cause food-borne sickness (Savini, 2016). This group causes two main types of disease syndrome. Firstly, diarrhoea is caused by the production of heat-labile enterotoxins in the host's small intestine during the growth of vegetative cells. The infective dose is between 10^4 and 10^9 cells per gram of food (Dubey *et al.*, 2021). This syndrome is mild and characterised by stomach cramps and diarrhoea that continue for 6 to 12 hours following an incubation period of 8 to 16 hours. The sickness is known as the "long-incubation" or diarrhoeal form of the disease (Logan and Rodrigez-Diaz, 2006). Secondly, emetic syndrome, also known as "short-incubation" or the emetic version of the disease, is acute and more severe than diarrhoeal syndrome. The toxin cereulide causes emetic sickness, which is frequently generated in food before administration, resulting in food-borne poisoning (Fratamico *et al.*, 2010). This illness is caused by a short cyclic heat-stable peptide that induces vomiting 1 to 6 hours after ingestion. In the emetic type of sickness, the dosage is around 10^5–10^8 cells per gram (Logan and Rodrigez-Diaz, 2006; Dubey *et al.*, 2021). The principal mechanism of transmission is by the intake of *B. cereus* via contaminated food. The emetic type of food poisoning has been linked to rice and pasta consumption, whereas the diarrhoeal variety has been linked to milk products, vegetables, and meat (Logan and Rodrigez-Diaz, 2006).

The symptoms of a *Bacillus cereus* infection mostly go away on their own in a few days. In extreme circumstances, supportive treatment such as oral rehydration therapy, may be necessary to control symptoms and avoid dehydration (Meldrum, 2009; McDowell, 2022]. Since *Bacillus cereus* is frequently resistant to several antibiotics, medications are not typically advised for treating *Bacillus cereus* infection (McDowell, 2022).

2.2.3.6 *Yersinia* spp.

Yersinia enterocolitica is a Gram-negative coccus belonging to the family Enterobacteriaceae. Based on biochemical and serological characteristics, *Yersinia* has three species (*Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis*), six biovars and 70 serovars which are responsible for food-borne sickness (Fredriksson-Ahomaa, 2017; Bottone, 2018). Yersiniosis epidemics have been linked to food items like vegetables, dairy products, poultry, seafood and fermented products (Fig 2.2) (Bursová *et al.*, 2017; Martins *et al.*, 2018). The bacterial cell infects the small intestine at the terminal ileum and the proximal colon, which are the primary infection sites. Bacteria are thought to colonise the intestine via virulent gene products, and as the temperature rises to 37°C, they promote the expression of the virulent gene pYV, which secretes virulent components that elicit immune responses that promote fluid secretion in an affected host (Shoaib *et al.*, 2019). The treatment of *Yersinia* species varies with the serotype susceptibility pattern of isolates. However, they are mostly resistant to first-generation cephalosporin and macrolides (Valenti *et al.*, 2021).

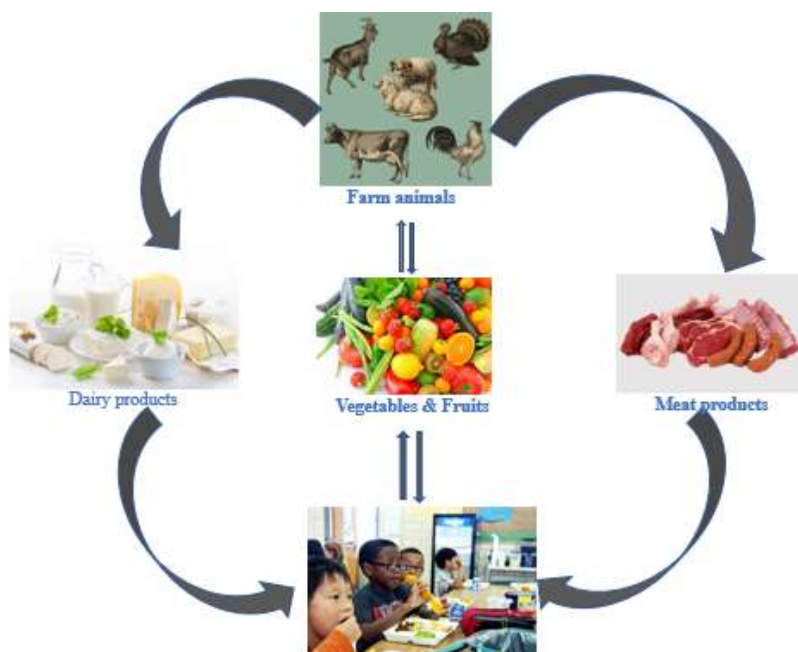

Fig 2.2: Yersinia *enterocolitica* transmission cycle (adapted from Shoaib *et al.,* 2019)

2.2.3.7 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, rod-shaped bacterium that is facultatively anaerobic but does not sporulate. *Listeria monocytogenes* causes listeriosis, and symptoms include fever, headache, diarrhoea, stomach ache and vomiting in susceptible hosts (Li, 2017). Despite its low number of cases, listeriosis has a significant fatality rate, and is a serious public health concern (Authority European Food Safety, 2021). *Listeria* spp. are extensively distributed in water, contaminated dairy foods, soil, faeces of mammals and birds and can infiltrate food-processing facilities through incoming raw materials or equipment mobility (Dhama *et al.*, 2015). Other than *L. monocytogenes*, *Listeria* spp. are seldom pathogenic and are not regarded as medically significant, but they can be utilised as hygiene indicators (Cairo *et al.*, 2021). Noninvasive *Listeria* gastroenteritis often resolves entirely within 2 to 3 days, especially in healthy individuals (Mehmood *et al.*, 2017). In other cases, penicillin is usually the drug of choice (Ooi and Lorber, 2005).

2.2.3.8 *Clostridium* spp.

Clostridia are rod-shaped, anaerobic, Gram-positive organisms that produce spores. The gastrointestinal microbiota of healthy and ill animals and humans contains these widespread soil-borne bacteria (Kiu and Hall, 2018). Despite this, representatives of this genus are generally recognised as human and animal gastrointestinal pathogens (Songer, 1996). Clostridial enterocolitis, enterotoxaemia, overeating disease, enterotoxemic jaundice, pulpy kidney disease, yellow lamb disease, braxy (bradsot), struck, lamb dysentery, clostridial abomasitis and clostridial enteritis are all prevalent gastrointestinal disorders in ruminant cattle (Simpson *et al.*, 2018). Except for braxy, which is caused by *C. septicum*, all these diseases are caused by *C. perfringens* subgroups (Simpson *et al.*, 2018). Since this agent has been discovered in both diarrhoeic and healthy calves, it is unclear whether *C. difficile* is a pathogen linked with diarrhoea in calves, or whether calves serve as reservoirs for human infections (Magistrali *et al.*, 2015). It is not preferred for a healthy carrier to use any antibiotics. The manifestation of the infection is usually through the presence of *Clostridium* toxin in faeces of diseased individuals, and vancomycin is usually the first line antibacterial agent (Bouza *et al.*, 2005).

2.2.3.9 *Salmonella* spp.

Salmonella species are Gram-negative, facultative anaerobes that were first isolated from pig intestines. Based on variations in their 16S rRNA sequences, *S. enterica* and *S. bongori* are the two major species with more than 2 500 serotypes (Eng *et al.*, 2015). *Salmonella enterica* can further be split into six different subtypes. In dairy cattle and other animals, *S. enterica* subspecies *enterica* is the most important species (Eng *et al.*, 2015; Holschbach and Peek, 2018). This subtype is responsible for 99 % of *Salmonella* infections in humans and warm-blooded animals. Over 2 500 serovars have been identified based on flagellar (H), somatic (O) and capsular (Vi) antigens; however, only a few are of clinical relevance (Holschbach and Peek, 2018). *Salmonella enterica* serovars such as *Salmonella* Typhimurium, *Salmonella* Newport, and *Salmonella* Dublin (a special bovine-adapted serotype) are the primary causes of diarrhoea in calves. The diarrhoea can range from watery to mucoid, containing fibrin and blood, and is frequently accompanied by fever and loss of appetite (Habing *et al.*, 2011; Delling and Daugschies, 2022). A variety of *S. enterica* serovars can infect sheep. Among other bacteria, *S.* Typhimurium and *S.* Dublin are important causes of diarrhoea in lambs (Methner and Moog, 2018). Antibiotics, including ampicillin, ciprofloxacin, and azithromycin are frequently used to treat *Salmonella* infections (Mathole *et al.*, 2017). The particular strain of *Salmonella* and its sensitivity to antibiotics determines the best antibiotic to use. Supportive treatment, such as hydration and electrolyte replenishment, and intravenous antibiotics in severe instances, may be administered (Kotloff, 2022).

2.2.3.10 *Enterococcus faecalis*

Gram-positive bacteria known as enterococci are present in the intestines of mammals, insects, reptiles, birds, and other creatures. Out of the 58 known enterococcal species, *E. faecalis* and *E. faecium*, which are the prevalent enterococcal species in the gut microbiota of man, causing majority of human's enterococcal infections. Other species include *E. gallinarum, E. hirae, E. avium*, *E. casseliflauvus, E. mundtii*, *E. durans* and *E. raffinosus* (Thaden *et al.*, 2014; Parte, 2018). However, enterococci become intestinal wall invaders when the delicate host-commensal interaction is disrupted due to antibiotic usage, abdominal surgery or host immune compromise (Butler, 2006). Ampicillin, vancomycin and gentamicin are common antibiotics used to treat *Enterococcus faecalis* infections. The choice of antibiotic is based on the infection's severity and the patient's general state of health (Kotloff, 2022).

2.2.3.11 *Escherichia coli*

Escherichia coli is a facultative anaerobic, Gram-negative bacillus that inhabits the bowel of humans and warm-blooded animals (Ewing, 1986). Most *E. coli* strains reside in the colon without causing disease in healthy species. However, they are the most common bacteria found in epidemic and endemic outbreaks and have been linked to both acute and moderate-to-severe diarrhoea in both healthy and immunocompromised hosts (Lindsay *et al.*, 2015). *Escherichia coli* strains that cause diarrhoea are termed diarrhoeagenic *E. coli* (DEC). Diarrhoeagenic *Escherichia coli* uses an interacting collection of virulence determinants to induce infections, which are adapted to the interaction of a certain bacteria with a specific host, resulting in a distinct pathotype (Chandra *et al.*, 2013). The major pathotypes of DEC are enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), shiga toxin *E. coli* (STEC) enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) (Harrington *et al.*, 2006). The severity of the infection determines the best therapy for diarrhoeal sickness caused by *E. coli*. The sickness often self-limits and passes on its own in a few days. Antibiotics, however, can be needed in extreme circumstances to treat diarrhoea (Imdad *et al.*, 2018). Additionally, maintaining hydration is essential for treating the sickness since dehydration can result from diarrhoeal illnesses linked to *E. coli* (Kotloff, 2022).

2.2.3.11.1 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* is distinguished by its capacity to produce one or both of two enterotoxins, which are known as heat-stable (ST) and heat-labile (LT) enterotoxins. Except for Southeast Asia, ETEC has traditionally been considered the most prevalent bacterial cause of diarrhoea worldwide (Duan *et al.*, 2019).

2.2.3.11.2 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* is commonly linked to watery diarrhoea. Enteropathogenic *E. coli* virulence genes are encoded in the chromosomal locus of enterocyte effacement (LEE) pathogenicity island (Schmidt, 2010). Enteropathogenic *E. coli* is identified via the LEE-encoded intimin gene eae. Some EPEC strains additionally carry the bundle-forming pilus gene bfp through the EPEC adherence factor plasmid (pEAF). (Gomes *et al.*, 2016). Bundle-forming pilus+ strains are known as typical EPEC (tEPEC), whereas bfp strains are known as atypical EPEC (aEPEC) (Alikhani *et al.*, 2006).

2.2.3.11.3 Shiga toxin *E. coli* (STEC)/Enterohaemorrhagic E. coli (EHEC)

Shiga toxic *E. coli* causes mild to bloody diarrhoea, usually accompanied by a fever and vomiting. Enterohemorrhagic *E. coli* (EHEC), a STEC subtype, is the source of outbreaks of food-borne illnesses that can be fatal. Additional virulence factors present in EHEC strains can cause haemorrhagic colitis (bloody diarrhoea) and, in rare instances, haemolytic uremic syndrome, a potentially fatal condition (Tarr *et al.*, 2005). The presence of the phage-encoded Shiga toxin distinguishes STEC bacteria, while EHEC strains have additional virulence components whose production causes haemorrhagic colitis (HUS). All STEC are EHEC; however, not all EHEC are STEC. Shiga toxic *E. coli* is diagnosed by looking for Shiga toxin variations (stx genes) and additional virulence genes, such as indicators expressed by the LEE pathogenicity island, which is also seen in EPEC (Jesser and Levy, 2020). The EHEC strains are usually diagnosed using plasmid-encoded haemolysin genes (Ud-Din and Wahid, 2014).

2.2.3.11.4 Enteroinvasive *E. coli* (EIEC)

The virulence mechanisms and clinical presentation of EIEC are identical to those initiated by closely related *Shigella* spp. The pINV F-type plasmid, which contains the genes necessary for enteroinvasive pathogenesis, is carried by both EIEC and *Shigella* bacteria. (Ud-Din and Wahid, 2014). Biochemical features or molecular tests which detect lacY gene which is specific to *E. coli*, used to identify EIEC isolates from *Shigella* spp. (Dhakal *et al.*, 2018).

2.2.3.11.5 Enteroaggregative *E. coli* (EAEC)

The extra-intestinal infectious pathogen called EAEC has the potential to cause diarrhoea. Infected EAEC cells form a stacked-brick or honeycomb shape when they adhere to one another and to the intestinal epithelial cells of the host (Kaur *et al.*, 2010). Several chemical targets have also been utilised to identify the EAEC pathotype; however, the pathotype is quite diverse. Enteroaggressive *E. coli* marker genes include the plasmid-encoded biofilm-forming gene aatA, the transcriptional activator egg, and the gene on a genomic island aaiC with a type-VI secretion system (Dudley *et al.*, 2006).

2.2.3.11.6 Diffusely adherent *E. coli* (DAEC)

Although epidemiological data links DAEC to diarrhoea in children in low-income countries, its position as a diarrhoeal disease agent is questionable due to inconclusive challenge

experiments and large rates of asymptomatic carriage (Servin, 2005). DAEC detection methodologies are not well-defined. Diffusely adherent *E. coli* strains were identified based on the presence of adhesion genes expressed by the physically and functionally similar afa, dra, or daa operons (Le Bouguénec and Servin, 2006). Although there are tests to identify the afa, dra, and daa operons' daaC, daaE, afaB, and afaC genes, however, they cross-react with known EAEC genes. (Servin, 2014).

2.3 Management of diarrhoea

Damage to the intestinal mucosa is caused by the common pathogenic agents involved in diarrhoea, resulting in mixed malabsorptive and secretory diarrhoea. Even if therapy against the causative bacteria were available, the pathophysiological process would make it unlikely that the duration of diarrhoea could be modified significantly (Foster and Smith, 2009).

2.3.1 Oral rehydration therapy

In uncomplicated diarrhoea, replacing fluid and electrolyte losses remains the single most critical therapy approach (Lorenz *et al.*, 2011). Oral rehydration therapy, which was first created in human medicine to treat cholera, is widely regarded as one of the most significant medical breakthroughs of the twentieth century (Victora *et al.*, 2000). An effective oral rehydration solution (ORS) must be easily digested, be able to normalise the extracellular fluid volume, and rectify acidosis (Michell, 2005). The ORS does not halt the diarrhoea episode but rather makes the absorption of water and solutes exceed their secretion (Kelly *et al.*, 2018).

2.3.2 Antidiarrhoeal drugs

Antidiarrhoeal therapy such as loperamide is the most often used antimotility medication, and it is probably most effective when used in conjunction with an antibiotic (Murphy *et al.*, 1993). The introduction of an enkephalinase inhibitor, racecadotril, with an absorptive effect due to its capacity to enhance endogenous enkephalins in the colon, has been a significant advancement in antidiarrhoeal treatment. This is a good way to reduce bowel movement frequency (Kelly, 2019).

2.3.3 Probiotics

Probiotics are living microbes that provide a health benefit to the host when taken in adequate quantities (FAO and WHO, 2002). Their mode of action includes: (i) maintenance of the

gastrointestinal barrier function (ii) regulation of intestinal microbiota homeostasis, inhibition of procarcinogenic enzymes and (iii) interference with the ability of pathogens to colonise and infect the mucosa, immunomodulatory effects, inducement of enzymatic activities to promote nutrient absorption and production of bacteriocins (Timmerman *et al.*, 2005; Mazmanian *et al.*, 2008). Antibiotics and other pharmaceutical compounds were widely utilised in the past, primarily to alter the intestinal flora in humans and animals, but also to increase production and animal development. Long-term usage of such drugs has resulted in the development of drug-resistant bacteria, providing a health risk to consumers, and having a detrimental environmental impact (Biernasiak *et al.*, 2010). This led to the ban on antibiotic-based growth stimulators by the European Union in January 2006. Animal intestinal pathogens such as *Campylobacter, Salmonella, Listeria* and *Yersinia* are a direct source of food contamination and zoonotic infections. As a result, new animal breeding methods are being adopted to improve meat quality and safety while also considering animal welfare and environmental protection (Markowiak and Śliżewska, 2018). The most significant genera used as probiotics are *Lactobacillus, Enterococcus, Bifidobacterium, Streptococcus, Leuconostoc* and *Lactococcus* (Vasiljevic and Shah, 2008). Probiotics such as *Lactobacillus rhamnosus*, *L. reuteri, L. casei* and *Bifidobacterium lactis* have been demonstrated in well-controlled clinical studies to reduce the duration of severe diarrhoea (Isolauri *et al.*, 2002; Das *et al.*, 2022). However, probiotics used in human medicine, animal feed and feed additives must fulfil certain requirements as indicated in Fig 2.4. Probiotics are rapidly gaining acceptance as a viable alternative to traditional antibiotic medication, owing to their low cost and lower risk of resistance due to their diverse mode of action. They have also been shown to improve the immunological response, reduce harmful bacterial colonisation, lower serum cholesterol, and prevent colon cancer, in addition to their usefulness in diarrhoea control (Narayan *et al.*, 2010).

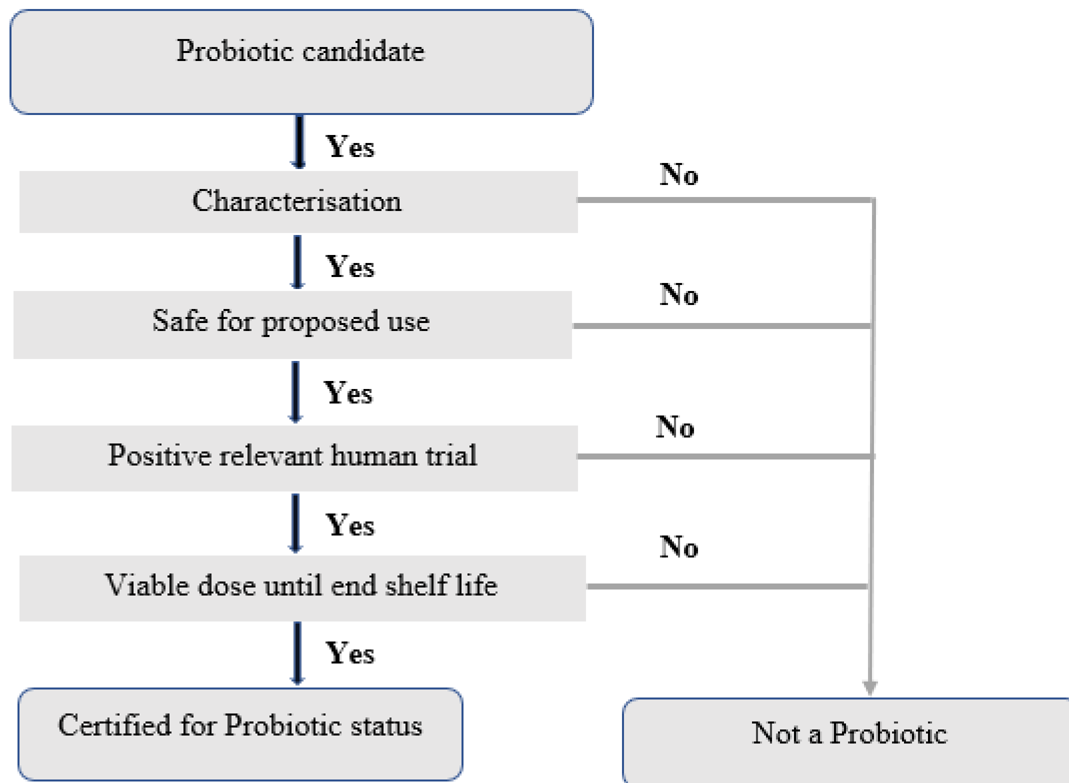

Fig 2.3: Fulfilment criteria for a probiotic candidate (adapted from (Binda *et al.*, 2020))

2.3.4 Antibiotics

Antibiotics are antimicrobial substances that can inhibit or kill bacteria. Any substance used against microorganisms are broadly referred to as antibiotics (Russell, 2004). They are thus recommended only in extreme cases or life threatening conditions because of the proliferation of antimicrobial resistance (Kelly, 2019).

2.3.4.1 History and usage of antibiotics

In 1947, S. A. Waksman proposed a definition of the term "antibiotic" as "A chemical compound created by microorganisms that holds back bacteria and other microorganisms, and even kill them" (Waksman, 1947). Currently, antibiotics are referred to as any natural (microbial origin) or synthesized organic compound that inhibits or inflicts a cidal effect on pathogenic microbes (Laskin *et al.*, 2003). Antibiotics are generally used for treating infections, as preventive measures (prophylaxis) and as feed additives for animal growth promotion. In 1928, Alexander Fleming discovered penicillin which spurred the development of antibiotics (Fig 2.4). After 17 years, penicillin was used on a large scale to treat bacterial infection. Prontosil, a sulfonamide developed by German chemical scientist Gerhard Domagk, was the

first extensively used antibiotic. The period between 1940-1962 was considered the golden era of antibiotics when the vast majority of the antibiotic classes that exist till date were established and introduced to the market. During the pharmacologic era, attempts were undertaken to better understand and use antibiotics by dosage, administration, and other means. During the biochemical period, research into the biochemical actions of antibiotics and resistance mechanisms led to functionalisation studies to avoid resistance. Initiatives for producing novel chemical compounds were driven by the genetic research, mechanism of action and target era. During the genomic/HTS era, genomic sequencing was used to foresee important objectives for use in high-performance screening tests. Because of the failure of their significant investment in genome-based techniques, several firms terminated their discovery endeavours during the so-called "disenchantment phase" (Davies, 1996; Rahman *et al.*, 2022).

2.3.4.2 Antimicrobial stewardship

The extensive use of antibiotics led to the concept of antimicrobial stewardship. Antimicrobial stewardship is a concerted effort toward educating and encouraging antimicrobial prescribers to follow practical guidelines to mitigate antibiotic misuse and, as a result, antimicrobial resistance (Dyar *et al.*, 2017). This strategy has been applied in a variety of settings, ranging from clinic-based antimicrobial stewardship to veterinary antimicrobial stewardship policy, one-health antimicrobial stewardship program, and the World Health Organization's (WHO) global stewardship framework (Dyar *et al.*, 2015; Dyar *et al.*, 2017; Powell *et al.*, 2017). This concept is a much-needed plan to prevent the overuse of antibiotics in humans and animals.

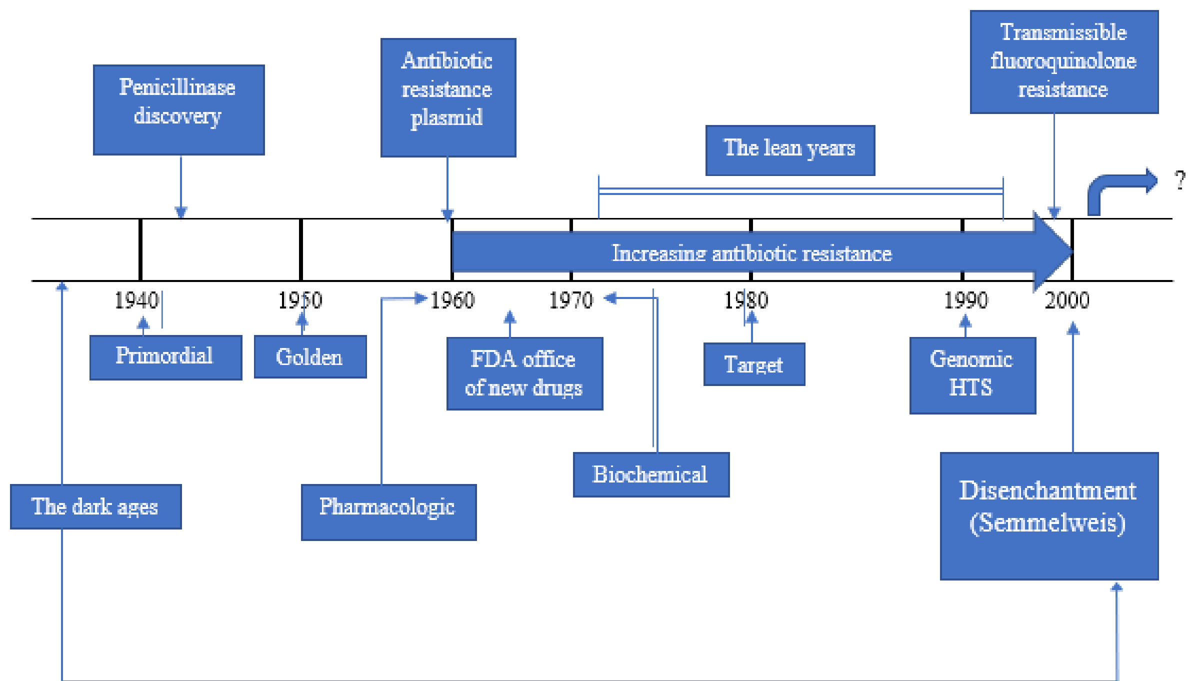

Fig 2.4: History of antibiotic discovery and development of resistance (adapted from (Rahman *et al.*, 2022))

2.3.4.3 Mechanism of antimicrobial action

Antibiotic discovery and development is one of the most significant medical breakthroughs since they have drastically hampered and battled bacterial infections that are usually severe and devastating (Bonafede and Rice, 1997). Antibiotics can repress bacterial growth via the following mechanisms: inhibition of cell wall synthesis, protein synthesis inhibition, inhibition of cell membrane function, inhibition of the nucleic acid synthesis pathway and inhibition of essential metabolic pathways (Fig 2.5) (Sengupta *et al.*, 2013).

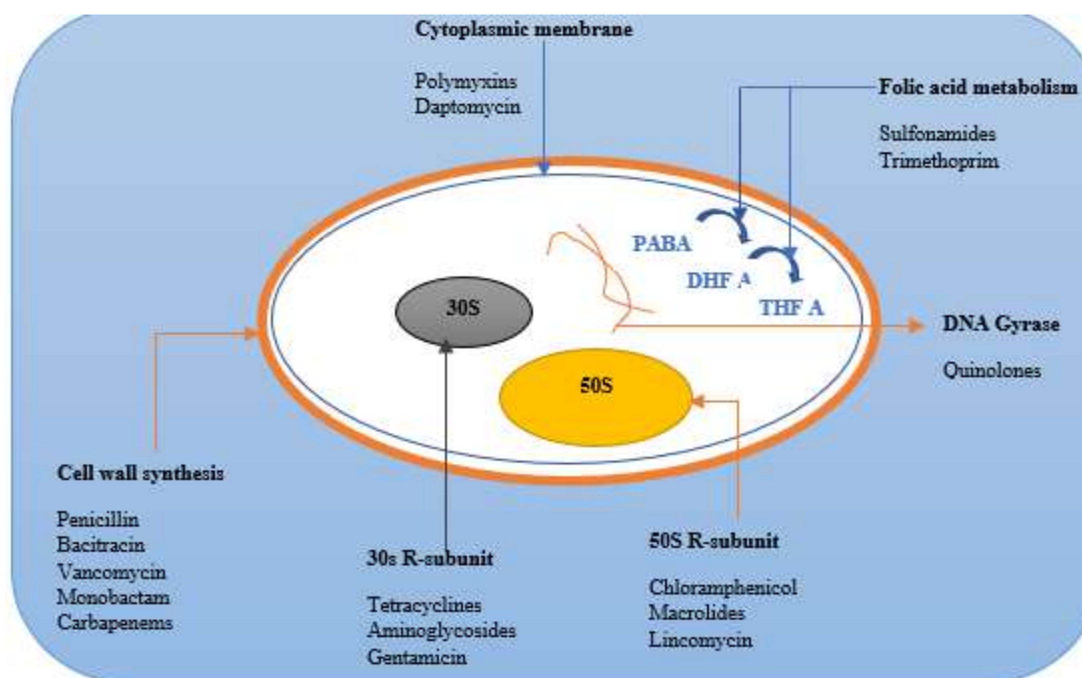

Fig 2.5: Mechanism of action of common antimicrobial agents adapted from(Johnson, 2011)

2.3.4.4 Antimicrobial resistance in the treatment of diarrhoea

Antibiotic resistance began to rise due to the continual and uncontrolled use of antibiotics, partially owing to the intense selective pressure on bacterial cells (Theuretzbacher, 2013). Antibiotic-resistant pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) have become a major threat, as has the emergence of superbugs which have now become a serious global public health challenge (Silva *et al.*, 2005). Bacteria have adopted different mechanisms to circumvent antimicrobial agents, including inactivating them, efflux pumping of drugs, altering the drug's target site and restriction of drug intake (Reygaert, 2018). Drug resistance has been a major impediment to treating several bacterial infections, necessitating new techniques in developing antibiotics for the future generation (Kebede *et al.*, 2021). This prompted researchers to investigate novel antibacterial compounds by screening and investigating plant extracts, fractions and isolated compounds for antimicrobial potency (Infectious Diseases Society of America, 2004).

2.4 Biofilm formation and quorum sensing interplay in bacteria

Biofilms are complex structures created when bacteria colonise an extracellular matrix (ECM), resulting in permanent attachment to biotic and abiotic components, offering protection and boosting antimicrobial resistance (Jamal *et al.*, 2018). The syndicate of sessile cells formed is bonded by electrostatic forces, van der Waal forces and hydrogen bonds (Peña *et al.*, 2011). This kind of cell arrangement is crucial in limiting antibiotic absorption and mitigates the effects of severe environmental conditions (Srinivasan *et al.*, 2021). Biofilms aid bacterial growth, antibiotic resistance, immune cell evasion, and genetic material transfer. Intercellular communication, also known as quorum sensing (QS), is directly linked to biofilms. Gene expression is controlled by diffusible signalling molecules termed autoinducers, making them difficult to eliminate by antimicrobial drugs or host immune cells (Kai, 2018). Biofilm-mediated multidrug resistance genes have been linked with the production of enzymes or proteins that interfere or degrade drug molecules.

2.5 Medicinal plants as alternative remedies

Plants are known to possess antibacterial compounds which have been widely investigated and used in traditional medicine to treat various ailments from time immemorial (Sakunpak and Panichayupakaranant, 2012). Medicinal plants may be defined as herbs with one or more organs contains bioactive compounds with therapeutic, beneficial physiological properties or as precursor for effective pharmaceutical products (WHO, 2013b). A large percentage of the human population relies primarily on these medicinal plants for therapeutic purposes (Seth and Sharma, 2004). Plant extracts are usually obtained from whole plants, roots, leaves, fruits and flowers as alternatives therapy. In addition, the extracts of the bark, roots and other components of woody plants are often used. Medicinal plants contain substantial amounts of secondary metabolites, which are biologically active compounds that play a vital role in promoting the therapeutic effects on numerous diseases (van Wyk and Wink, 2015). These compounds may also act as natural immune system stimulators (Taylor *et al.*, 2001). In recent times, due to the rise in antimicrobial resistance, there has been a greater interest in therapeutic benefits, as well as the reduction in efficiency and side effects of some synthetic drugs (Rao *et al.*, 2008; Cheesman *et al.*, 2017).

2.5.1 Medicinal plants used in the treatment of diarrhoea in South Africa

South Africa has a diverse temperate flora, with over 24 000 species accounting for over 10% of the world's vascular plants (Germishuizen and Meyer, 2003). The majority of the population patronises traditional healers for medical services or to complement orthodox treatments (Van Wyk *et al.*, 1997). In the Republic of South Africa, particularly in the Eastern Cape region, where more than 50% of the population lives below the poverty line, diarrhoea is one of the prominent ailments treated and controlled by traditional treatments (Thornton, 2009). Northern Maputaland in the KwaZulu-Natal province of South Africa is another area with significant poverty levels, where diarrhoea is a big issue and herbal remedies are commonly utilised to control and cure diarrhoea (van Vuuren *et al.*, 2015). The use of herbal regimens is also a common practice in the animal health sector to forestall production loss due to morbidity and mortality (Masika *et al.*, 2000).

. Plant species of the Fabaceae, Anacardiaceae and Asteraceae families are notably used in treating different medical conditions, especially gastrointestinal disorders (McGaw and Eloff, 2008; Ahmed, 2012; McGaw *et al.*, 2020). In addition, twenty plant species from sixteen families are used by the Bapedi traditional healers of South Africa's Limpopo Province to treat diarrhoea. Of them, 5 species (or 25%) are foreign and are grown in backyard gardens as food plants, while 15 species (or 75% of them) are native to the Limpopo Province in South Africa (Semenya and Maroyi, 2012). Some medicinal plants used in treating diarrhoea in South Africa include *Elephantorrhiza elephantina, Punica granatum*, *Gymnosporia senegalensis*, *Elephantorrhiza burkei, Indigofera daleoides, Ozoroa insignis*, *Syzygium cordatum* and *Spirostachys africana* (Mathabe *et al.,* 2006). Several of these plants were extracted using methanol, ethanol, acetone, and hot water from various plant components, including leaves, roots, bark, and stem rhizomes (Mathabe *et al.*, 2006). The anti-diarrhoeal properties of herbal plants can be attributed to tannins, flavonoids, saponins, alkaloids, steroids, and terpenoids (Ojewole *et al.*, 2008; Teke *et al.*, 2010).

Some of the antidiarrhoeal herbal medicines have been reported to possess antispasmodic properties, which delay gastrointestinal processes, increase water absorption, reduce gut motility and electrolyte secretion in the process. These biological effects may describe the benefits of their use in the management of diarrhoea (Maroyi, 2016).

2.5.2 Plant secondary metabolites and their mechanisms of action

For hundreds of years and in many regions of the world, medicinal plants have been utilised as traditional therapies for a variety of human and animal ailments. Natural products obtained from medicinal plants have been shown to be a rich source of physiologically active molecules (phytochemicals), with many of them being used to develop novel pharmaceutical chemicals (Palombo, 2006). These heterogeneous compounds found in medicinal plants are categorised as primary or secondary metabolites based on their role in plant metabolism (Saxena *et al.*, 2013). Phytochemicals are secondary metabolites and are not basically involved in the growth of plants but are produced in other metabolic pathways to protect plants from infestation and external damage contributing to the colour, scent, and flavour of the plant (War *et al.*, 2012). Over 4 000 phytochemical compounds have been catalogued and partially studied (Saxena *et al.*, 2013). These pharmacological compounds accumulate in different segments of the plants, such as fruits, seeds, leaves, flowers, roots and stems (Costa *et al.*, 1999). Investigation of the bioactivity and effects on the intestinal function of plants that have historically been used as diarrhoea remedies. The investigation showed that plant extracts could reduce electrolyte release, delay gastrointestinal transit, lower gut motility, stimulate water adsorption and antispasmodic activity (Palombo, 2006). This justifies the use of plants in the treatment of diarrhoea. Plant bioactive compounds can best be categorised based on their chemical classes and biochemical pathways (Mazid *et al.*, 2011). Some of the important categories are described briefly below.

2.5.2.1 Alkaloids

Alkaloids are natural products with heterocyclic nitrogen atoms that have a basic nature. They are usually very bitter in taste with potent biological activity (Aniszewski, 2007). They can be classified based on the heterocyclic ring system, such as quinoline, isoquinoline, pyridine and pyrrolidine alkaloids. Examples of previously isolated alkaloids are atropine, berberine (antidiarrhoeal compound), quinine (antimalaria), morphine, codeine, nicotine and heroine (Aniszewski, 2007). Numerous pharmacological activities such as antibacterial, antimalarial, antifungal and analgesic effects have been linked to alkaloids (Saxena *et al.*, 2013). However, their usage is controlled, owing to their cytotoxic and addictive tendencies. Furthermore, most alkaloids are employed as semi-synthetic analogues rather than in their pure form (Saxena *et al.*, 2013).

2.5.2.2 Phenolics

Phenolic compounds are the most abundant and extensively distributed phytochemicals in the kingdom Plantae (Aniszewski, 2007; Saxena *et al.*, 2013; Dey *et al.*, 2020). They are distinguished by the attachment of substituent groups such as hydroxy (-OH) or methoxyl (O-OCH3) groups to aromatic and non-aromatic ring structures. Flavonoids, phenolic acids, and polyphenols are the three most significant types of dietary phenolics. Phenol is the simplest, while flavonoids are this group's largest and most investigated. The widely diverse hydroxybenzoic and hydroxycinnamic acids are among the several phenolic acids. Tannins are polymers of phenol compounds with high molecular weight and are classified into two categories known as condensed and hydrolysable tannins (Saxena *et al.*, 2013).

2.5.2.3 Terpenoids

Terpenoids are a class of natural compounds derived from isoprene, a five-carbon unit (Anulika *et al.*, 2016). Most terpenoids have multicyclic structures with functional groups and carbon skeletons that differ from one another. These natural lipids may be found in all forms of living creatures, making them the most diverse collection of natural compounds (Barton and Meth-Cohn, 1999). Terpenoids have significant commercial relevance in food and cosmetics due to their strong flavours and fragrances; examples include borneol, linalool, sclareol and menthol. Terpenoids are grouped based on the number of isoprene units present. Terpenoids can be hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids (Saxena *et al.*, 2013). Other phytochemical compounds classified as terpenoids are essential oils and saponins. This phytochemical compound class has varied bioactive effects such as antibacterial, antiviral, antimalarial, gastrointestinal stimulant, anti-ulcer and anti-carcinogenic activities (Böhme *et al.*, 2014; Tiwari and Shukla, 2020).

2.6 Some biological effects of medicinal plants with relevance to the current study

2.6.1 Antioxidant activity

Medicinal herbs are well-known and popular for various health advantages, including blood pressure reduction, resolution of diarrhoea, cardiovascular disease prevention and cancer risk reduction due to their antioxidant activity (Škrovánková *et al.*, 2012). Research has demonstrated an inverse relationship between the consumption of natural antioxidants from medicinal herbs and the occurrence of certain illnesses due to their ability to eliminate free

radicals (Škrovánková *et al.*, 2012). Antioxidant compounds are abundant in plants and these are mostly extracted with the use of polar solvents such as methanol, water and ethanol for lower molecular weight polyphenol and acetone for higher molecular weight polyphenols (Grzegorczyk *et al.*, 2007). Phenolics, such as phenolic acids, flavonoids, tannins, terpenoids, tocopherols, ascorbic acid (vitamin C), and a group of carotenoids are among the prominent antioxidant components isolated from medicinal plants. Examples of plant-associated antioxidant compounds include gallic, coumaric, ferulic, rosmarinic acid, quercetin, kaempferol, apigenin, cumin, fennel, rosmanol and ascorbic acid, amongst others (Škrovánková *et al.*, 2012). The mechanisms of antioxidant compounds include: reduction in localised oxygen concentration; decomposition of peroxides to prevent reversible conversion to radicals; binding of catalysts to avert initiating radical generation; chain breaking to discontinue hydrogen abstraction by radicals; and scavenging of active radicals to prevent chain initiation (Dorman *et al.*, 2003).

2.6.2 Anti-inflammatory activity

Plants have been utilised to cure a variety of ailments, from infectious agents like bacteria and fungi to physiological and neurological diseases. Inflammation is the body's response to tissue damage caused by a diverse range of factors such as physical, chemical or microbial sources (Klopfleisch and Jung, 2017). Inflammation is part of the nonspecific immune response to injuries and is a vital component in many inflammatory-related disorders such as diarrhoea. Inflammation in cells includes the stimulation of pro-inflammatory cytokines by leukocytes, endothelial cells, and macrophages. Inflammation is also linked to the production of anti-inflammatory cytokines like IL-10 in addition to pro-inflammatory cytokines (Li *et al.*, 2005). Nitric oxide synthase (iNOS) and cyclooxygenase (COX) are two pro-inflammatory enzymes released by inflamed cells. These are in charge of raising nitric oxide (NO) and prostaglandin (PGE2) levels. Nitric oxide and PGE2 are recognised to have a role in various chronic disorders, such as multiple sclerosis and colon cancer (Yan *et al.*, 2007).

Inflammation has traditionally been treated using a variety of medicinal plants (Taylor *et al.*, 2001). Extracts, fractions and purified active compounds from different medicinal plants have been identified to have anti-inflammatory activities (Bernstein *et al.*, 2018). *In-vitro* study revealed that flavonoids, a sub-class of polyphenols, have anti-inflammatory activities (Bernstein *et al.*, 2018). An example is aspirin, a plant-inspired semi-synthetic compound based on salicylic acid found naturally in *Salix* species (willow bark) used locally to treat pain and

fever (Taylor *et al.*, 2001). Quercetin, found in apples, onions and berries, inhibits lipopolysaccharide (LPS)-induced TNF-α production in macrophages (Xaus *et al.*, 2000). Curcumin derived from turmeric is capable of inhibiting NF-κB activated by many inflammatory responses (Singh and Aggarwal, 1995). Curcumin also downregulates enzymes such as COX-2, LOX-5 and other pro-inflammatory cytokines (Kunnumakkara *et al.*, 2017). Other examples include *p*-coumaric acid, rosmarinic acid, rosmanol and colchicine, which were reportedly able to inhibit inflammatory responses in different pathways (Bernstein *et al.*, 2018).

2.6.3 Immunomodulation

Immunomodulators are natural or synthetic medicines that impact a therapeutic benefit by modulating the immune system (Jantan *et al.*, 2015). Traditional medicine has a lengthy history in several parts of the world till today. Medicinal herbs, such as *Astronium urundeuva*, *Cochlospermum vitifolium, Thymus vulgaris* and *Salix alba* are common plants used for immunomodulation (Akram *et al.*, 2014). Gallic acid, abrine, phytocidin, quercetin and apigenin are common plant constituents with reported immunomodulatory activity. The mechanisms of activities include enhancement of Th1 and Th2 helper T cells, delayed hypersensitivity response by suppressing antibody titre, enhancement of cell immunoglobulin levels, immunostimulation of phagocytic cells, among others (Aly and Mohamed, 2010; Nakano *et al.*, 2012; Akram *et al.*, 2014).

2.6.4 Toxicity

A vast majority of communities in southern Africa rely on the use of rich and diverse flora as therapy for different medical challenges and food security (Fennell *et al.*, 2004). As a defence mechanism, these plants produce a plethora of biochemical compounds, hence the possibility of those compounds being poisonous to animal and humans (Wink and Van Wyk, 2008). The survey of Wink and Van Wyk (2008) established that about 750 toxic substances exist in about 150,000 of the different metabolites obtained from approximately 1000 plant species. Extracts such as *Helleborus viridis, Petroselium cripsum* and *Bryonia dioica* are reportedly rich in cytotoxic alkaloids. From earlier studies, it is understood that *Bauhinia* plants are non-toxic (Barata, 2005). It is therefore necessary to re-affirm some of the claims to establish their safety for development of a herbal remedy.

2.7 Medicinal plants as a source of natural antibiofilm and anti-quorum sensing agents

Plant based natural products have been identified to possess antimicrobial, quorum sensing inhibition (QSI) and antibiofilm formation (ABF) functions (Famuyide *et al.*, 2019; Lu *et al.*, 2019). The antibiofilm activity of natural products could involve the inhibition of cellular attachment, repression of polymer matrix formation, interruption of extracellular matrix initiation and blocking of signalling molecules (Fig 2.6). Medicinal plants like *Agathosma betulina, Sphedamnocarpus pruriens, Coptis chinensis* and *Herba patriniae* have been reported to possess antibiofilm activity (Lu *et al.*, 2019). Patriniae, quercetin, and phloretin are compounds identified as natural anti-biofilm agents (Table 2.1). The mechanism of ABF and QSI effects include inhibition of Las A, cell adhesin, protease and other metabolic activities, acyl homoserine lactones (AHL), biomass formation, and pyocyanin production (Table 2.1). Considering that there are hundreds of medicinal plants available and that traditional medical herbs have a long history of treating numerous medical conditions, medicinal herbs might be a rich source of novel products to combat biofilm formation *vis-à-vis* quenching of quorum sensing (Lu *et al.*, 2019).

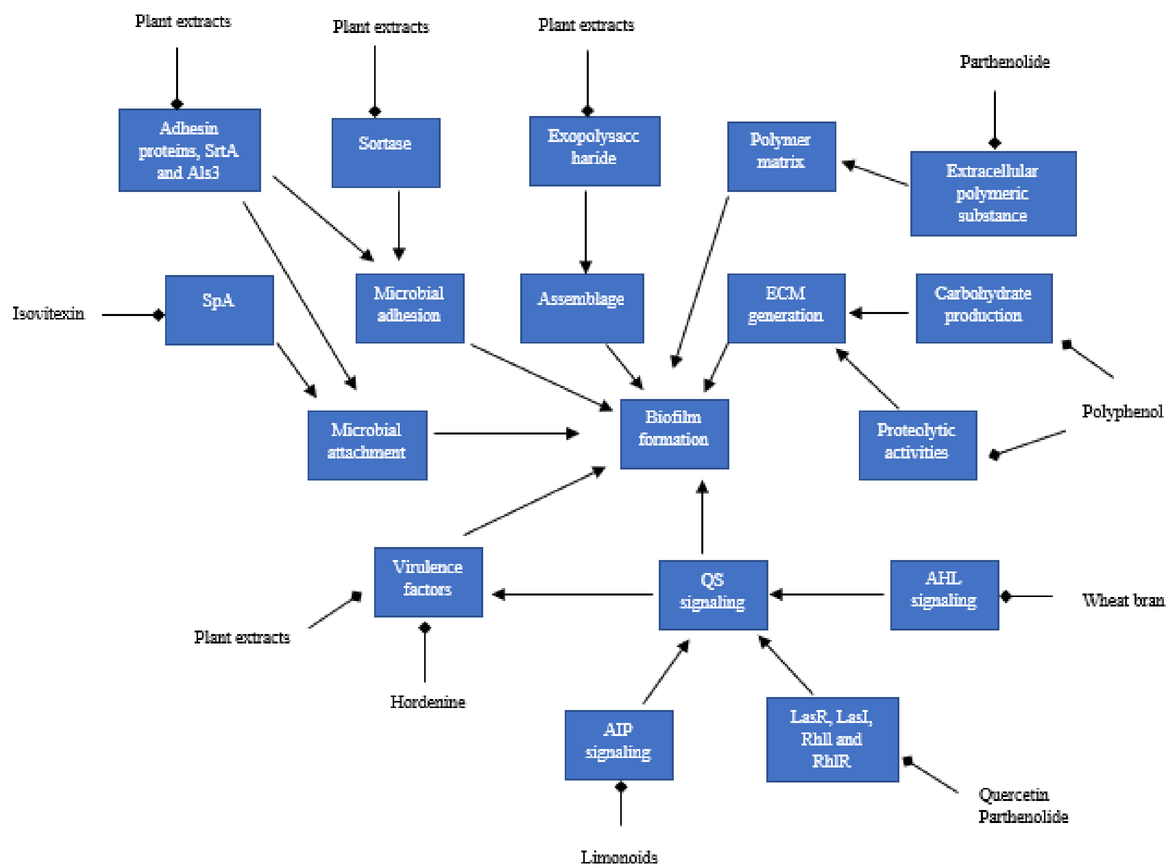

Fig 2.6: Potential mechanism of antibiofilm activity of natural products (adapted from Lu *et al.*, 2019)

Table 2.1: Natural agents and their mechanism of biofilm inhibition

Plant extract/compounds	Mechanism of ABF/AQS effects	Target organism	References
Ethanol (dried plant extract) *Agathosma betulina* (P.J.Bergius) Pillans. (Rutaceae)	Repression of pyocyanin and Las B Elastase production	*Pseudomonas aeruginosa*	(Cosa and Chenia, 2014)
Ethanol leaf extract of *Agave sisalana* Perrine. (Agavaceae)	Inhibition of Las A protease activity and biomass formation	*P. aeruginosa, Staphylococcus aureus* and *Candida albicans*	(Al-Refi, 2017)
Ethanol (leaf extract) *Cuminum cyminum* L. (Apiaceae)	Biofilm biomass reduction and pyocyanin production inhibition	*P. aeruginosa, S. aureus* and *C. albicans*	(Al-Refi, 2017)
Ethanol (leaf extract) *Boswellia serrata* Triana & Planch. (Burseraceae)	Total inhibition of biofilm formation	*P. aeruginosa*	(Cosa and Chenia, 2014)
Dichloromethane/methanol (leaf extract) *Echinacea angustifolia* DC. (Asteraceae)	Inhibition of cell adhesin and metabolic activity	*Listeria monocytogenes* ATCC 19111	(Sandasi *et al.*, 2011)
Methanol rhizome *Glycyrrhiza glabra* L. (Fabaceae)	Reduction of *A. baumannii* biomass and inhibition of acyl homoserine lactones (AHLs)	*Acinetobacter baumannii*	(Bhargava *et al.*, 2015)

Ethanol (leaf extract) *Sphedamnocarpus pruriens* (A.Juss) Szyszyl. (Malpighiacea)	Inhibition of biofilm biomass formation	*Mycobacterium smegmatis*	(Oosthuizen *et al.*, 2019)
Linalool	Prevention of bacteria adhesion and biofilm formation and interference with QS system	*A. baumannii* and *Salmonella typhimurium*	(Alves *et al.*, 2016; Prakash *et al.*, 2019)
Limonene	Biofilm formation inhibition in concentration dependent manner	*Streptococcus pyogenes* (SF370)	(Subramenium *et al.*, 2015)
L-carvone	Inhibition of QS activity by reducing AHLs production and reduction of biofilm formation	*Hafnia alvei*	(Li *et al.*, 2019)
Carvacrol	Prevention of biofilm formation, inhibition of violacein and pyocyanin production. Repression of lasR expression	*Chromobacterium violaceum, P. aeruginosa, Escherichia coli* and *S. aureus*	(Nostro *et al.*, 2012; Burt *et al.*, 2014; Tapia-Rodriguez *et al.*, 2017; Tapia-Rodriguez *et al.*, 2019)
Ginkgolic acids	Repression of biofilm formation	*E. coli* O157:H7	(He *et al.*, 2013; Lee *et al.*, 2014)
Phloretin	Inhibition of biofilm formation (at low concentration in S. aureus) and fimbria production	*E. coli* O157:H7, *S. aureus* RN4220 and SA1199B	(Lee *et al.*, 2011; Lopes *et al.*, 2017)

Quercetin	Blockage of SrtA, and sialic acid Production, impair biofilm formation and expression of virulent factors and biofilm pH distruption	*S. pneumoniae, P. aeruginosa, Enterococcus faecalis*	(Krishnan *et al.*, 2012; Wang *et al.*, 2018; Zeng *et al.*, 2019)
Isolimonic acid	Cell-to-cell signalling interference and inhibition of biofilm formation	*Vibrio harveyi*	(Wang *et al.*, 2018)
Patriniae	Inhibition of exopolysaccharide production and biofilm formation	*P. aeruginosa*	(Fu *et al.*, 2017)
Wheat-bran	Inhibition of biofilm formation and QS activity by downregulating AHLs production	*S.aureus*	(González-Ortiz *et al.*, 2014)
Parthenolide	Inhibition of QS genes such as *LasI/LasR* and *RhlI/RhlR* and Downregulation of extracellular polymeric substance	*P. aeruginosa* PAO1	(Kalia *et al.*, 2018)

2.8 Expression of virulence genes in bacteria

The ability of an organism to infect a host and cause disease is referred to as virulence. The chemical molecules that help the bacteria invade the host at the cellular level are known as virulence factors. These substances are either secretory, membrane-bound, or cytosolic (Sharma *et al.*, 2017). The cytosolic factors help the bacteria adjust quickly to changes in metabolism, morphology and physiological states. The virulence factors linked with the membrane assist the bacteria in attachment and evasion of the host cell. The secretory factors are essential components of the bacterial arsenal that aid the bacterium in navigating the host's innate and adaptive immune responses. The secretory virulence factors work together to destroy host cells in extracellular infections (Sharma *et al.*, 2017). Many virulence factors are generated only by aggressive bacterial strains (e.g., enterotoxins for some *E. coli* strains). The interplay of virulence factors and the host's response typically determines the clinical course of a disease. The infection finally occurs when the balance between virulence factors and host response is favourable (Winzer and Williams, 2001; Denzer *et al.*, 2020).

For some diseases, the bacterial population has a significant impact on the outcome of the host-bacterium interaction. When virulence factors are produced in response to cell population density (biofilm formation), the host does not have enough time to establish an effective defence against such coordinated attack. This technique relies on a bacterial cell's capacity to detect other members of the same species and differently express certain sets of genes in response. Quorum sensing is a type of cell-to-cell communication that includes the direct or indirect stimulation of a response regulator by a tiny diffusible signal molecule (Winzer and Williams, 2001).

2.8.1 Effect of plant extracts/compounds on virulence gene expression

In the last few decades, extensive research has shown that naturally occurring compounds have an antibacterial effect against pathogenic organisms and also have other biological activities. Although the antimicrobial properties of plants that target bacterial cellular viability have been widely explored previously (Ushimaru *et al.*, 2007; Upadhyay *et al.*, 2014). Few studies have focused on the effects of these compounds on modulating various aspects of bacterial virulence genes, which is an important factor influencing pathogenesis in the host (El-Azzouny *et al.*, 2018).

2.9 Plant selection

Nine plant species were chosen for this study based on ethnobotanical records, availability and antimicrobial potency. All the selected plant species belong to three families, namely Fabaceae, Asteraceae and Anacardiaceae. Their phytochemistry and biological activities are briefly described below.

2.9.1 Fabaceae

2.9.1.1 *Bauhinia* genus

The *Bauhinia* genus consists of more than 300 plant species largely distributed in tropical areas of Africa, South America and Asia. They are commonly called cow's hoof due to the shape of their leaves. *Bauhinia* species are used to treat various diseases, including diarrhoea, diabetes, general discomfort, inflammation and infections in folklore medicine (Ahmed *et al.*, 2012). In recent years, scientific understanding of their biological characteristics and active components has advanced dramatically due to their therapeutic potential. Although many of the chemical components listed for the genus *Bauhinia* may also be present in other plant species, the secondary metabolites generated by this genus, notably flavonoids, make these plants a valuable reservoir of potential phytotherapeutic or medicinal agents (de França Bonilha *et al.*, 2015). Other phytocompounds found in the genus include flavonoids, quinones, alkaloids, aromatic acids and terpenes are reportedly found in this group of plants (Mali *et al.*, 2007).

2.9.1.1.1 *Bauhinia bowkeri* (Harv.) A.Schmitz

The common name of *B. bowkeri* is Kei white Bauhinia in English, Keibeesklou in Afrikaans and umDlandlovu in Xhosa. The leaves are distinctly butterfly-like (two rounded fused semicircular lobes). This tall plant is a drought-resistant shrub with a greyish brown stem and white flowers (Jaarsveld and Notten, 2001). It is currently on the red list of South African plants threatened by agricultural activities. *Bauhinia bowkeri* is a rare endemic species in the bushveld region of the Eastern Cape and winter rainfall area of the Western Cape (Jaarsveld and Notten, 2001). White Bauhinia is used locally to treat gastrointestinal infection and induce vomiting, as well as for bathing and steaming (Ndawonde *et al.*, 2007). It exhibited potent antioxidant, anti-inflammatory and antimicrobial activities (Ahmed *et al.*, 2012).

Fig 2.7: The leaves and stem of *Bauhinia bowkeri*. (Credited to R.O Adeyemo)

2.9.1.1.2 *Bauhinia galpinii* N.E.Br.

Bauhinia galpinii (Fig 2.8) is known as Pride of De Kaap in English and Vlam-van-die-Vlakte in Afrikaans. It is a medium to large drought-resistant shrub with a brick red flower, and it sometimes behaves like a climber especially in the wild state. The plant derived its name from the De Kaap valley, Nelspruit, Mpumalanga Province, South Africa. It is also widely distributed across KwaZulu-Natal, North West, and Limpopo Provinces (Hankey, 2001). The leaves, stem bark and seeds of *B. galpinii* are traditionally used in treating gastrointestinal disorders, infertility, amenorrhoea, inflammation and other infectious diseases (Samie *et al.*, 2009). Earlier reports established that the acetone extracts and compounds such as 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone, 3, 5, 7, 3', 4', 5'- hexahydroxyflavone quercetin-3-*O*-galactopyranoside, 2″-*O*-rhamnosylvitexin, myricetin-3-*O*-galactopyranoside from this plant exhibit potent antimicrobial, antioxidant, anti-inflammatory, antimutagenic and cytotoxic activities (Reid *et al.*, 2006; Aderogba *et al.*, 2007; Ahmed *et al.*, 2012).

Fig 2.8: The leaves of *Bauhinia galpinii* (Credited to R.O Adeyemo)

2.9.1.1.3 Bauhinia variegata L.

Bauhinia variegata (Fig 2.9) known as Mountain ebony in English, is a medium sized, sun-loving deciduous plant with hairy branches and a small dark trunk. It has green smooth alternate leaves with parallel venation. Mountain ebony has elongated pod-like fruit (Irchhaiya *et al.*, 2014). It usually grows on rocky or hilly slopes (acidic soil) and originated from India and China. *Bauhinia variegata* is used locally for treating diarrhoea, haemorrhoids and dysentery (Dey and Das, 1988). The plant's bark is used to treat fever (tonic), skin infections and goitre. In addition, the root is used locally as anti-snake venom (Golwala and Patel, 2009). Previous findings have shown that the plant contains reducing sugars, glycosides, octacosanol (from stem back), flavonones (root bark), heptatriacontan-12,13-diol and dotetracont-15-en-9-ol (leaves) (Reddy *et al.*, 2003; Yadava and Reddy, 2003). Pharmacological studies have shown that the ethanolic leaf extract of *B. variegata* possesses anti-tumour activity (Rajkapoor *et al.*, 2003). A flavonol compound isolated from the root has anti-inflammatory activity and the

methanolic extract of the leaves exhibited antibacterial and antifungal activities (Sharma and Saxena, 1996; Yadava and Reddy, 2003).

Fig 2.9: The leaves and stem of *Bauhinia variegata* (Credited to Alejandro Bayer Tamayo, CC BY-SA 2.0)

2.9.2 Anacardiaceae

2.9.2.1 *Searsia* genus

This genus, formerly known as *Rhus*, consists of over 250 species of shrubs and deciduous plants belonging to the family Anacardiaceae. The genus is widely distributed in the temperate, tropic and subtropic regions of the world (Phongkrathung *et al.*, 2016). They have small flowers and trifoliate leaves with a resinous smell and produce fruit that belongs to the drupe type. *Searsia* species commonly grow in infertile soil and are used by different indigenous cultures for numerous purposes. *Rhus chinensis* extracts are used to treat diarrhoea, cough, snakebite, malaria, fever, jaundice (Chen *et al.*, 2009); *R. glabra* extracts are used to treat rectal bleeding, vaginal discharge, sore mouth, and sore throat (Moerman, 1998; Abdel-Mawgoud *et al.*, 2019); *R. javanica* extracts are sometimes used to treat dysentery, spermatorrhoea, malaria and diarrhoea (Ouyang *et al.*, 2008). *Rhus tripartita, R. trilobata, R. typhina, R. succedanea* extracts are also used to treat diarrhoea, dysentery, wounds, sore throat, ulcer (Kirtikar and Basu, 2000; Abbassi and Hani, 2012; Kirby *et al.*, 2013). *Rhus* species are reported to have antimicrobial, anti-inflammatory, antioxidant, antimalarial and antimutagenic bioactivities (Opiyo *et al.*, 2021). Tannins are very common in most *Rhus* species, and other classes of phenolics found are coumarins and quinones. Previous findings reveal that flavonoids and tannins possess antibacterial, antiviral and antidiarrhoeal effects (Maroyi, 2013; Farjana *et al.*, 2014). However, not all species identified have been fully explored for their bioactive components and potential biological activities

2.9.2.1.1 *Searsia batophylla* (Codd) Moffett

Searsia batophylla is commonly called bramble currant and has distinctive bicoloured bramble-like leaves (trifoliate) with a leaf stalk, making it simple to recognize. It is a drought-resistant evergreen shrub. Bramble currant is widely distributed in mountain areas of the Limpopo province in South Africa (SANBI, 2013). There is not enough information available on this plant's ethnobotanical, pharmacological and biological activities.

Fig 2.10: The leaves and stem of *Searsia batophylla* (Credited to R.O Adeyemo)

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

Searsia lancea (Fig 2.11), formerly *Rhus lancea*, is a tree native to South Africa. It is called Karee in English, and Mosabele, Rooikaree, Inhlangutshane, Mushakaladza in local languages in South Africa (Madzinga and Kritzinger, 2020). The Karee is an evergreen, drought-resistant tree that may be found in most sections of southern Africa (Aganga and Mosase, 2001). In South Africa, the leaves are used to cure headaches, fevers, colds, pustules and papules (Mulaudzi *et al.*, 2012; Kose *et al.*, 2015). Also, the leaves and fruits of this plant are claimed to be used to cure diabetes and herpes. To treat diabetes, the leaves and fruits are combined with milk from a young cow (Kose *et al.*, 2015). Historically, the decoction is also used to wash animals with skin problems and to bathe infants with diseases associated with smallpox symptoms (Mabogo, 1990; Madzinga and Kritzinger, 2020). *Searsia lancea*t contains tannins and flavonoids (Van der Merwe *et al.*, 2001). According to Nair *et al.* (1983), the flavonoid compound myricetin 7,4-dimethyl ether and its 3-O-galactoside have been isolated from the

ethanolic extract of *S. lancea*. In addition, the essential oil from *S. lancea* contains benzene, δ-3-carene, α-pinene, trans-caryophyllene and isopropyl toluene (Gundidza *et al.*, 2008). The essential oil from *S. lancea* leaves was shown to possess potent antibacterial and antifungal activities against tested bacteria and fungi species with the most significant activity against *E. coli* (Gundidza *et al.*, 2008). The findings of Fouché *et al.* (2008) revealed that dichloromethane extracts of the fruit and stem exhibit moderate anticancer activity against breast, renal and melanoma cell lines. Other reported biological activities are anti-inflammatory, antioxidant and anti-cholinesterase activities (Gundidza *et al.*, 2008; Mulaudzi *et al.*, 2012).

Fig 2.11: The leaves, stem and flowers of *Searsia lancea* (Credited to R.O Adeyemo)

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

This drought-resistant shrub is known as mountain Karee (Fig 2.12). It is also called klepkaree, Mohlwehlwe, Inhlangushane in Afrikaans, Sotho and Siswati respectively. Mountain Karee is distributed across the Northern region of South Africa and surrounding nations like Lesotho, Botswana, Zimbabwe, and Mozambique (Smith *et al.*, 2004). It has bright green, tiny textured, trifoliate leaves with a resinous smell when crushed. *Searsia leptodictya* differs from other *Searsia* species because its two lateral leaves are at a right angle to the middle leaflet. *Searsia leptodictya* is reportedly used locally to treat gastrointestinal disorders (Sebothoma, 2010). This plant possesses excellent biological activities such as antioxidant, antimicrobial and anti-inflammatory activities (Ahmed *et al.*, 2014; Mtunzi *et al.*, 2017).

Fig 2.12: The leaves, fruits and stem of *Searsia leptodictya* (credited to R.O Adeyemo)

2.9.2.1.4 *Searsia pendulina* (Jacq.) Moffett

Searsia pendulina is called White Karee, River Karee and Willow Karee in English (Fig 2.13), Wilderosyntjieboom in Afrikaans and Mosilabele in Sotho (SANBI, 2005). It is an evergreen, drought-tolerant semi-deciduous plant. It grows naturally along the Orange River and its tributaries. White Karee has a trifoliate leaf with edible fruit and according to Coates-Palgrave (2002), locally is used locally to treat gastrointestinal disorders and as an enema in children. *Searsia pendulina* is reported to possess antimicrobial, antioxidant and cytotoxic activities (Ahmed *et al.*, 2014).

Fig 2.13: The leaves, fruits and stem of *Searsia pendulina*. (Credited to R.O Adeyemo)

2.9.2.1.5 *Searsia gueinzii* (Sond.) F.A Barkley

Searsia gueinzii is often referred to as thorny Karee in the Afrikaans language. It is a resinous shrub with trifoliate leaves. There is a paucity of information on *S. gueinzii.* However, previous studies have revealed that members of the genus *Searsia* possess desirable bioactivities such as antimicrobial, antioxidant, anti-inflammatory activities, as well as antimutagenicity (Elgorashi *et al.*, 2003; Rayne and Mazza, 2007; Ahmed *et al.*, 2014).

2.9.3 Asteraceae

2.9.3.1 *Brachylaena* genus

The Asteraceae family consists of diverse shrubs, trees and herbs which are widely distributed worldwide in arid and semi-arid climate regions. Plants belonging to the Asteraceae family contain compounds such as saponins, alkaloids, stilbenes, terpenoids, phenolic acids, lignans, polyphenolic compounds and essential oils (Wegiera *et al.*, 2012). Flavonoids, polyacetylenes, caffeoylquinic acids, acetophenones, chalcone, and phloroglucinols are also common phytochemical substances found in the Asteraceae family. Among the Asteraceae family, one of the most significant sources of traditional remedies is the genus *Brachylaena*. *Brachylaena* has fifteen species and subspecies across Southern and Eastern Africa, nine of which are in South Africa. Common species are *B. elliptica, B. transvaalensis, B. ilicifolia,* amongst others. The name *Brachylaena* comes from the Greek words for "shorter" and "cloak," referring to the flowers' length relative to the involucre (Beentje, 2000). Sesquiterpene lactones, colourless lipophilic compounds, exist naturally in *Brachylaena* species and other Asteraceae. Sesquiterpene lactones have been widely studied because of their biological properties,

including anti-inflammatory, anticancer, neurocytotoxic and antitumor activities. Others include antimalarial and analgesic. It is also use in the treatment of burns, flu and diarrhoea (Chadwick *et al.*, 2013).

2.9.3.1.1 *Brachylaena transvaalensis* E. Philips and Schweick

Brachylaena transvaalensis (Fig 2.14) is evergreen and sometimes deciduous, depending on the location. It has dark green glossy leaves with dense whitish flowers. It is commonly called Malabar silver oak in English, Malbaar (Afrikaans), Umphahla (Zulu). It is an indigenous South African plant used in treating diarrhoea in humans and animals (Hutchings and Johnson, 1986; de Wet *et al.*, 2010). *Brachylaena transvaalensis B. transvaalensis*, *B. ilicifolia* and *B. elliptica* are historically used as therapy for diabetes by the Xhosa and Zulu people of South Africa (Deutschländer *et al.*, 2009). This antidiabetic activity could be attributed to saponins, flavonoids, alkaloids, phenols and other compounds (Adam, 2017). The methanolic, aqueous and dichloromethane leaf extracts have shown good antimicrobial activity when combined with other plant species (Adam, 2017).

Fig 2.14: The leaves and stem of *Brachylaena transvaalensis* (Credited to R.O Adeyemo)

Chapter 3: Antibacterial, anti-biofilm and anti-quorum sensing activities of selected South African plants traditionally used to treat diarrhoea

Preface

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Abstract

The development of resistance of microorganisms to conventional antibiotics is a major global health concern, hence the increasing interest in medicinal plants as therapeutic options. This study aimed to evaluate the antibacterial, anti-biofilm and anti-quorum activities of crude extracts prepared using various solvents of nine indigenous South African plants used locally for the treatment of diarrhoea.

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method and the crystal violet assay was used to test the anti-biofilm activity of the extracts against a panel of bacteria. Anti-quorum sensing activity of the extracts was assessed via inhibition of violacein production in *Chromobacterium violaceum* ATCC 12472.

Preliminary screening of extracts against *E. coli* ATCC 25922 revealed that the acetone extracts had significant activity, with MIC values ranging from 0.04 to 0.63 mg/mL. Further screening against a panel of bacterial pathogens showed that the acetone extract of *Bauhinia bowkeri* was the most active with MIC of 0.01 mg/mL against *Salmonella* Enteritidis, followed by *Searsia lancea* with MIC of 0.03 mg/mL against *Bacillus cereus*. All the plant extracts prevented the attachment of biofilms by more than 50% against at least one of the tested bacteria. However, only the mature biofilm of *B. cereus* was susceptible to the extracts, with 98.22% eradication by *Searsia pendulina* extract. *The* minimum quorum sensing inhibitory concentration of the extracts ranged from 0.08 to 0.32 mg/mL with *S. lancea* having the most significant activity.

The extract of *S. lancea* had the best half maximal violacein production inhibitory value of 0.17 mg/mL.

Overall, the results obtained indicate that acetone extracts of *S. leptodictya, S. lancea, S. batophylla, S. pendulina, B. galpinii* and *B. bowkeri* possess antibacterial and anti-biofilm activities and can modulate quorum sensing through the inhibition of violacein production. Therefore, these results signify the potential of the selected plant extracts in treating diarrhoea through inhibition of bacterial growth, biofilm formation inhibition and quorum sensing antagonism, supporting their traditional medicinal use.

3.1. Introduction

Diarrhoea is the disruption of normal morphological and physiological functioning of the gastrointestinal tract resulting in an abnormal increase in stool volume, frequency, and fluidity. Diarrhoea is a major cause of infant death after pneumonia, especially in developing countries, accounting for about 10% of total deaths in children (WHO, 2017). It is the third highest cause of death in South Africa. In animal production, diarrhoea causes significant loss in yield, leading to major economic setbacks (Arnaiz *et al.*, 2021). Bacterial infectious diarrhoea is primarily caused by members of the *Enterobacteriaceae*, a family of Gram-negative opportunistic bacteria responsible for enormous infections (Li *et al.*, 2022). The majority form part of the normal intestinal flora. *Escherichia coli* is the most frequently isolated microbe from enteric diseases, and is referred to as diarrhoeagenic *Escherichia coli* (DEC) (Snehaa *et al.*, 2021). Over 80% of human microbial infections are associated with a syndicate of bacterial cells called biofilms. Biofilms are complex structures formed when bacterial colonies group together within an extracellular matrix, leading to the irreversible attachment to biotic and abiotic components, providing protection and aiding antimicrobial resistance (Jamal *et al.*, 2018). This group of cells has clinical importance in preventing the uptake antibiotics and avoiding the effect of harsh environmental conditions. Biofilms enhance bacterial growth, antibiotic resistance, immune cell evasion, and genetic material transfer (Jamal *et al.*, 2018). Some bacteria species like *E. coli* and *Shigella* species have reportedly been linked to diarrhoea virulence gene expressions and biofilm formation (Mohamed *et al.*, 2007). Biofilms are closely linked to inter-cellular communication, otherwise called quorum sensing (QS). With the help of diffusible signaling molecules called auto-inducers, gene expressions are regulated, making them difficult to eradicate by antimicrobial agents or host immune cells (Kai, 2018). Hence,

there is a need for alternative or complementary remedies to combat the menace of antibiotic resistance assisted by biofilm formation.

Plants have been used traditionally to treat infectious diseases for centuries. Antimicrobial resistance to conventional antibiotics, poor medical facilities, and poverty, especially in low-income countries, contributed to the use of medicinal plants as therapy (Kebede *et al.*, 2021). Medicinal plants contain bioactive secondary metabolites like alkaloids, saponins, tannins and flavonoids with numerous therapeutic functions such as antibacterial, anti-inflammatory, antioxidant and immune cells stimulator (van Wyk and Wink, 2015).

Plant extracts have been widely tested for direct antibacterial activity, however, no novel antibiotics derived from a plant have succeeded to become commercialised. There is therefore a necessity to look beyond microbial growth destruction by plants but rather the use of plants as biofilm disruptors and quorum sensing inhibitors, thus promoting eradication of infections without pressure of antibiotic resistance. However, there is a need for empirical investigations to establish the effectiveness of plant extracts. *Brachylaena transvaalensis, Searsia batophylla*, *S. pendulina*, *S. leptodictya, S. lancea, S. gueinzii, Bauhinia galpinii, B. bowkeri, B. variegata* were selected based on ethnobotanical records, previous findings of antimicrobial activity in our laboratory and available published information on the plants. This study aimed to evaluate the antibacterial, anti-biofilm and anti-quorum sensing activities of these indigenous South African plants.

3.2 Materials and Methods

3.2.1 Plant material and extraction

The leaves of the selected plants were collected at the Lowveld National Botanical Gardens in Nelspruit, Mpumalanga, South Africa, in July 2019. Voucher specimens were prepared and deposited in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria and voucher specimen numbers (PRU) were obtained (Table 2.1). Two voucher specimens were recorded as duplicates of those from the Lowveld National Botanical Gardens, lodged in the South African National Herbarium in Pretoria (PRE) as plant material was collected from the same trees. The collected plant materials were dried in a well-ventilated room at room temperature and ground into powder using a Janke and Künkel Model A10 mill. The powders were stored in an air-tight polythene sack and kept in the dark until use. Acetone, ethanol, 70% methanol,

methanol: dichloromethane (50:50) and hot water were used as solvents for extraction. Ten grams of powdered plant leaves were soaked separately in 100 mL of respective solvent. After 24 h, the supernatants were filtered through Whatman No 1 filter paper into previously weighed glass jars. This process was repeated thrice on the same plant material. The filtrates were then dried under a stream of cold air and extract yields were calculated.

3.2.2 Antibacterial screening

3.2.2.1 Bacterial strains and culture conditions

Strains of *Escherichia coli* O157:H7 (ATCC 43888), *E. coli* (ATCC 25922), *E. coli* (ATCC 35218), *Bacillus cereus* (ATCC 21366), *Staphylococcus aureus* (ATCC 29213), *Salmonella enterica* subsp. *Enterica* serovar Typhimurium (*S*. Typhimurium, ATCC 39183), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enterica* subsp. *Enterica* serovar Enteritidis (*S*. Enteritidis, ATCC 13076) were used for the antibacterial assay. They were maintained in Mueller Hinton agar.

3.2.2.2 Determination of minimum inhibitory concentration (MIC)

A simple two-fold serial dilution microplate method was used to determine the minimum inhibitory concentration (MIC) (Eloff, 1998b). Bacterial cultures grown overnight in Mueller Hinton broth (Sigma Aldrich, SA) were adjusted to McFarland standard 0.5, equivalent to 1.5 x 10^8 CFU/mL. A 100 µL aliquot of sterile distilled water was added to all the wells of a 96-well microtitre plate. The prepared extracts (10 mg/mL stock concentrations) were added to the first row of the microplate and serially diluted in a 1:1 ratio. After that, 100 µL of adjusted bacterial cultures were added to each well. The bacteria were exposed to the extracts of final concentrations ranging between 2.5 and 0.01 mg/mL. Acetone and gentamicin served as negative and positive controls, respectively. The plates were then incubated at 37°C for 18-24 h. Following incubation, 40 µL (0.2 mg/mL) of p-iodonitrotetrazolium violet (INT) was added to each well and incubated for 1 h. The MIC was taken as the lowest extract concentration to show growth inhibition, visible in terms of a decrease in red colour generated by conversion of the INT to a red product by actively respiring bacteria. *Escherichia coli* ATCC 25922 was used to evaluate preliminary antibacterial potential of all the nine plants extracted with five solvents. The extracts with the best antibacterial activity were selected for further screening against the other bacterial strains.

3.2.3 Cytotoxic activity

The cytotoxic evaluation of the acetone plant extracts was done using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay (Mosmann, 1983) modified by McGaw *et al.* (2007) on Vero (African green monkey kidney) cells (ATCC® CCL-81™). Cell growth inhibition for each extract was expressed in terms of LC_{50} values, defined as the concentration that caused 50% cell lethality. The experiments were carried out in triplicate and repeated thrice. The selectivity index (SI) values of extracts were calculated by dividing the LC_{50} values by the MIC values ($SI = LC_{50}/MIC$).

3.2.4 Biofilm forming ability assay

The ability of the bacteria to form biofilm was determined using the modified method of (Stefanović, 2018). Briefly, 0.5 McFarland standard was prepared from an overnight culture of test bacteria (approximately 1.5x 10^8 CFU/mL) grown in Mueller Hinton broth supplemented with 2% glucose. The standardised bacteria were further diluted 1:100 in culture media to obtain an approximately 1.5 x 10^6 CFU/mL inoculum. A 100 µL aliquot of the diluted inoculum was dispensed into the well, and 100 µL of the culture medium was added. The plates were covered and incubated for 24 h and 48 h. The biofilm formation of the bacteria was determined quantitatively using a crystal violet stain. The plates were washed gently three times with sterile distilled water to eliminate planktonic cells. The plates were dried at 60°C for 45 min. Sessile cells were stained with 100 µL of 0.1% crystal violet for 15 min. The plates were washed to remove excess stain. A 150 µL aliquot of ethanol was added to destain the crystal violet bound cells attached to the wells, then 100 µL of the destained ethanol was transferred into a fresh microplate and absorbance was read at 590 nm wavelength using a Biotek plate reader. The biofilm-forming ability was then classified based on the following: (a) Non-biofilm former if $OD_{test} \leq ODc$, (b) weak biofilm former if $ODc < OD_{test} \leq 2\times ODc$, (c) moderate biofilm former if $2\times ODc < OD_{test} \leq 4\times ODc$, (d) strong biofilm former if $OD_{test} > 4\times ODc$.Where ODc is the mean $OD_{media\ ctrl}$ + (3 x standard deviation of $OD_{media\ ctrl}$) and OD_{test} is the mean optical density of the tested bacterial strain OD_{test} - $OD_{media\ ctrl.}$ Only the bacteria with moderate to strong biofilm forming capacity were considered for biofilm formation inhibition and eradication of preformed biofilm tests.

3.2.5 Anti-biofilm assay

3.2.5.1 Inhibition of biofilm formation

The method of Stefanović (2018) was used to investigate the ability of the acetone extracts to prevent the formation of bacterial cell mass and attachment. Briefly, 100 μL (at half MIC concentration) of plant extracts and antibiotic were added in twelve replicates into the wells of 96-well microtitre plates. Then 100 μL aliquots of standardised concentration of bacterial cultures (OD_{590nm} = 0.02 equivalent to 1.0 x 10^6 CFU/mL) grown in Mueller Hinton broth supplemented with 2% glucose were added and incubated at 37°C for 24 h. Following incubation, the biofilm biomass was assayed using the crystal violet (CV) staining assay as described for the biofilm formation assay in section 3.2.4. The percentage of biofilm inhibition was determined using the following formula (1):

$$\% \text{ Inhibition} = [(OD_{\text{Negative control}} - OD_{\text{Sample}})/OD_{\text{Negative control}}] \times 100$$

Biofilm inhibition was rated between 0 and 100%. Values below 0% were categorised as biofilm growth enhancement; between 0-50% indicated weak anti-biofilm activity, and above 50% represented good biofilm inhibition.

3.2.5.2 Eradication of preformed biofilm

The ability of plant extracts to prevent the further formation and or destruction of cell mass was also investigated. A standardised concentration of bacterial cell cultures (100 μL) with OD_{590} = 0.02 (1.0 x 10^6 CFU/mL) of test bacteria were aliquoted into flat bottomed 96-well microtitre plates and incubated at 37°C for 24 h without shaking. This was followed by adding 100 μL aliquots of plant extracts and antibiotic (half-MIC) into the wells of a 96-well microtitre plate. The plates were further incubated at 37°C for 24 h. The biofilm biomass was quantified, and the percentage of biofilm eradication was determined as described in section 3.2.5.1.

3.2.6 Anti-quorum sensing

3.2.6.1 Inoculum preparation

A single colony of the pigment-producing bacterial strain *Chromobacterium violaceum* ATCC 12472 was cultured in Luria-Bertani (LB) broth was inoculated into 10 mL of LB broth, cultured overnight in a shaker incubator at 30°C with shaking at 0.76 g. The working bacterial suspension was prepared by further diluting the overnight grown culture with LB broth to

obtain an absorbance of 0.1±0.02 at a wavelength of 590 nm to match McFarland standard 0.5 (1.5×10^8).

3.2.6.2 Quantitative detection of violacein inhibition in the presence of plant extracts

Using the method of (Ahmad *et al.*, 2015) varying concentrations of plant extracts ranging from 2.5 to 0.02 mg/mL were added to eight of ten test tubes containing 5 mL of LB broth. Then 100 µL of inoculum was added to each test tube. Acetone and vanillin were added to different test tubes as negative and positive controls respectively. The last test tube was not treated; this served as the culture control and also as the reference to determining the percentage of violacein inhibition. All the tubes were incubated at 30°C overnight, shaking at 0.76 g. Anti-quorum sensing was evaluated based on the growth of the biosensor organism and the reduction of purple pigment production in the test tubes containing culture and extract of different concentrations. The lowest extract concentration with visible growth (turbid) and no purple pigment production was interpreted as the minimum quorum sensing inhibitory concentration (MQSIC). This was further confirmed by aliquoting medium from a test tube without turbidity and purple colouration onto an LB agar plate and incubating for 24 h to detect visible growth.

3.2.6.3 Violacein detection

After incubation as described in section 3.2.6.2, a 1 mL aliquot was transferred from each test tube to a 15 mL centrifuge tube and centrifuged at 978.26 x g for 10 min to allow the violacein bacteria to form a pellet, and the supernatant was discarded. The pellets in the test tubes were resuspended in DMSO and vortexed until the pellet was completely solubilised. The tubes were centrifuged again at 978.26 x g for 7 min to separate the bacteria from the solution. Then, 200 µL of the supernatant in each of the tubes was dispensed in wells of a 96-well microtitre plate in duplicate, and the absorbance was measured at 595 nm using a Biotek microplate reader. The percentage of violacein inhibition was calculated using the below formula;

(1): % violacein inhibition % Inhibition = $[(OD_{control} - OD_{test})/OD_{control}] \times 100$

The extract concentrations at which 50% of the violacein produced was inhibited (IC_{50}) were obtained using a regression line between the percentage violacein inhibition and their respective concentrations.

3.2.7 Statistical analysis

Data were entered and collated in Microsoft Excel 356 version, and GraphPad Prism version 6.0 was used for data analysis using one-way analysis of variance (ANOVA) and Tukey's post hoc test where appropriate, with a significance level of $p < 0.0001$ and $p < 0.05$ in Table 3.3 and Fig 3.2 respectively.

3.3 Results

The characteristics of the investigated plant species are presented in Table 3.1.

3.3.1 Minimum inhibitory concentration (MIC)

The preliminary antibacterial activity of the plants extracted with five different solvents is recorded in Table 3.2. The MIC values against the *E. coli* ATCC 25922 strain ranged from 0.04-0.37 mg/mL for acetone extracts, from 0.08-1.87 mg/mL for ethanol extracts, 0.32-2.5 mg/mL for hot water extracts, from 0.29-2.50 mg/mL for methanol/dichloromethane extracts and 0.16-2.50 mg/mL for methanol/water extracts. Therefore, the acetone extracts which had the best overall antibacterial efficacy were selected for further antibacterial screening against eight other bacterial strains related to those implicated in causing diarrhoea. The data presented in Table 3 showed that *B. bowkeri* was the most active with MIC = 0.01 mg/mL against *Salmonella* Enteritidis, followed by *S. lancea* which had MIC value of 0.03 mg/mL against *Bacillus cereus,* while *S. pendulina* had MIC = 0.05 mg/mL against *E. coli* (ATCC 38152). In addition, *B. cereus* was the most resistant strain, susceptible to only S. *lancea* and *S. leptodictya* at 0.03 and 0.16 mg/mL respectively.

3.3.2 Cytotoxicity and selectivity index of tested extracts

Compared to doxorubicin, a reference cytotoxic compound, all the plant extracts had significantly higher LC_{50} values against the Vero kidney cell line (Table 3.3). The LC_{50} values varied from 0.03 mg/mL to >1 mg/mL for extracts. *B. galpinii* was the least cytotoxic with LC_{50} >1 mg/mL, followed by *B. bowkeri* with 0.51 mg/mL, while *S. pendulina* had the lowest value of 0.03 mg/mL, indicating the highest toxicity to the cells.

Table 3.1: Characteristics of the plant species investigated

Plant name (Family name)	Common name (language)	Traditional use	Part used	Previous pharmacological activities	Voucher number
Searsia pendulina (Jacq.) Moffett (Anacardiaceae)	Witkaree (Afrikaans), Garas (Nama), mosilabele (South Sotho)	Stomach ailments, enema in children (Coates-Palgrave, 2002)	leaves	Cytotoxicity, antioxidant, and antimicrobial activities (Ahmed *et al.*, 2014)	PRU 127997
Searsia leptodictya (Diels) T.S.Yi, A.J.Mill. & J.Wen (Anacardiaceae)	Mountain karee (English), Klipkaree (Afrikaans), Mohlwehlwe (North Sotho)	Gastrointestinal disorder (Sebothoma, 2010)	leaves	Cytotoxicity, antioxidant, and antimicrobial activities (Ahmed *et al.*, 2014)	PRU 70151
Searsia gueinzii (Sond.) F.A Barkley (Anacardiaceae)	Thorny karee (English)	Gastrointestinal infections (Elgorashi *et al.*, 2003)	Root	Mutagenicity, antimutagenicity (Elgorashi *et al.*, 2003)	PRE 586271
Searsia lancea L.f. F.A.Barkley (Anacardiaceae)	Rooikaree (Afrikaans), Mokalabata (N.Sotho), inhlangutshane (Siswati), Mosilabele (Thwana and S. Sotho) Mushakaladza (Venda).	Diarrhoea and gall sickness (McGaw and Eloff, 2008)	Bark and leaves	Antibacterial, antihelmintic and cytotoxicity (McGaw *et al.*, 2007)	PRU 126859

Searsia batophylla (Codd) Moffett (Anacardiaceae)	Bramble currant (English) Braamtaaibos (Afrikaans)	/	/	/	PRE 0611152
Bauhinia galpinii N.E.Br. (Syn *Bauhinia galpinii* var. *ungulata* L.) (Fabaceae)	Pride of De Kaap (English), Vlaam-van-die-Vlakte (Afrikaans)	Gastrointestinal disorder, infertility, amenorrhoea, inflammation and infectious diseases (Samie *et al.*, 2010)	Leaves, bark, and seed	Antibacterial, and antioxidant, antimutagenic, cytotoxic activities (Reid *et al.*, 2006; Aderogba *et al.*, 2007)	PRU 28944
Bauhinia bowkeri (Harv.) A.Schmitz (Fabaceae)	White Bauhinia (English), Kiebeesklou (Afrikaans), umDlandlovu	Gastrointestinal infection, induce vomiting, bathing, and steaming (Ahmed *et al.*, 2012)	Leaves and bark	Antiinflammatory, antibacterial and antifungal activity (Ahmed *et al.*, 2012)	PRU 127998
Bauhinia variegata L. (Fabaceae)	Mountain ebony, butterfly tree, orchid tree (English)	Diarrhoea, dysentery, goitre, diabetes (Parekh and Chanda, 2007)	Leaves and bark	Anti-inflammatory (Rao *et al.*, 2008), Immunomodulatory (Ghaisas *et al.*, 2009)	PRU 38533
Brachylaena transvaalensis E. Philips and Schweick (Asteraceae)	Forest Silver-oak (English), vaalboom (Afrikaans), Mufhata (Venda), iPhahla (Siswati)	Diarrhoea (de Wet *et al.*, 2010)	Leaves and bark	Antimicrobial activity (van Vuuren *et al.*, 2015)	PRU 126858

PRU: H.G.W.J. Schweickerdt Herbarium, University of Pretoria; PRE: Pretoria National Herbarium, South African National Biodiversity Institute; /: Not reported

Table 3.2: Minimum inhibitory concentration (in mg/mL) of different extracts against *Escherichia coli* ATCC 25922

Plant name	**Minimum inhibitory concentration of extract (mg/mL)**				
	Methanol /water	Methanol/D CM	Hot water	Ethanol	Acetone
Searsia pendulina	2.50	0.63	0.63	1.25	0.08
Searsia leptodictya	0.16	1.25	0.63	1.25	0.08
Searsia gueinzii	1.14	2.08	2.50	1.87	0.37
Searsia lancea	0.24	0.29	0.63	0.08	0.04
Searsia batophylla	0.32	0.29	ND	0.47	0.11
Brachylaena transvaalensis	1.25	1.25	0.63	0.83	0.63
Bauhinia galpinii	1.25	1.25	0.63	0.83	0.08
Bauhinia bowkeri	1.25	2.50	1.14	0.63	0.07
Bauhinia variegata	0.32	0.63	0.32	0.47	0.21
Gentamicin (Control)			0.005		

DCM: dichloromethane

Table 3.3: Minimum inhibitory concentration (in mg/mL), cytotoxicity and selectivity index (in brackets) of acetone plant extracts

Plant	*E. coli* 25922	*E. coli* 35218	*E. coli* O157:H7	*S.* Enteritidis	*B. cereus*	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*S.* Typhimurium	LC_{50} (mg/mL)
Searsia	0.08	0.05	0.06	0.06	0.33	0.09	0.06	0.06	0.08	0.03±0.00****
pendulina	(0.37)	(0.56)	(0.48)	(0.44)	(0.09)	(0.31)	(0.43)	(0.45)	(0.37)	
Searsia	0.08	0.06	0.08	0.08	0.16	0.09	0.07	0.13	0.16	0.11±0.00****
leptodictya	(1.33)	(1.62)	(1.33)	(1.33)	(0.67)	(1.09)	(1.41)	(0.80)	(0.67)	
Searsia	0.04	0.08	0.09	0.13	0.03	0.04	0.04	0.05	0.06	0.20±0.02****
lancea	(5.82)	(2.55)	(2.19)	(1.53)	(6.11)	(4.36)	(5.09)	(3.52)	(3.05)	
Searsia	0.11	0.08	0.18	0.08	0.21	0.08	0.08	0.20	0.08	0.15±0.01****
batophylla	(1.32)	(1.90)	(0.82)	(1.90)	(0.63)	(1.80)	(1.71)	(0.73)	(1.90)	
Bauhinia	0.08	0.08	0.08	0.04	0.32	0.20	0.10	0.14	0.13	> 1
galpinii	(**14.48**)	(**14.48**)	(**14.48**)	(**25.18**)	(3.37)	(4.83)	(**10.86**)	(7.90)	(8.69)	
Bauhinia	0.07	0.08	0.09	0.01	0.27	0.10	0.06	0.15	0.06	0.51±0.04****
bowkeri	(8.46)	(6.34)	(5.19)	(**50.75**)	(1.83)	(4.96)	(7.61)	(3.36)	(7.61)	
Gentamicin	0.005	0.005	0.001	0.010	0.005	0.002	0.005	0.005	0.002	nd
Doxorubicin					nd					0.01±0.00

nd= Not determined, bold values indicate interesting SI values, **** $p < 0.0001$

3.3.3 Biofilm forming ability

The nine bacterial strains used in this study were evaluated for their biofilm-forming capacity at two different incubation times, 24 h and 48 h. Results presented in Fig 3.1 show that after 24 h, seven of the nine bacteria were able to form biofilm. Three strains (*B. cereus*, *P. aeruginosa*, and *S.* Typhimurium) were classified as strong biofilm formers. Three strains (*E. coli* ATCC 35218, *E. faecalis*, *E. coli* ATCC 25922) were moderate biofilm formers, while *E. coli* 0157:H7 was the only poor biofilm forming strain. *Bacillus cereus* and *P. aeruginosa* had the strongest biofilm-forming ability with optical densities of 0.52 and 0.50 at wavelength 590 nm after 24 h and 48 h respectively.

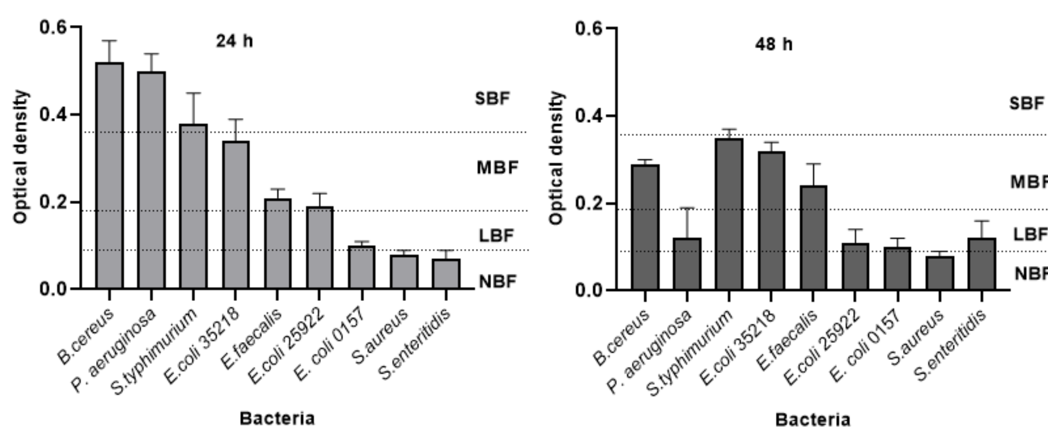


Fig 3.1: Biofilm formation ability of tested bacteria at 24 h and 48 h, NBF=non-biofilm former, LBF= low biofilm former, MBF= moderate biofilm former and SBF= strong biofilm former.

After 48 h incubation, none of the bacterial strains appeared to be strong biofilm formers since all the recorded biofilm formation abilities were below the threshold of strong biofilm former capacity. *B. cereus, S.* Typhimurium, *E. faecalis* and *E. coli* 35218 were moderate biofilm formers. Others produced weak or no biofilm.

3.3.4 Anti-biofilm activity

The anti-biofilm effect of the acetone extracts was investigated using moderate and strong biofilm forming strains. The percentage of biofilm inhibition and eradication is represented in Table 3.4. An inhibition percentage above 50% was considered as good anti-biofilm activity, while those with an inhibition percentage between 0 and 50% were considered poor anti-

biofilm activity, and values < 0 were regarded as having no anti-biofilm activity and instead considered as biofilm formation enhancers (Sandasi *et al.*, 2011).

All the extracts had various levels of biofilm inhibitory activity against the bacteria. The extracts showed more than 50% biofilm inhibition against *S*. Typhimurium, *P. aeruginosa* and *E. coli* ATCC 25922. On the other hand, there was no biofilm formation inhibition >50 % of *E. coli* ATCC 35218 biofilm by all the plant extracts. Except for *S. lancea* and *B. galpinii* which showed poor biofilm inhibitory activity, all other extracts had good biofilm inhibitory activity against *B. cereus* biofilm.

In the biofilm eradication test (treatment after 24 h), negative percentage inhibition of plant extracts was noted for all the tested bacteria except *B. cereus*. However, all the extracts had good biofilm eradication activity against *B. cereus* strains. Also, *S. leptodictya* had good biofilm eradication activity against *P. aeruginosa* with a percentage of biofilm eradication of 79.85%. However, *S. pendulina, S. lancea, B. galpinii,* and *B. bowkeri* had weak biofilm eradication activity against *S.* Typhimurium with percentages lower than 50. Similar results were obtained with *S. batophylla* against *E. faecalis* and *B. galpinii* against *E. coli* ATCC 35218 (Table 3.4).

Table 3.4: Percentage of biofilm inhibition and eradication by acetone plant extracts

Plant species	Biofilm formation inhibition (%)						Biofilm eradication					
	E. coli 35218	*S.* Typhimurium	*B. cereus*	*P. aeruginosa*	*E. faecalis*	*E. coli* 25922	*E. coli* 35218	*S.* Typhimurium	*B. cereus*	*P. aeruginosa*	*E. faecalis*	*E. coli* 25922
S. pendulina	22.53	123.96	77.54	78.06	31.65	81.63	-83.59	25.03	98.22	-99.60	-25.77	40.29
S. leptodictya	13.31	112.30	72.89	111.47	21.20	124.28	-74.90	-7.93	69.15	79.85	-47.34	-74.46
S. lancea	6.46	65.44	22.67	60.02	24.14	76.97	-74.94	1.55	51.20	-199.40	-75.07	-95.23
S. batophylla	9.35	110.59	65.70	110.65	94.81	85.99	-35.39	-68.31	55.62	-224.57	34.44	-270.56
B. galpinii	0.85	91.71	26.20	71.06	78.04	54.60	11.13	13.13	42.50	-213.29	-27.88	-253.80
B. bowkeri	6.26	78.27	73.52	108.47	92.94	124.35	-84.19	32.82	70.29	-24.33	-16.56	-39.49
Gentamicin (control)	81.88	103.66	100.05	100.76	74.73	105.26	62.27	74.25	53.79	73.87	67.93	51.93

3.3.5 Quorum sensing inhibition

The ability of plant extracts to inhibit quorum sensing (QSI) was tested using a biosensor bacterium, *Chromobacterium violaceum* (ATCC 12472). The minimum quorum sensing inhibition concentration (MQSIC) was defined as the lowest concentration characterised by growth (turbidity) and no purple ring formation indicating bacterial growth without violacein pigment. All the plant extracts showed inhibition of quorum sensing at varying concentrations by inhibiting violacein production. *Searsia lancea* had the most significant MQSIC of 0.08 mg/mL (Fig 3.2). All other plants had MQSIC lower than vanillin (positive control) except *S. leptodictya*. The minimum inhibitory concentration was taken as the lowest concentration, characterized by no growth or turbidity and purple ring formation. *Searsia lancea* had the lowest MIC value of 0.16 mg/mL, while *S. leptodictya* had the highest MIC value of 0.63 mg/mL (Fig 3.2).

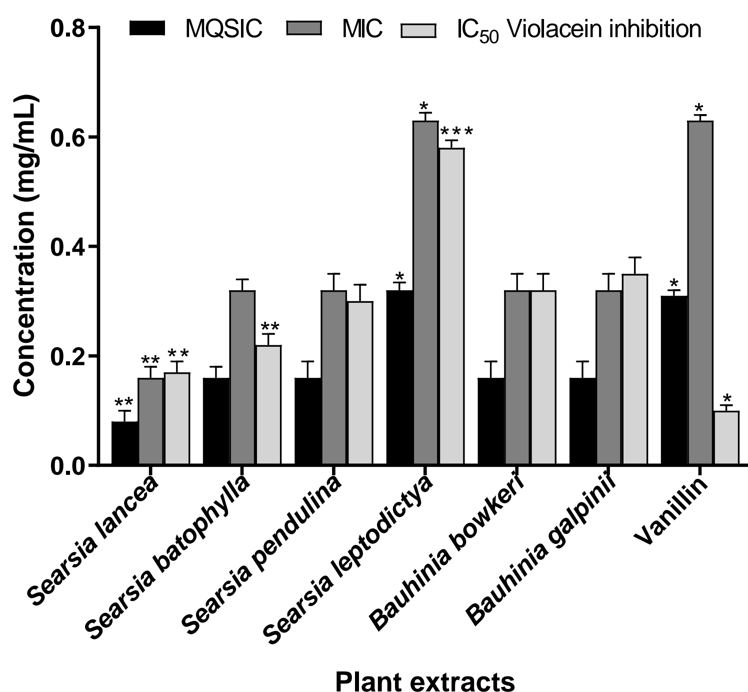


Fig 3.2: Quorum sensing inhibitory activity of acetone extracts of selected plant species in *Chromobacterium violaceum*. MQSIC: minimum quorum sensing inhibitory concentration, MIC: minimum inhibitory concentration, IC_{50}: 50% inhibitory concentration of violacein production.

The plant extracts were tested against *C. violaceum* to determine the concentration having 50% violacein production inhibition (IC_{50}). The IC_{50} value for all the plant extracts was obtained from the standard graph of concentration against % violacein inhibition and thus ranged

between 0.17 and 0.58 mg/mL (Fig 3.2). The lower the IC_{50} value the better the antiquorum sensing ability to inhibit violacein production. Statistically, *S. lancea* (IC_{50} = 0.17 mg/mL) and *S. batophylla* (IC_{50} = 0.22 mg/mL) had the best ability to prevent the production of violacein among the plant extracts, while *S. leptodictya* had the least violacein inhibition (IC_{50} = 0.58 mg/mL).

3.4 Discussion

Many conventional interventions such as oral rehydration therapy (ORT), antisecretory or pro-absorptive agents and probiotics (post antibiotics administration) are used to treat diarrhoea (Kelly, 2011). However, medicinal plants have also been used to treat various medical conditions including diarrhoea (Ahmed *et al.*, 2012). Research interest in plant products has been increasing because of the increase in antibiotic resistance which is often linked with biofilm formation (Jamal *et al.*, 2018), presence of numerous biologically active phytochemicals which targets different microbial metabolic pathways, consequently inhibiting microbial growth and survival (Adnan *et al.*, 2021). Despite the antimicrobial potency of medicinal plant products, there are scarce in-depth analyses on their ability to inhibit biofilm formation and quorum sensing (Al-Shabib *et al.*, 2017).

In this study, nine indigenous South African plants were selected based on ethnobotanical record of use in treating diarrhoea, literature, and antibacterial activity in preliminary studies. The antimicrobial, antibiofilm and anti-quorum sensing potential of the selected plants was investigated. *E. coli* ATCC 25922, a recommended reference strain for antibacterial susceptibility testing, and other bacteria based on their association with causing diarrhoea episodes and ability to form biofilms were selected in this study (Kelly, 2011; WHO, 2017; Li *et al.*, 2022).

The selected plants were screened for antibacterial, anti-biofilm and quorum-sensing inhibition activities. Five different solvents with varying polarities were used for extraction and extracts were tested against the *E. coli* ATCC 25922 reference strain to determine the extractant with the promising antibacterial activity. Acetone extracts had the most significant antibacterial activity against the tested bacteria, and thus conform with the previous report on acetone as the best extractant for the screening and isolation of antimicrobial components from plants(Eloff, 1998a). According to Eloff (2021), an MIC value of < 0.02 mg/mL is regarded as outstanding activity, 0.021-0.04 mg/mL as excellent activity, 0.041-0.08 as very good activity, 0.081-0.16 mg/mL as good activity, $0.16 \leq MIC \leq 0.32$ mg/mL as moderate or average activity, while MIC

values above 0.32 are considered weak activity. All the acetone plant extracts had good antibacterial activity against all three of the *E. coli* strains, with only *S. batophylla* having a slightly weaker MIC value (0.18 mg/mL) above the cut-off point (0.16 mg/mL) against *E. coli* 0157:H7. The acetone extract of *B. bowkeri* had outstanding antibacterial activity against *S.* enteritidis, an organism that causes gastrointestinal disorders (Chang *et al.*, 2022). The antibacterial potency of *B. bowkeri* in this study was better when compared with previous findings (Ahmed *et al.*, 2012). This may be due to variation in the phytochemical constituents of the plant because of the difference in geographical location and period of the year the leaves were harvested for investigation. Little information is available on the antibacterial activity of some of the plants; however, petroleum ether, dichloromethane, ethyl acetate and water extracts of *S. lancea* have been reported to possess good bacterial inhibitory activity when tested against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* (Mulaudzi *et al.*, 2012). The acetone extract of *Bauhinia galpinii* has also been reported to have good antibacterial and antifungal activities and is known to be phenolic-rich in content, which probably accounts for the antimicrobial action (Ahmed *et al.*, 2012), and this supports the outcome of this work.

For medicinal plants to have clinical relevance, the preparation should be selective in its toxicity. Therefore, a cell-based *in vitro* assay for cytotoxic evaluation against Vero monkey kidney cells was used to determine the toxicity of the plant extracts. Our findings showed that the toxicity value obtained for all the plant extracts was above the toxic cut off level of 0.02 mg/mL (Kuete *et al.*, 2011). The toxicity values were statistically lower than that of doxorubicin, a toxic drug. This suggests that the observed antibacterial activity of the plant acetone extracts may not be due to a toxic metabolic effect.

The selectivity index (SI) is the ratio of toxicity to bioactivity and is often used to evaluate the degree of selective activity of a substance (Kutsuno *et al.,* 2015). Selectivity index values greater than 1 indicate that there is greater toxicity against bacteria or infectious agents than to host cells. Preparations with SI values greater than 10 are often considered to be valuable in pursing product development (Katsuno *et al.*, 2015). The antibacterial activities of the acetone extracts of both *B. galpinii* and *B. bowkeri* were excellent and were coupled with low cytotoxicity, thus revealing good prospects for product development. Excellent SI values above 25 for these two plant extracts were obtained against *S.* enteritidis, which is extremely promising and deserving of further research.

Developing a non-biocidal strategy to combating microbial infections is of paramount importance because use of antibiotics commonly leads to drug resistance, which is of global medical concern. Biofilm forming bacteria have shown resistance to broad-spectrum antibiotics, making the treatment of biofilm-related infections very difficult (da Silva *et al.*, 2021). The findings of Mohamed *et al.* (2007) showed a link between biofilm formation and virulence gene expression that could enhance attachment of diarrhoea pathogens to mucosal surfaces of the large and small intestine resulting in immune cells evasion and prolong infectious diarrhoea (Costerton *et al.*, 1999). In this study, we found that the optimum biofilm formation time for the bacterial strains studied was 24 h. This result is contrary to the finding of Al-kafaween *et al.* (2019) who found that three days of incubation led to the highest formation of biofilms for *Pseudomonas aeruginosa*. This may be due to loss of exopolysaccharides which activates biofilm detachment from surface wall or because of the different strains used (da Silva *et al.*, 2021). *Salmonella* enteritidis, *E. coli* O157:H7 and *S. aureus* did not form substantial biofilms. Our aim was to determine the ability of the extracts to either inhibit biofilm formation or to eradicate preformed biofilms, or both, so a concentration of half-MIC was used to ensure that the experiment was conducted at appropriate concentrations that did not completely inhibit bacterial cell growth. All plant extracts showed good biofilm inhibition activity, however most of them were unable to destroy preformed biofilm but rather promoted further development of the established biofilms (negative % inhibition values). Only *B. cereus* preformed biofilm was eradicated with values $> 50\%$ by all the plant extracts, except *B. galpinii.* The present anti-biofilm results showed that prevention of biofilm formation is easier than eliminating the existing biofilm. A similar observation was also made by Erhabor and colleagues in their study of *in vitro* bioactivity of *Combretum elaeagnoides* leaf extract against selected foodborne pathogens (Erhabor *et al.*, 2021). This could be due to the ability of plant extracts to curtail binding forces that promote cell attachments as suggested by (Famuyide *et al.*, 2019). According to a report by Taufiq and Darah (2020), the negative value suggests that the bacteria reacted to the change in the environment, consequently producing a large amount of biofilm to annul the effect of the perceived unfavourable environmental condition. In addition, it is also suggested that the difficulty in total eradication of biofilm is because the consortium of microbial growth is formed by the interaction of multiple species. The biofilm inhibition results thus suggest that some of the selected plants could be considered in the design of a good alternative therapy for preventing microbial colonization of surfaces and epithelial layers prior to infections. It is

therefore necessary to further investigate with clinical trials and their probable mechanisms of biofilm inhibition.

Quorum sensing (QS) is a bacterial intercellular communication system that allows the control of specific processes such as biofilm formation (Brackman and Coenye, 2015). QS has been linked to some virulence expression in both Gram-positive and Gram-negative bacteria. Gram-positive bacteria often secrete autoinducer peptides (AIPs) which in high concentration activate genes expression such as toxin and degradative enzymes. Gram-negative bacteria on the other hand, usually produce autoinducer homoserine lactones (AHLs). Increase concentration in the bacteria environment promotes expression of specific virulence genes such as adhesins and proteases (Lu *et al.*, 2019). Therefore, targeting this system is another way to curtail infectious agents' propagation. Therefore, we also investigated the ability of the acetone extracts to interfere with QS signalling process. The tested acetone extracts were able to disturb the quorum sensing processes mediated by the inhibition of violacein production. All the plant extracts except *S. leptodictya* had minimum quorum sensing inhibitory concentration (MQSIC) values that were statistically better than vanillin, the positive control at a significant level of $p < 0.05$. The recorded MQSIC values were lower than their respective MICs; this implies that the QSI was not because of cell growth inhibition; it rather indicates that the plant extracts could regulate virulence factors by inhibiting violacein pigment formation at sub-MIC. Previous research findings have reported anti-quorum sensing (AQS) activity of medicinal plants against *C. violaceum* and other bacteria (Itumeleng *et al.*, 2022). *Bauhinia galpinii* and *B. bowkeri* acetone extracts were previously reported to contain phenolic compounds, and compounds rich in phenol have been shown to be capable of inhibiting the synthesis of N-decanoyl-homoserine lactone, downregulate QS mediated metabolite (ethanolamine), reduced production of violacein and haemolysin, repress QS related gene expression (*cvil* and *cviR*) in *C. violaceum* (Cheng *et al.*, 2020).

Thus, QS and biofilm formation inhibition ability of the studied plant extracts may play an important role in reducing bacterial biofilm formation and therefore mitigate diarrhoeal infections as well as the development of antimicrobial resistance. However, there is a need to further investigate the exact mechanism of quorum signal inhibition as well as the compounds responsible for the observed activity.

3.5 Conclusion

Acetone extracts of *Searsia leptodictya, S. lancea, S. batophylla, S. pendulina, Bauhinia galpinii* and *B. bowkeri* had very good antibacterial activity against a panel of bacteria implicated in causing diarrhoea symptoms. In general, acetone was the most successful in extracting antibacterial compounds. *Bauhinia bowkeri* and *B. galpinii* had excellent selectivity index values of 50.75 and 25.18 respectively against *S. enteritidis* and are thus prospective candidates for product development. This study also showed that the acetone extracts of the selected plants had significant inhibitory activity against biofilm formation and quorum sensing mediated violacein pigment production at sub-MIC values. Disruption of preformed biofilms was difficult to achieve, indicating that the plant extracts had little efficacy against established bacterial biofilms. The plants with good activity have the potential to be developed as antibacterial remedies but further studies, particularly *in vivo*, are recommended to investigate potential pharmaceutical applications.

Purification and characterisation of the bioactive compounds from the most promising plant species, including antibacterial, anti-biofilm and anti-quorum sensing principles would be useful. In addition to possibly serving as framework molecules for development of novel chemicals to treat diarrhoea and related symptoms, these may serve as potential chemical markers that can be used to standardise plant-based preparations derived from the plant species of interest.

Chapter 4: Isolation of antibacterial compounds and their in vitro safety from *Searsia batophylla* (Codd) Moffett. acetone crude leaf extract effective against *Escherichia coli*.

Preface

This chapter has been submitted for publication in MDPI-Metabolites and is under review.

Abstract: *Searsia* species (Anacardiaceae) are used for different medicinal purposes in southern Africa, including gastrointestinal disorders, diarrhoea and gall sickness. *Searsia* spp reportedly possess antimicrobial, anthelmintic, antioxidant and cytotoxic activities. The present study aimed to evaluate the bioactivity and cytotoxicity of fractions and isolated compounds from the leaves of *Searsia batophylla* (Codd) Moffett. The crude acetone extract was partitioned with water, ethyl acetate, hexane, methanol, butanol, and chloroform. A two-fold serial dilution assay was used to determine the antibacterial activity of fractions and compounds against *Escherichia coli* ATCC 25922 and an *E. coli* isolate from a clinical case of diarrhoea in cattle. The free radical scavenging ability of samples was determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays. Cytotoxicity was investigated against Vero monkey kidney cells.

Bioassay-guided fractionation of the chloroform fraction yielded three compounds. The crude extract and the partitioned fractions had good antioxidant activity in both ABTS and DPPH assays with most of their IC_{50} values lower than 50, however, the chloroform fraction had poor radical scavenging ability. Structure elucidation was conducted using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and gas chromatography-mass spectrometry (GC-MS). Fractions obtained from the column chromatography had varying Minimum inhibitory concentration (MIC) values ranging from 0.04 to 2.50 mg/mL. Dodecanamide, 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione (α, β-unsaturated ketone), and 3-oxo-olean-18-en-28-oic acid were isolated from *S. batophylla* leaves. Although the chloroform fraction had a MIC value of 0.16 mg/ml, the purified compounds had MIC values ranging from 0.63 to 0.93 mg/mL. The compounds had more than 50% cell viability at the highest concentration tested of 0.2 mg/mL against Vero cells. This study shows that the crude extract of *S. batophylla* had better antibacterial activity than the isolated compounds, suggesting synergistic antibacterial activity of several components. In addition, the extract and compounds had low cytotoxicity to Vero cells. Further studies are recommended to elucidate other

mechanisms of anti-diarrhoeal efficacy of the compounds, such as biofilm and quorum sensing inhibitory activities.

Keywords: *Searsia batophylla;* structure elucidation; antibacterial; cytotoxicity; antioxidant

4.1 Introduction

Diarrhoea refers to incessant bowel movements or the passage of usually soft or watery faeces. Infections of the gastrointestinal tract (GIT) by viruses, bacteria or parasites primarily cause diarrhoea and are transmitted through contaminated water by faeces (Kelly, 2019). Symptoms of diarrhoea usually resolve in a few days, but there may be loss of body fluids, salts and nutrients in severe cases, leading to dehydration and, ultimately, death (Shamkuwar, 2013; Levine *et al.*, 2020). Diarrhoea is common in underdeveloped countries, particularly affecting children below the age of five due to restricted access to potable drinking water and lack of environmental sanitation. According to WHO (2013), about 760 000 deaths occur across the globe yearly owing to diarrhoea. In South Africa, about 43 000 annual deaths of adults can be attributed to diarrhoea, with HIV/AIDS as a contributing factor (Mara, 2001). Diarrhoea is also notorious in farm animals, affecting productivity, quality of farm products such as milk and meat and eventual death of farm animals if not properly managed (Windeyer *et al.*, 2014; Arnaiz *et al.*, 2021). Infectious bacterial agents of diarrhoea originate predominantly from the Enterobacteriaceae family but also include *Staphylococcus aureus* and *Campylobacter* species (Wiemer *et al.*, 2011; Li *et al.*, 2022). Apart from the economic implications of diarrhoeal treatment, bacterial resistance to antibiotics comprises a major global health concern that needs urgent intervention. Traditionally, medicinal plants have been relied upon for treating various ailments, including diarrhoea, because of their effectiveness, accessibility and availability (Singh and Bharadvaja, 2021). Many people in developing nations depend on using plants as therapy, with their therapeutic effect owing to the existence of several bioactive compounds, including tannins, terpenoids, flavonoids, carbohydrates, essential oils and phenols (Ibrahim and Kebede, 2020).

Furthermore, empirical evidence from literature has established the potential of medicinal plants as therapy in treating various diseases, although no antimicrobial drug has been developed from plants owing to various challenges (Vaou *et al.*, 2021). These include the varying composition of plant compounds, inadequate empirical toxicological and clinical studies to establish safety and potency, difficulty in mapping out the complex interaction between compounds in herbal medicine from plants, poor storage conditions, inconsistency in preparation and, very importantly, the interest of pharmaceutical industries in developing drugs for chronic diseases because it is more economically rewarding than drug products that cure

patients within a few days (Calixto, 2000; Holford and Burne, 2006; Radulovic *et al.*, 2013). However, plant-based preparations with diverse bioactive effects could be an alternative to reduce the adverse effects of microbial infectious agents.

The family Anacardiaceae contains about 800 different species that grow in tropical and subtropical regions (Phongkrathung *et al.*, 2016). The genus *Searsia*, previously known as *Rhus*, was renamed after Paul B. Sears, a former leader of Yale School of Botany (Moffett, 1994). Species of this genus are mostly shrubs and resinous trees with trifoliolate leaves, although some bear simple leaves with a resinous smell when mashed. *Searsia* is a widespread and abundant genus in Southern Africa. (Qiu *et al.*, 2016). *Searsia* species have been used indigenously to treat cough, gastrointestinal and cardiovascular disorders, diarrhoea, gall sickness, fever, malaria, snakebite, jaundice, paralysis and obesity (Chen *et al.*, 2009; Alimi *et al.*, 2013; Ahmadi *et al.*, 2017). Studies conducted on extracts of this genus have shown that they possess desirable biological activities such as antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, antiviral, antifungal, antimalarial, antitumorigenic, cytotoxic, antimutagenic and antifibrogenic (Rayne and Mazza, 2007; Opiyo *et al.*, 2021). Compounds isolated from *Searsia* species have anti-HIV activities (Wang *et al.* (2008), as well as antidiabetic and antioxidant activities, indicating interesting prospects for novel drug development (Rayne and Mazza, 2007; Alqasoumi *et al.*, 2016).

Biologically active compounds have been previously isolated and identified from *Searsia* species such as 3-(*Z*)-heptadec-13-enyl), rhuschromone, benzene-1,2-diol, 2',4’-dihydroxychalcone-(4-*O*-5''')-4'',2''',4’’’-trihydroxychalcone from *S. natalensis* root bark Mwangi *et al.* (2013), quercetin and fisetin from S. *mysorensis* leaves (Opiyo *et al.* (2010), pinoresinol and moronic acid isolated from *S. javanica*, quercetin-3-*O*-β-D-glucopyranoside from *S. parviflora*, 7-*O*-methylluteolin isolated from *S. retinorrhea*, gallic acid from *S. glabra* (Saxena *et al.*, 1994; Lee *et al.*, 2005; Rayne and Mazza, 2007; Zhou *et al.*, 2017), 2'',3’’-dihydrohinokiflavone isolated from *R. tripartita* stem bark (Alqahtani *et al.*, 2019). Because of the importance of the *Searsia* genus in traditional medicine, it is necessary to investigate the phytochemical and bioactive compounds present in this under-studied species (Opiyo *et al.* (2021) and as a follow-up study on previous findings which show outstanding antibacterial activity of *S. batophylla* (Adeyemo *et al.*, 2022). The aim of this work was to assess the antibacterial and antioxidant activity of fractions and the in vitro safety of compounds isolated from *S. batophylla* leaves.

4.2 Materials and Methods

4.2.1 Preparation of plant materials

Leaves of *Searsia batophylla* were collected during the winter of July 2019 at the Lowveld National Botanical Garden in Nelspruit, Mpumalanga, South Africa. The voucher specimen was lodged in the South African National Biodiversity Institute (SANBI) National Herbarium, Pretoria, South Africa. The plant leaves were air-dried at room temperature in a ventilated room. The dried leaves were crushed into fine powder using a Janke and Künkel Model A10 mill and stored in covered glass jars in the dark at room temperature.

4.2.2 Fractionation of bioactive fractions

4.2.2.1 Reagents and solvents

Acetone, methanol, ethyl acetate, hexane and butanol were obtained from Minema Chemicals, South Africa. Ethanol and chloroform were purchased from Sugar Illovo limited and Associated Chemical Enterprises, South Africa, respectively

4.2.2.2 Bulk extraction and solvent-to-solvent fractionation

Powdered leaves of *Searsia batophylla* (500 g) were extracted with 5 000 mL of fresh acetone (subjected to vigorous mixing on a Labotec shaker (Labotec, South Africa). This process was repeated until the extract turned pale, indicating exhaustive extraction. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated using a Büchi R-114 rotary evaporator (Büchi, Germany). The process was repeated five times, and 37.31 g of crude extract was obtained.

The acetone extract (35 g) was dissolved in a 1:1 combination of chloroform and water (320 mL each) in a separating funnel to obtain chloroform and water fractions. The water fraction was further partitioned with 320 mL of *n*-butanol, hexane, and ethyl acetate. The chloroform fraction was returned to the separating funnel and partitioned with 35% methanol. The fractions were evaporated to dryness with a rotary evaporator (Büchi, Germany).

4.2.3 Thin Layer Chromatography (TLC) and TLC Bioautography

The qualitative screening was done for the fractions of chloroform, ethyl acetate, 35% methanol, butanol, water, and hexane using the method of (Kotze and Eloff, 2002). The phytochemical fingerprint of the fractions was determined by using aluminium-backed thin-layer chromatography (TLC) silica gel plates (10 × 20 cm, 60 F254, Merck, United States). A

10 μL solution of fractions dissolved to a concentration of 10 mg/mL was spotted on the TLC plate, developed with a solvent system of varying degrees of polarities, namely benzene: ethanol: ammonium hydroxide (BEA) in the ratio 90:10:1 (non-polar, basic); chloroform: ethyl acetate: formic acid (CEF) in the ratio 5:4:1 (intermediate polarity, acidic) and ethyl acetate: methanol: water (EMW) in the ratio 40:5.4:5 (polar, neutral), respectively. On the TLC plate, fractions were put in a band with a width of 1 cm, and visible bands were marked faintly under ultraviolet light (Camag) of 254 and 365 nm wavelengths. The TLC plates were sprayed with a freshly prepared vanillin-sulphuric acid solution (0.1 g vanillin was dissolved in 28 mL methanol and 1 mL sulphuric acid). After that, plates were heated in an oven (about 110°C) until visible colour developed.

Bioautography was done to ascertain the presence of active compound(s) in the fractions. The TLC plates were developed as described earlier, except for the vanillin spray. The plates were allowed to dry in cold air to get rid of residual eluents. Furthermore, plates were sprayed with a standardized overnight culture of test culture of *E. coli* (ATCC 25922) until wet in a biosafety class II fume cabinet (ESCO, South Africa). The wet plates were allowed to dry and incubated for 24 h at 37^{0}C to enhance the growth of sprayed test bacteria. After incubation, the plates were sprayed with freshly prepared p-iodonitrotetrazolium (INT) with a concentration of 2 mg/mL dissolved in sterile distilled water and further incubated for 1-2 h. A clear zone against the pink/red background denotes the inhibition of bacterial growth by the separated compounds on the plate.

4.2.4 Antioxidant activity

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

The free radical scavenging activity was determined using the ABTS cation decolourization method of Re *et al.* (1999) with slight modifications. ABTS radical solution (7 μM) was prepared by dissolving 76.81 mg of ABTS in 20 mL methanol solution and 26.49 mg of potassium persulphate ($K_2S_2O_4$) in 20 mL of methanol. The solutions were mixed and kept in the dark for 18 h at room temperature. The ABTS radical solution was further diluted with methanol to an absorbance of 0.7-0.8 at a wavelength of 734 nm. A 40 μL aliquot of fractions of the 1 mg/mL stock solution was prepared in different concentrations in 96-well microtitre plates, and 160 μL of ABTS radical solution was added to each well. Ascorbic acid, Trolox, and methanol were used as positive and negative controls, respectively. After 5 min of

incubation, the absorbance was read using a microplate reader (Epoch-BioTek, USA). The percentage of radical scavenging activity was calculated with the formula below.

ABTS scavenging activity (%) = [(A1-A2/A1)] x 100.

Where A1= absorbance of control reaction, A2= absorbance of the sample.

The 50% inhibitory concentration (IC_{50}) values were extrapolated from the graph obtained by plotting percentage inhibition against the concentrations. Each experiment was repeated thrice.

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

The method of Brand-Williams *et al.* (1995), with modification, was used to determine the DPPH radical scavenging ability of the fractions. Ascorbic acid and Trolox served as the positive control and methanol as the negative control. In brief, samples were dissolved in methanol to a concentration of 1 mg/mL. After that, 40 µL of samples and both positive controls, Trolox and ascorbic acid, were prepared in different concentrations in a 96 well-microtitre plate. One hundred and sixty (160) µL of DPPH solution was added. The mixture was allowed to incubate for 30 min in the dark, and the absorbance of the microtitre plate was measured with a microplate reader (Epoch, Biotek) at 517 nm. The percentage of DPPH scavenging activity was determined using the expression:

DPPH scavenging activity (%) = [(A1-A2/A1)] x 100.

Where A1= absorbance of control reaction, A2= absorbance of the sample.

From the graph plotted as a percentage of inhibition versus the concentrations, the IC_{50} values were extrapolated. Each experiment was repeated thrice.

4.2.5 Isolation of bioactive compounds

Isolation of bioactive compounds from the chloroform fraction was done using open mobile column chromatography with a stationary phase of silica gel (Merck silica gel 60). Silica gel powder (150 g) was mixed with *n*-hexane and then transferred into a column. The column was allowed to settle overnight. The chloroform fraction (15 g) was dissolved in 45 mL of acetone and mixed with 30 g of silica gel. The mixture was air-dried overnight and loaded on the packed column. Hexane/ethyl acetate and ethyl acetate/methanol were used as mobile phases to elute the fractions starting with 100% hexane, followed by a gradient increase of ethyl acetate up to 100%. After this, a gradient increase of methanol up to 100% followed. Collections of fractions were made in volumes of 250 mL, yielding 72 fractions (before TLC analysis).

Nine subfractions were obtained based on similarities in the TLC fingerprint (F1-F9) based on the three solvent systems (BEA, CEF and EMW). The fractions F6 and F7 were combined based on their antimicrobial activity and similarity in terms of TLC profiles. The combined

fractions (F6 and F7, G) were further fractionated using a smaller column with the same mobile phase gradients described above to elute the fractions.

From the combined fraction (G), a total of 57 fractions were collected in 250 mL honey jars. Fractions were analysed using TLC, and six fractions were obtained (G1-G6) after combining fractions with similar profiles. Fractions G2, G3 and G4, had good antibacterial activity. The fractions G2 and G3 were combined (Gc) because their profiles were similar on the TLC plate using hexane: ethyl acetate (4:1) as the solvent system.

Both Gc and G4 were further purified using preparative thin-layer chromatography (20 cm by 20 cm silica gel PTLC plates) manufactured by Merck, Germany, using the solvent system hexane: ethyl acetate (4:1). Two compounds, Sb-6 and Sb-7 were isolated from Gc and from G4 compound Sb-14 was obtained. The schematic representation of fractionation of the plant extract to achieve the compounds is shown in **Fig 4.1**.

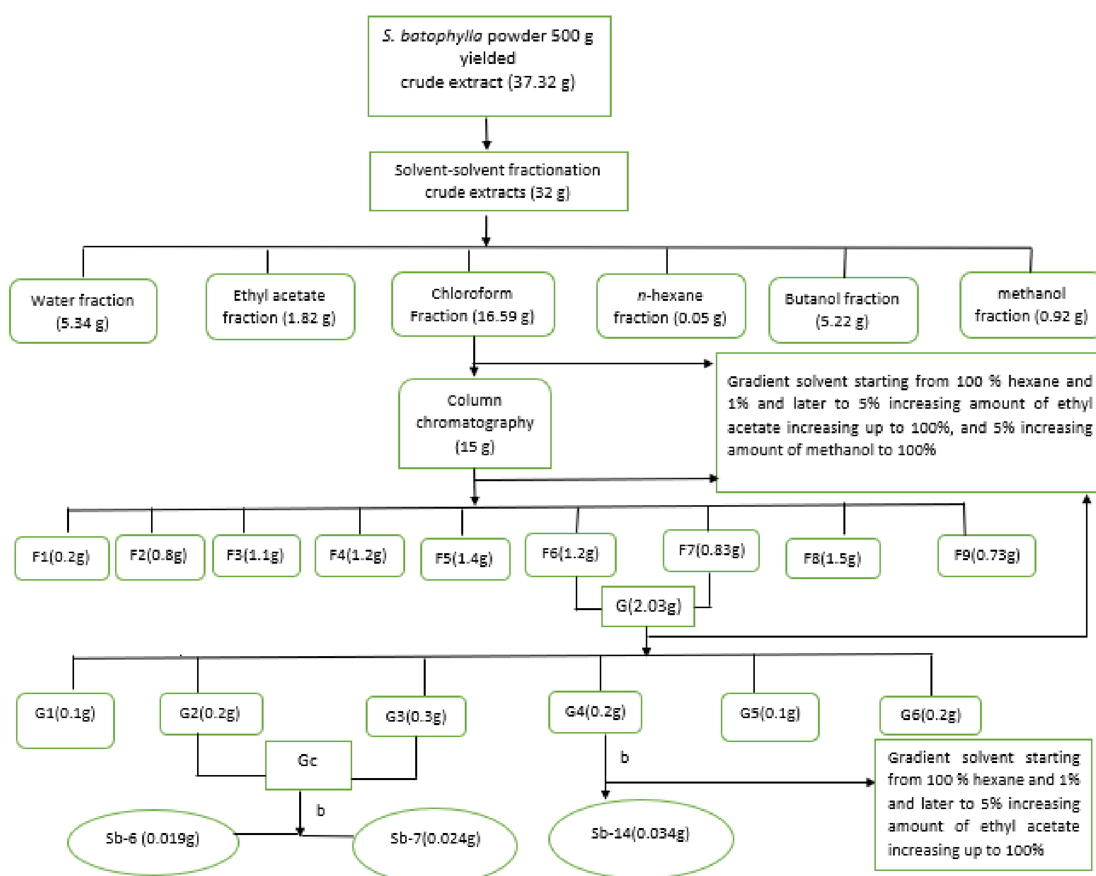


Fig 4.1: Schematic extraction and fractionation towards compound isolation from *Searsia batophylla*

4.2.5.1 Liquid chromatography-mass spectrometry (LC-MS)

Compound separation and detection were performed using a Waters® Synapt G2 high definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, USA).

The system comprises a Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument. The system was operated with MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) for data acquisition and processing. An internal lock mass control standard, 2 pg/µL solution leucine enkephalin (*m/z* 555.2693), was directly infused into the source through a secondary orthogonal electrospray ionization (ESI) probe allowing intermittent sampling. The internal control was used to compensate for instrumental drift, ensuring good mass accuracy throughout the duration of the runs. The instrument was calibrated using sodium formate clusters and Intellistart functionality (mass range 112.936 – 1 132.688 Da). Resolution of 20 000 at *m/z* 200 (full width at half maximum (FWHM)) and mass error within 0.4 mDa were obtained.

4.2.5.2 Gas chromatography-mass spectrometry (GC-MS)

A LECO Pegasus 4D GC-TOFMS (LECO Africa (Pty) Ltd., Kempton Park, South Africa) GC column Rxi-1MS 30 m x 0.25 mm ID x 0.2 µm film thickness (Restek, Bellefonte, PA, USA) was used. Injection volume 1uL, splitless injection, splitless time 30s GC inlet 250°C GC oven temperature programme 40 °C (hold for 3 min) at 10°C/min to 300°C (hold for 5 min). Carrier gas UHP Helium (Afrox, South Africa) at 1 ml/min, constant flow mode Mass acquisition range 40-550 Da. MS transfer line temperature 280°C Ion source temperature 230°C MS solvent delay 5 min Electron energy 70 eV in the electron ionization mode (EI+) Data acquisition rate 10 spectra/s Detector voltage 1750 V.

4.2.6 Antibacterial activity of fractions and compounds

A twofold serial dilution microplate method (Eloff, 1998) was used to evaluate the minimum inhibitory concentration (MIC) of all the fractions and compounds against *Escherichia coli* ATCC 25922 and *E. coli* isolated from a clinical case of bovine diarrhoea. Before the experiment, 18 - 24 h old bacterial culture was grown in Mueller Hinton broth (Merck, South Africa), incubated at 37 °C in an orbital shaking incubator at 150 rpm (United Scientific, South Africa). After that, the *E. coli* culture was adjusted to McFarland standard 1 (approximately 3.7×10^8 colony forming unit per mL, CFU/mL) using a spectrophotometer (Epoch-Biotek, USA) measured at a wavelength of 560 nm. A 100 µL aliquot of 10 mg/mL (fractions) and 5 mg/mL (compounds) were added in triplicate to the first row of the microplate (Lasec, South Africa) and serially diluted in 1:1 ratio with sterile distilled water. Bacterial culture (100 µL) was added to each well. The antibacterial activity was tested at concentrations ranging between 2.5 to 0.02 mg/mL for fractions and 1.25 to 0.01 mg/mL after two-fold dilution. The antibiotic gentamicin (Virbac, South Africa) and solvents in which the samples were dissolved served as

positive and negative controls, respectively. Broth and water served as sterility controls. The microplates were covered and incubated in an oven (IncoTherm, Labotec) at 37° C for 24 h. A 40 µL aliquot of 0.02 mg/mL p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, South Africa) was added to each well and incubated for an hour. The presence of bacterial growth was indicated by the formation of red/pink-coloured formazan, which appeared following the reduction of INT by bacterial cells. The MIC was taken as the lowest concentration with no red/pink-coloured formazan formation. The experiment was performed thrice for fractions and twice for compounds.

4.2.7 Cytotoxic analysis and selectivity index of the isolated compounds

The cytotoxicity of the isolated compounds was determined against Vero African Green monkey kidney cells (ATCC® CCL-81TM). The MTT (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide) in vitro assay (Mosmann, 1983) modified by (McGaw et al., 2007) was used. The cells were cultured and maintained in Minimal Essential Medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% of gentamicin (Virbac) and 5% fetal calf serum (Adcock-Ingram). Cell suspensions were seeded from 70-80% confluent monolayer cultures, plated at a density of 1 x 10^4 into a flat-bottomed 96-well microtitre cell culture plate and incubated for 24 h at 37 ºC in 5% CO2 for cell attachment. Following incubation, the cells were aspirated, and fresh MEM was introduced. A 100 µL aliquot of the compounds was added to the wells containing the cells in varying concentrations. Doxorubicin (Pfizer) and DMSO were used as positive and negative controls, respectively. The plates were again incubated for 48 h in the CO_2 incubator. After incubation, the wells were washed with phosphate buffered saline (PBS, Sigma-Aldrich, South Africa), and fresh medium (200 µL) was added. After that, 30 µL of MTT (5 mg/mL in PBS) was added to all the wells and later incubated at 37oC for 4 h. The medium was aspirated from the cells, and 50 µL of DMSO was added to each well to dissolve crystallised MTT formazan. The absorbance was measured to determine the amount of reduced MTT at 570 nm absorbance using a microtitre plate reader (Bio Tek Synergy). Each selected concentration was tested in quadruplicate, and the assay was repeated twice. The plant extract concentration of 50% viable cell inhibition was calculated. Vero cell growth inhibition was calculated in terms of LC_{50} values. The selectivity index (SI) value of all extracts was determined by dividing the cytotoxicity LC_{50} value by the MIC value.

4.2.8 Statistical analysis

Data were entered and collated in Microsoft Excel 356 version. Graphpad Prism version 9.0 was used for data analysis using one-way analysis of variance (ANOVA) and Tukey's post hoc test, with a significance level of $p < 0.05$.

4.3 Results

4.3.1 Extraction and fractionation yield

The extraction of 500 g of finely ground *Searsia batophylla* leaves yielded 37.31 g (7.46%) of crude acetone extract. About 32 g was used for solvent-solvent partitioning to yield five different fractions of *n*-butanol (5.22 g), hexane (0.12 g), ethyl acetate (1.82 g) and 35% methanol (0.92 g). The water and chloroform fractions yielded 5.34 g and 16.59 g, respectively. The total mass recovered was 30.01 g, which denotes 93.78% recovery from the 32 g used for partitioning. The percentage recovery for each fraction was 16.31% for *n*-butanol, 0.53% for hexane, 5.68% for ethyl acetate, 2.87% for 35% methanol, 16.68% for water and 51.84% for chloroform, as shown in **Table 4.1**.

Table 4.1: Percentage yield, minimum inhibitory concentration (MIC) and total antibacterial activity (TAA) of S. batophylla fractions against E. coli ATCC 25922 and clinical strain

		***E. coli* ATCC**		***E. coli* NW**	
Sample	**% Yield**	**MIC (mg/mL)**	**TAA**	**MIC (mg/mL)**	**TAA**
Sb-cr	7.46	0.16 ± 0.05	608.69	**0.06** ± 0.02	1166.67
Sb-m	2.87	0.53 ± 0.10	48.27	0.63 ± 0.00	57.50
Sb-c	51.84	**0.11** ± 0.00	3240.23	**0.06** ± 0.02	7776.56
Sb-w	16.68	1.25 ± 0.00	133.50	0.63 ± 0.00	264.88
Sb-h	0.53	0.32 ± 0.00	16.60	0.83 ± 0.29	6.34
Sb-b	16.31	0.63 ± 0.00	258.92	0.63 ± 0.00	258.92
Sb-Et	5.68	**0.08** ± 0.00	710.93	**0.08** ± 0.00	710.93
F1	1.30	2.50 ± 0.00	5.2	2.50 ± 0.00	5.20
F2	5.33	2.50 ± 0.00	21.32	2.50 ± 0.00	21.32
F3	7.32	2.50 ± 0.00	29.28	2.50 ± 0.00	29.28

F4	7.99	2.50 ± 0.00	31.96	2.50 ± 0.00	31.96
F5	9.33	0.32 ± 0.00	291.56	0.32 ± 0.00	291.56
F6	7.99	**0.03** ± 0.00	2663.33	**0.04** ± 0.00	1997.50
F7	5.53	**0.04** ± 0.00	1382.50	**0.04** ± 0.00	1382.50
F8	9.99	0.32 ± 0.00	312.18	0.32 ± 0.00	312.18
F9	4.86	2.50 ± 0.00	19.44	1.25 ± 0.00	38.88
G1	4.92	2.50 ± 0.00	19.68	2.50 ± 0.00	19.68
G2	9.85	**0.14** ± 0.03	703.57	0.32 ± 0.00	307.81
G3	14.77	**0.13** ± 0.03	1136.15	0.32 ± 0.00	461.56
G4	9.85	**0.16** ± 0.00	615.62	0.24 ± 0.00	410.41
G5	4.92	2.50 ± 0.00	19.68	1.25 ± 0.00	39.36
G6	9.85	2.50 ± 0.00	39.4	1.14 ± 0.23	86.40
Gentamicin	NA	0.005 ± 0.00	NA	0.016 ± 0.00	NA

Sb, *Searsia batophylla*, Sb-cr, S. batophylla crude extract; Sb-m, methanol fraction; Sb-c, chloroform fraction; Sb-w, water; Sb-h, hexane; Sb-b, butanol; Sb-Et, ethyl acetate fractions; F1-F9, first column fractions, G1-G6, second column fractions. Values in bold indicate significant antibacterial activity.

4.3.2 Antibacterial activity by TLC bioautography of fractions

The TLC (plate 1) and TLC bioautography (plate 2) of the fractions showed a clear zone of inhibition in the hexane (Sb-H), chloroform (Sb-C), and ethyl acetate (Sb-Et) fractions as well as in the crude extract (Sb-cr) using BEA with retention factor (Rf) value of 0.31 and CEF with Rf value of 0.21, but no zone of inhibition was observed in the EMW eluent **(Fig 4.2)**. The TLC fingerprint (A) and the bioautography (B) of the chloroform combined fractions (F1-F9) showed no visible zone of inhibition **(Fig 4.3)**. Also, the TLC fingerprint of F6 and F7 combined (G) fractions as well as the TLC-bioautography on hexane: ethyl acetate (4:1) system showed a zone of inhibition with an Rf value of 0.33 on G2 and G3 **(Fig 4.4)**.

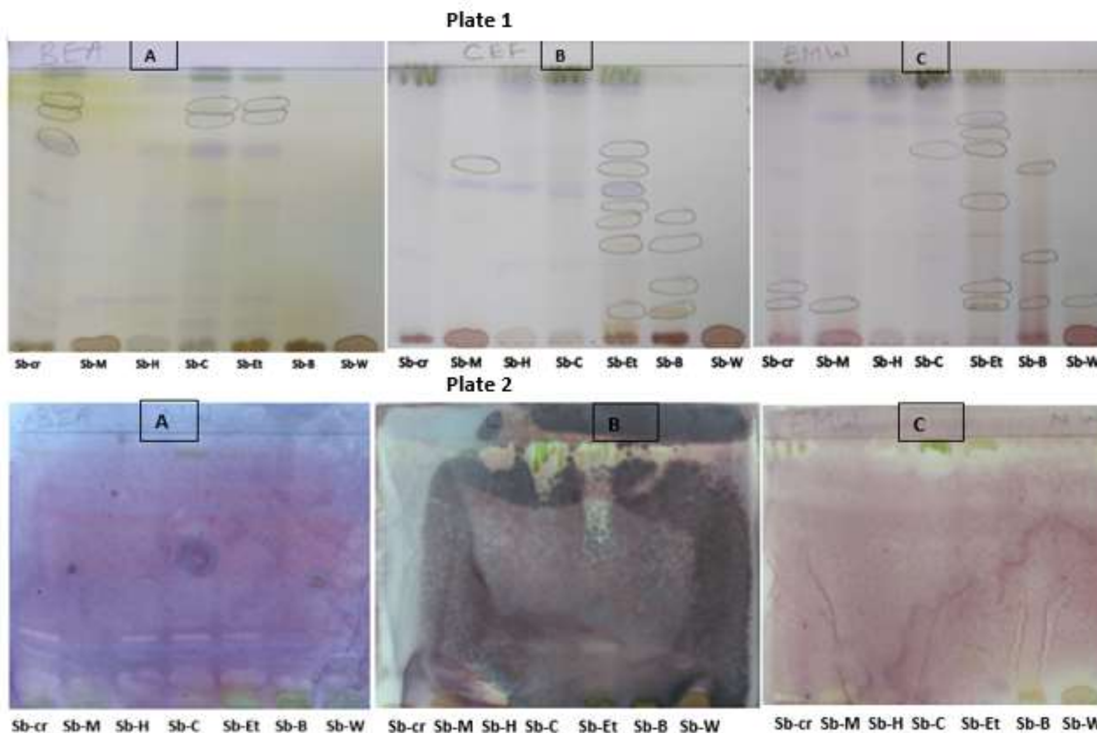


Fig 4.2 Plate 1: Chromatogram developed in (A) BEA [benzene: ethanol: ammonium hydroxide (90:10:1)], (B) CEF [chloroform: ethyl acetate: formic acid (7:3:0.5)] and (C) EMW [ethyl acetate: methanol: water (40:5.4:4)] Plate 2; Bioautography of clinical strain *E. coli*-TLC plate developed in (A) BEA (18:2:0.2), (B) CEF (5:4:1) and (C) EMW (40:5.4:5). Sb-cr, *S. batophylla* crude extract; Sb-M, methanol fraction, Sb-H, hexane fraction; Sb-C, chloroform fraction; Sb-Et, ethyl acetate fraction; Sb-B, butanol fraction; Sb-W, water fraction.

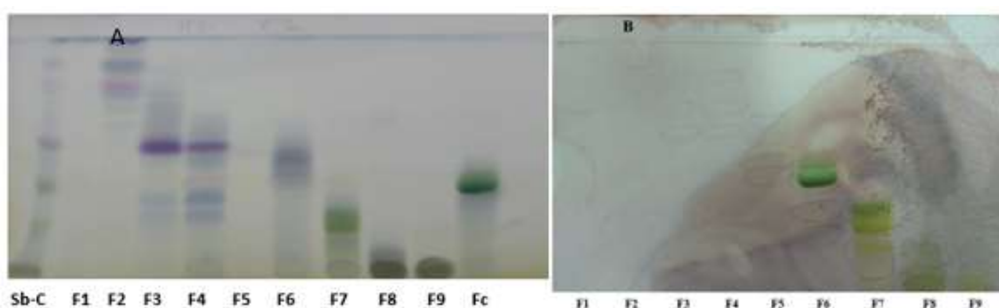


Fig 4.3: (A) TLC fingerprint and (B) TLC-bioautography of the first column chloroform fractions on hexane: ethyl acetate, 8:2, solvent system

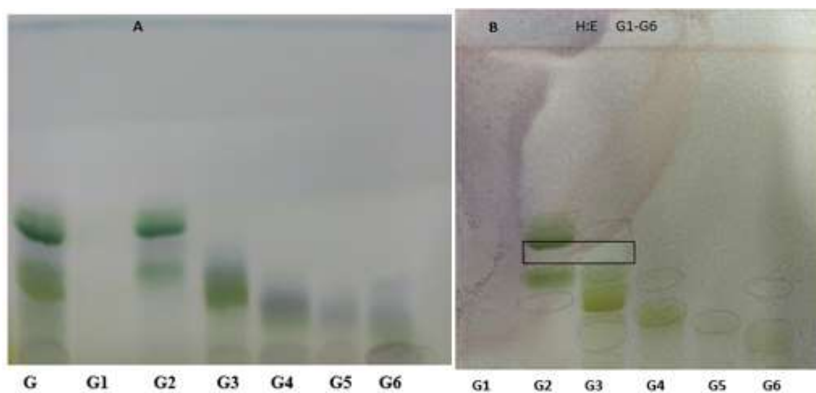


Fig 4.4: (A) TLC fingerprint and (B) TLC-bioautography of the second column chloroform fractions on hexane: ethyl acetate, 8:2 solvent system

4.3.3 Antioxidant activity

The ability of the *S. batophylla* fractions to scavenge free radicals was assessed using the ABTS and DPPH techniques. This assessment is based on how much the ABTS and DPPH solution's colour changed (Saleem *et al.*, 2016). The results were compared with Trolox and ascorbic acid (vitamin C). The lower the IC_{50} value, the better the ability to eliminate free radicals. In ABTS, the IC_{50} value ranged from 0.35 to 64.35 µg/mL for the partitioned fractions. The ethyl acetate fraction gave the best result, with its IC_{50} value lower than that of Trolox (2.02 µg/mL) but less than that of vitamin C (0.05 µg/mL). Statistically, there was no significant difference between the obtained antioxidant value of *S. batophylla* crude extract, methanol, butanol, water and ethyl acetate fractions (**Table 4.2**). However, column fractions of *S. batophylla* chloroform partition fraction exhibited poor antioxidant activity with IC_{50} values greater than 100 µg/mL. As with ABTS, the partition fractions were relatively good radical scavengers of DPPH with IC_{50} values ranging from 0.82 to 62.22 µg/mL, with the *S. batophylla* hexane fraction having the best antioxidant activity. The statistical evaluation showed no significant difference between the standards used (Vitamin C and Trolox) and *S. batophylla* methanol, butanol, water and ethyl acetate partition fractions. However, chloroform column fractions showed poor scavenging power.

Table 4.2: Antioxidant activity of *S. batophylla* extract and fractions

Sample	ABTS IC_{50} (µg/mL)	DPPH (µg/mL)
Sb-cr	$0.54±0.11^{a}$	$35.03±7.65^{b}$
Sb-C	$64.35±1.59^{b}$	$56.99±5.23^{b}$
Sb-H	$15.19±0.95^{a}$	$62.22±8.28^{b}$
Sb-M	$0.37±0.04^{a}$	$2.36±0.17^{a}$
Sb-But	$0.07±0.00^{a}$	$0.82±0.08^{a}$
SB-W	$0.53±0.11^{a}$	$2.42±0.03^{a}$
Sb-Et	$0.35±0.07^{a}$	$3.02±0.10^{a}$
F1	$133.83±1.84^{cd}$	$186.62±5.66^{d}$
F2	$123.51±1.72^{c}$	$106.75±7.87^{c}$
F3	$173.68±1.54^{d}$	$192.75±6.09^{d}$
F4	$188.93±10.45^{de}$	$170.98±13.10^{d}$
F5	$169.43±13.88^{d}$	$126.30±12.68^{c}$
F6	$132.98±7.12^{cd}$	$166.12±3.58^{d}$
F7	$199.79±8.98^{e}$	$129.55±8.01^{c}$
F8	$132.29±6.06^{cd}$	$198.25±9.64^{d}$
F9	$131.08±1.14^{cd}$	$181.55±4.46^{d}$
G1	ND	ND
G2	$106.91±8.05^{c}$	$180.40±17.74^{d}$
G3	$114.46±1.04^{c}$	$146.46±6.03^{d}$
G4	$149.23±8.45^{cd}$	$116.04±25.68^{c}$
G5	$133.60±0.14^{cd}$	$128.53±9.32^{cd}$
G6	$161.14±6.88^{d}$	$194.14±4.82^{d}$
G7	ND	ND
TRO	$2.02±0.17^{a}$	$4.70±0.02^{a}$
AA	$0.05±0.00^{a}$	$1.86±0.01^{a}$

ND- not determined, F1-F9; fractions of *S. batophylla* (chloroform fraction), G1-G7; fractions of F6 and F7 combined, TRO; Trolox, AA; ascorbic acid. Values with different letters indicate significant difference at $p < 0.0001$.

4.3.4 Structure elucidation and characterisation

Three compounds were isolated from the chloroform fraction and coded as Sb-6, Sb-7 and Sb-14. GC-MS and UPLC-MS were used to identify the compounds isolated by comparing with computer library data. Specifically, the GC-MS was done because the LC-MS could not detect some of the compounds. The molecular weight, CAS number, molecular formula, percentage area and similarity of the compounds Sb-6 and Sb-7 are recorded in **Table 4.3** Compound Sb-6 was identified as dodecanamide based on GC-MS analysis **(Fig 4.5a)**, with proposed structure and calculated molecular mass of 199.3336 g/mol presented in **Fig 4.5b**. This compound has percentage area and similarity of 30.0 and 90.3, respectively. Dodecanamide appeared as a very light-yellow band on ultraviolet (UV) light, however, no visible colour was observed on the TLC plate (R_f value = 0.75) when sprayed with vanillin **(Fig 4.6)**.

Table 4.3: GC-MS analysis for dodecanamide and 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione

Compound	Molecular weight (g/mol)	Molecular formular	CAS number	Similarity	Percentage area
Dodecanamide	199.3336	$C_{12}H_{25}NO$	1120-16-7	90.3	30.0
7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione	276.3724	$C_{17}H_{24}O_3$	82304-66-3	91.9	27.4

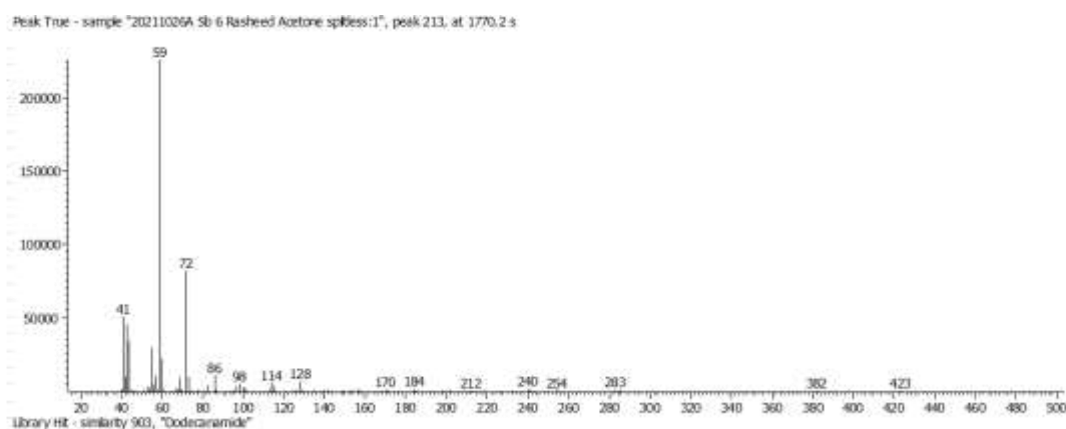
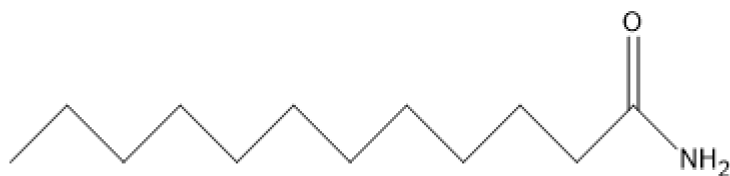


Fig 4.5a. GC-MS for compound dodecanamide



Dodecanamide
$C_{12}H_{25}NO$
Molecular weight 199.3336

Fig 4.5b: Structure of compound Sb-6 (dodecanamide)

Compound Sb-7 was elucidated as 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione based on GC-MS data **(Fig 4.7a)**. The percentage area and similarity index of the compound 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione are 27.4 and 91.9 respectively **(Table 4.3)**. The proposed structure and calculated molecular mass of 276.3724 g/mol are represented in **Fig 4.7b**. It appeared as a light grey band (R_f value = 0.45) on UV light but no distinct colour after spraying with vanillin **(Fig 4.6)**.

The compound Sb-14 was identified as 3-oxo-olean-18-en-28-oic acid based on UPLC-MS spectra in positive **(Fig 4.8a)** and negative **(Fig 4.8b)** modes. The structure and calculated molar mass of 454.6862 g/mol are shown in **Fig 4.8c**. The compound looked greyish black under UV (R_f value = 0.36) with no obvious colour after spraying with vanillin **(Fig 4.6).**

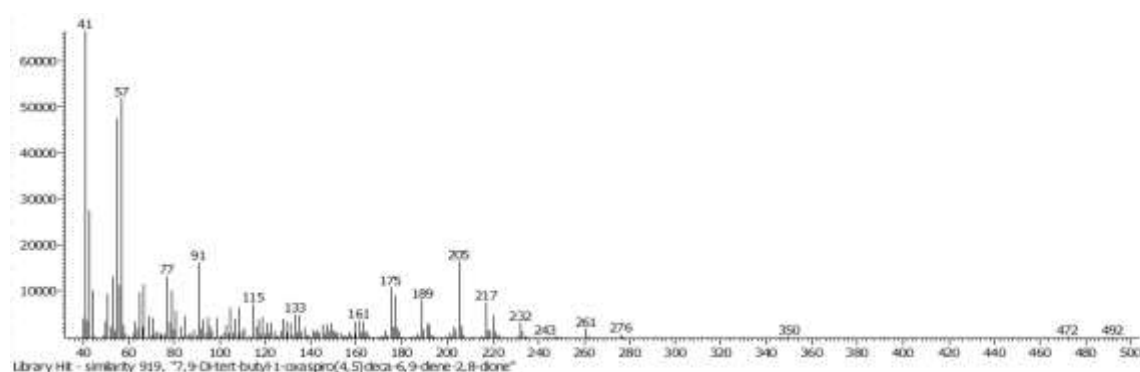
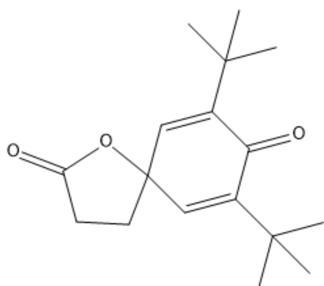


Fig 4.6a: GC-MS for compound 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione



7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
$C_{17}H_{24}O_3$
Molecular weight 276.3724

Fig 4.6b: Structure of 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione

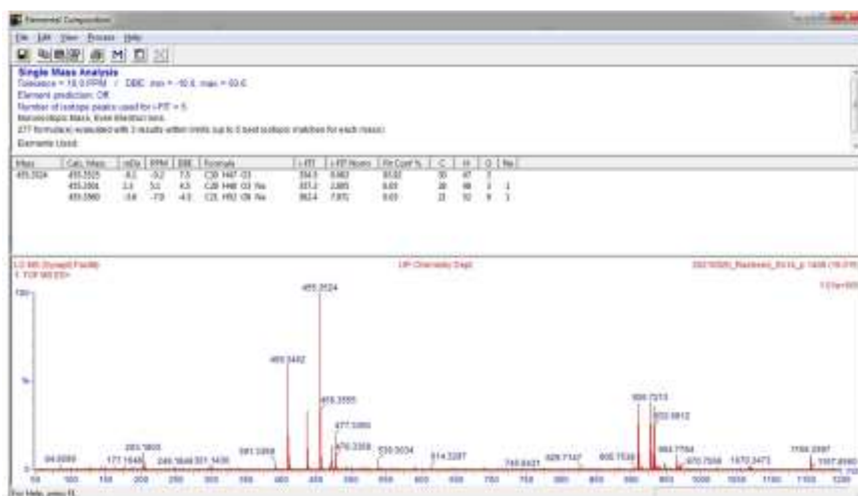


Fig 4.7a: UPLC-MS for compound 3-oxo-olean-18-en-28-oic acid (Positive mode)

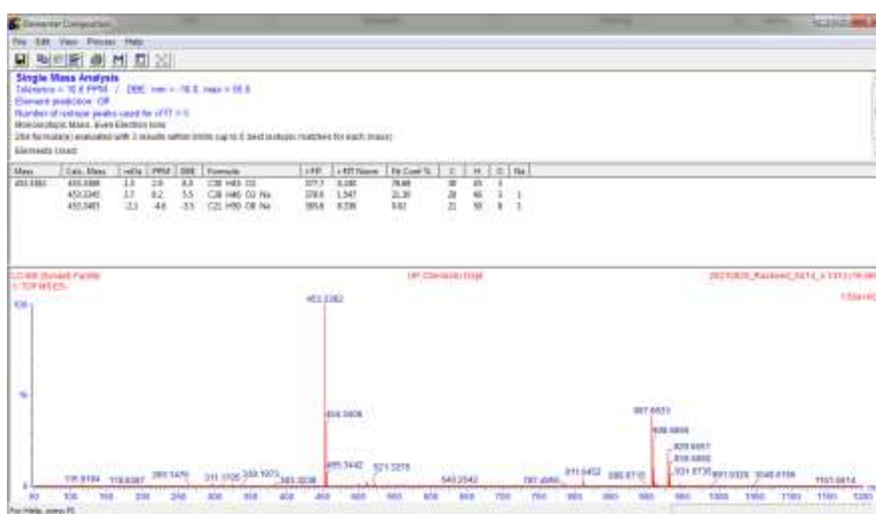


Fig 4.7b: UPLC-MS for compound 3-oxo-olean-18-en-28-oic acid (Negative mode)

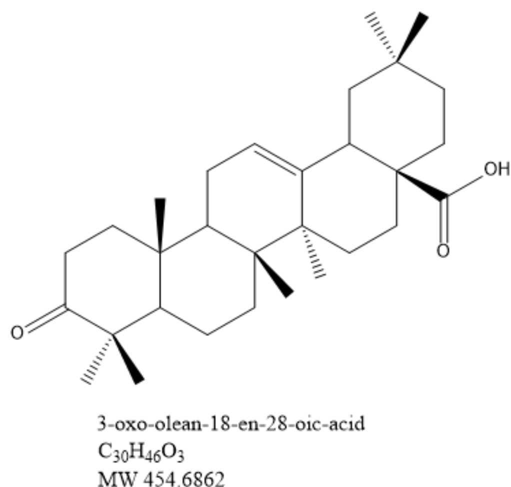


Fig 4.7c: Structure of 3-oxo-olean-18-en-28-oic acid

4.3.5 Antibacterial activity of fractions and total activity

Based on the previously determined antibacterial activity (MIC= 0.06-0.16 mg/mL) of the acetone crude extract of *Searsia batophylla* against the tested strains of *E. coli*, this species was selected for isolation of active compounds. The previous findings of Adeyemo *et al.* (2022 2022) reported that the ethyl acetate fraction of *S. batophylla* leaf acetone extract had the best antibacterial efficacy with MIC of 0.08 mg/mL, followed by the chloroform fraction with MIC value of 0.16 mg/mL. The chloroform fraction yield of 51.84% was the highest yield of all the solvents used for the partitioning and was therefore selected for the isolation of active compounds (**Table 4.1**). There was no significant difference in the MIC value of the chloroform fraction (MIC = 0.16 mg/mL) and the crude acetone extract (MIC = 0.11 mg/mL). Better antibacterial activity was also observed in the column fractions of chloroform F6 and F7 (MIC = 0.03 and 0.04 mg/mL). There was a decline in the antibacterial activity of fractions of the second column (G2, G3 and G4), with MIC values ranging from 0.13-2.5 mg/mL (**Table 4.1**). The positive control, gentamicin, had MIC values of 0.005 mg/mL and 0.016 mg/mL against the *E. coli* ATCC and clinical strain, respectively.

4.3.6 Cytotoxicity and selectivity index

All the compounds had significantly higher LC_{50} values and, therefore, lower cytotoxicity against Vero kidney cells compared to doxorubicin (**Table 4.4**). They had more than 50% cell viability at the highest tested concentration of 0.2 mg/mL. The selectivity index (SI) of the isolated compounds could not be determined exactly because their LC_{50} values were greater than the highest concentration tested, so the highest concentration was taken as being the LC_{50} for purposes of calculating approximate SI values.

Table 4.4: Antibacterial activity of the isolated compounds against *E coli* ATCC 25922, *E. coli* NW and cytotoxicity against Vero cells

Compound	Cytotoxicity (mg/mL)	*E. coli* ATCC		*E. coli* NW	
		MIC (mg/mL)	Selectivity index	MIC (mg/mL)	Selectivity index
Dodecanamide	0.20 ± 0.00	0.63 ± 0.00	0.31	0.63 ± 0.00	0.31
7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	0.20 ± 0.00	0.63 ± 0.00	0.31	0.63 ± 0.00	0.31
3-oxo-olean-18-en-28-oic acid	0.20 ± 0.00	0.93 ± 0.34	0.21	0.73 ± 0.25	0.27
Gentamicin	NA	0.005 ± 0.00	NA	0.005 ± 0.00	NA
Doxorubicin	0.01 ± 0.00	NA	NA	NA	NA

NA: not applicable

4.4 Discussion

Antimicrobial resistance development and restrictions on the use of antibiotics call for alternative sources to conventional antimicrobial agents. Over time, medicinal plants have shown great potential to halt the growth and pathogenicity of bacterial infections as well as to treat chronic diseases in humans and animals (Duraipandiyan *et al.*, 2006). The preliminary study revealed that *S. batophylla* acetone extract had promising biological activities such as antibacterial, antibiofilm and anti-quorum sensing effects (Adeyemo *et al.*, 2022 2022), which makes it a good candidate for the presence of potentially bioactive compounds. Therefore, this study aimed to assess the antibacterial activity of fractions and isolated compounds, antioxidant properties of fractions, as well as to assess the level of toxicity of isolated compounds from the leaf material.

The non-polar compounds in the chloroform fraction separated well using the BEA eluting solvent system. According to Adamu *et al.* (2014), non-polar compounds from plant extracts generally have better antibacterial activity than polar compounds. The antibacterial activity of

the chloroform fraction suggests that the active compounds present may be relatively non-polar. However, the qualitative antibacterial assay of the chloroform fraction (second column) showed no zone of inhibition. This could be because further separation reduced the synergistic effects of the compounds' antibacterial activity, or photo-oxidation on TLC leading to breakdown of antibacterial compounds.

Fractionation of the acetone crude extract potentiated antibacterial activity after the first column (F6 and F7). However, fractions from further fractionation showed a decrease in antibacterial activity, which implies that the antibacterial activity resulted from the synergistic effect of the compounds and other components of the extract and fractions (Madikizela and McGaw, 2018).

The crude extract had significant antioxidant activity in both ABTS and DPPH assays. The fractions exhibited potent antioxidant activity except for the chloroform fraction in the ABTS antioxidant assay. In the DPPH assay, both the chloroform and hexane fractions had good activity, while other fractions had more potent antioxidant activity. According to Phongpaichit *et al.* (2007), radical scavenging IC_{50} values of <100 µg/mL are considered good, while values <50 µg/mL are considered potent. All fractions from column chromatography had poor antioxidant activity with IC_{50} values > 100 µg/mL. The normal physiological processes of the gastrointestinal tract (GIT) produce reactive oxygen species (ROS) which, in excess, can cause damage to intestinal cells and favours GIT disorders (Halliwell, 1993). Hence, the crude extract and the partitioned fractions of *S. batophylla* could be beneficial in managing and treating diarrhoea by lowering the incidence of reactive oxygen species.

4.4.1 Structure elucidation and characterisation

Dodecanamide is a saturated nitrogenous compound (fatty acid) isolated from *S. batophylla* acetone extract in this study. Previously, dodecanamide has been isolated from the ethyl acetate extract of wild *Rostellularia diffusa* and the aqueous extract of *Camellia chinensis* (Chinese green tea) (Beltagy, 2016; Uduman *et al.*, 2017). In this study it had weak antibacterial activity (MIC = 0.63 mg/mL). There are no antimicrobial activity reports on this compound with MIC lower than 0.1 mg/mL. This suggests that dodecanamide does not possess potent antimicrobial ability and that the antibacterial activity of precursor fractions might be due to other compounds or the interplay with other compounds present.

Cytotoxicity testing showed that the compound had no toxic effect on the Vero kidney cells at the highest concentration tested. Dodecanamide has been shown to possess selective antiproliferation and antioxidant activities (Hayashi *et al.*, 2009; Anjum *et al.*, 2017). Due to the low quantity isolated, the antioxidant activity of this compound could not be evaluated.

The flavonoid compound 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione (α,β-unsaturated ketone) is an oxaspiro compound with an enone, cyclic ketone and a lactone that belongs to a flavonoid group (ChemicalBook, 2020). The compound has tert-butyl substituents at two positions, 7 and 9 (ChemicalBook, 2020). This bioactive compound has been previously identified from the whole plant methanolic extract of *Euphorbia pulcherrima* (poinsettia), a plant used in folklore medicine to treating intestinal disorders, gonorrhoea, migraine, warts and skin infections (Sharif *et al.*, 2015). It is also present in the ethyl acetate extract of *Manilkara hexandra* (Roxb.) Dubard stem bark and the ethanolic extract of *Chara baltica* as a phytoactive component (Monisha and Vimala, 2018). In addition, 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione was previously isolated from the ethanol extract of the marine algal species *Clathria baltica* and from the methanol extract of *Chnoospora minima* (Gunathilaka *et al.*, 2019; Tatipamula *et al.*, 2019). The compound possessed good antioxidant activity (Gunathilaka *et al.*, 2019). In this study, the compound 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione had poor antibacterial activity and this is the first antibacterial report to the best of our understanding. The poor antibacterial activity observed may have resulted from the breakdown of the compound due to its instability or that the antimicrobial activity observed from the penultimate fraction was due to a combination or synergistic effect between the components. Prajna *et al.* (2016) showed that the compound has good antioxidant activity that supports its use for therapeutic purposes. Due to the low yield of this compound, antioxidant activity could not be investigated in this study.

The compound 3-oxo-olean-18-en-28-oic acid also referred to as oleanonic acid is a member of the class triterpenoids, a common group of naturally occurring compounds (Ferreira *et al.*, 2019). Terpenes are one of the most common components of plant essential oils. They are often categorised as mono-, di-, tri- and sesquiterpenes, depending on the number and arrangement of isoprene units present (Langenheim, 1994). The compound 3-oxo-olean-18-en-28-oic acid is a triterpene insoluble in water, weakly acidic and is found naturally in grapes, apples, tomatoes and berries. Based on the current results, it has weak antibacterial activity and minimal toxicity against Vero cells. This compound has been isolated from the ethyl acetate extract of the root powder of *Waltheria viscosissima* (Soares *et al.*, 1998; Kwon *et al.*, 2011). In addition, the compound was isolated from the ethanolic extract's hexane fraction of the stem bark of *Mimosa caesalpiniifolia* (Monção *et al.*, 2015). The crude ethanol extract and fractions from *W. viscosissima* were tested for larvicidal effect on *Aedes aegypti* larvae resulting in lethargy, compromised mobility, complete paralysis and mortality (LC_{50} = 38.37 mg/mL) (Ferreira *et al.*, 2019). Although the larvicidal potential of the compound was not determined, oleanonic acid could be speculated to be one of the active ingredients responsible for the

activity. In addition, conjugates of 3-oxo-olean-18-en-28-oic acid such as oleanonic diamine and oleanolic acid polyamine, possess antimicrobial activity against *Staphylococcus aureus, Bacillus cereus, Streptococcus faecalis, E. coli* and *Pseudomonas aeruginosa*, as well as antioxidant, anti-inflammatory and hepatoprotective activities (Xu *et al.*, 2014; Khusnutdinova *et al.*, 2022). This implies that the compound could be a good precursor in the development of beneficial herbal preparations in the treatment of diarrhoea. To the best of our knowledge, this is the first report of the isolation of antibacterial compounds from *S. batophylla*.

4.4.2 Antibacterial activity of fractions and total activity

S. batophylla had significant antibacterial activity against tested *E. coli* strains. The crude extract of *S. batophylla* was active against a panel of bacteria, and the total activity of the chloroform fraction was better than that of the crude extract.

The isolated compounds had poor antibacterial efficacy compared to the crude extract and fractions. The activity of compounds with MIC < 0.01 mg/mL is considered good, $0.01 <$ MIC > 0.1 mg/mL as moderate, and MIC > 0.1 mg/mL as poor. A weaker bacterial inhibitory effect of the isolated compounds was obtained against the tested *E. coli* when compared with that of the crude extract and fractions. This is often the case after purification, as established by (Lewis and Ausubel, 2006). It is, therefore, safe to say that the inhibitory effect of the extract and fraction is the resultant effect of synergism amongst the constituting compounds.

4.4.3 Cytotoxicity and selectivity index of isolated compounds

A good potential candidate for further research and development should have relatively low cytotoxicity and a high selectivity index. Therefore, the IC_{50} value for cytotoxicity should be greater than 0.02 mg/mL (Zirihi *et al.*, 2005). All the isolated compounds had low toxicity to Vero cells, even at the highest concentration. The SI value is important in determining the safety of a drug. An SI value greater than 1 indicates that the antimicrobial agent or drug is more toxic to the infectious agent than the cell (Makhafola *et al.*, 2012). Although the SI of the isolated compounds dodecanamide, 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione and 3-oxo-olean-18-en-28-oic acid could not be specifically obtained due to their high LC_{50} values, this suggests that the compounds were not toxic to Vero cells. Therefore, they may be further explored for their biological properties like anti-biofilm, anti-quorum sensing and anti-inflammatory activities.

4.5 Conclusion

In this study, the column fractions of the chloroform fraction of the crude acetone extract of *S. batophylla* leaves had considerable antibacterial activity, poor antioxidant activity and low toxicity to Vero kidney cells. Dodecanamide, 7,9-di-tert-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione and 3-oxo-olean-18-en-28-oic acid, amongst other compounds, were isolated and are known to possess some biological activities. Therefore, the extract and fractions have the potential to be developed into useful herbal products as complementary antimicrobial preparations rather than the isolated constituents. Further studies including anti-biofilm and anti-quorum sensing activities, as well as *in vivo* toxicity and animal feed trials are recommended to confirm the efficacy and safety of the plant extract or fractions as useful supplements to prevent or treat diarrhoea.

Chapter 5: Phytochemical analyses, *in vitro* antioxidant, anti-inflammatory activities and antibacterial activity against *Escherichia coli* isolates of leaf extracts of selected plants used to treat diarrhoea in South African ethnomedicine.

5.1 Introduction

Medicinal herbs are well-known and popular for various health advantages, including blood pressure reduction, resolution of diarrhoea, cardiovascular disease prevention, and cancer risk reduction due to their antioxidant activity (Škrovánková *et al.*, 2012). These herbs contain a range of pharmacologically important phytochemicals such as flavonoids, alkaloids, phenolics, proanthocyanidins and vitamins. These bioactive compounds serve as the basic raw materials for many pharmaceuticals, herbal remedies, nutraceuticals and functional foods (Susanna *et al.*, 2022).

Most biological systems generate reactive oxygen species (ROS) and other highly reactive free radicals. ROS can potentially cause oxidative damage to lipids, proteins and nucleic acids, resulting in genetic alterations and degenerative illnesses (Jarosz *et al.*, 2017). These free radicals can be trapped by antioxidants, reducing ROS. Antioxidants include phenolic acids, polyphenols and flavonoids, which may scavenge free radicals like hydroxyl, hydroperoxide, and lipid peroxyl radicals. Antioxidants could also quench hydrogen peroxide and superoxide anion, preventing the oxidative damage that causes degenerative illnesses (Sevgi *et al.*, 2015; Nasab *et al.*, 2020). Research has demonstrated an inverse relationship between the consumption of natural antioxidants from medicinal herbs and the occurrence of certain illnesses, due to the ability of such antioxidants to eliminate free radicals (Škrovánková *et al.*, 2012).

Inflammation in cells includes the production of pro-inflammatory cytokines by leukocytes, endothelial cells and macrophages. It is also associated with the production of anti-inflammatory cytokines like IL-10 and pro-inflammatory cytokines (Li *et al.*, 2005). Nitric oxide synthase (iNOS) and cyclooxygenase (COX) are two pro-inflammatory enzymes released by inflamed cells. These enzymes are responsible for raising nitric oxide (NO) and prostaglandin (PGE2) levels. Plants have been utilised to cure physiological, neurological and inflammatory-related diseases (Yuan *et al.*, 2016). Extracts, fractions and purified active compounds from different plants have been identified to have anti-inflammatory activities

(Bernstein *et al.*, 2018). *In vitro* studies have revealed that flavonoids, a sub-class of polyphenols, have anti-inflammatory activities (Bernstein *et al.*, 2018).

With more than 20,000 plant species, South Africa boasts a varied temperate flora that makes up more than 10% of all vascular plants in the world (Germishuizen and Meyer, 2003). To supplement or receive therapy, the vast majority of the population visits traditional practitioners (Van Wyk *et al.*, 1997). Plants belonging to the Fabaceae, Anacardiaceae and Asteraceae families are notably used in treating different medical conditions, especially gastrointestinal disorders (McGaw and Eloff, 2008). Diarrhoea is a significant barrier to profitable livestock farming and hence the supply of animal products. Diarrhoea is a disruption in the normal morphology and physiological process of the digestive tract (GIT) leading to imbalance in intestinal fluid equilibrium and alterations in GIT motility in both man and animals. Animal diarrhoea is caused by infections, gastroenteritis, distorted intestinal oxidative homeostasis, allergies, or symptoms to some diseases. Infectious diarrhoea is mainly caused by *Escherichia coli*, *Salmonella typhi*, *Shigella flexneri,* (enteric coliforms) *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa, Entamoeba histolytical, Giardia intestinalis*, *Cryptosporidium parvum* and some viruses (Lowy, 1998; Bányai *et al.*, 2018; Rawat *et al.*, 2020). The effect of diarrhoea in production animals can be very devastating, with low productivity and quality of eggs, meat, milk and other products. This may lead to morbidity, weight loss, abortion and ultimately death of animals (Fairbrother *et al.,* 2005).

Due to the cost of orthodox medicine and the problem of antimicrobial resistance, diarrhoea is one of the major disorders treated and controlled by traditional remedies in the Republic of South Africa (Thornton, 2009; van Vuuren *et al.*, 2015). The use of herbal regimens is also common in animal health to forestall production loss due to morbidity and mortality (Masika *et al.*, 2000). Some herbal remedies for diarrhoea have been said to possess antibacterial and antispasmodic activities, slowing the gastrointestinal processes, decreasing gut motility, increasing water absorption, and lessening electrolyte output. These biological actions may explain the advantages of using plants to treat diarrhoea. (Maroyi, 2016).

Bauhinia bowkeri, Bauhinia galpinii, Searsia batophylla, Searsia lancea, Searsia leptodictya and *Searsia pendulina* were selected for this work based on previous evaluation of their antibacterial, antibiofilm and quorum sensing inhibition of their acetone extracts (Adeyemo *et al.*, 2022). However, little is known about the other biological activities and phytochemical components of these selected plants. Therefore, this study aimed to determine the antioxidant, anti-inflammatory and antibacterial activities of the selected South African plant species against clinical isolates as well as the presence of potential bioactive compounds that could be

responsible for their biological activities via Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

5.2 Materials and methods

5.2.1 Collection, drying and storage of plant materials

Leaves of the selected plants were collected from the Lowveld National Botanical Garden in Nelspruit, Mpumalanga, South Africa. Some voucher specimens were deposited in the HGWJ Schweickerdt Herbarium at the University of Pretoria, Pretoria, South Africa (PRU) and others at the National Herbarium of the South African National Biodiversity Institute (SANBI), Pretoria, South Africa (PRE). The plant species were identified by staff of the Department of Plant and Soil Science, University of Pretoria, and SANBI. The plant species were *Bauhinia bowkeri* (PRU 127998), *Bauhinia galpinii* (PRU 28944), *Searsia batophylla* (PRE 0611152), *Searsia lancea* (PRU 126859), *Searsia leptodictya* (PRU 70151) and *Searsia pendulina* (PRU 127997).

The plants were dried and stored using standard protocols previously developed in the Phytomedicine Programme. In brief, the leaves of the selected plants were carefully separated from the stems and transported to the laboratory in open-weave nylon bags to allow ventilation. The leaves were further dried under shade at room temperature for not less than two weeks. The dried leaves were later ground into a fine powder using a Janke and Künkel Model A10 mill and stored in air-tight glass jars in the dark and at room temperature until use.

5.2.2 Extraction solvents

The solvents used for this research project were obtained from Minema Chemicals, South Africa, Sugar Illovo limited, and Associated Chemical Enterprises, South Africa.

5.2.3 Extraction

Acetone was used as an extraction solvent. The powder (g): solvent (mL) ratio was 1:10 for all the plants and extracts were vigorously mixed on a Labotec shaker (Labotec, South Africa). The supernatants were filtered after 24 h through Whatman No 1 filter paper into previously weighed glass jars. This process was carried out thrice on the same plant material for maximum extraction of constituent compounds. A Büchi R-114 rotary evaporator (Büchi, Germany) was used to concentrate the filtrates. Stock solutions of 10 mg/mL concentration were prepared in acetone immediately prior to the assays.

5.2.4 Antibiogram

The antimicrobial susceptibility of the clinical *E. coli* isolates was done using Kirby-Bauer's disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). Pure colonies of *E. coli* were grown on freshly prepared Mueller Hinton Agar (MHA). A single distinct colony was transferred into 5 ml of sterile distilled water and incubated for two hours. The turbidity was then adjusted to 0.5 McFarland standard (equivalent to 1.5 x 10^8 cfu/ml). A sterile swab was used to streak bacteria on the entire agar surface of the plate. A panel of ten antimicrobial discs of animal and human health importance used were: Gentamicin (CN; 10 µg/disk), Amikacin (AK; 30 µg/disk), Colistin sulphate (CT; 30 µg/disk), Ampicillin (AMP; 10 µg/disk), Tetracycline (TE; 30 µg/disk), Amoxicillin/clavulanic acid (AMC; 30 µg/disk), Sulphamethoxazole/trimethoprim (SXT; 25 µg/disk), Ceftiofur (EFT; 30 µg/disk), Doxycycline (D0; 30 µg/disk) and Chloramphenicol (C; 30 µg/disk) (Oxoid, UK). The antimicrobial discs were gently placed aseptically on the streaked agar plate with centres at least 24 mm apart. The discs were allowed to sit on the agar for an hour before inversion and incubation at 35-37°C for 18-24 h. The diameter of the zone of inhibition was then measured using a vernier calliper.

5.2.5 Phytochemical profiling by thin layer chromatography

The phytochemical profiling of the plant extracts was conducted by thin layer chromatography (TLC) on aluminium-backed silica gel plates (10 × 20 cm, 60 F254, Merck, United States). A 10 µL aliquot of each plant extract at a concentration of 10 mg/mL was loaded on the plate and then developed with solvent systems of varying degrees of polarities, namely benzene: ethanol, and ammonium hydroxide (BEA) in the ratio 90:10:1 (non-polar, basic); chloroform: ethyl acetate: formic acid (CEF) in the ratio 5:4:1 (intermediate polarity, acidic) and ethyl acetate: methanol: water (EMW) in the ratio 40:5.4:5 (polar, neutral) respectively. The extracts were loaded on TLC plates (1 cm width), and visible bands were marked faintly under ultraviolet light at 254 and 360 nm wavelengths. The plate was then sprayed with freshly prepared 0.1 g vanillin dissolved in 28 mL of methanol and 1 mL of sulphuric acid. The plates were thereafter heated in an oven (about 110°C) until visible colour development.

5.2.6 Antibacterial activity

5.2.6.1 Determination of minimum inhibitory concentration (MIC)

A simple two-fold serial dilution microplate method was used to determine the minimum inhibitory concentration (MIC) (Eloff, 1998b). Ten bacterial isolates of *E. coli* from clinical

cases of bovine diarrhoea were obtained from the Antimicrobial Resistance and Phage Biocontrol Research Laboratory, Department of Microbiology, School of Biological Sciences, Faculty of Natural and Agricultural Sciences, North West University, South Africa.and the isolates were used to evaluate the antibacterial potential of all the selected acetone crude extracts. Bacterial cultures grown overnight in Mueller Hinton broth (Sigma Aldrich, SA) were adjusted to McFarland standard 0.5 (equivalent to 1.5 x 10^8 CFU/mL). A 100 µL aliquot of sterile distilled water was added to all the wells of a 96-well microtitre plate. The prepared extracts (10 mg/mL stock concentrations) were added to the first row of the microplate and serially diluted twofold with water down the columns. After that, 100 µL of adjusted bacterial cultures were added to each well. The bacteria were exposed to the extracts of final concentrations ranging between 2.5 and 0.01 mg/mL. Acetone and gentamicin served as negative and positive controls, respectively. The plates were then incubated at 37°C for 18-24 h. Following incubation, 40 µL (0.2 mg/mL) of p-iodonitrotetrazolium violet (INT) was added to each well and incubated for 1 h. The MIC was taken as the lowest extract concentration to show growth inhibition, visible in terms of a decrease in red colour generated by conversion of the INT to a red product by actively respiring bacteria.

5.2.6.2 Antibacterial activity by bioautography

Bioautography was done to visualize the presence of active compound(s) in the plant extracts. The TLC plates were developed as described in section 5.2.5 but were not sprayed with vanillin spray reagent. The plates were left to dry in cold air to remove residual eluents. The TLC plates were then sprayed until wet in a biosafety class II fume cabinet (ESCO, South Africa) with a standardised overnight culture of *Escherichia coli* O157: H7 (ATCC 43888), *E. coli* (ATCC 25922) and *E. coli* (ATCC 35218) grown in Mueller-Hinton broth (Merck, South Africa). One of the donated clinical isolates from North West University, South Africa, was also included. The wet plates were allowed to dry and incubated for 24 h at 37 ^{0}C in a humidified, closed plastic container to enhance the growth of the sprayed test bacteria. After incubation, the plates were sprayed with freshly prepared p-iodonitrotetrazolium (INT) with a concentration of 2 mg/mL dissolved in sterile distilled water and incubated further for 1-2 h. A clear zone against the pink/red background denotes the inhibition of bacterial growth by the separated compounds on the plate. The zones of inhibitions were measured, and retention factors (R_f) were calculated.

5.2.7 Antioxidative activity

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

The free radical scavenging activity of acetone plant extracts was determined using the ABTS cation decolourisation method of Re *et al.* (1999) with slight modifications. ABTS radical solution was prepared by dissolving 26.49 mg of potassium persulphate ($K_2S_2O_4$) in 20 mL of methanol and 76.81 mg of ABTS in 20 mL of methanol solution. The solutions were combined and kept in the dark for 18 h at room temperature. The ABTS radical solution was further diluted with methanol to an absorbance of between 0.7-0.8 at a wavelength of 734 nm. A 40 µL aliquot of extracts and fractions (1 mg/mL) were prepared in different concentrations in 96 well-microtitre plates, and 160 µL of ABTS radical solution was added to each well. Ascorbic acid and Trolox were used as positive controls, and methanol served as the negative control. After incubation for 5 min, the absorbance was read using a microplate reader (Epoch-BioTek, USA). The percentage of radical scavenging activity was calculated with the formula .

ABTS scavenging activity (%) = [(A1-A2/A1)] x 100

Where A1= absorbance of control reaction, A2= absorbance of the sample.

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

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

The method of Brand-Williams *et al.* (1995), with slight modification, was used to determine the DPPH radical scavenging ability of the fractions. Ascorbic acid and Trolox served as the positive controls, while methanol was used as the negative control. In brief, samples were dissolved in methanol (1 mg/mL), and 40 µL of samples and both positive controls, Trolox and ascorbic acid, were prepared in different concentrations in a 96 well-microtitre plate. One hundred and sixty (160) µL of DPPH solution was added. The mixture was covered with foil paper and allowed to incubate in the dark for 30 min, and the absorbance of the microtitre plate was measured with a plate reader (Epoch-Biotek, USA) at 517 nm. The percentage of DPPH scavenging activity was determined using the formula:

DPPH scavenging activity (%) = [(A1-A2/A1)] x 100

Where A1= absorbance of control reaction, A2= absorbance of the sample.

The 50% inhibitory concentration (IC_{50}) values for each extract were calculated from the graph plotted as percentage inhibition against the concentrations. Each experiment was done in triplicate and thrice.

5.2.8 Anti-inflammatory assays

5.2.8.1 Soybean lipoxygenase inhibition assay

The experiment was done using the method of Pinto *et al.* (2007) with slight modifications. The assay functions to measure the inhibitory potential of 15-lipoxygenase enzyme (15-LOX) activity by the crude extracts of the plants in the presence of linoleic acid, by forming the complex Fe3+/xylenol orange, which can therefore be measured in a spectrophotometer at an absorbance of 560 nm. All extracts were prepared to the concentration of 10 mg/mL in DMSO and further diluted to 2 mg/mL in Tris-HCl buffer containing 20% DMSO. Concisely, 40 µl of 15-LOX enzyme (Sigma-Aldrich, Germany) was incubated with 20 µl of different concentrations (100 to 0.78 µg/mL) of the extracts and quercetin (positive control) at 25°C for 5 min. Furthermore, 40 µl of linoleic acid (substrate) was added and incubated at 25°C for 20 min in the dark. The reaction was discontinued with the addition of 100 µl of freshly prepared FOX reagent, which is made up of sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM) in methanol/water (9:1). The negative control consisted of 15-LOX solution and Tris-HCl buffer, substrate, and FOX reagent. The blank, on the other hand, contained 15-LOX and buffer, and substrate (linoleic acid) was added after the FOX reagent. The lipoxygenase inhibitory action of the extracts was obtained by calculating the inhibition percentage of hydroperoxide production from the changes in absorbance values measured with a plate reader (Epoch-Biotek, USA) at 560 nm after 30 min at 25°C using the formula:

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

5.2.8.2 Inhibition of nitric oxide (NO) production assay

The macrophage cell line (RAW 264.7) of the American Type Culture Collection (ATCC, CRL-2278™) (Rockville, MD, USA) was employed to determine the potential of the plant extracts to inhibit nitric oxide production when cells were stimulated with lipopolysaccharide (LPS, Sigma). The NO inhibition was measured in terms of the amount of nitrite produced by the cells. Macrophages were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin/fungizone (PSF) at 37°C with 5% CO_2 in 75 cm^2 flasks. The cells were sub-cultured at 75-80% confluence twice a week. The cells were afterwards seeded at a concentration of 2×10^6 cells/mL in 96-well microtitre plates and incubated overnight at 37°C with 5% CO_2 to promote attachment. The cells were then activated by incubating in a medium containing 2 µg/mL of only LPS (negative control) and treated simultaneously with different concentrations of the extracts. Quercetin (positive control) was dissolved in DMSO (concentration did not exceed 0.2%), and cells were incubated for 24 h at 37°C with 5% CO_2.

5.2.8.2.1 Nitrite measurement

The nitrite produced by the RAW macrophage cells was determined by measuring the nitrite concentration in the supernatant with Griess reagent (Griess, 1879). In summary, after the plates were incubated for 24 h, 100 µL of cell supernatant from each well was removed and put in a new 96-well microtitre plate, and 100 µL of Griess reagent (Sigma) was added. The mixture was covered with foil and incubated in the dark at room temperature for 15 min, and the absorbance was determined using a microtitre plate reader (Epoch-Biotek, USA) at 550 nm. A standard curve from regression analysis of the different concentrations of sodium nitrite was used to calculate nitrite concentrations. Nitric oxide (NO) has a short half-life and high interference with metabolites and hence is very unstable. An indirect NO quantification was done by measuring nitrite (NO_2) production, which is the oxidized form of NO. The percentage inhibition of nitric oxide (inhibition of NO_2) was calculated by the ability of nitric oxide production inhibition in macrophage cells by extracts relative to the negative control (cells treated with LPS only).

5.2.8.2.2 Determination of cell viability

To establish that the inhibition of nitric oxide was not because of cell death due to toxicity, the MTT assay was used to determine the extracts' toxicity to macrophage cells. Briefly, the supernatants were removed from the extract- and quercetin-treated macrophage cells from the above-described assay. The cells were washed twice with phosphate-buffered saline (PBS), and 200 µL of culture medium and 30 µL of MTT solution (5 mg/mL) were added to the wells. The plates were left to incubate for 4 h at 37°C with 5% CO_2. The medium was removed gently, and 50 µL DMSO was added to all wells to dissolve the formed formazan salts. A microplate reader (Biotek Synergy, USA) was used to determine the absorbance at 570 nm. The percentage of the viable cells was calculated by relating the absorbance of extract-treated wells to the negative control (100% viability).

5.2.9 Quantification of phenolic constituents of the extracts

5.2.9.1 Total phenolic content determination

The plant extracts' total phenolic content was determined using the Folin-Ciocalteu method (Wong-Paz *et al.*, 2014). In brief, the reaction mixture of 20 µL of 1 mg/mL of the plant extracts dissolved in methanol and 20 µL of Folin-Ciocalteu reagent (10 %) were incubated for 5 min. Then 20 µL of sodium bicarbonate (7.5 %) was added and then incubated for a further 30 min. After this, 160 µL of distilled water was added and absorbance was read at 765 nm using a microplate reader (Epoch-Biotek). A standard curve was obtained with gallic acid at concentrations ranging from 0.0078-1.0 mg/mL. The data were presented as GAE/g (gallic acid equivalent per g) of extract.

5.2.9.2 Total flavonoid content determination

The method of Ordonez *et al.* (2006) with slight modification was used to determine the total flavonoid content. A 100 µL aliquot (1 mg/mL) of extracts dissolved in methanol was added to 100 µL of 1 mg/mL of $AlCl_3$ (2%) and incubated for 1 hour. A yellow colouration implies the presence of flavonoids. The absorbance was read at 430 nm. A standard curve of quercetin was obtained with a concentration range of 0.0078-1.0 mg/mL. Data were presented in QE/g (quercetin equivalent per g) of extract.

5.2.10 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry of the plant extracts was investigated to identify dominant compounds present. This was carried out at the Department of Chemistry, University of Pretoria, using a LECO Pegasus 4D GC-TOFMS (LECO Africa (Pty) Ltd., Kempton Park, South Africa) GC column Rxi-1MS 30 m x 0.25 mm ID x 0.2 µm film thickness (Restek, Bellefonte, PA, USA). Injection volume 1uL, splitless injection, splitless time 30s GC inlet 250°C GC oven temperature programme 40°C (hold for 3 min) at 10°C/min to 300°C (hold for 5 min). Carrier gas UHP Helium (Afrox, South Africa) at 1 ml/min, constant flow mode Mass acquisition range 40-550 Da. MS transfer line temperature 280°C Ion source temperature 230°C MS solvent delay 5 min Electron energy 70 eV in the electron ionisation mode (EI+) Data acquisition rate 10 spectra/s Detector voltage 1750 V.

5.2.11 Statistical analysis

Data were gathered in Microsoft Excel version 365, presented as mean ± standard error of the mean. GraphPad Prism version 9.0 statistical software was used to analyse data using one-way analysis of variance and Tukey's post hoc test. A value of $p < 0.0001$ was used to establish significant levels.

5.3 Results

5.3.1 Antibiotic susceptibility testing of the E. coli isolates

Antimicrobial sensitivity tests such as disc diffusion, agar well diffusion and broth microdilution methods have been used since the discovery of antibiotics to determine the pattern of susceptibility or resistance of microorganisms to antibiotics (Balouiri *et al.*, 2016). The antibiogram test was done using discs of nine different antibiotics, and results were categorised as resistant, moderate and susceptible based on EUCAST standards. All the isolates were susceptible to sulphamethoxazole. Except for *E. coli* 1, all other isolates were susceptible to chloramphenicol. Also, all the tested *E. coli* strains were susceptible to gentamicin except for *E. coli* 9. The two most susceptible strains were *E. coli* 4 and *E. coli* 8, with a single antibiotic resistance to amoxicillin and colistin sulphate, respectively (**Fig 5.1**)

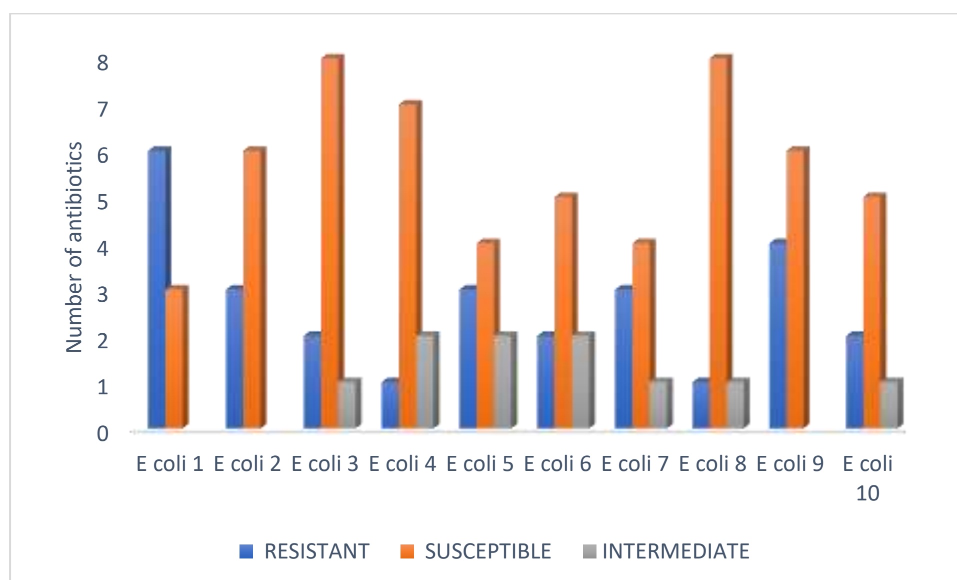


Fig 5.1: Antibiogram test on *E. coli* strains isolated from cattle with clinical signs of diarrhoea

5.3.2 Antibacterial activity

5.3.2.1 Antibacterial activity by microplate method

In the previous study, the microdilution assay was used to evaluate the antibacterial activity of the acetone extract of the selected plants against nine different ATCC bacterial strains associated with gastrointestinal tract infections (Adeyemo *et al.*, 2022). This follow-up study assessed the efficacy of the previously identified promising plant extracts against ten *E. coli* clinical isolates. All six plant extracts had good bacterial growth inhibition ability with minimum inhibitory concentration (MIC) values ranging from 0.04 to 0.16 mg/mL (**Table 5.1**). *E. coli* 1 was the most susceptible among all the tested strains, with the extracts having an average MIC value of 0.06 mg/mL. On the other hand, *Searsia lancea,* with an average MIC of 0.06 mg/mL, and *S. batophylla* and *Bauhinia galpinii,* with an average MIC of 0.07 mg/mL, had the best antibacterial activity (**Table 5.1**).

Table 5.1: Cytotoxicity, minimum inhibitory concentration (in mg/mL), and selectivity index (in brackets) of acetone plant extracts against *E. coli* clinical strains

Plant	LC_{50} (mg/mL) (Adeyemo *et al.*, 2022)	*E. coli* 1	*E. coli* 2	*E. coli* 3	*E. coli* 4	*E. coli* 5	*E. coli* 6	*E. coli* 7	*E. coli* 8	*E. coli* 9	*E. coli* 10	Average MIC
Searsia pendulina	0.03±0.00****	0.04 (0.64)	0.05 (0.58)	0.07 (0.40)	0.12 (0.24)	0.08 (0.37)	0.08 (0.37)	0.12 (0.23)	0.08 (0.34)	0.08 (0.37)	0.08 (0.40)	0.08
Searsia leptodictya	0.11±0.00****	0.07 (1.45)	0.13 (0.80)	0.12 (0.88)	0.12 (0.88)	0.10 (1.00)	0.14 (0.72)	0.14 (0.72)	0.10 (1.00)	0.16 (0.66)	0.14 (0.44)	0.13
Searsia lancea	0.20±0.02****	0.06 (3.16)	0.06 (3.39)	0.07 (2.77)	0.08 (2.54)	0.08 (2.54)	0.05 (3.59)	0.05 (3.59)	0.08 (2.54)	0.07 (2.77)	0.06 (2.77)	0.06
Searsia batophylla	0.15±0.01****	0.06 (2.52)	0.08 (1.89)	0.08 (1.89)	0.09 (1.62)	0.07 (2.06)	0.07 (2.06)	0.06 (2.35)	0.08 (1.89)	0.08 (1.89)	0.06 (2.00)	0.07
Bauhinia galpinii	> 1	0.05 (> **20.44**)	0.07 (> **15.79**)	0.07 (> **15.79**)	0.06 (> **19.30**)	0.07 (> **14.89**)	0.04 (> **24.80**)	0.05 (> **20.44**)	0.05 (> **23.16**)	0.10 (> **10.85**)	0.06 (> **11.58**)	0.07
Bauhinia bowkeri	0.51±0.04****	0.06 (8.45)	0.08 (6.34)	0.06 (8.45)	0.05 (8.95)	0.07 (6.92)	0.08 (5.85)	0.12 (4.22)	0.07 (6.61)	0.10 (4.75)	0.08 (5.70)	0.08
Average MIC	**NA**	0.06	0.07	0.08	0.08	0.08	0.08	0.09	0.08	0.10	0.10	NA
Gentamicin	NA	0.005	0.005	0.001	0.005	0.005	0.002	0.005	0.005	0.005	0.001	0.004
Doxorubicin	0.01±0.00	NA										

NA= Not applicable, bold values indicate interesting SI values, LC_{50} values (Adeyemo *et al.,* 2022), **** $p < 0.0001$

5.3.2.2 Antibacterial activity by bioautography

The benzene: ethanol: ammonium hydroxide (BEA) solvent system gave the best separation of the components of the acetone plant extracts. The antibacterial bioautography revealed that all the selected plant extracts had components with antibacterial activity against *E. coli* ATCC 25922, most prominently when separated with the BEA solvent system (**Fig 5.2 A'**). With the eluents CEF and EMW, only the *S. lancea* extract showed good bacterial inhibition (**Fig 5.2 B' and C'**) with a potential active compound situated at R_f values of 0.66 and 0.87, respectively.

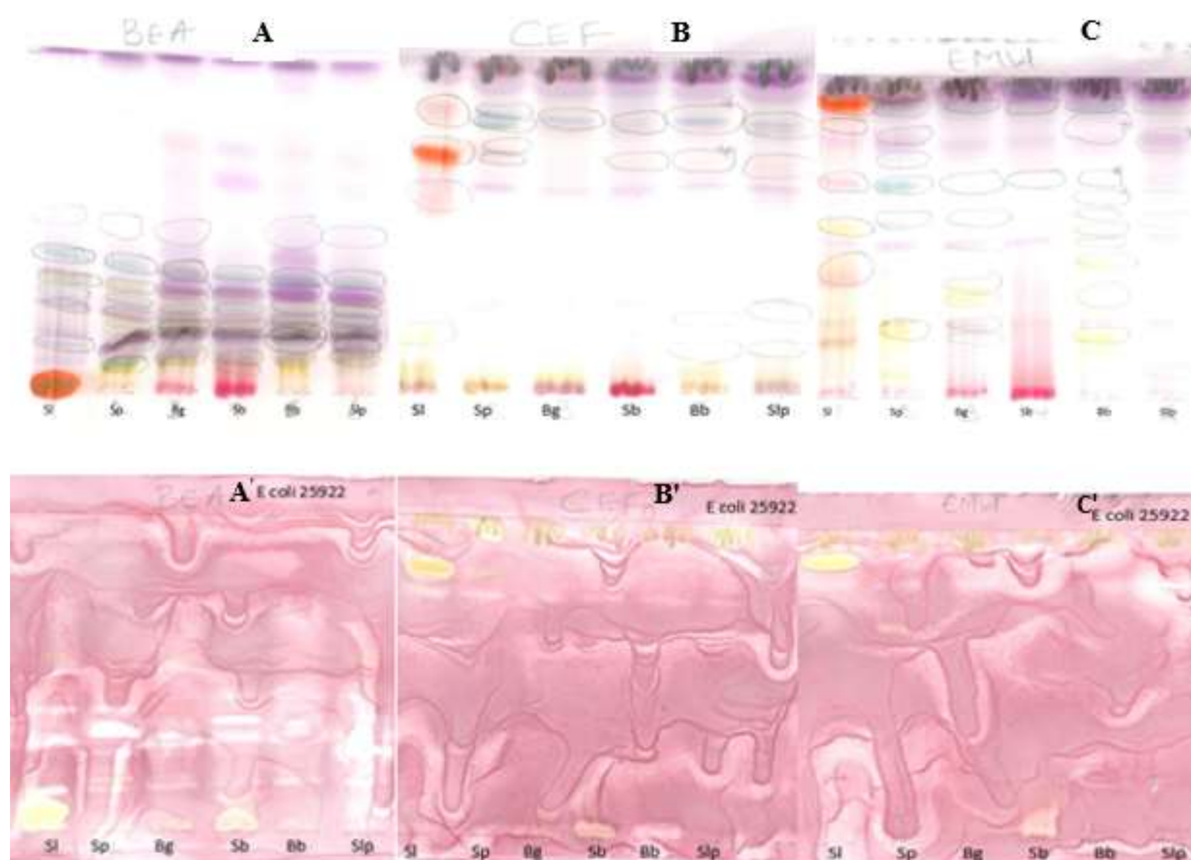


Fig 5.2: (A) Chromatogram of crude extracts developed in BEA [benzene: ethanol: ammonium hydroxide (90:10:1)] sprayed with vanillin. (B) Chromatogram of plant crude extracts developed in CEF [chloroform: ethyl acetate: formic acid (5:4:1)] sprayed with vanillin. (C) Chromatogram of crude extracts developed in EMW [ethyl acetate: methanol: water (40:5.4:5)] sprayed with vanillin. (A') Bioautography of *E. coli* 25922-TLC plate developed in BEA benzene: ethanol: ammonium hydroxide (90:10:1)] (B') Bioautography of *E. coli* 25922-TLC plate developed in CEF [chloroform: ethyl acetate: formic acid (5:4:1) (C') Bioautography of *E. coli* 25922-TLC plate developed in EMW [ethyl acetate: methanol: water (40:5.4:5)].

5.3.3 Selectivity index of antibacterial extracts

Clinical significance for medicinal plant preparations requires toxicity targeted at the pathogens, not the host cells. The cytotoxicity of the selected plants against mammalian Vero cells has been previously reported, and *Searsia pendulina* had the highest cytotoxic effect, with an LC_{50} value of 0.03 mg/mL (Adeyemo *et al.*, 2022). The selectivity index (SI), which is the ratio of a toxic dose to a therapeutic dose, is often used to evaluate the selectivity of active ingredients and the SI was calculated for the active extracts. *Bauhinia galpinii* had the highest SI values ranging from 11.58 to 23.16, followed by *B. bowkeri* with SI values ranging from 4.22 to 8.95 across the tested bacteria (**Table 5.1**).

5.3.4 Antioxidant activity

The ability of the crude extracts to scavenge free radicals was determined by the ABTS and DPPH methods. The evaluations are based on the extent of colour change in both the ABTS and DPPH solutions, corresponding to scavenging of the free radicals (Saleem *et al.*, 2016). The results were compared with positive controls, vitamin C (ascorbic acid) and Trolox. The IC_{50} value is inversely proportional to the ability to scavenge free radicals (Phongpaichit *et al.*, 2007). In the ABTS assay, all tested plant extracts had an IC_{50} value lower than 50 µg/mL. *Searsia batophylla* extract had the best scavenging ability with an average IC_{50} value of 0.54 µg/mL, which is even lower than the IC_{50} values of Trolox and ascorbic acid (1.44 and 1.16 µg/mL, respectively) (**Fig 5.3**). This was followed by *S. lancea* extract with an average IC_{50} of 4.10 µg/mL. Statistically, there was no significant difference between the antioxidant capacity of *S. batophylla* and *S. lancea* and the standard antioxidant agents ascorbic acid and Trolox. In the DPPH assay, all the plant extracts exhibited good antioxidant ability ($IC_{50} < 100$ µg/mL). *Bauhinia bowkeri* had the best scavenging ability ($IC_{50} = 14.31$), followed by *S. batophylla* ($IC_{50} = 35.03$ µg/mL). Other plant extracts had comparatively lower antioxidant ability (IC_{50} ranged between 45.77 and 88.16 µg/mL). Overall, there were significant differences between all the acetone plant extracts and controls ($P < 0.0001$).

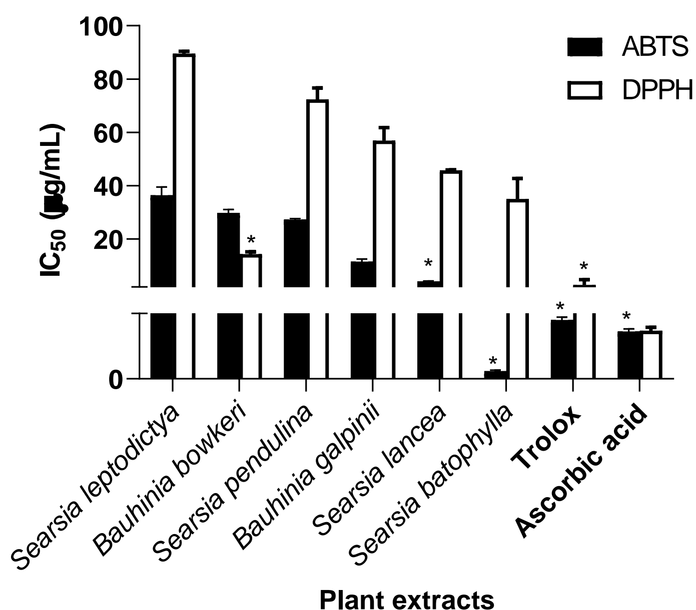


Fig 5.3: Antioxidant effect (IC_{50} in µg/mL) of plant extracts

5.3.5 Anti-inflammatory activity

5.3.5.1 Soybean lipoxygenase inhibition assay

The anti-inflammatory activities of the plant extracts were assessed based on their ability to inhibit 15-lipoxygenase (LOX) enzyme activity and nitric oxide (NO) production in lipopolysaccharide (LPS) activated RAW 264.7 macrophages. *Searsia batophylla* showed outstanding inhibition of 15-LOX (IC_{50} = 4.12 µg/mL), and no significant difference in the observed activity by this plant and quercetin with IC_{50} = 11.90 µg/mL (P = 0.24), a standard anti-inflammatory agent. All other plant extracts had anti-inflammatory activity with IC_{50} values less 100 µg/mL (**Table 5.2**)

5.3.5.2 Inhibition of nitric oxide (NO) production assay

All the plant extracts showed concentration-dependent NO production inhibition. All the tested plant extracts at the highest concentration (100 µg/mL)had percentage inhibition above 50% except for *B. bowkeri,* which had NO inhibition IC_{50} value of 129.26 µg/mL. *Searsia batophylla* had the lowest NO inhibition IC_{50} value of 48.86 µg/mL. The plant extracts' toxicity was determined using the MTT cell viability assay. Only *S. pendulina* extract exhibited toxicity toward the macrophage cells (cell viability of 16.49%) (**Table 5.2**). NO is a toxic radical produced in response to pro-inflammatory conditions and agents like lipopolysaccharides by

macrophages via oxidation of inducible nitric oxide synthase (iNOS) (Mollace *et al.*, 2005; Aro *et al.*, 2016).. In this study, there were significant differences in the NO inhibitory IC_{50} values of all the tested acetone plant extracts and quercetin, the positive control. However, no statistical difference was observed between the IC_{50} values for NO inhibition of *S. batophylla, S. pendulina, S. leptodictya* and *S. lancea* at $P < 0.0001$.

Table 5.2: Inhibitory activities of nitric oxide production in lipopolysaccharide activated RAW 264.7 macrophage cells, anti-lipoxygenase, and quantitative phytochemical content analysis of the acetone leaf extracts of the selected plants.

Plant name	Concentration (µg/mL)	% NO inhibition	% Cell viability	NO inhibition IC_{50} (µg/mL)	15-Lox IC_{50} (µg/mL)	Total phenolic content (TPC) (GAE mg/g)	Total flavonoid content (TFC) (QE mg/g)
Sl	1.6	12.99 ± 1.78	101.52 ± 7.84	60.58 ± 7.40^{b}	64.74 ± 1.48^{b}	348.97 ± 23.94^{b}	253.72 ± 14.36^{a}
	12.5	29.60 ± 3.02	87.06 ± 4.23				
	50	45.52 ± 3.71	80.85 ± 11.05				
	100	64.23 ±1.79	67.76 ± 10.55				
Sp	1.6	17.06 ± 1.68	92.16 ± 8.84	49.64 ± 4.58^{b}	69.92 ± 2.66^{b}	220.17 ± 9.89^{d}	151.84 ± 17.80^{b}
	12.5	22.83 ± 2.69	72.02 ± 10.46				
	50	56.35 ± 3.47	45.79 ± 5.16				
	100	63.44 ± 2.84	16.49 ± 0.69				
Slp	1.6	9.55 ± 1.43	87.90 ± 8.57	59.44 ± 10.90^{b}	74.31 ± 5.86^{b}	251.72 ± 6.40^{c}	23.68 ± 1.53^{d}
	12.5	15.59 ± 1.77	77.44 ± 7.39				
	50	48.95 ± 6.77	74.20 ± 12.81				
	100	65.02 ± 3.34	68.04 ± 13.18				
Bg	1.6	11.87 ± 0.81	88.70 ± 5.54	92.23 ± 1.28^{c}	69.55 ± 6.42^{b}	227.20 ± 20.30^{c}	19.35 ± 1.30^{d}

	12.5	14.35 ± 2.42	74.36 ± 5.46				
	50	31.86 ± 2.96	75.07 ± 11.37				
	100	53.96 ± 0.93	75.10 ± 7.99				
Bb	1.6	13.09 ± 1.15	106.37±12.07	129.26 ± 22.18[d]	95.75 ± 8.74[c]	171.24 ± 20.14[d]	56.96 ± 5.37[c]
	12.5	13.73 ± 1.26	99.11 ± 7.31				
	50	25.13 ± 4.32	98.96 ± 7.60				
	100	46.43 ± 5.88	97.55 ± 0.61				
Sb	1.6	8.35 ± 1.08	117.40 ± 15.17	48.82 ± 0.86[b]	4.27 ± 0.82[a]	443.36 ± 24.65[a]	13.65 ± 1.44[d]
	12.5	11.94 ± 1.21	114.78 ± 7.22				
	50	52.58 ± 5.01	114.16 ± 12.69				
	100	74.79 ± 5.06	114.25 ± 5.7				
Quercetin	1.6	43.91 ± 3.46	99.00 ± 9.29	3.33 ± 0.05[a]	11.90 ± 0.21[a]	NA	NA
	12.5	64.66 ± 5.55	81.10 ± 4.38				
	50	94.53 ± 4.99	54.90 ± 4.57				
	100	**96.15 ± 0.79**	**31.52 ± 3.28**				

Key: Sp= *Searsia pendulina*, Slp= *Searsia leptodictya,* Sl= *Searsia lancea* Sb= *Searsia batophylla,* Bg= *Bauhinia galpinii,* Bb= *Bauhinia bowkeri.* Quercetin= positive control (anti-inflammatory agent),NA= not applicable, values with different letters indicate significant difference at $p < 0.0001$.

5.3.6 Total phenolic content and total flavonoid content (TPC)

The total phenolic content was expressed in terms of gallic acid equivalent (GAE). The TPC of the selected plant acetone extracts ranged from 171.24 to 443.36 mg GAE/g. Statistical analysis showed that *S. batophylla* had the highest TPC, followed by *S. lancea,* while *B. bowkeri* and *S. pendulina* had the lowest TPC (**Table 5.2**). A similar TPC result of 225 mg GAE/g was reported for *B. galpinii* by Ahmed (2012), however, the same could not be said of the TPC result of *B. bowkeri, S. leptodictya* and *S. pendulina* as the obtained values from the current finding were lower than those of the previous study. This may be due to acidified acetone used by Ahmed (2012) as solvent of extraction compared to 100% acetone used in this work. The extraction process is an integral factor in the assessment of the bioactivity of plant extracts (Berlin and Berlin, 2005). Other reasons may be differences in the source plant material used to prepare the extracts.

On the other hand, total flavonoid content (TFC) was determined as a function of quercetin equivalent (QE). The TFC also ranged from 13.65 to 253.72 mg QE/g. Based on the statistical evaluation, *S. lancea* had the highest TFC value, followed by *S. pendulina*. However, no significant difference between the TFC values obtained for *S. leptodictya, B. galpinii* and *S. batophylla* ($P < 0.0001$) (**Table 5.2**). The TFC values obtained from the present study on *S. pendulina, S. leptodictya, B. galpinii* and *B. bowkeri* were higher than that of the previous work of Ahmed (2012), which again may be due to slight variation in the solvent used (acidified acetone).

5.3.7 Phytochemical analysis of plant acetone extracts using GC-MS

The GC-MS analyses of the six plant acetone extracts are presented in **Appendix 8.1 to 8.6**, showing the molecular name, molecular formula, percentage peak area and similarity of the compounds present. The results were depicted by comparing the peaks to a library with known compounds. The percentage area and similarity were obtained. However, compounds with both high percentage area and similarity were recorded.

5.4 Discussion

The observed susceptibility pattern in the pathogens may occur because of antibiotics misuse or over use. The potency of the antimicrobial agents used and possibility of acquired resistance genes from related and distantly related species, a common phenomenon with intestinal pathogens (Bonfiglio *et al.*, 2002; Asrat, 2008). Resistance to antimicrobials is a major global concern because of the difficulty in the treatment of infectious diseases. The problem of antimicrobial resistance which may lead to increases in death rate and low economic productivity due to prolonged hospital stays. Therefore, there is a need to support the one health initiative to combat the growing rate of global antimicrobial resistance.

The bioautography method shows how separated components of the extracts inhibit bacterial growth and contribute to the total activity of the plant extract (Masoko and Eloff, 2005). This is unlike the dilution method, which evaluates the antimicrobial activity of unseparated components of the extract. The interaction of components of the crude extracts can either mask the bioactivity of specific compounds or enhance their bioactive effects by synergism (Junio *et al.*, 2011). Such interaction is however prevented by this method. In this study, *S. lancea* showed a prominent zone of inhibition against the *E. coli* (ATCC 25922) culture in all three solvent systems. This suggests that the *S. lancea* extract has at least one component with promising inhibitory effect on the growth of *E. coli*. In addition, all the acetone plants extract used for this work had at least one compound in the fingerprint with an inhibitory effect on *E. coli* ATCC 25922 on BEA system. This result is consistent with our previous work on antimicrobial activity against the tested bacteria with MIC values of < 0.16 mg/mL (Adeyemo *et al.*, 2022)**.** However, the bioautography method has limitations of only applying to microorganisms that can grow satisfactorily on TLC plates.

Based on the evaluation of Eloff (2021), MIC values lower than 0.02 mg/mL are classified as outstanding activity, 0.021-0.04 mg/mL excellent activity, 0.041-0.08 mg/mL very good activity, 0.081-0.16 mg/mL good activity, 0.161-0.32 mg/mL average activity and MIC greater than 0.32 mg/mL is weak activity. The (MIC) values obtained in this study were noteworthy (≤ 0.16 mg/mL). The bioautography result of *S. lancea* is congruent with the MIC value. The MIC and TLC bioautography data obtained from this study showed the antibacterial potential of *S. lancea*. These results are in consonance with the findings of Adeyemo *et al.* (2022) with *S. lancea* leaf extract having MIC values ranging from 0.04-0.09 mg/mL against different ATCC *E. coli* strains. However, the slight variation in MIC values (0.05-0.08 mg/mL) may be due to the different bacteria used in the current evaluation (clinical strains) Many researchers

have explored using natural products such as medicinal plants and their compounds as antimicrobial agents to impede the growth of microbes (McGaw *et al.*, 2007; Akande *et al.*, 2022). Suggestions have been made that plant components like alkaloids, terpenoids and phenolic compounds could compromise the integrity of the microbial cell membrane by interacting with some enzymes and proteins, leading to the drifting of protons towards the cell exterior, consequently inducing inhibition of essential amino acids and ultimately, cell death (Gill and Holley, 2006; Mostafa *et al.*, 2018). In addition, the hydrophobic character of some natural products allows them to interact with vital organelles like mitochondria, altering their functions (Tiwari *et al.*, 2009).

The SI values greater than 1 imply that the drug or antimicrobial agent is more toxic to the pathogens, in this case bacterial cells, than to normal mammalian cells (Katsuno *et al.,* 2015). In pursuit of product development, active ingredients with SI values higher than 10 are often regarded as valuable (Katsuno *et al.*, 2015). This study reveals that *B. galpinii* extract, with SI value as high as 24.80, has excellent prospects for herbal product development.

Based on the evaluation of Omisore *et al.* (2005), extracts with $IC_{50} < 50$ µg/mL are said to possess potent antioxidant effects, while 50 µg/mL $< IC_{50} <$ 100 µg/mL implies that the extract merely has good antioxidant activity. The gastrointestinal tract is an important site where pro-oxidants are produced due to the abundance of bacterial cells and food components interacting with the immune cells (Lee, 2015). In diarrhoea episodes, oxidative stress may arise from the imbalance between microbial-induced production of pro-oxidant species and antioxidant defences (Brown and Mayer, 2007). As a result, research findings suggest that dietary antioxidant compounds, especially from natural products, could confer protection by modulating cellular events in the gastrointestinal tract (Ozdal *et al.*, 2016; Khan *et al.*, 2017; Gil-Cardoso *et al.*, 2019). Therefore, the selected plant extracts, particularly *S. batophylla,* may be beneficial in treating acute diarrhoea and other chronic inflammation.

The isomeric enzyme 15-LOX plays a vital role in the synthesis of leukotrienes from arachidonic acids, which mediate pro-inflammatory reactions. Therefore the inhibition of leukotrienes would be of paramount importance for therapeutic purposes (Schneider and Bucar, 2005). These plant extracts and, more specifically, *S. batophylla,* could be relevant in treating various inflammatory disorders. Anti-inflammatory mediators modulate the activities of pro-inflammatory components.

S. batophylla had the highest macrophage cell viability of 114.25% at the highest tested concentration of 100 µg/mL. In addition, macrophage cell viability was more than 50% at the highest tested concentration of 100 µg/mL for all the other plant extracts, except for *S. pendulina,* with low cell viability of 16.49% (Table 5.2). This implies that the suppression of NO production observed for *S. pendulina* may be due to toxicity. The NO production inhibitory activity may be due to the inhibition of iNOS enzyme activity (Ryu *et al.*, 2003). Previous findings have established plant products as a good repressor of iNOS expression in LPS-activated macrophages (Son *et al.*, 2000; Akande *et al.*, 2022). The interplay between microbes and immune cells of the GIT may promote NO production, which may exacerbate infectious diarrhoea episodes (Brown and Mayer, 2007). The build-up of NO in the living system is crucial to various disease developments, therefore, NO production repression is significant in treating inflammation-related disorders. Therefore, these plant extracts, except for *S. pendulina* and *B. bowkeri,* could be useful in reducing NO-induced inflammation disorders.

Plant products are natural sources of different phytochemical constituents such as phenols, tannins, alkaloids and flavonoids. Phenolic and flavonoid compounds are notably responsible for antioxidant, antisecretory, antimotility, antispasmolytic and other pharmacological activities (Scalbert *et al.*, 2005; Douho Djimeli *et al.*, 2022). In this study, *B. galpinii* had TPC of 227 mg GAE/g. Similarly, the TPC result of 225 mg GAE/g was reported for *B. galpinii* by Ahmed *et al.* (2012). However, the same could not be said of the TPC result of *B. bowkeri, S. leptodictya* and *S. pendulina* as the obtained values from the current finding were lower than those of the previous study. This may be due to acidified acetone used by Ahmed *et al.* (2012) as solvent of extraction compared to 100% acetone used in this work. The extraction process is an integral factor in the assessment of the bioactivity of plant extracts (Berlin and Berlin, 2005). Other reasons may be differences in the source plant material used to prepare the extracts. The earlier work of Elisha *et al.* (2016) on some indigenous South African plants used as therapy for arthritis also showed that their phenolic content could be responsible for the observed excellent antioxidant activities. In addition, phenolic compounds as natural antioxidants act as single oxygen scavengers, reducing agents and metal chelators (Bulugahapitiya, 2020). Phenol-rich extracts have been previously linked to many physiological and health benefits (Ahmed (2012) hence, they are a likely contributing factor to the observed anti-inflammatory and antioxidant activities. The TFC values obtained from the present study on *S. pendulina, S. leptodictya, B. galpinii* and *B. bowkeri* were higher than that of the previous work of Ahmed *et al.* (2012), which again may be due to slight variation in the solvent used (acidified acetone). In addition, flavonoids also have many useful

pharmacological activities such as free radical scavenging activity (Scalbert *et al.*, 2005). However, their antioxidant effect depends on the presence of electron donor and electron acceptor substituents on their ring structure, the arrangement of their hydroxyl groups and the extent of structural conjugation (Miliauskas *et al.*, 2005). Other beneficial roles, such as inhibition of inflammatory mediators, have also been reported (Feng *et al.*, 2020). Hence, flavonoids are likely to also be a contributing factor to the antioxidant and anti-inflammatory effects of the tested acetone plant extracts.

The GC-MS data revealed that compounds such as xylene, docosene, dodecanamide, dodecene, squalene, phytol, phenol,3-pentadecyl, phenol, diisooctyl phthalate, 4-(1,1-)dimethylpropyl, 9-octadecenamide, (Z)- and benzene derivatives were common to all the selected plants. The most abundant compounds found in acetone crude extracts of the selected plants were: 9-octadecenamide in *S. batophylla* and *S. lancea*, 3-((4Z,7Z)-heptadeca-4,7-dien-1-yl)phenol in *S. leptodictya*, n-hexadecanoic acid in *S. pendulina*, squalene in *B. bowkeri* and n-hexadecanoic acid in *B. galpinii*. These compounds could be responsible for their various biological activities. For example, the compound 9-octadecenamide was previously isolated from the methanolic extract of *Diaporthe schini* and *Cinnamomum verum* bark and reportedly possessed antioxidant, antibacterial and antifungal activities against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Aspergillus flavus* (Hameed *et al.*, 2016; Dos Reis *et al.*, 2019). The findings of Elufioye and Berida (2018) suggested that 3-((4Z,7Z)-heptadeca-4,7-dien-1-yl)phenol could be responsible for the significant antioxidant activity of methanolic extracts of whole fruit and stem bark of *Spondias purpurea* due to its relative abundance. The compound *n*-hexadecanoic acid, the predominant compound in *S. pendulina* and *B. galpinii,* is an inhibitor of phospholipase A_2 (Aparna *et al.*, 2012). Squalene, a triterpenic compound, has been sourced from plants such as*Vitis vinifera*, *Olea europaea*, rice, peanut, amaranth and corn (Lozano-Grande *et al.*, 2018). Several studies have established the biological, nutritional, and medicinal benefits of squalene. It is considered a potent antioxidant because of its ability to scavenge free oxygen radicals, inhibit tumour growth, particularly in skin, colon, breast and lungs and immune stimulatory activity (Newmark, 1997; Reddy and Couvreur, 2009; Güneş, 2013; Lozano-Grande *et al.*, 2018). Squalene also possess antimicrobial activity, with the mechanism of action speculated to be cell membrane disruption (Mendoza *et al.*, 1997; Arunkumar and Muthuselvam, 2009).

The GC-MS phytochemical analysis identified some similarities between the chemical constituents of the plants, although in varying amounts. This could be due to their closeness in

classification, explaining their content of related compounds. The observed similarity is a pointer toward selecting plants based on their chemotaxonomy in the search for novel compounds in the drug development process (Pauw and Eloff, 2014).

5.5 Conclusion

This study reveals the biological relevance of acetone leaf extracts of selected *Bauhinia* and *Searsia* species with GC-MS analysis showing their various phytochemical constituents. Compounds such as 9-octadecenamide, 3-((4Z,7Z)-heptadeca-4,7-dien-1-yl)phenol, n-hexadecanoic acid and squalene may contribute to the observed antimicrobial, antioxidant, anti-inflammatory and other activities. Therefore, purifying these compounds from the individual plants may be worthwhile, as well as further investigating their biological capability in *in vivo* studies.

Also, the observed antibiotic resistance in the clinical isolates of *E. coli* used in this work which were found to be susceptible to most plant extracts tested, points to the prospect of these plant extracts having potential value in the development of phytogenic feed additives to prevent diarrhoea.

Chapter 6 General discussion and conclusions

6.1 Summary of the study

One of the world's major health conditions, particularly in developing nations, is diarrhoea. The issue is made worse by the rise of HIV infections, opportunistic infections and other health issues that present diarrhoea as one of the symptoms. In animal production, diarrhoea epidemics usually result in significant economic losses due to high fatality rates, high treatment costs, and reduced animal productivity levels. Serious challenges in treating and managing diarrhoea include the emergence of more virulent species due to drug-resistant bacteria and the apparent adverse effects of several standard medications already in use. In addition to promoting effective antimicrobial stewardship, many nations, notably those in the European Union, have outlawed the addition of conventional antimicrobial agents and growth promoters to animal feeds due to concerns of antimicrobial resistance. Others, particularly in developing nations like South Africa, have yet to implement this prohibition. To meet the needs of the rising global population, there is a dire need to develop alternative therapies or drugs derived from medicinal plants or other sources with few or no side effects, good efficacy, and cost-effectiveness in place of conventional antibiotics. For years, plant preparations have been used to treat diarrhoea locally, even though their potencies, safety and mechanisms of action have not been proven empirically.

Overall, this thesis was designed to investigate indigenous South African plants that may be of therapeutic importance in the place of antimicrobial agents in treating diarrhoea in humans and animals. The antibacterial potency of plant extracts against diarrhoeagenic bacteria was investigated. Probable modes of action of plant extracts and bioactive species were also assessed using different methods. Plant species were selected from native South African flora from the family Anacardiaceae, Asteraceae and Fabaceae, and specifically from the genera *Searsia, Brachylaena* and *Bauhinia*, respectively. The choice of plants selected for evaluation of biological activities in this study was based on the ethnomedicinal use of the plants in the treatment of diarrhoea, preliminary antibacterial assessment of the medicinal plant species in the Phytomedicine laboratory of the Department of Paraclinical Sciences, Faculty of Veterinary Sciences (University of Pretoria) and paucity of available reports on some of the plants and their biological activities. The selected plants species were *Searsia pendulina*, *Searsia leptodictya*, *Searsia gueinzii*, *Searsia lancea*, *Searsia batophylla*, *Bauhinia galpinii*,*Bauhinia*

bowkeri, *Bauhinia variegata*, *Brachylaena transvaalensis*. To achieve the aim of this study, the following objectives were established and summarized below.

6.2 Antibacterial, anti-biofilm and anti-quorum sensing activities of selected South African plants traditionally used to treat diarrhoea

Investigation of the antibacterial, anti-biofilm and anti-quorum sensing activities of crude extracts of nine indigenous South African plants against diarrhoeagenic bacteria was a primary goal. The acetone extracts showed significant activity in a preliminary test of all extracts prepared using different solvents against a reference strain of *E. coli* (ATCC 25922). The obtained MIC values ranged from 0.04 to 0.63 mg/mL. Further testing against other bacterial pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium*, Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Salmonella enterica* were done. The MIC results revealed that the acetone extract of *Bauhinia bowkeri* was most potent against *Salmonella* Enteritidis with an MIC value of 0.01 mg/mL. On the other hand, *S. lancea* had MIC value of 0.03 mg/mL and was most active against *B. cereus*.

All the plant extracts prevented biofilm attachment by more than 50% for at least one of the studied bacteria. However, in the destruction of pre-formed biofilm test, only *B. cereus* biofilm was susceptible to *S. pendulina* extracts with 98.22% eradication. The minimum quorum sensing inhibitory concentration (MQSIC) of the plant extracts ranged from 0.08 to 0.32 mg/mL, with *S. lancea* being the best. The optimum half maximum violacein production inhibitory value was 0.17 mg/mL for the *S. lancea* extract. The highest half maximum violacein production inhibitory value was 0.17 mg/mL for the *S. lancea* extract. Overall, the findings showed that acetone extracts of *S. pendulina, S. batophylla, S. leptodictya, S. lancea, B. bowkeri* and *B. galpinii* have antibacterial and anti-biofilm actions as well as the ability to modify quorum sensing by reducing violacein production.

This study further revealed that at concentrations below the MIC, the acetone extracts of the investigated plants significantly inhibited biofilm formation and quorum sensing-mediated violacein pigment synthesis. It was challenging to disrupt pre-existing biofilms, demonstrating that the plant extracts were mostly ineffective at disrupting bacterial biofilms that had already developed. The most potent plant extracts could therefore be developed as alternative preventative antibacterial remedies. However, more study, especially *in vivo* research, is required to confirm possible pharmaceutical uses. Purification and elucidation of active compounds with antibacterial, anti-biofilm formation and anti-quorum sensing activities from

these plants could be of importance, in terms of their potential as precursors for the synthesis of novel compounds to treat diarrhoea and associated symptoms.

6.3 Isolation of antibacterial compounds from *Searsia batophylla* acetone crude extract effective against *Escherichia coli*

Bioassay-guided fractionation of the chloroform fraction of *S. batophylla* yielded three compounds. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and gas chromatography-mass spectrometry (GC-MS) were used for the elucidation of the structures of the compounds. Fractions from the column chromatography had MIC ranging from 0.04 to 2.50 mg/mL against *E. coli*. Three compounds, namely7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione (unsaturated ketone), dodecanamide, and 3-oxo-olean-18-en-28-oic acid were isolated from *S. batophylla* leaves. The minimum inhibitory concentrations (MIC) of the compounds ranged from 0.63 to 0.93 mg/mL, whereas the MIC value for the chloroform fraction was 0.16 mg/mL. At the highest concentration of 0.2 mg/mL against Vero cells, the compounds showed more than 50% cell viability.

This study showed that compounds extracted from *S. batophylla* had low cytotoxicity to Vero cells. The compounds had improved antibacterial activity when they were together with other components, compared to when separated. However, more research is advised to clarify alternative antibacterial mechanisms of the compounds and fractions of *S. batophylla* leaf extracts. Studies like inhibitory effects on biofilm and quorum sensing, or effect on bacterial membrane permeability would be useful.

In this work, fractions of the chloroform fraction of the crude acetone extract of *S. batophylla* leaves had significant antibacterial activity. The fractions also had low toxicity to Vero kidney cells and poor antioxidant activity. Hence, extracts or fractions from the *S. batophylla* plant could be useful to develop alternative herbal products rather than the individual compounds. Further studies are required to evaluate their antibiofilm and anti-quorum sensing activities, as well as *in vivo* studies to assess their safety and biological activities.

6.4 *In vitro* antibacterial, antioxidant and anti-inflammatory activity, phytochemical composition and GC-MS profiling of selected South African plants used to treat diarrhoea

In this study, the MIC values ranged from 0.04 to 0.16 mg/mL against *E. coli* strains isolated from clinical cases of bovine diarrhoea, and *S. pendulina* had the best antibacterial activity.

Bioautograms using the BEA solvent system as eluent revealed the antibacterial effect of the plant extracts against *E. coli* ATCC 25922. Some of the clinical isolates employed in this study had multiple resistance to different antibiotics. The most resistant strain among all the isolates was *E. coli* 1, which was resistant to six different antibiotics. All the tested acetone plant extracts were observed to possess outstanding antibacterial activity against the multidrug resistant strain *E. coli* 1, hence, they are potential sources of novel antimicrobial agents. In addition, the acetone extracts were seemingly non-toxic to Vero cells. *Bauhinia galpinii* had the best selectivity index value, greater than 10, which is considered valuable when selecting potential therapeutic products for development. *S. batophylla* and *B. bowkeri* had the best radical scavenging ability with IC_{50} of 0.19 µg/mL and 14.31 µg/mL in ABTS and DPPH, respectively.

Searsia batophylla was the best extract to inhibit the activity of the 15-LOX enzyme, with an IC_{50} value of 4.27 µg/mL. The NO production by LPS-activated macrophages was suppressed in a concentration-dependent manner. *Searsia batophylla* extract showed the best inhibition of NO production with an IC_{50} value of 48.81 µg/mL with cell viability of 114.25 % at the highest concentration of 100 µg/mL. *Searsia batophylla* had the highest total phenolic content (TPC), with a value of 443.36 mg GAE/g. This could be a major contributing factor to its observed radical scavenging and anti-inflammatory activities. *Searsia lancea* had the highest total flavonoid content with a value of 253.72 mg QE/g. In general, the selected plant acetone extracts possess various significant biological activities that could be ascribed partly to their phenolic content. Hence, they could be potential candidates to be considered for developing herbal products from natural sources. This study establishes some biological relevance of the selected acetone leaf extracts. The GC-MS analysis showed their various phytochemical constituents. The presence of known bioactive compounds such as 9-octadecenamide, 3-((4Z,7Z)-heptadeca-4,7-dien-1-yl) phenol, n-hexadecanoic acid and squalene may be responsible for the observed antimicrobial, antioxidant anti-inflammatory and other biological activities. However, other phytochemical analyses such as LC-MS may also be needed to annotate potential bioactive compounds. Isolating and purifying these compounds from the selected plants may be worthwhile, as well as further investigation of their biological capabilities, especially *in vivo* studies.

6.5 Summary

This study contributed to knowledge on nine indigenous South African plant species, namely *S. batophylla, S. lancea, S. leptodictya, S. pendulina, S. gueinzii, B. galpinii, B. bowkeri, B.*

variegata and *B. tranvaalensis*with regard to their antibacterial activity. The inhibitory activity was against reference strains of *Escherichia coli* O157: H7 (ATCC 43888), *E. coli* (ATCC 25922), *E. coli* (ATCC 35218), *Bacillus cereus* (ATCC 21366), *Staphylococcus aureus* (ATCC 29213), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. typhimurium*, ATCC 39183), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S. enteritidis*, ATCC 13076). Ten different *E. coli* clinical isolates were also included in the antibacterial evaluation, and anti-biofilm, antioxidant, anti-quorum sensing, and anti-inflammatory activities were also investigated. Three compounds, namely dodecanamide 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione (unsaturated ketone) and 3-oxo-olean-18-en-28-oic acid were isolated from the *S. batophylla* acetone leaf extract.

Based on this work, many of the investigated plant extracts had promising biological activities. Three of the plant extracts had particularly high biological activities, which may be promising for the development of preventative or treatment preparations against diarrhoea. *Bauhinia bowkeri* and *Searsia lancea* showed outstanding antibacterial activity against both typed culture and clinical *E. coli* isolates. However, *Bauhinia galpinii* had the best selectivity index due to its good antibacterial activity and low toxicity. *Searsia batophylla* extracts had the best radical scavenging and anti-inflammatory activities. Furthermore, *S. batophylla* was selected for isolation and three compounds, namely dodecanamide, 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione (unsaturated ketone) and 3-oxo-olean-18-en-28-oic acid, were elucidated.

Overall, this work provides sufficient rationale to carry out further studies on the selected plant species. The study chiefly highlighted the excellent *in vitro* antibacterial, antioxidant and anti-inflammatory activities of extracts of *Searsia batophylla,* from which three compoudns were identified. This provides motivation for further research on this plant species in terms of its development as a phytogenic feed additive for livestock to reduce the occurrence of diseases with which diarrhoea is associated.

6.6 Future research perspectives

It is recommended that the plant products highlighted as being active in this study, including extracts, fractions and compounds, should be explored further by testing their ability to repress the expression of virulence genes, which is vital in bacterial pathogenicity. Identification of the effect of the plant derivatives on phenotypic expression of certain properties of bacteria, such

as drug resistance or substrate metabolism, could be investigated using for instance the Biolog Phenotype MicroArray system.

Future research should also involve the following:

1. Isolation of more biologically active compounds from *Searsia* and *Bauhinia* species.
2. Evaluation of the potency of selected plant extracts, fractions, and isolated compounds against highly drug resistant bacterial and fungal microbes associated with diarrhoea, as this study focused only on *E. coli*. The presence of broad spectrum activity needs to be identified.
3. Investigate the mechanism of anti-diarrhoeal activity of the most active plant extracts using *ex vivo* studies, such as tissue and organ baths.
4. Toxicity assessment of the promising plant extracts on other mammalian cells, and *in vivo*.
5. *In vivo* trials of the most active plants as feed additives or herbal preparations to prevent and treat diarrhoea, particularly in weanlings who are most susceptible.
6. Investigate the synergistic antimicrobial effect of the active plants, fractions and compounds used in this study in combination with each other and with currently used antibiotics against diarrhoeagenic microbes.

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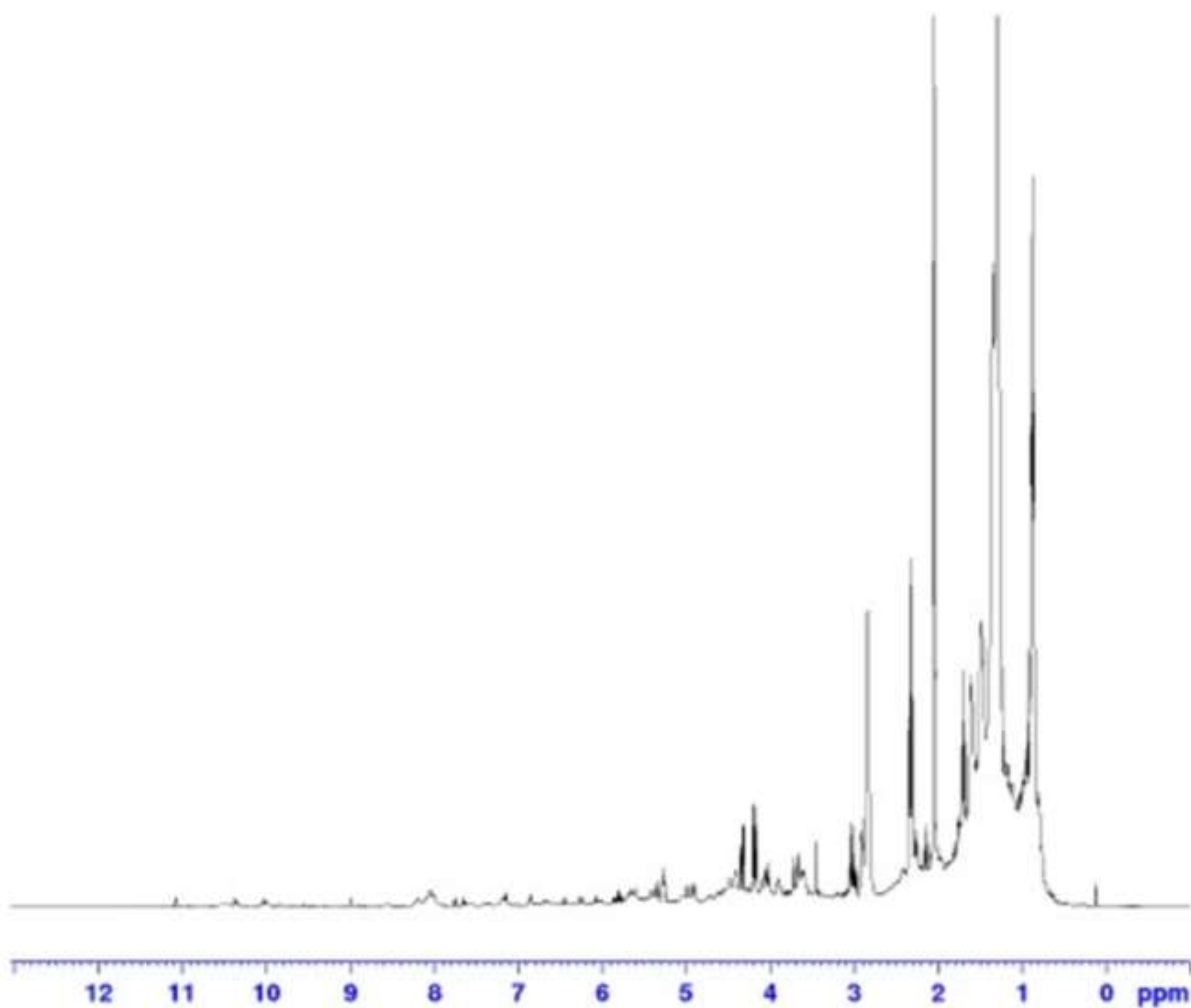
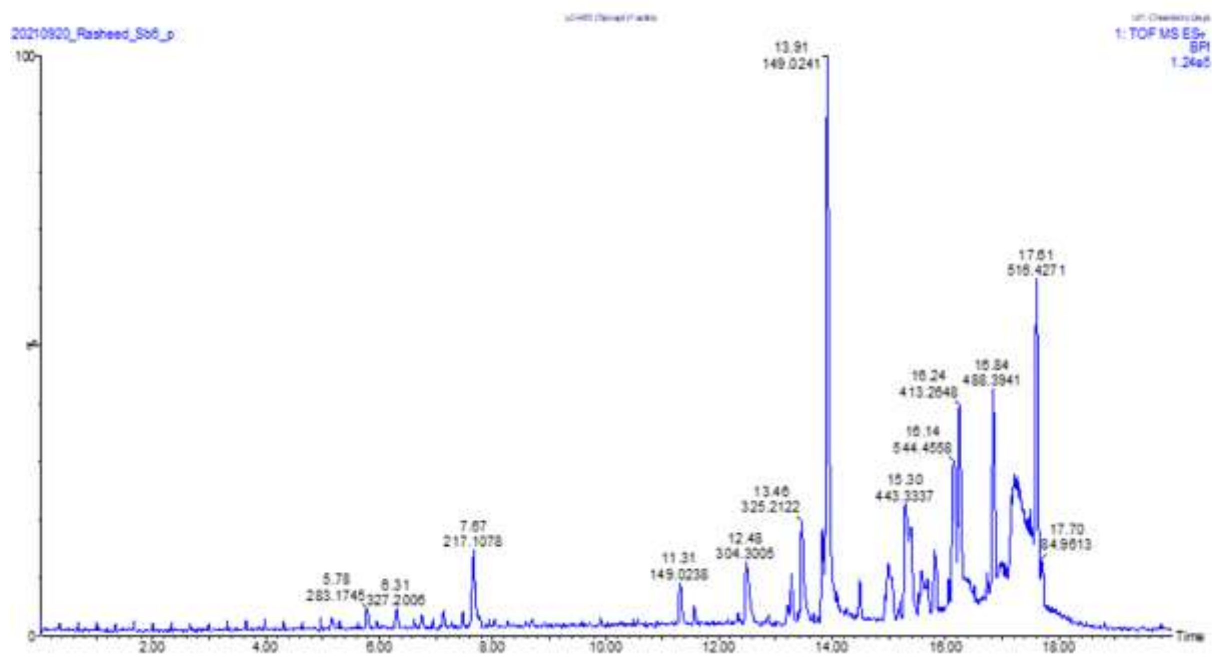
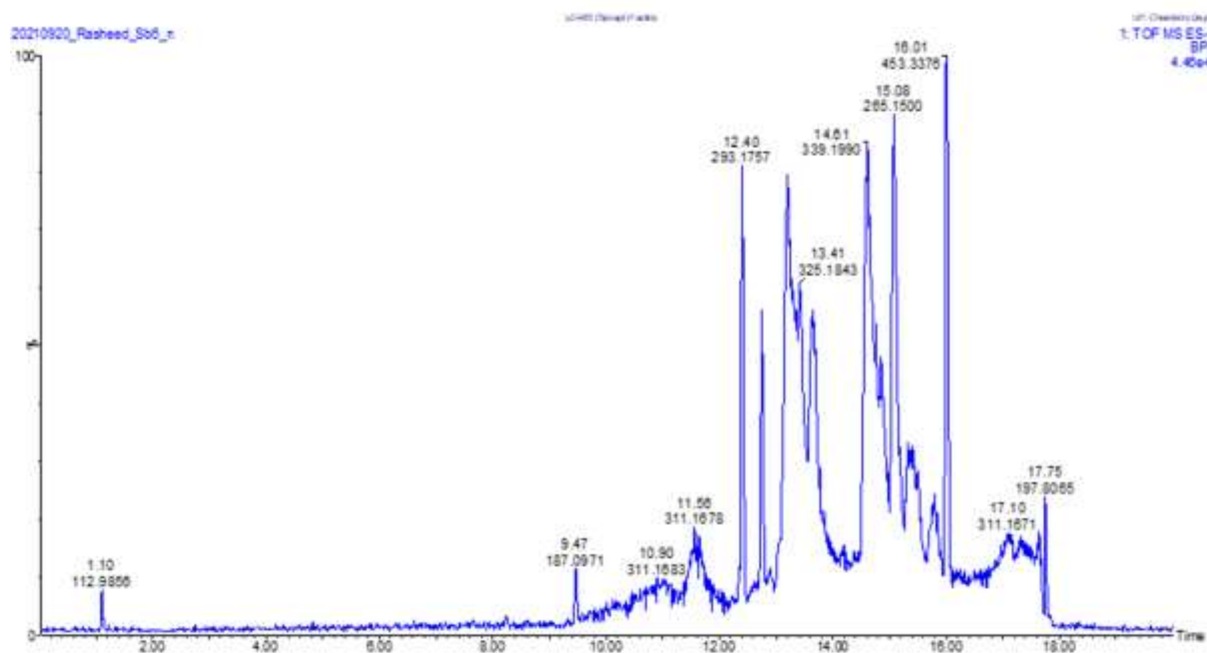
Appendix



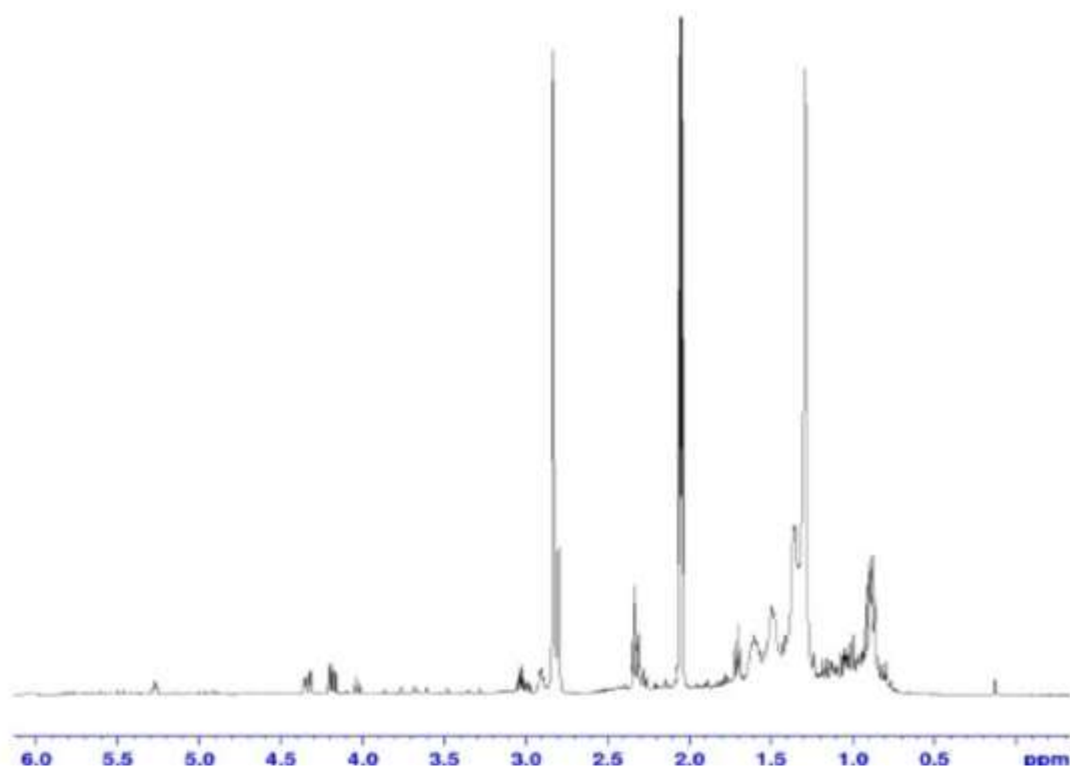
Appendix 1: ^{1}H NMR spectra for dodecanamide (Sb-6)



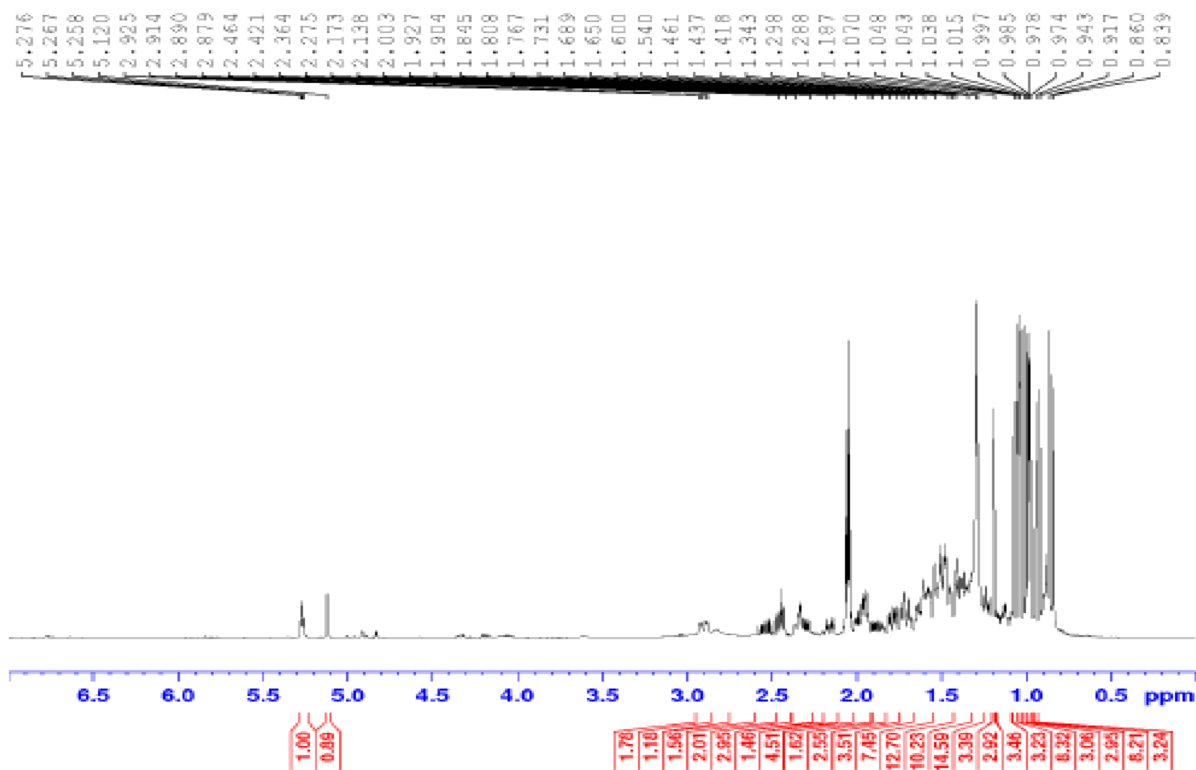
Appendix 2: UPLC-MS for compound dodecanamide (Sb-6) (Positive mode)



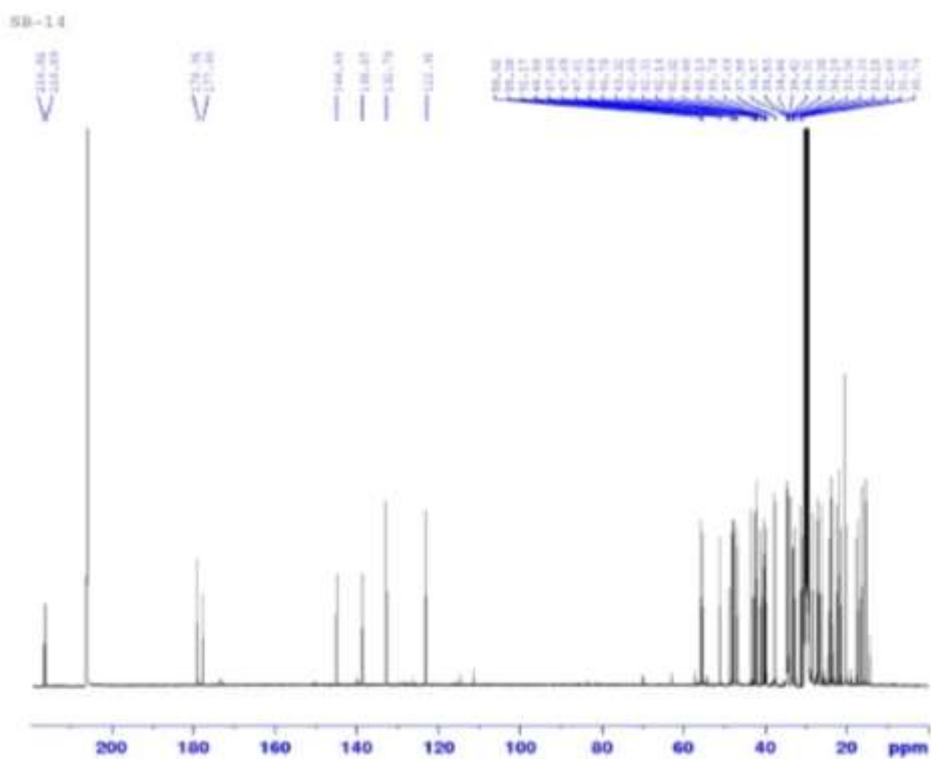
Appendix 3: UPLC-MS for compound dodecanamide (Sb-6) (Negative mode)



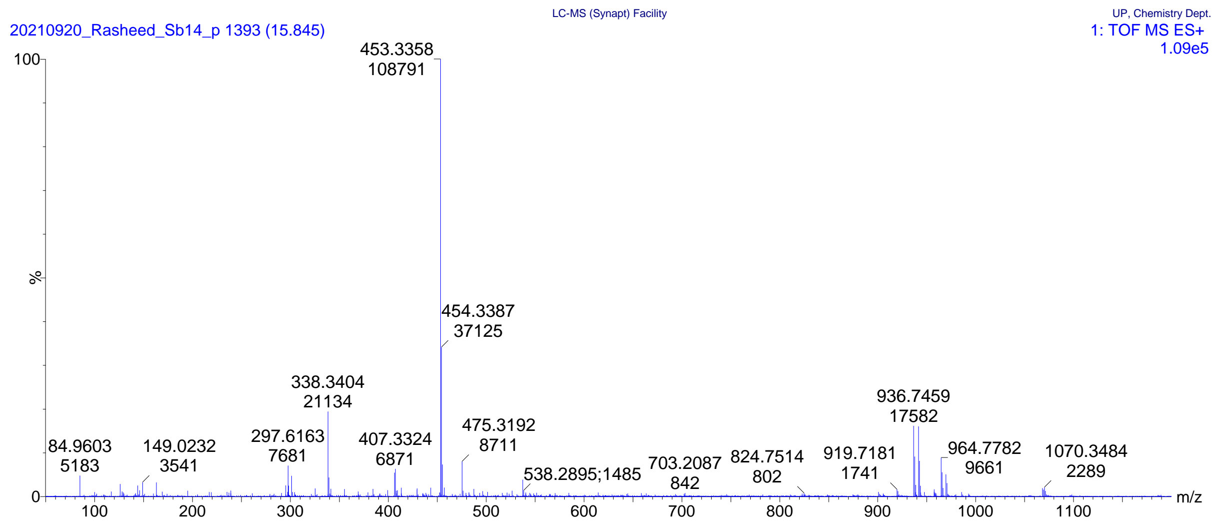
Appendix 4: ^{1}H NMR Spectrum of 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (Sb-7)



Appendix 5: ^{1}H NMR spectrum of 3-oxo-olean-18-en-oic-acid



Appendix 6: ^{13}C NMR spectrum of 3-oxo-olean-18-en-oic-acid



Appendix 7: UPLC-MS for compound 3-oxo-olean-18-en-oic-acid (Negative mode)

Appendix 8.1: List of compounds identified from *S. batophylla* acetone extract

Name	Similarity	Area %	Formula
1-Docosene	95.60	0.87	$C_{22}H_{44}$
p-Xylene	95.20	0.58	C_8H_{10}
o-Xylene	95.00	0.31	C_8H_{10}
Benzene, 1,2,3-trimethyl-	94.90	0.82	C_9H_{12}
1,3,5,7-Cyclooctatetraene	94.90	0.81	C_8H_8
Dibutyl phthalate	93.40	0.17	$C_{16}H_{22}O_4$
Cetene	93.00	1.90	$C_{16}H_{32}$
9-Octadecenamide, (Z)-	92.70	6.82	$C_{18}H_{35}NO$
n-Hexadecanoic acid	92.70	2.30	$C_{16}H_{32}O_2$
Dotriacontane	92.50	1.30	$C_{32}H_{66}$
Mesitylene	92.50	0.39	C_9H_{12}
Dodecanamide	91.40	1.79	$C_{12}H_{25}NO$
3-Tridecene, (E)-	91.30	0.93	$C_{13}H_{26}$
Benzamide, N-propyl-	91.30	0.27	$C_{10}H_{13}NO$
Nonane, 3-methyl-	91.10	0.53	$C_{10}H_{22}$
Decane	90.50	1.10	$C_{10}H_{22}$
2-Pentanone, 4-hydroxy-4-methyl-	90.20	4.88	$C_6H_{12}O_2$
Ethylbenzene	90.10	0.10	C_8H_{10}
Diisooctyl phthalate	90.00	0.21	$C_{24}H_{38}O_4$

Appendix 8.2: List of compounds identified from *S. lancea* acetone extract

Name	Similarity	Area %	Formula
p-Xylene	95.80	0.33	C_8H_{10}
1-Docosene	95.40	0.93	$C_{22}H_{44}$
Benzene, 1-ethyl-2-methyl-	95.20	0.58	C_9H_{12}
Benzene, 1,2,3-trimethyl-	95.10	0.61	C_9H_{12}
1-Docosene	95.00	0.63	$C_{22}H_{44}$
p-Xylene	93.90	0.19	C_8H_{10}
Bicyclo[4.2.0]octa-1,3,5-triene	93.30	0.43	C_8H_8
9-Octadecenamide, (Z)-	93.00	8.72	$C_{18}H_{35}NO$
Cetene	93.00	1.67	$C_{16}H_{32}$
Hentriacontane	92.70	7.48	$C_{31}H_{64}$
n-Hexadecanoic acid	92.70	1.71	$C_{16}H_{32}O_2$
Dotriacontane	92.40	0.48	$C_{32}H_{66}$
1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	92.00	0.07	$C_{20}H_{30}O_4$
1-Docosene	91.90	0.11	$C_{22}H_{44}$
Dodecanamide	91.40	4.09	$C_{12}H_{25}NO$
Nonane, 3-methyl-	91.30	0.47	$C_{10}H_{22}$
Heptacosane	91.10	0.37	$C_{27}H_{56}$
2-Pentanone, 4-hydroxy-4-methyl-	90.90	4.67	$C_6H_{12}O_2$

Appendix 8.3: List of compounds identified from *S. leptodictya* acetone extract

Name	Similarity	Area %	Formula
Phenol, 3-pentadecyl-	96.80	1.27	$C_{21}H_{36}O$
p-Xylene	95.70	0.13	C_8H_{10}
Benzene, 1-ethyl-4-methyl-	95.10	0.11	C_9H_{12}
Benzene, 1,2,3-trimethyl-	94.80	0.28	C_9H_{12}
Dodecanamide	94.50	2.91	$C_{12}H_{25}NO$
Benzene, 1-ethyl-3-methyl-	94.30	0.26	C_9H_{12}
1-Docosene	94.30	0.78	$C_{22}H_{44}$
Ethylbenzene	94.10	0.04	C_8H_{10}
Benzene, propyl-	94.10	0.06	C_9H_{12}
Tributyl acetylcitrate	93.80	0.07	$C_{20}H_{34}O_8$
Hentriacontane	93.80	0.40	$C_{31}H_{64}$
Diisooctyl phthalate	93.00	0.08	$C_{24}H_{38}O_4$
9-Octadecenamide, (Z)-	92.90	2.93	$C_{18}H_{35}NO$
n-Hexadecanoic acid	92.80	1.65	$C_{16}H_{32}O_2$
5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	92.80	0.11	$C_{13}H_{22}O$
Cetene	92.40	0.97	$C_{16}H_{32}$
Oleic Acid	92.30	0.66	$C_{18}H_{34}O_2$
1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	92.30	0.03	$C_{20}H_{30}O_4$
9-Octadecenoic acid (Z)-, methyl ester	92.00	0.08	$C_{19}H_{36}O_2$
3-Dodecene, (Z)-	92.00	0.14	$C_{12}H_{24}$
Dotriacontane	91.90	0.05	$C_{32}H_{66}$
3-Tetradecene, (E)-	91.70	0.49	$C_{14}H_{28}$
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	91.70	0.01	$C_{10}H_{14}$

9-Nonadecene	91.60	0.05	$C_{19}H_{38}$
Undecane	91.50	0.04	$C_{11}H_{24}$
Heptadecane	91.50	0.10	$C_{17}H_{36}$
Phytol	91.40	0.90	$C_{20}H_{40}O$
2-Pentanone, 4-hydroxy-4-methyl-	91.40	1.58	$C_6H_{12}O_2$
Tetradecanoic acid	91.30	0.36	$C_{14}H_{28}O_2$
Hexadecane	91.30	0.10	$C_{16}H_{34}$
cis-2-Methyl-7-octadecene	91.30	0.07	$C_{19}H_{38}$
Squalene	91.20	0.12	$C_{30}H_{50}$
3-((4Z,7Z)-Heptadeca-4,7-dien-1-yl)phenol	91.10	5.46	$C_{23}H_{36}O$
Phenol, 4-(1,1-dimethylpropyl)-	91.00	0.01	$C_{11}H_{16}O$
Benzene, 1-methyl-4-propyl-	91.00	0.03	$C_{10}H_{14}$
Benzene, 4-ethyl-1,2-dimethyl-	90.90	0.01	$C_{10}H_{14}$
9,12,15-Octadecatrienoic acid, methyl ester, Z,Z,Z)-	90.90	0.19	$C_{19}H_{32}O_2$
9,12-Octadecadienoic acid, methyl ester	90.80	0.07	$C_{19}H_{34}O_2$
Heptacosane	90.70	0.14	$C_{27}H_{56}$
Nonane, 3-methyl-	90.50	0.16	$C_{10}H_{22}$
Neophytadiene	90.40	0.18	$C_{20}H_{38}$
Decanedioic acid, dibutyl ester	90.20	2.91	$C_{18}H_{34}O_4$

Appendix 8.4: List of compounds identified from *S. pendulina* acetone extract

Name	Similarity	Area %	Formula
p-Xylene	96.70	0.10	C_8H_{10}
Phenol, 3-pentadecyl-	96.30	0.24	$C_{21}H_{36}O$
Benzene, propyl-	96.20	0.04	C_9H_{12}
Benzene, 1-ethyl-2-methyl-	94.90	0.19	C_9H_{12}
1-Docosene	94.80	0.48	$C_{22}H_{44}$
Benzene, 1,2,3-trimethyl-	94.80	0.19	C_9H_{12}
1-Docosene	94.50	0.64	$C_{22}H_{44}$
Cetene	94.10	0.57	$C_{16}H_{32}$
p-Xylene	93.80	0.04	C_8H_{10}
Hentriacontane	93.60	0.54	$C_{31}H_{64}$
Phenol, 3-pentadecyl-	93.40	0.18	$C_{21}H_{36}O$
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	93.20	0.45	$C_{19}H_{32}O_2$
5-Eicosene, (E)-	93.10	0.04	$C_{20}H_{40}$
Dotriacontane	93.00	0.73	$C_{32}H_{66}$
Heptacosane	93.00	0.40	$C_{27}H_{56}$
Benzene, 1-ethyl-3-methyl-	93.00	0.12	C_9H_{12}
9-Octadecenamide, (Z)-	92.80	2.08	$C_{18}H_{35}NO$
5-Eicosene, (E)-	92.60	0.73	$C_{20}H_{40}$
n-Hexadecanoic acid	92.40	3.89	$C_{16}H_{32}O_2$
Dotriacontane	92.30	0.48	$C_{32}H_{66}$
4-Dodecene, (E)-	92.10	0.09	$C_{12}H_{24}$
Dodecanamide	91.90	0.69	$C_{12}H_{25}NO$
Nonane, 3-methyl-	91.80	0.12	$C_{10}H_{22}$
Phytol	91.60	0.84	$C_{20}H_{40}O$
3-Tetradecene, (E)-	91.60	0.44	$C_{14}H_{28}$

2-Pentanone, 4-hydroxy-4-methyl-	91.40	3.07	$C_6H_{12}O_2$
Heptacosyl acetate	91.10	0.20	$C_{29}H_{58}O_2$
Tributyl acetylcitrate	91.10	0.05	$C_{20}H_{34}O_8$
Phenol, 4-(1,1-dimethylpropyl)-	91.10	0.02	$C_{11}H_{16}O$
1-Docosene	91.00	0.08	$C_{22}H_{44}$
3-((4Z,7Z)-Heptadeca-4,7-dien-1-yl) phenol	90.90	0.73	$C_{23}H_{36}O$
Tridecanoic acid, methyl ester	90.90	0.10	$C_{14}H_{28}O_2$
Benzene, 1-methyl-4-propyl-	90.80	0.02	$C_{10}H_{14}$
Diisooctyl phthalate	90.70	0.06	$C_{24}H_{38}O_4$
Benzene, 1-methyl-4-propyl-	90.70	0.01	$C_{10}H_{14}$
Neophytadiene	90.30	0.38	$C_{20}H_{38}$
Decane	90.30	0.25	$C_{10}H_{22}$
Tetradecanoic acid	90.30	0.18	$C_{14}H_{28}O_2$
Heptacosane	90.30	0.06	$C_{27}H_{56}$

Appendix 8.5: List of compounds identified from *B. bowkeri* acetone extract

Name	Similarity	Area %	Formula
Phenol, 3-pentadecyl-	96.60	0.26	$C_{21}H_{36}O$
Benzene, 1-ethyl-2-methyl-	95.20	0.29	C_9H_{12}
1-Docosene	94.80	0.64	$C_{22}H_{44}$
Benzene, 1,2,3-trimethyl-	94.80	0.29	C_9H_{12}
1-Docosene	94.60	0.79	$C_{22}H_{44}$
o-Xylene	94.60	0.06	C_8H_{10}
p-Xylene	94.40	0.15	C_8H_{10}
Cetene	93.80	1.04	$C_{16}H_{32}$
1-Docosene	93.80	0.17	$C_{22}H_{44}$
Heptacosane	93.70	0.16	$C_{27}H_{56}$
9-Octadecenamide, (Z)-	93.30	1.26	$C_{18}H_{35}NO$
Benzene, 1-ethyl-3-methyl-	93.20	0.16	C_9H_{12}
Cetene	92.90	1.03	$C_{16}H_{32}$
Benzene, propyl-	92.80	0.05	C_9H_{12}
n-Hexadecanoic acid	92.60	2.16	$C_{16}H_{32}O_2$
Phytol	92.20	0.65	$C_{20}H_{40}O$
Diisooctyl phthalate	92.10	0.13	$C_{24}H_{38}O_4$
Benzene, 1,2,3-trimethyl-	91.80	0.06	C_9H_{12}
Squalene	91.70	3.75	$C_{30}H_{50}$
Bicyclo[4.2.0]octa-1,3,5-triene	91.60	0.32	C_8H_8
2-Tridecene, (E)-	91.50	0.42	$C_{13}H_{26}$
Heptacosane	91.40	0.30	$C_{27}H_{56}$
2-Pentanone, 4-hydroxy-4-methyl-	91.30	1.70	$C_6H_{12}O_2$

Octacosanol	91.20	0.28	$C_{28}H_{58}O$
Dodecanamide	90.50	2.23	$C_{12}H_{25}NO$
Hexadecane	90.50	0.12	$C_{16}H_{34}$
3-((4Z,7Z)-Heptadeca-4,7-dien-1-yl) phenol	90.40	0.49	$C_{23}H_{36}O$
Tetradecanoic acid	90.40	0.30	$C_{14}H_{28}O_2$
Nonane, 3-methyl-	90.40	0.17	$C_{10}H_{22}$
Heptane, 3-ethyl-2-methyl-	90.20	0.17	$C_{10}H_{22}$

Appendix 8.6: List of compounds identified from *B. galpinii* acetone extract

Name	Similarity	Area %	Formula
1-Docosene	95.70	0.89	$C_{22}H_{44}$
p-Xylene	95.10	0.18	C_8H_{10}
Benzene, 1,2,3-trimethyl-	95.00	0.46	C_9H_{12}
Dodecanamide	94.50	2.38	$C_{12}H_{25}NO$
Cetene	93.80	1.13	$C_{16}H_{32}$
9-Octadecenamide, (Z)-	93.50	2.08	$C_{18}H_{35}NO$
Bicyclo[4.2.0]octa-1,3,5-triene	93.500	0.46	C_8H_8
Heptacosane	93.20	0.26	$C_{27}H_{56}$
n-Hexadecanoic acid	92.70	2.70	$C_{16}H_{32}O_2$
Benzene, 1-methyl-3-propyl-	92.40	0.03	$C_{10}H_{14}$
Phenol, 3-pentadecyl-	92.40	0.13	$C_{21}H_{36}O$
Phytol	92.00	1.42	$C_{20}H_{40}O$
Dotriacontane	91.80	0.23	$C_{32}H_{66}$
Squalene	91.80	0.95	$C_{30}H_{50}$
2-Pentanone, 4-hydroxy-4-methyl-	91.50	2.20	$C_6H_{12}O_2$
2-Tridecene, (E)-	91.50	0.57	$C_{13}H_{26}$
4-Dodecene, (E)-	91.50	0.18	$C_{12}H_{24}$
Nonane, 3-methyl-	91.50	0.28	$C_{10}H_{22}$
Dibutyl phthalate	91.30	0.08	$C_{16}H_{22}O_4$
Benzeneacetaldehyde, à-methyl-	91.20	0.03	$C_9H_{10}O$
Octacosanol	91.10	0.55	$C_{28}H_{58}O$
Oleic Acid	90.80	0.81	$C_{18}H_{34}O_2$
Benzene, (1-methylethyl)-	90.60	0.02	C_9H_{12}

Diisooctyl phthalate	90.20	0.14	$C_{24}H_{38}O_4$
Tributyl acetylcitrate	90.20	0.05	$C_{20}H_{34}O_8$
Tetradecanoic acid	90.00	0.34	$C_{14}H_{28}O_2$

Appendix 9: Antibiotic susceptibility testing of isolated *Escherichia coli*

Bacteria	Antibiotics									
	AMP	**C**	**TE**	**AMC**	**AK**	**SXT**	**CN**	**DO**	**EFT**	**CT**
E. coli 1	NZ	9.80	NZ	NZ	18.54	29.20	25.54	7.60	ND	19.42
E. coli 2	NZ	23.70	16.60	8.38	9.98	25.70	20.50	20.50	21.70	12.10
E. coli 3	NZ	24.50	21.78	13.98	16.12	31.00	23.90	27.20	23.00	15.00
E. coli 4	NZ	25.96	21.96	13.90	18.98	29.20	17.60	23.38	21.19	11.58
E. coli 5	NZ	24.46	NZ	12.76	15.76	31.58	23.52	13.98	ND	19.04
E. coli 6	NZ	23.48	21.00	NZ	19.44	34.22	27.90	ND	ND	19.28
E. coli 7	10.10	26.38	NZ	14.40	13.48	35.30	27.10	ND	ND	19.10
E. coli 8	23.96	31.56	31.60	27.06	25.36	26.68	16.58	21.09	19.60	9.30
E. coli 9	NZ	25.80	35.14	34.48	15.20	34.62	NZ	38.60	30.98	NZ
E. coli 10	NZ	25.90	21.38	14.82	15.52	33.34	25.58	ND	ND	17.40

AMP= Ampicillin, C= Chloramphenicol, TE= Tetracycline, AMC= Amoxycillin/Clavulanic acid, AK= Amikacin, SXT= Sulphamethoxazole, CN= Gentamicin, DO= Doxycycline, EFT= Ceftiofur, CT= Colistin Sulphate. NZ= no zone of inhibition, ND= not determined