An antimicrobial and phytochemical validation of southern African plants used as soap substitutes and formulation of herbal soap

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DECLARATION

I, Nyiko Fortunate Mzimba, declare that this dissertation is my own work. It is being submitted for the Master of Pharmacy degree at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any other degree or examination at this or any other University.

14/06/23

N.F Mzimba **Date**

DEDICATION

This dissertation is dedicated to my loving parents Vivian and Solani Mzimba and brothers Rifumo and Mahlori Mzimba. Thank you for your support and encouragement.

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ABSTRACT

A major proportion of the population in southern Africa relies on medicinal plants commonly known as soapy plants for bathing and washing. There is limited scientific research that assess the effectiveness of southern African soap plants. Therefore, this study investigated the phytochemistry, antimicrobial activity, and toxicity of plants used in southern Africa as soap substitutes. Thereafter, an effective antimicrobial herbal soap was formulated and assessed for its antimicrobial efficacy.

A comprehensive literature review was conducted to gather information on plants used as soap substitutes in southern Africa. A total of 59 plant species were identified to be used for bathing and washing. A total of 26 plant species were collected based on availability at Walter Sisulu Botanical Garden, Random Harvest Indigenous Nursery and University of Johannesburg herbarium and University of the Witwatersrand storage. The organic and aqueous extracts were prepared and screened for the presence of alkaloids, terpenoids, and saponins. Methanol and acetone were the optimal solvents to extract alkaloids from 62.07% of plant extracts. Terpenoids were best extracted with ethanol (75.86% of plant extracts), followed by methanol (68.97% of plant extracts). Saponins were highly detectable using water (93.10% of plant extracts) and ethanol (82.76% of extracts). The qualitative evaluation of saponins using thin layer chromatography displayed a variety of saponins, including steroidal saponins that had Rf-values comparable to diosgenin (a steroidal aglycone used as a standard). *Sideroxylon inerme* subsp. *inerme* (16.3%) had a high percentage yield of saponins. *Hermannia cuneifolia* displayed the highest saponin content (262.41 ± 1.90 mg/g), followed by *Sideroxylon inerme* subsp. *inerme* (71.34 ± 1.01 mg/ml), *Acalypha glabrata* (70.48 ± 2.05 mg/g), and *Noltea africana* (68.53 ± 2.43 mg/g).

The organic and aqueous extracts of each of the selected soap plants were tested for their antimicrobial activity against skin-relevant pathogens. *Pelargonium peltatum* demonstrated the best antimicrobial activity against *Brevibacterium linens* and *Cutibacterium acnes* with an MIC value of 0.06 mg/ml. *Calodendrum capense* (leaves), *Noltea africana* (leaves), *Olea europaea* (leaves), *Pelargonium peltatum* (leaves), *Plectranthus ciliatus* (leaves), *Ptaeroxylon obliquum*

(bark), and *S. inerme* subsp. *inerme* (leaves) organic extracts displayed noteworthy antimicrobial activity against the pathogen *C. acnes* with an MIC value of 0.06 mg/ml. The plants that demonstrated notable broad-spectrum activity against most of the tested pathogens were *Calodendrum capense* (leaves), *Pelargonium peltatum*, *Plectranthus ciliatus*, and *Ptaeroxylon obliquum* (bark).

The toxic profiles of the organic and aqueous extracts were evaluated to assess the safety of the plant species using brine-shrimp lethality assay (BSLA). Aqueous plant extracts were more toxic (65.52%) compared to organic plant extracts (62.07%). *Acalypha glabrata* (leaves), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Deinbollia oblongifolia* (leaves), *Ledebouria luteola* (bulb), *Pouzolzia mixta* (leaves), and *Sideroxylon inerme* subsp. *inerme* (leaves) organic and aqueous extracts demonstrated the lowest toxic effects at 24 and 48 h. *Aristaloe aristata* (leaves), *Calodendrum capense* (leaves) and *P. obliquum* (bark) organic extracts were non-toxic, and *Hermannia cuneifolia* (leaves), *Plectranthus ciliatus* (leaves), and *Ptaeroxylon obliquum* (leaves) aqueous extracts were non-toxic. *Crinum bulbispermum* (bulb), *Haemanthus albiflos* (bulb) and *Ilex mitis* (leaves) were highly toxic, with LC_{50} values > 250 µg/ml after 48 h. *Pelargonium peltatum* displayed low toxicity at a concentration of 125 µg/ml.

The extracts of *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* were then used for soap-making by the basic saponification reaction, and the physicochemical parameters and antimicrobial activity of the soaps were evaluated. The *Calodendrum capense* herbal soap had the lowest pH (10.79), moisture content (28%), and free caustic alkali (0.03%). *Pelargonium peltatum* and *Calodendrum capense* herbal soaps were categorized as first-grade soaps (84 and 80%, respectively). The antimicrobial efficacy of the soaps was determined by inoculating selected skin micro-organisms on agar containing soap formulations using the multipoint inoculator. Gram-positive micro-organisms were inhibited (MIC values of ≤ 1.57 mg/ml). All the tested micro-organisms except for *Enterobacter cloacae* were inhibited at a concentration of 12.5 mg/ml, which is comparable to the control, Protex® commercial soap. The findings herein of the antimicrobial properties, phytochemistry, and toxicity contribute to the knowledge gaps that exist in the ethnobotanical literature of some southern African soap plants and provide evidence for their incorporation into soap formulation.

PRESENTATIONS ARISING FROM THIS STUDY

NF Mzimba, A Moteetee, S van Vuuren. Antimicrobial and phytochemical validation of southern African plants used as soap substitutes. South Africa. 24th Indigenous Plant forum 2022. 4-7 July 2022. University of Johannesburg. [online presentation, abstract Appendix A1].

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ATCC	American-type culture collection
BSLA	Brine-shrimp lethality assay
CC_{50}	The 50% cytotoxic concentration
CI	Confidence intervals
COVID-19	Coronavirus disease
CV	Crystal violet
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EICA	Encyclopaedia of industrial chemical analysis
FCA	Free caustic alkali
HPLC	High-performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
ISO	International standard organisation
JRAU	University of Johannesburg herbarium
LC_{50}	Median lethal concentration
MDCK	Madin-Darby canine kidney cells
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NCSS	Number cruncher statistical systems
pH	Potential hydrogen
Rf	Retention factor
RHIN	Random harvest indigenous nursery
TFM	Total fatty matter
TLC	Thin layer chromatography
TSA	Tryptone soya agar
TSB	Tryptone soya broth

TSC	Total saponin content
UPLC	Ultra-pressure liquid chromatography
UV	Ultraviolet
UV/VIS	Ultraviolet-visible
WHO	World Health Organisation
WSBG	Walter Sisulu Botanical Gardens

CHAPTER 1
GENERAL INTRODUCTION

1.1 Overview of the human skin

1.1.1 Skin microbiota

The largest organ in the human body is the skin which regulates temperature and maintains fluids through eccrine glands (Grice and Segre, 2011). The skin contains sebaceous glands which secrete lipid-rich substances called sebum which assists in preventing the desiccation of the skin and in sealing moisture within the skin. The sebaceous glands also contribute to the production of most lipidic compounds on the skin surface through the sebum's assistance (Hoover *et al.*, 2022). The sebum moderately regulates the skin pH since the skin pH is dependent on the skin moisture, i.e., high moisture areas such as the axilla have a higher pH (Schmid-Wendtner and Korting, 2006). Therefore, the characteristics of sebum contribute to creating an ecosystem which accommodates symbiotic bacteria as well as viruses, fungi, and mites on the skin (Boxberger *et al.*, 2021). A collection of these symbiotic micro-organisms colonises the skin to form the skin microbiota that protects the skin against pathogenic micro-organisms. The T-cells on the skin and immune cells interact with these symbiotic micro-organisms, then respond to pathogenic micro-organisms that cause skin infections. They reinforce and repair the external barrier formed by the skin (Grice and Segre, 2011; Eisenstein, 2020). According to Byrd *et al.* (2018), the human skin is predominantly colonised by Gram-positive bacteria such as *Corynebacterium* spp., *Cutibacterium* spp., *Enhydrobacter* spp., *Micrococcus* spp. and *Veillonella* spp. Although Gram-positive bacteria make up a large proportion of the skin microbiota, there are also other Gram-negative commensal bacteria included (Boxberger *et al.*, 2021). These include *Acinetobacter* spp., *Enterobacterium* spp., *Moraxella osloensis*, *Pantoea septica*, *Roseomonas mucosa*, and *Pseudomonas* spp. (Myles *et al.*, 2016). Other commensal micro-organisms include *Cryptococcus* spp., *Debaryomyces* spp., *Demodex* mites, and *Malassezia* spp. (Grice and Segre, 2011).

The colonization of the skin by a symbiotic bacterial community is primarily dependent on the physiology of the skin site. The abundance of microbial communities is associated with dry, moist,

or sebaceous environments (Grice and Segre, 2011; Byrd *et al.*, 2018). *Corynebacterium* spp., *Cutibacterium* spp., and *Staphylococcus aureus* colonize the moist sites of the human skin. These bacteria colonize bodily locations with hair follicles such as the axilla (underarm) and external genitalia, as well as other sites such as the bends of elbows and feet (Byrd *et al.*, 2018; Rudden *et al.*, 2020). Grice and Segre (2011) recorded that there is low diversity of bacteria in sebaceous sites (behind the ear, inside the nostrils, face, back, and chest), which shows what conditions are not favourable for most bacteria. However, lipophilic *Cutibacterium acnes* is dominant on these sites because *C. acnes* degrades the lipids of sebum, releasing free fatty acids that aid in the colonisation of the skin surface at a pH of 5 (Grice and Segre, 2011). In contrast to the bacterial community, the fungal community does not depend on physiological skin sites for colonization. *Malassezia* spp. colonizes the armpits, while a combination of *Aspergillus* spp., *Cryptococcus* spp., *Epicoccum* spp., and *Rhodotorula* spp. colonises the core body (Hrestak *et al.*, 2022).

1.1.2 Pathogenesis of micro-organisms that usually form part of the skin microbiome

Although micro-organisms found in the skin microbiota are normally commensal, at times they can cause skin infections and/or disorders reflecting the pathogenicity of the relative micro-organisms. Skin infections or disorders often occur when micro-organisms from the skin microbiome invade other sites that they do not normally colonize. Furthermore, skin infections occur when they manage to overcome the normal host defence mechanism (Chiller *et al.*, 2001). When these micro-organisms invade other sites, the balance of the microbiota is disturbed, thus, resulting in the development of skin infections such as boils, erythrasma, folliculitis, and impetigo (Aly, 1996). On healthy human skin, pathogens such as coryneform bacteria, *Staphylococcus aureus*, and *Streptococcus pyogenes* are inhibited by an acidic pH on the skin surface, encouraging the growth of *Corynebacteria*. However, during a time when the skin is occluded, the pH is elevated, thus, the growth of pathogenic micro-organisms is favoured (Grice and Segre, 2011). Table 1.1 shows the micro-organisms that are part of the microbiome and also have potential pathogenesis on the skin. Pathogens that are not usually found on the skin can nonetheless cause skin infections and an unpleasant body odour.

Table 1.1: The roles of selected pathogens in contaminating the skin, causing skin infection and bad body odour.

Pathogen	Classification	Role	Pathogenesis
Acinetobacter baumannii [a]	Gram-negative	Skin contaminant (Meneghetti *et al.*, 2018).	It causes a severe type of cellulitis and other skin and soft tissue infections (Ali *et al.*, 2014).
Brevibacterium agri	Gram-positive	Commensal micro-organisms on the human skin (Van Vuuren *et al.*, 2019).	Digests dead skin and causes foot odour (methane thiol). They are also the leading cause of bromodosis (Van Vuuren *et al.*, 2019).
Brevibacterium epidermidis			
Brevibacterium linens			
Candida albicans	Yeast	Resides on the skin surface as a commensal (Kühbacher *et al.*, 2017).	It causes cutaneous candidiasis, skin thickening, erythema, diaper rash, and hyperkeratosis (Kühbacher *et al.*, 2017; Palese *et al.*, 2018).
Corynebacterium minutissimum	Gram-positive	Found on the uppermost layers of the epidermis (Forouzan and Cohen, 2020).	It causes erythrasma or interdigital erythrasma. Found on the toe webs, co-infecting with *C. albicans* and/or *Trichophyton* spp. (Forouzan and Cohen, 2020).
Corynebacterium xerosis	Gram-positive	A commensal micro-organism that is normally present on the skin and nasopharynx (Chiller *et al.*, 2001).	It causes skin infections when associated with other *Corynebacterium* species (Hernández-León *et al.*, 2016).

Pathogen	Classification	Role	Pathogenesis
Cutibacterium acnes	Gram-positive	Part of the normal skin microbiome (Findley and Grice, 2014).	It is part of the normal flora of the skin that forms biofilms within the body, causing intense body odour (Elston *et al*., 2019). It plays a role in the development of Acne vulgaris in teenagers (Mayslich *et al*., 2021).
Cutibacterium avidum	Gram-positive	A skin commensal micro-organism is found in regions containing eccrine sweat glands (Corvec, 2018).	It is associated with intense malodour (Lam *et al.*, 2018) and acts as either superficial or invasive infections such as breast infections and skin abscesses (Corvec, 2018).
Enterobacter cloacae	Gram-negative	An opportunistic skin pathogen and a skin contaminant (Meneghetti *et al.*, 2018).	It causes wound infections and is also a secondary causative agent of bacterial folliculitis (Ni Riain, 2013; Buckle, 2015; Meneghetti *et al.*, 2018).
Escherichia coli	Gram-negative	Skin contaminant (Afsar and Khanam, 2016).	It causes cellulitis localized to the lower or upper (Petkovšek *et al.*, 2009).
Klebsiella pneumoniae	Gram-negative	Skin contaminant (Meneghetti *et al.*, 2018).	It gains access to the skin through breaks in the skin leading to bacterial infection (Chang *et al.*, 2008).
Malassezia species	Yeast	It is a fungus found on many	It is an infectious agent causing atopic dermatitis tinea

Pathogen	Classification	Role	Pathogenesis
		human skin sites (Findley and Grice, 2014).	versicolor, dandruff, and to a lesser extent psoriasis (Findley and Grice, 2014).
Pseudomonas aeruginosa	Gram-negative	Skin contaminant (Afsar and Khanam, 2016).	It infects damaged skin, such as burns, wounds, or sores, and causes bacterial folliculitis and toe web infections (Findley and Grice, 2014; Afsar and Khanam, 2016).
Staphylococcus aureus	Gram-positive	It colonizes the moist site of the human skin (Byrd *et al.*, 2018).	It is the most common skin pathogen that causes superficial and deep dermal infections. (Van Vuuren *et al*., 2014). It also causes infections such as impetigo, folliculitis, and cellulitis (Ni Riain, 2013).
Staphylococcus capitis	Gram-positive	It is a commensal micro-organism on the skin of the human scalp, forehead, and ears (Kamalakannan *et al.*, 2004).	It has been implicated in biofilm-related infections. Additionally, it causes skin and soft tissue infections such as folliculitis and the formation of cysts (Natsis and Cohen, 2018).
Staphylococcus epidermidis	Gram-positive	It is part of the normal flora of the skin (Meneghetti *et al.*, 2018).	It degrades leucine which is present in perspiration and responsible for an unpleasant odour (Kanda *et al.*, 1990). Cause skin and soft tissue infections such as abscesses,

Pathogen	Classification	Role	Pathogenesis
			folliculitis, cellulitis, formation of boils, and cysts (Natsis and Cohen, 2018).
Staphylococcus haemolyticus	Gram-positive	It is part of the normal flora of the skin (Meneghetti *et al*., 2018).	It causes skin and soft tissue infections called felon, which is an infection that occurs on the pulp of the fingertips (Natsis and Cohen, 2018).
Staphylococcus hominis	Gram-positive	It colonises the skin for several weeks or months, found under the arms (Lam *et al.*, 2018).	It is associated with malodour. It produces thioalcohol compounds that contribute to unpleasant body odours (Lam *et al.*, 2018).
Staphylococcus lugdunensis	Gram-positive	It is part of the normal flora of the skin (Meneghetti *et al*., 2018).	It causes skin and soft tissue infections such as paronychia, felon, formation of cysts, abscesses, and boils (Natsis and Cohen, 2018).
Streptococcus pyogenes	Gram-positive	It is part of the normal skin microbiome (Grice and Segre, 2011).	It causes skin infections such as impetigo, cellulitis, and erysipelas (Grice and Segre, 2011).
Trichophyton mentagrophytes	N/A	It is part of the normal skin microbiome (Grice and Segre, 2011).	It is responsible for infections affecting the body, face, and feet such as ringworm, onychomycosis, and tinea

Pathogen	Classification	Role	Pathogenesis
			pedis (Findley and Grice, 2014).

Key – [a] micro-organisms in bold are micro-organisms selected for the study.

1.1.3 Bacterial skin infection:

1.1.3.1 Impetigo

Impetigo is a superficial epidermal infection which is common in children and caused by Gram-positive bacteria *Staphylococcus aureus* or *Streptococcus pyogenes* (Chiller *et al.*, 2001). There is primary impetigo, which occurs on healthy skin (skin without infection), and secondary impetigo, which occurs on skin that previously had an underlying infection or an injury (Motswaledi, 2011). It is highly contagious; however, practising personal hygiene by washing the injuries and sores of infected patients with water and soap can limit the spread to healthy people. It is classified as one of the skin conditions that can lead to life-threatening complications (Abrha *et al.*, 2020). According to Abrha *et al.* (2020), approximately 2% of the world's population gets affected by impetigo. This includes isolated communities in first-world and third-world countries or unprivileged tropical countries. This is due to poor hygiene practices and the lack of adequate housing (Abrha *et al.*, 2020).

1.1.3.2 Folliculitis

Folliculitis is a skin condition that causes the skin to become abnormally red due to inflammation of the hair follicles. The inflammation is caused by bacterial and fungal species (Winters and Mitchell, 2022). *Staphylococcus aureus* commonly causes superficial bacterial folliculitis, which occurs on the upper portion of the hair follicle. This infection is frequently found in the axillae, beard, and buttocks. The bacterial infection can extend deeper into the hair follicle, resulting in furuncles, commonly called boils. This leads to swelling and perifollicular inflammation. Gram-negative bacterial folliculitis is commonly associated with pseudomonal infections from

contaminated water. It is usually caused by *P. aeruginosa*; however, it can also be caused by *Enterobacter* and *Klebsiella* spp. (Ni Riain, 2013). A form of folliculitis that is caused by fungal (yeast) species is called Pityrosporum folliculitis. *Malassezia* species are the most common causative agents. It usually occurs in adolescent patients since there is increased activity in the sebaceous glands (Suzuki *et al.*, 2016). According to Winters and Mitchell (2022), one of the recommended ways to manage folliculitis is to maintain good skin hygiene by bathing with antimicrobial soap and clean water. This is also important as it prevents the reoccurrence of infection.

1.1.3.3 Cellulitis and erysipelas

Cellulitis is an acute inflammatory skin infection that naturally spreads on the subcutaneous tissue and lower dermis. The primary causative agents are *S. aureus* and Streptococci (*S. pyogenes*) which enter the dermis through a break in the skin such as wounds, injuries, bites, and burns (Maitre, 2006; Phoenix *et al.*, 2012). A rash develops on the skin (most likely on the leg), resulting in swelling around the affected area. If not treated with antibiotics, the rash can expand, and the infection will get worse (Han *et al.*, 2020). Unlike cellulitis which affects the deeper skin tissues, erysipelas is the more superficial form of cellulitis that occurs on the upper layers of the skin. It can be distinguished from cellulitis by the well-developed margins and bright red colour of the affected area. Similar to cellulitis, this superficial bacterial infection occurs due to a crack in the skin as a result of an injury, a bite, a wound, or a burn (Michael and Shaukat, 2022). According to Henry *et al.* (2004), cellulitis and erysipelas can be prevented by practising good personal hygiene and using antiseptics or antimicrobial soap adequately when there are breaks on the skin.

1.1.4 Fungal (yeast) skin infections

The skin microbiota includes fungi such as *Candida*, *Cryptococcus*, *Malassezia*, and *Rhodotorula* species; however, some of these species are pathogenic. According to Havlickova *et al.* (2008), an estimated 20-25% of the world's population is affected by fungal skin infections. *Candida albicans* is often responsible for symptomatic skin infections among the *Candida* species, and the common symptoms include redness of the skin and increased thickness of the outer layer of the skin. Skin

infections caused by *Candida* species occur in closed regions where there is constant friction, accumulation of carbon dioxide, and humidity (Kühbacher *et al.*, 2017). Fungal skin pathogens are classified into two classes, namely, dermatophytes and yeast infections. Dermatophytes cause a group of skin infections broadly referred to as dermatomycoses. This group of fungal infections is caused by the *Trichophyton* species. One of the infections is tinea corporis which is commonly known as ringworm. It is characterised by circular lesions with clear skin in the centre, scaling, and redness of the skin. It usually occurs on the legs, scalp, neck, and arms (O'Dell, 1998; Yee and Al Aboud, 2022). Candidal intertrigo is one of the skin infections caused by *Candida* species. It is an inflammatory skin condition that develops on the skin due to reduced air circulation, increased friction, and humidity. It is characterised by the development of satellite lesions, crusts, exudative erosions, and fissures after presenting itself as mild erythematous papillae (Kalra *et al.*, 2014). The inflammation of the skin is usually caused by treating the skin with harsh ointments, harsh antimicrobial soaps, or topical steroids. Preventative measures that can be taken to avoid recurrent infections include maintaining good hygiene by keeping the infected areas clean, dry, and cool (Metin *et al.*, 2018).

1.2 Skin hygiene

Good hygiene is a vital principle that must be exercised to prevent skin infections and malodour (Afsar and Khanam, 2016). Odourless precursors of sweat, such as fatty acids, glycerol, and lactic acid are naturally secreted on the skin's surface by apocrine glands, then decomposed by bacteria into human body odour (Lam *et al.*, 2018; Rudden *et al.*, 2020). Body odour is normal; however, can lead to discomfort and negative outcomes. Good skin hygiene can be achieved by bathing with commercial soaps (Felhaber and Mayeng, 1997). Soap is a chemical mixture of potassium or sodium salt with fatty acids that results in a saponification reaction (Mak-Mensah and Firempong, 2011; Nchimbi, 2020).

One of the principles for maintaining good hygiene is handwashing because hands are a critical vector that transmits micro-organisms faster between objects and/or individuals. Cross-transmission of pathogens happens when hands are not washed effectively or often enough (Edmonds-Wilson *et al.*, 2015). Recently, there has been a global effort to prevent the spread of

COVID-19, and one of the measures that have been strongly recommended is handwashing with soap and water for 20 seconds. The reason is that soap dissolves the lipid bilayer of the self-assembled virus, causing it to disassemble (Türsen *et al.*, 2020). Other alternatives such as alcohol-based hand sanitisers are used when soap and water are not available (Alzyood *et al.*, 2020). The COVID-19 pandemic revealed that it is important to maintain personal hygiene; however, according to the World Health Organisation (WHO), approximately 43% of the world's population does not have access to water and soap (Unicef, 2020).

The frequent use of antiseptic soaps can damage the skin by changing the composition of the skin flora (Weaver, 2005). This allows Staphylococci and other pathogenic Gram-negative bacteria to colonise the skin and cause skin conditions such as cellulitis, erysipelas, and impetigo (Stulberg *et al.*, 2002). Furthermore, the overuse of alcohol-based sanitisers results in antimicrobial resistance as micro-organisms tend to mutate through the natural process due to repeated exposure to disinfectants (Mahmood *et al.*, 2020). A study by Hayat and Munnawar (2016) reported that almost all Gram-negative bacteria are resistant to commercial hand sanitisers. Furthermore, 48% of *Escherichia coli* and 64% of *Pseudomonas aeruginosa* were resistant to all the sanitisers on the Pakistani market (Hayat and Munnawar, 2016). Therefore, a more naturally based approach should be considered.

Plants have been used across the globe as soap substitutes by indigenous people for centuries. In southern Africa most of the population uses medicinal plants for bathing and washing. However, their use has only gained importance in recent years as the cornerstone of non-pharmaceutical interventions in combatting skin infections and COVID-19 (Sindhu *et al.*, 2019; Kunatsa and Katerere, 2021). According to Solanki (2011), herbal soaps have about 60-80% antimicrobial activity; thus, they can inhibit micro-organisms from causing skin infections. Given that soap plants are natural, utilising them might be a promising route for achieving good health with fewer side effects and they are also more cost-friendly (Sindhu *et al.*, 2019). However, the toxicity of some of the soap plants must be evaluated to ensure that the plants are safe to use (Mohlakoana, 2020).

1.3 Alternative: plants used as soap substitutes in southern Africa

In the past, indigenous people used plants as soap substitutes because when agitated in an aqueous solution, there is a formation of natural lather and foam (Levey, 1954; Akuaden *et al.*, 2019; Mohlakoana, 2020). According to Akuaden *et al.* (2019), lather and foam are formed due to a group of naturally occurring plant steroids or triterpenoid glycosides called saponins. Saponins are characterised by their strong foam-forming properties in water; hence, plants containing substantial amounts of saponins have rich lather and are regarded as a natural soap base (Man *et al.*, 2010). They are also recognised for their ability to rupture erythrocytes (Kunatsa and Katerere, 2021). Apart from saponins, lather-forming plants are rich in phytochemicals such as alkaloids, tannins, flavonoids, terpenoids, and phenolic compounds which give the plants the ability to exert antimicrobial activity against skin pathogens. Furthermore, they have medicinal properties such as relieving pain and promoting wound healing (Van Wyk, 2008; Hulley and Van Wyk, 2019). Most soapy plants originate from the genus *Saponaria* L. since they are saponin-rich. Families such as Agavaceae, Dioscoreaceae, and Liliaceae (monocotyledons) are major sources of steroidal saponins, while families such as Fabaceae, Araliaceae, and Caryophyllaceae (dicotyledons) are major sources of triterpenoid saponins (Oleszek and Hamed, 2010; Rai *et al.*, 2021). According to Kunatsa and Katerere (2021), families such as Aceraceae, Aloaceae, Fabaceae, Hippocastanaceae, Lamiaceae, Malvaceae, Pedaliaceae, Rhamnaceae, Sapindaceae, and Tiliaceae have saponin-rich species that are used in Southern Africa as soap substitutes.

Herbal soap substitutes have been used globally for centuries. An example of such a plant is *Yucca schidigera* Roezl ex Ortgies used by Native Americans as a soap substitute and is currently used in a commercial soap (Oleszek and Hamed, 2010). The roots and rhizomes of *Saponaria officinalis* L. commonly known as soapwort, are also used as a soap substitute or as a detergent in Europe and Asia due to their cleaning abilities (Kregiel *et al.*, 2017). Besides being a natural emulsifier and detergent, soapwort also has high biological activities (Jurek *et al.*, 2019). Other soap plants with substantial amounts of saponins used internationally include *Chlorogalum pomeridianum* (DC.) Kunth, *Sapindus saponaria* L., *Polygala* L. and *Primula* L. spp. (Kregiel *et al.*, 2017). Soap plants are also popular in Africa. In Angola, before the production of commercial soaps was introduced, plants were used as soap to cleanse the body (Bossard, 1993). In Nigeria, an open

market manufactures soap locally by mixing several plants to make lye. Some of the plants include *Ageratum conyzoides* Linn., *Aloe vera* (L.) Burm.f. and *Theobroma cacao* L. These soaps are used to treat wounds, ringworm, and body odours (Moody *et al.*, 2004; Igbeneghu, 2013). In southern Africa, plants used as soap include *Aloe maculata* All., *Artemisia afra* Jacq. ex Willd and *Ilex mitis* (L.) Radlk. var. *mitis*. A list of southern African plants used as soap substitutes can be found in Table 1.2.

1.4 Chemistry and toxicity of plants used as soap substitutes

The two distinctive moieties of saponins after hydrolysis are glycan and aglycone. Glycan is water-soluble and made up of sugar molecules, namely hexoses and pentoses. Aglycone has two types of carbohydrate moieties, namely triterpenoidal and steroidal aglycones which bring about diversity in the structure of saponins (Desai *et al.*, 2009; Kunatsa and Katerere, 2021). Aglycone has a hydrophobic backbone that makes it non-soluble in water; thus, the hydrophilic glycan and hydrophobic aglycone give saponins their emulsifying and lathering properties. Saponins can be classified based on the carbon skeleton (–H, –COOH, or –CH3) on the aglycone as either triterpenes, steroids, or steroidal alkaloids (Oleszek and Hamed, 2010; Sandeep, 2020). Triterpenoid saponins are widely distributed in the plant kingdom, hence they are further subcategorized into dammarane, oleanane and ursolic acid saponins (Böttcher and Drusch, 2017). Steroidal saponins are subcategorized into furostanol and spirostanol. Alkaloidal saponins have a steroid-like structure; however, instead of having a pyranose ring in its structure, it has a piperidine. They usually occur in plant families such as Solanaceae (El Aziz *et al.*, 2019; Rai *et al.*, 2021). Besides saponins, there are also other secondary metabolites found in soap plants, namely alkaloids and terpenoids. In the past, alkalis used in soap-making were obtained from plants or ash (Levey, 1954). Such plants produce a soap-like substance due to the presence of alkaloids; however, most of them have low amounts of saponins (Mohlakoana, 2020). *Psilocaulon absimile* N.E.Br and *Sceletium tortuosum* (L.) N.E.Br. are some of the plants with low saponin content; nonetheless, they have soap-like sap due to alkaloids (Watt and Breyer-Brandwijk, 1962).

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Table 1.2: Plants used traditionally in southern Africa as soap substitutes.

Medicinal plants	Common name	Family	Use	Part used	Reference
***Acalypha glabrata* Thunb.**[a]	Umthombothi (X), forest false nettle (Eng.)	Euphorbiaceae	Used to clean the skin	Bark	(Afolayan *et al.*, 2014)
Agathosma capensis (L.) Dümmer	Spicy buchu (Eng.)	Rutaceae	Used to wash the body or as lotion	Not specified	(Hulley and Van Wyk, 2019)
***Albizia versicolor* Welw. Ex Oliv.**	Muvhambangoma (V), large-leaved albizia (Eng.)	Fabaceae	Used as a soap substitute	Bark, root, and leaves	(Magwede *et al.*, 2019)
***Aloe ferox* Mill.**	Inhlaba (Z), bitter aloe (Eng.)	Asphodelaceae	Used for cleansing the skin	The juice gel and the leaves	(Afolayan *et al.*, 2014)
***Aloe maculata* All.**	Soap aloe (Eng.), lekhala (S)	Asphodelaceae	Used as a soap substitute	Leaves	(Felhaber and Mayeng, 1997; Ngalo, 2010)
***Aristaloe aristata* (Haw.) Boatwr. & J.C. Manning**	Umathithibala (X), lace aloe (Eng.)	Asphodelaceae	The juice from the leaves is mixed with water to wash the body	Leaves	(Watt and Breyer-Brandwijk, 1962)
***Artemisia afra* Jacq. ex Willd**	Umhlonyane (X), African wormwood (Eng.)	Asteraceae	Used as a body wash	Leaves	(Hutchings *et al*., 1996; Rabe and Van Staden, 1997)
***Bauhinia bowkeri* Harv.**	Umdlandlovu (X), kei white bauhinia (Eng.)	Fabaceae	Used for steaming and bathing	Leaves and bark	(Ndawonde *et al*., 2007)

Medicinal plants	Common name	Family	Use	Part used	Reference
Caesalpinia decapetala (Roth) Alston	Luanakha (V), Mauritius-thorn (Eng.)	Fabaceae	Used as a soap substitute	Fruit sap or seeds	(Magwede *et al.*, 2019)
***Calodendrum capense* (L.f.) Thunb.**	Umemezi omhlophe (Z), cape chestnut (Eng.)	Rutaceae	Used to clean the skin; used in soap; externally used to lighten skin, as a moisturizer, and to treat pimples.	Fruits or seeds and Bark	(Philander, 2011; Afolayan *et al.*, 2014)
***Carica papaya* L.**	Pawpaw (Eng.)	Caricaceae	Used as a soap substitute	Leaves	(Watt and Breyer-Brandwijk, 1962)
Citrus assamensis R.M Dutta & Bhattacharya	Tshikavhavhe (V)	Rutaceae	Used as a facial wash	Fruit sap	(Ndhlovu *et al.*, 2019)
***Crinum bulbispermum* (Burm.f) Milne-Redh. & Schweick**	Orange river lily (Eng.), umnduze (Z)	Amaryllidaceae	Used as a soap substitute	Bulb	(Felhaber and Mayeng, 1997; Mohlakoana and Moteetee, 2021)
***Cussonia paniculata* Eckl. & Zeyh.**	Umsengembuzi (Z), mountain cabbage tree (Eng.)	Araliaceae	Tree sap is mixed with water for bathing	Tree sap	(Mogale *et al.*, 2019)
Cyathula cylindrica Moq.	Bohome-bo-boholo (S)	Amaranthaceae	Used as a soap substitute	Roots	(Moffett, 2010)
***Cyathula uncinulata* (Schrad.) Schinz**	Bohome-ba-lipoli (S)	Amaranthaceae	Used as a soap substitute	Roots	(Watt and Breyer-Brandwijk, 1962; Moffett, 2010)

Medicinal plants	Common name	Family	Use	Part used	Reference
***Deinbollia oblongifolia* (E. Mey. ex Arn.) Radlk.**	Dune soapberry (Eng.), umbangabanga (X)	Sapindaceae	Used as a soap substitute	Seed	(Naidoo, 2009; Würger, 2010)
Dianthus crenatus Thunb.	Iningizimu (Z), wild pink (Eng.)	Caryophyllaceae	With *Tephrosia lurida* Sond. as a facial face wash (froth is produced)	Roots	(Watt and Breyer-Brandwijk, 1962)
Dianthus thunbergii (Thunb.) S.S. Hooper	Ungcane (X), wild pink (Eng.)	Caryophyllaceae	Used to remove body odour	Leaves	(Afolayan *et al.*, 2014)
Dicerocaryum eriocarpum (Decne.) Abels	Devil's thorn (Eng.), intekelane (N)	Pedaliaceae	Used as soap and shampoo	Leaves and flowers	(Watt and Breyer-Brandwijk, 1962; Hoveka, 2017)
Dicerocaryum senecioides (Klotzsch) Abels	Museto/dinda (V)	Pedaliaceae	Applied topically as a substitute for soap	Leaves	(Ndhlovu *et al.*, 2019)
Dicerocaryum zanguebaricum Merrill	Museto (V)	Pedaliaceae	Used as a soap substitute	Leaves and flowers	(Watt and Breyer-Brandwijk, 1962)
Dissotis princeps Triana	Purple dissotis (Eng.), kalwerbossie (Afr.)	Melastomataceae	Used as a soap substitute	Roots	(Watt and Breyer-Brandwijk, 1962)
Dysphania ambrosioides (L.)	Imboya (X), Mexican tea (Eng.)	Amaranthaceae	Used to clean the skin	Leaf lotion	(Afolayan *et al.*, 2014)

Medicinal plants	Common name	Family	Use	Part used	Reference
Mosyakin & Clements					
Entada phaseoloides Merr.	Box bean (Eng.)	Fabaceae	Used as a soap substitute	Bark	(Watt and Breyer-Brandwijk, 1962)
Foeniculum vulgare Mill.	Imbozisa (X), fennel (Eng.)	Apiaceae	Used as a body wash	Not specified	(Mhlongo and Van Wyk, 2019)
***Haemanthus albiflos* Jacq.**	Umathunga (X), white paintbrush (Eng.)	Amaryllidaceae	Remove body odour	Leaf decoction	(Afolayan *et al.*, 2014)
Helinus integrifolius (Lam.) Kuntze	Mupupuma (V)	Rhamnaceae	Applied topically as a substitute for soap	Whole herb	(Ndhlovu *et al.*, 2019)
***Hermannia cuneifolia* Jacq.**	Doll's roses (Eng.), poprosie, moederkappie (Afr.)	Malvaceae	Used for external wash	Not specified	(Hulley and Van Wyk, 2019)
***Ilex mitis* (L.) Radlk. var. mitis**	Mollo-oa-phofu (S), African holly (Eng.)	Aquifoliaceae	Produces a soap-like lather used to wash the body	Leaves	(Phillips, 1917; Watt, 1927)
Ipomoea simplex Thunb.	Igontsi (X), seakhoe (S)	Convolvulaceae	Used to clean the skin	Leaves	(Afolayan *et al.*, 2014)
Kedrostis capensis (Sond.) A. Meeuse	Sesepa-sa-linoha (S)	Cucurbitaceae	Used as soap	Not specified	(Phillips, 1917; Watt and Breyer-Brandwijk, 1962; Moffett, 2010)

Medicinal plants	Common name	Family	Use	Part used	Reference
***Ledebouria* sp. [*Ledebouria apertiflora* (Baker) Jessop]**	Umathunga (Z), Sekanama (S), giant African hyacinth (Eng.)	Hyacinthaceae	Wash wounds, rubbed on painful parts of the body, mixed with bathing water, or used as soap	Bulb	(Komoreng *et al.*, 2017; Mogale *et al.*, 2019)
***Merwilla plumbea* (Lindl.)**	Wild squill (Eng.)	Hyacinthaceae	It is considered a soap plant	Bulb	(Street and Prinsloo, 2012; Mohlakoana, 2020)
Mesembryanthemum crystallinum L.	Crystalline ice plant (Eng.), brakslaai (Afr.)	Aizoaceae	Used as a soap substitute	Leaves	(Watt and Breyer-Brandwijk, 1962)
Morella serrata (Lam.) Killick	Malokela (S), lance-leaf waxberry (Eng.)	Myricaceae	Used as a soap substitute	Branches	(Guillarmod, 1971; Hutchings and Van Staden, 1994)
***Noltea africana* (L.) Endl.**	Soap glossy-leaf (Eng.), umkhuthuhla (X)	Rhamnaceae	Used as a soap substitute	Leaves and twigs	(Watt and Breyer-Brandwijk, 1962)
***Olea europaea* L. subsp. africana (Mill.) P.S. Green**	Umquma (X), wild olive (Eng.)	Oleaceae	Used to remove body odour	Leaves	(Afolayan *et al.*, 2014)
***Pelargonium peltatum* (L.) L'Hér.**	Ityolo (X), ivy-leaved pelargonium (Eng.)	Geraniaceae	Used to clean the skin	Tubers	(Afolayan *et al.*, 2014)
Piliostigma thonningii	Camel's foot (Eng.)	Fabaceae	Used in soap making	Unripe pods	(Ayisere *et al.*, 2009; Ibrahim *et al.*, 2019)

Medicinal plants	Common name	Family	Use	Part used	Reference
(Schumach.) Milne-Redh.					
***Plectranthus ciliatus* E. Mey.**	Lephelephele (S), speckled spur-flower (Eng.)	Lamiaceae	Used as a substitute for soap	Leaves	(Phillips, 1917; Watt and Breyer-Brandwijk, 1962; Guillarmod, 1971)
***Pouzolzia mixta* Sohm**	Soap-nettle (Eng.), murovhadembe (V)	Urticaceae	Used as a soap substitute to wash hands and clothes	Leaves	(Masupa, 2013)
Psilocaulon sp.cf. *coriarium* (Burch. ex N.E.Br.) N.E.Br.	Asbos (H.)	Mesembryanthemaceae	The ash is placed in water, the solution is used to wash the hair; used to make soap	Stems	(De Beer and Van Wyk, 2011)
***Ptaeroxylon obliquum* (Thunb.) Radlk.**	Umthathi (X), sneezewood (Eng.)	Rutaceae	Remove body odour	Bark	(Afolayan *et al.*, 2014)
Pterocarpus angolensis DC.	Mutondo (V)	Leguminosae	Wash or clean the skin	Stems	(Ndhlovu *et al.*, 2019)
Rhynchosia caribaea (Jacq.) DC.	Monya-mali (S), snoutbean (Eng.)	Fabaceae	Soap substitute	Leaves	(Guillarmod, 1971; Hutchings and Van Staden, 1994; Moffett, 2010)
Salsola aphylla L. f.	Ganna bush (Eng.)	Chenopodiaceae	Used to supply the alkali for soap-making	Leaves	(Watt and Breyer-Brandwijk, 1962)
Senna obtusifolia (L.) H.S. Irwin & Barneby.	Mutsheketsheke (V)	Leguminosae	Soap substitute	leaves	(Ndhlovu *et al.*, 2019)

Medicinal plants	Common name	Family	Use	Part used	Reference
Sesbania sesban (L.) Merr. subsp. *sesban*	Mugunwa (V), frother (Eng.)	Fabaceae	Soap substitute	Leaves	(Magwede *et al.*, 2019)
Sida rhombifolia L.	Pretoria bossie (Afr.), arrow leaf sida (Eng.)	Malvaceae	Bathing and shampooing	Shoot bark	(Mehta and Bhatt, 2007; Kunatsa and Katerere, 2021)
Sideroxylon inerme* L. subsp. *inerme	Unqwashu (X), white milkwood (Eng.)	Sapotaceae	Remove body odour	Leaves	(Afolayan *et al.*, 2014)
Solanum aculeastrum Dunal subsp. *aculeastrum*	Bitter apple (Eng.),	Solanaceae	Used as a soap substitute	Fruits	(Watt and Breyer-Brandwijk, 1962; Welman, 2004)
Solanum tomentosum L.	Slangbessiebos (Afr.)	Solanaceae	Wash body	Leaves	(De Beer and Van Wyk, 2011)
Stoebe capitatum P.J. Bergius	Groen slangbos (Afr.)	Asteraceae	Douche and a base for soap	Foliage	(Philander, 2011)
Stoebe plumosa (L.) Thunb.	Grys slangbos (Afr.)	Asteraceae	As a base for soap	Foliage	(Philander, 2011)
Talinum caffrum (Thunb.) Eckl. & Zeyh.	Porcupine root (Eng.), impunyu (Z)	Talinaceae	Used as soap	Leaves	(Watt and Breyer-Brandwijk, 1962)
Taraxacum officinale F.H. Wigg	Ikhokhoyi (X), common dandelion (Eng)	Asteraceae	Remove body odour	Leaves	(Afolayan *et al.*, 2014)

Medicinal plants	Common name	Family	Use	Part used	Reference
Withania somnifera (L.) Dunal	Geneesbos (Afr.), winter cherry (Eng.)	Solanaceae	Used as a soap wash and douche	Foliage	(Philander, 2011)

Key – Afr. (Afrikaans); Eng. (English); H. (Hantam); S (Southern Sotho); V (Venda); X (Xhosa); Z (Zulu); [a] species names in **bold** = species selected for this study.

Although these phytochemicals exhibit antimicrobial activity and other medicinal properties, they can also be toxic to humans and animals. *Merwilla plumbea* (Lindl.) Speta is one of the soap plants found in southern Africa that has been reported to have elevated levels of toxicity (Fennell *et al.*, 2004). According to Notten (2001), it causes skin irritation and itching. However, it is used traditionally as soap and in an ointment for wounds (Street and Prinsloo, 2012). *Carica papaya* L. is traditionally used as a soap substitute and skin-lightening agent due to the presence of papain and chymopapain. The saponins found in *Carica papaya* can heal wounds by boosting collagen production, and carpaine alkaloids serve as a detox agent within the body (Nugrahaningsih *et al*., 2019). According to Aravind *et al*. (2013), latex causes skin irritation, and if the leaves are ingested, they can cause severe gastritis. The phytochemical analysis of *Crinum* L. spp. has yielded more than 150 Amaryllidaceae alkaloids, which are highly toxic. *Crinum bulbispermum* (Burm.f) Milne-Redh. & Schweick is traditionally used as a soap substitute (Felhaber and Mayeng, 1997); however, it has been reported to be highly lethal due to the crinamine and isoquinoline alkaloids found in the plant (Van Wyk *et al.*, 2002; Maroyi, 2016). *Haemanthus albiflos* has elevated toxicity due to the presence of toxic alkaloids called homolycorine and albomaculine, thus it is important to assess potential soap plants for toxicity (Crouch et al., 2005).

1.5 Antimicrobial activity of plants used as soap substitutes

Saponins have a similar chemical structure as soaps and detergents, with hydrophilic heads and hydrophobic tails on both ends (Rai *et al*., 2021). The hydrophobic tails interact with the lipids of bacterial envelopes, causing them to rupture and release their contents. In the same way that soaps form micelles around dirt, proteins from damaged envelopes are enveloped in saponin molecules. In fungi, saponin contact with cell membranes induces cell content leakage, which leads to disintegration (Dong *et al*., 2020). *Medicago arabica* (L.) Huds. is one of the plants used as a soap substitute, and it was reported to have good antimicrobial activity against Gram-positive bacteria. This is due to phytochemicals such as alkaloids, saponins, and terpenoids (Avato *et al.*, 2006). Saponins extracted from *Sorghum bicolor* L. Moench are active against Gram-positive bacteria (Soetan *et al*., 2006).

1.6 Formulation of herbal soap

Historical studies show that the evidence for soap formulation dates back more than 6000 years, and it was mostly utilised by the Egyptians and Babylonians (Levey, 1954). According to Watt (1946), the Gauls were the original inventors of soap making by combining goats' fat with the ashes of beech trees. This was found to produce crude soap that cleaned and washed grease effectively. According to Babayemi *et al.* (2010), when wood or plant material is burned by fire into ash, there are compounds (potassium hydroxide and sodium hydroxide) that are conserved into potash (potassium carbonate) and soda ash (sodium carbonate). These major alkali components (combined with oil or animal fats for the effect of saponification) were used for washing and cleaning (Levey, 1954). Potash and soda are two remarkably similar chemicals; hence, the difference was only recognised in the nineteenth century by Lewkowitsch (1894). Lewkowitsch (1894) was able to differentiate between the two alkalis and that when using caustic soda, a hard soap will be produced, while when using caustic potash, a soft or soluble soap will be produced. This still applies where caustic potash (from wood) is used to make liquid soap and caustic soda, or lye is used to make hard bar soaps. Fatty acids (usually vegetable oils) are added to an alkaline (sodium or potassium salt) solution, resulting in the saponification reaction where glycerine and soap are produced (Akuaden *et al.*, 2019).

Excellent quality soap must produce lather; hence, foam retention and height are some phytochemical parameters to assess after the formulation. The formulated soap must have the moisturising ability and be compatible with the skin (Mak-Mensah and Firempong, 2011; Sindhu *et al.*, 2019). This is assessed by determining the strength and purity of the alkali and the moisture content in the soap. Free caustic alkali in soap prevents it from being oily; however, excess free alkali (caused by incomplete saponification) causes clothes to wear out and the skin to itch. Formulated soap must have a good fragrance, colour, and storage stability (Sindhu *et al.*, 2019). The percentage of chlorine is vital since excess amounts can cause the soap to crack. It is also important to assess the pH of the soap since healthy skin has a pH range of 5.4-5.9 (Mak-Mensah and Firempong, 2011). Skin products are expected to have a pH close to this range to reduce skin irritation (Akuaden *et al.*, 2019). Finally, yet importantly, the soap should have antimicrobial activity against skin pathogens (Igbeneghu, 2013).

1.7 Aim and objectives

The study aimed to investigate the antimicrobial activity, phytochemistry, and toxicity of southern African plants used as soap substitutes; thereafter, an effective antimicrobial herbal soap was formulated and assessed for efficacy. The following objectives were implemented to align with the respective research chapters which are four in total:

- To do undertake preliminary phytochemical screening, a qualitative screening (thin layer chromatography (TLC)) and quantitative screening to determine the presence of saponins.
- To determine the antimicrobial activity of the selected plants using the minimum inhibitory concentration assay (MIC).
- To determine the toxicity of the selected soap plants using the brine-shrimp lethality assay.
- To formulate herbal soaps by the saponification process and assess the physicochemical parameters of the formulations by determining the percentage of free caustic alkali, moisture content, pH, foam retention, and total fatty matter, as well as determining the antimicrobial efficacy.

CHAPTER 2
PHYTOCHEMICAL ANALYSIS OF SELECTED SOUTHERN AFRICAN PLANTS USED AS SOAP SUBSTITUTES

2.1 Introduction

A review conducted on the ethnobotanical literature (Chapter 1; Table 1.2) revealed that 59 plant species are used in southern Africa as soap substitutes. Although the phytochemistry of several plants listed in the review has been previously studied, there is still limited scientific data to assess the presence and quantity of saponins in selected plant species. Therefore, investigating the phytochemical properties of selected southern African soap plants may provide some evidence for the traditional use of the plants as soap substitutes. Terpenoids are the largest and most diversified class of secondary metabolites, produced from the five-carbon molecule isoprene, and subdivided according to the number of isoprene units present in the plant (Adefegha *et al.*, 2022). Triterpenes are one of the subgroups classified in the terpenoid class. Triterpenes have active glycosylation sites used to convert the subgroup into triterpene glycoside, one of the major groups of saponins (Perveen, 2018; El Aziz *et al.*, 2019). Historical studies show evidence that the ashes of plants were used as a detergent because they became soapy when mixed with fat or vegetable oil (Watt, 1946). According to Kurek (2019), the alkalinity of alkaloids is caused by the nitrogen atoms in their structures. Hence, the plants that are used to make detergents have alkali-like properties. Thus, detecting alkaloids, terpenoids, and saponins in soap plants could help distinguish true soap plants from those with high alkaline content that act as surfactants. This chapter aimed to determine the presence of alkaloids, terpenoids, and saponins in selected plants. Furthermore, the quantity of saponins in the plants was determined. The following objectives were designed:

- To carry out a preliminary phytochemical screening for alkaloids, terpenoids, and saponins.
- To determine the presence of saponins using thin layer chromatography.
- To determine the quantity of saponins using the vanillin-sulphuric acid assay.

2.2 Methods and materials

2.2.1 Selection of plants

The ethnobotanical information about plants used as soap substitutes was gathered from various sources, such as medicinal plant-based books (Watt and Breyer-Brandwijk, 1962; Felhaber and Mayeng, 1997), journal articles, dissertations, and theses. Databases such as EBSCOHost, JSTOR, PubMed, ProQuest Central, SABINET Online, Science Direct, Scopus, SpringerLink, Taylor & Francis Journals, Wiley Online Library, and Web of Science were utilised to search for ethnobotanical literature. Keywords such as "Ethnobotanical survey," "Medicinal plant inventory," "Indigenous knowledge," "soapy plants," "saponins," "skin wash," "herbal soap," and "medicinal plants used for skin ailments" were used to search for ethnobotanical literature. The generated list of plant names (Chapter 1, Table 1.2) was sent to Mr Andrew Hankey, the chief horticulturist at Walter Sisulu Botanical Gardens (WSBG) who indicated which plants on the list were available for collection. The list was also sent to Random Harvest Indigenous Nursery (RHIN) and the University of Johannesburg Herbarium (JRAU). The plants that were available at these locations were selected for the study.

2.2.2 Collection of plants

Selected plants were collected from various locations, as indicated in Table 2.1. Mr Andrew Hankey, the chief horticulturist, granted permission and assisted in plant identification and collection from the Walter Sisulu Botanical Gardens (WSBG). Professor Sandy van Vuuren signed the documents to transfer plant material to the University of the Witwatersrand for research purposes. The two plants (*Aloe ferox* and *Crinum bulbispermum*) that were not collected from WSBG, were purchased from the Random Harvest Indigenous Nursery (RHIN), and two other plants (*Cyathula uncinulata* and *Deinbollia oblongifolia*) were obtained from the University of Johannesburg. The co-supervisor and herbarium curator at the University of Johannesburg (JRAU), Professor Annah Moteetee, assisted in plant identification and confirmation. The plant samples were dried and housed at the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

Table 2.1: List of plants that were used in this study, the parts used, voucher numbers, supplier and when it was collected.

Plant name	Plant part collected	Place obtained	Voucher number
Acalypha glabrata	Bark and leaves	WSBG[a]	SVV249
Albizia versicolor	Bark	WSBG	SVV240
Aloe ferox	Leaves	RHIN[b]	SVV264
Aloe maculata	Leaves	WSBG	SVV251
Aristaloe aristata	Leaves	WSBG	SVV250
Artemisia afra	Leaves	WSBG	SVV172
Bauhinia bowkeri	Leaves	WSBG	SVV252
Calodendrum capense	Leaves and bark	WSBG	SVV253
Carica papaya	Leaves	WSBG	SVV239
Crinum bulbispermum	Bulb	RHIN	SVV265
Cussonia paniculata	Leaves	WSBG	SVV271
Cyathula uncinulata	Roots	JRAU[c]	MRM4
Deinbollia oblongifolia	Leaves	JRAU	MRM5
Haemanthus albiflos	Bulb	WSBG	SVV256
Hermannia cuneifolia	Leaves	De Rust	SVV983
Ilex mitis	Leaves	WSBG	SVV145
Ledebouria luteola	Bulb	WSBG	SVV255
Ledebouria zebrina	Bulb	WSBG	SVV257
Merwilla plumbea	Bulb	WSBG	SVV254
Noltea africana	Leaves	JRAU	MRM8
Olea europaea	Leaves	WSBG	SVV238
Pelargonium peltatum	Leaves	WSBG	SVV258
Plectranthus ciliatus	Leaves	WSBG	SVV259
Pouzolzia mixta	Bark	WSBG	SVV260
Ptaeroxylon obliquum	Bark and leaves	WSBG	SVV261

Plant name	Plant part collected	Place obtained	Voucher number
Sideroxylon inerme subsp. *inerme*	Leaves	WSBG	SVV262

Key – [a] Walter Sisulu Botanical Gardens; [b] Random Harvest Indigenous Nursery; [c] University of Johannesburg herbarium.

2.2.3 Plant sample preparation

The plants were stored separately based on which part of the plant is traditionally used as a soap substitute. Leaf, root, and bark were left to dry at room temperature for 7–14 days, whereas the bulb and succulent leaves were left to dry in a warm air oven (Memmert, Germany) at 37°C for 7–14 days. The dried leaves, roots, and bulbs were ground into a fine powder using an electric grinder (Mellerware, Johannesburg), whereas the bark was crushed manually using a pounder (purchased at Faraday supermarket, Johannesburg).

2.2.4 Preliminary phytochemical screening of alkaloids, terpenoids and saponins

A qualitative phytochemical analysis was undertaken to identify the presence of secondary phytochemical constituents found in abundance in particular plant species that either have toxic or beneficial effects on a living system. Standard detection methods are used to test for the presence of phytochemicals such as saponins, alkaloids, and terpenoids (Harborne, 1984). According to Tiwari *et al.* (2011), the type of solvent used for extraction is vital in determining the biologically active compounds in the plant material. Hence, acetone, ethanol, methanol, and distilled water were used in the current study to extract and determine the presence of alkaloids, terpenoids, and saponins. All phytochemical screening tests were performed following methods by Tiwari *et al.* (2011) and Solomon *et al*. (2013).

2.2.4.1 Preparation of plant extracts

The plant material was extracted using four different solvents, namely distilled water, acetone (Associated Chemical Enterprise, South Africa), 70% ethanol (Univar, USA), and methanol

(Sigma-Aldrich, Germany). The extraction was done using a solvent-to-sample ratio of 10:1 (v/w). The solutions were left to stand at room temperature for a extraction 24 h. Thereafter, the solution was filtered twice using the Whatman No.1 filter paper. Thereafter, the filtrate was used for phytochemical screening of alkaloids, terpenoids, and saponins following Wagner's reagent test, Salkowski's test, and the froth test, respectively.

2.2.4.2 Detection of alkaloids using Wagner's test

Wagner's reagent was prepared by making up 1.27 g of iodine (BDH Chemicals Ltd, Poole England) and 2 g of potassium iodide (BDH Chemicals Ltd, Poole England) in 100 ml of distilled water. Five drops of Wagner's reagent were used to treat a small fraction of each plant filtrate. The formation of a brown-reddish precipitate indicated the presence of alkaloids. The potassium metal ion from Wagner's reagent covalently bonds with nitrogen on an alkaloid compound to form a potassium-alkaloid complex, thus the formation of the brown-reddish precipitate (Parbuntari *et al.*, 2018).

2.2.4.3 Detection of terpenoids using Salkowski's test

Salkowski's test detects sterols and terpenoids by yielding a reddish-brown precipitate on the bottom layer when treated with chloroform and concentrated sulphuric acid. Two millilitres of each extract were added to test tubes, altogether with 1 ml of chloroform (Associated Chemical Enterprise, South Africa) and a few drops of sulphuric acid (Rochelle Chemicals, South Africa). The formation of a reddish-brown precipitate indicated the presence of terpenoids.

2.2.4.4 Detection of saponins using the froth test

In test tubes containing 2 ml of plant filtrates each, 18 ml of distilled water was added. The test tubes were then shaken vigorously for 15 mins. The formation of a 1 cm layer of foam indicated the presence of saponins.

2.2.5 Qualitative evaluation of saponins

2.2.5.1 Preparation of plant extracts

A method by Makkar *et al.* (2007) was followed with modifications. Ten grams of ground plant material was defatted using 100 ml of hexane (Associated Chemical Enterprise, South Africa) and left overnight. The solvent was filtrated, and the residue was left to dry under a fume hood. After the residue was dry, 100 ml of 50% aqueous methanol was added, and the solution was left on a magnetic stirrer overnight at room temperature. The contents were centrifuged at 3000 *x*g for 10 min, and the supernatant was collected. The extraction process was repeated using the same solvent by stirring on a magnetic stirrer overnight and then centrifuging. The first and second supernatants were combined and filtrated. Utilizing a rotary evaporator (Buchi, Switzerland), methanol was evaporated from the solution under a vacuum at approximately 42℃. Water-insoluble components were removed from the aqueous phase by centrifuging at 3000 *x*g for 10 mins. The aqueous phase was transferred to a separating funnel and extracted three times with an equal volume of chloroform (Associated Chemical Enterprise, South Africa) to remove pigments. Thereafter, concentrated saponins were extracted from the aqueous solution (twice) with an equal volume of n-butanol (Rochelle Chemicals, South Africa) using a separating funnel. The solvent (n-butanol) was evaporated under a vacuum at a temperature not exceeding 45℃. The dried fraction containing saponins was dissolved in 40 ml of distilled water, and the solution was transferred into a pre-weighed container. The fraction was then lyophilized for 4-7 days using a freeze-dryer (Virtis, California), and the percentage recovery of saponins was calculated using Equation 2.1.

$$\%\ \text{yield of saponins} = \frac{W_1 - W_2}{W_3} \times 100$$

Equation 2.1

W_1 = weight of the vessel, W_2 = weight of the vessel and freeze-dried saponins and W_3 = weight of plant material.

2.2.5.2 Thin layer chromatography (TLC)

A mixture of chloroform, methanol (Associated Chemical Enterprise, South Africa), and distilled water (65:35:10, v/v/v) was prepared as the developing solution. Thereafter, 120 ml of the

developing solution was poured into a chromatographic tank, and the tank was saturated overnight. The sample was prepared by dissolving 5 mg of freeze-dried crude saponin residue in 1 ml of 50% aqueous methanol. The TLC plates were marked with a pencil, 2.5 cm from the bottom of the plate (20 cm × 20 cm, silica gel 60; Merck catalogue No. 1.05721, South Africa). Each sample was applied on the plate using capillary tubes and allowed to dry. After the spots were dry, the TLC plates were inserted into the saturated chromatographic tank containing the developing solution. When the developing solution had reached 1 cm below the top of the TLC plate, the plate was carefully removed and allowed to dry at room temperature. The vanillin-perchloric acid reagent was prepared by making up 1% vanillin in ethanol (w/v) and 2% perchloric acid in ethanol in separate bottles. Thereafter, equal volumes of 1% vanillin (Merck, South Africa) and 2% perchloric acid (Merck, South Africa) were combined. The vanillin-perchloric acid reagent was sprayed on the plates. A different set of plates were also sprayed with 10% sulphuric acid (Associated Chemical Enterprise, South Africa). The plate was heated at 100℃ for 5 mins. Violet- or blue-coloured spots were visually located on the plates as saponins (Oleszek, 2002). The retention factor (Rf) values were calculated using Equation 2.2.

$$\mathrm{Rf} = \frac{\text{Distance moved by the solute/compound (mm)}}{\text{Distance moved by the solvent front (mm)}}$$

Equation 2.2

2.2.6 Quantitative evaluation of saponins

2.2.6.1 Sample and reagent preparation

The vanillin-sulphuric acid assay developed by Hiai *et al.* (1976) was used to determine the total saponin content (TSC). The reaction of oxidised triterpene saponins with vanillin is the basic principle of the assay (Cheok *et al.*, 2014). A review by Kunatsa and Katerere (2021) recorded that plants with a saponin concentration of 40 mg/g and above were considered to be saponin-rich. In this study, the same benchmark was used. The freeze-dried saponin residues (Section 2.2.5.1) were prepared to a concentration of 5 mg/ml using 80% aqueous methanol. The standard saponin solution was prepared by dissolving 10 mg of diosgenin in 80% methanol. The final concentration of diosgenin in the solution was 0.5 mg/ml. The vanillin reagent was prepared by dissolving 800

mg in 10 ml of 99.5% ethanol. Thereafter, 72% (v/v) sulphuric acid was made up by carefully adding 72 ml of sulphuric acid to 28 ml of distilled water.

2.2.6.2 Preparation of standard curve

Diosgenin was used as a standard compound, and the total saponin content was expressed as mg/g diosgenin equivalents. Different volumes (0, 50, 100, 150, 200, and 250 µl) of diosgenin standard solution were made up to 0.25 ml with 80% aqueous methanol. The vanillin reagent was added at a volume of 2.5 ml. Thereafter, 2.5 ml of 72% sulphuric acid was slowly added to oxidize triterpene saponins (Cheok *et al.*, 2014). The samples were mixed well using a vortex (Benchmixer V2) and transferred to a water bath (Labcon (Pty) Ltd, South Africa) set at 60°C for 10 mins. Thereafter, the tubes were placed in ice-cold water for 3-4 min to cool. The distinctive purple colour indicates indicated that the oxidation reaction was complete (Cheok *et al.*, 2014). The absorbance was measured using a UV/VIS spectrophotometer (Biochrom WPA Lightwave II) at 544 nm against a reagent blank containing 0 µl of diosgenin standard solution. A standard curve was generated. The linear relationship between the concentration of the standard (diosgenin) and the absorbance value is demonstrated in Figure 2.1.

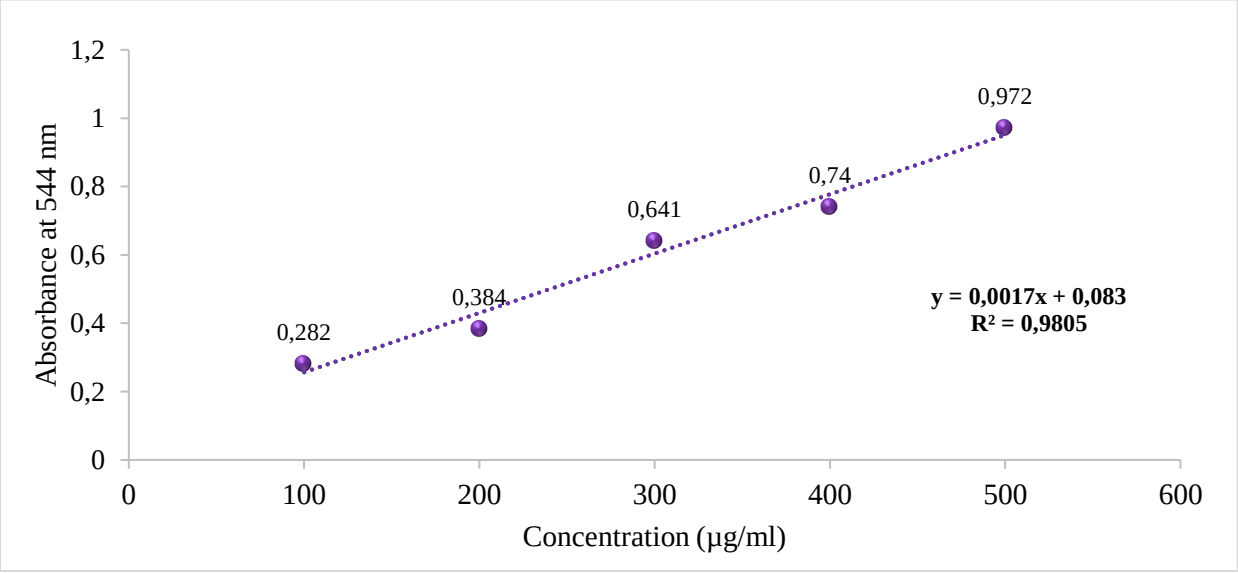


Figure 2.1: The standard curve showing the absorbance of diosgenin at various concentrations.

2.2.6.3 Determining the absorbance of the samples

A mixture of 0.25 ml of sample extracts and reagent blank, together with 0.25 ml of 8% vanillin reagent and 2.50 ml of 72% sulphuric acid, was incubated for 10 min at 60℃ in a water bath, with the reagent blank made up of the solvent used for extracting the plant samples (methanol) (Table 2.1). After cooling in ice water for 3-4 min, the absorbance of the extracts was measured at 544 nm. This was done in triplicate.

Table 2.2: The vanillin-sulphuric acid assay setup procedure for the reagent blank, standard, and sample.

Component	Reagent blank (ml)	Standard (ml)	Sample (ml)
Extraction solvent (80% methanol)	0.25	-	-
Diosgenin in 80% methanol	-	0.25	-
Sample in 80% methanol	-	-	0.25
8% vanillin in ethanol	0.25	0.25	0.25
72% sulphuric acid	2.50	2.50	2.50

The unknown concentration of the samples was calculated from the regression equation (Equation 2.3) from the calibration curve of diosgenin.

$$A = 0{,}0017C + 0{,}083$$

Where A = absorbance of sample, C = unknown sample concentration (µg/ml)

Equation 2.3

Thereafter, the TSC was calculated using Equation 2.4, where C = concentration of the sample (µl/ml), V = volume of extract in the test tube (ml), the mass of extract (g)

$$\text{TSC } (\text{mg/g}) = C \times 10^{-3} \times \frac{V}{W}$$

Equation 2.4

2.3 Results and discussion

2.3.1 The presence of alkaloids, terpenoids and saponins in soap plants

The preliminary phytochemical screening results of 26 plant species are presented in Table 2.3. The results revealed that most of the alcoholic and aqueous extracts contained terpenoids and saponins. Saponins were detected in high concentrations in aqueous extracts (93.10% of plant extracts), followed by ethanol extracts (82.76% of plant extracts). Saponins are naturally occurring amphiphilic glycosides that contain a glycosidic linkage between a lipophilic non-polar aglycone and a hydrophobic polar sugar chain (glycone moieties). Therefore, the surfactant properties observed when saponins are agitated in water are attributed to the amphiphilic structure (El Aziz *et al.*, 2019; Rai *et al.*, 2021). According to Oleszek and Hamed (2010), saponins with one sugar chain (monodesmoside) have the best foaming properties, while those with two or three sugar chains (bidesmoside and tridesmoside) have decreased foaming abilities. Some saponins have no foaming properties in water solutions; however, they are still considered saponins due to their chemical structure (Oleszek and Hamed, 2010). Thus, this explains the high presence of saponins in aqueous extracts. Furthermore, the high concentration of saponins in the aqueous extracts validates the traditional use of 93.10% of the selected plants as soap substitutes.

Methanol and acetone were the optimal solvents to extract alkaloids from 62.07% of plant extracts. Terpenoids were best extracted with ethanol (75.86% of plant extracts), followed by methanol (68.97% of plant extracts), and distilled water (65.52% of plant extracts). A review by Tiwari *et al.* (2011) highlights that methanol and distilled water are the best solvents for the extraction of terpenoids and saponins, while ethanol is recorded as a good solvent for extracting terpenoids and alkaloids. Acetone is a good extractant for phenols and flavanols; hence, terpenoids and saponins were not detected in most acetone plant extracts. The results observed in this study are comparable to those obtained by Truong *et al.* (2019), where alcoholic solvents and distilled water proved to be the best extraction solvents for saponins and terpenoids.

A few previous studies conducted preliminary phytochemical screening for some of the plants investigated in this study. In a study by Kane *et al.* (2019), the ethanol extract of *Artemisia afra* contained terpenoids and alkaloids, while the aqueous extract displayed the presence of saponins.

These results corroborate those of the current study. The presence of saponins in ethanol and aqueous *Carica papaya* extracts and alkaloids in the ethanol extract is consistent with findings by Singh *et al.* (2018) and Nduche *et al.* (2019). The presence of alkaloids and saponins in *Aloe ferox* was reported by Wintola and Afolayan (2011). Choi *et al.* (2015) did a preliminary screening of *Aloe maculata* leaf and reported that the extract displayed the presence of terpenoids and saponins. These results are comparable with those in the current study. The presence of terpenoids and saponins in *Crinum bulbispermum*, *Cyathula uncinulata*, *Deinbollia oblongifolia*, *Ilex mitis*, *Merwilla plumbea*, *Noltea africana*, and *Plectranthus ciliatus* was previously reported by Mohlakoana and Moteetee (2021), and the results correlate with those in the current study. A previous study (Nahal *et al*., 2012) showed that *Olea europaea* does have saponins.

2.3.2 Qualitative analysis: thin layer chromatography (TLC)

The retention factor (Rf) values for 29 extracts are presented in Table 2.3. The vanillin-perchloric acid and 10% sulphuric acid reagent were used as visualisation reagents. The saponin compounds in the plant samples displayed similar mobilities. The extracts in this study were not hydrolysed, hence the similar mobilities. When the plates were sprayed with vanillin-perchloric acid reagents (Appendix C1 and C2), the Rf values ranged from 0.10 to 0.97, whereas when sprayed with 10% sulphuric acid (Appendix C3 and C4) the Rf values ranged from 0.06 to 0.98. The compounds found in most of the extracts were considered less polar since the Rf values were higher due to a wider migration distance. *Hermannia cuneifolia* displayed a total of six different bands. Out of the six bands, three were present on plates sprayed with vanillin-perchloric acid (Rf values = 0.23; 0.40; 0.60), and three were on plates sprayed with 10% sulphuric acid (Rf values = 0.76; 0.85; 0.95). Out of the six bands, three were present on plates sprayed with vanillin-perchloric acid (Rf values = 0.23; 0.40; 0.60) and three were on plates sprayed with 10% sulphuric acid (Rf values = 0.76; 0.85; 0.95). *Acalypha glabrata* (bark), *Aloe ferox* (leaves), *Calodendrum capense* (leaves), and *Sideroxylon inerme* subsp. *inerme* (leaves) extracts displayed two bands on each plate (Table 2.2).

Table 2.3: Preliminary phytochemical screening results and Rf values of 26 southern African soap plants.

Plant sample	Extraction solvents												Vanillin-perchloric acid	10% Sulphuric acid
	Acetone			Ethanol			Methanol			Aqueous				
	A	T	S	A	T	S	A	T	S	A	T	S	Rf values	
Acalypha glabrata (leaves)	+	-	+	+	+	+	+	+	+	-	+	+	0.76; 0.98	0.93
Acalypha glabrata (bark)	-	-	+	-	+	++	-	+	-	-	-	+	0.35; 0.60	0.79; 0.95
Albizia versicolor	+	-	-	-	+	+	+	-	-	-	+	-	0.97	0.79
Aloe ferox	++	+	-	-	-	++	++	++	-	++	++	++	0.73; 0.95	-
Aloe maculata	-	+	-	-	++	+	++	++	-	-	++	+	-	0.88
Aristaloe aristata	-	-	-	-	-	+	-	-	-	-	+	+	0.33	0.92
Artemisia afra	+	+	-	++	+	+	-	+	-	+	++	+	0.33; 0.91	0.79; 0.88
Bauhinia bowkeri	++	-	-	-	+	++	-	+	-	-	+	+	0.26	0.07; 0.82
Calodendrum capense (leaves)	+	-	-	+	+	+	-	+	-	-	-	++	0.18; 0.51	0.14; 0.93
Calodendrum capense (bark)	-	-	-	+	-	+	+	+	-	-	-	++	0.77; 0.90	0.84
Carica papaya	+	-	-	-	+	++	+	-	+	-	-	+	0.19; 0.42	0.90
Crinum bulbispermum	-	+	+	-	-	-	+	+	-	-	-	+	0.27	0.06; 0.69
Cussonia paniculata	+	+	-	-	-	+	+	+	-	-	-	+	0.10	0.95
Cyathula uncinulata	-	-	-	-	+	++	-	-	+	-	+	++	0.24	0.98
Deinbollia oblongifolia	+	-	-	-	-	++	-	+	+	-	-	+	0.82; 0.93	0.82
Haemanthus albiflos	+	-	-	+	+	+	-	+	-	+	+	++	0.79	0.95
Hermannia cuneifolia	+	-	-	-	+	++	-	+	-	+	+	+	0.23; 0.40; 0.60	0.76; 0.85; 0.95
Ilex mitis	-	-	-	-	+	++	+	+	+	++	+	+	0.90	-

Plant sample	Extraction solvents												Vanillin-perchloric acid	10% Sulphuric acid
	Acetone			Ethanol			Methanol			Aqueous				
	A	T	S	A	T	S	A	T	S	A	T	S	Rf values	
Ledebouria luteola	+	-	-	-	-	++	+	-	-	++	++	+	0.84	0.70; 0.84
Ledebouria zebrina	-	-	-	-	++	++	-	-	-	+	-	+	0.30	0.93
Merwilla plumbea	-	-	-	-	+	-	++	+	++	+	+	++	0.19	0.82
Noltea africana	++	-	-	-	+	++	+	++	-	++	+	++	0.35; 0.64	0.61
Olea europaea	-	+	++	-	+	++	++	-	++	-	-	++	0.97	-
Pelargonium peltatum	++	-	-	-	+	+	+	-	-	-	-	++	0.92	0.79
Plectranthus ciliatus	+	-	-	-	+	+	+	+	+	-	++	+	-	0.70
Pouzolzia mixta	-	+	-	-	+	-	-	+	-	++	+	-	0.91	0.52
Ptaeroxylon obliquum (leaves)	+	-	+	-	+	+	++	++	-	-	+	++	0.94	0.85
Ptaeroxylon obliquum (bark)	+	+	-	-	+	-	+	+	-	-	+	++	0.91	0.82
Sideroxylon inerme subsp. *inerme*	++	-	-	-	+	++	+	++	+	++	++	++	0.13; 0.73	0.73; 0.76
Diosgenin (standard saponin)			++			++			++			++	0.21; 0.61; 0.76	0.18; 0.79

Key – A: alkaloids, T: terpenoids, S: saponins; highly present: ++ (as determined by the intensity of brown-reddish precipitate for the presence of alkaloids, reddish-brown precipitate for the presence of terpenoids and formation of a foam layer greater than 1 cm for the presence of saponins), slightly present/present: +, not present: -

Compounds separated from extracts of *Aristaloe aristata* (Rf value = 0.33), *Artemisia afra* (Rf value = 0.33), *Calodendrum capense* (leaves) (Rf value = 0.18), *Carica papaya* (Rf value = 0.19; 0.42), *Crinum bulbispermum* (Rf values = 0.06; 0.27), *Cussonia paniculata* (Rf value = 0.10), *Cyathula uncinulata* (Rf value = 0.24), *Hermannia cuneifolia* (Rf values = 0.23; 0.40), *Noltea africana* (Rf value = 0.35) and *Sideroxylon inerme* subsp. *inerme* (Rf value = 0.13) were considered polar since they had lower Rf values. According to Oleszek *et al.* (2008), the number of sugars in an extract determines the polarity of the compounds, which can range widely.

Diosgenin showed three violet-coloured bands on the vanillin-perchloric acid plate, with Rf values of 0.21, 0.61, and 0.76, respectively. Two bands with Rf values of 0.18 and 0.79 were observed on a TLC plate sprayed with 10% sulphuric acid. Amir *et al.* (2012) stated that a typical TLC chromatogram of diosgenin has an Rf value of 0.76, which supports the results obtained in this study. Diosgenin is a non-polar steroidal aglycone formed from the hydrolysis of a saponin compound (dioscin), and it is often extracted from the tubers of *Dioscorea villosa* L. (wild yam). It is the precursor for the commercial synthesis of steroids such as cortisone, progesterone, and pregnenolone (Kregiel *et al.*, 2017).

2.3.3 Quantitative analysis: vanillin-sulphuric acid assay

The extraction yield and total saponin content (TSC) results of the 29 plant extracts (prepared from 26 plant species) are presented in Table 2.4. As recommended by Kunatsa and Katerere (2021), plants with a saponin concentration of 40 mg/g and above were considered to be saponin-rich. In this study, the same benchmark was used. The extraction yield of saponins was expressed in percentages.

The bark of *Acalypha glabrata* had a TSC of 70.48 ± 2.05 mg/g; however, the leaf had a TSC of 4.02 ± 0.36 mg/g. According to Kunatsa and Katerere (2021), the saponin content can vary in different plant parts. Ncube *et al.* (2011) stated that the concentration in the different plant parts correlates with the levels and sites of pathogenic stimulation. *Aloe maculata*, commonly known as soap aloe, was found to contain a TSC of 43.49 ± 1.05 m g/g, which was within the 40 mg/g saponin threshold (Kunatsa and Katerere, 2021). Choi *et al.* (2015) conducted a detailed analysis

of the saponin concentration on each part of the *Aloe maculata* leaf, and the highest saponin concentration obtained was 1.71 ± 0.05 mg/g. The saponin content of the previous study does not correlate with the current study. The difference in saponin contents might have been affected by the choice of extraction method and differences in the selection of reagent, varied standards, and wavelength (Cheok *et al.*, 2014).

Bauhinia bowkeri had a TSC of 61.10 ± 0.64 mg/g and a saponin extraction yield of 12.80%. It is a species of the family Fabaceae. According to Rai *et al.* (2021), species of the Fabaceae are major sources of triterpenoid saponins. Therefore, using a chromatographic method such as high-performance liquid chromatography (HPLC) to separate and purify the saponins found in this species would help to identify the specific triterpenoid saponin compounds found in the plant. The high saponin content validates the traditional use of the plant as a soap substitute.

Hermannia cuneifolia showed the highest TSC of 262.41 ± 1.91 mg/g and a saponin extraction yield of 10.60%. No study has previously reported on the saponin content of this species. *Hermannia cuneifolia* is classified in the Malvaceae family (Chapter 1, Section 1.3), which is reported to have saponin-rich species (Hulley and Van Wyk, 2019; Kunatsa and Katerere, 2021). Therefore, this corroborates the high saponin content that was found in *Hermannia cuneifolia.*

The TSC of *Merwilla plumbea* was found to be 43.10 ± 1.18 mg/g, and an extraction yield of 8.60% was recorded. The saponin content of *Merwilla plumbea* had been previously analysed by Ncube *et al.* (2011) and Mohlakoana and Moteetee (2021), whereby a TSC of 20 mg/g and 25.59 ± 0.83 mg/g were recorded. Previous studies reported lower TSC compared to the current findings. According to Kunatsa and Katerere (2021), factors such as age, physiological state, and environmental conditions impact the concentration of phytochemicals present within a plant. Furthermore, seasons and the biogeographical location influence the chemical diversity and presence of saponins, hence the inconsistent saponin concentration obtained in the current study and previous studies (Ncube *et al.*, 2011; Kibungu *et al.*, 2021).

The TSC of *Noltea africana* was found to be 68.53 ± 2.43 mg/g, and the extraction yield was 5.10%. In a study by Mohlakoana and Moteetee (2021), *Noltea africana* had a TSC of 52.65 ±

6.81 mg/g. It is commonly known as the soap dogwood due to the soapy properties it possesses and the traditional use of the plant as a soap substitute (Mohlakoana and Moteetee, 2021). It is classified in the family Rhamnaceae (Chapter 1, Section 1.3), which is reported to have saponin-rich species that are used in southern Africa as soap substitutes (Kunatsa and Katerere, 2021). This validates the traditional use of the plant as a soap substitute.

Pelargonium peltatum had a TSC of 43.61 ± 1.65 mg/g and an extraction yield of 5.60%. The presence of saponins in *Pelargonium peltatum* has been previously reported by Scott *et al*. (2004) using preliminary phytochemical screening methods and HPLC. *Sideroxylon inerme* subsp. *inerme* had the highest percentage extraction yield of 16.30% with a TSC of 71.34 ± 1.01 mg/g. To the best of our knowledge, there are no previous reports that highlight the saponin content of *Sideroxylon inerme* subsp. *inerme*.

The nomenclature of *Aloe maculata*, *Deinbollia oblongifolia*, *Noltea africana*, and *Pouzolzia mixta* is based on their soapy properties, such that their English common names include "soap" (soap aloe, dune soapberry, soap glossy leaf, and soap nettle, respectively), to emphasise the traditional use and soapy properties of the plant (Kunatsa and Katerere, 2021). These plants are used for washing, bathing, and hair shampooing since they form a lather when agitated in water. In this study, *Aloe maculata* (43.49 ± 1.05 mg/g) and *Noltea africana* (68.53 ± 2.43 mg/g) were found to have high saponin content. However, *Deinbollia oblongifolia* (6.94 ± 0.49 mg/g) and *Pouzolzia mixta* (18.11 ± 0.36 mg/g) had low saponin contents. The saponin content of *Deinbollia oblongifolia* had been previously reported by Mohlakoana and Moteetee (2021), whereby a higher concentration of 16.02 ± 2.26 mg/ml was obtained. Although it is higher than the concentration in the current study, it is still lower than the selected benchmark for saponin-rich plants. According to Kunatsa and Katerere (2021), although some plants are used as soap substitutes, the saponin content varies from low to high. The saponin concentration in a plant can also be affected by the extraction procedures. According to Rai *et al.* (2021), the maceration extraction used in this study produces a low saponin yield. Therefore, further analysis should be done using more environmentally friendly green technologies (advanced extraction methods) such as ultrasound-assisted extraction and microwave-assisted extraction, as they have been reported to have high precision and produce higher saponin yields.

Table 2.4: The percentage yield of saponins and total saponin content of 26 plant species used as soap substitutes.

Plant sample	Extraction yields (%)	Total saponin content (mg/g)
Acalypha glabrata (leaves)	6.00	4.02 ± 0.36
Acalypha glabrata (bark)	5.60	**70.48 ± 2.05[a]**
Albizia versicolor	9.50	4.71 ± 0.67
Aloe ferox	9.90	16.62 ± 1.43
Aloe maculata	6.70	**43.49 ± 1.05**
Aristaloe aristata	6.60	26.04 ± 1.08
Artemisia afra	12.80	20.22 ± 0.92
Bauhinia bowkeri	12.80	**61.10 ± 0.64**
Calodendrum capense (leaves)	10.67	24.59 ± 0.25
Calodendrum capense (bark)	6.50	32.21 ± 0.38
Carica papaya	1.97	16.70 ± 0.75
Crinum bulbispermum	9.69	31.55 ± 0.75
Cussonia paniculata	2.70	11.16 ± 0.63
Cyathula uncinulata	12.10	30.40 ± 1.54
Deinbollia oblongifolia	14.50	6.94 ± 0.49
Haemanthus albiflos	9.50	29.5 ± 1.74
Hermannia cuneifolia	10.60	**262.41 ± 1.91**
Ilex mitis	8.60	30.87 ± 0.35
Ledebouria luteola	5.30	27.07 ± 0.60
Ledebouria zebrina	3.70	31.18 ± 1.20
Merwilla plumbea	8.60	**43.10 ± 1.18**
Noltea africana	5.10	**68.53 ± 2.43**
Olea europaea	10.10	23.93 ± 0.99
Pelargonium peltatum	5.20	**43.61 ± 1.65**
Plectranthus ciliatus	7.40	15.83 ± 0.96
Pouzolzia mixta	1.50	18.11 ± 0.36

Plant sample	Extraction yields (%)	Total saponin content (mg/g)
Ptaeroxylon obliquum (leaves)	10.00	17.87 ± 0.66
Ptaeroxylon obliquum (bark)	2.80	17.28 ± 0.76
Sideroxylon inerme subsp. *inerme*	16.30	**71.34 ± 1.01**

Key – [a] values in **bold** = TSC < 40 mg/g (saponin-rich)

2.4 Summary

- Saponins were detected in high concentration in 93.10% of the aqueous extracts using the froth test.
- *Sideroxylon inerme* subsp. *inerme* (16.3%) had a high extraction yield of saponins.
- Eight soap plants, namely *Acalypha glabrata* (bark), *Aloe maculata*, *Bauhinia bowkeri*, *Hermannia cuneifolia*, *Merwilla plumbea*, *Noltea africana* and *Sideroxylon inerme* subsp. *inerme* were found to contain TSC of more than 40 mg/g.
- *Hermannia cuneifolia* showed the highest total saponin content (262.41 ± 1.90 mg/g), followed by *Sideroxylon inerme* subsp. *inerme* (71.34 ± 1.01 mg/ml), and *Acalypha glabrata* bark (70.48 ± 2.05 mg/g).

CHAPTER 3
THE ANTIMICROBIAL ACTIVITY OF SELECTED SOUTHERN AFRICAN PLANTS USED AS SOAP SUBSTITUTES

3.1 Introduction

While some of the antimicrobial effects of several selected soap plants for this study (Chapter 1, Table 1.2) have been previously studied, there is limited scientific data to assess the antimicrobial efficacy of these plants against neglected skin pathogens such as *Corynebacterium xerosis*, *Staphylococcus capitis*, *Staphylococcus haemolyticus*, and *Staphylococcus lugdunensis*. Therefore, investigating the antimicrobial properties against a wider range of skin-relevant pathogens for selected southern African plants used as soap substitutes will provide more in-depth value for the possibility of these plants being used as soap alternatives. Furthermore, the findings obtained will also promote a more natural, affordable, and effective personal hygiene option for people in rural areas (Tura *et al.*, 2017). The ability of the soap plants to inhibit bacterial or fungal growth causing skin infectious diseases was measured by antimicrobial susceptibility tests (Reller *et al*., 2009). The MIC assay was used to measure this ability (Reller *et al*., 2009). Minimum inhibitory concentration (MIC) assay is the lowest concentration that an antimicrobial sample or an antibiotic can inhibit microbial visible growth after incubation. It is used as a research tool to determine new antimicrobials' in vitro activity against various pathogens (Andrews, 2001). This chapter aimed to investigate the antimicrobial activity of organic and aqueous plant extracts against pathogens that cause skin infections or odour, and that are common contaminants of the skin. This was achieved by following these objectives:

- To prepare organic (dichloromethane: methanol) and aqueous (sterile water) extracts.
- To select skin-relevant pathogens and prepare viable and pure cultures.
- To undertake minimum inhibition concentration (MIC) assays to test the antimicrobial activity.

3.2 Methods and materials

3.2.1 Preparation of organic and aqueous plant extracts

The method by Van Vuuren and Viljoen (2006) was used to prepare the plant extracts. A total of 26 plant species selected based on availability. In preparing the organic plant extracts (29), ground plant material was submerged in twice the quantity of organic solvents at a ratio of 1:1 (Dichloromethane (DCM) (Merck, South Africa): methanol (Associated Chemical Enterprise, South Africa)). Thereafter, the extracts were placed in a shaking incubator (Labcon, Krugersdorp) for 24 h at 37°C. The samples were filtered using autoclaved cotton wool and left in the fume hood to allow evaporation. The residue from the first extraction was used for re-extraction for 24 h in the shaking incubator at 37°C. The supernatant was mixed with previously collected supernatant and then placed in the fume hood for evaporation for three days. To prepare aqueous plant extracts, weighed ground plant material was submerged in twice the quantity of sterile water in previously weighed conical flasks. The extracts were placed in a shaking incubator for 24 h at 30°C for extraction. Thereafter, the extracts were filtered and stored at -80°C overnight to eliminate possible microbial contaminants. The aqueous extracts were then lyophilised for 4-7 days using a freeze-dryer (Virtis, California). Thereafter, the samples were placed under ultraviolet light for 24 h. The plant samples were prepared at a concentration of 32 mg/ml, using acetone (Associated Chemical Enterprise, South Africa) for organic extracts and sterile water for aqueous extracts.

3.2.2 Selection of test micro-organisms and preparation of cultures

In this study, sixteen micro-organisms (American Type Culture Collection (ATCC) strains from Davies Diagnostics, South Africa) were selected based on prevalence to cause odour, skin infections, or act as common contaminants of the skin (Table 3.1). An ethics waiver to use micro-organisms was applied for and approved by the University of the Witwatersrand Human Research Ethics Committee (Reference No. W-CBP-210408-01, Appendix B). The bacterial and fungal strains (16 micro-organisms in total) were cultured and incubated according to the growing conditions as indicated in Table 3.1.

Table 3.1: Pathogens and culturing conditions for antimicrobial screening.

Cultures to be used in this study		Growing conditions for cultures	
Pathogen name and strain	**Gram-positive/ Gram-negative**	**Medium**	**Incubation conditions**
Acinetobacter baumannii (ATCC 19606)	Gram-negative	Tryptone Soya agar and broth (Oxoid, UK)	37°C for 18–24 h
Brevibacterium agri (ATCC 51663)	Gram-positive	Tryptone Soya agar and broth	37°C for 18–24 h
Brevibacterium epidermidis (ATCC 20660)			
Brevibacterium linens (ATCC 20425)	Gram-positive	Tryptone Soya agar and broth	30°C for 4 days
Candida albicans (ATCC 10231)	N/A	Tryptone Soya agar and broth	37°C for 48 h
Corynebacterium xerosis (ATCC 373)	Gram-positive	Brain heart infusion agar and broth (Oxoid, UK)	30°C for 24–48 h
Cutibacterium acnes (ATCC 11827)	Gram-positive	Thioglycolate broth (Oxoid, England)	37°C for 5–7 days
Enterobacter cloacae (ATCC 13047)	Gram-negative	Tryptone Soya agar and broth	37°C for 18–24 h
Escherichia coli (ATCC 8739)			
Klebsiella pneumoniae (ATTC 13883)	Gram-negative	Tryptone Soya agar and broth	37°C for 18–24 h
Pseudomonas aeruginosa (ATCC 27853)			
Staphylococcus aureus (ATCC 25923)	Gram-positive	Tryptone Soya agar and broth	37°C for 18–24 h
Staphylococcus capitis (ATCC 146)			
Staphylococcus epidermidis (ATCC 12228)	Gram-positive	Tryptone Soya agar and broth	37°C for 18–24 h

Cultures to be used in this study		Growing conditions for cultures	
Pathogen name and strain	**Gram-positive/ Gram-negative**	**Medium**	**Incubation conditions**
Staphylococcus haemolyticus (ATCC 29970)			
Staphylococcus lugdunensis (ATCC 49576)			

3.2.3 Minimum inhibition concentrations (MIC) assay

In this study, the antimicrobial activities of plants used as soap substitutes were evaluated. Methods by Eloff (1998) and Van Vuuren *et al.* (2019) were used with modifications. In each well of the 96-well microtitre plate (Monitoring and Control Laboratories (Pty) Ltd., South Africa), 100 µl of sterile broth specific to the micro-organism was aseptically added. Thereafter, 100 µl of the respective plant samples at a concentration of 32 mg/ml to be tested were introduced in the first row in duplicate. Positive controls (0.01 mg/ml ciprofloxacin (Sigma Aldrich, South Africa) for bacteria and amphotericin B (Sigma Aldrich, South Africa) for *C. albicans*) were used to assess the microbial susceptibility, whereas the negative controls (optimal media and acetone) were used to ensure that the acetone (the solvent used for re-suspension) has a minimal or no antimicrobial effect. The broth with the pathogen was used as a culture control to ensure that microbial growth can be supported by the broth chosen. To get a 0.5 McFarland Turbidity Standard, 1 ml of a 24 h growing culture was transferred into approximately 10 ml sterile broth until it appeared just turbid. Then the stock culture was made at a ratio of 1: 100 (i.e., 20 ml of sterile TBS in 200 µl of the McFarland standard). In all wells, 100 µl of the pathogen (stock culture) was added, and the plates were sealed with a sterile adhesive sealer and then incubated at optimum incubation conditions for each pathogen (Table 3.1). A streak plate of the stock culture was prepared and incubated at optimum conditions to check for any contamination. After the appropriate incubation period for each pathogen, 40 µl of a previously prepared 0.4 mg/ml p-iodonitrotetrazolium violet (INT; Sigma Aldrich, South Africa) solution was introduced into each well to act as an indicator for growth. The change in colour to purple-pink was observed, which indicated microbial growth. This reaction is based on the transfer of electrons from NADH (a product of threonine dehydrogenase

from fungal or bacterial growth) to INT during the active growth of the micro-organisms (Masoko, 2007). The lowest dilution with the absence of the purple-pink colour indicated the MIC end-point value, the lowest concentration at which the pathogen was inhibited. The plates were read after 6 h, except for *C. albicans* which was read after a further 24 h. The study was performed in triplicate and on consecutive days to ensure accuracy.

3.3 Results and discussion

The antimicrobial effects of 29 organic and aqueous extracts (prepared from 26 plant species) were evaluated against ten Gram-positive bacteria, five Gram-negative bacteria, and *Candida albicans*. The MIC values that were ≤ 0.16 mg/ml were considered noteworthy, while values that were between 0.16-1.00 mg/ml were considered to have moderate activity, and MIC values that were greater than 1.00 mg/ml indicated weak activity (Van Vuuren and Holl, 2017; Seleteng-Kose *et al.*, 2019). The organic extracts displayed superior antimicrobial activity compared to aqueous extracts. Several plant-based antimicrobial research studies support these observations (Van Vuuren and Naidoo, 2010; Mabona *et al.*, 2013; Shirinda *et al.*, 2019). As stated by Das *et al.* (2010), most of the constituents that are soluble in water, such as flavonoids and water-soluble phenols do not possess antimicrobial significance, hence the aqueous extracts demonstrated weak antimicrobial activity. The poor antimicrobial activity of the aqueous extracts can also be due to inadequate quantities of active constituents in the extract (Ibrahim and Kebede, 2020). As no significant activity was observed for the aqueous extracts (Tables 3.2-3.3), only organic extracts are discussed further.

Table 3.2: The average MIC values of 26 plant species against Gram-positive bacteria.

Plant extract	Pathogen (mean MIC values mg/ml)																			
	***B. agri* (ATCC 51663)**		***B. epidermidis* (ATCC 20660)**		***B. lines* (ATCC 20425)**		***C. xerosis* (ATCC 373)**		***C. acnes* (ATCC 11827)**		***S. aureus* (ATCC 25923)**		***S. capitis* (ATCC 146)**		***S. epidermidis* (ATCC 12228)**		***S. haemolyticus* (ATCC 29970)**		***S. lugdunensis* (ATCC 49576)**	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Acalypha glabrata (leaves)	4.00	≥ 8.00	3.00	≥ 8.00	*1.00*[a]	≥ 8.00	*1.00*	≥ 8.00	1.50	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00
Acalypha glabrata (bark)	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	*1.00*	≥ 8.00	1.60	8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Albizia versicolor	4.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	3.00	≥ 8.00
Aloe ferox	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.80	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	3.00	≥ 8.00
Aloe maculata	3.00	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	3.00	≥ 8.00
Aristaloe aristata	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	3.00	8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Artemisia afra	2.00	8.00	4.00	8.00	*0.75*	8.00	*1.00*	≥ 8.00	2.50	8.00	*1.00*[a]	≥ 8.00	2.00	≥ 8.00	4.00	8.00	2.00	≥ 8.00	2.00	≥ 8.00
Bauhinia bowkeri	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	*0.75*	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Calodendrum capense (leaves)	2.00	≥ 8.00	1.50	≥ 8.00	*0.38*	≥ 8.00	*1.00*	≥ 8.00	0.06[b]	≥ 8.00	*0.50*	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	*0.75*	≥ 8.00	2.00	≥ 8.00
Calodendrum capense (bark)	4.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	*1.00*	≥ 8.00	1.25	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Carica papaya	4.00	≥ 8.00	4.00	≥ 8.00	*1.00*	≥ 8.00	*1.00*	≥ 8.00	1.40	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	1.50	≥ 8.00	2.00	≥ 8.00
Crinum bulbispermum	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	1.67	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00

Plant extract	Pathogen (mean MIC values mg/ml)																			
	B. agri **(ATCC 51663)**		***B. epidermidis*** **(ATCC 20660)**		***B. lines*** **(ATCC 20425)**		***C. xerosis*** **(ATCC 373)**		***C. acnes*** **(ATCC 11827)**		***S. aureus*** **(ATCC 25923)**		***S. capitis*** **(ATCC 146)**		***S. epidermidis*** **(ATCC 12228)**		***S. haemolyticus*** **(ATCC 29970)**		***S. lugdunensis*** **(ATCC 49576)**	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Cussonia paniculata	4.00	8.00	4.00	≥ 8.00	*1.00*[a]	≥ 8.00	*1.00*	≥ 8.00	1.50	8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	3.50	≥ 8.00	2.00	≥ 8.00
Cyathula uncinulata	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00
Deinbollia oblongifolia	4.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	1.75	≥ 8.00	1.25	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Haemanthus albiflos	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	1.40	≥ 8.00	*0.44*	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00
Hermannia cuneifolia	4.00	8.00	4.00	8.00	2.00	8.00	1.40	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	8.00	4.00	≥ 8.00	3.00	≥ 8.00
Ilex mitis	3.00	8.00	4.00	8.00	1.50	≥ 8.00	*1.00*	≥ 8.00	1.38	≥ 8.00	2.00	8.00	2.00	≥ 8.00	4.00	8.00	4.00	≥ 8.00	4.00	≥ 8.00
Ledebouria luteola	3.00	≥ 8.00	4.00	≥ 8.00	1.60	≥ 8.00	*1.00*	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00
Ledebouria zebrina	6.00	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	1.40	8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00
Merwilla plumbea	4.00	≥ 8.00	4.00	≥ 8.00	1.60	≥ 8.00	*0.70*	≥ 8.00	1.40	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00
Noltea africana	4.00	≥ 8.00	2.00	≥ 8.00	1.80	≥ 8.00	*0.75*	≥ 8.00	0.06	4.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	3.00	≥ 8.00	3.00	≥ 8.00
Olea europaea	3.00	8.00	4.00	8.00	1.40	≥ 8.00	*1.00*	≥ 8.00	0.06	≥ 8.00	2.00	8.00	2.00	≥ 8.00	4.00	8.00	4.00	≥ 8.00	3.00	≥ 8.00
Pelargonium peltatum	2.00	≥ 8.00	2.00	≥ 8.00	**0.06**[b]	≥ 8.00	*0.35*	≥ 8.00	0.06	8.00	*0.50*	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	1.50	≥ 8.00	1.50	≥ 8.00
Plectranthus ciliatus	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	*0.38*	≥ 8.00	0.06	≥ 8.00	*1.00*	≥ 8.00	1.50	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Pouzolzia mixta	4.00	≥ 8.00	4.00	≥ 8.00	*1.00*	≥ 8.00	*0.88*	≥ 8.00	*0.22*	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00

Plant extract	Pathogen (mean MIC values mg/ml)																			
	B. agri **(ATCC 51663)**		***B. epidermidis*** **(ATCC 20660)**		***B. lines*** **(ATCC 20425)**		***C. xerosis*** **(ATCC 373)**		***C. acnes*** **(ATCC 11827)**		***S. aureus*** **(ATCC 25923)**		***S. capitis*** **(ATCC 146)**		***S. epidermidis*** **(ATCC 12228)**		***S. haemolyticus*** **(ATCC 29970)**		***S. lugdunensis*** **(ATCC 49576)**	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Ptaeroxylon obliquum (leaves)	4.00	≥ 8.00	3.00	8.00	*1.00*	≥ 8.00	*1.00*	≥ 8.00	0.06	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Ptaeroxylon obliquum (bark)	2.00	≥ 8.00	2.00	≥ 8.00	*0.50*	≥ 8.00	*0.27*	≥ 8.00	0.06	≥ 8.00	*0.38*	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	*1.00*	≥ 8.00
Sideroxylon inerme subsp. *inerme*	4.00	8.00	6.00	≥ 8.00	1.40	8.00	*0.75*	≥ 8.00	0.06	≥ 8.00	2.00	8.00	2.00	≥ 8.00	4.00	8.00	4.00	≥ 8.00	3.00	≥ 8.00
Culture control	> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00	
Positive control (ciprofloxacin)	**0.00125**		**0.00063**		**0.00125**		0.00131		0.00063		0.00063		0.00031		0.00063		0.00063		0.00063	
Negative control (acetone)	> 8.00		> 8.00		> 8.00		4.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00	

Key – [a] values in *italics* = MIC values 1.00 ≤ MIC > 0.16 mg/ml (moderate activity); [b] values in **bold** = MIC values ≤ 0.16 mg/ml (noteworthy activity); O = organic extracts; A = aqueous extracts

Table 3.3: The average MIC values of 26 plant species against Gram-negative bacteria and *Candida albicans*.

Plant extract	Pathogen (mean MIC values mg/ml)											
	A. baumannii **(ATCC 19606)**		***E. cloacae*** **(ATCC 13047)**		***E. coli*** **(ATCC 8739)**		***K. pneumoniae*** **(ATTC 13883)**		***P. aeruginosa*** **(ATCC 27853)**		***C. albicans*** **(ATCC 10231)**	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Acalypha glabrata (leaves)	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*[a]	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Acalypha glabrata (bark)	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	*0.50*	≥ 8.00

Plant extract	Pathogen (mean MIC values mg/ml)											
	***A. baumannii* (ATCC 19606)**		***E. cloacae* (ATCC 13047)**		***E. coli* (ATCC 8739)**		***K. pneumoniae* (ATTC 13883)**		***P. aeruginosa* (ATCC 27853)**		***C. albicans* (ATCC 10231)**	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Albizia versicolor	4.00	≥ 8.00	2.00	≥ 8.00	4.00	8.00	1.40	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Aloe ferox	4.00	≥ 8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Aloe maculata	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	1.50	≥ 8.00	1.75	≥ 8.00	2.00	≥ 8.00
Aristaloe aristata	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	4.00	≥ 8.00
Artemisia afra	2.00	8.00	2.00	≥ 8.00	2.00	8.00	*1.00*	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Bauhinia bowkeri	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	4.00	≥ 8.00	*1.00*	≥ 8.00
Calodendrum capense (leaves)	1.50	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00	1.50	≥ 8.00	1.50	≥ 8.00
Calodendrum capense (bark)	3.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00
Carica papaya	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Crinum bulbispermum	4.00	≥ 8.00	*1.00*	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Cussonia paniculata	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Cyathula uncinulata	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00
Deinbollia oblongifolia	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	1.50	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00
Haemanthus albiflos	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	8.00	2.00	≥ 8.00	1.33	≥ 8.00
Hermannia cuneifolia	4.00	≥ 8.00	2.00	≥ 8.00	3.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Ilex mitis	4.00	≥ 8.00	2.00	≥ 8.00	4.00	8.00	2.00	8.00	4.00	≥ 8.00	*1.00*	≥ 8.00
Ledebouria luteola	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00
Ledebouria zebrina	3.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	8.00	4.00	≥ 8.00	1.50	≥ 8.00

Plant extract	Pathogen (mean MIC values mg/ml)											
	***A. baumannii* (ATCC 19606)**		***E. cloacae* (ATCC 13047)**		***E. coli* (ATCC 8739)**		***K. pneumoniae* (ATTC 13883)**		***P. aeruginosa* (ATCC 27853)**		***C. albicans* (ATCC 10231)**	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Merwilla plumbea	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00
Noltea africana	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	8.00	*1.00*	≥ 8.00	1.50	≥ 8.00
Olea europaea	4.00	8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	8.00	4.00	≥ 8.00	1.60	≥ 8.00
Pelargonium peltatum	1.50	≥ 8.00	*0.75*	≥ 8.00	2.00	≥ 8.00	*1.00*	4.00	*0.50*	≥ 8.00	*0.50*	≥ 8.00
Plectranthus ciliatus	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	1.50	8.00	*1.00*	≥ 8.00	*0.25*	≥ 8.00
Pouzolzia mixta	4.00	≥ 8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*1.00*	≥ 8.00
Ptaeroxylon obliquum (leaves)	4.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	8.00	4.00	≥ 8.00	1.50	≥ 8.00
Ptaeroxylon obliquum (bark)	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	2.00	≥ 8.00	*0.25*	≥ 8.00	*1.00*	≥ 8.00
Sideroxylon inerme subsp. *inerme*	4.00	8.00	2.00	≥ 8.00	4.00	≥ 8.00	2.00	8.00	4.00	≥ 8.00	1.50	8.00
Culture control	> 8.00		> 8.00		> 8.00		> 8.00		> 8.00		> 8.00	
Positive control	**0.00063[b]**		**0.00016[b]**		**0.00063[b]**		**0.00031[b]**		**0.00125[b]**		**0.00004[c]**	
Negative control (acetone)	> 8.00		> 8.00		> 8.00		> 8.00		8.00		4.00	

Key – [a] values in *italics* = MIC values 1.00≤ MIC > 0.16 mg/ml (moderate activity); [b] Ciprofloxacin, [c] Amphotericin; O = organic extracts; A = aqueous extracts

3.3.1 Antimicrobial activity of the organic extracts against Gram-positive bacteria

Pelargonium peltatum (leaves) demonstrated the best antimicrobial activity against *Brevibacterium linens* with an MIC value of 0.06 mg/ml. There are no previous studies highlighting the plant species' antimicrobial potential against *B. linens*. Moderate antimicrobial activity against *B. linens* was also observed for *Acalypha glabrata* (bark and leaves) (MIC = 1.00 mg/ml), *Artemisia afra* (leaves) (MIC = 0.75 mg/ml), *Calodendrum capense* (bark and leaves) (MIC = 1.00 mg/ml and 0.38 mg/ml, respectively), *Carica papaya* (leaves), *Cussonia paniculata* (leaves), *Deinbollia oblongifolia* (leaves), *Plectranthus ciliatus* (leaves), *Pouzolzia mixta* (leaves) (MIC = 1.00 mg/ml) and *Ptaeroxylon obliquum* (bark and leaves) (MIC = 0.50 mg/ml and 1.00 mg/ml, respectively). To the best of our knowledge, no studies have been published that highlight the antimicrobial activity of *Acalypha glabrata*, *Artemisia afra*, *Calodendrum capense*, *Cussonia paniculata*, *Pouzolzia mixta*, and *Ptaeroxylon obliquum* against *B. linens*. *Plectranthus ciliatus* has previously been shown to possess an antimicrobial effect against *B. linens* with an MIC value of 0.50 mg/ml (Mohlakoana, 2020). Mohlakoana (2020) also reported on the antimicrobial effects of *Carica papaya* and *Deinbollia oblongifolia*; however, weak antimicrobial activity was observed (MIC values of 2.00 mg/ml).

Calodendrum capense (leaves), *Noltea africana* (leaves), *Olea europaea* (leaves), *Pelargonium peltatum* (leaves), *Plectranthus ciliatus* (leaves), *Ptaeroxylon obliquum* (bark), and *Sideroxylon inerme* subsp. *inerme* (leaves) exhibited noteworthy antimicrobial activity (MIC values of 0.06 mg/ml) against *Cutibacterium acnes*. Mohlakoana (2020) reported that *Noltea africana* (MIC = 1.00 mg/ml) and *Plectranthus ciliatus* (MIC = 0.25 mg/ml) displayed moderate activity against *C. acnes*. The variation between MIC values observed in the prior study and the current study may be attributable to seasonal variation and geographical variation. According to Kibungu *et al.* (2021), seasons influence the production of bioactive compounds responsible for antimicrobial properties in plant species. In addition, the biogeographical location influences the chemical diversity and presence of bioactive compounds, thereby impacting the antimicrobial activity of the plant species. Regarding the antimicrobial properties of *Calodendrum capense*, *Olea europaea*,

Pelargonium peltatum, *Ptaeroxylon obliquum*, and *Sideroxylon inerme* subsp. *inerme* against *C. acnes*, no previous studies were found.

The antimicrobial activity of *Cyathula uncinulata* (root) (MIC = 1.00 mg/ml), *Haemanthus albiflos* (bulb) (MIC = 0.44 mg/ml), *Ledebouria luteola* (bulb) (MIC = 1.00 mg/ml), and *Pouzolzia mixta* (leaves) (MIC = 0.22 mg/ml) against *C. acnes* was moderate. The results obtained for *Cyathula uncinulata* (1.00 mg/ml) are congruent with the findings from a previous study by Mohlakoana (2020). Previous research on the antimicrobial activity of *Haemanthus albiflos* and *Pouzolzia mixta* (McGaw *et al.*, 2005; Samie *et al.*, 2005; Thibane *et al.*, 2019) focused on other skin pathogens apart from *C. acnes*. The sensitivity of the organic extracts to *C. acnes* validates the traditional usage of these plant species as soap substitutes, as adequate hygiene must be maintained to prevent the commensal microorganism from becoming pathogenic (Chiller *et al.*, 2001).

Moderate antimicrobial activity against *C. xerosis* was displayed by *Bauhinia bowkeri* (leaves) (MIC = 0.75 mg/ml), *Merwilla plumbea* (bulb) (MIC = 0.70 mg/ml), *Noltea africana* (leaves) (MIC = 0.75 mg/ml), *Pelargonium peltatum* (leaves) (MIC = 0.35 mg/ml), *Plectranthus ciliatus* (leaves) (MIC = 0.38 mg/ml), *Pouzolzia mixta* (leaves) (MIC = 0.88 mg/ml), *Ptaeroxylon obliquum* (bark) (MIC = 0.27 mg/ml) and *Sideroxylon inerme* subsp. *inerme* (leaves) (MIC = 0.75 mg/ml). *Acalypha glabrata* (bark and leaves), *Artemisia afra* (leaves), *Calodendrum capense* (bark and leaves), *Carica papaya* (leaves), *Cussonia paniculata* (leaves), *Ilex mitis* (leaves), *Ledebouria luteola* (bulb), *Olea europaea* (leaves), and *Ptaeroxylon obliquum* (leaves) also demonstrated moderate antimicrobial activity (MIC values of 1.00 mg/ml). To the best of our knowledge, these plants have not been studied previously against the pathogen *C. xerosis*. *Corynebacterium xerosis* is a commensal Gram-positive micro-organism typically found on the skin causing various infections, particularly skin infections. *Corynebacterium xerosis* enters the body of immunocompromised persons through wounds, injuries, and cuts, thus leading to infections such as septicaemia (Kim and Reboli, 2020).

Artemisia afra (leaves) (MIC = 1.00 mg/ml), *Calodendrum capense* (leaves) (MIC = 0.50 mg/ml), *Plectranthus ciliatus* (leaves) (MIC = 1.00 mg/ml), *Pelargonium peltatum* (leaves) (MIC = 0.50 mg/ml), and *Ptaeroxylon obliquum* (bark and leaves) (MIC = 0.38 mg/ml and 1.00 mg/ml)

displayed moderate antimicrobial activity against *Staphylococcus aureus* (Table 3.2b). Similar results for *Calodendrum capense* and *Plectranthus ciliatus* have been previously reported with MIC values of 0.32 mg/ml and 1.00 mg/ml, respectively (Nielsen *et al.*, 2012; Sakong, 2012; Mohlakoana, 2020). Singab *et al.* (2015) reported on the antimicrobial effects of *Pelargonium peltatum*, where zones of inhibition (15 mm-20 mm) were observed against *S. aureus* using the disc diffusion bioassay. The results reported support the moderate activity of *Pelargonium peltatum* against *S. aureus*. In previous studies, *Artemisia afra* and *Ptaeroxylon obliquum* exhibited noteworthy antimicrobial activity against *S. aureus*, with MIC values ranging from 0.03-0.16 mg/ml (Buwa and Afolayan, 2009; Nielsen *et al.*, 2012; Ramadwa *et al.*, 2019). The difference between the MIC values obtained in the previous study and the current study may be due to the different extraction solvents used. According to Paiva *et al.* (2010), the biological features of a plant extract derived from solvents of different characteristics are diverse. Differences in the polarity of the solvents are among the characteristics that contribute to the unique biological qualities (Paiva *et al.*, 2010).

The antimicrobial activities displayed by *Acalypha glabrata* (bark) (MIC = 1.00 mg/ml), *Calodendrum capense* (leaves) (MIC = 0.75 mg/ml), and *Ptaeroxylon obliquum* (bark) (MIC = 1.00 mg/ml) were moderate against *S. haemolyticus*. Furthermore, *Aloe ferox* (leaves), *Calodendrum capense* (leaves), *Crinum bulbispermum* (bulb), *Pelargonium peltatum* (leaves) and *Ptaeroxylon obliquum* (bark) all displayed moderate antimicrobial activity (MIC = 1.00 mg/ml) against *S. capitis*, while *Ptaeroxylon obliquum* (bark) (MIC = 1.00 mg/ml) showed moderate activity against *S. lugdunensis.* Since the antibacterial activity of the plants against *S. capitis*, *S. haemolyticus*, and *S. lugdunensis* has not been previously investigated, past data cannot be compared.

3.3.2 Antimicrobial activity of the organic extracts against Gram-negative bacteria

Aloe ferox (leaves) (MIC = 1.00 mg/ml), *Crinum bulbispermum* (bulb) (MIC = 1.00 mg/ml) and *Pelargonium peltatum* (leaves) (MIC = 0.75 mg/ml) demonstrated moderate antimicrobial activity against *Enterobacter cloacae* (Table 3.3). There has been no previous research undertaken on the antimicrobial activity of *Aloe ferox*, *Crinum bulbispermum* and *Pelargonium peltatum* (leaves)

against *E. cloacae*. *Acalypha glabrata* (leaves), *Artemisia afra* (leaves), *Bauhinia bowkeri* (leaves), *Calodendrum capense* (leaves), and *Pelargonium peltatum* (leaves) all displayed moderate efficacy against *Klebsiella pneumoniae*, with MIC values of 1.00 mg/ml. Buwa and Afolayan (2009) highlighted the antimicrobial properties of the dichloromethane extract of *Artemisia afra* extract having an MIC value of 0.39 mg/ml, which supports the moderate activity observed in this study. To the best of our knowledge, *Acalypha glabrata*, *Bauhinia bowkeri*, *Calodendrum capense*, and *Pelargonium peltatum* have not been previously studied for their antibacterial activity against *K. pneumoniae*.

Acalypha glabrata (bark) (MIC = 1.00 mg/ml), *Noltea africana* (leaves) (MIC = 1.00 mg/ml), *Pelargonium peltatum* (leaves) (MIC = 0.50 mg/ml), *Plectranthus ciliatus* (leaves) (MIC = 1.00 mg/ml), and *Ptaeroxylon obliquum* (bark) (MIC = 0.25 mg/ml) exhibited moderate activity against *P. aeruginosa*. The results for *Noltea africana* and *Plectranthus ciliatus* (MIC values of 1.00 mg/ml) are comparable with those of a previous study (Mohlakoana, 2020). The antimicrobial effects of several organic extracts of *Pelargonium peltatum* were highlighted in a study by Singab *et al.* (2015), where zones of inhibition (14 mm-20.5 mm) were observed against *P. aeruginosa* when tested in the disc diffusion assay. According to Singab *et al.* (2015), *Pelargonium peltatum* extracts possess moderate activity against *P. aeruginosa*, which validates the findings of the current study.

3.3.3 Antimicrobial activity of the organic plant extracts against *C. albicans*

Candida albicans was moderately susceptible to *Acalypha glabrata* (bark) with an MIC = 0.50 mg/ml, *Bauhinia bowkeri* (leaves) with an MIC = 1.00 mg/ml, *Calodendrum capense* (bark) with an MIC = 1.00 mg/ml, *Cyathula uncinulata* (root) with an MIC = 1.00 mg/ml, *Deinbollia oblongifolia* (leaves) with an MIC = 1.00 mg/ml, *Ilex mitis* (leaves) with an MIC = 1.00 mg/ml, *P. peltatum* (leaves) with an MIC = 0.50 mg/ml, *Plectranthus ciliatus* (leaves) with an MIC = 0.25 mg/ml, *Pouzolzia mixta* (leaves) with an MIC = 1.00 mg/ml, and *Ptaeroxylon obliquum* (bark) with an MIC = 1.00 mg/ml) (Table 3.3). *Acalypha glabrata*, *Calodendrum capense*, and *Pouzolzia mixta* have not previously been investigated for their antimicrobial efficacy against *C. albicans*. In a study by Mohlakoana (2020), *Cyathula uncinulata*, *Deinbollia oblongifolia*, *Ilex mitis*, and

Plectranthus ciliatus had weak activity (MIC values above 2.00 mg/ml) against *C. albicans*, which does not correspond with the findings in the current study. The variation in collection sites and seasons may account for the disparity between the antimicrobial properties observed in the prior study and the current one. According to Ncube *et al.* (2011), the bioactive compounds that contribute to the plant's antimicrobial efficacy vary with the different seasons of the year, thus resulting in fluctuating antimicrobial activity. In a study by Ahmed *et al.* (2012) and Singab *et al.* (2015), *Bauhinia bowkeri* (MIC values of 0.63 mg/ml) and *Pelargonium peltatum* (15 mm) showed moderate activity against *C. albicans*; thus, previous results correspond with those in the current study.

3.3.4 Overall antimicrobial effects of organic extracts on Gram-positive and Gram-negative micro-organisms

The Gram-positive bacteria with the greatest susceptibility to the tested organic extracts were *C. xerosis* (22%), followed by *B. linens* (16%) and *C. acnes* (15%), whereas the Gram-negative bacteria with the highest susceptibility were *P. aeruginosa* (6%) and *K. pneumoniae* (6%) (Figure 3.1). The difference in susceptibility of Gram-positive and Gram-negative bacteria to organic extracts is attributed to the presence of structural lipopolysaccharide components on the outer phospholipidic membrane in Gram-negative bacteria. Gram-negative bacterial cell walls become impermeable to lipophilic solutes, and porins on the outer membranes act as a selective barrier to hydrophilic solutes. On the contrary, Gram-positive bacteria only have an outer peptidoglycan layer, which is not an effective permeability barrier (Paiva *et al.*, 2010).

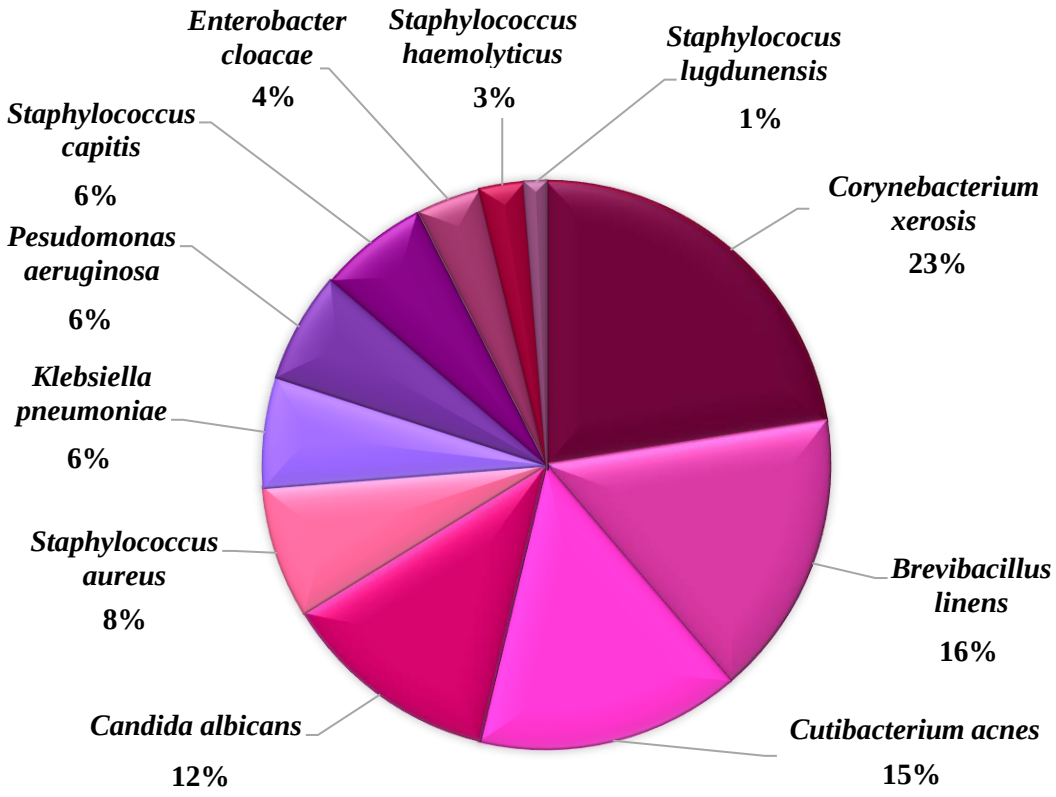


Figure 3.1: A summary of the susceptibility of different micro-organisms (≤ 1.00 mg/ml).

3.3.5 Antimicrobial activity of plants that demonstrated broad-spectrum activity

Pelargonium peltatum is traditionally used as a soap substitute to clean the skin (Afolayan *et al.*, 2014). According to Adams (2005), the petals are used to make a cleanser for greasy skin since they are astringent. The leaves have exhibited broad-spectrum activity against Gram-positive bacteria (*B. linens*, *C. acnes*, *C. xerosis*, *S. aureus*, and *S. capitis*) and Gram-negative bacteria (*E. cloacae*, *K. pneumoniae*, and *P. aeruginosa*), as well as *C. albicans*. Singab *et al.* (2015) observed that the leaves demonstrated activity against Gram-positive bacteria, Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *S. aureus*), and *C. albicans*. Although *E. coli* was not susceptible to the organic extract in the current study, *P. aeruginosa* was susceptible, indicating that the extract can disrupt the microbial membranes of Gram-negative bacteria and influence their permeability (Khameneh *et al.*, 2019).

The bark of *Ptaeroxylon obliquum* (bark) has been reported to be traditionally used to remove body odour and to wash wounds (Soyelu and Masika, 2009; Afolayan *et al.*, 2014). In this study, the bark of *Ptaeroxylon obliquum* organic extracts exhibited broad-spectrum activity against both Gram-positive (*C. acnes*, *B. linens*, *C. xerosis*, *S. aureus*, *S. capitis*, *S. haemolyticus*, and *S. lugdunensis*) and Gram-negative (*P. aeruginosa*) bacteria, as well as *C. albicans*. A previous study (Nielsen *et al.*, 2012) examined the antimicrobial effects of the bark and found that *C. albicans*, *E. coli*, and *S. aureus* were susceptible to the bark organic extract (MIC values of 0.16 mg/ml, 0.078 mg/ml, and 0.16 mg/ml, respectively). Therefore, this shows that the plant can inhibit both Gram-negative and Gram-positive bacteria, together with yeast. According to Ramadwa *et al.* (2019), the antimicrobial activity of the extract is influenced by compounds that have been isolated from the bark, such as pyrogallol-type tannins (hydrolysable tannin), peucenin, and coumarins (prenyletin and scopoletin).

Calodendrum capense (leaves) displayed broad-spectrum activity against Gram-positive bacteria (*B. linens*, *C. xerosis*, *C. acnes*, *S. aureus*, *S. capitis*, and *S. haemolyticus*) and a Gram-negative bacterium (*K. pneumoniae*) (Figure 3.2). In a study by Sakong (2012), *Calodendrum capense* leaves demonstrated broad-spectrum activity against a variety of Gram-negative and Gram-positive bacteria as well as *C. albicans*, thereby validating the findings in the current study. Traditionally, the leaves of *Calodendrum capense* are used in soap preparations and as a face mask (Mapunya *et al.*, 2012; Lall and Kishore, 2014). Therefore, the observed broad-spectrum activity justifies the use of the leaves as soap substitutes.

Traditionally, *Plectranthus ciliatus* is used to wash clothing and the skin to maintain personal hygiene (Watt and Breyer-Brandwijk, 1962; Busch, 2013). The organic extract of *Plectranthus ciliatus* exhibited broad-spectrum activity against *C. acnes*, *C. xerosis*, and *S. aureus*, a Gram-negative bacterium (*P. aeruginosa*), and *C. albicans*. Mohlakoana (2020) found that the leaves of *Plectranthus ciliatus* inhibited *C. acnes*, *P. aeruginosa*, and *C. albicans*, validating the findings in this study.

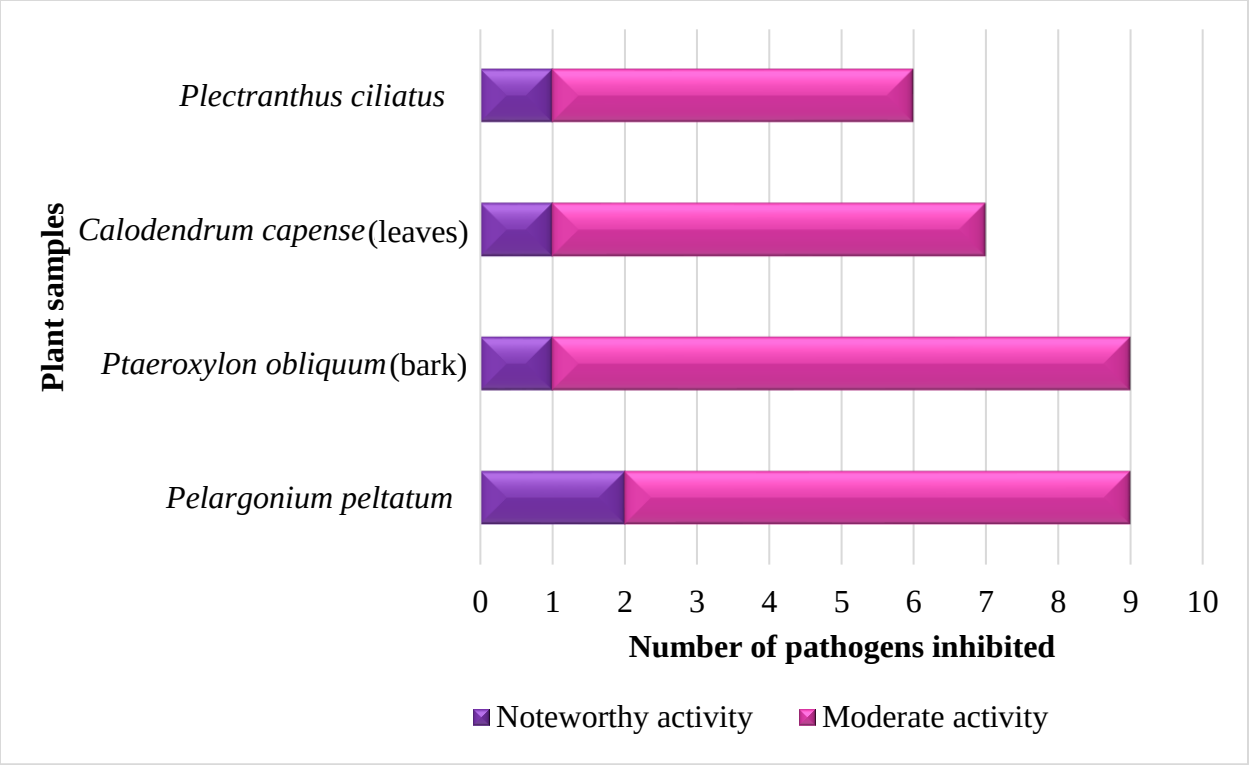


Figure 3.2: A summary of plant extracts that exhibited broad-spectrum activity.

3.4. Summary

- *Pelargonium peltatum* demonstrated the best antimicrobial activity against *B. linens* and *C. acnes* with an MIC value of 0.06 mg/ml.
- The organic extracts of *Calodendrum capense* (leaves), *Noltea Africana* (leaves), *Olea europaea* (leaves), *Pelargonium peltatum* (leaves), *Plectranthus ciliatus* (leaves), *Ptaeroxylon obliquum* (bark), and *Sideroxylon inerme* subsp. *inerme* (leaves) displayed noteworthy antimicrobial activity against the pathogen *C. acnes* with an MIC value of 0.06 mg/ml.
- Plants that demonstrated notable broad-spectrum activities are *Calodendrum capense*, *Pelargonium peltatum*, *Plectranthus ciliates*, and *Ptaeroxylon obliquum*.

CHAPTER 4
TOXICITY ANALYSIS OF SELECTED SOUTHERN AFRICAN PLANTS USED AS SOAP SUBSTITUTES

4.1 Introduction

Most herbal medicine is considered safe to use; however, some plants produce phytochemicals that are toxic for defence purposes and hence possess adverse effects on humans (Bose *et al*., 2021). Additionally, not all soap plants are primarily used to wash the skin. For example, *Noltea africana, Plectranthus ciliatus* and *Pouzolzia mixta* are soap plant that are also used in to wash clothes and sheep skin (Watt and Breyer-Brandwijk, 1962; Masupa, 2013). The importance of toxicity thus requires investigating to ensure safety on the skin. Therefore, regardless of their traditional use, this necessitates the need to conduct a toxicity study to assess the safety of these plants. In this study, the brine-shrimp lethality assay (BSLA) was used to determine the toxicity of the selected soap plants (Chapter 1, Table 1.2). The BSLA is a preliminary toxicity screening tool used to test the toxicity of plant extracts and bioactive compounds (Sarah *et al.*, 2017). It is an assay based on plant extracts or bioactive samples being able to kill *Artemia fransiscana* (brine-shrimp), thus the mortality rate is used to determine the toxicity of the plant extracts (Wu, 2014; Banti and Hadjikakou, 2021). This method is often used because it is a simple, easy-to-perform assay at a microscale and does not require expensive equipment and materials (Rahmatullah *et al.*, 2010). In this study, the assay outlined by Hübsch *et al.* (2014) was used. This chapter aimed to investigate the toxicity of selected 26 southern African plants used as soap substitutes. This was achieved by following these objectives:

- To make 2 mg/ml samples of an organic extract with 2% DMSO and 2 mg/ml samples of an aqueous extract with sterile water.
- To set up optimal growth conditions for *A. fransiscana*.
- To perform a toxicity response to *A. fransiscana* and determine the LC_{50} values.
- To determine a dose-response for plant samples demonstrating toxicity.

4.2 Methods and materials for Brine shrimp lethality bioassay

Artificial seawater was prepared by dissolving 16 g of Tropic Marine® salt in 500 ml of distilled water. Thereafter, 0.5 g of dried brine shrimp (*A. franciscana*) eggs (Ocean Nutrition™) were added to the artificial seawater. The mixture of brine shrimp eggs and artificial seawater was exposed to a light source and aerated using a rotary pump (Kiho) to promote better hatching. The mixture was also left at ambient temperature (25℃) for 24-48 h to allow the eggs to hatch. All the plant extracts were prepared to a concentration of 2 mg/ml using sterile water for aqueous extracts and 2% (v/v) DMSO for organic extracts. A starting concentration of 1 mg/ml was achieved after dilution.

The artificial seawater containing the hatched shrimp was transferred into a shallow, four-sided container, and then a lamp was placed next to the container, allowing maximum light exposure. This allowed the shrimp to gather in one place for easy collection. A volume of 400 µl artificial seawater containing brine shrimp (40–60) was added to each well of a 48-well micro-titre plate. Before adding the plant extracts, the feasibility of the brine shrimp was confirmed by observing it under a light microscope (Leica). Thereafter, a volume of 400 µl plant extract (organic and aqueous) was added in triplicate. Artificial seawater at a concentration of 32 mg/ml was used as the negative control since it simulates natural seawater. Furthermore, 1.6 mg/ml potassium dichromate (Sigma Aldrich) was used as the positive control. The initial number of dead shrimp after 24 and 48 h was recorded. After the 48-hr count, 50 µl glacial acetic acid (lethal to brine shrimp) was added to each well so that the number of shrimp in each well could be counted and the percentage mortality calculated using Equation 4.1. A percentage above 50 was regarded as toxic (Bussmann *et al.*, 2011).

$$\% \text{ mortality} = \frac{\text{dead shrimp at 48 hours (before acetic acid)-dead shrimp (time 0)}}{\text{dead shrimp (after acetic acid)}} \times 100$$

Equation 4.1

All the samples that had a percentage mortality greater than 50 were considered biologically toxic and were tested at concentrations of 1, 0.5, 0.25, 0.125, and 63 µg/ml to determine the highest concentrations at which the extracts would be non-toxic. Each of the dilutions was tested in triplicate in two separate experiments. The positive and negative controls were also included. The results from all experiments were averaged, and the data was represented in graphs and tables in Microsoft Excel 2019 (Version 2211).

The results were further analysed by determining the median lethal concentration (LC_{50}) at 95% confidence intervals (CI). The LC_{50} value is the concentration at which a test plant material possesses a toxic effect on 50% of the tested shrimp (Shirinda, 2019). The LC_{50} was estimated according to the method of Finney (1971) by plotting the mean results of brine shrimp mortality against the logarithms of concentrations using the probit analysis tool of the NCSS statistical software package. The LC_{50} values below 249 µg/ml were categorised as highly toxic, the values between 250 and 499 µg/ml as moderately toxic, and the values between 500 and 1000 µg/ml as having a low toxicity. Values greater than 1000 µg/ml were deemed non-toxic (Hübsch *et al.*, 2014).

4.3 Results and discussion

The results for the brine-shrimp lethality of 29 organic and aqueous extracts are shown in Table 4.1. At 24 h, 55.17% of the organic extracts were non-toxic, and 48.28% of the aqueous extracts were non-toxic. At 48 h, 37.93% of the organic extracts were non-toxic, whereas 34.48% of the aqueous extracts were non-toxic. In general, the aqueous extracts proved to be more toxic compared to the organic extract. The lowest percentage mortality was displayed by *Pouzolzia mixta* (leaves) organic and aqueous extracts (0.00%) at 24 h and 48 h. This is similar to the findings reported by McGaw *et al.* (2007).

Acalypha glabrata (leaves), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Deinbollia oblongifolia* (leaves), *Ledebouria luteola* (bulb), and *Sideroxylon inerme* subsp. *inerme* (leaves) had the lowest toxic effect for both organic and aqueous extracts at 24 and 48 h (Table 4.1). There are no previous toxicity studies that report on the toxicity of *Acalypha glabrata* and *Ledebouria*

luteola. Seleteng-Kose *et al.* (2019) support the non-toxic effect displayed by *Aloe maculata*, whereby the percentage mortalities of 4.82% (aqueous extract) and 13.23% (organic extract) after 24 h were reported. A study by Mohlakoana (2020) corroborated the findings herein, where a non-toxic effect was displayed by *Deinbollia oblongifolia*. The toxicity of *Bauhinia bowkeri* and *Sideroxylon inerme* subsp. *inerme* on brine-shrimp has not been evaluated in previous studies; however, the plant extracts have been tested on monkey kidney cell lines and mouse melanocytes, respectively (Momtaz *et al.*, 2008; Adeyemo *et al.*, 2022). Adeyemo *et al.* (2022) reported low toxicity for *B. bowkeri* (LC_{50} = 510 µg/ml), and Momtaz *et al.* (2008) reported low toxicity for *Sideroxylon inerme* subsp. *inerme* at a concentration lower than 500 µg/ml. Therefore, the results from previous studies corroborate with those of the current study, albeit with different toxicity studies.

Table 4.1: The average percentage mortality of 26 plant species extracts at 24 and 48 h.

Plant name	Organic extracts		Aqueous extracts	
	Average % mortality at 24 h	**Average % mortality at 48 h**	**Average % mortality at 24 h**	**Average % mortality at 48 h**
Acalypha glabrata (leaves)	**14.07[a]**	**16.82**	**10.84**	**19.24**
Acalypha glabrata (bark)	**25.45**	100	**11.97**	93.34
Albizia versicolor	**25.06**	**32.54**	100	100
Aloe ferox	**19.34**	96.01	**21.76**	89.70
Aloe maculata	**17.11**	**37.58**	**7.33**	**13.30**
Aristaloe aristata	**0.006**	**0.079**	100	100
Artemisia afra	75.70	98.09	61.01	100
Bauhinia bowkeri	**21.5**	**37.67**	**2.48**	**6.01**
Calodendrum capense (leaves)	**16.63**	**17.32**	73.41	100
Calodendrum capense (bark)	84.88	96.25	99.09	100
Carica papaya	**31.715**	69.62	100	100
Crinum bulbispermum	85.08	100	**43.75**	100
Cussonia paniculata	100	100	100	100
Cyathula uncinulata	**39**	95.83	70.19	98.82

Plant name	Organic extracts		Aqueous extracts	
	Average % mortality at 24 h	**Average % mortality at 48 h**	**Average % mortality at 24 h**	**Average % mortality at 48 h**
Deinbollia oblongifolia	**20.31**	**25.99**	**32.60**	**40**
Haemanthus albiflos	98.5	99.73	100	100
Hermannia cuneifolia	70.45	96.30	**0.61**	**31**
Ilex mitis	100	100	100	100
Ledebouria luteola	**20.72**	**23.81**	**7.58**	**28.07**
Ledebouria zebrina	64.67	97.94	100	100
Merwilla plumbea	89.44	99.54	76.34	100
Noltea africana	83.11	99	100	100
Olea europaea	85.06	98.80	**13.63**	87.70
Pelargonium peltatum	100	100	100	100
Plectranthus ciliatus	**37.69**	62.30	**2.38**	**2.98**
Pouzolzia mixta	**0**	**0**	**0**	**0**
Ptaeroxylon obliquum (leaves)	100	100	**8.38**	**17.41**
Ptaeroxylon obliquum (bark)	**32.12**	**42.63**	100	100
Sideroxylon inerme subsp. *inerme*	**21.88**	**22.68**	**17.22**	**33**
Positive control[b]	100	100	100	100
Negative control[c]	**0**	**0**	**0**	**0**

Key – [a] values in **bold** denote mortality less than 50% and are considered non-toxic; [b] 1.6 mg/ml potassium dichromate; [c] artificial seawater.

4.3.1 The LC_{50} values of plant samples that possess a toxic effect on 50% of the brine-shrimp

The LC_{50} values of organic extracts and aqueous extracts that possess a toxic effect on 50% of the brine-shrimp are presented in Table 4.2. The LC_{50} values below 249 µg/ml were categorised as highly toxic, the values between 250 and 499 µg/ml as moderately toxic, and the values between 500 and 1000 µg/ml as low toxicity. The values greater than 1000 µg/ml were deemed non-toxic

(Bussmann *et al.*, 2011). The organic and aqueous extracts of *Acalypha glabrata* (bark) and *Aloe ferox* (leaves) displayed toxicity after 48 h. *Acalypha glabrata* organic extract displayed no toxic effect with LC_{50} values of 4963.48 at 24 h and 4545.87 µg/ml at 48 h. The aqueous extract displayed low toxicity (LC_{50} = 842.33 µg/ml) at 24 h and moderate toxicity (LC_{50} = 377.28 µg/ml) at 48 h. *Aloe ferox* organic extract displayed no toxicity (LC_{50} = 1581.29 µg/ml) at 24 h; however, it became slightly toxic (LC_{50} = 578.71 µg/ml) when the exposure time was increased to 48 h. After 48 h, the aqueous extract was non-toxic, with an LC50 value of 1149.87 µg/ml. There are no toxicity studies that report the toxicity of *Acalypha glabrata*. In a study by Ghuman *et al.* (2016), *Aloe ferox* displayed an LC_{50} value of 1930 µg/ml at 24 h which corresponds with the findings in the current study. *Aloe ferox* preparation is generally considered safe; however, adverse effects such as hypersensitivity have previously been described (Chen *et al.*, 2012).

Table 4.2: The LC_{50} values of organic and aqueous plant extracts that displayed toxic effects at 24 and 48 h.

Plant name	**LC_{50} (µg/ml)**			
	Organic extracts		**Aqueous extracts**	
	24 h	**48 h**	**24 h**	**48 h**
Acalypha glabrata (bark)	4963.48	4545.87 (500)[a]	842.33	*377.28* (250)
Albizia versicolor	-[b]	-	760.71	570.04 (500)
Aloe ferox	1581.29	578.71 (500)	4071.19	1149.87 (250)
Aristaloe aristata	-	-	1694.12	*390.36* (250)
Artemisia afra	718.84	689.68 (500)	622.11	574.29 (250)
Calodendrum capense (leaves)	-	-	1009.60	863.57 (500)
Calodendrum capense (bark)	2549.65	1355.87 (500)	994.13	868.99 (500)
Carica papaya	8805.50	5700.50 (500)	1001.31	754.72 (500)
Crinum bulbispermum	**192.87**[c]	**89.81** (63)	**186.78**	**134.41** (125)
Cussonia paniculata	*368.26*	**42.18** (250)	1038.47	578.51 (500)
Cyathula uncinulata	732.44	*369.17* (250)	**217.10**	**148.56** (125)
Haemanthus albiflos	**190.88**	**109.19** (63)	522.73	**168.04** (125)
Hermannia cuneifolia	1642.79	722.13 (500)	-	-
Ilex mitis	*316.62*[d]	**175.60** (125)	*267.39*	**197.35** (125)

Plant name	LC_{50} (µg/ml)			
	Organic extracts		Aqueous extracts	
	24 h	48 h	24 h	48 h
Ledebouria zebrina	2217.27	767.25 (500)	777.81	682.32 (250)
Merwilla plumbea	627.73	588.28 (500)	*466.32*	**246.20** (125)
Noltea africana	2927.39	2105.59 (500)	*479.99*	**117.44** (63)
Olea europaea	1045.29	*363.12* (500)	1000.00	784.63 (500)
Pelargonium peltatum	*441.81*	**158.40** (125)	*343.61*	**180.90** (125)
Plectranthus ciliatus	1278.59	931.04 (500)	-	-
Ptaeroxylon obliquum (leaves)	*481.66*	*385.71* (500)	-	-
Ptaeroxylon obliquum (bark)	-	-	1134.19	*445.74* (250)

Key – [a] values in brackets reflect the highest non-toxic concentration when tested in the dose-response assay**; [b] (-)** = mortality less than 50% and are considered non-toxic; **[c]** values in **bold** signify highly toxic medicinal plants ($LC_{50} \leq$ 249 µg/ml); **[d]** values in *italics* signify moderately toxic (LC_{50} = 250-499 µg/ml)

The organic extracts of *Albizia versicolor* (bark) and *Aristaloe aristata* (leaves) displayed no toxic effect at 24 and 48 h; however, the aqueous extracts were toxic at 24 and 48 h. *Albizia versicolor* aqueous extract displayed low toxicity at 24 and 48 h (LC_{50} values of 760.71 µg/ml and 570.04 µg/ml, respectively). In a study by McGaw *et al.* (2005), the organic extracts of *Albizia versicolor* displayed no toxic effects against brine-shrimp after 24 h. *Aristaloe aristata* displayed a non-toxic effect at 24 h (LC_{50} = 1694.12 µg/ml) and moderate toxicity (LC_{50} = 390.36 µg/ml) at 48 h. These results are supported by Ghuman *et al.* (2016), where *Aristaloe aristata* had an LC_{50} value of more than 2000 µg/ml at 24 h. *Artemisia afra* (leaves) organic (LC_{50} = 718.84 µg/ml and 689.68 µg/ml) and aqueous (LC_{50} = 622.11 µg/ml and 574.29 µg/ml) extracts displayed low toxicity at 24 and 48 h. The leaf is reported to contain a toxic ketone called thujone (Wink and Van Wyk, 2008; Ndhlala *et al.*, 2013), hence the slight toxic effects that the plant extracts displayed in this study.

Calodendrum capense (leaves) displayed no toxic effect at 24 and 48 h; however, the aqueous extracts were toxic at 24 and 48 h. *Calodendrum capense* (leaves) aqueous extract displayed low toxicity (LC_{50} = 863.57 µg/ml). The brine-shrimp lethality assay has not been used in previous studies to test the lethality of *Calodendrum capense* (leaves). A previous study by Sakong (2012) reported that the acetone extracts of *Calodendrum capense* (leaves) exhibited low toxicity in the

C3A cells (LC_{50} = 83.08 µg/ml). The LC_{50} values of the bark organic *Calodendrum capense* extract displayed no toxicity (LC_{50} values of 2549.65 µg/ml at 24 h and 1355.87 µg/ml at 48 h), whereas the aqueous extracts displayed low toxicity at both 24 and 48 h (LC_{50} = 994.13 µg/ml and 868.99 µg/ml, respectively). The toxic effects of *Calodendrum capense* (bark) have not been determined previously.

Carica papaya (leaves) organic extract displayed no toxic effect at 24 and 48 h with LC_{50} values of 8805.50 µg/ml and 5700.50 µg/ml, respectively (Table 4.2b). The calculated LC_{50} values for the aqueous extract indicated that *Carica papaya* aqueous extract was non-toxic at 24 h (LC_{50} = 1001.31 µg/ml) and moderately toxic (LC_{50} = 754.72 µg/ml) at 48 h. In previous studies (Awolola *et al.*, 2010; Madjos and Luceño, 2019), *Carica papaya* aqueous extract displayed high toxicity (LC_{50} = 196.49 and 29.48 µg/ml, respectively). *Crinum bulbispermum* exhibited high toxic effects at 24 and 48 h. The organic extract displayed LC_{50} values of 192.87 µg/ml and 89.81 µg/ml, whereas the aqueous extract displayed LC_{50} values of 186.78 µg/ml and 134.41 µg/ml at 24 and 48 h. The toxicity of *Crinum bulbispermum* has been previously reported by Mohlakoana (2020). The whole plant is considered toxic due to alkaloids called crinamine and isoquinoline alkaloids, which are regarded as highly lethal (Van Wyk *et al.*, 2002; Ndhlala *et al.*, 2013). In a study by Aboul-Ela *et al.* (2004), the butanol fraction and alkaline extract demonstrated toxic effects with LD_{50} values of 63.1 µg/ml and 73 µg/ml, respectively. Therefore, the previous study supports the toxic effects of *Crinum bulbispermum* observed in this study.

The aqueous and organic extracts of *Cussonia paniculata* displayed a high percentage of mortality at 24 and 48 h. These findings correlate with previously recorded findings by Mugwena (2020), whereby *Cussonia paniculata* had a percentage mortality of 92.58% (organic) and 87.91% (aqueous) against brine-shrimp. The LC_{50} values indicate that the organic extract was moderately toxic (368.26 µg/ml) at 24 h and highly toxic (42.18 µg/ml) at 48 h. The aqueous extract demonstrated no toxicity at 24 h and low toxicity at 48 h with LC_{50} values of 1038.47 µg/ml and 578.51 µg/ml, respectively. According to Maroyi (2019), *Cussonia paniculata* is considered toxic since previous studies (Adedapo *et al.*, 2008; De Villiers *et al.*, 2010) reported that it caused 80% mortality in rats and moderate toxicity against a human T-cell leukaemia (Jurkat) cell line.

The aqueous extract of *Cyathula uncinulata* was toxic after 24 and 48 h, while the organic extract was toxic after 48 h. When the LC_{50} was calculated, the organic extract displayed moderate toxicity (LC_{50} = 394.26 µg/ml), whereas the aqueous extract demonstrated high toxicity (LC_{50} = 148.56 µg/ml) at 48 h. Since *Cyathula uncinulata* is toxic at higher concentrations (>125 µg/ml), lower doses (≤ 125 µg/ml) should be considered when the plant is used in traditional medicine. *Haemanthus albiflos* organic and aqueous extracts were extremely toxic, with LC_{50} values of 109.19 µg/ml and 168.04 µg/ml, respectively. The high toxic effects of *Haemanthus albiflos* are due to the presence of toxic alkaloids called homolycorine and albomaculine (Crouch *et al.*, 2005). The LC_{50} values displayed by *Ilex mitis* indicate that the organic and aqueous extracts were moderately toxic (316.62 µg/ml and 267.39 µg/ml) at 24 h and highly toxic (175.60 µg/ml and 197.35 µg/ml) at 48 h.

The aqueous extracts of *Hermannia cuneifolia* (leaves) displayed no toxic effect at 24 and 48 h; however, the organic extracts displayed toxic effects at 24 and 48 h. The calculated LC_{50} value for *Hermannia cuneifolia* demonstrated low toxicity (722.13 µg/ml), at 48 h. There are no previous studies that report on the toxic effects of *Hermannia cuneifolia* against brine shrimp. The low toxicity of *Hermannia cuneifolia* extracts is supported by a previous study, whereby *Hermannia cuneifolia* was non-toxic at an IC_{50} of >200 µg/ml on human kidney epithelium cells (Essop *et al.*, 2008). The high mortality rate displayed by *Ilex mitis* has been reported previously by Mohlakoana (2020), whereby the aqueous and organic extracts had a mortality percentage of 80.84% and 60.46%, respectively. The organic and aqueous extracts demonstrated moderate toxicity, with LC_{50} values of 316.62 and 267.39 µg/ml at 24 h. At 48 h, the extracts displayed high toxicity, with LC_{50} values of 175.60 and 197.35 µg/ml.

The organic and aqueous extracts of *Ledebouria zebrina* demonstrated low toxicity, with LC_{50} values of 767.25 µg/ml and 682.32 µg/ml, respectively. The toxicity of *Ledebouria zebrina* had not been previously tested in other studies. *Merwilla plumbea* organic extract displayed low toxicity, with LC_{50} values of 627.73 µg/ml and 588.28 µg/ml at 24 and 48 h, respectively. The aqueous extract was moderately toxic (LC_{50} = 466.32 µg/ml) at 24 h and highly toxic (117.44 µg/ml) at 48 h. *Merwilla plumbea* contains the toxic cardiac glycoside transvaalin, which is one of the compounds that contribute to the toxicity of the plant (Street and Prinsloo, 2012). The bulb has

been reported to cause itchiness and extreme irritation to the skin. Furthermore, the sap released from the plant can burn human skin (Notten, 2001; Street and Prinsloo, 2012). Therefore, precautions should be taken when the plant is used for its medicinal purposes, especially if considered for washing purposes.

Noltea africana organic extract displayed no toxic effects (2927.39 µg/ml and 2105.59 µg/ml) at 24 and 48 h; however, the aqueous extract displayed moderate toxicity (479.99 µg/ml) at 24 h and high toxicity (117.44 µg/ml) at 48 h. In a study conducted by Gado *et al.* (2021), *Noltea africana* was reported to have moderate toxicity against African green monkey kidney (Vero) cells with an LC_{50} value of 370 µg/ml. At 24 h, the LC_{50} of the *Olea europaea* organic extract displayed no toxicity (1045.29 µg/ml); however, at 48 h, moderate toxicity (LC_{50} = 363.12 µg/ml) was displayed. In a study by Rahman *et al.* (2015), the organic extract displayed moderate toxicity on brine shrimp with an LC_{50} value of 263.4 µg/ml. Therefore, previous results correlate with those of the current study.

The organic and aqueous extracts of *Pelargonium peltatum* displayed a percentage mortality rate of more than 50% at 24 and 48 h. The LC_{50} values revealed that at 24 h, the extracts were moderately toxic (organic = 441.81 µg/ml and aqueous = 343.61 µg/ml), and at 48 h, the extracts were highly toxic (organic = 158.40 µg/ml and aqueous = 180.90 µg/ml). The toxicity of *Pelargonium peltatum* has not been previously tested on brine-shrimp; however, it has been tested on MDCK cellular lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) toxicity assay. Moderate cytotoxicity was observed at < 100 µg/ml (Coronado-López *et al.*, 2018).

The aqueous extracts of *Plectranthus ciliatus* (leaves) displayed no toxic effect at 24 and 48 h; however, the organic extracts displayed toxic effects at 24 and 48 h. The calculated LC_{50} value for *Plectranthus ciliatus* demonstrated low toxicity (931.04 µg/ml) at 48 h. The non-toxic effects demonstrated by *Plectranthus ciliatus* aqueous extracts at 48 h and the organic extract at 24 h correspond with the results from a previous study (Mohlakoana, 2020), where percentage mortalities of 3.34% and 0.00% were obtained.

The aqueous extracts of *Ptaeroxylon obliquum* (leaves) displayed no toxic effects at 24 and 48 h; however, the organic extracts displayed toxic effects at 24 and 48 h. At 24 and 48 h, the bark of *Ptaeroxylon obliquum* did not have any toxic effects; however, the aqueous extracts were toxic at both 24 and 48 h. The calculated LC_{50} value for *Ptaeroxylon obliquum* leaves displayed moderate toxicity, with LC_{50} values of 385.71 µg/ml and 445.74 µg/ml, respectively, at 48 h. There are no previous studies that report on the toxic effects of *Ptaeroxylon obliquum* against brine shrimp. The low toxicity of *Ptaeroxylon obliquum* extracts is supported by previous studies, whereby *Ptaeroxylon obliquum* was non-toxic at a CC_{50} of 106.5 µg/ml on human kidney epithelium cells (Ramadwa *et al.*, 2021).

4.3.2 Dose-response of organic and aqueous extracts that demonstrated toxicity

A dose-response assessment was done on organic and/or aqueous extracts that displayed a percentage mortality that was above 50%. The results are presented in Appendix D1 and E2. The organic extracts of *Acalypha glabrata* (bark) and *Aloe ferox* (leaves) were non-toxic at a concentration of 500 µg/ml (Appendix D1), whereas the aqueous extracts were non-toxic at a concentration of 250 µg/ml (Appendix D2). At a concentration of 500 µg/ml, the organic extract of *Artemisia afra* displayed a low mortality rate, whereas the aqueous extract displayed a low mortality at a concentration of 250 µg/ml. *Albizia versicolor* (bark) and *Calodendrum capense* (leaves) aqueous extracts displayed no toxic effect against the brine-shrimp at 500 µg/ml. *Plectranthus ciliatus* (leaves) organic extract displayed no toxic effect at 500 µg/ml. The organic and aqueous extracts of *Calodendrum capense* (bark), *Carica papaya* (leaves) and *Olea europaea* (leaves) displayed a low mortality rate at 500 µg/ml.

After a dose-response assessment was conducted, the *Cussonia paniculata* organic extract displayed a low mortality percentage at a concentration of 250 µg/ml, while the aqueous extract displayed no toxic effect at a concentration of 500 µg/ml. Similar results were reported previously by Mugwena (2020). *Crinum bulbispermum* and *Haemanthus albiflos* organic extracts exhibited low mortality at a concentration of 63 µg/ml, and the aqueous extract displayed low toxicity against brine-shrimp at a concentration of 125 µg/ml. *Cyathula uncinulata* organic extract displayed no toxic effect at a concentration of 250 µg/ml, and the aqueous extract displayed no toxicity at a

concentration of 125 µg/ml after 48 h. Similar results were reported when Mohlakoana (2020) investigated the toxic effects of *Crinum bulbispermum* and *Cyathula uncinulata*. *Hermannia cuneifolia* (leaves), *Ledebouria zebrina* (bulb), *Merwilla plumbea* (bulb), *Noltea africana* (leaves), and *Ptaeroxylon obliquum* (leaves) organic extracts demonstrated low mortality at a concentration of 500 µg/ml. Mohlakoana (2020) corroborated the results obtained for *Merwilla plumbea. Ilex mitis* and *Pelargonium peltatum* organic and aqueous extracts were non-toxic at a concentration of 125 mg/ml after 48 h. At a concentration of 250 µg/ml, *Aristaloe aristata* (leaves), *Ledebouria zebrina* (bulb), and *Ptaeroxylon obliquum* (bark) aqueous extracts displayed a low mortality percentage (Appendix D2). The aqueous extracts of *Merwilla plumbea* and *Noltea africana* displayed no toxic effects at 125 µg/ml and 63 µg/ml, respectively.

4.4 Summary

- The aqueous plant extracts were more toxic (65.52%) compared to the organic plant extracts (62.07%).
- *Pouzolzia mixta* organic and aqueous extracts were the least toxic (0.00%) at 24 and 48 h against the brine-shrimp.
- *Acalypha glabrata* (leaves), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Deinbollia oblongifolia* (leaves), *Ledebouria luteola* (bulb), and *Sideroxylon inerme* subsp. *inerme* (leaves) were non-toxic at 24 and 48 h.
- At 48 h, *Aristaloe aristata* (leaves), *Calodendrum capense* (leaves), and *Ptaeroxylon obliquum* (bark) organic extracts were non-toxic, and *Hermannia cuneifolia* (leaves), *Plectranthus ciliatus* (leaves), and *Ptaeroxylon obliquum* (leaves) aqueous extracts were non-toxic.
- *Crinum bulbispermum*, *Haemanthus albiflos*, *Ilex mitis*, and *Pelargonium peltatum* were highly toxic, with LC_{50} values > 250 µg/ml after 48 h.
- *Calodendrum capense* (leaves) and *Ptaeroxylon obliquum* (bark) organic extracts demonstrated no toxic effect at 24 and 48 h against the brine-shrimp and were thus considered for soap formulation.
- *Pelargonium peltatum* demonstrated non-toxicity at a concentration of 125 µg/ml and was also considered for soap formulation.

CHAPTER 5
HERBAL SOAP FORMULATION OF THREE SELECTED PLANTS USED AS SOAP SUBSTITUTES

5.1 Introduction

Most commercially available soaps in southern Africa are infused with plants such as *Aspalathus linearis* (Burm.f.) R. Dahlgren and *Cyclopia genistoides* (L) R. Br., which are not plants traditionally used as soap substitutes (Madzinga *et al*., 2018). Hence, the formulation of a natural herbal soap using southern African soap plants will promote public awareness about soap plants and encourage wider usage. Herbal soaps are prepared by adding dried plants or plant extracts to a basic soap base (Devipriya *et al.*, 2021). As soaps aid in general body hygiene, herbal soaps should possess the ability to remove micro-organisms adhering to the skin. *Calodendrum capense* (leaves), *Pelargonium peltatum* (leaves) and *Ptaeroxylon obliquum* (bark) organic extracts exhibited broad-spectrum antimicrobial activity (Chapter 3) against most of the tested pathogens, least toxicity (except for *Pelargonium peltatum* which exhibited a dose-response with no toxicity exhibited at 125 µg/ml) (Chapter 4) and moderate saponin content (Chapter 2). These plant organic extracts were selected to formulate three herbal soaps. This chapter aimed to formulate herbal soaps containing *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* extracts and then analyse the physicochemical and antimicrobial properties of the formulations. The following objectives were designed:

- To formulate basic and herbal soaps.
- To assess the physicochemical parameters of the formulations.
- To assess the antimicrobial efficacy of the formulated herbal soaps.

5.2 Methods and materials

5.2.1 Basic and herbal soap formulation

Methods by Mak-Mensah and Firempong (2011) and Ruckmani *et al.* (2014) were followed with modifications. The basic soap was prepared by adding 3.5 g of sodium hydroxide pellets (Associated Chemical Enterprise, South Africa), 12.5 ml of distilled water, and 12.5 ml of ethanol (Associated Chemical Enterprise, South Africa) together and stirring until dissolved. The solution was mixed with 10 g of preheated (100℃) coconut oil (Pick n Pay, South Africa). The mixture was stirred gradually for 20-30 min until the saponification process was complete. The mixture was allowed to cool and then filtered with Whatman No. 2 filter paper (Lasec). Impurities were removed by washing the soap paste with 300 ml of saturated sodium chloride (Merck, South Africa). The basic soap base was allowed to solidify for an hour for consistency. The process was performed in triplicate.

One soap bar was made at a higher concentration (10 mg/ml), and one was made at a lower concentration (5 mg/ml) of the plant extract. To make the soap at a higher concentration, 0.75 g of organic plant extract (Chapter 3, Section 3.2.1) was added to 75 ml of melted soap base, and to make a bar soap at a lower concentration, 0.38 g of organic extract was added to 75 ml of soap base. Thereafter, 0.033 g of stearic acid (Saarchem Holpro Analytic (Pty) Ltd., South Africa) was added to 1 ml of hot water and poured into each bar of melted soap. A solution was made from 1 g of sodium lauryl sulphate (Saarchem Holpro Analytic (Pty) Ltd., South Africa) and 5 ml of distilled water. A few drops of the solution were added to the melted soap. This was done to enhance the performance and stability of the soap. The mixture was stirred for 30 min; thereafter, the paste was moulded into a circular shape and allowed to solidify at room temperature. A commercial herbal soap (Protex®) was used as a positive control, and the formulated basic soap (without plant extract) was used as a negative control.

5.2.2 Validation of the physicochemical properties of the soap

The colour and appearance were checked by visual inspection against a white background. The odour was evaluated for any undesirable qualities. The pH was determined by dissolving 1 g of

the formulated soap in 10 ml of distilled water and measuring it using a previously calibrated pH metre (Orion Star A212; Thermo Fisher Scientific Inc., South Africa). These parameters of the herbal soap formulation were assessed in duplicate on consecutive days to assess the stability and standard of the formulation (Akuaden *et al.*, 2019).

5.2.2.1 Determining foam height and foam retention

The height of the foam was determined by dispersing 0.50 g of soap in 25 ml of distilled water. The solution was transferred into a 100 ml measuring cylinder, and the volume was made up to 50 ml with distilled water. The measuring cylinder was covered and shaken 25 times, and the foam height above the aqueous volume was measured. The foam retention was determined by dissolving 1 g of soap in 100 ml of distilled water, and then 25 ml was transferred into a 100 ml measuring cylinder. The cylinder was covered and shaken ten times. Thereafter, the foam retention was recorded at a one-minute interval for 10 min.

5.2.2.2 Determining moisture content and total fatty matter content

The moisture content was determined by weighing 5 g of soap and transferring it to a pre-weighed evaporating dish. The evaporating dish was placed in an oven (Memmert, Germany) at 101℃ for 2 h and repeated until a constant weight was reached. The moisture content was calculated in percentage using Equation 5.1, where W_S = weight of evaporating dish and sample, W_E = weight of the evaporating dish, and W_L = weight of evaporating dish with sample after evaporation:

$$\% \text{ moisture} = \frac{W_S - W_L}{W_S - W_E} \times 100$$

Equation 5.1

The total fatty matter (TFM) was determined using a method by Betsy *et al.* (2013). Five grams of soap was dissolved in 100 ml of hot distilled water. Thereafter, 40 ml of 0.5 N nitric acid (Merck (Pty) Ltd., South Africa) was added to the solution to make it acidic. The solution was heated until the visualisation of a dispersed layer of fatty acids. Thereafter, the solution was placed in ice water

to solidify the fatty acids, which were then placed in a pre-weighed beaker. The aqueous layer was treated with 50 ml of chloroform (Associated Chemical Enterprise, South Africa) to remove the remaining fatty acids. The fatty matter (bottom layer) was separated using a separating funnel and then mixed with the previously separated fatty acids. The excess solvent was evaporated, and the yield was recorded. The TFM was calculated as a percentage using Equation 5.2.

$$\% \text{ fatty matter} = \frac{(\text{weight of beaker and soap after drying-weight of the beaker})}{\text{weight of soap sample}} \times 100$$

Equation 5.2

5.2.2.3 Free caustic alkali and percentage chloride

Free caustic alkali (FCA) was determined by dissolving 5 g of soap in 30 ml of ethanol. A few drops of phenolphthalein indicator (Merck (Pty) Ltd., South Africa) were added to the solution, along with 10 ml of 20% barium chloride (Merck Chemicals (Pty) Ltd., South Africa). The solution was titrated against 0.05 M sulphuric acid (Associated Chemical Enterprise, South Africa). The FCA was calculated as a percentage using Equation 5.3.

$$\text{Free caustic alkali (\%)} = \frac{0.31}{\text{weight of soap}} \times \text{volume of acid}$$

Equation 5.3

The percentage of chloride in the soap was determined by dissolving 10 g of soap in 100 ml of distilled water. Thereafter, 20 ml of 15% calcium nitrate (Associated Chemical Enterprise, South Africa) was added to the soap to ensure that the soap was completely dissolved. The solution was then made up to 250 ml with distilled water. Using a Whatman No. 2 filter paper, the solution was filtered, and a few drops of methyl orange (Merck (Pty) Ltd, South Africa) were added to 100 ml of filtrate. Thereafter, the solution was titrated against 10 N sulphuric acid until a pink colour was obtained. The pink solution was titrated against 0.1 N silver nitrate (Sigma-Aldrich, South Africa) using potassium dichromate (Sigma-Aldrich, South Africa) as an indicator until a brink red colour was obtained. The percentage of chloride was calculated using Equation 5.4.

$$\% \text{ chloride} = \frac{\text{titre volume}}{\text{weight of soap}} \times 0.585$$

Equation 5.4

5.2.3 Validation of the antimicrobial efficacy

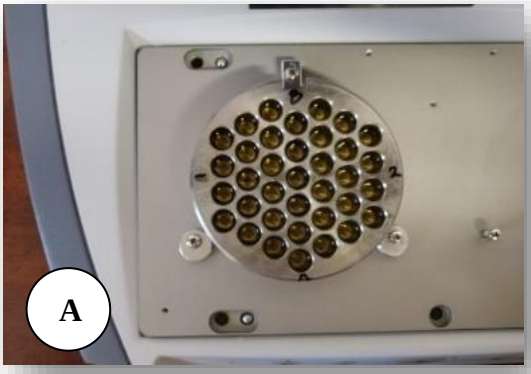
The MIC of the herbal soap was assessed using the agar dilution method. This method involves the incorporation of serially diluted concentrations of the antimicrobial agent into the agar medium using two-fold serial dilutions, followed by inoculation of the selected micro-organisms on the agar plate. The lowest concentration at which the antimicrobial agent can completely inhibit the growth of the micro-organism is recorded as the MIC endpoint (Balouiri *et al.*, 2016).

5.2.3.1 Preparation of the soap-agar plates

A method by Beech *et al.* (1955) was used with modifications. The herbal soaps, basic soap, and commercial soap were weighed in different quantities to achieve the different concentrations (25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.13 mg/ml, and 1.57 mg/ml) and then added to 20 ml of TSA (Oxoid, UK). Forty grams of TSA was suspended in 1 L of sterilised distilled water and then autoclaved for 15 min at 121℃. Thereafter, 20 ml of molten agar was poured into a beaker with the soap and placed on the Bunsen burner. The solution was stirred until the soap completely melted and there was homogenous distribution. The agar-soap solution was then poured into labelled Petri dishes and allowed to cool.

The test micro-organisms were inoculated onto the different soap concentrations containing agar plates using the multipoint-elite inoculator (Mast Group Ltd., UK). A multipoint inoculator is an equipment that can inoculate multiple micro-organisms on an agar plate simultaneously with high precision (Kapen *et al.*, 2019). One millilitre of a 24-hr growing culture (Chapter 3, Table 3.1) was transferred into approximately 10 ml of sterile broth until appearing just turbid to get a 0.5 McFarland turbidity standard. Thereafter, the stock culture was made at a 1: 100 ratio; that is, 100 µl of the McFarland standard in 10 ml of sterile TBS. The 1:100 cultures were each inoculated on

the labelled agar-soap plate, as indicated in Figure 5.1. The plates were left to stand for 30 min to ensure the inoculum spots were absorbed into the agar-soap plates before incubation. All the agar-soap plates inoculated with the pathogens highlighted in Figure 5.2 were incubated at 37°C for 18-24 h. The agar-soap plates inoculated with *B. linens* were incubated at 30°C for four days, while the plates inoculated with *C. xerosis* were incubated at 30°C for 48 h. Plates inoculated with *C. albicans* were incubated at 37°C for 48 h. Culture control plates were inoculated first and last to ensure the growth of the test micro-organisms. The MICs were done in triplicate to ensure accuracy.



One milliliter of each of the 1:100 culture was added to the wells in triplicate.

The agar-soap plate was placed on the petri dish holder while the metal inoculators were in starting position.

The metal inoculators were submerged into the inoculum.

The inoculum was transferred onto the agar-soap plate.

Figure 5.1: Method of how the cultures were inoculated on the surface of the agar-soap plates.

5.3 Results and discussion

The formulated herbal soaps containing *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* are shown in Figure 5.2. A colour change was observed when the plant extracts were added to the basic soaps. The soap with *Calodendrum capense* changed from white to green colour, while the *Pelargonium peltatum* soap changed to a light brown colour, and the *Ptaeroxylon obliquum* soap changed to a creamy white colour. The texture and appearance of *Calodendrum capense* and *Pelargonium peltatum* were equivalent to commercially bought soaps, whereas *Ptaeroxylon obliquum* soap had a soft texture compared to commercially bought soap. The soaps had a pleasant smell that would be favourable for use. It was important to investigate each soap independently since the parameters vary between the different plants when incorporated into the basic soap base.

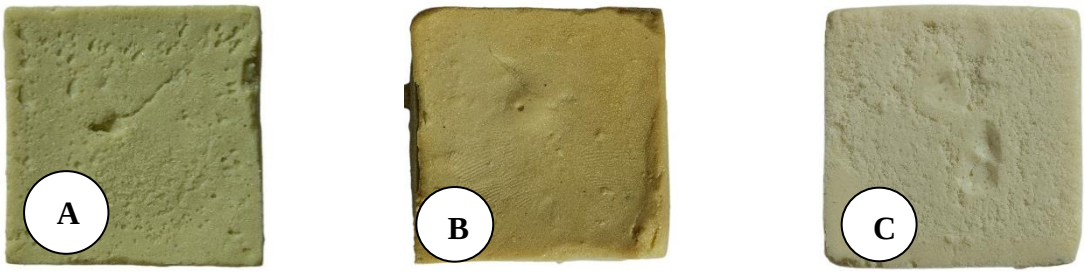


Figure 5.2: The three formulated herbal soaps. **A** - *Calodendrum capense* herbal soap, **B** - *Pelargonium peltatum* herbal soap and **C** - *Ptaeroxylon obliquum* herbal soap.

5.3.1 Physicochemical properties of the formulated herbal soap

The results of the evaluated physicochemical parameters are given in Table 5.1. The *Ptaeroxylon obliquum* herbal soap had the highest pH value of 11.49, followed by *Pelargonium peltatum* herbal soap with a pH value of 10.86, and *Calodendrum capense* herbal soap with a pH value of 10.79. The pH of the basic soap was 10.76, slightly lower than the pH of the herbal soaps, thus suggesting that adding the plant extracts did not significantly alter the pH of the basic soap. The pH of *Ptaeroxylon obliquum* herbal soap is comparable with the pH of the control commercial soap, Protex (11.05). The pH values of *Pelargonium peltatum* and *Calodendrum capense* are similar to

those obtained by Vivian *et al.* (2014) for commercial soaps such as Sunlight (10.80) and Ndume (a commercial soap sold in Kenya) (10.65). Previous studies (Ruckmani *et al.*, 2014; Akuaden *et al.*, 2019; Sindhu *et al.*, 2019) noted that formulated herbal soaps reported that the pH ranged from 6.50-11.9. According to Mak-Mensah and Firempong (2011), the normal pH range for soap is 8-11.

Foam height was assessed to determine the ability of the soaps to form foam. Foam formation indicates soap stability and detergency (Sindhu *et al.*, 2019). The foam height for the *Pelargonium peltatum* and *Calodendrum capense* soaps was consistent with the foam height for the control soap, Protex, which was 20 mm. *Ptaeroxylon obliquum* soap had the lowest foam height, which indicated that the soap was the least stable of the three that were formulated. Foam retention is the time for which the foam produced by a soap is retained. The longer the foam is retained, the better the soap's stability and foaming capacity (Akuaden *et al.*, 2019). The herbal soaps had a foam retention time of 10 min while the basic soap had the lowest foam retention time of 8 min which indicated that the foam of the herbal soaps was more stable. The foam retention time observed for the herbal soaps is similar to that of a herbal soap formulated by Sindhu *et al.* (2019).

Moisture content is a characteristic used to determine the shelf life of the soap (Vivian *et al.*, 2014). *Pelargonium peltatum* and *Ptaeroxylon obliquum* herbal soaps had the highest level of moisture content (30%), followed by *Calodendrum capense* herbal soap (28%). According to Sindhu *et al.* (2019), the greater the moisture content, the faster the soap will deteriorate. This is because the excessive moisture content in the soap would allow excess water to react with un-saponified fat to produce free fatty acids and glycerol during storage, in a process called hydrolysis (Akuaden *et al.*, 2019). The recommended percentage limits for moisture content in soaps, according to the Encyclopaedia of Industrial Chemical Analysis (EICA), are from 10% to 15% (Vivian *et al.*, 2014; Akuaden *et al.*, 2019). Although the moisture content of the herbal soaps does not fall within the limits of EICA, the results are supported by a previous study (Ogunsuyi and Akinnawo, 2012), where a moisture content of 29.05% was obtained.

The total fatty matter (TFM) represents the quality and shelf life of the soap. *Pelargonium peltatum* herbal soap had the highest TFM of 84%, while *Calodendrum capense* herbal soap had a TFM of

80%. The high TFM observed supports the high moisture content (30%) that the herbal soaps displayed. A TFM of 80% is good for dry skin since it rehydrates the skin, making it smooth. The excess oil in soap acts as a lubricant that lasts on the skin throughout the day (Mak-Mensah and Firempong, 2011). *Ptaeroxylon obliquum* herbal and basic soap had the lowest TFM (52 and 50%, respectively). According to Mak-Mensah and Firempong (2011), low TFM is due to residual sodium hydroxide in the soap. The International Standard Organisation (ISO) recommends that soaps with TFM above 76% are classified as the first grade, while soaps with TFM between 65% and 75% are classified as second grade, and soaps with TFM below 65% are classified as third grade (Betsy *et al.*, 2013; Mwanza and Zombe, 2020). Based on the recommended grading, *Pelargonium peltatum* and *Calodendrum capense* herbal soaps fall under the first grade, and *Ptaeroxylon obliquum* falls under the third grade.

The free caustic alkali is a parameter used to determine the ability of the soap to irritate due to an incomplete saponification process (Mak-Mensah and Firempong, 2011). In the current study, *Ptaeroxylon obliquum* herbal soap had the highest free caustic alkalinity (FCA) value of 0.16%, followed by *Pelargonium peltatum* herbal soap with an FCA value of 0.06%, and then *Calodendrum capense* herbal soap with an FCA value of 0.03%. According to Mwanza and Zombe (2020), a high-quality soap should have minimal or no free caustic alkali. Therefore, these results explain the low quality observed for *Ptaeroxylon obliquum* herbal soap. Excessive free alkali irritates the skin, resulting in scaly skin that is vulnerable to fungal infection. Mwanza and Zombe (2020) proposed that the free alkali content of a skin-care soap should be less than 0.1%. As a result, all the formulated soaps and control soap fall under the recommended value (less than 0.1%), except for the *Ptaeroxylon obliquum* herbal soap.

Excess amounts of chloride in the soap cause it to crack, thus it is important to determine the percentage of chloride levels. In this study, *Pelargonium peltatum* soap had high levels of chloride content (0.18%), followed by *Ptaeroxylon obliquum* soap (0.12%), and then *Calodendrum capense* soap (0.06%). The results are similar to those of Akuaden *et al.* (2019) for herbal soaps. The high chloride content in *Pelargonium peltatum* herbal and basic soap is caused by excess chloride retained in the soap when the sodium hydroxide pellets were dissolved during the saponification process (Ruckmani *et al.*, 2014).

Table 5.1: Physicochemical properties of formulated herbal soaps.

Type of soap	pH	Foam height (cm)	Foam retention (mins)	Moisture content (%)	Total fatty matter	Free caustic alkali (%)	% Chloride
Calodendrum capense herbal soap	**10.79[a]**	20	10	28	**80[c]**	**0.03[d]**	**0.06[e]**
Pelargonium peltatum herbal soap	**10.86**	20	10	30	**84**	**0.06**	0.18
Ptaeroxylon obliquum herbal soap	11.49	8	10	30	52	0.16	0.12
Negative control (Basic soap)	10.76	20	8	18	50	**0.03**	0.29
Positive control (Protex)	11.05	20	15	**12[b]**	**82**	**0.06**	0.15

Key – [a] pH in **bold**: falls within the pH range for herbal formulated soaps in other publications; **[b]** Moisture content in **bold**: falls within the EICA limits. **[c]** TFM in **bold**: first-grade soap; **[d]** FCA values in **bold** are less than 0.1%; **[e]** %Cl in bold is less than 0.1%

5.3.2 Antimicrobial efficacy of formulated herbal soaps

The mean MIC values of each formulated soap and the controls are depicted in Table 5.2. The lowest feasible weight was 0.063 g to make up a concentration of 1.57 mg/ml. Hence, concentrations lower than 1.57 mg/ml were not investigated to determine the MIC endpoint for herbal soaps and were thus represented as <1.57 mg/ml. Figure 5.3 indicates the growth of tested Gram-positive and Gram-negative bacteria on a plate that contains no soap. Figure 5.4 indicates inhibition of all the tested micro-organisms except for *Enterobacter cloacae,* at the concentration of 12.5 mg/ml which was the same when compared to the control, Protex commercial soap. The three herbal soaps were active against *A. baumannii* at MIC values of ≤ 1.57 mg/ml. Hence, the potency of the *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* herbal soaps against *A baumannii* could be used to inhibit this micro-organism implicated as the most prevalent causative agent of severe cellulitis and other skin and soft tissue infections (Ali et al., 2014).

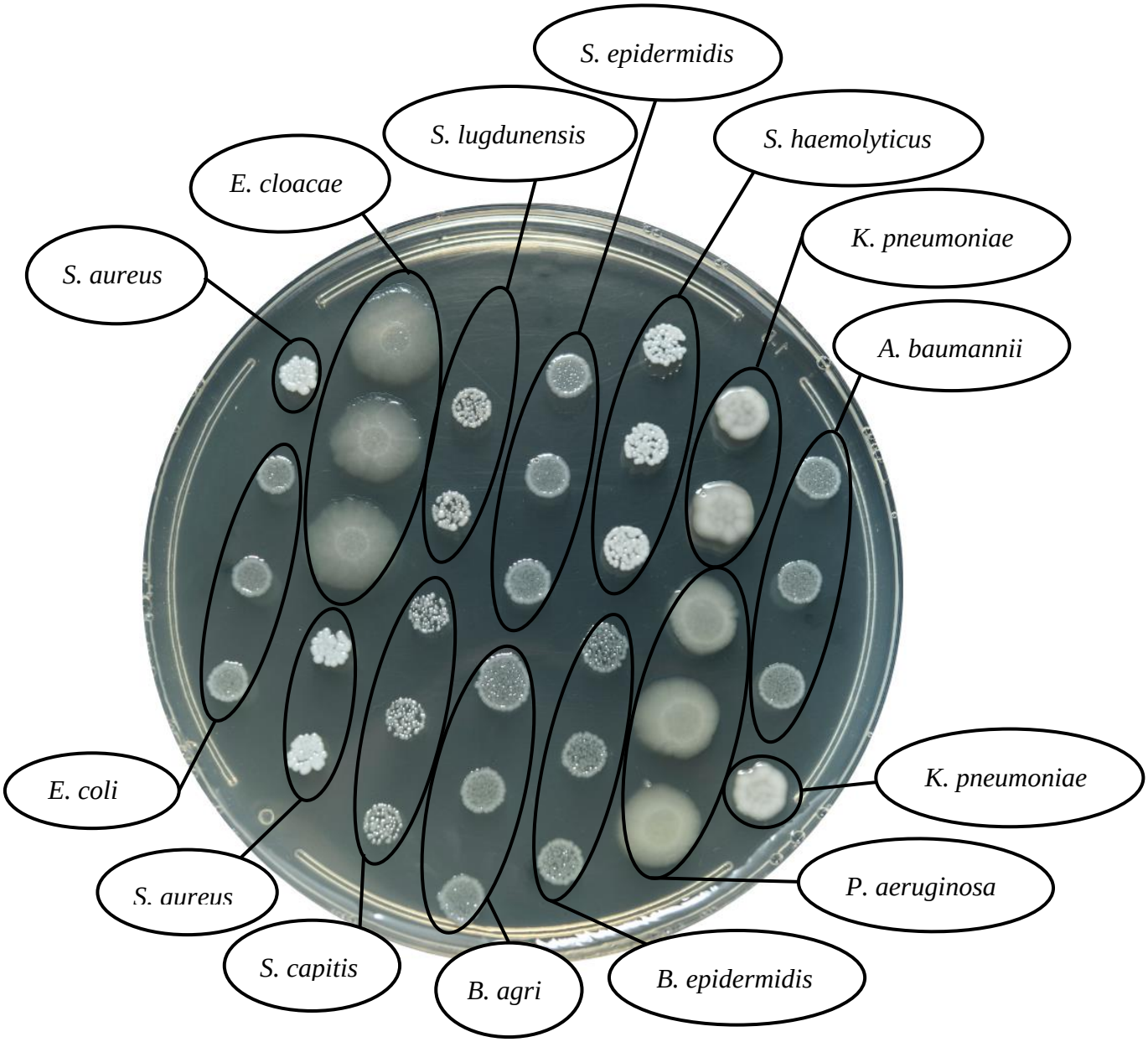


Figure 5.3: Indication of how the micro-organisms grew on the culture control agar-soap plate.

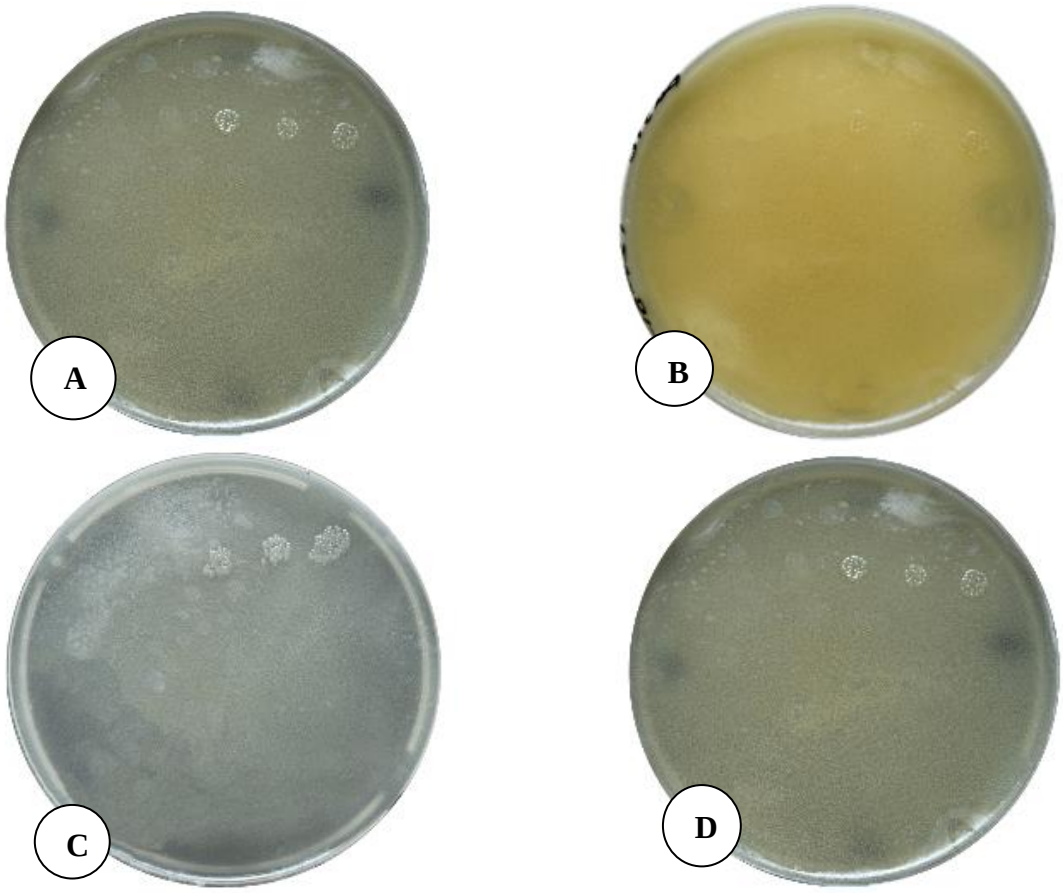


Figure 5.4: The herbal soaps inhibited all the tested micro-organisms at the concentration of 12.5 mg/ml except for *E. cloacae* which is the same for the control (Protex).

Key – **A:** *Calodendrum capense* herbal soap in agar; **B:** *Pelargonium peltatum* herbal soap in agar; **C:** *Ptaeroxylon obliquum* herbal soap in agar; **D:** Positive control (Protex).

Candida albicans was inhibited by all three herbal soaps (MIC values of ≤ 1.57 mg/ml). *Candida albicans* resides on the skin as a commensal micro-organism; however, when there is an accumulation of carbon dioxide, increased friction, and humidity, it can cause superficial infections of the skin (Mayer *et al.*, 2013; Kühbacher *et al.*, 2017). Therefore, using herbal soaps on the skin will aid in general body hygiene by removing or inhibiting *C. albicans* that adheres lightly to the skin, thus reducing the incidence of Candida-related skin infections (Igbeneghu, 2013).

All Gram-positive micro-organisms were inhibited (MIC values of ≤ 1.57 mg/ml) except for *Staphylococcus aureus*. The MIC values displayed by the herbal soaps against *S. aureus* ranged

from 3.13–12.50 mg/ml. The notable antimicrobial activity of herbal soaps is significant as skin infections such as acne, impetigo, furuncles, and carbuncles are caused by Gram-positive micro-organisms (Igbeneghu, 2013). The incorporation of the plant extracts in the basic soap enhanced the activity of the soaps against Gram-positive bacteria because the basic soap displayed MIC values of 3.13 mg/ml and the herbal soaps displayed lower MIC values of ≤ 1.57 mg/ml. Ayobami *et al.* (2017) observed similar results after incorporating plant extracts into a basic soap. The results observed for all the herbal soaps against *B. epidermidis* and *S. capitis*, *S. epidermidis*, *S. haemolyticus*, and *S. lugdunensis* were comparable with the results obtained for the positive control (MIC values of ≤ 1.57 mg/ml). The three herbal soaps were found to have superior antimicrobial activity against *B. linens* and *C. xerosis* compared to the positive control (Protex).

Studies that have previously investigated the antimicrobial activity of formulated herbal soaps have used the agar well diffusion method. However, the focus has been on a few skin pathogens, namely, *C. albicans*, *E. coli*, *P. aeruginosa*, and *S. aureus*. The current study was more comprehensive since multiple pathogens, such as *Corynebacterium xerosis, Staphylococcus capitis, Staphylococcus haemolyticus,* and *Staphylococcus lugdunensis* were tested, and the herbal soaps were able to display broad-spectrum activity. In a study by Ruckmani *et al.* (2014), Gram-positive bacteria (*S. aureus*) were more susceptible to a formulated herbal soap that contained *Vitex negundo* Linn. extract compared to the Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Additionally, *C. albicans* was also susceptible. These results support the susceptibility of the Gram-positive bacteria and yeast in the current study. To the best of my knowledge, this is the first time that *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* were successful incorporated into soap with broad-spectrum antimicrobial properties.

Table 5.2: The antimicrobial activity of three formulated herbal soaps.

Micro-organism	Type of soap							
	Calodendrum capense		***Pelargonium peltatum***		***Ptaeroxylom obliquum***		**Positive control (Protex)**	**Negative control (Basic soap)**
	Plant extract concentration (mg/ml)							
	5	**10**	**5**	**10**	**5**	**10**		
	Minimum inhibitory concentration (mg/ml)							
Acinetobacter baumannii (ATCC 19606)	3.13	**< 1.57**[a]	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**1.57**	3.13
Brevibacterium agri (ATCC 51663)	4.69	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**1.57**	3.13
Brevibacterium epidermidis (ATCC 20660)	**< 1.57**	**< 1.57**	**1.57**	**< 1.57**	**1.57**	**< 1.57**	**1.57**	3.13
Brevibacterium linens (ATCC 20425)	**1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	12.50	3.13
Candida albicans (ATCC 10231)	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	6.25	3.13
Corynebacterium xerosis (ATCC 373)	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	6.25	3.13
Enterobacter cloacae (ATCC 13047)	25.00	6.25	25.00	12.50	25.00	6.25	25.00	12.50
Escherichia coli (ATCC 8739)	12.50	6.25	12.50	12.50	12.50	6.25	25.00	12.50
Klebsiella pneumoniae (ATTC 13883)	12.50	6.25	12.50	12.50	6.25	6.25	25.00	12.50
Pseudomonas aeruginosa (ATCC 27853)	12.50	6.25	12.50	12.50	6.25	6.25	18.75	12.50
Staphylococcus aureus (ATCC 25923)	6.25	4.69	12.50	9.38	6.25	4.69	3.13	9.38
Staphylococcus capitis (ATCC 146)	**1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	3.13
Staphylococcus epidermidis (ATCC 12228)	**3.13**	**< 1.57**	**1.57**	**< 1.57**	**1.57**	**< 1.57**	**< 1.57**	3.13
Staphylococcus haemolyticus (ATCC 29970)	**1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**1.57**	3.13
Staphylococcus lugdunensis (ATCC 49576)	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	**< 1.57**	3.13

Key – [a] values in **bold** denote noteworthy activity

Several studies (Ruckmani *et al.*, 2014; Afsar and Khanam, 2016; Sindhu *et al.*, 2019) have investigated the formulation of herbal soaps using various medicinal plants. In a study by Sindhu *et al.* (2019), the antimicrobial properties of a herbal soap at various concentrations against *E. coli* and *S. aureus* were determined. The results revealed that the zones of inhibition increased with the increased concentration of the soap in the agar (MIC values ranged from 12 to 21 mm for *E. coli* and from 14 to 29 mm for *S. aureus*). A study by Afsar and Khanam (2016) determined the antimicrobial properties of formulated herbal soaps containing various concentrations of plant extracts against *E. coli*, *S. aureus*, and *P. aeruginosa*. The herbal soaps that contained higher concentrations of plant extract demonstrated greater zones of inhibition. This supports the increase in antimicrobial activity exhibited by herbal soaps with 10 mg/ml plant extracts in the current study. *Cassia alata* Linn. is traditionally used in eastern Nigeria as a soap substitute since it possesses lathering properties due to secondary metabolites such as saponins, alkaloids, and flavonoids (Esimone *et al*., 2007; Madzinga *et al*., 2018). In a study by Esimone *et al*. (2007), *Cassia alata* ethanolic extract was infused in a soap, and the antimicrobial activity of the herbal soap was tested against *Bacillus subtilis*, *E. coli*, *C. albicans*, *P. aeruginosa*, and *S. aureus*. The soap displayed noteworthy activity against the test pathogens.

5.4 Summary

- The plant extracts *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* were successfully used to make three herbal soaps.
- When the physical and chemical parameters were evaluated and compared to what was expected, *Pelargonium peltatum* herbal soap came out on top, followed by *Calodendrum capense* herbal soap and *Ptaeroxylon obliquum* herbal soap.
- *Pelargonium peltatum* herbal soap proved to have better quality and shelf life, followed by *Calodendrum capense* herbal soap, and then *Ptaeroxylon obliquum* herbal soap.
- When examining the antimicrobial activity, all three herbal soaps inhibited the pathogens at a concentration of 12.50 mg/ml except for *E. cloacae.*

CHAPTER 6
SUMMARY AND CONCLUSION

6.1 Overview of study

A literature review was conducted, and over 59 plants from southern Africa were identified for bathing and washing the skin. Saponin-rich plants had been previously reported to have exceptional physicochemical and biological attributes, making them a potential source of natural surfactants for both research and commercial applications (Rai *et al.*, 2021). However, it was noted that limited scientific data validates the phytochemical and antimicrobial activity of several plants identified in the literature review. It was also observed from the ethnobotanical literature that the toxicity of several plants had not previously been assessed. Therefore, this study was undertaken to assess the antimicrobial activity, toxicity, and phytochemistry of selected plant species. Thereafter, a selection of plant species with favourable properties was formulated into effective antimicrobial herbal soaps and assessed for efficacy.

All the objectives were achieved and summarized as follows:

Objective 1: To undertake a preliminary phytochemical screening, a qualitative screening (thin layer chromatography (TLC)) and quantitative screening to determine the presence of saponins.

Saponins were detected in the aqueous (27 extracts) and ethanol (24 extracts). Methanol and acetone were the optimal solvents to extract alkaloids from 18 extracts. Ethanol extracted terpenoids best (22 extracts), followed by methanol (20 extracts). Using TLC, steroidal saponins that had displayed a variety of saponins Rf-values comparable to diosgenin (a steroidal aglycone used as a standard) were observed. *Acalypha glabrata* (bark), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Hermannia cuneifolia* (leaves), *Merwilla plumbea* (bulb), *Noltea africana* (leaves), *Pelargonium peltatum* (leaves), and *Sideroxylon inerme* subsp. *inerme* (leaves) were found to have a high saponin content (> 40 mg/g).

Objective 2: To determine the antimicrobial activity of the selected plants using the MIC assay.

Gram-positive bacteria were more susceptible to organic plant extracts than Gram-negative bacteria. *Brevibacterium linens* and *Cutibacterium acnes* were susceptible to *Pelargonium peltatum* organic extracts at an MIC value of 0.06 mg/ml. The organic extracts of *Calodendrum capense* (leaves), *Noltea africana*, *Olea europaea* (leaves), *Pelargonium peltatum* (leaves), *Plectranthus ciliatus* (leaves), *Ptaeroxylon obliquum* (bark), and *Sideroxylon inerme* subsp. *inerme* (leaves) displayed noteworthy antimicrobial activity against the pathogen *C. acnes* with an MIC value of 0.06 mg/ml. *Calodendrum capense* (leaves), *Pelargonium peltatum*, *Plectranthus ciliatus*, and *Ptaeroxylon obliquum* demonstrated broad-spectrum activity against most of the tested pathogens.

Objective 3: To determine the toxicity of the selected soap plants.

The organic plant extracts displayed less toxic (62.07%) compared to the aqueous plant extracts (65.52%). At 24 and 48 h, *Acalypha glabrata* (leaves), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Deinbollia oblongifolia* (leaves), *Ledebouria luteola* (bulb), *Pouzolzia mixta* (leaves), and *Sideroxylon inerme* subsp. *inerme* (leaves) organic and aqueous extracts demonstrated the lowest toxic effects. Furthermore, the organic extracts of *Aristaloe aristata* (leaves), *Calodendrum capense* (leaves), and *Ptaeroxylon obliquum* (bark) were non-toxic, and the aqueous extracts of *Hermannia cuneifolia* (leaves), *Plectranthus ciliatus* (leaves), and *Ptaeroxylon obliquum* (leaves) were non-toxic. The extracts that were highly toxic with LC_{50} values > 250 µg/ml after 48 h are *Crinum bulbispermum* (bulb), *Haemanthus albiflos* (bulb), *Ilex mitis* (leaves), and *Pelargonium peltatum* (leaves). The dose-response results revealed that at lower concentrations, the toxic plants have a low mortality percentage.

Objective 4: To formulate herbal soaps by the saponification process and assess the physicochemical parameters of the formulations by determining the percentage of free caustic alkali, moisture content, pH, foam retention, and total fatty matter, as well as the antimicrobial efficacy.

The plant species selected for soap formulation were dependent on optimum antimicrobial activity, low activity, and notable saponin content. *Acalypha glabrata* (bark), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Hermannia cuneifolia* (leaves), and *Sideroxylon inerme* subsp. *inerme* (leaves) displayed non-toxic effects and high saponin content; however, exhibited narrow-spectrum antimicrobial activity. Although the plants had high saponin concentrations and the least toxicity, due to their lack of antimicrobial activity, the plants were not selected for herbal soap formulation. *Merwilla plumbea* (bulb) and *Noltea africana* (leaves) displayed high saponin content, moderate toxicity, and narrow-spectrum antimicrobial activity. These results show that high saponin concentrations do not equate to noteworthy antimicrobial activity or low toxicity; hence, these plants were also not used in soap formulation.

Calodendrum capense (leaves), *Pelargonium peltatum* (leaves), and *Ptaeroxylon obliquum* (bark) demonstrated broad-spectrum activity against most of the tested pathogens. *Calodendrum capense* and *Ptaeroxylon obliquum* organic extracts were non-toxic and had moderate saponin content, whereas *Pelargonium peltatum* had a high saponin content of 43.61 ± 1.65 mg/g and was non-toxic at a concentration of 125 µg/ml. These plants were selected for herbal soap formulation as they exhibited the most ideal properties from the selection studied. Table 6.1 shows the overall summary of the phytochemistry, antimicrobial activity, and toxicity of these three selected plants for soap formulation.

Traditionally, *Calodendrum capense* is used in soap preparations, as an ingredient in skin ointments, and for skin-hyperpigmentation problems. It is also traditionally known for its use to lighten and clean the skin (Mapunya *et al.*, 2012). In Xhosa communities, the bark of *Ptaeroxylon obliquum* is traditionally used to remove body odour. The bark infusion is applied through bathing (Soyelu and Masika, 2009; Afolayan *et al.*, 2014). *Pelargonium peltatum*, commonly known as ivy geranium, is a perennial herb that is traditionally used to clean the skin (Afolayan *et al.*, 2014). According to Adams (2005), the petals are astringent and are used to make a wash for greasy skin. Furthermore, the leaves are used as an antiseptic for burns, grazes, scratches, and wounds (Adams, 2005). Previous studies have highlighted the antimicrobial activity of these plants against a few pathogens, namely *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*.

Table 6.1: Overall summary of the phytochemistry, antimicrobial activity and toxicity of the three most ideal plants for soap formulation.

Plant name	Phytochemistry				Antimicrobial activity	Toxicity		Soap formulation
	Qualitative			Quantitative				
	A	T	S	Saponins		Dose-response	LC_{50} (µg/ml)	
Calodendrum capense (leaves)	Yes	Yes	Yes	24.59 ± 0.25	Displayed broad-spectrum activity (MIC values ≤ 1.00 mg/ml) against *B. linens*, *C. xerosis*, ***C. acnes***[a], *K. pneumoniae*, *S. aureus*, *S. capitis* and *S. haemolyticus.*	Aqueous extract – non-toxic at 500 µg/ml Organic extract – non-toxic at 1000 µg/ml	Aqueous extract – low toxicity (LC_{50}: 754.72)	pH – 10.79; FR – stable; Moisture – 28%; TFM – 80% (first grade); FCA – 0.03%; %Cl – 0.06%. Displayed broad-spectrum activity (MIC values ≤ 1.57 mg/ml) against *B. epidermidis*, *B. linens*, *C. albicans*, *C. xerosis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus* and *S. lugdunensis.*
Pelargonium peltatum (leaves)	Yes	Yes	Yes	43.61 ± 1.65	Displayed broad-spectrum activity (MIC values ≤ 1.00 mg/ml) against ***B. linens***,	Aqueous and organic extract – non-	Aqueous extract – high toxicity	pH – 10.86; FR – stable Moisture – 30%; TFM – 84% (first grade); FCA – 0.03%; %Cl – 0.18%.

Plant name	Phytochemistry				Antimicrobial activity	Toxicity		Soap formulation
	Qualitative			Quantitative				
	A	T	S	Saponins		Dose-response	LC_{50} (µg/ml)	
					C. acnes, *C. albicans*, *C. xerosis*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. capitis*.	toxic at 125 µg/ml	(LC_{50}: 180.90) Organic extract – high toxicity (LC_{50}: 158.40)	Displayed broad-spectrum activity (MIC values ≤ 1.57 mg/ml) against *A. baumannii*, *B. agri*, *B. epidermidis*, *B. linens*, *C. albicans*, *C. xerosis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus* and *S. lugdunensis.*
Ptaeroxylon obliquum (bark)	Yes	Yes	Yes	17.28 ± 0.76	Displayed broad-spectrum activity (MIC values ≤ 1.00 mg/ml) against *B. linens*, ***C. acnes***, *C. xerosis*, *P. aeruginosa*, *S. aureus*, *S. capitis*, *S. haemolyticus* and	Aqueous extract – non-toxic at 500 µg/ml Organic extract – non-toxic at (1000 µg/ml)	Aqueous extract – moderate toxicity (LC_{50}: 445.74)	pH – 11.49; FR – stable; Moisture – 30%; TFM – 52% (third grade); FCA – 0.16%; %Cl – 0.12% Displayed broad-spectrum activity (MIC values ≤ 1.57 mg/ml) against *A. baumannii*,

Plant name	Phytochemistry				Antimicrobial activity	Toxicity		Soap formulation
	Qualitative			Quantitative				
	A	T	S	Saponins		Dose-response	LC_{50} (µg/ml)	
					S. lugdunensis.			*B. agri*, *B. epidermidis*, *B. linens*, *C. albicans*, *C. xerosis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus* and *S. lugdunensis.*

Key – [a] micro-organisms in **bold** = plant demonstrated noteworthy activity against it; A: alkaloids, T: terpenoids, S: saponins; FR: foam retention, TFM: total fatty matter, FCA: free caustic alkali, %Cl: percentage of chloride.

The current study investigated more skin-relevant pathogens, thereby contributing to the knowledge of the antimicrobial properties of these plants. Furthermore, according to our knowledge, this study highlighted for the first time the toxicity of these plants against brine shrimp and their concentration of saponins.

The plant extracts *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum* were successfully used to make three herbal soaps. *Pelargonium peltatum* herbal soap proved to have better quality and shelf life, followed by *Calodendrum capense* herbal soap, and then *Ptaeroxylon obliquum* herbal soap. All the tested micro-organisms except for *Enterobacter cloacae* were inhibited at a concentration of 12.5 mg/ml, which is the same when compared to the control, Protex commercial soap.

6.2 Limitations

Firstly, undertaking this study under COVID-19 lockdown restrictions was found to be challenging, as access to laboratory facilities was at times restricted and at other times delayed. The maceration extraction method produces a low saponin yield; hence, the yield and saponin content were not directly proportional. Since the vanillin-sulphuric acid assay quantifies the total saponins from the reaction of oxidised triterpene saponins with vanillin, a standard that is in the triterpenoid saponin group (oleanolic acid, soya saponin, Quillaja saponin, and ginsenoside) should be used; however, only a diosgenin (steroid saponins) was available for this study. Another limitation was that the antimicrobial activity of the three formulated soaps on *C. acnes* couldn't be tested since the soap had to be melted in agar for the method used to test antimicrobial activity. The pathogen does not grow readily on agar.

6.3 Recommendations

Saponins are important secondary metabolites, especially in plants used as soap substitutes. To confirm the presence of different types of saponins, acid hydrolysis should be carried out before TLC evaluation. This is so that aglycones, glycosides, and sugar molecules can be separated and visualised by their different TLC mobilities, and the type of saponins present can be determined.

This requires acid, bases, or various enzymes to effect hydrolysis. Additionally, advanced chromatographic equipment such as HPLC and ultra-pressure liquid chromatography (UPLC) should be used to identify specific saponins found in the plants. The extracts contain a variety of compounds other than saponins, compared to saponin-enriched extracts. Future studies should be done to evaluate the antimicrobial activity of saponin-enriched extracts such as *Acalypha glabrata* (bark), *Aloe maculata* (leaves), *Bauhinia bowkeri* (leaves), *Hermannia cuneifolia* (leaves), and *Sideroxylon inerme* subsp. *inerme* (leaves). The effects of the southern African soap plants in combination should also be considered, as they might yield promising antimicrobial results that would culminate in an ideal mixture for soap formulation. The BSLA is a simple, easy-to-perform preliminary assay; however, other assays, such as the MTT, should be considered to assess plant toxicity on cell lines. The interaction of soap with skin surfaces and the efficacy of soap plants in removing micro-organisms (biofilm) from skin surfaces should be further analysed by using the crystal violet (CV) staining assay. In terms of soap formulation, the three selected plant species were considered for inclusion as a three-plant mixture in the soap; however, it was important to determine if ideal parameters first existed for the soaps individually before merging the extracts in one soap. In this study, *Ptaeroxylon obliquum* soap was not ideal since it had a high pH, low TFM, and high FCA. Future studies should consider combining *Calodendrum capense* and *Pelargonium peltatum* to determine if a synergistic effect is apparent between the two plant species that would enhance the properties of a better soap formulation. Herbal soaps aid in general body hygiene, and they should possess the ability to remove micro-organisms adhering slightly to the skin.

6.4 Conclusion

The results obtained in this study from the vanillin-sulphuric acid assay support the use of several southern African plants as soap substitutes. The plants that exhibited high saponin concentrations (*Acalypha glabrata*, *Aloe maculata*, *Bauhinia bowkeri*, *Hermannia cuneifolia*, and *Sideroxylon inerme* subsp. *inerme*) were also non-toxic. Out of the three favourable plants, namely *Calodendrum capense*, *Pelargonium peltatum*, and *Ptaeroxylon obliquum*, the *Pelargonium peltatum* organic extract inhibited most pathogens. Furthermore, the *Pelargonium peltatum* herbal soap parameters were within acceptable limits, proving that the soap had better quality and shelf life compared to *Calodendrum capense* and *Ptaeroxylon obliquum* herbal soaps. This study

contributes to the knowledge of the antimicrobial properties, phytochemical properties, and toxicity of plants used in southern Africa as soap substitutes. The positive findings will aid in future endeavours investigating the feasibility of soap plants used in southern Africa.

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APPENDIX A: PRESENTATIONS

A1: The 24th Indigenous Plant Use Forum (IPUF) conference 2022 – University of Johannesburg, South Africa. 4–7 July 2022. [online presentation].

Antimicrobial and phytochemical validation of southern African plants used as soap substitutes.

Nyiko F. Mzimba[a], Annah Moteetee[b] and Sandy van Vuuren[a]

[a] *Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa*

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Abstract: A human body's primary defence against micro-organisms is the skin. It is colonized by harmless symbiotic micro-organisms. However, some pathogens invade the skin and cause infections. Therefore, good hygiene is an important principle that has to be exercised to prevent skin infection. Soaps are regarded as good surfactants that can inhibit invading pathogens. The study aimed to investigate the antimicrobial activity, phytochemistry and toxicity of plants used in southern Africa as soap substitutes. In addition, a soap formulation was made to assess efficacy. Organic and aqueous extracts were prepared from 26 species and antimicrobially screened against 16 pathogens. The minimum inhibitory concentration (MIC) was determined. *Calodendrum capense* (L.f.) Thunb., *Pelargonium peltatum* (L.) L'Hér. and *Ptaeroxylon obliquum* (Thunb.) Radlk. were found to display the most noteworthy broad-spectrum activity (MIC values ≤ 1.00 mg/ml). Qualitative phytochemical analysis was conducted to determine the presence of saponins and alkaloids in the plant species. Using the froth test, *Acalypha glabrata* Thunb. and *Olea europaea* L. subsp. *africana* (Mill.) P.S. Green displayed a positive saponin reaction. The presence of alkaloids was detected in *Aloe ferox* Mill., *Noltea africana* (L.) Endl., and *Sideroxylon inerme* L. subsp. *inerme* acetone, methanol and aqueous extracts using Wagner's test. The brine-shrimp lethality assay was used as a toxicity screening tool. *Acalypha glabrata*, *Bauhinia bowkeri* Harv.

and *Ptaeroxylon obliquum* organic and aqueous extracts are non-toxic. A soap formulation containing the three most suitable species (*Calodendrum capense*, *Pelargonium peltatum* and *Ptaeroxylon obliquum*) and efficacy was noted and enhanced compared to the commercial control soap purchased. This study comprehensively validates the use of selected southern African medicinal plants as soap substitutes.

A2: Faculty of Health Sciences Research Day, 15 September 2022, University of the Witwatersrand, Johannesburg.

Validating the antimicrobial activity, phytochemistry and toxicity of southern African plants used as soap substitutes.

Nyiko F. Mzimba[a], Annah Moteetee[b] and Sandy van Vuuren[a]

[a] *Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa*

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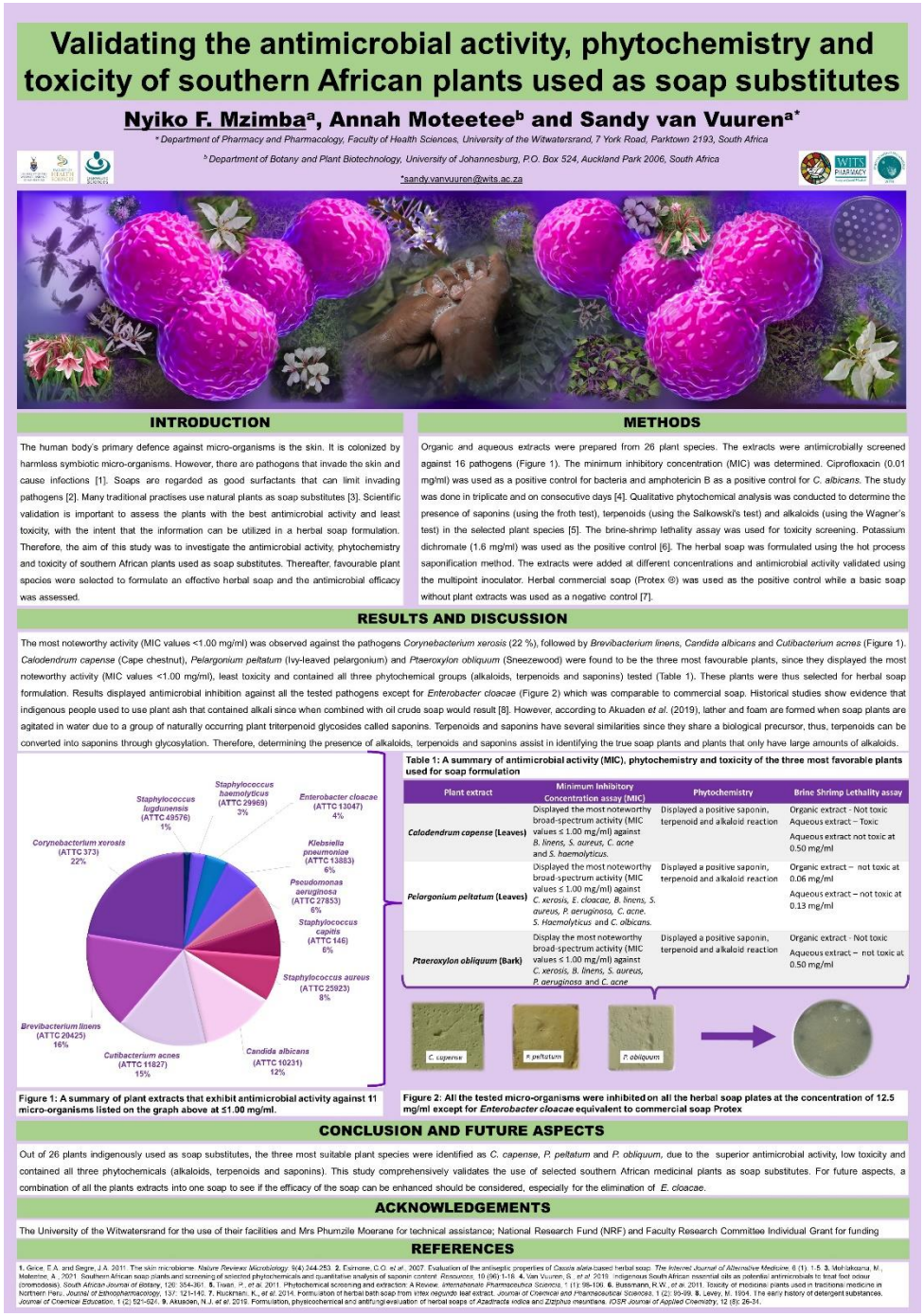
The human body's primary defence against micro-organisms is the skin. It is colonized by harmless symbiotic micro-organisms. However, some pathogens invade the skin and cause infections. Soaps are regarded as good surfactants that can inhibit invading pathogens. Many traditional practices use natural products as soap substitutes. This study aimed to investigate the antimicrobial activity, phytochemistry and toxicity of plants used in southern Africa as soap substitutes. In addition, a soap formulation was made to assess efficacy. Organic and aqueous extracts were prepared from 26 species and antimicrobially screened against 16 pathogens. The minimum inhibitory concentration (MIC) was determined. *Calodendrum capense* (Cape chestnut), *Pelargonium peltatum* (Ivy-leaved pelargonium) and *Ptaeroxylon obliquum* (Sneezewood) were found to display the most noteworthy broad-spectrum activity (MIC values ≤ 1.00 mg/ml). Qualitative phytochemical analysis was conducted to determine the presence of saponins and alkaloids in the plant species. Using the froth test *Acalypha glabrata* (Forest false nettle) and *Olea europaea* subsp. *africana* (Wild olive) displayed a positive saponin reaction. The presence of alkaloids was detected using Wagner's test in *Aloe ferox* (Soap aloe), *Noltea africana* (Soap bush), and *Sideroxylon inerme* subsp. *inerme* (White milkwood) extracts made up of acetone, methanol, and aqueous solvents. The brine-shrimp lethality assay was used as a toxicity screening tool. *Acalypha glabrata*, *Bauhinia bowkeri* (Kei White Bauhinia), and *P. obliquum* organic and aqueous extracts were found to be non-toxic. A soap formulation containing the three most suitable species (*Calodendrum capense*, *Pelargonium peltatum* and *Ptaeroxylon obliquum*) was made and efficacy

was noted and enhanced compared to the commercial control soap purchased. This study comprehensively validates the use of selected southern African medicinal plants as soap substitutes.

Keywords: Soap plants, skin, soap substitutes, antimicrobial activity



Validating the antimicrobial activity, phytochemistry and toxicity of southern African plants used as soap substitutes

Nyiko F. Mzimba[a], Annah Moteetee[b] and Sandy van Vuuren[a*]

[a] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa

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INTRODUCTION

The human body's primary defence against micro-organisms is the skin. It is colonized by harmless symbiotic micro-organisms. However, there are pathogens that invade the skin and cause infections [1]. Soaps are regarded as good surfactants that can limit invading pathogens [2]. Many traditional practises use natural plants as soap substitutes [3]. Scientific validation is important to assess the plants with the best antimicrobial activity and least toxicity, with the intent that the information can be utilized in a herbal soap formulation. Therefore, the aim of this study was to investigate the antimicrobial activity, phytochemistry and toxicity of southern African plants used as soap substitutes. Thereafter, favourable plant species were selected to formulate an effective herbal soap and the antimicrobial efficacy was assessed.

METHODS

Organic and aqueous extracts were prepared from 26 plant species. The extracts were antimicrobially screened against 16 pathogens (Figure 1). The minimum inhibitory concentration (MIC) was determined. Ciprofloxacin (0.01 mg/ml) was used as a positive control for bacteria and amphotericin B as a positive control for *C. albicans*. The study was done in triplicate and on consecutive days [4]. Qualitative phytochemical analysis was conducted to determine the presence of saponins (using the froth test), terpenoids (using the Salkowski's test) and alkaloids (using the Wagner's test) in the selected plant species [5]. The brine-shrimp lethality assay was used for toxicity screening. Potassium dichromate (1.6 mg/ml) was used as the positive control [6]. The herbal soap was formulated using the hot process saponification method. The extracts were added at different concentrations and antimicrobial activity validated using the multipoint inoculator. Herbal commercial soap (Protex ®) was used as the positive control while a basic soap without plant extracts was used as a negative control [7].

RESULTS AND DISCUSSION

The most noteworthy activity (MIC values <1.00 mg/ml) was observed against the pathogens *Corynebacterium xerosis* (22 %), followed by *Brevibacterium linens*, *Candida albicans* and *Cutibacterium acnes* (Figure 1). *Calodendrum capense* (Cape chestnut), *Pelargonium peltatum* (Ivy-leaved pelargonium) and *Ptaeroxylon obliquum* (Sneezewood) were found to be the three most favourable plants, since they displayed the most noteworthy activity (MIC values <1.00 mg/ml), least toxicity and contained all three phytochemical groups (alkaloids, terpenoids and saponins) tested (Table 1). These plants were thus selected for herbal soap formulation. Results displayed antimicrobial inhibition against all the tested pathogens except for *Enterobacter cloacae* (Figure 2) which was comparable to commercial soap. Historical studies show evidence that indigenous people used to use plant ash that contained alkali since when combined with oil crude soap would result [8]. However, according to Akuaden *et al.* (2019), lather and foam are formed when soap plants are agitated in water due to a group of naturally occurring plant triterpenoid glycosides called saponins. Terpenoids and saponins have several similarities since they share a biological precursor, thus, terpenoids can be converted into saponins through glycosylation. Therefore, determining the presence of alkaloids, terpenoids and saponins assist in identifying the true soap plants and plants that only have large amounts of alkaloids.

Figure 1: A summary of plant extracts that exhibit antimicrobial activity against 11 micro-organisms listed on the graph above at ≤1.00 mg/ml.

Table 1: A summary of antimicrobial activity (MIC), phytochemistry and toxicity of the three most favorable plants used for soap formulation

Plant extract	Minimum Inhibitory Concentration assay (MIC)	Phytochemistry	Brine Shrimp Lethality assay
***Calodendrum capense* (Leaves)**	Displayed the most noteworthy broad-spectrum activity (MIC values ≤ 1.00 mg/ml) against *B. linens, S. aureus, C. acne* and *S. haemolyticus.*	Displayed a positive saponin, terpenoid and alkaloid reaction	Organic extract - Not toxic Aqueous extract – Toxic Aqueous extract not toxic at 0.50 mg/ml
***Pelargonium peltatum* (Leaves)**	Displayed the most noteworthy broad-spectrum activity (MIC values ≤ 1.00 mg/ml) against *C. xerosis, E. cloacae, B. linens, S. aureus, P. aeruginosa, C. acne, S. Haemolyticus* and *C. albicans.*	Displayed a positive saponin, terpenoid and alkaloid reaction	Organic extract – not toxic at 0.06 mg/ml Aqueous extract – not toxic at 0.13 mg/ml
***Ptaeroxylon obliquum* (Bark)**	Display the most noteworthy broad-spectrum activity (MIC values ≤ 1.00 mg/ml) against *C. xerosis, B. linens, S. aureus, P. aeruginosa* and *C. acne*	Displayed a positive saponin, terpenoid and alkaloid reaction	Organic extract - Not toxic Aqueous extract – not toxic at 0.50 mg/ml

Figure 2: All the tested micro-organisms were inhibited on all the herbal soap plates at the concentration of 12.5 mg/ml except for *Enterobacter cloacae* equivalent to commercial soap Protex

CONCLUSION AND FUTURE ASPECTS

Out of 26 plants indigenously used as soap substitutes, the three most suitable plant species were identified as *C. capense*, *P. peltatum* and *P. obliquum*, due to the superior antimicrobial activity, low toxicity and contained all three phytochemicals (alkaloids, terpenoids and saponins). This study comprehensively validates the use of selected southern African medicinal plants as soap substitutes. For future aspects, a combination of all the plants extracts into one soap to see if the efficacy of the soap can be enhanced should be considered, especially for the elimination of *E. cloacae*.

ACKNOWLEDGEMENTS

The University of the Witwatersrand for the use of their facilities and Mrs Phumzile Moerane for technical assistance; National Research Fund (NRF) and Faculty Research Committee Individual Grant for funding

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APPENDIX B: ETHICS WAIVER

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms NF Mzimba
School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

E-mail: sisterfor.m@gmail.com

CC: Supervisor: Professor S van Vuuren <Sandy.vanVuuren@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 08/04/2021

REF: *R14/49*

PROTOCOL NO: W-CBP-210408-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *An antimicrobial validation of the efficacy of southern African plants used as soap substitutes and formulation of herbal soap*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

08/04/2021

Ref: W-CBP-210408-01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Ms NF Mzimba
Student No. (if appropriate): 1167113
Staff No. (if appropriate):

Supervisor: Professor S van Vuuren

School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

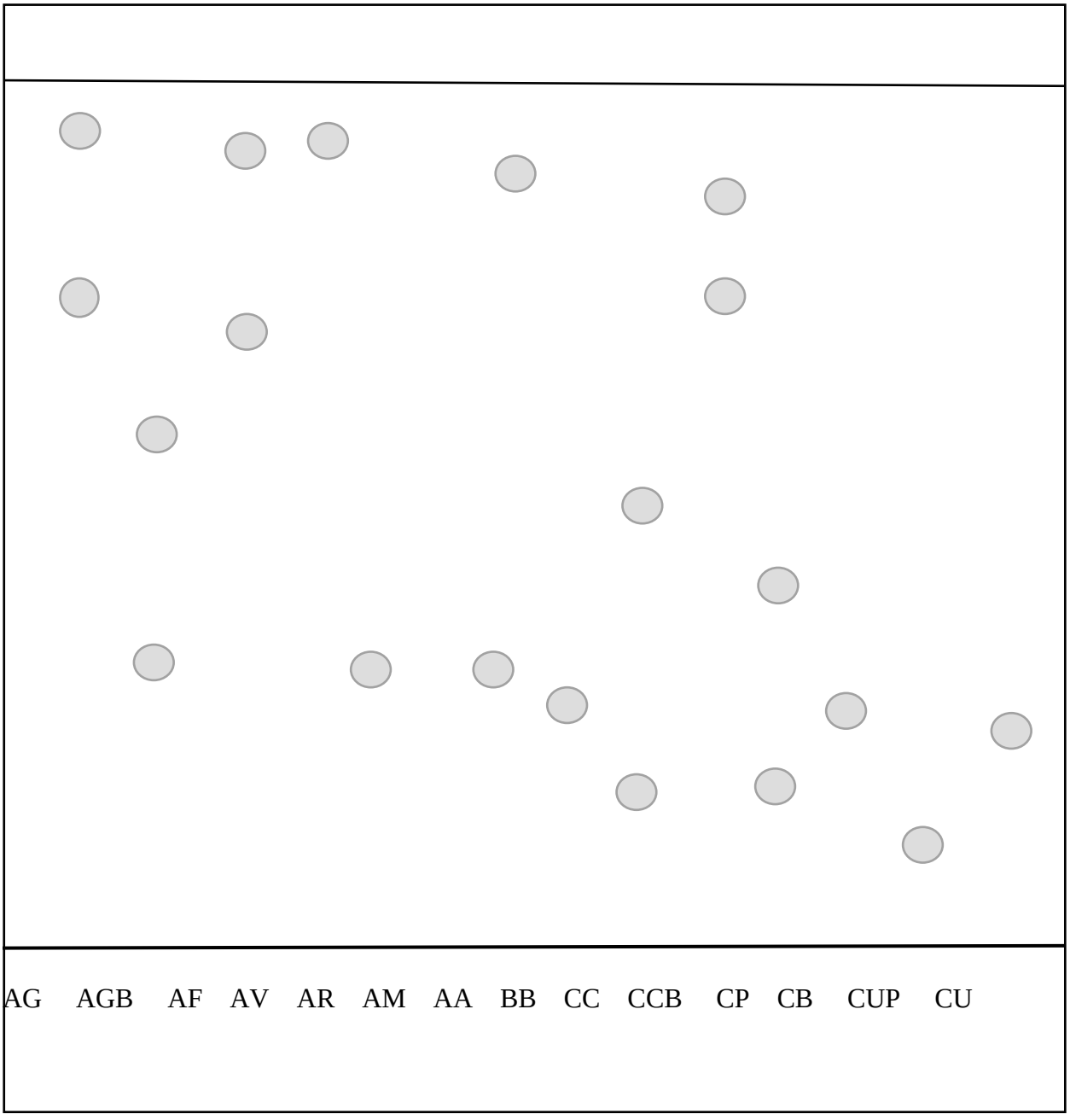
Project title: *An antimicrobial validation of the efficacy of southern African plants used as soap substitutes and formulation of herbal soap*

Reason: Laboratory study
No human participants will be involved in the study

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

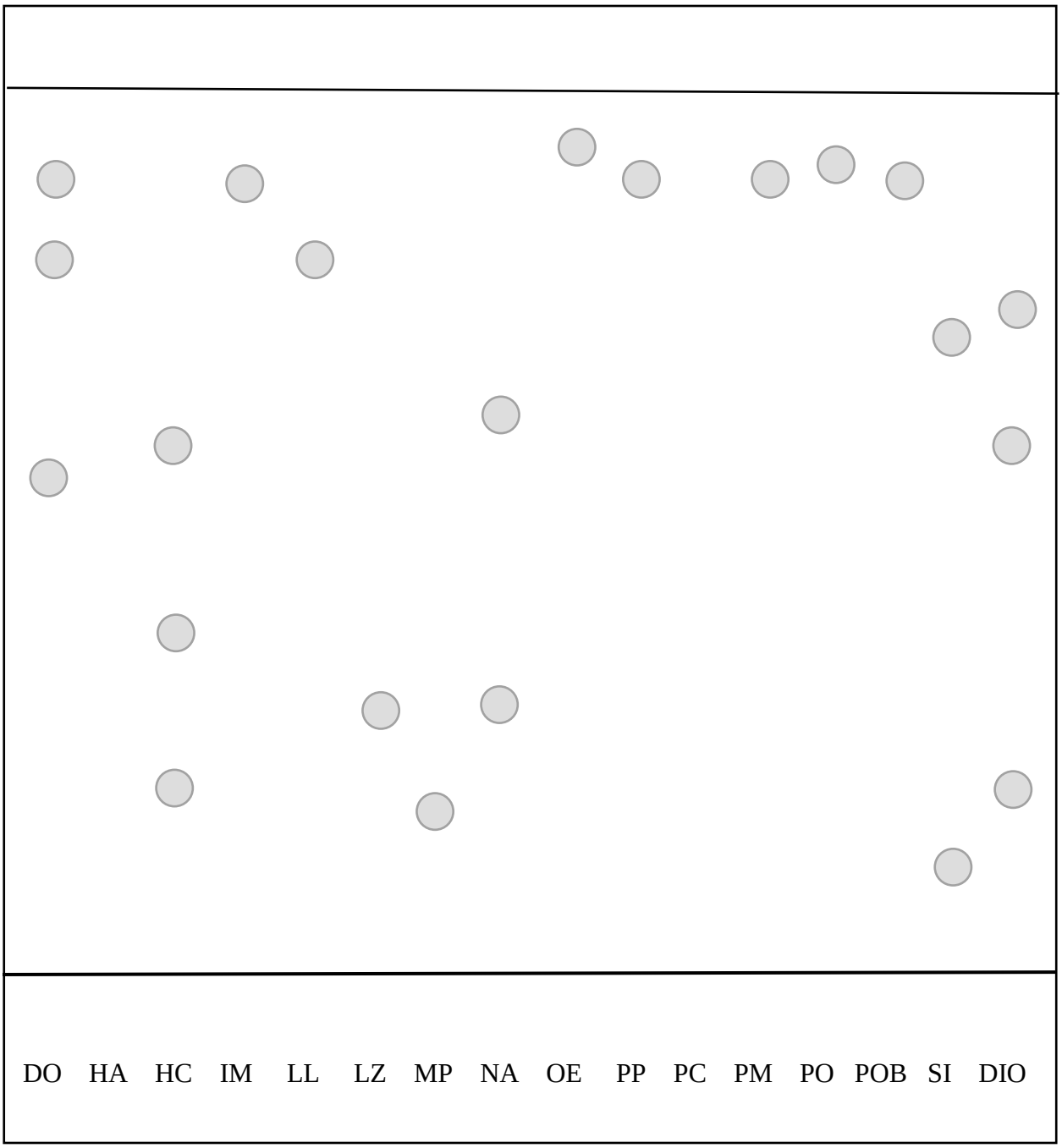
Research Office Secretariat:
Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
Postal address: Private Bag 3, Wits 2050
Tel Nos: +27 (0)11 717 1234/1252/2656/2700
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website:
https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

APPENDIX C: TLC PLATES



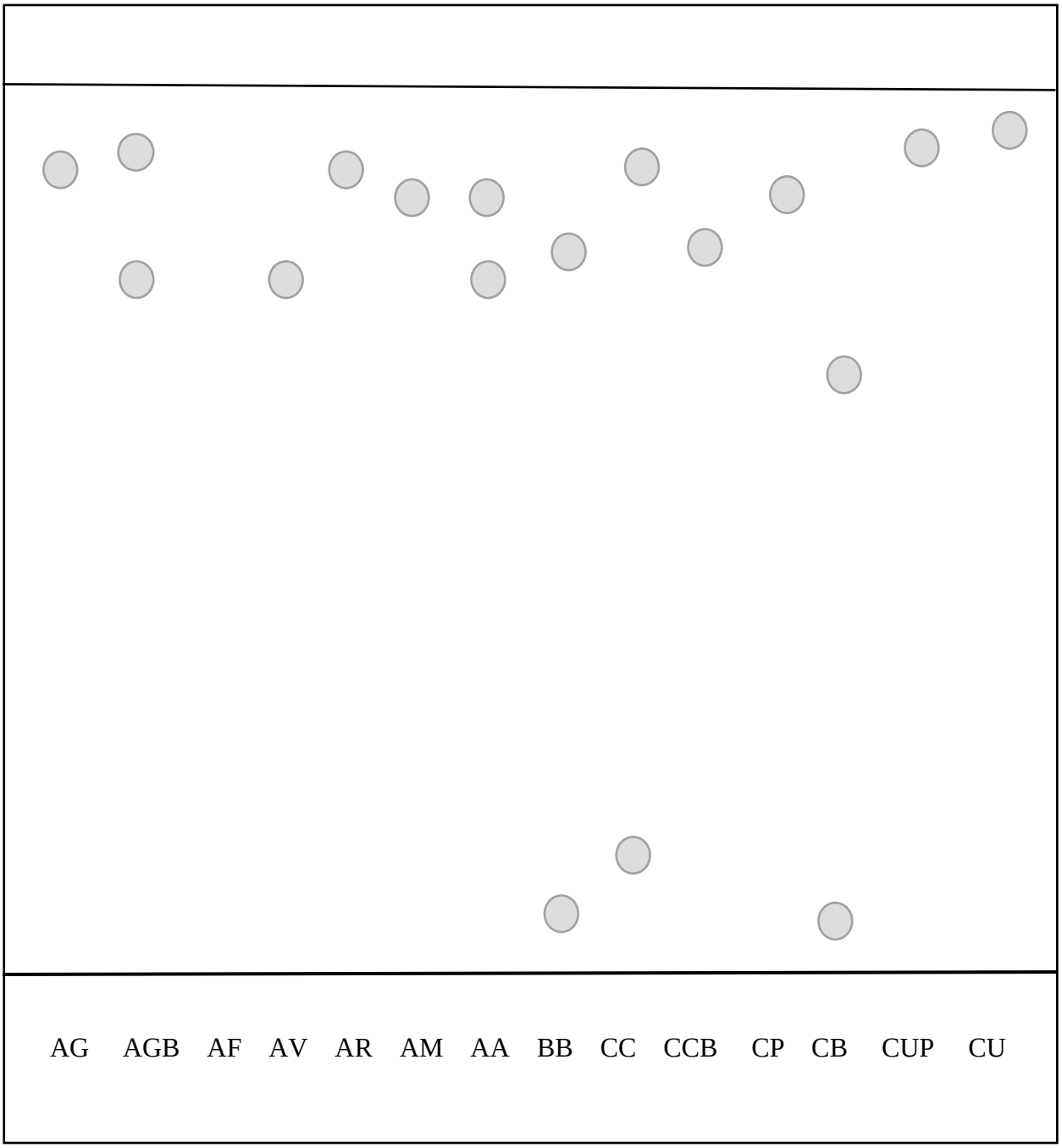
C1: TLC plate sprayed with a vanillin-perchloric acid reagent.

Key – AG: *Acalypha glabrata*, AGB: *Acalypha glabrata* bark, AF: *Aloe ferox*, AV: *Albizia versicolor*, AR: *Aristaloe aristata*, AM: *Aloe maculata*, AA: *Artemisia afra*, BB: *Bauhinia bowkeri*, CC: *Calodendrum capense* leaves, CCB: *Calodendrum capense* bark, CP: *Carica papaya*, CB: *Crinum bulbispermum*, CUP: *Cussonia paniculata*, CU: *Cyathula uncinulata*.



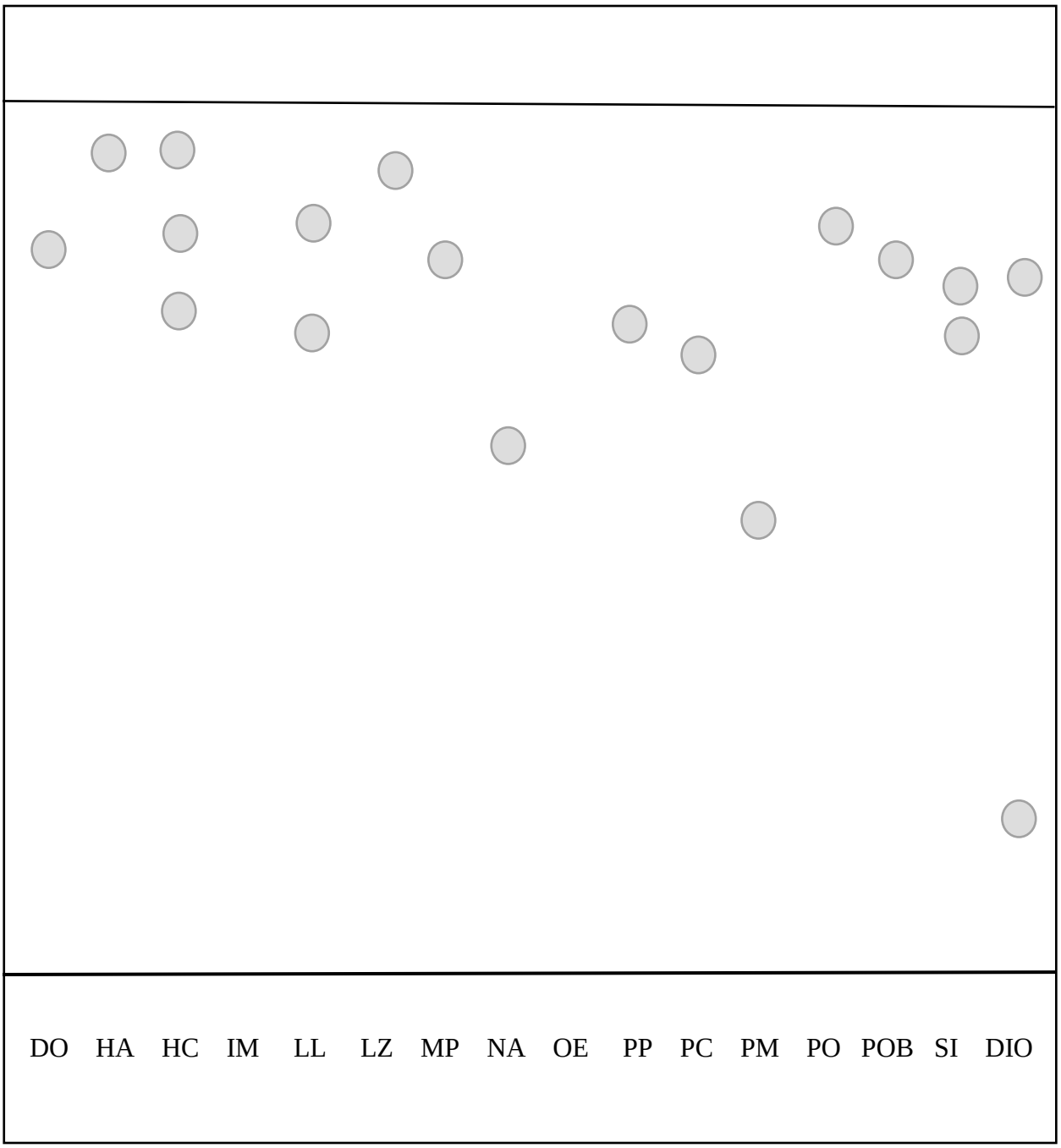
C2: TLC plate sprayed with a vanillin-perchloric acid reagent.

Key – DO: *Deinbollia oblongifolia*, HA: *Haemanthus albiflos*, HC: *Hermannia cuneifolia*, IM: *Ilex mitis*, LL: *Ledebouria luteola*, LZ: *Ledebouria zebrina*, MP: *Merwilla plumbea*, NA: *Noltea africana*, OE: *Olea europaea*, PP: *Pelargonium peltatum*, PC: *Plectranthus ciliatus*, PM: Pouzolzia mixta, PO: *Ptaeroxylon obliquum* leaves, POB: *Ptaeroxylon obliquum* bark, SI: *Sideroxylon inerme* subsp. *inerme*, DIO: Diosgenin (standard saponin).



C3: TLC plate sprayed with a 10% sulphuric acid reagent.

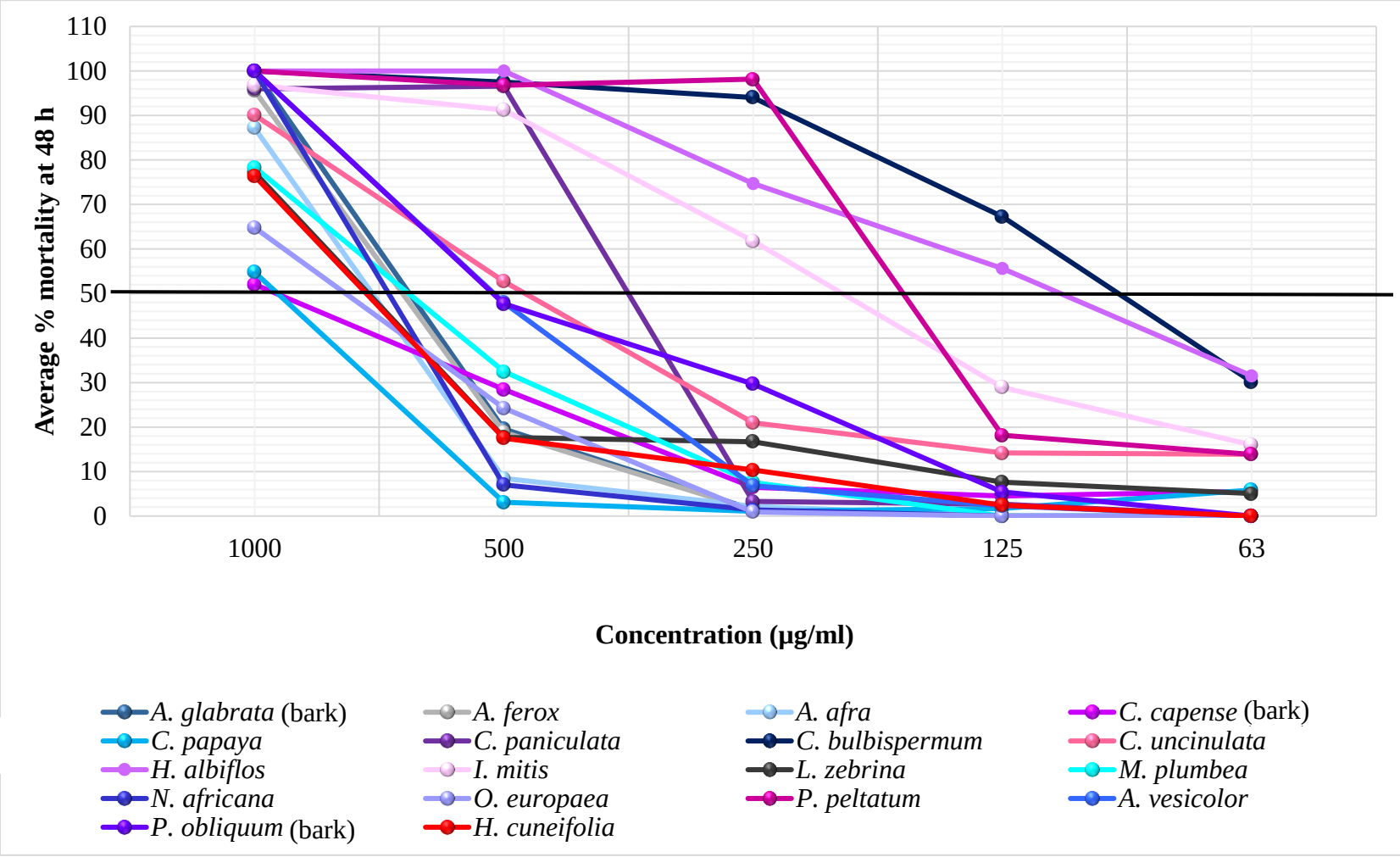
Key – AG: *Acalypha glabrata*, AGB: *Acalypha glabrata* bark, AF: *Aloe ferox*, AV: *Albizia versicolor*, AR: *Aristaloe aristata*, AM: *Aloe maculata*, AA: *Artemisia afra*, BB: *Bauhinia bowkeri*, CC: *Calodendrum capense* leaves, CCB: *Calodendrum capense* bark, CP: *Carica papaya*, CB: *Crinum bulbispermum*, CUP: *Cussonia paniculata*, CU: *Cyathula uncinulata*.



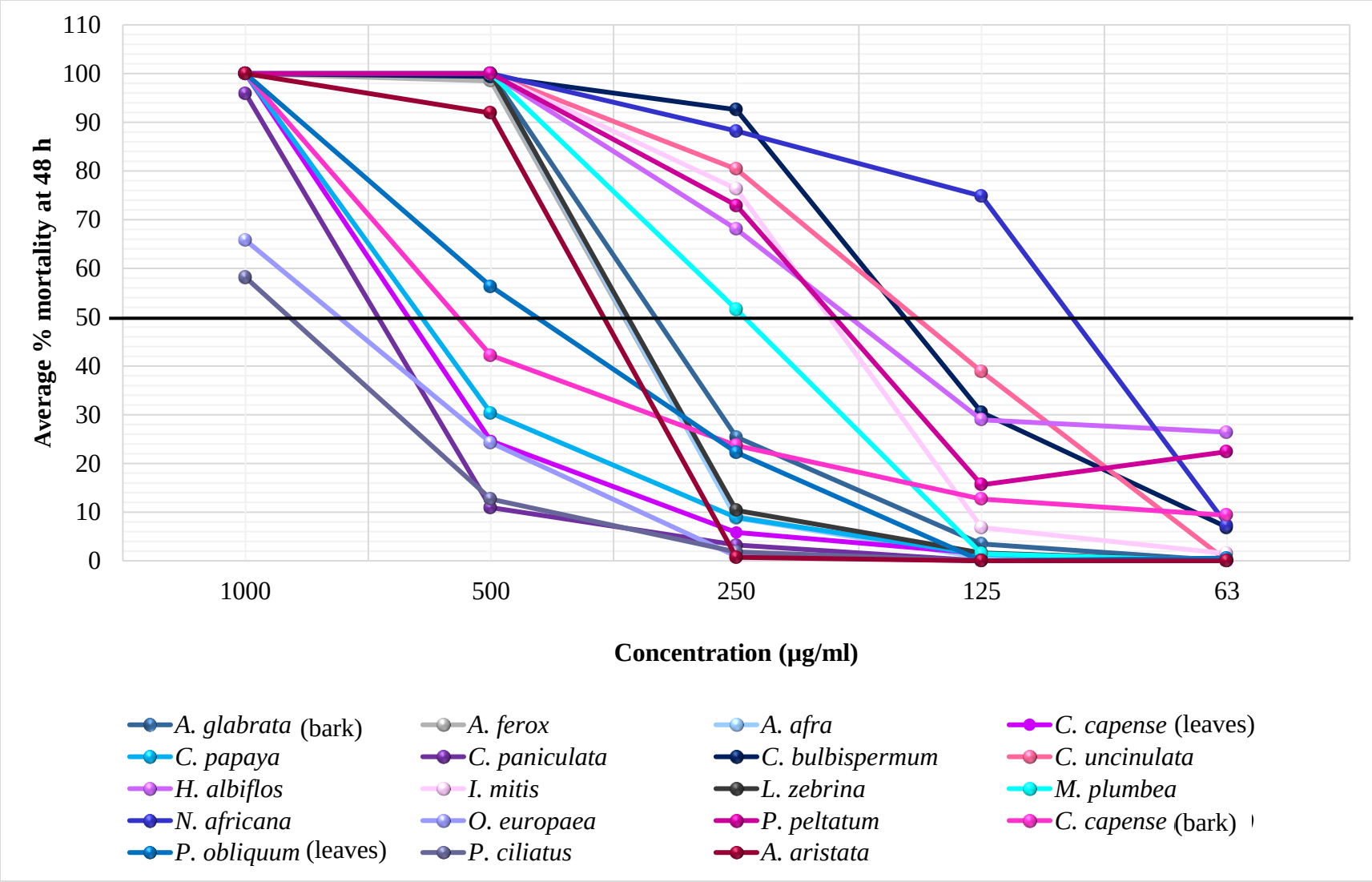
C4: TLC plate sprayed with 10% sulphuric acid reagent.

Key – DO: *Deinbollia oblongifolia*, HA: *Haemanthus albiflos*, HC: *Hermannia cuneifolia*, IM: *Ilex mitis*, LL: *Ledebouria luteola*, LZ: *Ledebouria zebrina*, MP: *Merwilla plumbea*, NA: *Noltea africana*, OE: *Olea europaea*, PP: *Pelargonium peltatum*, PC: *Plectranthus ciliatus*, PM: *Pouzolzia mixta*, PO: *Ptaeroxylon obliquum* leaves, POB: *Ptaeroxylon obliquum* bark, SI: *Sideroxylon inerme* subsp. *inerme*, DIO: Diosgenin (standard saponin).

APPENDIX D: DOSE-RESPONSE GRAPHS



D1: The dose-response for organic extracts that were considered toxic after 48 h.



D2: The dose-response for aqueous extracts that were considered toxic after 48 h.

ORIGINALITY REPORT

Nyiko Fortunate Mzimba Masters report

ORIGINALITY REPORT

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