

Validating the traditional use of medicinal plants in Maputaland to treat skin diseases

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Declaration

I, Sibongile Nciki declare that this dissertation is my own work. It is being submitted in fulfilment for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....

Sibongile Nciki

.....

Date

Dedication

To my loving mother and siblings, Nikeziwe, Mzee and Phiwe.

Thank you for your continual support, tireless faith and confidence in my abilities.

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Abstract

Medicinal plants are widely used as a source of primary health care by the rural inhabitants of northern Maputaland, KwaZulu-Natal, South Africa. A recent (2013) ethnobotanical study conducted specifically in four rural communities of Maputaland (Mabibi, Mbazwana, Mseleni and Tshongwe) revealed that lay people use plants individually and in combinations to treat various skin diseases. Based on the extensive use and lack of scientific evidence, the current study established the scientific validity of the documented plants by investigating their antimicrobial effects (individually and combinations) against dermatological relevant pathogens. The chemical profiles of the most antimicrobial active plants and the chemical compounds capable of permeating the skin were also investigated.

Aqueous and organic (1:1 dichloromethane-methanol) extracts were prepared from plants collected from Maputaland. Antimicrobial screening (micro-titre plate dilution assay) was performed on bacteria and fungi known to cause skin infections. Efficacy of the plants used in combinations was evaluated using the sum of Fractional Inhibitory Concentration (Σ FIC). The combinations that displayed mostly synergistic interactions when combined in equal ratios were studied further in varied ratio studies to determine possible interactions when combined in various mixtures. Qualitative chemical analysis of the most active plant extracts (aqueous and organic extracts) was performed using phytochemical screening tests, UV-Vis spectrometry and a reverse-phase High Performance Liquid Chromatography (RP-HPLC). The permeation of compounds through the skin was investigated *in vitro* on intact porcine skin using the ILC07 automated system and qualitatively analysed through RP-HPLC prior and after the permeation assay.

When investigated individually, all the organic extracts proved to be active (MIC<1.00 mg/ml) on at least four of the 12 test pathogens. Other plant species such as *Anonna senegalensis*, *Elephantorrhiza elephantina*, *Garcinia livingstonei*, *Kigelia africana*, *Ozoroa engleri*, *Parinari capensis* subsp. *capensis*, *Schotia brachypetala*, *Sclerocarya birrea*, *Syzygium cordatum*, *Terminalia sericea*, and *Zanthoxylum capense* demonstrated broad-spectrum activities giving the mean MIC values<1.00 mg/ml. The organic extract of *G. livingstonei* was found to be the most

antimicrobial active extract displaying a mean MIC of 0.27 mg/ml. It was worth noting that plants such as *A. senegalensis*, *O. Engleri* and *P. capensis* subsp. *capensis* investigated for the first time against skin pathogens demonstrated broad-spectrum antimicrobial effects. The efficacy of the aqueous extracts was mostly moderate with 66% of the extracts exhibiting pathogen specific noteworthy effects (MIC<1.00 mg/ml). *Staphylococcus aureus* proved to be the least susceptible pathogen while *T. mentagrophytes* was the most susceptible with 98% of organic extracts displaying noteworthy effects.

When the Σ FICs of plants combined in equal ratios was calculated, the combinations were found to display synergistic (27%), additive (31%), non-interactive (25%) and antagonistic (17%) interactions. The most synergistic effect was observed for the organic extract combination of *Hypoxis hemerocallidea* and *Solanum rigescens* against *Staphylococcus epidermidis* giving the Σ FIC value of 0.04. In varied ratio studies, the combinations combined in various mixtures mostly displayed additive interactions. The organic extract combination of *H. hemerocallidea* and *S. rigescens* displayed synergistic interaction regardless of the ratios when tested against *S. epidermidis*.

All the antimicrobially active topically applied plants (aqueous and organic extracts) phytochemically analyzed possessed anthraquinones, flavonoids, tannins and saponins. The UV-Vis spectra of all the tested extracts gave an initial absorption peak at 212-218 nm which might be characteristic of saponins. These compounds were detected in all the extracts. The second absorption maxima occurred between 250-268 for all the extracts and may confirm the presence of flavonoids that are usually absorbed between a range of 250 and 300 nm. From the *in vitro* permeation assay, it was observed that several compounds present in the crude extracts were able to diffuse across intact porcine skin. Both aqueous and organic extracts of *K. africana* proved to have more compounds capable of permeating the skin than the other tested extracts.

Overall, more than 80% of plants tested displayed a correlation between antimicrobial efficacy and the diseases they were reported to treat. Interesting antimicrobial effects were also noted from the plant combinations. Some compounds present in the extracts are capable of permeating the intact skin *in vitro*. Thus, the current investigation supported the ethnobotanical claims of the documented plants.

Publications and conference presentations

Research publication: De Wet, H., **Nciki, S.**, Van Vuuren, S.F., 2013. Medicinal plants used for the treatment of various skin disorders by rural community of northern Maputaland, South Africa. *Journal of Ethnobiology and Ethnomedicine* 9:51 (Appendix A).

Research publication: **S. Nciki**, S.F. van Vuuren, A.D van Eyk, H. de Wet, 2015. Plants used to treat skin diseases in northern Maputaland, South Africa: Antimicrobial activity and *in vitro* permeability studies. Submitted to the *Journal of Pharmaceutical Biology*, June 2015 (Appendix B for abstract).

Poster presentation: **S. Nciki**, H. de Wet and S.F van Vuuren. Validating the traditional use of medicinal plants in Maputaland to treat skin diseases. Postgraduate Botany Symposium 2013. University of Johannesburg, 29 October 2013 (Appendix C for abstract and Appendix D for a poster).

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Poster presentation: A.D. van Eyk, **S. Nciki**, S. van Vuuren, H. de Wet. *In vitro* diffusion characteristics of four crude medicinal plant extracts across intact porcine skin. Pharmacology and Toxicology Congress, Wits University, 31-August-02 September 2015 (Appendix F for abstract).

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Abbreviations

°C - Degrees celsius

% - Percentage

µm - Micrometer

µl - Microlitre

ΣFIC - The sum of the fractional inhibitory concentrations

AIDS - Acquired immune deficiency syndrome

ATCC - American type culture collection

ACE - Associated Chemical Enterprises

CFUs - Colony forming units

cm - Centimeter

D: M - Dichloromethane: methanol

DSM - Deutsche Sammlung von Mikroorganismen

g - Gram

GMRSA - Gentamycin and methicillin resistant *Staphylococcus aureus*

HIV - Human immunodeficiency virus

INT - *p*-Iodonitrotetrazolium violet

mAU - milli Absorbance Units

mg/ml - Milligrams per milliliter

MIC - Minimum inhibitory concentration

MRSA - Methicillin resistant *Staphylococcus aureus*

NCCLS - National Committee for Clinical Laboratory Standards

nm - Nanometer

PBS - Phosphate Buffered Saline

pH - Acidity or alkalinity of a solution

pKa - Ionization constant

RP-HPLC - Reverse phase High Performance Liquid Chromatography

TSA - Tryptone Soya agar

TSB - Tryptone Soya broth

UV - Ultra-violet

UV-Vis - Ultra-Violet visible

v/v - Volume per volume

w/v - Weight per volume

Chapter one: General introduction

1.1 Overview of skin diseases

The skin is one of the largest and most versatile organs of the body. It consists of three layers, the outer epidermis, the thick inner dermis and the underlying subcutaneous tissue (Hwa et al., 2011). Its complex and multi-layered nature serves as a mechanical barrier that protects the underlying cells from desiccation, chemical and mechanical injury as well as microbial and parasitic invasion (Evans et al., 2013). Apart from being an inert barrier, the skin also serves other important functions, such as percutaneous absorption, body temperature regulation, as a neuro-receptor and as an excretory organ (Abbasi et al., 2010). The skin also forms part of both specific and non-specific defences by virtue of Langerhans cells found in the mid epidermis (Weideman, 2005).

A large amount of different types of micro-organisms exist naturally on the skin and constitute the normal flora. Several bacterial species of the genera *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, *Micrococcus*, *Streptococcus*, *Brevibacterium*, *Acinetobacterium* and *Pseudomonas* are considered the normal microbiota of the skin. These resident (originating from the skin) and transient (originating from exogenous sources) micro-organisms are beneficial commensals to humans. When in the correct balance, the normal flora performs a protective function by competitively excluding harmful micro-organisms. However, the same micro-organisms can become pathogenic and fatal in the wrong place. The disturbance of the individual's flora by use of antibiotics or lotions may permit the overgrowth of pathogenic members which then interact with the host and cause a disease (Rosenthal et al., 2011). A breach in the integrity of the skin through cuts, burns or bites also provides an entry route for micro-organisms to infect the skin and underlying tissues (Weideman, 2005). Other factors such as immunodeficiency diseases, diabetes mellitus, poor hygiene and vascular insufficiency also contribute in the initiation of skin infections (Dinubile and Lipsky, 2004; Chanda and Baravallia, 2010). As a result, many microbial strains, considered as the normal skin flora such as *Staphylococcus aureus* and *Staphylococcus epidermidis* have emerged as multi-drug resistant skin pathogens (Rosenthal et al., 2011).

Skin infections are amongst the most common ailments that account for 34% of all occupational diseases (Abbasi et al., 2010). Micro-organisms responsible for skin infections can be bacterial, fungal, parasitic or viral in nature. These infections may arise spontaneously as primary infections or due to disorders such as eczema and psoriasis providing a means of entry for virulent microbes to initiate secondary infections. Such infections can be present for a few days or a week (acute) or persist for months (chronic), especially in cases of secondary infections (Dinubile and Lipsky, 2004).

1.1.1 Bacterial skin infections

Bacterial skin infections are more prevalent than any other skin infections. Such infections are particularly common in developing countries as they are often associated with poor hygienic conditions (Oumeish et al., 2001). Infections may cause irritation and some discomfort or may penetrate through the skin posing a severe effect on human health (Jamson et al., 2006). Thus, these infections vary in importance ranging from mild to severe and life threatening. Mild infections are usually attributed to *Staphylococcus aureus* and *Streptococcus pyogenes* and may occur in any parts of the body. They range from small pimples to very large lesions accompanied by tissue destruction and abscess formation. Common bacterial infections associated with these two strains are impetigo, folliculitis, erysipelas and cellulitis (Bhagavutta and Powell, 2010; Torok and Conlon, 2013). Complications from burns and wounds are normally associated with *S. aureus* and *Pseudomonas aeruginosa* (Gul et al., 2012).

Impetigo is the most contagious skin infection that commonly affects children between two and five years of age (Bhagavutta and Powell, 2010). It is the third most common infection in children after dermatitis and viral warts. Overcrowding, hot and humid living conditions accelerate an impetigo infection. The causative agents are *S. aureus* and group A *Streptococci*. The most susceptible body parts are the face, hands and areas around nose and mouth, where the infection may manifest in a bullous or non-bullous form (Dawson et al., 2012). The infection begins as intra-epidermal vesicles containing clear fluid which eventually change to pustules or bullae. The pustules burst and become encrusted causing lesions which harbour a large number of bacteria that are highly contagious. As the lesions rupture, the bacteria spread to other parts of the body and initiate further infection (Lim, 1998; Oumeish et al., 2001). In some cases the

lesions may penetrate the epidermis and form ulcerations called ecthyma (Dawson et al., 2012). Ecthyma usually affects the lower limbs and is mostly associated with trauma, insect bites, eczema pediculosis, diabetes and immune compression (Bhagavutta and Powell, 2010; Torok and Conlon, 2013).

Folliculitis originates through the Staphylococcal infection of the hair follicle, with pus accumulating in the epidermis. The infection may extend into the subcutaneous tissue to form furuncles commonly known as boils (Dawson et al., 2012). In some instances, a deep-seated infection of several contagious hair follicles may occur where infected follicles become interconnected by subcutaneous abscesses, resulting in the formation of carbuncles. Commonly affected body parts are the buttocks, neck, axilla and the scalp. Adolescents are most susceptible and poor hygiene, hyperhidrosis as well as obesity are predisposing factors (Bhagavutta and Powell, 2010).

An erysipelas infection presents as bright red edematous patches that appear on the skin (Abbasi et al., 2010). This Streptococcal infection of the dermis also involves cutaneous lymphatics. Other pathogens such as *Klebsiella pneumoniae*, *Haemophilus influenza*, *Yersinia enterocolitica* and *Moraxella* species have also recently been described as the causative agents of erysipelas. Cellulitis, like erysipelas is also a Streptococcal infection, but goes deeper and causes inflammation across the dermis and subcutaneous tissue. Cellulitis is characterized by erythema and edema and usually occurs as a result of skin disruption either by injury or when the pathogens breach the skin due to primary infections such as ulcers, eczema, psoriasis or ringworm. It affects the limbs and the resulting lesions are not well demarcated as with erysipelas (Bhagavutta and Powell, 2010; Dawson et al., 2012; Torok and Conlon, 2013).

Apart from skin infections associated with poor hygienic conditions, a number of bacterial pathogens are also associated with wound and burn infections, leading to severe inflammation and delayed healing. Commonly isolated pathogens are the Gram-negative *Escherichia coli* and *P. aeruginosa*, as well as the Gram-positive *S. aureus*. *Escherichia coli* is frequently isolated in surgical wounds while *S. aureus* infects diabetic wounds and foot ulcers. Infected wounds are usually characterized by a colour change and formation of lesions around the injured area. In the case of *P. aeruginosa*, the infected wounds are manifested by green pigment which later changes

to black (Lekganyane et al., 2012). *Pseudomonas aeruginosa* infections are fatal especially in victims with third-degree burns (Chiller et al., 2000).

1.1.2 Fungal skin infections

Fungal skin infections occur in all environments and may be superficial, subcutaneous or systemic (Jamison et al., 2006). Certain fungi are opportunistic such as *Candida albicans*, affecting the immune compromised individuals while others are true pathogens such as *Epidermophyton*, *Microsporum* and *Trichophyton* species, affecting immune competent candidates. With superficial infections, the pathogen infects and remains limited to the stratum corneum of the skin (Schwartz, 2004). In subcutaneous infections such as chromomycosis and sporotrichosis, the pathogen affects the subcutaneous tissue and the dermis and often extends to the bones. Systemic mycoses such as blastomycosis and cryptococcosis occur when the pathogen is disseminated into the bloodstream. Most fungi responsible for systemic mycoses usually affect Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome (HIV/AIDS) patients. However, true systemic mycoses affect normal individuals when the pathogen penetrates the body and spreads via the bloodstream to other organs including the skin (Rivitti and Aok, 1999). Although systemic infections rarely occur, they have been associated with considerable morbidity and mortality (Hay, 2006).

Superficial mycoses are the most common fungal infections affecting 20 to 25% of the world's population. They are predominantly caused by the dermatophytes belonging to the genera, *Epidermophyton*, *Microsporum* and *Trichophyton* (Ameen, 2010). These pathogens typically affect the stratum corneum resulting in tinea or ringworm. Susceptible body parts are the body skin (*tinea corporis*), scalp (*tinea capitis*), beard (*tinea barbae*), hands (*tinea manus*), nails (*tinea unguium*) and feet (*Tinea pedis*). *Tinea corporis* manifests as small circumscribed lesions which slowly form an inflamed ring with a clear centre. The inflamed borders become scaly and may be patched with blisters (Lim, 1998; Bhagavutta and Powell, 2010).

Tinea capitis, ringworm of the scalp is the most contagious fungal infection caused by *Microsporum canis* and *Trichophyton tonsurans*. It commonly infects children and is spread from child to child particularly in schools or from animals to children. *Tinea capitis* produces highly inflammatory lesions with suppuration followed by complete or partial loss of hair

accompanied by scaling of the scalp. In some children, the infected scalp becomes bald with localised patches. These patches are erythematous, postulated and may become elevated into a boggy mass (Jamson et al., 2006; Bhagavutta and Powell, 2010).

Tinea pedis, also called athlete's foot, is an infection of the feet and is also caused by the fungal species of the genus *Scytalidium*. In *Scytalidium* infections, the infected feet are presented by fractured and inflamed interspaces between the toes (Jamson et al., 2006). The infection may also affect the palms of hands and cause interdigital scaling. When the infection spreads to the nails, this may result in extensive onycholysis and fracture of the nails. The presented symptoms resemble those of *Trichophyton rubrum* infections and may be prone to secondary bacterial infections (Hay, 2006).

Cutaneous Candidiasis is another class of fungal skin infection predominately caused by a yeast-like fungus, *Candida albicans*. Other species such as *C. parapsilosis*, *C. tropicalis* and *C. glabrata* may also occasionally cause cutaneous candidiasis. *Candida albicans* is a normal flora of the mouth, vagina and gastrointestinal tract. However, in HIV infected individuals or patients undergoing prolonged antibiotic therapy, *C. albicans* overgrow and may initiate infection. The infection manifests as acute or chronic mucocutaneous candidiasis. The former presents as satellites, which are red and edematous pustules with creamy exudates. Such pustules usually occur in moist folded parts of the skin. The chronic mucocutaneous candidiasis may last for years producing continual and periodical infections on the skin, mucous membranes and nails. Such conditions are severe and resistant to treatments (Staab and Wong, 2009; Watts et al., 2009). Cutaneous candidiasis also affects healthy neonates and young infants usually causing oral thrush and candidal diaper dermatitis. The latter affects moist macerated parts of the skin of the diaper region and usually starts on the perineum and spreads to the lower abdomen and thighs. The infection is manifested by scaly papules which become weeping eroded lesions associated with satellite pustules (Watts et al., 2009).

1.1.3 Viral skin infections

Common viral skin infections include chicken pox, measles, herpetic sores, warts and HIV related infections. Chicken pox, also called varicella, is usually encountered during childhood via the respiratory tract. In most cases, the condition may not be severe and resolves spontaneously.

However, in some cases, the virus causing the infection may remain viable and in latent form in nerve cells. It then reactivates as herpes zoster or shingles later in life (Lim, 1998; Jamson et al., 2006). In immunocompromised individuals, the virus can also be disseminated to the lungs, liver, adrenal glands and other internal organs (Arvin et al., 1996).

Measles or rubella, caused by the measles virus, is a highly infectious disease that is also encountered through the respiratory tract and sometimes through the conjunctiva. The virus then enters the lymphatic system and disseminates to the reticuloendothelial system where the skin and respiratory tract become infected. Such infection results in the development of the measles rash (Duke and Mgone, 2003). German measles is similar to measles but is not severe and was believed to be harmless for many years. It has, however, been found to cause congenital defects in infants, collectively known as congenital rubella syndrome. Fever blisters or cold sores caused by the Herpes Simplex Type 1 virus are also acquired during childhood through oral or respiratory secretions. The virus remains in the body following childhood disease and can reappear throughout one's lifetime as a result of diet, trauma, sunlight, cold or emotional disturbances (Lim, 1998).

Warts and warts of the genitalia are caused by human papilloma viruses that induce the growth of their host cells. Warts can also result from hyperkeratosis, an excessive cornification of the palms and the soles (Van Wyk and Wink, 2004).

HIV-related skin diseases are usually the Kaposi's sarcoma as well as the toxic epidermal necrolysis. Epidermal necrolysis is usually rare and induced by body's reaction to drugs. The condition is severe and resembles that of severe burns. It is life-threatening as it leaves the body susceptible to severe infections. Other HIV-related skin disease includes the itchy papular eruption or papular pruritic eruption which manifests itself with severe papules on the face and upper trunk that tingle (Jamson et al., 2006).

1.1.4 Inflammatory skin disorders

Acne vulgaris is among the most prevalent inflammatory skin condition. It is a chronic inflammatory disease of the pilosebaceous unit commonly affecting adolescents, but it may persist up to the age of 30 years or lifelong. The condition occurs as a result of an increase in

sebum production in certain body parts such as the face and upper trunk. This leads to the blockage of sebaceous gland ducts and the formation of comedones. The comedones associated with hair follicles form an anaerobic and lipid-rich environment for colonization by *Propionibacterium acnes*. Such colonization result in dysbiosis of the facial tissue. Consequently, comedones progress to inflammatory papules, pustules and nodules. Sometimes, the condition may become severe with acne lesions blending to form large inflammatory cysts with scars (Von Hees and Naafs, 2001; Schommer and Gallo, 2013).

Psoriasis is another chronic inflammatory skin disorder. It is non-infectious and occurs spontaneously as a result of genetic and environmental factors. However, Gram-positive bacteria usually colonize the diseased skin (Schommer and Gallo, 2013). Psoriasis is manifested by an abnormal fast turnover of the skin. The skin layers become extremely thick, especially the stratum corneum causing it to become scaly, erythematous and inflamed. The condition is usually localized on the scalp, elbows, knees and buttocks, but palms and soles may also be affected. They usually become thick, form scales and crack (Von Hees and Naafs, 2001).

Eczema, also known as dermatitis, is a non-infectious inflammation of the skin although in some cases secondary bacterial infection may occur. Eczema is influenced by irritants such as ointments, detergents, heat or stress. When the condition is acute, it is usually presented by red, edematous papules and blisters which ooze and crust. This may progress to a subacute phase where the skin becomes drier, scaler and the pigment changes. In the chronic stage, the skin thickens, excoriates and cracks. Itching is the major complaint in all the stages. Common types of eczema are atopic and seborrhic dermatitis (Von Hees and Naafs, 2001). Atopic dermatitis is common in infants and tends to occur on the face, neck, trunk, hands and feet. The condition occurs as a result of hypersensitivity reactions due to genetic pre-disposure to the disease. Contact with allergens and emotional stress worsen the disease. Affected individuals become susceptible to secondary infections with *S. aureus*, herpes and vaccinia viruses. Seborrhic dermatitis is characterized by red patches associated with greasy scaling. Commonly affected body parts are the eyebrows, chest and scalp. In HIV infected individuals, the condition may affect the entire skin and the patient may easily become infected. Sometimes, the condition appears concomitantly with atopic dermatitis leading to treatment complications (Schommer and Gallo, 2013).

1.1.5 Prevalence of skin diseases

Skin diseases are common worldwide. Most skin infections have been self-limiting with low mortality rates. However, these infections have recently been responsible for significant morbidity due to their association with HIV/AIDS (De Wet et al., 2013). Skin and mucosal infections affect more than 90% of HIV infected individuals (Steenkamp et al., 2007). Herpes Zoster, the causative agent of shingles affects 8 to 13% of patients with AIDS, while HIV infected individuals experience the recurrent episodes of Herpes Zoster throughout the course of infection. About 10% of AIDS patients develop fungal mucocutaneous lesions as well as deep fungal infections. Seborrheic dermatitis affects 20-40% of HIV patients and in those with AIDS, the prevalence is 40-80% (Tschachler et al., 1996). Such morbidity rates necessitate expensive treatments and result in a high number of hospitalizations as well as mortality in AIDS patients (Steenkamp et al., 2007).

The prevalence of skin infections also seems high even in immunocompetent individuals. In developed countries, for instance, secondary skin infections are common, predominantly caused by resistant microbial strains. For example, in the United States (US), Methicillin resistant-*Staphylococcus aureus* (MRSA) has been responsible for more than 50% cases of suppurative skin infections. Such infections are of concern due to potential deadliness of their manifestations which include necrotizing fasciitis and severe sepsis (Boucher et al., 2010). Quave et al. (2008) mentioned that MRSA skin and soft tissue infections are responsible for 126 000 hospitalizations and 18 650 deaths each year in the US, a rate which even exceeds that of AIDS. Atopic dermatitis is also prevalent in developed countries, affecting 15 to 20% of children (Tadeg, 2004). In northern Europe, atopic dermatitis affects 15.6% children of seven years of age (Williams et al., 1998).

In developing countries, bacterial and fungal skin infections are prevalent. The prevalence rate ranges from 20 to 80% (Al-Hoqail, 2012). These infections are usually exacerbated by a low socio-economic status, synonymous with unhygienic living conditions and overcrowded living spaces. Such conditions provide greater incidence of skin-skin contact and co-habitation with pets (Havlickova et al., 2008). A study done on outpatients in the Al-Majmaah region in India revealed that dermatological disorders were the leading causes of hospital visits. In the reported

study, eczema was the most common diagnosis, responsible for 15.8% of cases followed by acne (14.7%). Viral (13.3%), fungal (7.6%) and bacterial infections (16.96%) contributed frequently to the occurrence of infectious diseases (Al-Hoqail, 2012). In another study by Chen et al. (2008) involving school children in Taiwan, acne vulgaris was the most common dermatosis with a prevalence rate of 17.3%. Ephelides, atopic dermatitis, warts, keloids and vitiligo were also identified, comprising 4.3%, 2.8%, 0.3% and 0.09% prevalence respectively. In Nigeria, the prevalence of MRSA infections was found to be 75% and 51.4% on wounds and skin respectively (Udobi et al., 2013).

Dermatophytes alone affect more than 25 % of the population in African countries. Figuera et al. (1997), for instance, observed a high prevalence of cutaneous infections in a study on school children in Ethiopia. Dermatophytosis was reportedly common with *tinea capitis* being the most prevalent, followed by *favus* and *tinea corporis*. *Tinea capitis* is also common amongst black South African children especially those residing in rural areas, especially in the KwaZulu-Natal province which has a subtropical climate and is densely populated (De Wet et al., 2013).

Despite the prevalence of dermatophytes in South Africa, other skin infections are also quite prevalent. A study conducted in five academic hospitals in Johannesburg area examined 1029 patients in which the prevalence of eczema, acne and superficial fungal infections were found to be high affecting 76% of black patients. Eczema was also high in Indian patients followed by fungal infections and psoriasis accounting for 30.4%, 11.8% and 9.6% respectively (Hartshorne, 2003). Another study in 33 schools in Cape Town Metropolis revealed the frequent occurrence of impetigo, fungal infections, papular urticaria and scabies in black South African girls compared to the white girls (Mercer et al., 2004). Atopic eczema was also found to be prevalent among adolescents in the same geographical area which affected this age group's life quality (Zar et al., 2007).

The study by Hartshorne, (2003), also found an increase in the prevalence of HIV related skin infections among black patients compared to the other surveyed races. For instance, Herpes Zoster was found to affect 3.4% of the patients while Kaposi's sarcoma affected 74%, a rate which is 50% higher than the rate observed 30 years back.

1.2 Treatment of skin diseases

1.2.1 Conventional approaches and their limitations

The high incidence of skin infections and their association with HIV/AIDS necessitates the need for effective treatment options in healthcare centres around the world. In most cases, dermatologists rely on antimicrobial preparations to treat various skin conditions. Therapy may be topical or systemic, directed against the suspected pathogen or the affected part. Antibiotics are commonly prescribed for the treatment of bacterial infections. Topical antibiotics are used to treat superficial infections while systemic antibiotics such as tetracycline, doxycycline, minocycline, erythromycin and clindamycin are active against systemic or deep skin infections. Superficial fungal infections caused by dermatophytes may be treated using topically or orally administered antifungal agents. For Candidal infections, treatment is accomplished through topical applications of clotrimazole, miconazole, econazole, ketoconazole, exiconazole, nystatin or intravenous or intramuscular administration of amphotericin B (Tadeg, 2004).

There are few effective treatments available for viral infections. Acyclovir and valacyclovir are usually employed when treating herpes simplex and varicella infections. Condyloma warts are treated with podophyllin, and podofilox. Inflammatory skin diseases such as eczema and psoriasis are treated with anti-inflammatory topical steroids. In severe cases of eczema, a stronger steroid such as betamethasone valerate is required. Staphylococcal infected dermatitis is treated with systemic antibiotics (flucloxacillin or erythromycin) or corticosteroids. When antibiotics fail, cyclosporin A and azathioprine are employed. Acne vulgaris is usually treated with retinoids and tretinoin which help loosen and prevent the formation of new comedones. Antimicrobials such as erythromycin, clindamycin and benzoyl peroxide are also the mainstays of treating acne. However, such antimicrobials have been reported to be particularly effective when used in combination with retinoids. Systemic therapy with retinoids, antimicrobials and hormones are used when inflammatory lesions develop (Tadeg, 2004).

Although antimicrobials are important treatments for fighting microbial infections, they have become less effective against certain illness due to the emergence of multi-drug resistant pathogens. Infectious diseases caused by these drug resistant pathogens are of global concern (Rakholiya and Chanda, 2012). For example, *S. aureus* as well as group A *Streptococci* which

cause common infections such as impetigo, folliculitis, carbuncles and furuncles, as well as severe necrotizing fasciitis to mention a few, have developed resistance to methicillin and erythromycin (Torok and Conlon, 2013). Adwan et al. (2010) also reported the difficulties in treating severe and life threatening secondary skin infections caused by *P. aeruginosa* due to the emergence of multi-drug resistant strains. Even antifungal agents such as the azole drugs have been associated with treatment failures due to the development of resistant strains of *C. albicans* (Watts et al., 2009). Other fungal species of the genera *Candida* and *Trichosporin* which cause invasive infections have also developed resistance to amphotericin B (Masoko et al., 2007).

Apart from the problem of resistance, many antimicrobials are associated with undesirable side effects (Rakholiya and Chanda, 2012). Many patients with eczema require a prolonged treatment with topical glucocorticoides which leads to cutaneous atrophy while cessation of the therapy worsens the condition. The use of neomycin by patients with extensive skin damage results in systemic toxicity and contact allergy or deafness. Patients with severe psoriasis are treated with systemic retinoid etretinate, but the drug is contraindicated in pregnant women as it is a potent teratogen (Tadeg, 2004). Thus, the high prevalence of antibiotic resistance and the presence of unwanted side effects are further major factors necessitating the search for effective and safer alternatives to manage skin infections (Chanda and Baravallia, 2010).

1.2.2 Medicinal plants commonly used to treat skin afflictions

Medicinal plants have been found to play an important role in treatment of skin disease (Abbasi et al., 2010). The traditional treatment of dermatological conditions with plant-derived therapeutic preparations existed for centuries prior to the development of Western medicine. Currently, the use of medicinal plants is gaining popularity as people seek for alternative cheap and safe treatments for skin infections. Efficacy studies involving various plant species indicated that many species do indeed possess biological effects against various microbial infections (Steenkamp et al., 2004; Njoroge and Bussmann, 2007; Mabona et al., 2013). An infusion made from the roots of *Cucurbita pepo* for example, is used in Central America to treat syphilitic sores, herpes lesions, acne vulgaris and blackheads. *Allium cepa* is employed as an antibacterial treatment for boils, abscesses, acne and blackheads where it draws out the infection, decrease inflammation and promote healing (Aburjai and Natsheh, 2003). In Indian, they use *Avena sativa*

as well as *Calotropis procera* to treat warts. *Chelidonium majus* is also used in China for the same purpose (Bedi and Shenefelt, 2002).

In addition to their antimicrobial potential, medicinal plants are also popular alternative therapies of chronic inflammatory skin disorders such as eczema, which do not respond to the standard treatments (Tadeg, 2004). Plants species such as *Artemisia vulgaris* and *Artemisia absinthum* for instance, are used in the Philippines to treat eczema, ulcerative sores and purulent scabies. The powdered rhizome of *Curcuma longa* is employed for psoriasis and eczema (Aburjai and Natsheh, 2003). In China, traditional treatment of dermatitis and psoriasis is widely practiced as plants are believed to possess anti-inflammatory, antibacterial, antifungal and corticosteroid effects. *Aloe vera* and *Capsicum frutescens* are the potential treatments of psoriasis in China (Bedi and Shenefelt, 2002). *Centaurium erythraea* is a popular remedy in India for the treatment of eczema and all types of sores (Tadeg, 2004).

Apart from their possible antimicrobial properties, certain plants species are known to possess wound healing activities. The active ingredients present in these plants are responsible for stimulating healing by inducing skin cell proliferation (Agyare et al., 2009). In this instance, the healing of wounds or sores can be enhanced through topical application of medicinal plants that have antiseptic activity. Plants with mucilage seem to promote healing by providing a physical barrier that protects the wound or any open sore from drying out. In some cases, plants with analgesic activity are used in relieving pain from burns, boils and abscesses. In fresh bleeding wounds, plants with stringent tannins are used to promote shrinking of the tissues as well as blood clotting, where antimicrobial activity prevent wounds from being infected (Van Wyk and Gerick, 2000). Several plant species such as *Quercus infectoria*, *Catharanthus roseus*, *Lycopodium serratum*, *Sesamium indicum*, *Morinda citrifolia*, *Terminalia liabellirica* and *Moringa oleifera* are commonly known around the world as the potent treatment affording the healing of skin wounds (Rawat et al., 2012).

In South Africa, traditional medicinal plant use is also vital for the treatment of skin afflictions. Several species of the genus *Aloe* are commonly employed by South Africans to treat various skin diseases. One example is the *Aloe ferox* which is used in the Eastern Cape Province to treat skin cancer, burns, eczema and psoriasis. *Aloe aborescens* is used by the Zulu speaking people to

treat burns, cuts, scrapes, rashes and sores (Coopoosamy and Naidoo, 2013). *Artemisia afra* is also a popular remedy for skin ailments in South Africa, commonly employed to treat acne, boils and carbuncles. Another plant, *Borreria natalensis* is used in the KwaZulu-Natal province to cure leprosy and furuncles while *B. campacta* is used for eczema. *Plumbago zeylanica* is also extremely popular in South Africa as a treatment of various skin diseases such as leprosy, acne, scabies, sores and leg ulcers. The antimicrobial potential of this species is attributable to plumbagin, a compound which is affective to a wide spectrum of bacterial and fungal pathogens (Tadeg, 2004). *Senecio serratuloides* is also popularly used in South Africa to treat various skin conditions (Van Wyk et al., 2009; Pooley, 2003, De Wet et al., 2013).

1.2.3 Ethnobotanical and ethnopharmacological approaches to the healing of skin diseases

A number of ethnobotanical studies have been conducted around the world based on the traditional use of medicinal plants to treat skin ailments and have provided insight in traditional treatment of such ailments (Martinez and Barboza, 2010). Among such studies, were the studies on the plants that promote wound healing. An example is the study conducted by Adetutu et al. (2011) in south western Nigeria where 33 plant species were shown to have wound healing properties. In Mali, West Africa, 73 plant species have been documented which are used in the washing of wounds, extraction of pus, as a coagulants, as well as for the treatment of infected wounds (Inngjerdingen et al., 2004). In another survey by Agyare et al. (2009), 104 plant species used in wound healing have been recorded. In Pakistan, the survey conducted by Abbasi et al. (2010) recorded 66 plants that cure various skin ailments including boils, wounds, pimples, gums, scabies, warts, carbuncles, eczema, ringworms, abscesses, measles and leprosy. Quave et al. (2008), revealed a total of 38 plant species used to treat various dermatological conditions such as ulcerations, burns, wounds, inflammation, rash, dental abscesses, furuncle, dermatitis and other conditions. Saikia et al. (2006) documented 85 medicinal plants for 18 skin ailments in Assam, in northern India. In Kenya, 57 plants species have been documented which are used in the management of 14 skin conditions (Njoroge and Bussmann, 2007).

In addition to the ethnobotanical surveys, other studies have been conducted with the intention to validate the ethnotherapeutic claims of plants against skin associated pathogens. For example, in a study by Jeevan Ram et al. (2004) conducted in India, ethanol extracts from 23 plant species

were assayed for antimicrobial activity against bacterial and fungal pathogens with dermatological relevance. The selected plants exhibited antimicrobial activity against five tested micro-organisms. In Pakistan, Tadeq et al. (2005) screened hydro-alcoholic extracts of eight plant species for their antimicrobial potential against bacteria associated with skin infections. Two of the eight selected plant species showed a broad-spectrum antimicrobial activity. Other species were also found to have activity on at least one of the five tested microbial strains. In another study in North Punjab, 12 plants were selected, based on the ethnobotanical information from local people, for their use against skin diseases. The plants showed antimicrobial activity against 50% of the micro-organisms tested (Gul et al., 2012).

With most of the South African studies done on plants used to treat skin infections, the focus has been on plants that treat wounds and sores taking into account the possible impact of bacterial infections (Grieson and Afolayan 1999; Steenkamp et al., 2004; Mthethwa, 2009). In one such study conducted in the Eastern Cape Province, traditional healers as well as nurses and doctors were interviewed about the plants used to treat wounds. Four of the most commonly used plants were selected and tested against bacteria known to infect wounds. Of the four selected plants, three (*Grewia occidentalis*, *Polystichum pungens* and *Cheilanthes viridis*) showed broad-spectrum antibacterial activity against the tested strains (Grieson and Afolayan, 1999). Steenkamp et al. (2004) selected nine previously recorded plant species that promote wound healing and tested their antimicrobial activity. Two plant species namely, *Terminalia sericea* and *Gunnera perpensa* which showed broad-spectrum antimicrobial activity were further tested for antioxidant activities and effects on fibroblast growth. Mthethwa (2009) screened 18 plant species used to treat wounds and sores in the Ongoye area, KwaZulu-Natal, for their antibacterial activity against wounds and sores. Six plant species were found to be effective against the micro-organisms tested such as *E. coli*, *Klebsiella pneumoniae* and *S. aureus*. Even though these studies have shown antimicrobial potential of plants used against skin associated bacteria, they have, however, not been studied in great depth. In a review by Van Vuuren (2008) on antibacterial activity of South African medicinal plants, the need for further testing of plants used for skin afflictions against dermatophytic pathogens was emphasized. Even though these pathogens were included in the study by Mabona et al. (2013) on the antimicrobial activity South African medicinal with dermatological relevance, however, more than 80% of plants investigated in this study have never been investigated against the dermatophytic pathogens.

1.2.4 Plant combinations used to treat skin diseases

In most traditional medicine systems of the world including African traditional medicine, different plant species are often combined for better efficacy (Van Vuuren and Viljoen, 2011). The concept of combining different plant species to treat diseases have been practiced since ancient times in traditional medicine systems. With the global problem of antibiotic resistance, combination therapy is not only practiced in traditional medicine, but it is also employed in western medicine (Zonyane et al., 2013). Certain antimicrobial drugs may also be used in combination with bioactive plant extracts in order to enhance their effect. Synergistic effects resulting from such combinations have been beneficial in treating serious infections caused by drug resistant pathogens. In one study by Rakholiya and Chanda, (2012), a strong synergistic effect was observed between medicinal plants and standard antimicrobial drugs against *S. aureus*, *S. epidermidis* and other enteric pathogens. In another *in vivo* study, the combination of plant extracts with acyclovir demonstrated a combined therapeutic effect by suppressing the development of herpetic skin lesions in rats (Kurowaka et al., 1995).

Several *in vitro* antimicrobial reports have shown the role of synergism where certain plants are used in combinations to treat various infectious diseases (Van Vuuren and Viljoen, 2008; Van Vuuren et al., 2009; Suliman et al., 2010; York et al., 2012; Zonyane et al., 2013). However, studies dedicated in validating the use of South African plant species in combination against skin diseases are limited. In the study by Mabona et al. (2013) on the antimicrobial activity of southern African medicinal plants with dermatological relevance, the selected plant combinations showed antimicrobial activity against a number of skin associated pathogens. The information on the use of selected plants was, however, obtained from the readily available literature only. Thus, there is still a need for further scientific evidence on plant combinations used by the local ethnic people. Investigating the interactive efficacy of the ethno-directed plant combinations may serve as the foundation for novel chemotherapeutic agents effective against skin pathogens.

1.3 Phytochemical evaluation of topically administered plant preparations

Another aspect to be considered in the scientific evaluation of plants used against skin infections is the phytochemical investigation of antimicrobially active compounds that are capable of

permeating the skin. It is known that for most plant preparations, topical application is the convenient way of treating superficial skin conditions. For the treatment of deep skin infections such as furuncles and carbuncles, percutaneous absorption is necessary. Some studies have evaluated the *in vitro* permeability of certain pharmacological active compounds present in plant extracts. For instance, Heard et al. (2006) evaluated the transdermal delivery of caffeine, theobromine, theophylline and catechin (the active constituents of a plant *Pullinia cupana*) across pig's ear skin. The active constituents were found to penetrate the skin simultaneously with theobromine having a high permeability rate favoured by the delivery vehicle (water) used. Boonen et al. (2010) evaluated the transdermal permeation behaviour of an N-alkylamide spilanthol compound from *Spilanthes acmella* extract. The compound showed steady state permeation behaviour with 0.08-18.31% that permeated the skin after 24 hours of topical application. Park et al. (2012) compared the permeation profiles of the flavonoids, astragalin and kaempferol isolated from a plant *Suaeda asparagoides*. The degree of permeation of the compounds was found to increase linearly with kaempferol having greater penetration than astragalin after a 24 hour period of application. However, more studies are needed to identify chemical compounds permeating the skin after topical application. Phytochemical permeation investigations may provide insight on the pharmacokinetic characteristics of the chemical compounds present in topically applied plant preparations, and whether these compounds diffuse across the intact skin or not.

1.4 Overview of the study area and the context of this study

The study area, northern Maputaland is considered a region with low socioeconomic status in South Africa with 47% of unemployed population. The majority of people in this remote area live together in confined dwellings where infectious diseases are easily spread (York et al., 2011). Skin infections are problematic since the high HIV infection rate (15.2%), makes people susceptible to a wide range of skin pathogens (Statistic South Africa, 2007). Lack of proper infrastructure, humidity, heat and unsanitary living conditions facilitate bacterial and fungal skin infections. These factors are of concern in South African rural communities, where, for example, 78 million people in 2002 become infected with ringworm, *Tinea capitis* (Bank, 2002). The majority of the population in northern Maputaland also depend on wood fires for cooking which increases the risks of burn wounds while other wounds are inflicted during wood collection and

field cultivation (De Wet et al., 2013). Burns and wounds provide an entry route for pathogens such as *P. aeruginosa* to infect the underlying tissues and initiate deep skin infections (Weideman, 2005).

As in most developing countries, the indigenous people of South Africa especially in rural communities still depend on medicinal plants for their primary health care. Although health care is available free of charge in centres situated in rural communities, people still prefer traditional approaches of treating diseases due to cultural beliefs and ease of availability (Grieson and Afolayan, 1999). These observations were also apparent in northern Maputaland where skin infections are among the ailments treated traditionally using medicinal plants, despite the availability of 13 clinics and two hospitals in the area. In an ethnobotanical study by De Wet et al. (2013) in four rural communities (Mabibi, Mbazwana, Mseleni and Tshongwe) of northern Maputaland (Figure 1.1), lay people were interviewed about the plants they use to treat skin diseases. The focus was on plants that grow in and around the immediate vicinity of their homesteads. Forty seven plant species were documented as treatments against 11 skin ailments (abscesses, acne, boils, burns, incisions, rash, ringworm, shingles, sores, warts and wounds).

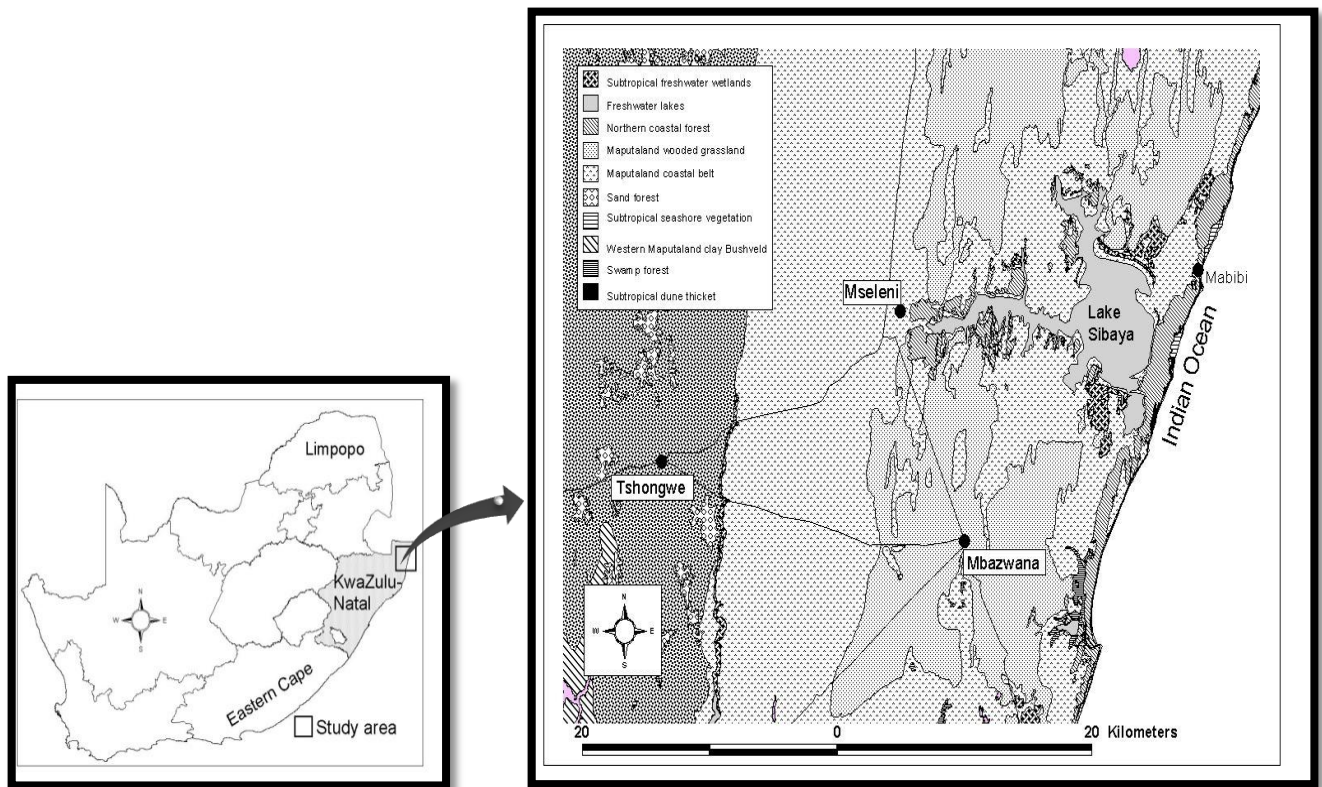


Figure 1.1: Map of the study area, northern Maputaland (De Wet et al., 2013).

From the survey by De Wet et al. (2013) nine plant species (*Acacia burkei*, *Brachylaena discolor*, *Ozoroa engleri*, *Parinari capensis* subsp. *capensis*, *Portulacaria afra*, *Sida pseudocordifolia*, *Solanum rigescens*, *Strychnos madagascariensis* and *Drimia delagoensis*) were documented for the first time in northern Maputaland as a treatment of skin infections. The anti-infective role of these nine plant species has not yet been explored.

The survey also revealed that some plants were used in combinations to treat six types of skin ailments (sores, shingles, burns, boils, acnes and ringworm). Such combinations involved the combination of two (e.g. *Sclerocarya birrea* and *Syzygium cordatum*), and even multiple plant species (e.g. *Brachylaena discolor*, *Euphorbia tirucalli*, *Hypoxis hemerocallidea*, *Ozoroa engleri* and *Senecio serratuloides*) per medication. The *in vitro* efficacy information on the interactive profiles of such plant combinations is lacking.

About 48% of the plant species documented by De Wet et al. (2013) are applied topically to treat both superficial and deep skin infections. As described in Section 1.3, the phytochemical evaluation of topically applied plant preparations is vital so as to comprehend the intended therapeutic effect of the chemical compounds present in such preparations. However, from the documented plants, specific information about the chemical profiles and the transdermal permeation behaviour of the compounds present in the crude extracts are not available. Thus, it was pivotal to also assess whether the chemical compounds present within the crude extracts are able to diffuse across the skin or not.

In this study, 37 plants (Table 1.1) from the study undertaken by De Wet et al. (2013) were considered for investigation. The documented plants were analyzed based on their ethnobotanical uses as claimed by the rural inhabitants of northern Maputaland. Figure 1.2 shows a diagrammatic summary of how this study was conducted starting from plant collection and identification. Thereafter, antimicrobial screening was undertaken for each plant species and those used in combinations. A selection of plants was further subjected to preliminary phytochemical analysis and evaluated in *in vitro* permeability experiments after exposure of intact porcine skin to the extracts for 120 min. The results obtained in this study would serve to scientifically validate the traditional therapeutic application of these herbal remedies as they contribute significantly to the primary health care system of people in rural Maputaland.

Table 1.1: Plants used to treat skin diseases by lay people of northern Maputaland as adapted from De Wet et al. (2013).

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Acacia burkei</i> Benth Fabaceae	Bark	Decoction used as enema	Sores	Combined with <i>Lippia javanica</i> , <i>Ozoroa engleri</i> , <i>Sclerocarya birrea</i> , <i>Syzygium cordatum</i> and <i>Tabernaemontana elegans</i>
		Decoction taken orally	Ringworm	<i>Kigelia africana</i>
<i>Albizia adianthifolia</i> (Schumach) W. Wight Fabaceae	Bark	Maceration used for bathing	Burning and itching rash	No combination
<i>Albizia vesicolor</i> Welw. ex Oliv Fabaceae	Bark	Decoction used as enema	Sores	No combination
<i>Annona senegalensis</i> Pers. Annonaceae	Roots	Decoction taken orally with soft porridge	Sores	No combination
<i>Brachyleana discolor</i> DC. Asteraceae	Twigs	Decoction taken orally	Sores	<i>Euphorbia tirucalli</i> , <i>Hypoxis hemerocallidea</i> , <i>Ozoroa engleri</i> and <i>Senecio serratuloides</i>
<i>Canthium inerme</i> (L.f) Kuntze Rubiaceae	Twigs	Decoction used for steaming	Acne vulgaris	<i>Dichrostachys cinerea</i>
<i>Dalbergia obovata</i> E. Mey. Fabaceae	Leaves	Paste applied topically	Burns	No combination

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Dialium schlechteri</i> Harms Fabaceae	Bark	Powder applied topically	Boils, burns and wounds	No combination
<i>Dichrostachys cinerea</i> (L.) Wight & Arm. Fabaceae	Twigs	Decoction used for steaming	Acne vulgaris	<i>Canthium inerme</i>
<i>Euphorbia tirucalli</i> L. Euphorbiaceae	Modified stems	Decoction taken orally	Sores	<i>Brachyleana discolor,</i> <i>Hypoxis hemerocallidea,</i> <i>Ozoroa engleri</i> and <i>Senecio serratuloides</i>
<i>Elephantorrhiza elephantina</i> (Burch.) Skeels Fabaceae	Roots	Decoction taken orally	Sores	<i>Cladostemon kirkii,</i> <i>Drimia delagoensis,</i> <i>Ficus sir, Sarcophyte</i> <i>sanguinea</i> and <i>Senecio</i> <i>serratuloides</i>
			Shingles	<i>Cladostemon kirkii,</i> <i>Drimia delagoensis,</i> <i>Sarcophyte sanguinea</i> and <i>Ranunculus</i> <i>multifidus</i>
<i>Garcinia livingstonei</i> T. Anderson Clusiaceae	Bark	Paste applied topically	Burns	No combination
<i>Hewittia malabarica</i> L. (Suresh) Convolvulaceae	Leaves	Paste applied topically	Abscesses and boils	No combination
<i>Hibiscus surratensis</i> L. Malvaceae	Roots	Paste applied topically	Burns	No combination
		Decoction taken orally	Sores	No combination

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Hypoxis hemerocallidea</i> Fisch., C.A. Mey. & Avelall. Hypoxidaceae	Corms	Decoction taken orally	Boils	<i>Solanum rigescens</i>
			Ringworm	<i>Ledebouria revoluta</i>
			Sores	<i>Brachyleana discolor</i> , <i>Euphorbia tirucalli</i> , <i>Ozoroa engleri</i> and <i>Senecio serratuloides</i>
<i>Kigelia africana</i> (Lam) Benth. Bignoniaceae	Bark	Decoction taken orally	New surgical incisions	No combination
	Fruits	Ash applied topically	Ringworm	<i>Acacia burkei</i> No combination
<i>Lippia javanica</i> (Burm. F.) Spreng Verbenaceae	Twigs	Decoction used as enema	Sores	<i>Acacia burkei</i> , <i>Ozoroa engleri</i> , <i>Sclerocarya birrea</i> , <i>Syzygium cordatum</i> and <i>Tabernaemontana elegans</i>
<i>Mormodica balsamina</i> L. Curcubitaceae	Leaves	Maceration used for bathing	Rash	No combination
		Paste applied topically	Ringworm	
<i>Ozoroa engleri</i> R.Fern. & A.Fern Anacardaceae	Bark	Decoction used as enema	Sores	<i>Acacia burkei</i> , <i>Lippia javanica</i> , <i>Ozoroa engleri</i> , <i>Sclerocarya birrea</i> , <i>Syzygium cordatum</i> and <i>Tabernaemontana elegans</i>

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Ozoroa engleri</i> R.Fern. & A.Fern Anacardaceae	Twigs	Decoction taken orally	Sores	<i>Brachyleana discolor</i> , <i>Euphorbia tirucalli</i> , <i>Hypoxis hemerocallidea</i> and <i>Senecio serratuloides</i>
<i>Parinari capensis</i> Harv. subsp. <i>capensis</i> Chrysobalanaceae	Rhizome	Decoction taken orally	Sores	No combination
<i>Portulacaria afra</i> Jacq. Portulacaceae	Leaves	Maceration used for bathing	Rash and chronic sores	No combination
<i>Ranunculus multifidus</i> Forssk. Ranunculaceae	Whole plant	Maceration taken orally	Shingles	No combination
		Decoction taken orally	Sores	<i>Cladostemon kirkii</i> , <i>Drimia delagoensis</i> , <i>Ficus sir</i> , <i>Sarcophyte sanguinea</i> and <i>Senecio serratuloides</i>
			Shingles	<i>Cladostemon kirkii</i> , <i>Drimia delagoensis</i> , <i>Sarcophyte sanguinea</i> and <i>Ranunculus multifidus</i>
<i>Schotia brachypetala</i> Sond. Fabaceae	Bark	Decoction used as enema	Sores	<i>Sclerocarya birrea</i>
<i>Sclerocarya birrea</i> (A. Rich.) Hotsch. Anacardaceae	Bark	Powder applied topically	Burns	<i>Syzygium cordatum</i>
			Boils	No combination

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Sclerocarya birrea</i> (A. Rich.) Hotsch. Anacardaceae	Bark	Decoction used as enema	Sores	<i>Acacia burkei</i> , <i>Lippia javanica</i> , <i>Ozoroa engleri</i> , <i>Syzygium cordatum</i> and <i>Tabernaemontana elegans</i>
<i>Senecio serratuloides</i> DC. Asteraceae	Leaves	Paste applied topically	Abrasions, burns, cuts and open sores	No combination
		Decoction taken orally	Rash	No combination
		Maceration used for bathing	Rash	<i>Drimia delagoensis</i>
		Decoction taken orally	Sores	<i>Cladostemon kirkii</i> , <i>Drimia delagoensis</i> , <i>Ficus sir</i> , <i>Elephantorrhiza elephantina</i> and <i>Sarcophyte sanguinea</i>
<i>Solanum panduriforme</i> E. Mey. Solanaceae	Fruits	Topically	Warts	No combination
<i>Solanum rigescens</i> Jacq. Solanaceae	Fruits	Decoction taken orally		No combination
				<i>Hypoxis hemerocallidea</i>
<i>Strychnos madagascariensis</i> Poir Strychnaceae	Leaves	Paste applied topically	Sores	<i>Strychnos spinosa</i>
	Bark	Powder applied topically	Sores and burns	No combination
		Decoction used topically	Ringworm	No combination

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Strychnos spinosa</i> Lam. Strychnaceae	Fruits	Topically	Warts	No combination
	Bark	Paste applied topically	Sores	<i>Strychnos madagascariensis</i>
<i>Syzygium cordatum</i> Hochst. Ex C. Krauss. Myrtaceae	Bark	Decoction taken orally	Sores	No combination
		Powder applied topically	Burns	<i>Syzygium cordatum</i>
		Decoction used as enema	Sores	<i>Acacia burkei, Lippia javanica, Ozoroa engleri, Sclerocarya birrea and Tabernaemontana elegans</i>
<i>Tabernaemontana elegans</i> Stapf. Apocynaceae	Leaves	Latex applied topically	Ringworm and new wounds	No combination
		Decoction used as enema	Sores	<i>Acacia burkei, Lippia javanica, Ozoroa engleri, Sclerocarya birrea and Syzygium cordatum</i>
<i>Terminalia sericea</i> Busch ex. DC Combretaceae	Leaves	Paste applied topically	Burns	No combination
<i>Waltheria indica</i> L. Malvaceae	Roots	Paste and powder applied topically	Burns and wounds	No combination
<i>Withania somnifera</i> (L.) Solanaceae	Roots	Paste applied topically and decoction taken orally	Shingles	No combination

Botanical name/ Family	Part(s) used	Modes of administration	Medical condition	Used in combination
<i>Ximenia caffra</i> Sond. Olacaceae	Twigs	Decoction taken orally	Sores	No combination
<i>Zanthoxylum capense</i> (Thunb.) Harv. Rutaceae	Roots	Wash	New wounds	No combination
<i>Ziziphus mucronata</i> Wild. Rhamnaceae	Leaves and roots	Powder applied topically	Boils	No combination

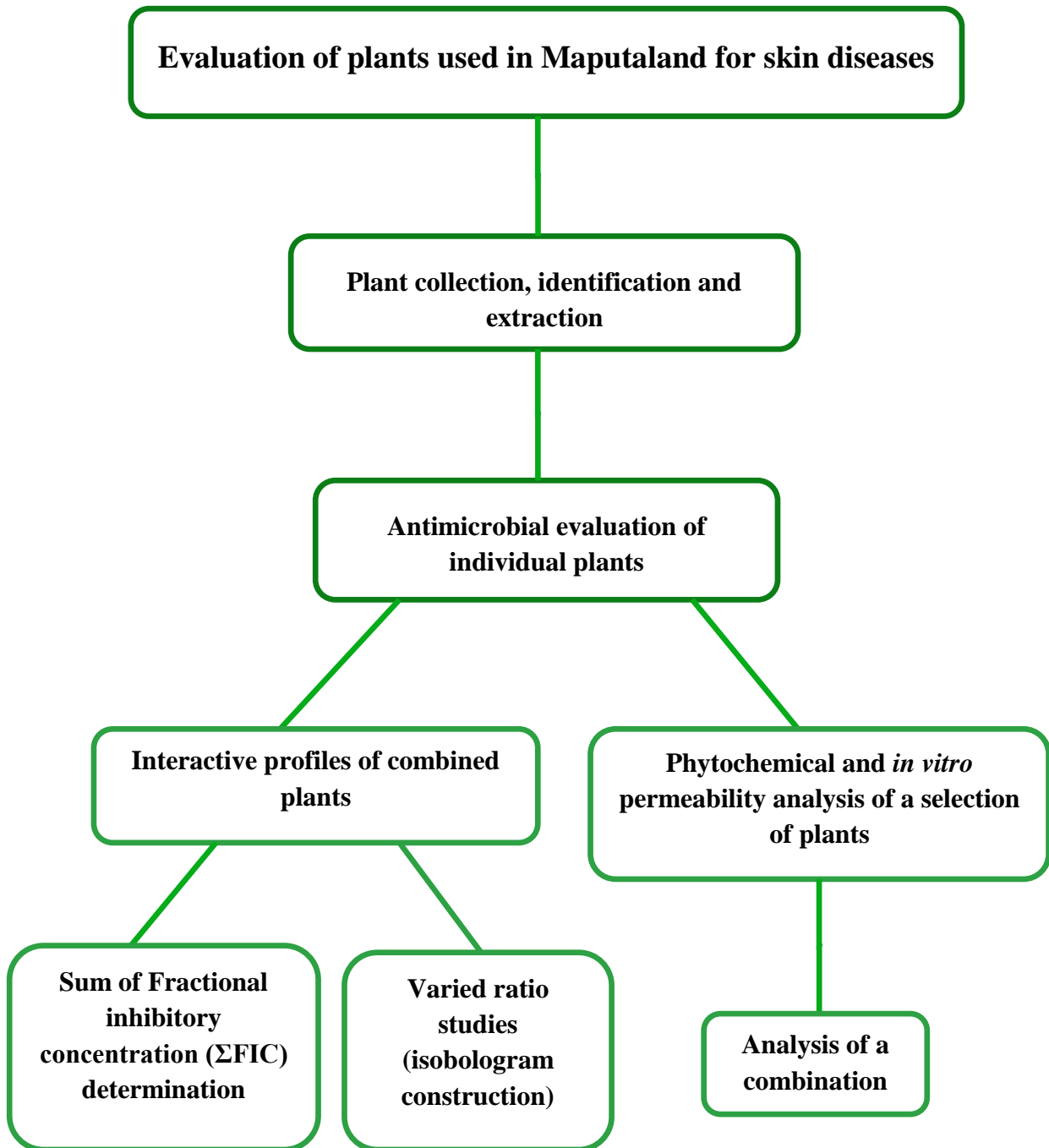


Figure 1.2: Diagrammatic summary of steps undertaken in validation of plants used in Maputaland for skin diseases.

1.5 Aims and objectives of the study

This study intended to validate the traditional use of medicinal plants by lay people of northern Maputaland against skin infections. Taking into consideration the ethnobotanical information documented by De Wet et al. (2013) and the lack of scientific information validating the ethnobotanical claims of the documented plants, the current study was designed to address these areas of concern. To achieve the main aim, the following objectives were set;

- To review an ethnobotanical study by De Wet et al. (2013) in order to obtain information related to the traditional use of plants in Maputaland to treat skin infections.
- To prepare aqueous and organic extracts of the relevant plant material collected from northern Maputaland, KwaZulu-Natal Province of South Africa.
- To evaluate the antimicrobial activity of the plants used independently and in combinations to treat skin infections using the Minimum Inhibitory Concentration (MIC) assay.
- To assess the interactions between the plants used in combinations using the sum of the fractional inhibitory concentration (Σ FIC) and varied ratio combination studies.
- To obtain the chemical profiles of the most antimicrobially active topically applied plant extracts (independent and combined samples) using Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) and indicate the number of chemical compounds capable of penetrating the skin after the *in vitro* permeability experiment using excised porcine skin.

Chapter two: Antimicrobial screening of individual plants used in northern Maputaland to treat skin diseases

2.1 Introduction

Apart from the ethnobotanical study by De Wet et al. (2013) in northern Maputaland where 47 medicinal plants were identified by lay people to have curative properties against various skin diseases (abscesses, acne, boils, burns, incisions, rash, ringworm, shingles, warts and wounds) (Chapter 1, Table 1.1), previous studies in the same geographical area by various authors further revealed the vast use of plants for the treatment of various infectious diseases not necessarily related to skin ailments. In this instance, the study by De Wet et al. (2010) identified 23 plant species which are used to treat diarrhoea, and such usage was validated by subjecting the plant extracts to diarrheal pathogens (Van Vuuren et al., 2015). Another study by York et al. (2011) documented 33 plant species used for the treatment of respiratory tract infections, and the species were evaluated against respiratory tract pathogens (York et al., 2012). De Wet et al. (2012) also identified 33 plant species used for the treatment of sexually transmitted infections (STIs), where Naidoo et al. (2013) assessed the plants against the pathogens associated with STIs. This chapter is consequently aimed at investigating the antimicrobial properties of 37 plant species selected from the 47 species listed by De Wet et al. (2013) against skin associated pathogens so as to scientifically support their ethnobotanical claims.

2.2 Materials and methods

2.2.1 Collection and preparation of plant material

Plant samples were collected from northern Maputaland, KwaZulu-Natal Province of South Africa during June-July 2012. Information obtained from the ethnobotanical study (De Wet et al., 2013) identified 47 plant species that were used for dermatological conditions, however, only 37 plants were considered for investigation in the present study based on the availability of the plant material during the survey. Plant parts used (such as roots, bark, leaves and fruits) from the identified plants were collected from around the vicinity of the homesteads during the interview

process with the lay people. Voucher specimens were then prepared on site (Figure 2.1) and are housed in the herbarium of the Department of Botany at the University of Zululand, South Africa. The plants were identified and authenticated by Dr. T.C.H. Morstert from the University of Zululand as well as Mr. Mkipheni Ngwenya from the South African National Biodiversity Institute (SANBI), Durban. Samples of the collected plant species used for identification together with their voucher specimen numbers are listed in Table 2.1. Collected bulk plant material was separated according to the parts used and dried at room temperature. The dried plant material was then ground into a fine powder using a Scientec RSA Hammer mill.



Figure 2.1: Collection of plant material and preparation of voucher specimens around northern Maputaland homesteads.

Table 2.1: Voucher specimen numbers, plant parts used and extracts yields of plants investigated.

Plant name/Family	Voucher specimen no.	Plant part(s) used	Extract yields (%)	
			Aqueous	Organic
<i>Acacia burkei</i> Benth. Fabaceae	S.Nciki 25	Bark	8.9	6.3
<i>Albizia adianthifolia</i> (Schumach.) W. Wight Fabaceae	S.Nciki 36	Bark	4.2	6.3
<i>Albizia versicolor</i> Welw. ex Oliv Fabaceae	S.Nciki 5	Bark	18.5	10.5

Plant name/Family	Voucher specimen no.	Plant part(s) used	Extract yields (%)	
			Aqueous	Organic
<i>Annona senegalensi</i> Pers. Annonaceae	S.Nciki 8	Roots	5.4	5.3
<i>Brachylaena discolor</i> DC. Asteraceae	S.Nciki 6	Twigs	6.7	4.8
<i>Canthium inerme</i> (L.f) Kuntze Rubiaceae	S.Nciki 3	Twigs	3.3	12.2
<i>Dalbergia obovata</i> E. Mey. Fabaceae	S.Nciki 9	Leaves	1.7	6.9
<i>Dialium schlechteri</i> Harms Fabaceae	S.Nciki 31	Bark	2.3	18.3
<i>Dichrostachys cinerea</i> (L.) Wight & Arn. Fabaceae	S.Nciki 10	Twigs	13.6	30.2
<i>Elephantorrhiza elephantina</i> (Burch.) Skeels Eurphobiaceae	S.Nciki 12	Rhizome	7.3	9.5
<i>Euphorbia tirucalli</i> L Fabaceae	T.York 37	Stems	6.3	10.4
<i>Garcinia livingstonei</i> T. Anderson Clusiaceae	S.Nciki 21	Bark	4.1	2.2
<i>Hewittia malabarica</i> L. (Suresh) Convolvulaceae	S.Nciki 14	Leaves	6.5	33.3
<i>Hibiscus surattensis</i> L. Malvaceae	S.Nciki 28	Roots	0.3	8.2
<i>Hypoxis hemerocallidea</i> Fisch., C.A. Mey. & Avé-Lall. Hypoxidaceae	Nzama 38	Corms	18.5	8.4
<i>Kigelia africana</i> (Lam.) Benth. Bignoniaceae	S.Nciki 27	Bark	15.0	6.7
		Fruits	1.6	7.0
<i>Lippia javanica</i> (Burm. F.) Spreng. Verbenaceae	S.Nciki 26	Twigs	9.2	8.0
<i>Momordica balsamina</i> L. Cucurbitaceae	S.Nciki 11	Leaves	19.2	18.1
<i>Ozoroa engleri</i> R.Fern. & A.Fern Anacardaceae	S.Nciki 37	Bark	12.1	17.8
		Leaves	5.6	9.4
<i>Parinari capensis</i> Harv. subsp. <i>capensis</i> Chrysobalanaceae	S.Nciki 7	Roots	2.4	3.7
<i>Portulacaria afra</i> Jacq. Portulacaceae	S.Nciki 4	Leaves	4.1	4.5
<i>Ranunculus multifidus</i> Forssk. Ranunculaceae	S.Nciki 23	Whole plant	12.4	11.8

Plant name/Family	Voucher specimen no.	Plant part(s) used	Extract yields (%)	
			Aqueous	Organic
<i>Schotia brachypetala</i> Sond. Fabaceae	S.Nciki 22	Bark	11.9	12.1
<i>Sclerocarya birrea</i> (A. Rich.) Hotsch. Anacardaceae	S.Nciki 17	Bark	9.7	5.4
<i>Senecio serratuloides</i> DC. Asteraceae	S.Nciki 1	Leaves	11.7	15.2
<i>Solanum panduriforme</i> E. Mey. Solanaceae	S.Nciki 38	Fruits	21.7	30.2
<i>Solanum rigescens</i> Jacq. Solanaceae	S.Nciki 39	Fruits	5.4	14.3
<i>Strychnos madagascariensis</i> Poir Strychnaceae	S.Nciki 35	Bark	7.6	5.8
		Leaves	5.9	13.6
<i>Strychnos spinosa</i> Lam. Strychnaceae	S.Nciki 34	Fruits	14.5	14.0
<i>Syzygium cordatum</i> Hochst. ex C. Krauss. Myrtaceae	S.Nciki 18	Bark	8.7	4.6
<i>Tabernaemontana elegans</i> Stapf Apocynaceae	S.Nciki 24	Fruits	10.2	25.6
		Leaves	16.8	20.7
<i>Terminalia sericea</i> Busch ex. DC Combretaceae	S.Nciki 16	Leaves	5.6	19.5
<i>Waltheria indica</i> L. Malvaceae	S.Nciki 29	Roots	1.5	12.1
<i>Withania somnifera</i> (L.) Dunal. Solanaceae	S.Nciki 15	Roots	20.4	4.2
<i>Ximenia caffra</i> Sond. Olacaceae	S.Nciki 30	Twigs	6.3	5.8
<i>Zanthoxylum capense</i> (Thunb.) Harv. Rutaceae	S.Nciki 33	Roots	20.0	20.7
<i>Ziziphus mucronata</i> Willd. Rhamnaceae	S.Nciki 32	Leaves	4.4	12.6

2.2.2 Extraction of plant material

Two types of extracts (organic and aqueous) were prepared from the ground plant material. The procedures followed to obtain both types of extracts are depicted in Figure 2.2. Organic extracts were prepared by submerging a known quantity (of approximately 30 g) of dried, powdered plant



(i) Weighing ground plant material



(ii) Adding solvents

(iii) Filtering solvents



(Dichloromethane: methanol)



(Aqueous)



Evaporating solvents



Freeze dried

Figure 2.2: Schematic representation of the extraction procedure for aqueous and organic extracts.

material in a 1:1 mixture of dichloromethane and methanol (CH₂Cl₂: MeOH) in approximately 200 ml (Merck). Dichloromethane and methanol were used as the solvents of choice as they extract both polar and non-polar compounds from plants. Each mixture was agitated at 37 °C in a shaking incubator for 24 hours to thoroughly mix plant material with organic solvents. Thereafter, the solution was filtered using sterile cotton wool. Extraction was repeated twice using the same plant material and adding the same amount of fresh solvent followed by agitation for another 24 hours. Both resulting filtrates were then combined in one vessel and air-dried to evaporate the solvent.

In the ethnobotanical study (De Wet et al., 2013) it was found that water was the traditionally used solvent for preparing the plant remedies. The lay people prepared the plant remedies as decoctions (mixing a known amount of plant material, e.g. two handfuls of leaves, with a certain amount of water, e.g. 2 litres, and bringing the mixture to boil) and macerations (soaking fresh or dried plant material in cold or warm water). Consequently, it was important to include aqueous extracts in the current study so as to imitate the actual preparations from the homesteads. Aqueous extracts were prepared by submerging a known amount of powdered plant material (approximately 30 g) in warm, sterile distilled water of approximately 200 ml. These were then left at ambient temperature overnight. Thereafter, they were filtered using Wattman No 1 filter paper and the filtrates were frozen at -80 °C before lyophilisation. All extracts were then left in a lyophiliser (Labcon) until totally dried. Extracts were thereafter subjected to ultraviolet (UV) light overnight to eliminate possible microbial contaminants. The percentage yields of all the dried crude extract residues were calculated (Table 2.1) and the extracts then were kept in sealed bottles at room temperature until further use.

2.2.3 Antimicrobial screening

2.2.3.1 Preparation of crude extracts for antimicrobial screening

For antimicrobial testing, plant extracts were prepared to a final concentration of 32 mg/ml. Acetone was used for reconstituting the dried crude organic extracts due to its almost negligible minimal antimicrobial effects (Eloff, 1998). Extracts that could not dissolve adequately in acetone were sonicated using a Labcon sonicator for approximately 10 minutes until thoroughly dissolved. Aqueous extracts were also prepared to a standard concentration of 32 mg/ml by

dissolving the freeze dried plant powder in sterile distilled water. The prepared extracts were then stored in a fridge at 4 °C.

2.2.3.2 Preparation of cultures selected for analysis

Culture and media preparations were performed according to the Clinical and Laboratory standards Institute guidelines (CLSI, 2012). American Type Culture Collection (ATCC) bacterial and fungal strains associated with skin infections were selected for analysis. The bacterial strains included the Gram-positive *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), gentamycin and methicillin resistant *Staphylococcus aureus* (GMRSA), *Staphylococcus epidermidis*, *Propionibacterium acnes* and *Brevibacterium agri*, as well as the Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli*. Two dermatophytes (*Trichophyton mentagrophytes* and *Microsporum canis*) and yeast (*Candida albicans*) were included as fungal pathogens. *Brevibacterium linens*, another bacterial strain from DSM (Deutsche Sammlung von Mikroorganismen), was also included. A waiver for the approval of the use of microbial cultures was obtained from the University of the Witwatersrand Human Research Ethics Committee (Appendix G). A summary of selected pathogens, their ATCC reference numbers, characteristics and associated skin pathogenesis is represented in Table 2.2.

Bacterial cultures were prepared by transferring 1 ml of inoculum from a frozen stock culture of each strain into Tryptone Soya broth (TSB) (Oxoid) followed by incubation at 37 °C for 24 hours. *Propionibacterium acnes*, a fastidious anaerobe, was grown in Thioglycolate broth (Oxoid), which was incubated at 37 °C for seven days under anaerobic conditions using a candle jar. The yeast, *Candida albicans*, was grown in TSB and incubated at 37 °C for 48 hours. The dermatophytes, *Trichophyton mentagrophytes* and *Microsporum canis*, were cultured in Sabouraud's Dextrose broth (Oxoid) and incubated at 35 °C for seven days under 100% relative humidity.

2.2.3.3 Minimum inhibitory concentration (MIC) assays

The micro-dilution technique was used to determine the minimum inhibitory concentration (MIC) values of the plant extracts. The technique involves the use of 96-well microtitre plates and tetrazolium salts to indicate microbial growth (Eloff, 1998). Conventional antimicrobial

Table 2.2: Summary of pathogens selected for investigation and their microbial characteristics

Selected pathogen	Microbial classification	Skin associated condition(s)	Growth medium	References
<i>Brevibacterium agri</i> ATCC ^a 51663	Gram-positive	Foot odour	Tryptone Soya broth/agar	Abramson, (1983).
<i>Brevibacterium linens</i> DSM ^b 20425	Gram-positive	Foot odour	Tryptone Soya broth/agar	Abramson, (1983).
<i>Staphylococcus aureus</i> ATCC 25923, Methicillin-resistant <i>Staphylococcus aureus</i> ATCC 43300 and Gentamycin-methicillin resistant <i>Staphylococcus aureus</i> ATCC 33592	Gram-positives	Abscesses, carbuncles, furunculosis, pyoderma, pustulosis, scalded scale syndrome, skin and soft tissue infections, skin necrosis, stye and sycosis barbae	Tryptone Soya broth/agar	Lim, (1998); Said-Salim et al. (2003); Daum, (2007); Quave et al. (2008); Boucher et al., (2010); Peterson, (2010).
<i>Staphylococcus epidermidis</i> ATCC 2223	Gram-positive	Minor abscesses	Tryptone Soya broth/agar	Lim, (1998).
<i>Propionibacterium acnes</i> ATCC 11827	Gram-positive	Acne vulgaris	Thioglycolate broth	Lim, (1998); Weckesser et al. (2007).
<i>Escherichia coli</i> ATCC 25922	Gram-negative	Cellulitis localized to lower and upper limbs, necrotizing fasciitis, surgical site infections and infection after burn injuries.	Tryptone broth/agar	Petkovsek et al. (2009); Masoud and Gouda, (2012).

Selected pathogen	Microbial classification	Skin associated condition(s)	Growth medium	References
<i>Pseudomonas aeruginosa</i> ATCC 27853	Gram-negative	Pyoderma, cutaneous folliculitis, wound and burn infections	Tryptone Soya broth/agar	Agger and Mardan, (1995); Lim, (1998); Peterson, (2010).
<i>Candida albicans</i> ATCC 10231	Yeast	Cutaneous candidiasis including intertrigo and perianal dermatitis.	Tryptone broth/agar	Lim, (1998); Weckesser et al. (2007).
<i>Microsporum canis</i> ATCC 36299	Dermatophyte	Ringworm (tinea)	Sabouraud's Detrose broth/agar	Beneke et al. (1984); Lim, (1998); Jamson et al. (2006).
<i>Trichophyton mentagrophytes</i> ATCC 9533	Dermatophyte	Ringworm (tinea)	Sabouraud's Detrose broth/agar	Beneke et al. (1984); Lim, (1998); Jamson et al. (2006).

^a American Type Culture Collection; ^b Deutsche Sammlung von Mikroorganismen.

agents were used as positive controls, i.e. ciprofloxacin (Sigma Aldrich®) and amphotericin B (Sigma Aldrich®). The former (at a starting concentration of 0.01 mg/ml dissolved in sterile water), was used as a positive control for bacteria, while the latter (at a starting concentration 0.1 mg/ml, initially dissolved in DMSO and thereafter diluted using sterile water), was used as a positive control for the yeast and dermatophytes. These controls were selected due to their known broad-spectrum activity against bacteria (ciprofloxacin) and fungi (amphotericin B), and were included in each assay to confirm the antimicrobial susceptibility of the test pathogens. Negative controls (solvent and growth medium) were also included. In this instance, acetone was diluted to a starting concentration of 32 mg/ml in sterile water as a solvent control and was included in all assays to determine if it posed any inhibitory effect on the growth of test pathogens. Growth medium was included as a culture control to confirm the presence of microbial growth in the absence of the antimicrobials.

For the antimicrobial analysis, sterile 96-well microtitre plates were aseptically prepared under a laminar flow cabinet unit (Labcon) by adding 100 µl of TSB into each well (Figure 2.3). A volume of 100 µl of each plant extract (32 mg/ml) and the controls [antibiotic (0.01 mg/ml or 0.1 mg/ml), acetone (32 mg/ml) and culture controls] respectively were transferred into the wells in the first row of the microtitre plates. Serial dilutions were performed longitudinally by transferring 100 µl of the well content and so diluting the extracts and controls with 50% each time. A 100 µl of the sub-culture (bacteria or yeast) was added to all the wells of each microtitre plate. Before use, the culture was first diluted in broth to a just-turbid consistency (0.5 McFarland standard) and diluted 1:100 with broth to give a density of approximately 1×10^6 colony forming units/ml (CFUs/ml). The final concentrations of the extracts thus ranged from 8.00 mg/ml from the first row to 0.06 mg/ml in the last row. Each microtitre plate was sealed with a sterile adhesive seal to prevent evaporation of the extracts. The plates were then incubated at 37 °C for 24 hours and 48 hours for bacteria and yeast respectively. To confirm purity of the culture used, each diluted pathogen-broth mixture was also streaked onto TSA and incubated overnight (for bacteria) and 48 hours (for *C. albicans*).

After incubation, 40 µl of 0.4 mg/ml of *p*-iodonitrotetrazolium (INT) dissolved in sterile water was added to all microtitre plate wells. INT was used as an indicator of microbial growth as it

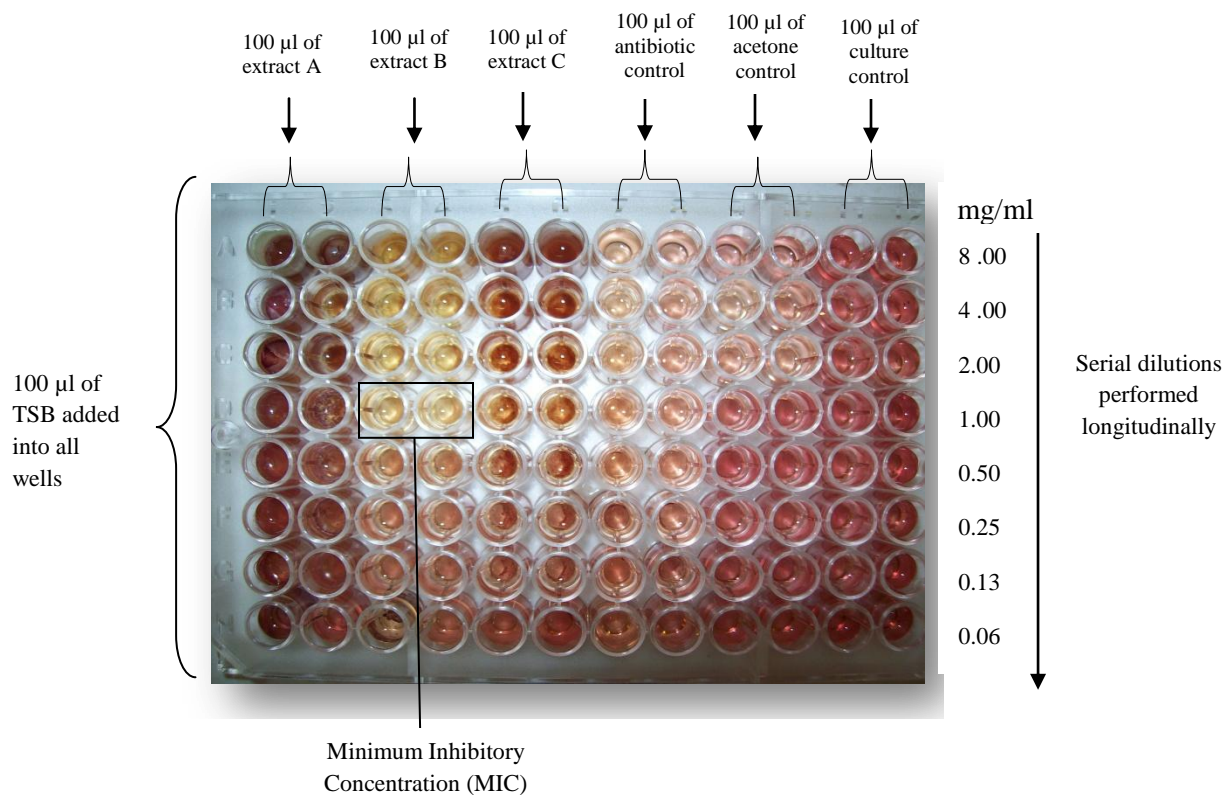


Figure 2.3: The microtitre plate dilution technique showing serial dilutions and corresponding concentration values of diluted plant extracts.

changes from colourless to red in the presence of biological active micro-organisms (Eloff, 1998). After the addition of INT, the plates were left at room temperature and assessed for the development of a red colour in the culture control columns which appeared after approximately 1-6 hours depending on the test pathogen. For *Candida albicans*, the plates were left at room temperature and the results were read after 48 hours. The MIC was reported as the lowest concentration of the extract that resulted in inhibition of microbial growth, represented by a clear colour in the wells. In cases where the MIC of certain plant extracts was not detected (i.e. MIC < 0.06 mg/ml), the extracts were diluted 10-fold to yield a starting concentration of 3.2 mg/ml and MIC assays repeated. Tests were performed in duplicate (or triplicate when inconsistent results were obtained) for each pathogen.

When performing antimicrobial screening of plant extracts against fastidious pathogens (*P. acnes*, *M. canis* and *T. mentagrophytes*), slight modifications to the standard MIC methods were employed. For *P. acnes*, the microtitre plates were incubated at 37 °C for seven days under

anaerobic conditions using a candle gas jar after which 50 µl of INT (growth indicator) was added. Since *P. acnes* does not grow readily on agar, the purity of the culture was determined by streaking the Thioglycolate broth culture onto a TSA plate which was then incubated under aerobic conditions. No growth could be observed on the TSA, indicating the absence of contaminants. For the dermatophytes (*M. canis* and *T. mentagrophytes*), after aseptically preparing the micro-titre plates, the plates were then transferred to the biohazard safety cabinet (Labcon) where 100 µl of culture was added. The culture was diluted to a ratio of 1:100 without first preparing a McFarland standard. The growth indicator (40 µl of INT) was added to the plates before incubation. The plates were then incubated at 35 °C for seven days under 100% relative humidity. For *M. canis*, the plates were incubated at 30 °C and the results were read when a red colour appeared in culture control columns, usually after four to seven days.

2.3 Results and discussion

The antimicrobial properties of 82 (41 organic and 41 aqueous) extracts (some plants had different plant parts studied) derived from 37 plants against dermatological relevant pathogens (six Gram-positive bacteria, two Gram-negative bacteria, two dermatophytes and yeast) were determined. The MIC values (expressed in mg/ml) of the extracts for which no end point could be found were given a value of > 8.00 mg/ml. The antimicrobial results of both the aqueous and organic (dichloromethane: methanol) extracts are given in Table 2.3 and 2.4 respectively. Extracts with MIC < 1.00 mg/ml (written in bold) were considered noteworthy (Gibbons, 2004; Rios and Recio, 2005; Van Vuuren, 2008). The negative controls (acetone, water and growth media) did not affect the growth of all tested pathogens as they all returned MIC > 8.00 mg/ml. For positive controls (conventional antimicrobials), the activities varied (MIC values ranged 0.01–1.25 µg/ml) depending on the susceptibility of the tested pathogens.

2.3.1 Overall antimicrobial effect of plant extracts against the skin pathogens

The overall antimicrobial profile of all the tested extracts (aqueous and organic) demonstrated that the organic extracts have higher inhibitory activity than the aqueous extracts (Figure 2.4). About 31.7% of the organic extracts displayed broad-spectrum antimicrobial effect while the rest (68.3 %) displayed noteworthy effects against one or more pathogens. The efficacy of the

aqueous extracts was mostly moderate with few (4.9 %) that displayed broad-spectrum activity while the majority (60.9 %) exhibited pathogen specific noteworthy effects (MIC<1.00 mg/ml).

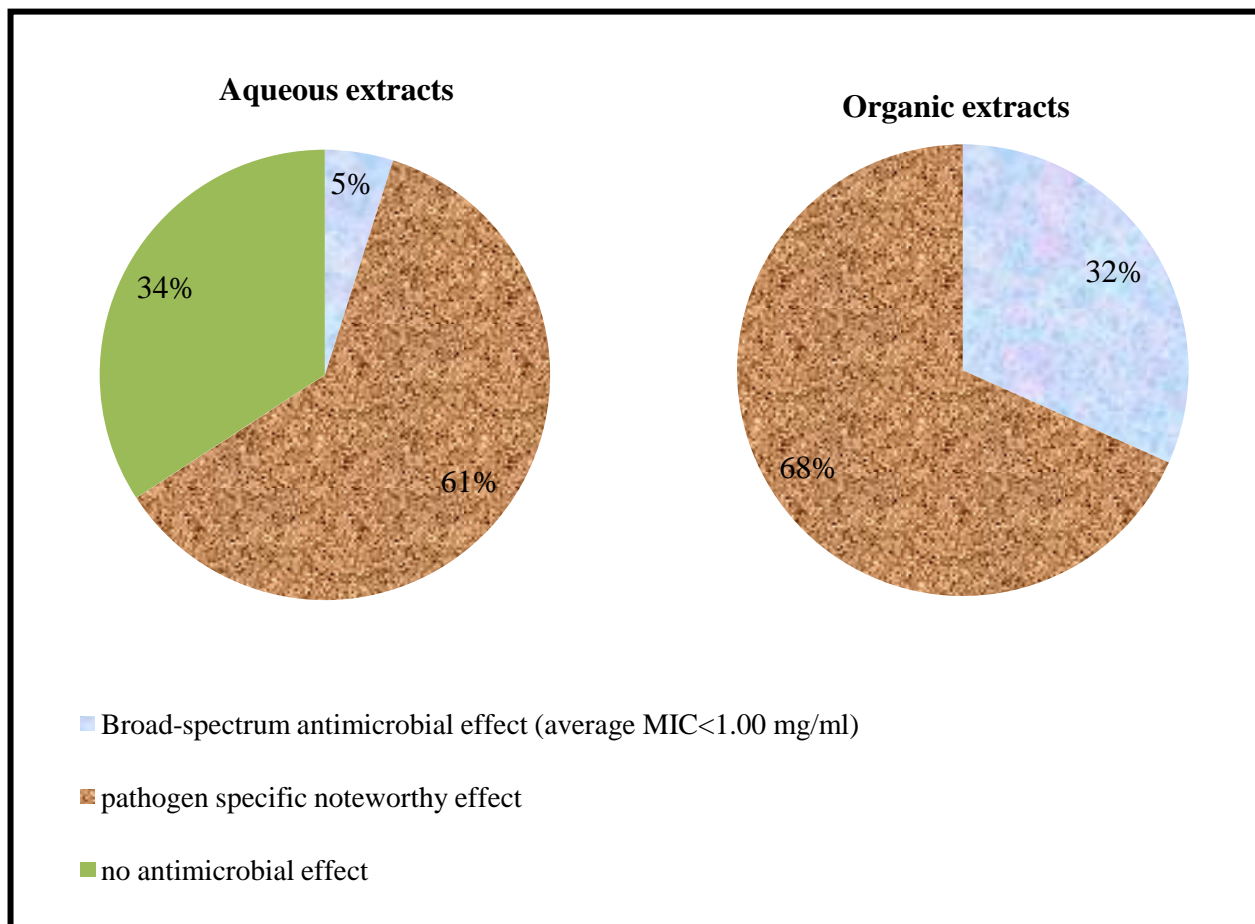


Figure 2.4: Representation of the overall antimicrobial effect of aqueous and organic (D:M) extracts against skin pathogens.

Trichophyton mentagrophytes was found to be the most susceptible pathogen with 98 % of the organic and 27 % of the aqueous extracts displaying noteworthy effects against this pathogen. These findings support the previous studies (Tadeg, 2004; Mabona et al., 2013) where *T. mentagrophytes* was also shown to be the most susceptible fungus towards plant extracts tested. *Microsporum canis* was further noted to be sensitive towards most (66 %) of the organic extracts. Amongst the Gram-positive bacteria, *B. agri* was the most susceptible, followed by *S. epidermidis* and *P. acnes*. Such findings were expected since the Gram-positive bacteria are

known to have less complex cell wall structures which make them more susceptible for antimicrobial attack (Nostro et al., 2000). Conversely, *S. aureus* which is usually the most susceptible Gram-positive bacterium in many studies was found to be the most resistant pathogen towards both aqueous and organic extracts. In this instance, only 5 % of the organic extracts exhibited noteworthy activity against *S. aureus*, while none of the aqueous extracts displayed activity (Figure 2.5).

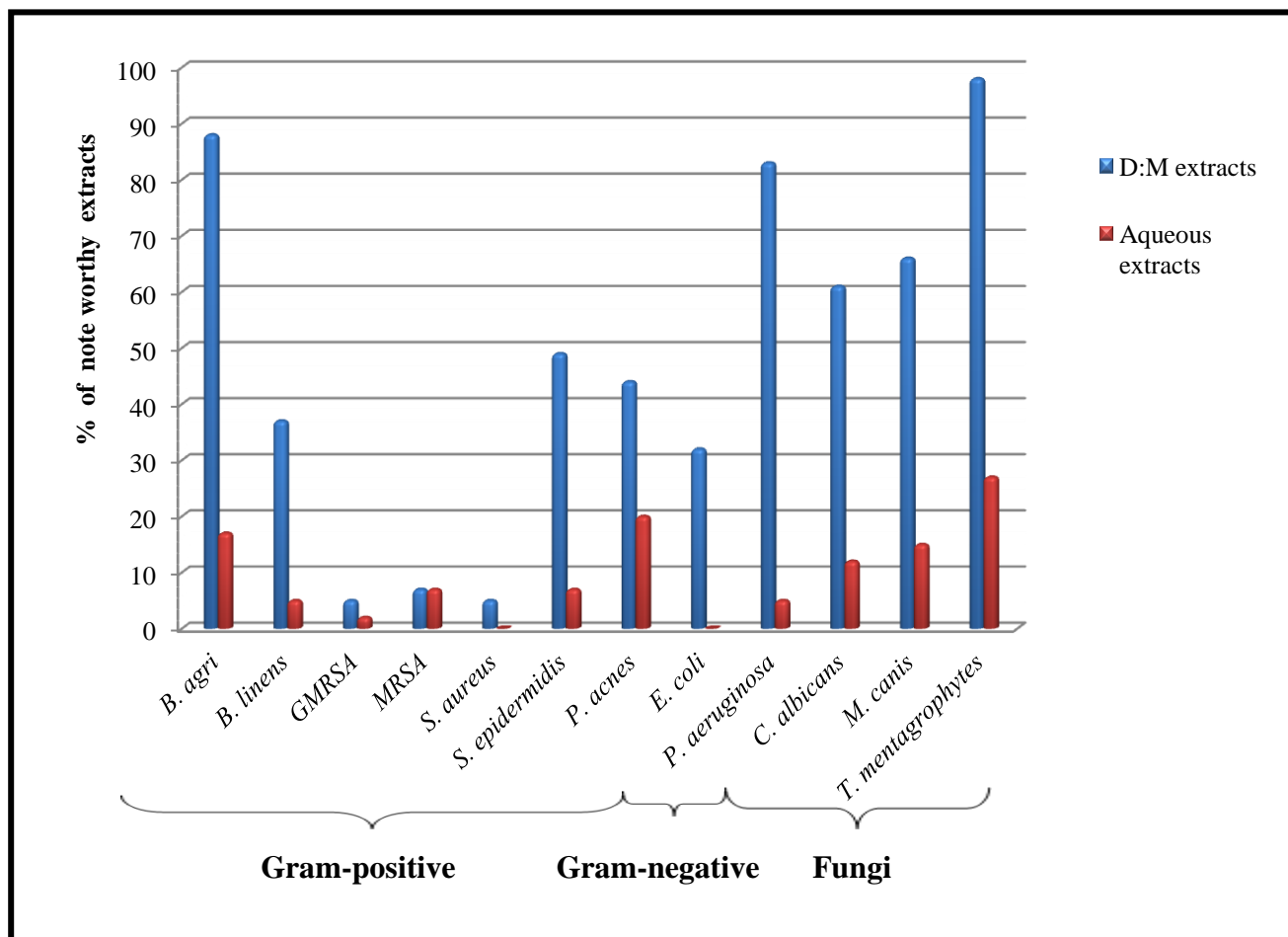


Figure 2.5: The percentage of aqueous and organic (D:M) extracts that displayed noteworthy activity (MIC<1.00 mg/ml) against each test pathogen in a group.

While several studies report that the Gram-negative bacteria are more resistant to various antimicrobials, the present study showed *P. aeruginosa* to be the most susceptible amongst all

the bacterial strains. In this instance, 83 % of the organic extracts displayed noteworthy activity against *P. aeruginosa*. In general, Gram-negative bacteria are known to have complex cell wall structures composed of lipopolysaccharides. These lipopolysaccharides reportedly act as diffusion barrier which make cell walls impermeable and less susceptible to the antimicrobial agents (Nostro et al., 2000). In spite of this permeability barrier, the organic extracts in the present study proved to be effective against Gram-negative bacteria as 32 % of the extracts displayed noteworthy activity against *E. coli* (another Gram-negative strain). These findings may justify the potential of these organic extracts in combating multi-drug resistant pathogens. It was, however, found that none of the aqueous extracts displayed noteworthy activity against *E. coli*.

2.3.2 Antimicrobial activity of the organic extracts

Although the organic solvents (dichloromethane and methanol) were not the traditionally used solvents, the extracts prepared from these solvents were the most active, exhibiting noteworthy activity against at least three or more of the tested pathogens. Only *A. vesicolor* showed the weakest activity by only having noteworthy activity against *T. mentagrophytes* (Table 2.3). Furthermore, some plant species displayed broad-spectrum antimicrobial activity giving average MIC values of < 1.00 mg/ml against all the pathogens. Plants that displayed broad-spectrum activities include *A. senegalensis*, *G. livingstonei*, *E. elephantina*, *K. africana*, *O. engleri*, *P. capensis* subsp. *capensis*, *S. brachypetala*, *S. birrea*, *S. cordatum*, *T. sericea* and *Z. capense*.

Annona senegalensis was reported to treat sores by the lay people of rural Maputaland (De Wet et al., 2013). Sores can be infected by various bacteria including *S. aureus*, GMRSA, MRSA, *S. epidermidis*, *P. aeruginosa* and sometimes be secondary infected by *E. coli*. This plant exhibited a broad-spectrum antimicrobial activity with an average MIC of 0.33 mg/ml. The plant was effective against MRSA (MIC value of 0.50 mg/ml), *S. epidermidis* (MIC value of 0.25 mg/ml), *E. coli* (MIC 0.50 mg/ml) and *P. aeruginosa* (MIC 0.25 mg/ml), the pathogens most likely to infect sores. Noteworthy antimicrobial properties were also observed against *B. agri*, *B. linens*, *C. albicans*, *M. canis* and *T. mentagrophytes* (MIC values between 0.06-0.50 mg/ml). From the literature, *A. senegalensis* have been reported to treat various skin diseases including leprosy

Table 2.3: The average MIC values (mg/ml) of organic (dichloromethane: methanol) extracts against 12 dermatological relevant pathogens.

Plant sample	Pathogens												Average MIC \pm σ^*
	<i>B. agri</i> ^a	<i>B. line</i> ^b	GMRSA ^c	MRSA ^d	<i>S. aure</i> ^e	<i>S. epid</i> ^f	<i>P. acne</i> ^g	<i>E. coli</i> ^h	<i>P. aeru</i> ⁱ	<i>C. albi</i> ^j	<i>M. cani</i> ^k	<i>T. ment</i> ^l	
<i>A. adianthifolia</i> bark	0.05	2.00	4.00	8.00	>8.00	1.00	1.50	1.50	1.00	1.00	0.13	0.06	1.84 \pm 2.33
<i>A. burkei</i> bark	0.40	0.50	2.00	1.00	4.00	1.00	0.38	1.00	0.25	2.00	0.13	0.25	1.06 \pm 1.12
<i>A. senegalensis</i> roots	0.13	0.13	1.00	0.50	>8.00	0.25	0.13	0.50	0.25	0.50	0.06	0.13	0.33 \pm 0.28
<i>A. vesicolor</i> bark	1.00	>8.00	3.00	2.00	>8.00	1.00	8.00	1.00	1.00	>8.00	1.00	0.25	2.03 \pm 2.37
<i>B. discolor</i> twigs	0.19	2.00	2.00	1.00	3.00	1.00	1.00	1.00	0.75	0.75	2.00	0.25	1.25 \pm 0.82
<i>C. inerme</i> twigs	0.03	0.50	4.00	2.00	4.00	0.25	2.00	1.00	0.25	0.75	1.00	0.25	1.34 \pm 1.23
<i>D. cinerea</i> twigs	1.00	1.00	2.00	2.00	4.00	0.19	1.00	0.75	0.25	2.00	0.25	0.50	1.25 \pm 1.10
<i>D. obovata</i> leaves	0.19	0.50	2.00	2.00	4.00	1.00	2.00	1.00	0.50	0.75	1.00	0.34	1.27 \pm 1.08
<i>D. schlechteri</i> bark	0.03	4.00	2.00	4.00	4.00	1.00	0.75	0.50	0.25	2.00	0.19	0.13	1.57 \pm 1.62
<i>E. elephantina</i> rhizome	0.25	1.50	2.00	4.00	1.00	1.00	0.25	0.13	0.50	0.13	0.25	0.25	0.94 \pm 1.14
<i>E. tirucalli</i> Modified stems	0.01	3.00	3.00	1.00	6.00	1.50	0.75	0.50	0.75	1.50	4.00	0.50	1.88 \pm 1.81
<i>G. livingstonei</i> bark	0.02	0.06	0.50	0.25	1.00	0.06	0.25	0.13	0.50	0.25	0.13	0.13	0.27 \pm 0.27
<i>H. hemerocallidea</i> corms	0.25	2.00	2.00	2.00	>8.00	2.00	1.50	1.00	2.00	1.00	1.00	0.13	1.35 \pm 0.72
<i>H. malabarica</i> leaves	0.03	3.00	2.00	2.00	8.00	0.50	1.00	1.00	0.50	0.50	0.75	0.50	1.65 \pm 2.17
<i>H. surratensis</i> leaves	0.75	>8.00	2.00	4.00	2.00	1.00	1.00	1.00	1.00	1.00	1.00	0.13	1.35 \pm 1.02
<i>K. africana</i> bark	0.08	2.00	1.00	2.00	>8.00	0.01	0.31	2.00	0.25	0.38	0.34	0.06	0.77 \pm 0.83

Plant sample	Pathogens												Average MIC \pm σ^*
	<i>B. agri</i> ^a	<i>B. line</i> ^b	GMRSA ^c	MRSA ^d	<i>S. aure</i> ^e	<i>S. epid</i> ^f	<i>P. acne</i> ^g	<i>E. coli</i> ^h	<i>P. aeru</i> ⁱ	<i>C. albi</i> ^j	<i>M. cani</i> ^k	<i>T. ment</i> ^l	
<i>K. africana</i> fruits	0.06	1.00	1.00	2.00	>8.00	0.13	0.31	2.00	0.25	0.38	0.38	0.06	0.68 \pm 0.72
<i>L. javanica</i> twigs	0.02	0.50	2.00	4.00	4.00	0.19	0.50	0.50	0.25	0.25	0.13	0.13	1.04 \pm 1.48
<i>M. balsamina</i> leaves	1.00	8.00	2.00	4.00	2.00	0.50	1.00	1.00	0.25	1.00	0.50	0.19	1.79 \pm 2.22
<i>O. engleri</i> bark	0.02	0.13	1.00	1.00	0.75	0.05	0.13	0.19	0.38	0.25	>8.00	0.13	0.37 \pm 0.37
<i>O. engleri</i> leaves	0.06	0.50	2.00	3.00	1.00	0.50	0.50	1.00	0.38	1.00	0.50	0.19	0.89 \pm 0.84
<i>P. afra</i> leaves	3.00	>8.00	1.00	0.38	1.00	3.00	8.00	2.00	0.50	2.00	0.19	0.25	1.94 \pm 2.26
<i>P. capensis</i> roots	0.06	0.50	0.50	2.00	2.00	0.03	0.19	1.00	0.25	0.25	0.38	0.06	0.60 \pm 0.70
<i>R. multifidus</i> whole plants	0.10	8.00	2.00	4.00	2.00	0.25	1.00	1.00	1.00	0.75	0.25	0.13	1.71 \pm 2.27
<i>S. birrea</i> bark	0.05	0.03	1.00	1.00	0.50	0.50	0.02	0.38	0.19	0.19	0.19	0.38	0.37 \pm 0.33
<i>S. brachypetala</i> bark	0.13	0.13	1.00	1.00	1.00	0.50	0.25	0.50	0.25	0.50	2.00	0.50	0.65 \pm 0.53
<i>S. cordatum</i> bark	0.25	0.06	2.00	2.00	2.00	1.00	0.31	0.75	0.25	0.38	0.50	>8.00	0.86 \pm 0.77
<i>S. madagascariensis</i> bark	0.05	8.00	4.00	>8.00	>8.00	>8.00	8.00	1.00	0.75	1.00	0.13	0.38	2.59 \pm 3.28
<i>S. madagascariensis</i> leaves	0.13	1.50	2.00	4.00	1.00	1.50	1.00	1.00	0.25	0.25	1.00	0.13	1.15 \pm 1.08
<i>S. panduriforme</i> fruits	0.06	2.00	2.00	2.00	2.00	0.50	>8.00	2.00	1.00	1.00	>8.00	0.50	1.31 \pm 0.77
<i>S. rigescens</i> fruits	0.06	1.00	2.00	2.00	4.00	0.15	2.00	1.00	0.50	0.13	0.19	0.13	1.20 \pm 1.21
<i>S. serratuloides</i> leaves	0.50	3.00	4.00	1.50	4.00	1.00	1.50	2.00	1.50	1.50	1.00	0.38	1.82 \pm 1.26
<i>S. spinosa</i> fruits	0.04	1.50	4.00	2.00	4.00	0.25	2.00	1.00	0.50	0.50	0.50	0.25	1.38 \pm 1.39
<i>T. elegans</i> fruits	0.06	2.00	2.00	4.00	>8.00	0.03	1.50	1.00	0.50	0.13	1.00	0.19	1.13 \pm 1.21

Plant sample	Pathogens												Average MIC \pm σ^*
	<i>B. agri</i> ^a	<i>B. line</i> ^b	GMRSA ^c	MRSA ^d	<i>S. aure</i> ^e	<i>S. epid</i> ^f	<i>P. acne</i> ^g	<i>E. coli</i> ^h	<i>P. aeru</i> ⁱ	<i>C. albi</i> ^j	<i>M. cani</i> ^k	<i>T. ment</i> ^l	
<i>T. elegans</i> leaves	0.38	3.00	2.00	4.00	4.00	1.00	6.00	2.00	0.38	0.75	0.13	0.13	1.98 \pm 1.90
<i>T. sericea</i> leaves	0.19	2.00	>8.00	>8.00	1.00	1.00	0.25	0.50	0.75	0.50	0.25	0.34	0.68 \pm 0.55
<i>W. indica</i> roots	0.31	0.50	4.00	3.00	2.00	1.00	0.75	1.00	0.25	1.00	>8.00	0.25	1.28 \pm 1.23
<i>W. somnifera</i> roots	0.31	1.00	2.00	4.00	2.00	1.00	1.50	1.00	0.38	2.00	0.50	0.25	1.33 \pm 1.07
<i>X. caffra</i> twigs	1.00	0.50	2.00	4.00	8.00	0.50	0.50	0.25	0.25	0.50	0.75	0.13	1.45 \pm 2.31
<i>Z. capense</i> roots	0.19	0.06	2.00	4.00	>8.00	1.00	1.00	1.00	0.50	0.13	0.50	0.13	0.96 \pm 1.32
<i>Z. mucronata</i> leaves	0.01	3.00	2.00	4.00	8.00	1.00	1.00	1.00	0.50	0.13	0.50	0.13	1.77 \pm 2.31
Positive control (Ciprofloxacin/ Amphotericin B μ g/ml)	0.18	0.13	0.63	0.31	0.63	0.34	1.25	0.16	0.16	0.01	0.13	0.01	0.33 \pm 0.24
Negative control (acetone)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00 \pm >8.00
Culture control (growth medium)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00 \pm >8.00

^a*Brevibacterium agri* ATCC 51663; ^b*Brevibacterium linens* DSM 20425; ^cGentamycin-methicillin resistant *Staphylococcus aureus* ATCC 33592; ^dMethicillin-resistant *Staphylococcus aureus* ATCC 43300; ^e*Staphylococcus aureus* ATCC 25923; ^f*Staphylococcus epidermidis* ATCC 2223; ^g*Propionibacterium acnes* ATCC 11827; ^h*Escherichia coli* ATCC 25922; ⁱ*Pseudomonas aeruginosa* ATCC 27853; ^j*Candida albicans* ATCC 10231; ^k*Microsporium canis* ATCC 36299; ^l*Trichophyton mentagrophytes* ATCC 9533; *MIC values >8.00 were not considered when calculating the average MIC and the standard deviation (σ) since no end point for MIC determination was obtained.

which is usually caused by *Mycobacterium leprae* (Abbiw, 1990), wounds (Hutchings et al., 1996), dermatitis and other unspecified skin infections (Kisangau et al., 2007). However, no studies have investigated the antimicrobial activity of this plant. Thus, the present study is the first to document the antimicrobial efficacy of *A. senegalensis* against skin pathogens.

Elephantorrhiza elephantina is well documented for its use against skin diseases. This plant is used as an astringent to new wounds (Pooley, 1998), and to treat acne (Pujol, 1990; Van Wyk et al., 2009) as well as dermatitis (Maphosa et al., 2012). The antimicrobial properties of *E. elephantina* have been reported by Mabona et al. (2013) where the organic extracts of the roots and rhizome showed noteworthy activity against 70% of the tested dermatological relevant pathogens giving MIC values of 0.50 mg/ml with notable activity observed against *P. acnes* (MIC 0.05 mg/ml). In the present study, *E. elephantina* rhizome was validated for its use against sores (De Wet et al., 2013). This plant exhibited broad-spectrum activity (average MIC of 0.94 mg/ml), which is congruent with the activity obtained by Mabona et al. (2013). However, the antimicrobial activity was more prominent against the Gram-negative *E. coli* (MIC 0.13 mg/ml) and *P. aeruginosa* (MIC 0.50 mg/ml) as well as fungal strains *C. albicans* (MIC 0.13 mg/ml), *M. canis* (0.25 mg/ml) and *T. mentagrophytes* (MIC 0.25 mg/ml) while in Mabona et al. (2013), the Gram-positive strains *S. aureus* (MIC 0.50 mg/ml), MRSA (MIC 0.50 mg/ml) and GMRSA (MIC 0.50 mg/ml) seemed most susceptible.

Garcinia livingstonei demonstrated the best broad-spectrum activity (average MIC of 0.27 mg/ml) when compared to the other organic extracts studied, with noteworthy activity against 11 of the 12 tested pathogens (MIC range from 0.02-0.50 mg/ml). Although, *G. livingstonei* was only reported to treat burns (De Wet et al., 2013), which are usually subjected to secondary infection by *E. coli* and *P. aeruginosa* (Lim, 1998), the broad-spectrum antimicrobial activity obtained may imply that the plant could be effective in treating both primary, secondary as well as fungal skin infections. The current findings also correlate with those reported by Van Vuuren et al. (2015) where the dichloromethane: methanol bark extract of *G. livingstonei* displayed noteworthy activity against all tested diarrheal pathogens (MIC values range 0.12-0.75 mg/ml). Some compounds (amentoflavone and 4'-monomethoxy amentoflavone) isolated from *G. livingstonei* acetone leaf extract also displayed noteworthy antimicrobial activity against *E. coli*, *S. aureus*, and *P. aeruginosa* with MIC values ranging from 8-100 µg/ml (Kaikabo et al., 2009).

These findings from literature and those documented in the current study demonstrate the potential of *G. livingstonei* in combating infectious diseases, especially skin infections.

The bark of *K. africana* was reported to treat incisions and ringworm while the fruits were reported to treat ringworm (De Wet et al., 2013). In addition to being effective against the dermatophytes *M. canis* (MIC 0.34 mg/ml) and *T. mentagrophytes* (MIC 0.06 mg/ml), the bark and fruit extracts were also effective against other pathogens (*B. agri*, *S. epidermidis*, *P. acnes*, *P. aeruginosa*, and *C. albicans*) displaying almost similar MIC values ranging from 0.01-0.38 mg/ml. These findings may imply the presence of similar compounds in both bark and fruits of *K. africana*. Chemical studies indicated the presence of an iridiod, vermonoside, which is commonly found in both bark and fruit of this plant species (Goudaa et al., 2003; Gabriel and Olubunmi, 2009; Van Wyk et al., 2009). Mabona et al. (2013) also investigated the dichloromethane: methanol fruit extracts of *K. africana* against the same skin pathogens investigated in the present study, but found it to exhibit low antimicrobial activity (MIC >1.00 mg/ml). This may be due to the phytochemical variation within the species due to different seasons (summer and winter) in which the plant material was collected. It is known that, the phytochemical concentration and composition of plants of the same species may vary with seasonal variation which may also influence the biological activity. Similarly, Naidoo et al. (2013) also reported the low antimicrobial activity of the aqueous and organic leaf extracts of *K. africana* against pathogens associated with sexually transmitted infections (STIs). In this instance, a possible explanation of such contradiction could be that different plant parts were used. In the current study fruits and bark were used while Naidoo et al. (2013) used leaves.

The antimicrobial properties of *O. engleri* against pathogens associated with STIs have also been reported by Naidoo et al. (2013). However, no previous studies were found related to the antimicrobial activity of *O. engleri* against pathogens associated with skin ailments. In the current study, *O. engleri* was validated for its use against sores (De Wet et al., 2013). Both bark and leaf extracts exerted broad-spectrum activity giving average MIC values of 0.37 and 0.89 mg/ml respectively. For the bark extract, notable antimicrobial effects were observed against *B. agri* (MIC 0.02 mg/ml) and *S. epidermidis* (MIC 0.05 mg/ml) while the leaf extract exerted an MIC of 0.06 mg/ml against *B. agri*. The more pronounced activity of the bark extract could be due to high concentrations of active ingredients found in bark (Van Wyk et al., 2009). In this

instance, the leaves could easily be substituted for the bark in traditional preparations as a precaution to prevent unsustainable harvesting of the bark.

Parinari capensis subsp. *capensis* was also reported to treat sores (De Wet et al., 2013). This plant species was found to give a broad-spectrum activity with average MIC of 0.60 mg/ml. Although there are no studies that have evaluated the antimicrobial properties of *P. capensis* against skin pathogens, a few studies reported the activity of this plant against other pathogens such as fungi. In this instance, the antifungal activity of *P. capensis* was tested against a plant pathogen, *Cladosporium cucumerinum*, where noteworthy activity (MIC 0.10 mg/ml) was reported (Garo et al., 1997). Uys et al. (2002) found the dichloromethane: methanol extract of *P. capensis* to be a promising antimalarial agent. York et al. (2012) studied its effect on respiratory tract pathogens, and reported that both the dichloromethane: methanol and aqueous extracts of *P. capensis* exhibited noteworthy activity against *S. aureus* with MIC values of 0.25 and 0.50 mg/ml respectively.

Even though the bark of *S. brachypetala* was reported to be effective against sores when used in combination with *S. birrea* (De Wet et al., 2013), the organic bark extract of *S. brachypetala* demonstrated noteworthy activities against eight pathogens independently with an average MIC of 0.65 mg/ml. Hutchings et al. (1996), documented *S. brachypetala* as a treatment for acne. To the best of our knowledge, no antimicrobial studies have been conducted in this regard. Its effects on diarrhoeal pathogens have, however been studied by Mathabe et al. (2006), where it was found that the methanol, ethanol, acetone and aqueous extracts of *S. brachypetala* were effective against 12 diarrhoeal pathogens tested (MIC values range 0.31-0.63 mg/ml). Interestingly, contradictory results have been reported in other studies where low activities against diarrheal pathogens were noted for the plant. For instance, Van Vuuren et al. (2015) found the dichloromethane: methanol extract of *S. brachypetala* to be only effective against *Shigella flexneri* and *Enterococcus faecalis* (MIC of 0.58 mg/ml and 0.63 mg/ml respectively) amongst five tested pathogens. Even compounds purified from the extract gave poor activity when tested against *Bacillus subtilis*, *E. coli*, *Klebsiella pneumoniae* and *S. aureus*, i.e. linoleic acid was least active (MIC values all >1 mg/ml) and methyl-5,11,14,17-eicosatetraenoate demonstrated weak activity against *B. subtilis* and *S. aureus* (MIC values of 0.78 mg/ml).

The antimicrobial properties of *S. birrea* have been studied and the bark extracts have been found to possess antibacterial activity against *S. aureus* (MIC 0.15 mg/ml) and *P. aeruginosa* (MIC 0.37 mg/ml) (Eloff, 2001). The antimicrobial activities of *S. birrea* bark have been further supported in recent studies (York et al., 2012, Naidoo et al., 2013, Van Vuuren et al., 2015). In the study by York et al. (2012) on respiratory tract pathogens, aqueous and dichloromethane: methanol bark extracts displayed broad-spectrum activity (average MIC value of 0.64 mg/ml). Naidoo et al. (2013) also found *S. birrea* bark extract to have broad-spectrum activity against pathogens associated with STIs including *Trichomonas vaginalis*, *Candida albicans*, *Oligella ureolytica* and *Ureaplasma urealyticum*, displaying an average MIC of 0.89 mg/ml. The same activity was also obtained by Van Vuuren et al. (2015) where dichloromethane: methanol extract of *S. birrea* exhibited notable activity against the diarrhoeal pathogens such as *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhimurium*, *Shigella flexneri* and *Staphylococcus aureus* (MIC range 0.20-0.95 mg/ml). In the present study, *S. birrea* bark was investigated against skin pathogens as the plant was reported to treat burns, boils and sores independently as well as in combination with other plants (De Wet et al., 2013). In this instance, the organic extract of the bark displayed noteworthy activity against 10 of the 12 tested pathogens (mean MIC 0.37 mg/ml). In our study, the best activities were noted against *B. agri*, *B. linens* and *P. acnes* with MIC values as low as 0.05, 0.03 and 0.02 mg/ml respectively. This validates the use of *S. birrea* as a potential effective antimicrobial agent against a large variety of pathogens all related to the various diseases the plant was reported to treat. However, the least antimicrobial activity observed against GMRSA and MRSA (1.00 mg/ml) indicates that *S. birrea* might not be an effective antimicrobial against resistant strains.

Syzygium cordatum was reported to treat sores and burns (De Wet et al., 2010) and was found to exhibit a broad-spectrum activity with the average MIC of 0.86 mg/ml in this study. Steenkamp et al. (2007) demonstrated that *S. cordatum* also possesses antifungal properties, where the methanolic bark extract displayed activity against two strains of *Candida albicans* (MIC value of 0.83 mg/ml). In the present study, MIC value obtained from the dichloromethane: methanol bark extract against *C. albicans* was even lower at 0.38 mg/ml. Other previous studies have further shown that *S. cordatum* bark is effective against diarrhoeal pathogens (Sibandze et al., 2010; Mulaudzi et al., 2011; Van Vuuren et al., 2015), respiratory tract pathogens (York et al., 2012) and pathogens associated with STIs (Van Vuuren and Naidoo, 2010; Naidoo et al., 2013).

Although dermatological uses for *S. cordatum* have been documented including the treatment of wounds (Hutchings et al., 1996), mouth sores and skin rashes (Corrigan et al., 2011), only its effect on *S. aureus* has been studied by York et al. (2012) where the organic and aqueous extracts exerted noteworthy activity with MIC values of 0.38 and 0.25 mg/ml respectively.

A previous antimicrobial study showed *Terminalia sericea* to exhibit broad-spectrum activity against dermatological relevant pathogens similar to the pathogens investigated in the present study, with noteworthy MIC values ranging between 0.03-0.80 mg/ml (Mabona et al. 2013). Our results show comparable findings in which *T. sericea* exhibited an average MIC value of 0.68 mg/ml against the test pathogens. This plant has also been found to possess broad-spectrum activity (average MIC<1.00 mg/ml) against diarrheal pathogens (Van Vuuren et al., 2015). The least activity for this species was however reported by Eloff (1999) against *S. aureus*, *E. coli* and *P. aeruginosa* (MIC range 1.00-6.00 mg/ml). Similar findings were also observed by Steenkamp et al. (2007) where *T. sericea* extract showed poor activity against *S. aureus* (MIC 5.00 mg/ml) and *S. epidermidis* (MIC 2.50 mg/ml). Such discrepancies could be attributed to the different solvents used. The present study and that of Mabona et al. (2013) employed dichloromethane and methanol while Eloff (1999) and Steenkamp et al. (2007) used methanol only. Consequently the active components may thus not be polar enough to completely dissolve in methanol.

Zanthoxylum capense was found to exhibit broad-spectrum antimicrobial activity with the average MIC of 0.96 mg/ml. The roots of this plant were reported to treat boils in an ethnobotanical study by De Wet et al. (2013). The poor antimicrobial activity was, however, observed against the Gram-positive bacteria GMRSA (MIC 2.00 mg/ml) MRSA (MIC 4.00 mg/ml), *S. aureus* (MIC >8.00 mg/ml) and *S. epidermidis* (MIC 1.00 mg/ml) which are most likely to be associated with boils. These findings contradict those obtained by Ndhlala et al. (2011) where the dichloromethane: methanol extracts of *Z. capense* roots exhibited noteworthy activity against *S. aureus* (MIC 0.19 mg/ml). The same study also demonstrated the activity of *Z. capense* against *E. coli* (MIC 0.78 mg/ml) which was found to be least susceptible in the present study giving a MIC value of 1.00 mg/ml. Such contradictions could be due to different localities where plants were sourced.

Even though the plant species with the most pronounced broad-spectrum activities have been highlighted, the other plant extracts also displayed a correlation between antimicrobial efficacy and their respective traditional uses. For instance, *M. balsamina*, specifically reported to treat ringworm, was found to be highly effective against the dermatophytes (MIC values of 0.50 and 0.19 mg/ml against *M. canis* and *T. mentagrophytes* respectively). Similarly, *T. elegans* leaves, reported to treat ringworm and wounds, also exhibited noteworthy activity against the dermatophytes (MIC of 0.13 mg/ml against both *M. canis* and *T. mentagrophytes*). Furthermore, the latter species was also found effective against *P. aeruginosa* (MIC value of 0.38 mg/ml) which is most likely to cause secondary wound infections.

Lippia javanica did not display broad-spectrum activity (mean MIC > 1.00 mg/ml), however, this plant exhibited notable antimicrobial activity against nine of the 12 tested pathogens. The best activity was observed against *B. agri*, *S. epidermidis*, *M. canis* and *T. mentagrophytes* with MIC values as low as 0.02, 0.19, 0.13 mg/ml respectively. Previous studies on *L. javanica* have focused on investigating the antimicrobial activity of the plant against respiratory tract pathogens since the plant is rich in volatile compounds that are known to be effective in treatment of various chest ailments (Manenzhe et al., 2004; Viljoen et al., 2005; York et al., 2012). Very little research has been done on the antimicrobial efficacy of *L. javanica* against skin associated pathogens. Shikanga et al. (2010) provided some antimicrobial data of this species against *S. aureus*, *E. coli* and *P. aeruginosa* where the plant was found to give noteworthy activities (MIC values range 0.13-0.42 mg/ml). In the present study, *L. javanica* was validated for its use to treat sores (De Wet et al., 2013), where the findings obtained evidenced the effectiveness of *L. javanica* against skin infections in addition to its well known effect against respiratory tract infections.

Ximenia caffra also did not display broad-spectrum activity. However, the plant was found to possess noteworthy activities against eight of the pathogens tested with an MIC ranging from 0.13-0.75 mg/ml. This plant has been evaluated against pathogens associated with STIs where it was also found to possess antigonococcal, antifungal and antibacterial activities (Mulaudzi et al., 2011; Naidoo et al., 2013). In the present study *X. caffra* was validated for its use in treatment of sores (De Wet et al., 2013). It was found to be less effective against Gram-positive GMRSA, MRSA and *S. aureus*, but more effective against the Gram-negative strains (*E. coli* and *P.*

aeruginosa) displaying MIC values of 0.25 mg/ml in both instances. Notable activity was also obtained against the dermatophyte, *T. mentagrophytes* (MIC value 0.13 mg/ml).

The findings presented in this study not only validate the tradition uses of these selected plant species, but also highlights the need to incorporate skin pathogens other than the commonly included pathogens such as *S. aureus*.

2.3.3 Antimicrobial activity of aqueous extracts

Even though water was the solvent used traditionally for the majority of the plants, the aqueous extracts exhibited moderate to poor activity (Table 2.4). While several studies reported water as a poor extractor of antimicrobial compounds from plant material, other studies have found the water extracts to be antimicrobially active displaying activities which are comparable or even superior to those from organically derived extracts. For example, in a study by Kumar et al. (2007), water extracts were found to be effective when investigated against *B. cereus*, *E. coli*, *Micrococcus luteus*, *P. aeruginosa* and *S. aureus*. Olajuyigbe and Afolayan, (2011) also found aqueous extracts to be antimicrobially active against pathogens associated with STIs with MIC values ranging between 0.16 and 0.63 mg/ml, the activities comparable to those exhibited by the ethanol extracts also investigated. Similar findings were further obtained by Naidoo et al, (2013), where the activities of the aqueous extracts were found to be equal and in some cases superior to the activities of the organic extracts against STI pathogens.

In the present study, 27 of the 41 aqueous extracts investigated were found to be active, exhibiting MIC values below 1.00 mg/ml for at least one or more of the pathogen(s) tested. Interestingly it was found that, although *A. vesicolor*, *K. africana* and *S. birrea* were not reported to treat acne, the aqueous extracts of these plants displayed notable activity against *P. acnes* with MIC values of 0.13, 0.19 and 0.13 mg/ml respectively. *P. capensis* and *T. elegans* displayed the best activity amongst the aqueous extracts against *T. mentagrophytes* (MIC 0.13 and 0.19 mg/ml respectively).

Certain plant species exhibited relevant activity (MIC values < 1.00 mg/ml) against the pathogens most likely to cause the skin infections they were reported to treat. In this instance, *A. burkei* was reported to treat ringworm (De Wet et al., 2013) and the aqueous extract showed noteworthy

Table 2.4: The average MIC values (mg/ml) of aqueous extracts against 12 dermatological relevant pathogens.

Plant sample	Pathogens												Average MIC \pm σ^*
	<i>B. agri</i> ^a	<i>B. line</i> ^b	GMRSA ^c	MRSA ^d	<i>S. aure</i> ^e	<i>S. epid</i> ^f	<i>P. acne</i> ^g	<i>E. coli</i> ^h	<i>P. aeru</i> ⁱ	<i>C. albi</i> ^j	<i>M. cani</i> ^k	<i>T. ment</i> ^l	
<i>A. adianthifolia</i> bark	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00 \pm >8.00
<i>A. burkei</i> bark	0.50	1.50	>8.00	2.00	3.00	2.00	0.50	>8.00	>8.00	>8.00	4.00	0.50	1.75 \pm 1.28
<i>A. senegalensis</i> roots	3.00	1.00	4.00	2.00	>8.00	4.00	1.00	2.00	>8.00	0.50	0.50	0.50	1.85 \pm 1.39
<i>A. vesicolor</i> bark	>8.00	>8.00	>8.00	8.00	>8.00	>8.00	0.13	>8.00	2.00	>8.00	4.00	0.25	2.88 \pm 3.28
<i>B. discolor</i> twigs	>8.00	>8.00	>8.00	0.25	6.00	>8.00	>8.00	>8.00	0.50	>8.00	4.00	1.00	2.35 \pm 2.53
<i>C. inerme</i> twigs	>8.00	8.00	>8.00	>8.00	8.00	>8.00	8.00	8.00	>8.00	>8.00	4.00	0.75	6.13 \pm 3.08
<i>D. cinerea</i> twigs	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	4.00	>8.00	>8.00	>8.00	>8.00	>8.00	4.00 \pm 0.00
<i>D. obovata</i> leaves	>8.00	4.00	3.00	>8.00	>8.00	1.00	>8.00	4.00	>8.00	>8.00	>8.00	>8.00	3.00 \pm 1.41
<i>D. schlechteri</i> bark	>8.00	2.00	4.00	3.00	4.00	8.00	1.50	3.00	4.00	4.00	0.75	4.00	3.48 \pm 1.33
<i>E. elephantina</i> rhizome	2.00	>8.00	4.00	4.00	6.00	4.00	2.00	1.00	2.00	2.00	0.50	2.00	2.68 \pm 1.62
<i>E. tirucalli</i> modified stems	>8.00	>8.00	0.50	0.25	>8.00	1.00	1.00	>8.00	>8.00	>8.00	>8.00	>8.00	0.69 \pm 0.37
<i>G. livingstonei</i> bark	0.75	8.00	2.00	1.50	2.00	4.00	>8.00	>8.00	>8.00	4.00	0.50	6.00	3.19 \pm 2.53
<i>H. hemerocallidea</i> corms	>8.00	>8.00	>8.00	1.00	2.00	0.25	2.00	2.00	4.00	>8.00	8.00	4.00	2.91 \pm 2.44
<i>H. malabarica</i> leaves	>8.00	>8.00	>8.00	>8.00	8.00	>8.00	>8.00	>8.00	>8.00	>8.00	0.50	8.00	5.50 \pm 5.59
<i>H. surratensis</i> leaves	>8.00	>8.00	2.00	4.00	>8.00	>8.00	8.00	>8.00	>8.00	8.00	4.00	4.00	5.00 \pm 2.45
<i>K. africana</i> bark	8.00	>8.00	4.00	4.00	8.00	4.00	6.00	4.00	>8.00	8.00	>8.00	>8.00	5.75 \pm 1.98
<i>K. africana</i> fruits	0.25	8.00	2.00	>8.00	>8.00	0.25	0.19	2.00	>8.00	4.00	2.00	1.00	2.19 \pm 2.50

Plant sample	Pathogens												Average MIC ± σ*
	<i>B. agri</i> ^a	<i>B. line</i> ^b	GMRSA ^c	MRSA ^d	<i>S. aure</i> ^e	<i>S. epid</i> ^f	<i>P. acne</i> ^g	<i>E. coli</i> ^h	<i>P. aeru</i> ⁱ	<i>C. albi</i> ^j	<i>M. cani</i> ^k	<i>T. ment</i> ^l	
<i>L. javanica</i> twigs	>8.00	>8.00	2.00	8.00	4.00	3.00	6.00	4.00	4.00	>8.00	6.00	8.00	5.00 ± 2.12
<i>M. balsamina</i> leaves	0.50	>8.00	1.00	1.00	>8.00	>8.00	8.00	>8.00	1.00	2.00	6.00	>8.00	2.79 ± 3.34
<i>O. engleri</i> bark	0.50	0.25	1.00	1.00	2.00	2.00	0.38	1.00	1.00	8.00	4.00	0.25	1.78 ± 2.22
<i>O. engleri</i> leaves	4.00	>8.00	8.00	4.00	4.00	2.00	0.25	1.00	1.00	8.00	4.00	2.00	3.45 ± 2.63
<i>P. afra</i> leaves	>8.00	>8.00	4.00	0.50	>8.00	>8.00	>8.00	2.00	>8.00	>8.00	8.00	0.50	3.00 ± 3.14
<i>P. capensis</i> roots	4.00	>8.00	8.00	1.00	>8.00	>8.00	>8.00	3.00	>8.00	>8.00	4.00	0.13	3.36 ± 2.77
<i>R. multifidus</i> whole plants	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	0.58	>8.00	>8.00	>8.00	>8.00	4.00	2.29 ± 3.34
<i>S. birrea</i> bark	2.00	0.50	2.00	2.00	2.00	2.00	0.13	3.00	2.00	1.00	2.50	2.00	1.76 ± 0.82
<i>S. brachypetala</i> bark	0.50	1.00	1.00	1.00	>8.00	2.00	0.50	8.00	>8.00	0.50	4.00	0.50	1.90 ± 2.40
<i>S. cordatum</i> bark	1.00	>8.00	2.00	2.00	2.00	4.00	8.00	1.00	4.00	2.00	4.00	>8.00	3.00 ± 2.37
<i>S. madagascariensis</i> bark	0.75	8.00	>8.00	2.00	>8.00	>8.00	8.00	>8.00	>8.00	>8.00	>8.00	8.00	5.35 ± 3.66
<i>S. madagascariensis</i> leaves	>8.00	>8.00	1.00	>8.00	>8.00	2.00	>8.00	>8.00	0.50	>8.00	6.00	8.00	3.50 ± 3.15
<i>S. panduriforme</i> fruits	>8.00	1.00	>8.00	8.00	4.00	>8.00	>8.00	>8.00	>8.00	0.25	8.00	0.38	3.61 ± 3.67
<i>S. rigescens</i> fruits	>8.00	4.00	>8.00	3.00	>8.00	0.50	8.00	>8.00	>8.00	>8.00	1.00	>8.00	3.30 ± 2.99
<i>S. serratuloides</i> leaves	>8.00	>8.00	8.00	>8.00	4.00	>8.00	8.00	8.00	>8.00	>8.00	8.00	1.00	6.17 ± 2.99
<i>S. spinosa</i> fruits	>8.00	>8.00	1.00	8.00	8.00	>8.00	>8.00	>8.00	>8.00	0.50	8.00	>8.00	5.10 ± 3.97
<i>T. elegans</i> fruits	8.00	4.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	1.00	0.19	3.30 ± 3.54
<i>T. elegans</i> leaves	>8.00	8.00	>8.00	>8.00	>8.00	2.00	1.00	>8.00	>8.00	8.00	4.00	>8.00	4.60 ± 3.29

Plant sample	Pathogens												Average MIC \pm σ^*
	<i>B. agri</i> ^a	<i>B. line</i> ^b	GMRSA ^c	MRSA ^d	<i>S. aure</i> ^e	<i>S. epid</i> ^f	<i>P. acne</i> ^g	<i>E. coli</i> ^h	<i>P. aeru</i> ⁱ	<i>C. albi</i> ^j	<i>M. cani</i> ^k	<i>T. ment</i> ^l	
<i>T. sericea</i> leaves	>8.00	>8.00	>8.00	>8.00	4.00	>8.00	>8.00	2.00	>8.00	>8.00	6.00	>8.00	4.00 \pm 2.00
<i>W. indica</i> roots	>8.00	>8.00	>8.00	2.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	1.00	1.00	1.33 \pm 0.58
<i>W. somnifera</i> roots	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	0.31	0.50	0.41 \pm 0.13
<i>X. caffra</i> twigs	2.00	1.00	4.00	4.00	2.00	4.00	1.00	1.00	4.00	0.25	2.00	8.00	2.77 \pm 2.18
<i>Z. capense</i> roots	>8.00	6.00	4.00	2.00	8.00	>8.00	>8.00	>8.00	8.00	8.00	8.00	1.00	5.38 \pm 2.94
<i>Z. mucronata</i> leaves	8.00	8.00	>8.00	4.00	4.00	3.00	8.00	8.00	>8.00	>8.00	>8.00	>8.00	6.14 \pm 2.17
Positive control (Ciprofloxacin/ Amphotericin B μ g/ml)	0.18	0.13	0.63	0.31	0.63	0.34	1.25	0.16	0.16	0.01	0.13	0.01	0.33 \pm 0.24
Negative control (water)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00 \pm >8.00
Culture control (growth medium)	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00 \pm >8.00

^a*Brevibacterium agri* ATCC 51663; ^b*Brevibacterium linens* DSM 20425; ^cGentamycin-methicillin resistant *Staphylococcus aureus* ATCC 33592; ^dMethicillin-resistant *Staphylococcus aureus* ATCC 43300; ^e*Staphylococcus aureus* ATCC 25923; ^f*Staphylococcus epidermidis* ATCC 2223; ^g*Propionibacterium acnes* ATCC 11827; ^h*Escherichia coli* ATCC 25922; ⁱ*Pseudomonas aeruginosa* ATCC 27853; ^j*Candida albicans* ATCC 10231; ^k*Microsporium canis* ATCC 36299; ^l*Trichophyton mentagrophytes* ATCC 9533; *MIC values >8.00 were not considered when calculating the average MIC and the standard deviation (σ) since no end point for MIC determination was obtained.

activity against *T. mentagrophytes* (MIC 0.50 mg/ml), i.e. a pathogen responsible for ringworm infections, thus validating its traditional use. The aqueous extract of *E. tirucalli* was reported to treat sores, and exhibited noteworthy activity against GMRSA and MRSA (MIC values of 0.50 and 0.25 mg/ml respectively), i.e. pathogens mostly associated with sores as well as skin and soft tissue infections. On the other hand, *A. senegalensis* which was also reported to treat sores, gave poor activity against the bacterial pathogens (MIC values >1.00 mg/ml) but exhibited noteworthy activity against all fungal pathogens, *C. albicans*, *M. canis* and *T. mentagrophytes* with MIC values of 0.50 mg/ml. These findings may favour the plant as a potential antimicrobial against ringworm infections.

2.4 Antimicrobial activity and traditional use

Eight plants species (*A. burkei*, *B. discolor*, *D. delagoensis*, *O. engleri*, *P. capensis* subsp. *capensis*, *P. afra*, *S. rigescens* and *S. pseudocordifolia*) were documented for the first time in the literature as a treatment of skin diseases (De Wet et al., 2013). With the exception of *D. delagoensis* and *S. pseudocordifolia*, six of these plants (*A. burkei*, *B. discolor*, *O. engleri*, *P. capensis* subsp. *capensis*, *P. afra* and *S. rigescens*) which were investigated in this study did show a correlation in antimicrobial efficacy and traditional use. For instance, *A. burkei* was reported to treat ringworm, and both aqueous and organic extracts of the plant were found to be active against the causative agents of ringworm, i.e. *M. canis* (MIC 0.13 mg/ml for the organic extract) and *T. mentagrophytes* (MIC values of 0.25 and 0.50 mg/ml for organic and water extracts respectively). *Ozoroa engleri*, also documented for the first time as a medicinal plant to treat sores by De Wet et al. (2013), displayed broad-spectrum activity with average MIC values of 0.37 and 0.89 mg/ml for the organic extracts of bark and leaves respectively. Noteworthy activity from *O. engleri* was observed with the organic bark extract against *S. aureus* (MIC 0.75 mg/ml), *S. epidermidis* (MIC 0.05 mg/ml), *E. coli* (MIC 0.19 mg/ml) and *P. aeruginosa* (MIC 0.38 mg/ml). These are all pathogens associated with sore infections. *Parinari capensis* subsp. *capensis*, also reported to treat sores, was among the plants that displayed a broad-spectrum antimicrobial activity against the tested pathogens with an average MIC of 0.60 mg/ml. For *P. afra* and *S. rigescens*, the antimicrobial activity was mostly moderate, displaying pathogen specific noteworthy activity against at least two or more test pathogens.

Even though some plant species (*A. senegalensis*, *G. livingstonei*, *P. capensis*, *S. brachypetala*, *T. sericea* and *Z. capense*) were rarely used by the lay people of rural Maputaland, their organic extracts were found to exhibit broad-spectrum antimicrobial activity. Other plant species which are most frequently used have, however, only exhibited moderate to poor activities. In this instance, *S. serratuloides* was the most cited plant (cited 17 times) by the interviewees as a treatment of skin diseases (De Wet et al., 2013). This plant is also well documented for its use in the treatment of various skin diseases including abscesses, boils (Van Wyk and Gericke, 2000), burns, cuts (Pooley, 2003), skin eruptions due to syphilis (Taylor et al., 2003) and STI sores (De Wet et al., 2012). The plant, however, displayed mostly poor antimicrobial activity when tested against the pathogens related to these skin ailments. This may imply that this plant could act by inducing other healing properties in a dermatological condition, i.e. it may act as an anti-inflammatory or for pain relief which would substantiate its traditional uses even though it is not necessarily an antimicrobial agent. Furthermore, it is known that *in vitro* studies may not always corroborate *in vivo* studies. It may thus be a case where *S. serratuloides* exhibits *in vivo* noteworthy activity even though its *in vitro* activity is questionable. The anti-inflammatory activity of *S. serratuloides* had been reported by Fawole et al. (2010), where the petroleum ether leaf extract exhibited good cyclooxygenase-1 (COX-1) enzyme inhibition (62%) while the methanol extract showed 71.6% COX-2 enzyme inhibition. Gould, 2014 also reported the *in vivo* wound healing properties of *S. serratuloides*, where the plant was found to be effective in treating deep partial thickness wounds in a porcine. Thus, future studies should investigate the inflammatory and other pharmacological properties of *S. serratuloides* apart from its antimicrobial effects. A similar incidence has been found by Naidoo et al. (2013) who also obtained the poor activity for the same plant against STI pathogens. Similarly, York et al., (2012) reported the poor antimicrobial activity of *L. javanica* which was frequently mentioned by the interviewees as a treatment of respiratory tract infections. York et al., (2012) concluded that, the frequency of a certain plant use does not necessarily correlate with high antimicrobial efficacy. Other plants that were frequently mentioned in the survey by De Wet et al. (2013) were *T. elegans* and *S. madagascariensis*, which again displayed moderate antimicrobial activity. *Sclerocarya birrea* which was also frequently mentioned did show good antimicrobial activity with broad-spectrum activity obtained from the organic extract (mean MIC 0.37 mg/ml).

2.5 Summary

- Organic extracts had better antimicrobial activity with more than 80% displaying correlation between antimicrobial efficacy and the pathogens most likely to be responsible for the specific skin infections they were reported to treat.
- The most antimicrobially active organic extract was *G. livingstonei* (mean MIC of 0.27 mg/ml), displaying noteworthy activity against 11 of the 12 tested pathogens with MIC values ranging from 0.02 to 0.50 mg/ml.
- For the aqueous extracts, the antimicrobial efficacy was mostly moderate with 24 extracts displaying pathogen specific noteworthy antimicrobial effects.
- *T. mentagrophytes* was the most susceptible pathogen with the majority (98%) of the organic extracts investigated exhibiting noteworthy activity (MIC values <1.00 mg/ml). Susceptibility was also pronounced against a Gram-negative, *P. aeruginosa* (83%) and a Gram-positive *B. agri* (88%) bacteria.
- For the resistant Staphylococcal strains, noteworthy effects were mostly observed. Activities between 0.25 - 0.50 mg/ml were observed for the plant species *A. senegalensis*, *G. livingstonei*, *P. afra*. and *E. tirucalli*.
- Six of the eight plant species (*A. burkei*, *B. discolor*, *O. engleri*, *P. capensis*, *P. afra*, and *S. rigescens*) documented for the first time in Maputaland as a treatment for skin inflictions, demonstrated susceptible antimicrobial effects.
- *O. engleri*, documented for the first time as a treatment for skin diseases demonstrated noteworthy activity against nine of the 12 tested pathogens displaying the mean MIC values of 0.37 and 0.89 mg/ml for the organic extracts of bark and leaves respectively.
- Organic and aqueous extracts of *A. burkei* (also documented for the first time to treat ringworm), exhibited noteworthy activity against the causative pathogen *T. mentagrophytes* with MIC values of 0.25 and 0.50 mg/ml respectively which validates its use as a treatment of ringworm.
- Similarly, *P. capensis* subsp. *capensis* reported to treat sores displayed a broad-spectrum activity (mean MIC 0.60 mg/ml) with noteworthy activity observed against MRSA, *P. aeruginosa* and *S. epidermidis* (activities between 0.25 – 0.50 mg/ml) which are the pathogens that are most likely to infect sores.

Chapter three: Interactive profiles of plants used in combinations to treat skin diseases

3.1 Introduction

The rationale of combining plants in a medication is the desire to produce a combination with improved therapeutic effect. The most desirable outcome in combination therapy is usually the additive or synergistic interaction. The former occurs when the effect of the combined plants equals the effect of each plant brought into combination. The latter occurs when the combined effect exceeds the effects of the individual plants simply added together (Che et al., 2013). Combination therapy is not only confined to traditional medicine. Even in clinical practice, critical diseases such as HIV/AIDS and cancer are treated more effectively with pharmaceutical combinations than they can be with single drugs (Wagner and Ulrich-Merzenich, 2009). In combination therapy, small quantities of medicines have to be used in order to obtain a specific outcome. Furthermore, when one of the plants used in a combination has toxic effects, the non-toxic plant is capable of reducing toxicity. However, this is not always the case as in some cases, Herb-herb interaction may result in increased toxicity even though the combination may give another pharmacologically desired synergistic effect. Such toxicity is not limited only to herbal preparations, even pharmaceutical drug-drug interactions are sometimes associated with toxic effects. Thus, caution should be adhered to when considering combination therapy. Even though synergism or an additive interaction is the desired outcome in combination therapy, however, non-interactive and even antagonistic interactions may also occur. The latter is when the inhibitory effect of the combination is decreased compared to the inhibitory effects of individual plants incorporated into a combination. This usually suggests that the plants forming part of the combination have opposing effects.

About 28 of the 47 plants documented in an ethnobotanical survey in northern Maputaland were combined to form 14 different combinations. Eight combinations (Table 3.1) were considered for investigation based on the availability of the plant material during the time of collection. While the independent antimicrobial efficacy of the plants used in combinations was determined in

Chapter 2, this Chapter first reports on the evaluation of the possible antimicrobial interactions of these plants when combined in equal ratios. In doing so, possible synergistic or antagonistic interactions against dermatological relevant pathogens were identified. The combinations were further analyzed in various ratios to determine the suitable ratios in which these remedies could be combined for better efficacy.

Table 3.1: Plant combinations considered for antimicrobial analysis adapted from De Wet et al. (2013).

Plant species in the combination	Part(s) used	Mode of administration	Medical condition
<i>Acacia burkei</i> and <i>Kigelia africana</i>	Bark	Decoction taken orally	Ringworm
<i>Acacia burkei</i> , <i>Ozoroa engleri</i> , <i>Sclerocarya birrea</i> , <i>Syzygium cordatum</i> , <i>Tabernaemontana elegans</i> and <i>Lippia javanica</i>	Bark and leaves	Decoction used as enema	Sores
<i>Brachylaena discolor</i> , <i>Euphorbia tirucalli</i> , <i>Hypoxis hemerocallidea</i> , <i>Ozoroa engleri</i> , <i>Senecio serratuloides</i>	Twigs, modified stems and corms	Decoction taken orally	Sores
<i>Canthium inerme</i> and <i>Dichrostachys cinerea</i>	Twigs	Decoction used for steaming	Acne
<i>Hypoxis hemerocallidea</i> and <i>Solanum rigescens</i>	Corms and fruits	Decoction taken orally	Boils
<i>Schotia brachypetala</i> and <i>Sclerocarya birrea</i>	Bark	Decoction used as enema	Sores
<i>Sclerocarya birrea</i> and <i>Syzygium cordatum</i>	Bark	Powder applied topically	Burns
<i>Strychnos madagascariensis</i> and <i>Strychnos spinosa</i>	Leaves and fruits	Paste applied topically	Sores

3.2 Materials and methods

3.2.1 Selection and preparation of microbial cultures

Three commonly tested pathogens associated with skin infections, i.e. two Gram-positive (*S. aureus* ATCC 25923 and *S. epidermidis* ATCC 2223) and a Gram-negative (*P. aeruginosa* ATCC 27853) strains, were selected to test the efficacy of all plant combinations. However, for some combinations, other additional pathogens were selected based on their relation to the traditional use for the particular combination. For instance, a combination reported to treat ringworm were evaluated against *M. canis* ATCC 36299 and *T. mentagrophytes* ATCC 9533 in addition to *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Furthermore, the combination reported to treat acne was investigated against *P. acnes* ATCC 11827 in addition to the commonly tested pathogens. The selected pathogens were then prepared according to specifications detailed in Chapter 2, Section 2.2.3.2.

3.2.2 Combination studies

3.2.2.1 Determination of the sum of Fractional Inhibitory Concentration (Σ FIC)

Microtitre plates were aseptically prepared by adding 100 μ l of the growth medium in all wells. For combinations of two plants, 50 μ l from each plant extract at a starting concentration of 32 mg/ml were combined adding up 100 μ l in the first row of the microtitre plate to make a 1:1 combination. Where five or six plants were combined, 20 or 16.6 μ l from each extract respectively, were combined to make up a volume of 100 μ l in each well in a 1:1:1:1:1 or 1:1:1:1:1:1 combination. Serial dilutions were performed on the extracts as detailed in Chapter 2, Section 2.2.3.3. Tests were undertaken in duplicates and triplicates with positive (conventional antimicrobials) and negative (acetone, water and sterile medium) controls included in each assay. The MIC values were determined for the plant combinations and the Fractional Inhibitory Concentration (FIC) was calculated for each combination. The FIC was calculated by dividing the MIC value of the combination by the MIC value of a particular plant extract placed in a combination (Equation 3.1). The Σ FIC was then calculated by adding together the FIC values.

Equation 3.1:
$$FIC_a = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}} \quad FIC_b = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

(a) and (b) in the equation represent different plants in the combination. Where five or six plants were combined, the equation also brought into account the number of plant species forming part of each combination. The value of the Σ FIC (also called the FIC index) was used to determine if the plants in a combination are synergistic, additive, non-interactive or antagonistic. The plants were regarded as synergistic if their Σ FIC value is equal or less than 0.50 mg/ml. The additive effect was observed when the Σ FIC was greater than 0.50 but less than or equal to 1.00 mg/ml. For the non-interactive interaction, the Σ FIC was greater than 1.00 but less than or equal to 4.00 mg/ml while antagonism occurred when the Σ FIC was greater than 4.00 mg/ml (Van Vuuren and Viljoen, 2011).

3.2.2.2 Varied ratio studies: Isobologram construction

Since the Σ FIC method is based on assumption that each extract in a combination gives half the antimicrobial effect of which is not always the case, the combinations were further studied using the isobole method. This method takes into account that the antimicrobial interaction may vary depending on the ratio in which the two plants are combined (Van Vuuren and Viljoen, 2011). Three combinations with two plants per combination were then combined in nine ratios i.e. 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90, with each ratio constituting the 100 μ l in the first row of the microtitre plate. The MIC was determined for all combinations using the MIC method detailed in Chapter 2, Section 2.2.3.3. Positive and negative controls were included in each assay and all assays were undertaken at least in duplicate. The mean MIC values of plant combinations were then plotted against independent plant MIC values on an isobologram as a ratio using a GraphPad Prism® version 5 software. This expressed the interactions of various ratio combinations on an isobole graph. The isobole graph was interpreted by examining the data points of the ratios falling within various quadrants or on either three of the adjoining lines (the 0.5:0.5 line, the 1.0:1.0 line and the 4.0:4.0 line) of the two axes (Figure 3.1). Synergistic activity was noted for points falling below or on the 0.5:0.5 line of the isobologram. An additive effect was expressed when ratio points fell between the 0.5:0.5 and 1.0:1.0 line or on the 1.0:1.0 line. Points falling between the 1.0:1.0 and 4.0:4.0 line expressed the non-interactive interaction while antagonism was noted for points falling above the 4.0:4.0 line (Van Vuuren and Viljoen, 2011).

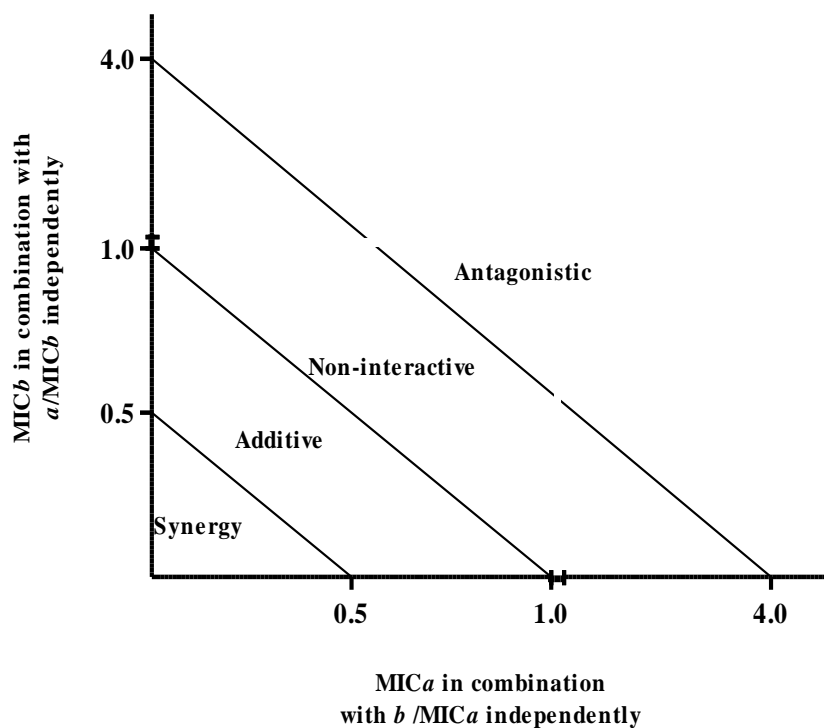


Figure 3.1: Isobologram interpretation showing synergy, additive, non-interactive and antagonistic interactions when two plants are combined in various ratios, where *a* is one plant and *b* is another (adapted from Van Vuuren and Viljoen, 2011).

3.3 Results and discussion

3.3.1 Σ FICs for plants combined in equal ratios

Eight plant combinations (with two to six plants per combination) were analyzed for their efficacy against *S. aureus*, *S. epidermidis* and *P. aeruginosa*. The mean MIC and the Σ FIC values for aqueous and organic (dichloromethane: methanol) extract combinations are depicted in Table 3.2. In cases where no end point for MIC determination was found either for a combination or individual plant(s) in a combination, the Σ FIC could not be calculated. In these cases, tentative interpretations (synergism, additive, non-interactive or antagonistic effect) were given based on the increase or decrease in MIC value of a combination when compared with the MIC values of the individual plants making up the combination.

Table 3.2: The mean MICs and Σ FICs of aqueous and organic plant extracts used in combinations to treat skin diseases.

Plant species and combinations		<i>S. aureus</i> ATCC 25923		<i>S. epidermidis</i> ATCC 2223		<i>P. aeruginosa</i> ATCC 27853	
		D:M	H ₂ O	D:M	H ₂ O	D:M	H ₂ O
<i>A. burkei</i>	MIC values (mg/ml)	4.00	3.00	1.00	2.00	0.25	>8.00
<i>K. africana</i>		>8.00	8.00	0.01	4.00	0.25	>8.00
<i>A. burkei</i> and <i>K. africana</i>	MIC value	0.50	>8.00	0.50	1.50	0.50	>8.00
	Σ FIC value	ND	ND	0.75	0.56	2.00	ND
ΣFIC interpretation		SYN	ANT	ADD	ADD	NON	ANT
<i>A. burkei</i>	MIC values (mg/ml)	4.00	3.00	1.00	2.00	0.25	>8.00
<i>O. engleri</i>		0.75	2.00	0.05	2.00	0.38	1.00
<i>S. birrea</i>		0.50	2.00	0.50	2.00	0.19	2.00
<i>S. cordatum</i>		2.00	2.00	1.00	4.00	0.25	4.00
<i>T. elegans</i>		4.00	>8.00	1.00	2.00	0.38	>8.00
<i>L. javanica</i>		4.00	4.00	0.19	3.00	0.25	4.00
<i>A. burkei</i> , <i>O. engleri</i> , <i>S. birrea</i> , <i>S. cordatum</i> , <i>T. elegans</i> and <i>L. javanica</i>	MIC value	0.75	6.00	0.50	1.00	0.50	1.00
	Σ FIC value	0.57	2.15	2.43	0.57	1.80	ND
ΣFIC interpretation		ADD	NON	NON	ADD	NON	ADD
<i>B. discolor</i>	MIC values (mg/ml)	3.00	6.00	1.00	>8.00	0.75	0.50
<i>E. tirucalli</i>		6.00	>8.00	1.50	1.00	0.75	>8.00
<i>H. hemerocallidea</i>		>8.00	2.00	2.00	0.25	2.00	4.00
<i>O. engleri</i>		1.00	4.00	0.50	2.00	0.38	1.00
<i>S. serratuloides</i>		4.00	4.00	1.00	>8.00	1.50	>8.00
<i>B. discolor</i> , <i>E. tirucalli</i> , <i>H. hemerocallidea</i> , <i>O.</i> <i>engleri</i> and <i>S. serratuloides</i>	MIC value	0.50	0.38	0.50	1.00	0.50	1.00
	Σ FIC value	ND	ND	0.52	ND	0.92	ND
ΣFIC interpretation		SYN	SYN	ADD	ADD	ADD	ADD

Plant species and combinations		<i>S. aureus</i> ATCC 25923		<i>S. epidermidis</i> ATCC 2223		<i>P. aeruginosa</i> ATCC 27853	
		D:M	H ₂ O	D:M	H ₂ O	D:M	H ₂ O
<i>C. inerme</i>	MIC values	4.00	8.00	0.25	>8.00	0.25	>8.00
<i>D. cinerea</i>	(mg/ml)	4.00	>8.00	0.19	>8.00	0.25	>8.00
<i>C. inerme</i> and <i>D. cinerea</i>	MIC value	0.50	>8.00	0.50	>8.00	0.75	>8.00
	ΣFIC value	0.19	ND	2.32	ND	3.00	ND
ΣFIC interpretation		SYN	ANT	NON	ANT	NON	ANT
<i>H. hemerocallidea</i>	MIC values	>8.00	2.00	2.00	0.25	2.00	4.00
<i>S. rigescens</i>	(mg/ml)	4.00	>8.00	0.15	0.50	0.50	>8.00
<i>H. hemerocallidea</i> and <i>S. rigescens</i>	MIC value	0.50	0.63	0.01	0.50	1.00	1.00
	ΣFIC value	ND	ND	0.04	0.56	2.00	ND
ΣFIC interpretation		SYN	ADD	SYN	ADD	NON	ADD
<i>S. brachypetala</i>	MIC values	1.00	>8.00	0.50	2.00	0.25	>8.00
<i>S. birrea</i>	(mg/ml)	0.50	2.00	0.50	2.00	0.19	2.00
<i>S. brachypetala</i> and <i>S. birrea</i>	MIC	0.50	2.00	0.25	2.00	0.25	0.25
	ΣFIC	0.75	2.15	0.34	1.00	1.16	ND
ΣFIC interpretation		ADD	NON	SYN	ADD	NON	SYN
<i>S. birrea</i>	MIC values	0.50	2.00	0.50	2.00	0.19	2.00
<i>S. cordatum</i>	(mg/ml)	2.00	2.00	1.00	4.00	0.25	4.00
<i>S. birrea</i> and <i>S. cordatum</i>	MIC value	0.50	0.25	0.25	4.00	0.25	0.50
	ΣFIC value	0.63	0.13	0.34	1.50	1.16	0.19
ΣFIC interpretation		ADD	SYN	SYN	NON	NON	SYN
<i>S. madagascariensis</i>	MIC values	1.00	>8.00	1.50	2.00	0.25	0.50
<i>S. spinosa</i>	(mg/ml)	4.00	8.00	0.25	>8.00	0.50	>8.00
<i>S. madagascariensis</i> and <i>S. spinosa</i>	MIC value	0.50	>8.00	0.13	>8.00	0.50	>8.00
	ΣFIC value	0.38	ND	0.29	ND	1.50	ND
ΣFIC interpretation		SYN	ANT	SYN	ANT	NON	ANT

D:M= dichloromethane: methanol (1:1) extracts, H₂O = aqueous extracts, ND = Not determined as no end point for MIC determination was obtained; SYN = Synergism (ΣFIC<0.50); ADD = Additive (ΣFIC>0.50-1.00); NON = Non-interactive (ΣFIC1.00->4.00); ANT = Antagonism (ΣFIC>4.00); Shaded = summary of results obtained in Chapter two; Bold = noteworthy and synergistic activity.

The combinations analyzed in equal ratios displayed synergistic (27%), additive (31%), non-interactive (25%) as well as antagonistic (17%) interactions against the tested pathogens. The majority of the combinations displayed synergistic interactions against *S. aureus* (44%), followed by *S. epidermidis* (25%) and then *P. aeruginosa* (12%). Additive effects of the combinations were more prominent against *S. epidermidis* (44%) while non-interactive interactions mostly occurred against *P. aeruginosa* (44 %) (Figure 3.2).

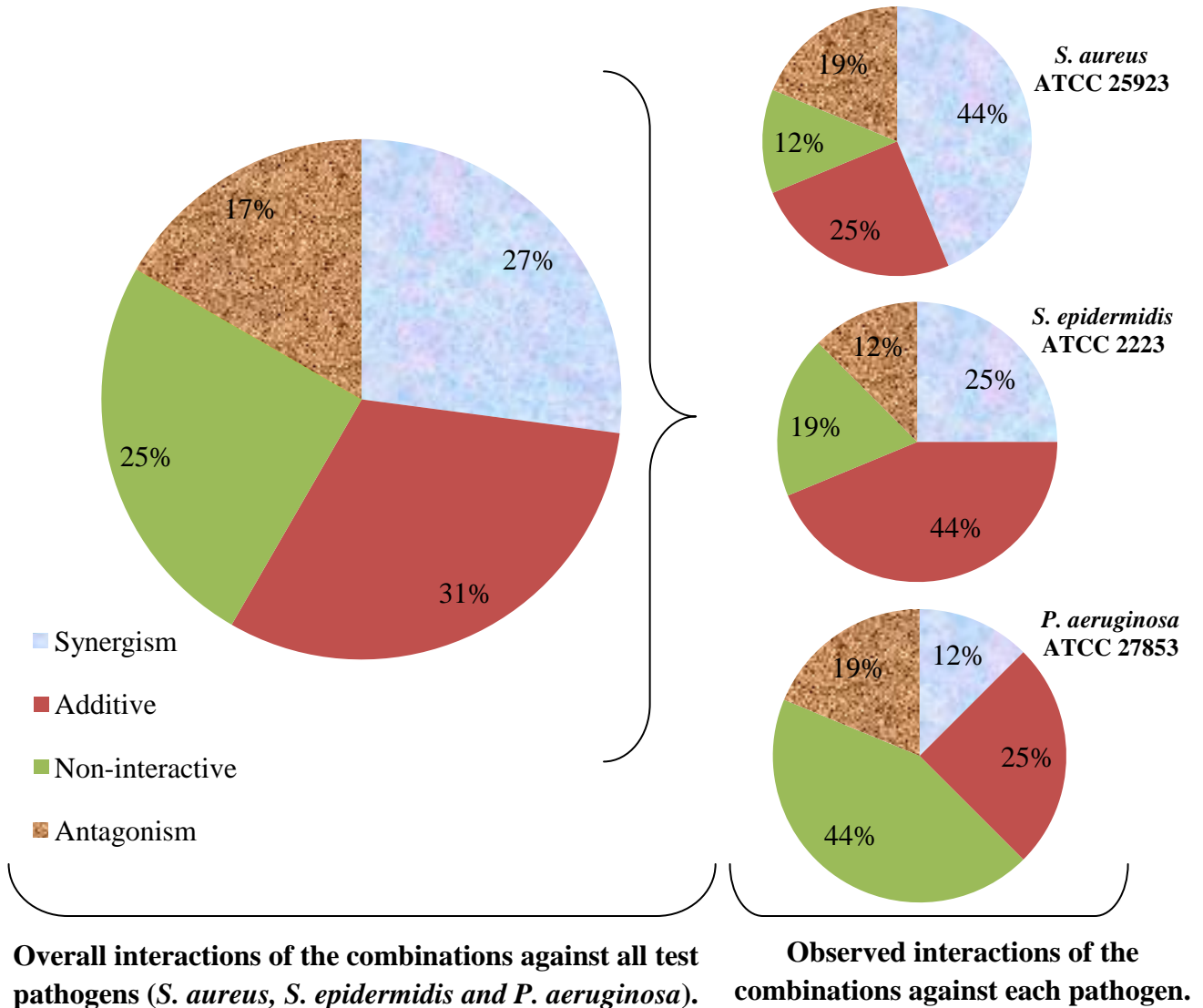


Figure 3.2: Antimicrobial interactions of plant combinations against the three tested pathogens.

The combination of *A. burkei* and *K. africana* was reported as a combined treatment for ringworm (De Wet et al., 2013). Mostly, additive interactions were observed when the

combination was investigated against *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Synergistic effect was only observed from the organic extract combination against *S. aureus* (based on tentative interpretation). When tested against *M. canis* and *T. mentagrophytes* (the causative agents of ringworm), both aqueous and organic extracts of the combination displayed non-interactive interaction against *M. canis* (Table 3.3). This was not expected especially for the organic extracts as they displayed noteworthy activity when investigated independently against *M. canis* with MIC values of 0.13 and 0.34 mg/ml for *A. burkei* and *K. africana* respectively (Chapter 2, Table 2.3). These findings may imply that the combination is not effective against *M. canis* or perhaps the plants may be combined for some other pharmacological purposes. Despite the non-interactive effect observed against *M. canis*, the aqueous and organic extracts of *A. burkei* and *K. africana* did exhibit synergistic effect against *T. mentagrophytes* with the highest display of synergism (Σ FIC value 0.17) noted for the aqueous extracts. The observed synergistic effect, especially from an aqueous extract combination, lends credibility to the use of this combination against ringworm.

Table 3.3: The mean MICs and Σ FICs of *A. burkei* in combination with *K. africana* investigated further in relation to its traditional uses.

Plants in a combination		Test pathogen			
		<i>M. canis</i> ATCC 36299		<i>T. mentagrophytes</i> ATCC 9533	
<i>A. burkei</i>	MIC value (mg/ml)	0.13	4.00	0.25	0.50
<i>K. africana</i>		0.50	4.00	0.06	1.00
<i>A. burkei</i> and <i>K. africana</i>	MIC value	0.38	8.00	0.13	0.16
	Σ FIC value	1.90	2.00	0.48	0.17
ΣFIC interpretation		NON	NON	SYN	SYN

D:M= dichloromethane: methanol (1:1) extracts, H₂O = aqueous extracts, ND = Not determined as no end point for MIC determination was obtained; SYN = Synergism (Σ FIC<0.50); ADD = Additive (Σ FIC>0.50-1.00); NON = Non-interactive (Σ FIC1.00->4.00); ANT = Antagonism (Σ FIC>4.00); Shaded = summary of results obtained in Chapter two; Bold = noteworthy and synergistic activity.

The aqueous and organic extracts of another poly-herbal combination (*A. burkei*, *O. engleri*, *S. birrea*, *S. cordatum*, *T. elegans* and *L. javanica*) also reported as a combined treatment of sores,

displayed mostly non-interactive interactions against the three test pathogens. The better interaction was an additive effect (Σ FIC 0.57) observed from the organic extracts combination against *S. aureus*. However, it was worth noting that the aqueous extracts of the combination interacted synergistically against *P. aeruginosa* (based on tentative interpretation). When considering the MIC data of the plants brought into this combination (Chapter 2, Table 2.3), the antimicrobial activity was mostly moderate (MIC values ranging 1.00 - >8.00 mg/ml). Thus, the synergistic effects observed indicated that the activity was enhanced when the plants were combined which supports the use of this combination for the treatment of sores caused by *P. aeruginosa* infections.

A combination with five plants (*B. discolor*, *E. tirucalli*, *H. hemerocallidea*, *O. engleri* and *S. serratuloides*) which was reported to treat sores showed synergistic or additive effects against the three pathogens (*S. aureus*, *S. epidermidis* and *P. aeruginosa*). Synergistic interactions were observed against *S. aureus* based on tentative interpretation since the organic extract of *H. hemerocallidea* had an average MIC value of >8.00 mg/ml. Therefore, the Σ FIC could not be calculated for the combination having *H. hemerocallidea*. However, the efficacy of the combined aqueous extracts was improved (showed by the tentative synergistic and additive effects) especially when considering the low activity obtained when the aqueous extracts of the combined plants were investigated individually. This was a promising result for the lay people as they use water as a solvent for medicinal preparations.

When *C. inermis* and *D. cinerea* were combined, mostly additive and non-interactive interactions were observed. Synergistic activity was only evident against *S. aureus* (Σ FIC 0.19 for the organic extract combination). Since the combined use of the plants was reported to treat acne (De Wet et al., 2013), the 1:1 combination was further investigated against *P. acnes* (the causative agent of acne) in which non-interactive (organic extracts and aqueous extracts) interactions were observed (Table 3.4). From the MIC data (Chapter 2, Table 2.2 and 2.3), the combined plants also displayed poor activity when investigated independently against *P. acnes*. Thus, these findings do not support the use of this combination for the treatment of *P. acnes*, but infections caused by *S. aureus* such as boils, carbuncles, pyoderma etc. may be treated successfully with this combination. Furthermore, the individual plants could have a potential in the treatment of other skin infections as they showed noteworthy activity against pathogens such as *B. agri* (MIC

value 0.03 mg/ml for *C. inerme*), *B. linens* (MIC value 0.50 mg/ml for *C. inerme*), *S. epidermidis* (MIC values 0.25 and 0.19 mg/ml for *C. inerme* and *D. cinerea* respectively), *P. aeruginosa* (MIC values 0.25 mg/ml for both *C. inerme* and *D. cinerea*) and *T. mentagrophytes* (MIC values 0.25 and 0.50 mg/ml for *C. inerme* and *D. cinerea* respectively).

Table 3.4: The mean MICs and Σ FICs of aqueous and organic extract combinations investigated further in relation to their traditional uses.

Plants in a combination		Test pathogen	
		<i>P. acnes</i> ATCC 11827	
		D:M	H ₂ O
<i>C. inerme</i>	MIC values (mg/ml)	2.00	8.00
<i>D. cinerea</i>		1.00	4.00
<i>C. inerme</i> and <i>D. cinerea</i>	MIC value	1.50	8.00
	Σ FIC value	1.13	1.50
ΣFIC interpretation		NON	NON
<i>H. hemerocallidea</i>	MIC values (mg/ml)	1.50	2.00
<i>S. rigescens</i>		2.00	8.00
<i>H. hemerocallidea</i> and <i>S. rigescens</i>	MIC value	0.09	8.00
	Σ FIC value	0.05	16.50
ΣFIC interpretation		SYN	ANT

D:M= dichloromethane: methanol (1:1) extracts, H₂O = aqueous extracts, ND = Not determined as no end point for MIC determination was obtained; SYN = Synergism (Σ FIC<0.50); ADD = Additive (Σ FIC>0.50-1.00); NON = Non-interactive (Σ FIC1.00->4.00); ANT = Antagonism (Σ FIC>4.00); Shaded = summary of results obtained in Chapter two; Bold = noteworthy and synergistic activity.

A strong synergistic activity (Σ FIC value 0.04) was evident from an organic combination of *H. hemerocallidea* and *S. rigescens* against *S. epidermidis*. When investigated independently, the organic extract of *H. hemerocallidea* showed moderate activity (2.00 mg/ml) against *S. epidermidis* while *S. rigescens* displayed noteworthy activity (0.15 mg/ml). This shows that *S. rigescens* contributed strongly to the enhanced activity of the combination. The aqueous extracts of this combination was also promising as it displayed additive effects against the three tested

pathogens (*S. aureus*, *S. epidermidis* and *P. aeruginosa*). Due to the observed positive results, this combination was investigated further using the isobole method. When further tested against *P. acnes* (Table 3.4), this combination displayed a strong synergistic effect with the Σ FIC value of 0.05 for the organic extract combination as opposed to a strong antagonistic effect of Σ FIC value of 16.50 by the aqueous extract combination. Such contradicting effects can be attributed to the different solvents used (water vs. dichloromethane: methanol) extracting different classes of active compounds (York et al., 2012).

The combined antimicrobial effect of *S. brachypetala* and *S. birrea* showed a synergistic interaction against *S. epidermidis* (organic extract combinations - Σ FIC 0.34) and *P. aeruginosa* (aqueous extract combination – tentative interpretation). An additive effect was observed from the organic extract combination against *S. aureus* (Σ FIC value 0.75) as well as with the aqueous extract combination against *S. epidermidis* (Σ FIC 1.00). When investigated independently, the organic extracts of *S. birrea* and *S. brachypetala* displayed noteworthy antimicrobial activity against *P. aeruginosa* with MIC values of 0.19 and 0.25 mg/ml respectively. However, when combined, the activity was not enhanced (Σ FIC value 1.16) as one would expect. The enhanced efficacy was only observed against *S. epidermidis* with synergistic effect (Σ FIC 0.34) from combined organic extracts and an additive effect (Σ FIC 1.00) from aqueous extracts.

Traditionally, *S. birrea* is combined with *S. cordatum* to treat sores (De Wet et al., 2013). When this combination was investigated against the pathogens *S. aureus*, *S. epidermidis* and *P. aeruginosa*, mostly synergistic interactions were evident with the Σ FIC values of 0.13 (aqueous extract combination), 0.34 (organic extract combination) and 0.19 (aqueous extract combination) respectively. Additive interaction (Σ FIC 0.63) from organic extracts against *S. aureus* and non-interactive effects (Σ FIC 1.50 and 1.16) against *S. epidermidis* and *P. aeruginosa* were also observed. This is the first report for the evaluation of this combination against skin pathogens. An evaluation against pathogens associated with sexually transmitted infections did, however, exhibit noteworthy interaction (aqueous extract combination) with the Σ FIC value of 0.42 against the pathogen *Oligella ureolytica* (Naidoo et al., 2013). These findings support the use of this combination in treatment of infectious diseases including skin infections, as claimed by the lay people of northern Maputaland.

When the combination of *S. madagascariensis* and *S. spinosa* was investigated against *S. aureus*, *S. epidermidis* and *P. aeruginosa*, the interaction was mostly non-interactive. The synergistic interaction was only observed for the organic extracts against *S. aureus* (Σ FIC 0.38) and *S. epidermidis* (Σ FIC 0.29). These synergistic interactions indicated an enhanced efficacy when the plants were combined compared with the low efficacy obtained when the plants were investigated independently. However, with *P. aeruginosa*, the combined efficacy of the organic extracts was reduced (Σ FIC 1.50) while the inhibitory effects of the independent extracts against this micro-organism (MIC values of 0.25 and 0.50 mg/ml for *S. madagascariensis* and *S. spinosa* respectively) were more noteworthy than those of the combined extracts.

3.3.2 Isobolograms for plants combined in various ratios

Some of the plant extract combinations showed promising synergistic interactions in a 1:1 ratio and were consequently selected for further analysis by means of the varied ratio method. However, the combinations that displayed synergistic interactions in a 1:1 ratio combination but contained more than two plants per combination were not investigated further in varied ratio studies. Each of the selected combination was investigated against the pathogen(s) in which synergistic activity was observed from the 1:1 combination (Σ FICs). Both aqueous and organic extracts of the selected combinations were assayed and the following plant combinations were examined:

- (a) *Acacia burkei* in combination with *Kigelia africana*, investigated against *S. aureus*.
- (b) *Canthium inerme* in combination with *Dichrostachys cinerea*, investigated against *S. aureus*.
- (c) *Hypoxis hemerocallidea* in combination with *Solanum rigescens*, investigated against *S. aureus* and *S. epidermidis*.
- (d) *Schotia brachypetala* in combination with *Sclerocarya birrea*, investigated against *P. aeruginosa* and *S. aureus*.
- (e) *Sclerocarya birrea* in combination with *Syzygium cordatum*, investigated against *P. aeruginosa*, *S. aureus* and *S. epidermidis*.
- (f) *Strychnos madagascariensis* in combination with *Strychnos spinosa*, investigated against *S. aureus* and *S. epidermidis*.

The isobolograms for all combinations [with each combination investigated against the relevant pathogen(s)] are shown in Figures 3.3-3.9 and are discussed in order of efficacy.

3.3.2.1 *Hypoxis hemerocallidea* in combination with *Solanum rigescens*

From the isobologram interactions of the aqueous and organic extract combinations of *H. hemerocallidea* and *S. rigescens* against *S. aureus* and *S. epidermidis* (Figure 3.3), the greatest synergistic interaction was observed from the organic extract combination against *S. epidermidis*, the interaction that was also observed from the 1:1 combination. The isobologram displayed a synergistic profile (all ratio points fell below the 0.5 line) irrespective of the ratio at which the two plants were combined. When investigated independently, the organic extract of *H. hemerocallidea* showed moderate activity (2.00 mg/ml) against *S. epidermidis* while *S. rigescens* displayed noteworthy activity (0.15 mg/ml). Thus, *S. rigescens* contributed strongly to the enhanced activity of the combination.

Even though *S. rigescens* and *H. hemerocallidea* have not been investigated as a combination against skin associated pathogens, *H. hemerocallidea* is well documented for its use against skin diseases (Hutchings et al., 1996; Elgorashi et al., 2003; Taylor et al., 2003; Van Wyk and Wink 2004; Van Wyk et al., 2009; Drewes et al., 2008; De Wet et al., 2012) while our earlier study (De Wet et al., 2013) documented *S. rigescens* for the first time as treatment of skin diseases. This brings into consideration the importance of validating the newly discovered plants. The aqueous extract combination however, displayed an additive effect from a 1:1 combination. The same pattern was also observed from the isobologram with all ratio points occurring in the additive region with exception of the 9:1 ratio point (where *H. hemerocallidea* was higher) returning a non-interactive result against *S. epidermidis*. Thus, it could be deduced that when the concentration of *H. hemerocallidea* increases, the efficacy of the aqueous combination decreases against *S. epidermidis*.

Where the activity against *S. aureus* is concerned, a synergistic interaction was exhibited from the organic extract combination, with eight ratio points (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) occurring on the 0.50:0.50 line. All the ratio points where the concentration of *H. hemerocallidea* was higher in relation to *S. rigescens* fell in the synergy region. Only one ratio point (9:1) occurred in the additive range where the concentration of *S. rigescens* was higher. On the other

hand, the aqueous extract combinations all exhibited an additive effect with three ratio points (9:1; 8:2 and 7:3) where the concentration of *H. hemerocallidea* was higher lying closer to the synergy line. From these results, it could be deduced that *H. hemerocallidea* contributed more to the observed synergistic effect against *S. aureus*.

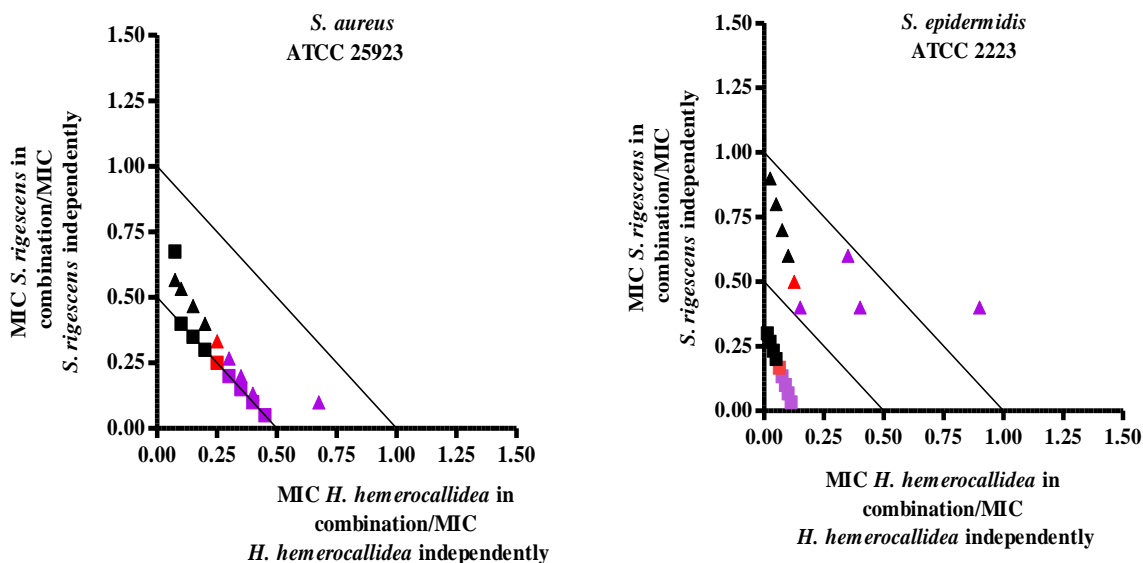


Figure 3.3: Isobologram representation of *H. hemerocallidea* in combination with *S. rigescens* against *S. aureus* and *S. epidermidis*. Where: the squares (\square) = organic extract combinations, the triangles (Δ) = aqueous extract combinations, \blacksquare \blacktriangle = 1:1 combination, \blacksquare \blacktriangle = combinations containing more *H. hemerocallidea*, \blacksquare \blacktriangle = combinations containing more *S. rigescens*.

3.3.2.2 *Schotia brachypetala* in combination with *Sclerocarya birrea*

When examining the interactive profiles of the aqueous and organic extracts of *S. brachypetala* combined with *S. birrea* (Figure 3.4), it was noted that the combinations mainly exhibited additive patterns against the tested pathogens. Some ratio points, however, did exhibit synergism, as in *P. aeruginosa* isobologram for aqueous extracts combinations. Even though the synergistic interaction was based on tentative interpretation in a 1:1 combination (Σ FIC), the majority of the ratio points where *S. brachypetala* was higher (9:1, 8:2, 7:3, and 6:4) did exhibit a synergistic effect. Thus, the observed synergy gave some support to the traditional use of this combination

and higher concentrations of *S. brachypetala* should be incorporated in order to obtain greatest effect against *P. aeruginosa*. For the organic extract combination, only one ratio point (9:1) with the majority of *S. brachypetala* fell in synergistic region. Thus, for the combined organic extracts to be effective against *P. aeruginosa*, they should be incorporated into a ratio of 9:1 (*S. brachypetala*: *S. birrea*). In *S. epidermidis* isobologram, two ratio points (6:4 and 5:5) of the organic extracts combination fell in the synergy region while the aqueous extracts ratio points occur in the additive region as was also observed from the Σ FICs. Even though *S. birrea* and *S. brachypetala* have been investigated for their independent antimicrobial properties against various other pathogens (as mentioned in Chapter 2, Section 2.3.2), no previous studies have investigated the interactive properties of this combination against dermatological relevant pathogens.

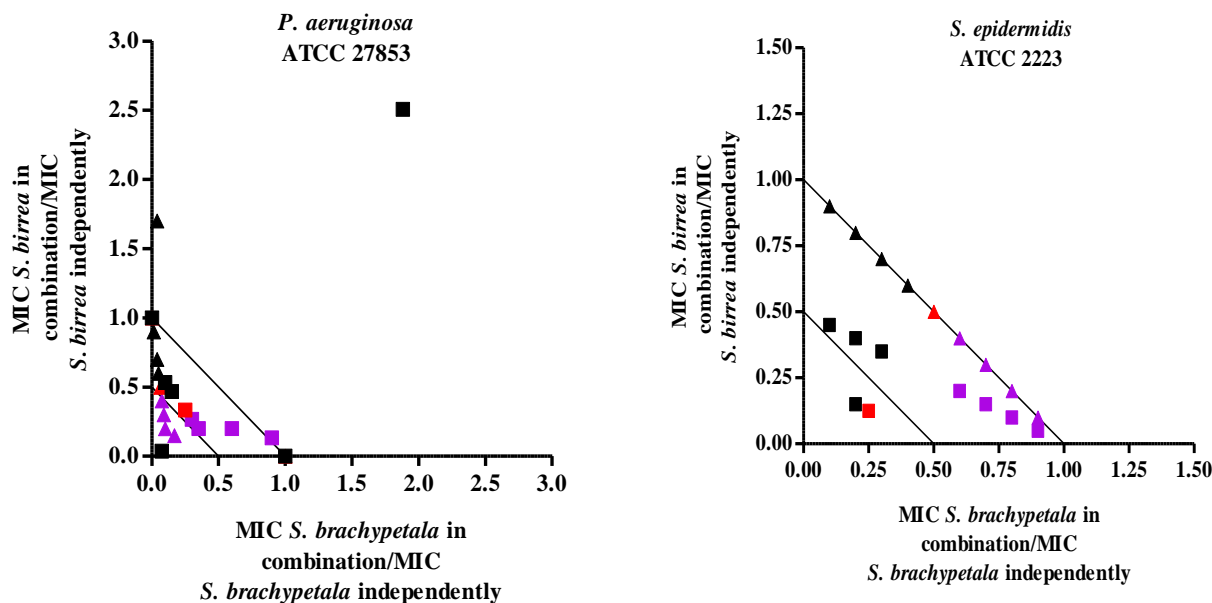


Figure 3.4: Isobologram representation of *S. brachypetala* in combination with *S. birrea* against *P. aeruginosa* and *S. epidermidis*. Where: the squares (\square) = organic extract combinations, the triangles (Δ) = aqueous extract combinations, \blacksquare \blacktriangle = 1:1 combination, \blacksquare \blacktriangle = combinations containing more *S. brachypetala*, \blacksquare \blacktriangle = combinations containing more *S. birrea*.

3.3.2.3 *Sclerocarya birrea* in combination with *Syzygium cordatum*

The aqueous extract combination of *S. birrea* and *S. cordatum* exhibited synergistic effect in a 1:1 combination (Σ FIC 0.13) investigated against *S. aureus*. When analyzed in various ratios (Figure 3.5), the Σ FIC evaluation of synergy was supported with five ratio points (2:8; 7:3; 6:4; 5:5 and 4:6, *S. birrea*: *S. cordatum*) occurring at the synergy region of the isobologram. Only one ratio point (where *S. cordatum* was in higher concentration) fell on a 0.5:0.5 line while the 9:1 point returned a non-interactive interaction. The majority of the organic extract ratio points, particularly where *S. birrea* was in majority displayed an additive effect, the effect that was also observed from the Σ FIC analysis. For *S. epidermidis*, the organic extract combination ratios did not display a specific pattern. Synergy (one point), additive (four points) and non- interactive interactions were observed for the organic extract. All the interactions occur where the concentrations of both plants were equal and it was worth noting that the 1:1 (50:50 μ l) ratio point fell in a synergy region, thus corresponding with the synergistic effect observed from the Σ FIC (0.34).

In a *P. aeruginosa* isobologram, the majority of ratio points for both aqueous and organic extract combinations displayed an additive pattern. Only two ratio points (1:1 and 2:8, *S. birrea*: *S. cordatum*) from the aqueous combination fell in the synergy region. The synergistic effect observed from the 1:1 (50:50) ratio point supported the Σ FIC evaluation (Σ FIC 0.19). One point (4:6 *S. birrea*: *S. cordatum*) from organic combination fell in the synergy region while a 1:1 point gave a non-interactive interaction which corresponded with the non-interactive interaction observed from the 1:1 combination (Σ FIC 1.16). The synergistic interaction observed from some ratio points of this combination particularly the aqueous combination, indicated that the antimicrobial efficacy was enhanced when comparing to their independent antimicrobial effects (Table 3.2). York et al. (2012) investigated the antimicrobial efficacy of these plants in a 1:1 combination against the respiratory tract pathogens where no synergy was depicted. However, when investigated against the pathogens associated with STIs, this combination exerted a synergistic effect in varied ratio studies (Naidoo et al., 2013).

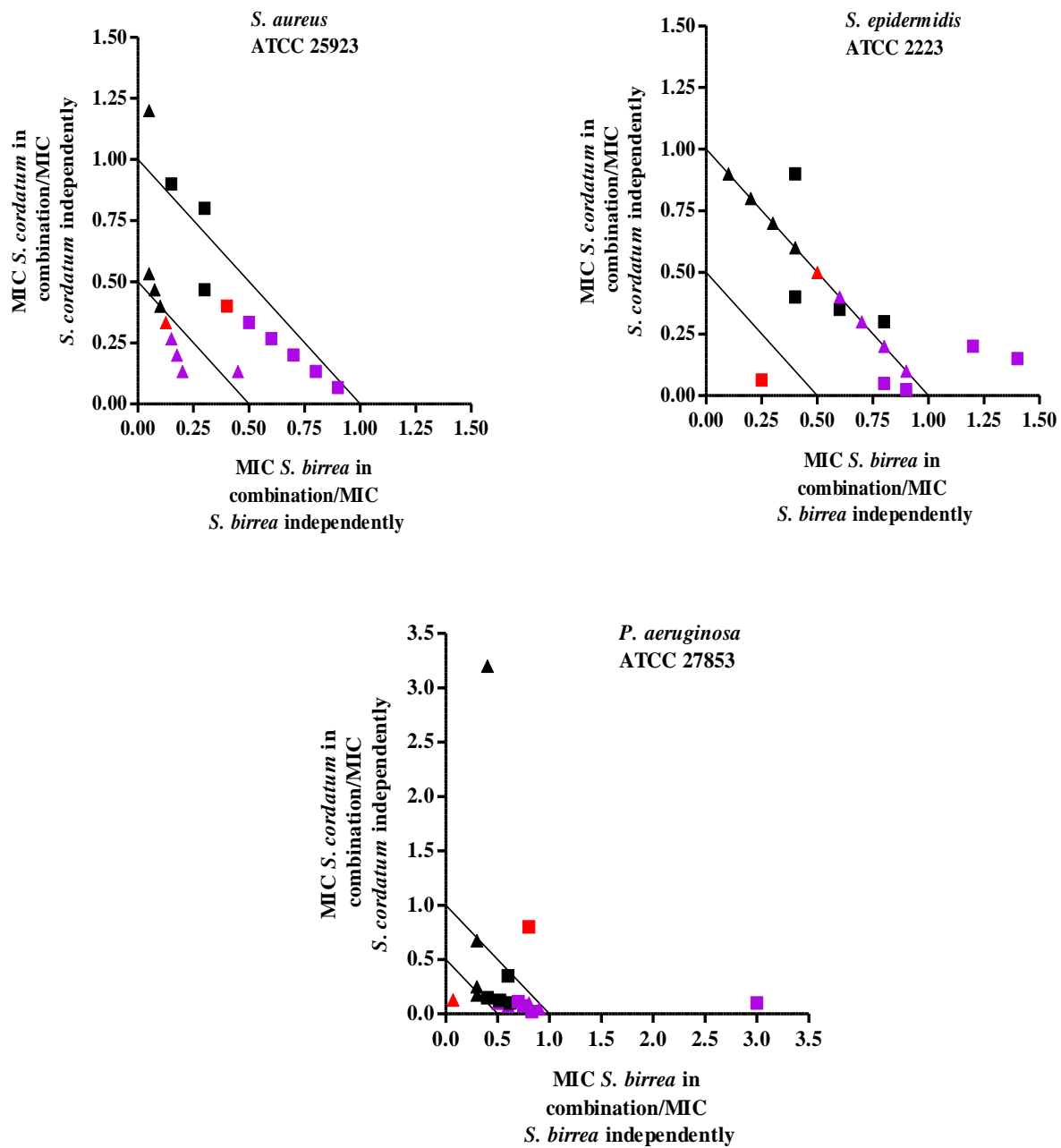


Figure 3.5: Isobologram representation of *S. birrea* in combination with *S. cordatum* against *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Where: the squares (□) = organic extract combinations, the triangles (▲) = aqueous extract combinations, ■ ▲ = 1:1 combination, ■ ▲ = combinations containing more *S. birrea*, ■ ▲ = combinations containing more *S. cordatum*.

3.3.2.4 *Strychnos madagascariensis* in combination with *Strychnos spinosa*

When varying concentrations of the organic extracts of these plants were combined (Figure 3.6), the Σ FIC evaluation of synergy (0.38) was supported against *S. aureus* with most of the ratio points (eight) occurring in a synergy region of the isobologram. Only one ratio point (6:4) where *S. spinosa* was in majority in relation to *S. madagascariensis* returned an additive interaction. Since all the ratio points where the concentration of *S. madagascariensis* was higher fell in a synergy region, it can be deduced that this plant greatly contributed to the observed synergistic effect. The ratio points of the aqueous extracts of the same combination against the same pathogen (*S. aureus*), mostly displayed an additive effect. Even though the effect of the combination was tentatively interpreted as non-interactive in the Σ FIC evaluation, only two ratio points (9:1 and 8:2 *S. spinosa*: *S. madagascariensis*) gave a non-interactive interaction. This indicates that, the efficacy of the combined plants may rely on the specific ratios in which the two plants are combined.

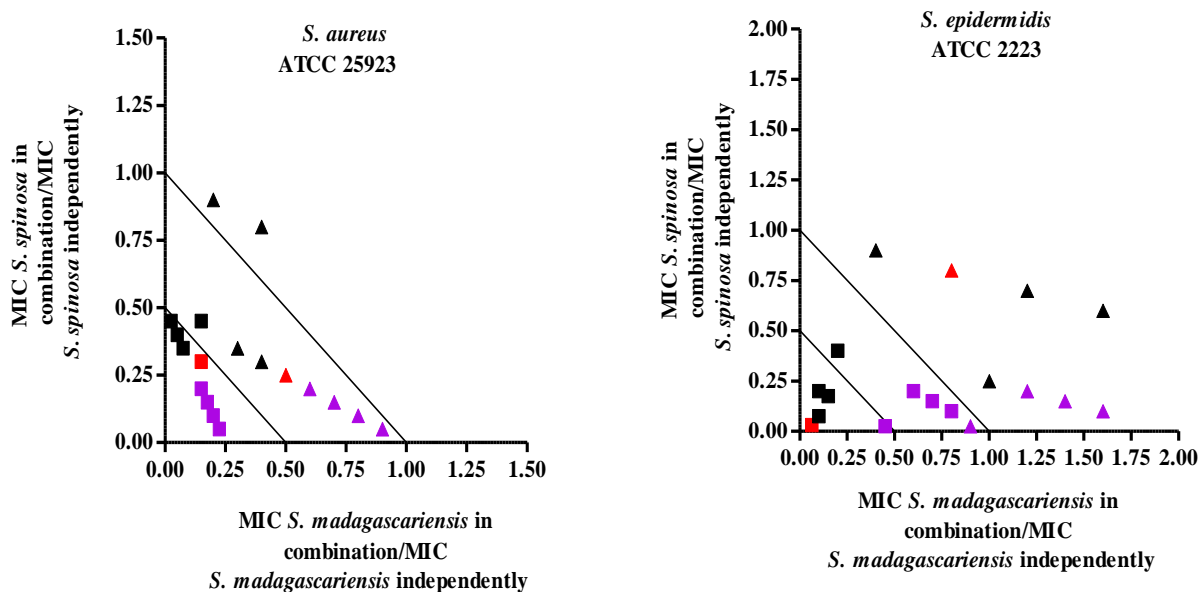


Figure 3.6: Isobologram representation of *S. spinosa* in combination with *S. madagascariensis* against *S. aureus* and *S. epidermidis*. Where: the squares (\square) = organic extract combinations, the triangles (Δ) = aqueous extract combinations, \blacksquare \blacktriangle = 1:1 combination, \blacksquare \blacktriangle = combinations containing more *S. madagascariensis*, \blacksquare \blacktriangle = combinations containing more *S. spinosa*.

When investigated against *S. epidermidis*, some ratio points from the organic extracts displayed a synergistic effect. Three ratio points (1:9; 3:7 and 4:6) where *S. spinosa* was higher gave a synergistic effect while only one point (9:1) where *S. madagascariensis* was higher gave a synergistic effect. The aqueous combination supported the Σ FIC evaluation with most of the ratio points occurring at the non-interactive region. Only one point (1:9) where *S. madagascariensis* was in majority gave an additive interaction. *S. spinosa* has been found to have poor activity when investigated individually against *C. albicans*, *E. coli*, *S. aureus* and *P. aeruginosa* (Kubmarawa et al., 2007). *S. madagascariensis* has only been documented for its use against inflammation and wounds (Norscia and Borgognini-Tarli, 2006) and has only been evaluated against *S. aureus* and *E. coli* in a diarrheal study by Van Vuuren et al. (2015), studies that have evaluated its antimicrobial efficacy in combination with *S. spinosa* against skin associated pathogens are limited.

3.3.2.5 *Canthium inerme* in combination with *Dichrostachys cinerea*

In varied ratio analysis of *C. inerme* and *D. cinerea* (Figure 3.7), two ratio points (5:5 and 4:6 *C. inerme*: *D. cinerea*) of organic extracts combination occurred at the synergy region of the isobologram while other points fell in the additive (1:9; 3:7 and 4:6) and the non-interactive region (9:1; 8: 2 and 7:3 *D. cinerea*: *C. inerme*). *D. cinerea* proved to contribute less effect since the majority of the ratio points containing *D. cinerea* in majority displayed a non-interactive interaction. The combination of the aqueous extract contributed equal effects as all ratio points occurred in an additive line. When investigated individually, *D. cinerea* ethanol extract gave poor activity against *C. albicans*, *E. coli*, *S. aureus* and *P. aeruginosa* (Kubmarawa et al., 2007). No antimicrobial study could be found for *C. inerme* against skin pathogens, this plant is only documented for its use in treatment of small pox lesions and body sores (Watt and Brandwijk, 1962). In the present study it was found that, a 5:5 and 4:6 organic combination of *C. inerme* in relation to *D. cinerea* can be a promising anti-staphylococcal.

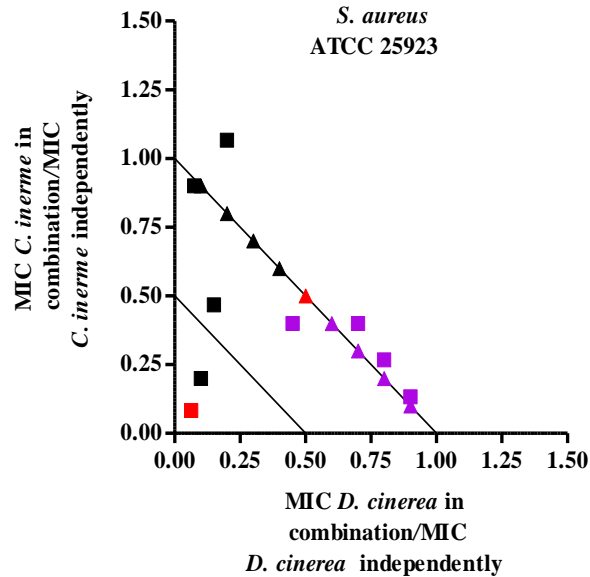


Figure 3.7: Isobologram representation of *C. inermis* in combination with *D. cinerea* against *S. aureus*. Where: the squares (\square) = organic extract combinations, the triangles (Δ) = aqueous extract combinations, \blacksquare \blacktriangle = 1:1 combination, \blacksquare \blacktriangle = combinations containing more *D. cinerea*, \blacksquare \blacktriangle = combinations containing more *C. inermis*.

3.3.2.6 *Acacia burkei* in combination with *Kigelia africana*

When combined in varying ratios, the aqueous and organic extracts of *A. burkei* in combination with *K. africana* exhibited both synergy, additive and non-interactive interactions (Figure 3.8). A 1:1 ratio point of the organic combination occurred in a synergy region of the isobologram, thus supporting the Σ FIC evaluation of synergy. Another ratio point (1:9, *A. burkei*: *K. africana*) also displayed a synergistic interaction. Furthermore, two ratio points (1:9 and 2:8, *A. burkei*: *K. africana*) from the aqueous combination displayed a synergistic interaction. The ratio points for both aqueous and organic extract combinations having *K. africana* in majority proved to be less effective giving only the additive and non-interactive interactions. Thus, for this combination to be effective against *S. aureus*, more *A. burkei* in relation to *K. africana* should be incorporated in 1:9 ratio for the organic combination and in 1:9; 2:8 ratios for the aqueous combination. The antimicrobial efficacy of *K. africana* against skin associated pathogens has been reported (Mabona et al., 2013) while *A. burkei* has only been investigated against *S. aureus* and *E. coli* in a previous study by Van Vuuren et al. (2015). No antimicrobial study has reported the efficacy of the combination of these two plants against skin associated pathogens.

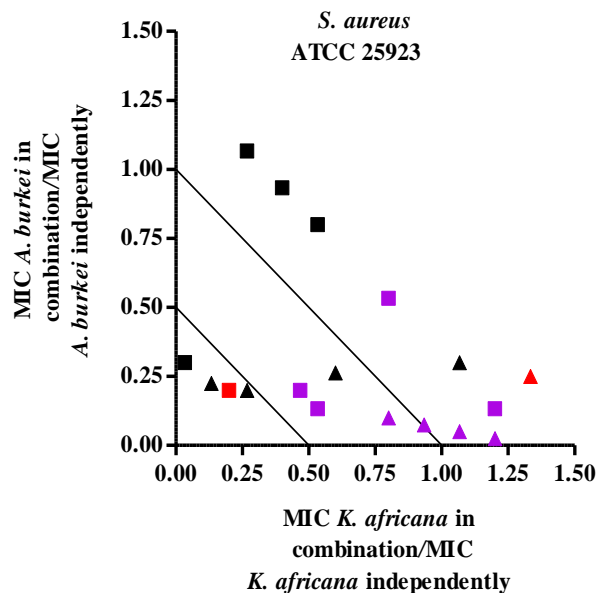


Figure 3.8: Isobologram representation of *A. burkei* in combination with *K. africana* against *S. aureus* Where: the squares (\square) = organic extract combinations, the triangles (Δ) = aqueous extract combinations, \blacksquare \blacktriangle = 1:1 combination, \blacksquare \blacktriangle = combinations containing more *K. africana*, \blacksquare \blacktriangle = combinations containing more *K. africana*.

3.4 Summary

- The overall Σ FIC analysis of the plant combinations indicated that some plants possess promising interactions when combined as 27% of the combinations were synergistic, 31% were additive, 25% were non-interactive and only 17% were antagonistic.
- *Staphylococcus aureus* proved to be the most susceptible pathogen with 44% of the combinations in 1:1 FIC combinations displaying synergistic effects against this micro-organism.
- The 1:1 organic extract combination of *H. hemerocallidea* and *S. rigescens* exhibited the best synergistic interaction (Σ FIC value 0.04) against *S. epidermidis*.
- The combination (1:1) of *A. burkei* and *K. africana* which was reported to treat ringworm by the lay people of northern Maputaland displayed a promising activity (synergism) against *T. mentagrophytes* which supports the traditional use of this combination.

- It was worth noting that the multi-plant combination of *B. discolor*, *E. tirucalli*, *H. hemerocallidea*, *O. engleri* and *S. serratuloides* displayed synergistic and additive effects (mostly based on tentative interpretation) against *S. aureus*, *S. epidermidis* and *P. aeruginosa* although the combination could not be investigated further in isobole method due to the number of plants combined.
- The isobologram method supported the findings from the 1:1 Σ FIC analysis where it was found that the majority of the combinations in various ratios displayed additive patterns.
- The various ratio combinations of the organic extracts of *H. hemerocallidea* and *S. rigescens* again exhibited the best synergistic effect against *S. epidermidis* with all ratio points plotted within the synergistic quadrant.

Chapter Four: *In vitro* diffusion characteristics of the antimicrobially active plant extracts across porcine skin

4.1 Introduction

The skin is a site of interest for application of drugs intended for local (on diseased skin) and systemic therapeutic effect (Sharma et al., 2011). However, the stratum corneum forms a barrier which determines the penetration or impermeability of a drug to or through the skin (Valenta and Auner, 2004). This layer is usually 10-20 μm thick depending on the body region being thicker on palmar and plantar regions (Mehta, 2004). It is composed of dead keratinized cells which form the outermost part of the avascularised epidermis (Figure 4.1). Beneath the stratum corneum are other layers of the viable epidermis namely, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The layer below the epidermis is a vascularised dermis in which epidermal appendages such as hair follicles, sebaceous and sweat glands originate. After penetrating the stratum corneum (a limiting barrier), a drug can diffuse relatively easy through the viable epidermis to the dermis for systemic absorption (Kwatra et al., 2013). Occurring beneath the epidermis is the subcutaneous tissue or hypodermis which is not necessarily involved in drug absorption as it occurs beneath the vascular system (Reddy et al., 2014).

The ability of a drug to penetrate the multilayered skin largely depends on its physicochemical properties such as lipophilicity, solubility, molecular weight or size, melting point and pH of the solution (Latheeshjlal et al., 2011; Gaikwad, 2013). Since the stratum corneum is lipophilic (composed of 40% of fats) drug molecules have to be lipophilic in order to partition within the lipid lamellae of the stratum corneum cells (corneocytes). However, if a drug is too lipophilic, it will be unable to diffuse through the aqueous dermis beneath and remain in the stratum corneum. Thus, a good drug candidate must have a low melting point in order to be adequately soluble in both fats and water. Drugs containing larger molecules cannot permeate the skin while those with a molecular weight of less than 500 Daltons are sufficiently mobile and permeate quickly. A

saturated drug formulation with a pH between five and nine also penetrates easily through the skin (Naik et al., 2000).

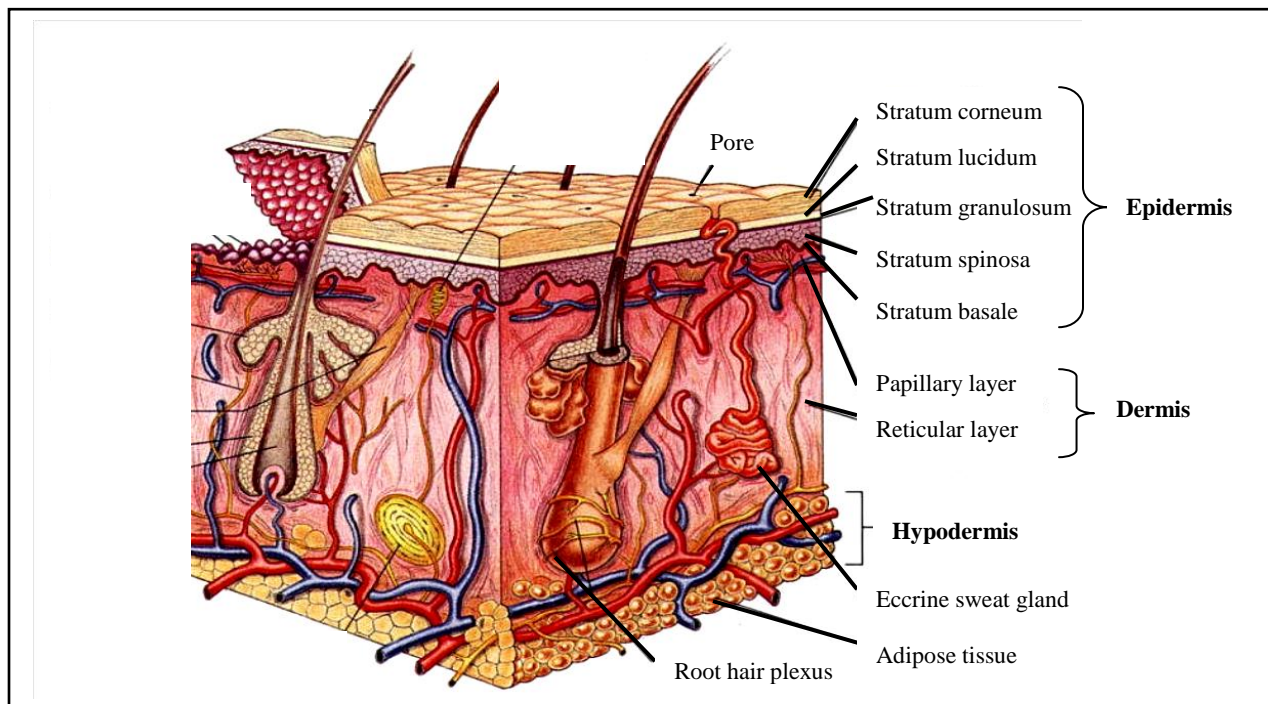


Figure 4.1: Schematic representation of the human skin (Trommer and Neubert, 2006).

Generally, drug molecules penetrate the skin by passive diffusion (Mehta, 2004). The molecules pass through the stratum corneum via appendigeal, intercellular or intracellular routes as depicted in Figure 4.2. Skin appendages provide a direct channel for drugs with larger molecules to penetrate the skin bypassing the stratum corneum. However, skin appendages occupy 0.1% of the skin surface which limits the area available for a drug to directly contact the skin. Consequently, skin appendages are not necessarily considered as routes of drug absorption (Kwatra et al., 2013). The intercellular and transcellular routes of drug diffusion across the stratum corneum depend on the physicochemical properties and partition coefficient of drug molecules. Small hydrophilic molecules diffuse passively (paracellular or intercellular) across the stratum corneum while lipophilic drugs follow the intracellular route of penetration. Most molecules diffuse through the lipid matrix between the corneocytes to the site of action (Gaikwad, 2013).

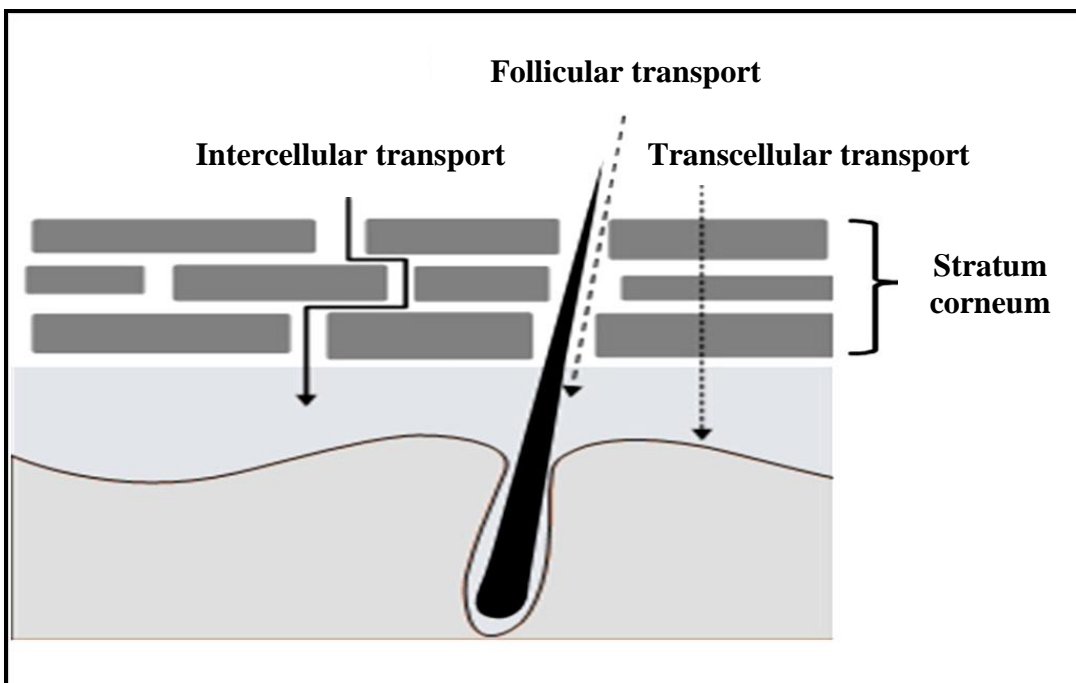


Figure 4.2: Routes of penetration through the stratum corneum of the skin (Rahimpour and Hamishehkar, 2012).

Plant preparations also possess a complex mixture of chemical compounds that when applied topically, exert local or systemic effects to a dermatological condition (Nidhal et al., 2005). Evaluating percutaneous absorption of these compounds is of prime importance in understanding their permeation behavior through the skin. Various *in vitro* and *in vivo* models are used in determining the permeation and mechanism of action of drugs in the skin. While the *in vivo* models give more reliable results, they are, however, expensive and require exposure of the animal or human being to a drug or compound. *In vitro* models are reproducible, easy to control and may provide preliminary observations needed for further *in vivo* observations (Sahaya et al., 2012), although the results may not always correlate with the *in vivo* state (Gonzalez, 2006). In most *in vitro* models, porcine skin is widely used as a model for the cutaneous barrier as it has been reported to give comparable results to human skin (Godin and Touitou, 2007). The technique involves topical application of a drug on one side of the excised skin placed between a donor and a receptor chamber as presented in Figure 4.3. Diffused compounds are collected in a

receptor chamber at various time points and assayed with, for example High Performance Liquid Chromatography.

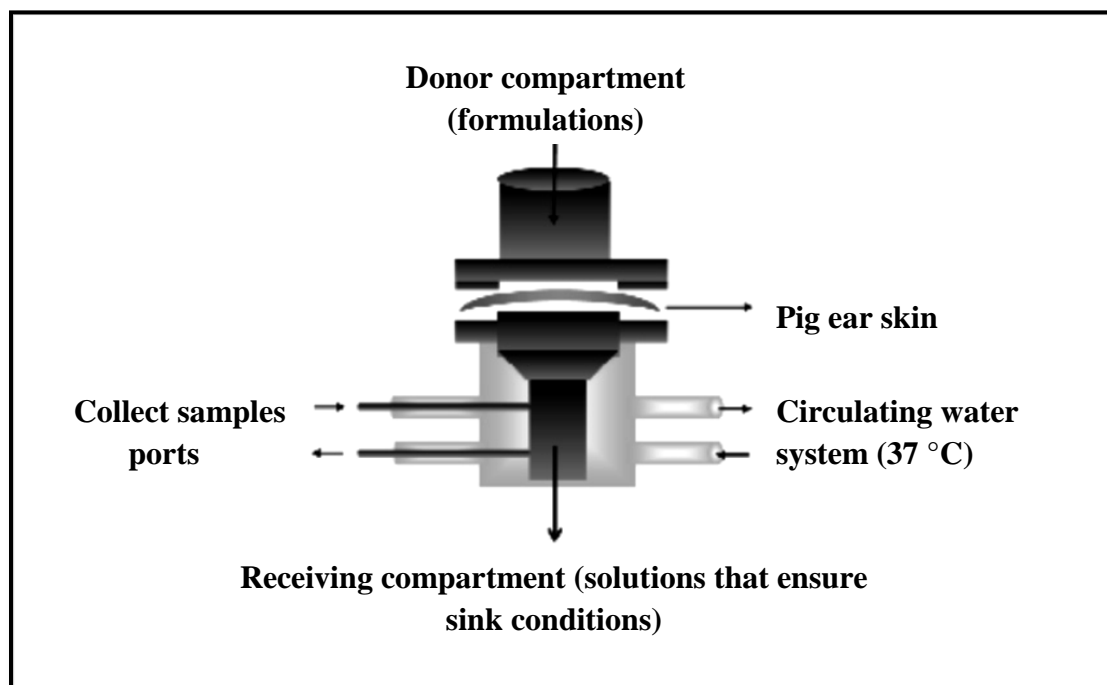


Figure 4.3: A flow-through diffusion cell system showing how the transdermal penetration of drug molecules is assessed *in vitro* (Silva et al., 2012).

Considering the lack of scientific information on the percutaneous absorption of compounds present in the topically applied plants studied in the present study, this chapter focuses on obtaining the chemical profiles and determining permeability properties of the chemical compounds from the most antimicrobially active plant extracts. Four independent plant extracts and one combination that were reported to be used topically by De Wet et al. (2013) were selected for *in vitro* diffusion studies based on the preliminary antimicrobial analysis. Dichloromethane: Methanol (D:M) extracts were used as a guide on selecting the extracts with broad spectrum antimicrobial activity (mean MIC < 1.00 mg/ml). Aqueous extracts of the same selected D:M extracts were also included so as to compare the permeation behavior of compounds present within these extracts. Selected plants (most antimicrobially active topically applied plants) as well as their mean MIC values are depicted in Table 4.1 (independent plants

adapted from Chapter 2, Table 2.3 and 2.4) and Table 4.2 (combination adapted from Chapter 3, Table 3.2).

Table 4.1: The most antimicrobially active topically applied plants selected for further analysis.

Botanical name	Part used	Disease(s) treated	*Average MIC values (mg/ml)	
			D:M	Aqueous
<i>Garcinia livingstonei</i>	Bark	Burns	* 0.27	*3.19
<i>Kigelia africana</i>	Fruits	Ringworm	0.68	2.19
<i>Sclerocarya birrea</i>	Bark	Burns, boils and sores	0.37	1.76
<i>Syzygium cordatum</i>	Bark	Burns and sores	* 0.86	*3.00

*Mean MIC values were calculated as the sum of MIC values of all tested pathogens divided by the total number of pathogens investigated against each plant extract. *There are MIC values that were not considered (MIC>8.00 mg/ml) since no end point was obtained for MIC determination.

Table 4.2: Most antimicrobially active topically applied plant combination.

Combination	ΣFIC values of test micro-organisms					
	<i>S. aureus</i> ATCC 25923		<i>S. epidermidis</i> ATCC 2223		<i>P. aeruginosa</i> ATCC 27853	
	D:M	Aqueous	D:M	Aqueous	D:M	Aqueous
<i>Sclerocarya birrea</i> and <i>Syzygium cordatum</i>	0.63	0.13	0.34	1.50	1.16	0.19
ΣFIC interpretation	Additive	Synergism	Synergism	Non- interactive	Non- interactive	Synergism

Highlighted in bold are synergistic interactions.

4.2 Materials and methods

4.2.1 Chemicals and reagents

All chemicals used were obtained from ACE (Associated Chemical Enterprises) Southdale Johannesburg, Sigma-Aldrich Pty (ltd) (St. Louis, MO, USA) and RC (Rochelle Chemicals) South Africa. Methanol and formic acid were of HPLC grade while all the other laboratory chemicals used were of analytical reagent grade and included the following: aluminum chloride, ammonium solution, benzene, bismuth (III) subnitrate, ferric chloride, 32% hydrochloric acid, L (+) – tartaric acid, Meyer’s reagent, petroleum ether, potassium hydroxide, potassium iodide, sodium azide and sulphuric acid. Other reagents or solutions were prepared using analytical grade chemicals and double distilled Millipore water. They included: Dragendorff’s reagent and phosphate buffered saline solution (PBS). Dragendorff’s reagent was prepared by mixing 1:1:1 ratio (v/v) of bismuth nitrate, tartaric acid and potassium iodide solutions (Bibi et al., 2012). PBS was prepared by mixing Sodium chloride (8 g), Potassium chloride (0.3 g), Potassium phosphate monobasic (0.2 g) and di- Sodium hydrogen orthophosphate 2-hydrate (0.73 g) in a liter of double distilled Millipore water and adjusting the pH to 7.4.

4.2.2 Equipment and apparatus

For UV/Vis spectra, a Ultra-Violet Visible Spectrophotometer (Thermo Scientific Multiskan GO) was used. Chemical profiling of extracts was performed using reverse-phase High Performance Liquid Chromatography (RP-HPLC). The analyses were performed utilizing a Flexar Perkin Elmer HPLC system containing a Perkin Elmer binary pump, a Flexar LC autosampler and a Flexar LC UV/Vis detector. The column used was a Brownlee Spheri-5 RP-C18, 5 μ , 100 x 4.6 mm. For the *in vitro* diffusion assay, an ILC07 automated system consisting of 7 in-line flow cells from PermeGear INC (Hellertown, PA, USA) was used. The system consisted of an ISCO Retriever IV fraction collector, an Ismatec IPC peristaltic pump and a Thermoscientific Haake SC100 water bath. Each flow cell of the system contained an orifice area of 0.039 cm².

4.2.3 Preliminary qualitative phytochemical screening

Various chemical tests were performed in order to determine the possible group of chemical compounds present in each of the selected plant extracts. The chemical tests were conducted using standard qualitative methods with slight modifications as described by Ahumuza and Kirimuhuzya, (2011) and Bibi et al. (2012) as follows:

Test for alkaloids: Each of the aqueous and organic powdered plant extracts (0.1 g) was added to 2 ml of Hydrochloric acid. The mixtures were warmed, filtered with Whatman filter paper and the filtrates were divided into 1 ml and 1 ml of each reagent (Dragendoff's and Meyer's reagent) was added. Turbidity or precipitation indicated the presence of alkaloids.

Test for anthraquinones: The extract (0.1 g) was mixed with 1% (v/v) hydrochloric acid in water and boiled. The boiled mixture was cooled, filtered with Whatman filter paper and the filtrate was shaken with 3 ml of benzene. The benzene layer was removed and 10% (v/v) ammonium solution was added. The appearance of a pink, violet or red colour in the lower phase of the ammonium solution indicated the presence of anthraquinones.

Test for coumarins: The plant extract (0.1 g), moistened with 1N sodium hydroxide was placed in a test tube and covered with a filter paper also moistened with the same solvent. The test tube was then placed in boiling water for a few minutes. The filter paper was removed and examined under UV light for yellow fluorescence which indicated the presence of coumarins.

Test for flavonoids: The extract (0.1 g) was shaken with 5 ml of petroleum ether. The fatty petroleum ether layer was then removed and the defatted residue was dissolved in 5 ml of 80% (v/v) ethanol in water. The solution was filtered using Whatman filter paper and the filtrate was divided into 2 ml which were then reacted separately with 1% (w/v) aluminum chloride solution and 1% (w/v) potassium hydroxide solution in methanol. Formation of a yellow colour in the filtrate that reacted with the aluminum chloride solution indicated the presence of flavones. In the filtrate that was reacted with potassium hydroxide solution, formation of a dark yellow colour indicated the presence of flavonoids.

Test for saponins: A small amount of plant (approximately 0.05 g) extract was dissolved in boiling water in a test tube. The mixture was allowed to cool and shaken thoroughly. Persistent frothing indicated the presence of saponins.

Test for tannins: Plant extract (0.1 g) was boiled with 2 ml of distilled water. The boiled mixture was cooled, filtered using a Whatman filter paper and the filtrate was added into 0.1% (w/v) iron chloride. Formation of a brown green or blue black colour indicated the presence of tannins.

Test for sterols: A small amount of plant extract was added into 2 ml of 92% (v/v) sulphuric acid in a test tube. A purple ring formed at the upper surface indicated the presence of sterols.

4.2.4 Ultraviolet-Visible spectrum analysis

The crude extracts (D: M and aqueous) were further subjected to ultraviolet-visible (UV-Vis) spectrum analysis in order to determine the absorption maxima of the compounds detected in the phytochemical screening investigation. The extracts were first dissolved in a Phosphate buffered saline (PBS) to a standard concentration of 32 mg/ml. Phosphate buffered solution was preserved with 0.1% (w/v) sodium azide for long-term use. It was maintained at a physiological pH of 7.4 to ensure that it does not interfere with the chemical composition of the extracts, for instance through ionization. Extracts were diluted further into 1:100 using PBS prior to obtaining the spectra in the range of 200 - 800 nm. PBS was used as the solvent blank.

4.2.5 Chemical profiling of extracts prior to the *in vitro* permeation assay

Chemical profiling of each extract was performed using the previously described HPLC system (Chapter 4, Section 4.2.2) prior to and after *in vitro* permeation assays. The column temperature of the HPLC was held constant at 25 °C. Plant extracts (32 mg/ml in PBS) were initially filtered through a syringe filter (0.45 µm) before chemical analysis. An aliquot (10 µl of the extract) was injected into the chromatographic system and the resultant chromatograms were used to indicate the peak spectrum of chemical compounds. PBS was used as a control.

The run time for all samples prior to and after *in vitro* permeability assays was 45 minutes at 1 ml/min. Different mobile phases were investigated in order to optimize the resulting

chromatogram peaks which included a gradient elution with a mixture of HPLC grade methanol (A): water (B) as well as HPLC grade methanol (A): water with 0.1% (v/v) formic acid (B). Better peak separation was obtained when using a mixture of methanol: water with formic acid (acidic aqueous solution) which was then selected as a mobile phase for the rest of the experiments. The chromatograms obtained when using methanol: water and methanol: water with formic acid are shown in appendix H1-H2. Double distilled Millipore water was first filtered through a 0.45 μm pore filter before being used. Chromatographic separation was performed by a gradient elution as seen in Table 4.3. The extracts were first monitored at different wavelengths including 210, 260, 280 and 360 nm based on absorption maxima wavelengths obtained under the UV-Vis spectrum analysis. A wavelength of 260 nm was then selected as a monitoring wavelength since there were no differences in chromatograms obtained under all the wavelengths investigated. Thus, only the chromatograms obtained at 260 and 360 nm were included in the appendices (appendix H1-H3).

Table 4.3: Elution gradient for 45 min runs at a flow rate of 1 ml/min.

Step time (min)	% A (Methanol)	% B (Water + 0.1% (v/v) formic acid)
0.5	1.0	99.0
10.0	5.0	95.0
5.0	15.0	85.0
5.0	20.0	80.0
20.0	60.0	40.0
5.0	1.0	99.0
10.0	1.0	99.0

4.2.6 *In-vitro* permeation assay

4.2.6.1 Collection and storage conditions of porcine skin tissue samples

An ethics waiver for the approval of the use of porcine skin tissue samples was obtained from the University of the Witwatersrand School of animal, plant and environmental sciences as well as the Human Research Ethics Committee (Appendix I). The excised porcine skin tissue samples were then harvested from animals (porcine) euthanized for other purposes at the central animal service unit at the University of the Witwatersrand Medical School. After collection, the skin were immediately placed in a PBS (pH 7.4) solution and transported to the laboratory within an hour. Excess fat and connective tissue were removed and the specimens were cut into 1x4 cm strips. The tissue strips were then placed in cryovials, snap frozen at -196 °C and stored in liquid nitrogen until further use.

4.2.6.2 *In vitro* permeability experiment

The permeability characteristics of the number of chemical compounds present in the plant extracts were determined using a 7 in-line (flow-through) diffusion system. A diagrammatic representation of the steps undertaken during each permeability experiment is depicted in Figure 4.4. Prior to each experiment, liquid nitrogen frozen skin tissue strips were thawed in PBS for 5 min at room temperature. The thawed skin tissue strips were cut into seven 1x1 cm diameter sections. The skin sections were mounted into the donor compartments of a flow-through diffusion system with the stratum corneum facing up in the direction of the donor compartments. Each compartment was then held together with a clamp wedging the skin between the donor and receptor compartments. The exposed surface area of the skin for diffusion in each flow cell was 0.039 cm². All air bubbles under the skin were removed and 500 µl of the plant extract with a concentration of 32 mg/ml was topically applied on the exposed skin surface in the donor compartment. The donor compartments were then covered with parafilm to prevent any evaporation and to keep a constant temperature within the chambers throughout the experiment. The temperature of the system was kept at 37 °C in a water bath. Each experiment was run for a total period of two hours at flow rate of 1.5 ml/h with liquid (containing the diffused compounds from the extracts) collected every 10 min in the fraction collector. Phosphate Buffered Saline (pH 7.4) was used as elution medium. All experiments were performed in sextuple for each



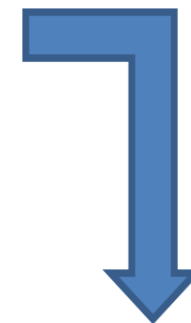
Defrosting skin tissue



Cutting skin into 1x1 cm pieces



Mounting the skin pieces



Putting skin tissues into donor chambers



Holding donor chambers with a clamp



Topically applying plant extracts on the skin tissues



The whole system with 7-in-line flow cells

Figure 4.4: Diagrammatic representation of how the permeability experiment was conducted.

extract with PBS used as a control. The liquid collected every 10 min was analyzed with RP-HPLC to determine whether the compounds present in the crude extracts were capable of diffusing from the extract across the skin.

4.3 Results and discussion

4.3.1 Preliminary qualitative phytochemical screening

Preliminary qualitative phytochemical screening (Table 4.4) revealed the predominant presence of flavonoids, saponins, tannins and anthraquinones in all tested extracts of the selected plants. Flavonoids are usually the main active ingredients used in medical preparations for the treatment of human ailments (Saravanakumar et al., 2009). As a result, flavonoids are the subject of anti-infective research because of their antibacterial, antifungal and antiviral properties. This class of phytochemical contains various compounds that may differ in their modes of action when targeting the microbial cell. For instance, some flavonoid compounds may interfere with nucleic acid while others may inhibit cytoplasmic membrane function and energy metabolism of the microbial cell (Cushnie and Lamb, 2005).

On the other hand, saponins are known to protect plants from microbial attack. Saponins usually act by disrupting the cell membrane resulting in irreversible damage of the lipid bilayer which account for their antimicrobial activity. This class of phytochemical also exhibit anti-inflammatory properties where they inhibit vascular permeability, the first stage of inflammation (Sparg et al., 2004). Anthraquinones isolated from plants also poses antibacterial and antifungal properties (De Barros et al., 2011). Tannin containing plants usually possess anti-inflammatory, anti-ulcer and antiparasitic properties. Tannins are also known to inhibit microbial growth by precipitating digestive proteins and thus hindering the availability of nutrients (Prasad et al., 2008). These compounds act in the same manner when exposed to burn wounds. They precipitate proteins of the exposed tissue in burn wounds and form granulation tissue.

These four classes of compounds (flavonoids, saponins, tannins and anthraquinones) were all present in the tested extracts and may account for their broad-spectrum antimicrobial activity, as observed in Chapter 2, Section 2.3.2, Table 2.3. In addition to the four common classes of compounds found above, *G. livingstonei* also indicated the presence of alkaloids and flavones,

Table 4.4: Preliminary qualitative phytochemical screening results.

Phytochemicals tested	Plants analysed							
	<i>G. livingstonei</i> Bark		<i>K. africana</i> Fruits		<i>S. birrea</i> Bark		<i>S. cordatum</i> Bark	
	D:M	H ₂ O	D:M	H ₂ O	D:M	H ₂ O	D:M	H ₂ O
Alkaloids	+++	+	-	-	-	-	+	+
Anthraquinones	+++	+++	+	+	+++	+++	+++	+++
Coumarins	-	-	++	+++	-	-	-	-
Flavones	++	++	+	+	-	-	-	-
Flavonoids	+++	+++	+++	+++	+++	+++	+++	+++
Saponins	+++	+++	+++	++	+++	+++	+++	+++
Sterols	-	-	++	+	++	++	-	-
Tannins	+++	+	++	+	++	++	++	++

D:M: Dichloromethane: methanol extract; H₂O: aqueous extract; +++ : resent in large amount; ++: moderately present; + : present in small amount; -: not detected.

while *K. africana* indicated the presence of coumarins, flavones and sterols. *S. birrea* indicated the presence of sterols while *S. cordatum* indicated the additional presence of alkaloids. The results obtained in the present study correspond mostly with previous studies performed on similar plant extracts. Previous studies indicated the presence of alkaloids, saponins, flavonoids, steroids and tannins in *K. africana* leaves and fruits (Priya et al., 2012; Agyare et al., 2013; Priya et al., 2013; Arkhipov et al., 2014; Solomon et al., 2014). Steroids, flavonoids, coumarins, tannins, alkaloids and saponins were also found to be present in the bark, leaf and fruit extracts of *S. cordatum* (Pallant and Steenkamp, 2008; Wanyama et al., 2011; Cordier et al., 2013; Deliwe and Amabeoku, 2013; Sidney et al., 2015). Leaf and bark extracts of *S. birrea* similarly indicated the presence of alkaloids, anthraquinones, flavonoids, saponins, steroids and tannins (Ojewole et al., 2010; Dabai et al., 2013; Baba et al., 2014), while steroids and flavonoids were mainly detected in *G. livingstonei* root and stem extracts (Magadula and Suleimani, 2010; Magadula and Tewtrakul, 2010).

4.3.2 UV-Vis spectrometry

The UV-Vis spectra of the selected plant extracts were performed at wavelengths ranging between 200-800 nm and their profiles displayed different absorption maxima between 210 and 330 nm (Figure 4.5). Three absorption peaks were recorded for *G. livingstonei* extracts (organic and aqueous). For the organic extract, the absorption peaks occurred at wavelengths of 218, 268 and 292 nm. With the aqueous extract, the first absorption peak (212 nm) was much lower than that observed from the organic extract which may imply the presence of more compounds in the organic extract than the aqueous extract at the absorption wavelength of 210 nm. The second peak (262 nm) displayed maximum absorption similar to that of the organic extract. All the other extracts (*K. africana*, *S. birrea* and *S. cordatum*) displayed two main peaks. *Kigelia africana* extracts had a major absorption peak at 216 nm accompanied by a weak shoulder at 254 nm. The first major peak of both *S. birrea* and *S. cordatum* were recorded at the same wavelength (224 nm) for both organic and aqueous extracts. The second peak for *S. birrea* occurred at 284 nm while that of *S. cordatum* was recorded at 260 nm.

From all the extracts, the first major peaks were detected at around 212, 216 and 218 nm which can be characteristic of saponins that are usually absorbed at a lower wavelength of nearly 210

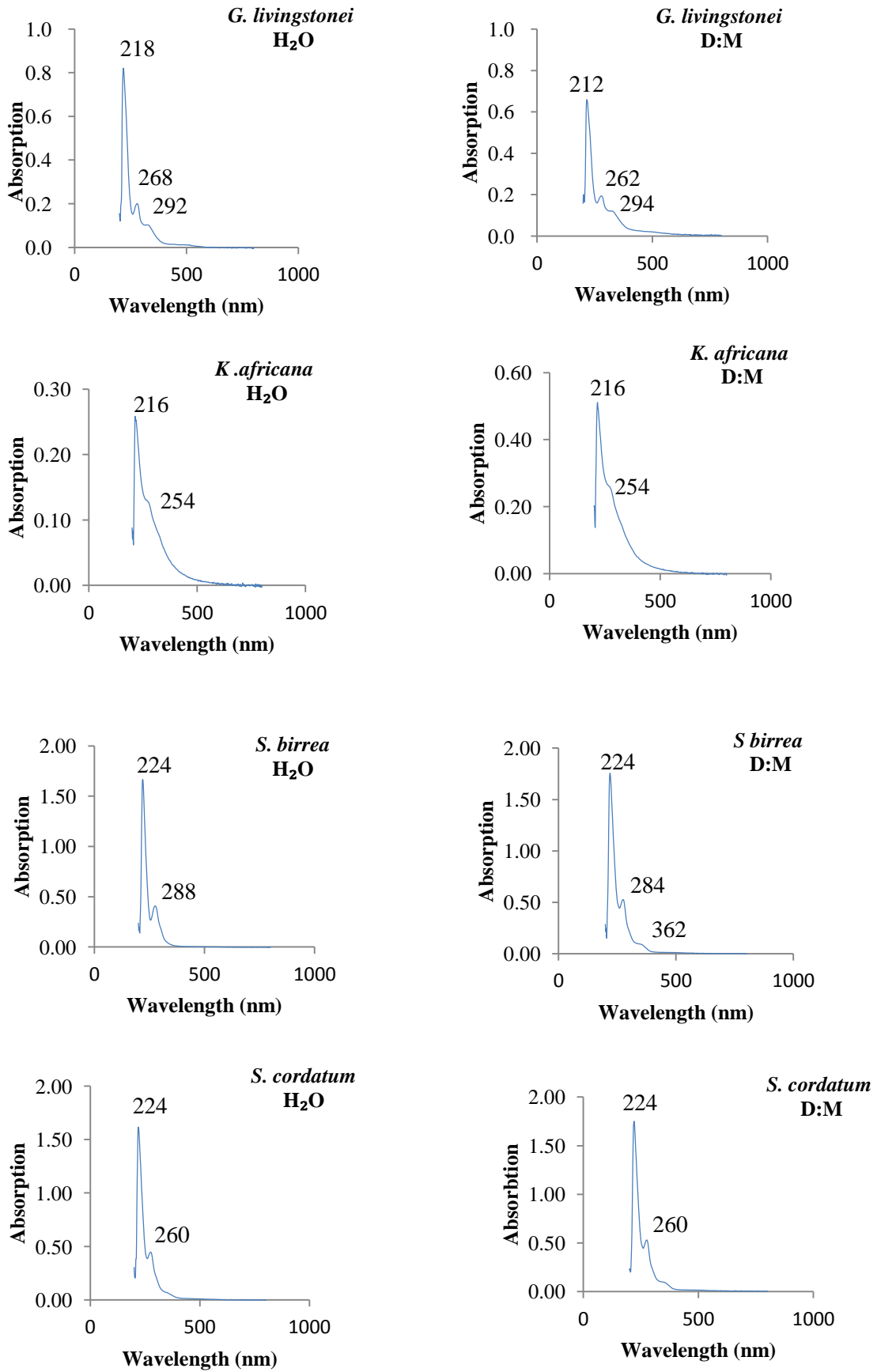


Figure 4.5: The UV/Vis spectra of the selected plant extracts analyzed at 200-800 nm.

nm (Oleszek, 2002). Saponins were present in all the extracts during phytochemical screening analysis. Furthermore, the second peaks from all extracts were detected at around 250-268 nm which may also confirm the presence of flavonoids (also detected in all extracts in phytochemical screening) since all flavonoids with aromatic chromophores are usually absorbed at a wavelength of 250 nm of the UV spectra. However, other flavonoids with carbonyl chromophores may also be slightly absorbed at the 300 nm regions (Sisa et al., 2010).

Garcinia livingstonei extracts exhibited peaks at 310 nm (aqueous extract) and 330 nm (organic extract). These peaks may indicate the presence of an anthraquinone moiety which usually gives maximum absorption at a wavelength between 257-341 nm (Osman et al., 2010) and anthraquinones were detected in *G. livingstonei* extracts. No other studies have analysed the UV-Vis spectra of these four plants investigated.

4.3.3 *In vitro* permeation of compounds from the most active plant extracts

Plant extracts are topically applied for the treatment of various skin diseases e.g. dermatitis, psoriasis, burns, inflammation and infections. The skin in these diseased states usually contains a compromised barrier function resulting in water loss and altered permeation of topically applied agents (Tsai et al., 2001). The active compounds in topically applied dermatological drugs may aim to act on the skin surface for superficial skin infections. They may also act on the dermal layer where they may penetrate the skin without systemic absorption for ailments affecting epidermal and dermal layers (Baert et al., 2007). Even though the topical administration of drugs is usually the most convenient since it is non-invasive and has a better compliance with patients, active compounds are, however, usually affected by the permeability of the stratum corneum (Baskar et al., 2012; Andrews et al., 2013). Thus, in most *in vitro* studies, the use of biological membrane systems which represent the stratum corneum are employed to study the diffusion profile of the active compounds from topical formulations. This allows the objective comparison of the mode of action of newly developed topical products whether they serve to act locally, dermally or transdermally on the skin. The same *in vitro* model (biological membrane system model) was used in this study to evaluate the diffusion of compounds present within the most antimicrobially active extracts through intact skin to provide a preliminary indication of the movement of these compounds through an intact skin barrier. Thawed intact porcine skin was

used as it is not always feasible to use freshly excised skin when conducting the diffusion experiments. Furthermore, the diffusion of compounds from the topically applied drugs usually occurs in the non-viable cells of the stratum corneum (Bartosova and Bajgar, 2012). It has also previously been found by Van der Bijl et al. (2001) that snap freezing tissues in liquid nitrogen within one hour of tissue removal only minimally affect diffusion of compounds across the tissue when compared to a freshly excised tissue.

Previous *in vitro* studies have investigated the permeation behavior of some known compounds present in topical formulations after certain time intervals with the mean flux values plotted as the standard curves (Heard et al., 2006; Boonen et al., 2010; Park et al., 2012). In the present study, all the groups of compounds present in each crude extract (chemical fingerprint) were evaluated for their permeation properties across the intact skin after 120 min of exposure and sampled after every 10 min interval. Thus, the results obtained were expressed as the HPLC chromatogram profile (chemical fingerprint) of each extract before and after the *in vitro* permeation assay. The results after the *in vitro* permeation assay were presented as chromatogram peaks (chemical fingerprint) after different time intervals which is 10, 60 and 120 min. PBS which was used as a control gave a single peak in all experiments and its RP-HPLC chromatogram peak and a retention time (1.02 min) and can be seen in Figure 4.6. The RP-HPLC chromatogram peaks of all the investigated extracts (before and after the *in vitro* permeability experiment) are depicted in Figures 4.7-4.16 with retention times of the major peaks detected shown in Tables 4.5-4.14. The retention times for some peaks before and after the permeability experiment, however, differed because the compounds may have co-eluted before the permeability experiment and separated after diffusion through the skin. Furthermore, some compounds present within the crude extracts (before the permeability experiment) giving retention times within 5 min could hardly be detected because of the low concentrations of these chemical compounds.

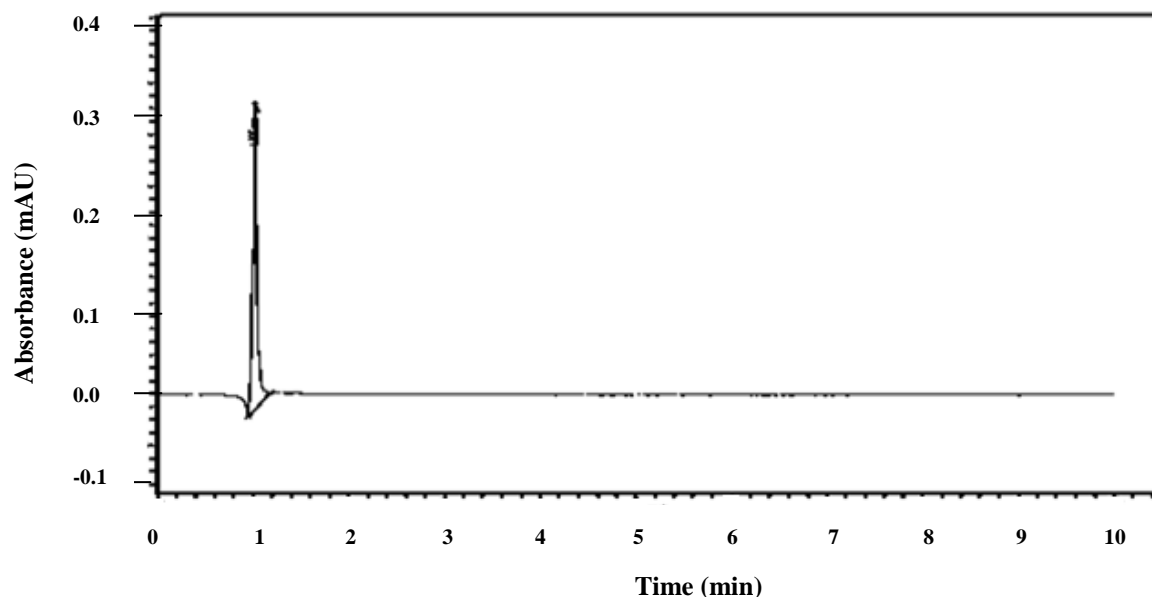


Figure 4.6: HPLC chromatogram for PBS (control) analyzed at a wavelength of 260 nm.

From all the extracts, *K. africana* possessed more compounds capable of permeating the skin. Approximately 19 peaks were detected in the crude organic extract (Figure 4.7A) while 14 were detected in the aqueous extract (Figure 4.8A) before the permeability assay. Agyare et al. (2013) detected eight and nine groups of chemical compounds from *K. africana* methanol bark and leaf extracts respectively. Such discrepancies in the number of groups of compounds detected may be due to the different plant parts analyzed and the extraction solvents used. In both aqueous and organic extracts analyzed in the present study, the compounds permeated in greater quantities after 10 min of exposure of the skin then decreased as the duration of exposure increased to 120 min as seen in Figures 4.7B-D and 4.8B-D and corresponding retention times in Tables 4.5-4.6. About nine peaks permeated the skin from the organic extract after 10 min. The number of peaks then decreased from 60 to 120 min (five peaks detected). For the aqueous extract, some of the compounds also rapidly diffused across the skin with four peaks detected after 10 min. However, the peaks became smaller in size from 60 to 120 min. The permeation behavior of such compounds in *K. africana* aqueous and organic extracts may indicate that the compounds may be small and slightly lipophilic and were able to diffuse rapidly through the stratum corneum. *K. africana* was reported to treat ringworm (De Wet et al., 2013) which is a superficial skin infection that does not require penetration of active compounds through the skin.

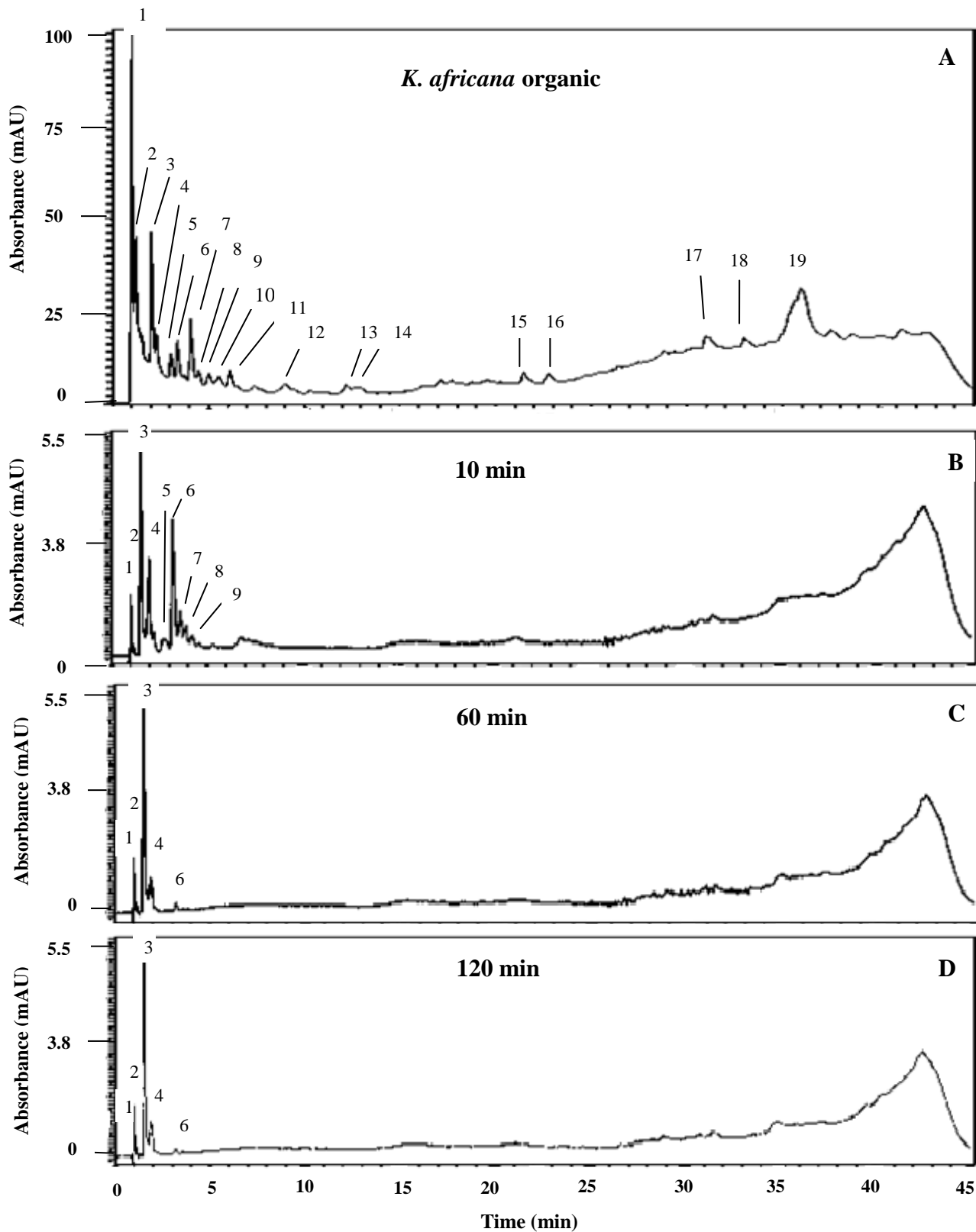


Figure 4.7: Chromatograms of *K. africana* organic extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260

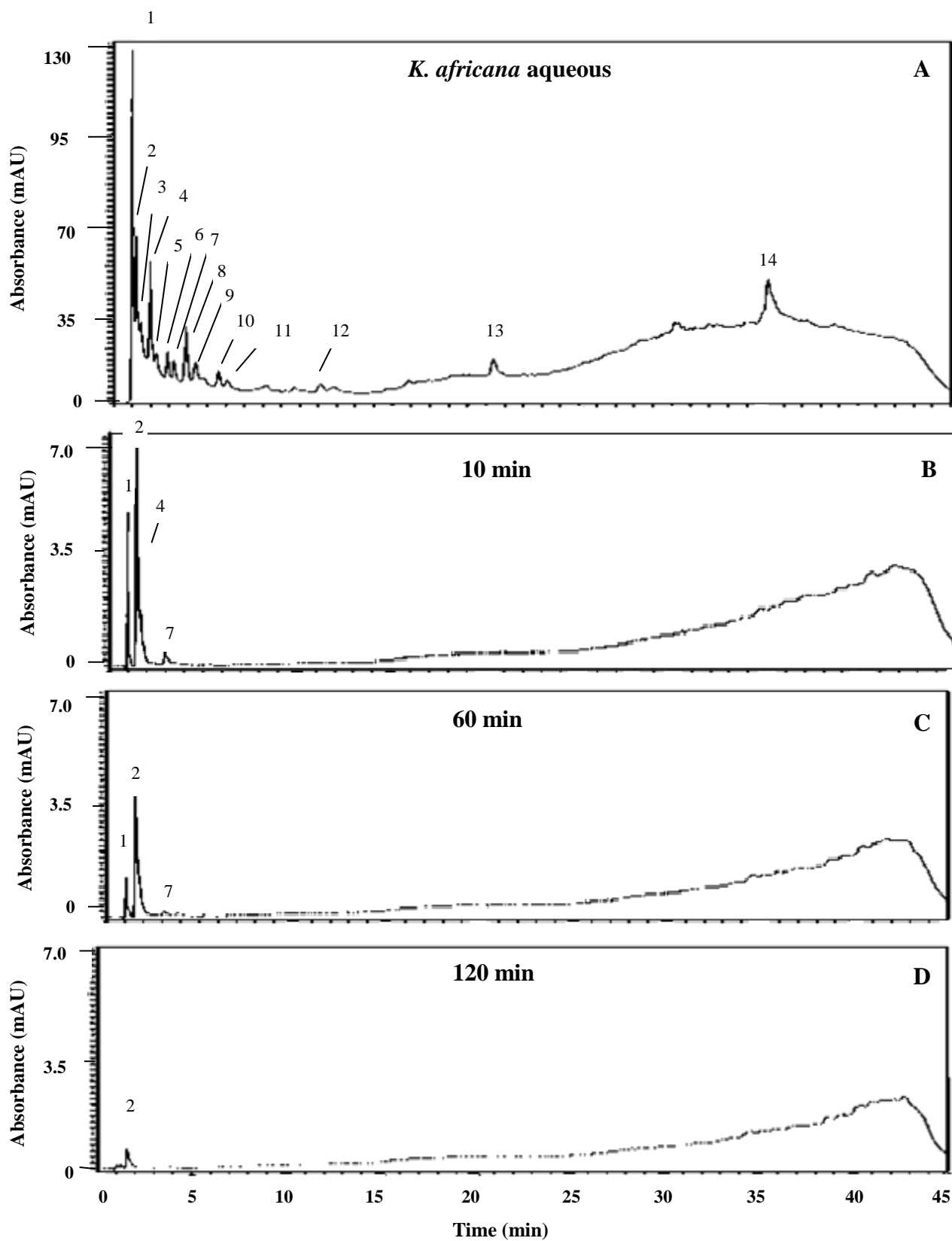


Figure 4.8: Chromatograms of *K. africana* aqueous extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

Table 4.5: Major peaks detected in *K. africana* organic extract (a) prior and (b) after the permeability assay with peak 1-9 detected after 10 min while only peak 1-3, 4 and 6 remained after 60 to 120 min. (a)

Peak number	Retention time
1	0.98
2	1.15
3	1.54
4	2.02
5	2.28
6	3.37
7	3.72
8	4.07
9	4.48
10	5.53
11	6.09
12	9.02
13	12.23
14	12.69
15	21.47
16	22.77
17	30.99
18	31.16
19	35.63

(b)

Peak number	Retention time
1	0.99
2	1.16
3	1.53
4	1.96
5	2.23
6	3.20
7	3.58
8	3.85
9	4.21

Table 4.6: Major peaks detected in *K. africana* aqueous extract (a) prior and (b) after the permeability assay with peak 1, 2, 4 and 7 detected after 10 min while peak 1, 2 and 7 remained up to 60 min and only peak 2 were detected at 120 min.

(a)

Peak number	Retention time
1	0.98
2	1.19
3	1.46
4	1.94
5	2.28
6	2.89

(b)

Peak number	Retention time
1	0.96
2	1.20
4	1.96
7	3.34

Peak number	Retention time
7	3.22
8	3.88
9	4.39
10	5.62
11	6.01
12	11.12
13	20.40
14	35.16

Shaded are the retention times of the group of compounds detected prior to and after the *in vitro* permeability assay. The numbers given to the peaks after the permeability experiment correspond to the peak numbers before the permeability experiment.

Thus, it can be concluded that the plant may be effective in treatment of both deep and superficial skin infections since some compounds were capable of permeating the skin while others may have accumulated within the skin as they could not go through because of their bigger size.

S. cordatum organic extract also possessed some compounds capable of diffusing across the skin. About 22 peaks were detected from the organic extract with 10 peaks detected after 10 min of the permeation assay (Figure 4.9A). The number of peaks detected decreased from 60 to 120 min with only four peaks detected (Figure 4.9B-D). For the aqueous extract (Figure 4.10A), 15 peaks were detected from the crude extract with three (10 min), five (60 min) and one peak (120 min) detected after the *in vitro* permeation assay (Figure 4.10B-D). The diffusion of compounds was rapid from the organic extract being high after 10 min and then decreased as the duration of skin exposure to the extract increased to 120 min. A similar trend was observed for the aqueous extract, with fewer peaks detected after the permeability experiment. Cock and Van Vuuren, (2015) detected approximately nine and 12 chromatogram peaks from the crude methanol and aqueous extracts of *S. cordatum* bark respectively, although the detected compounds were not investigated for their permeability across the skin. In the present study, the diffusion of compounds from *S. cordatum* extracts were analyzed as the plant was reported to treat burns and sores (De Wet et al., 2013). The causes of sores can be numerous and some may result in deep infections affecting the underlying skin layers such as cellulitis or leg ulcers. In such cases, the permeation of active compounds from a drug to the target site is of utmost importance. Thus, the

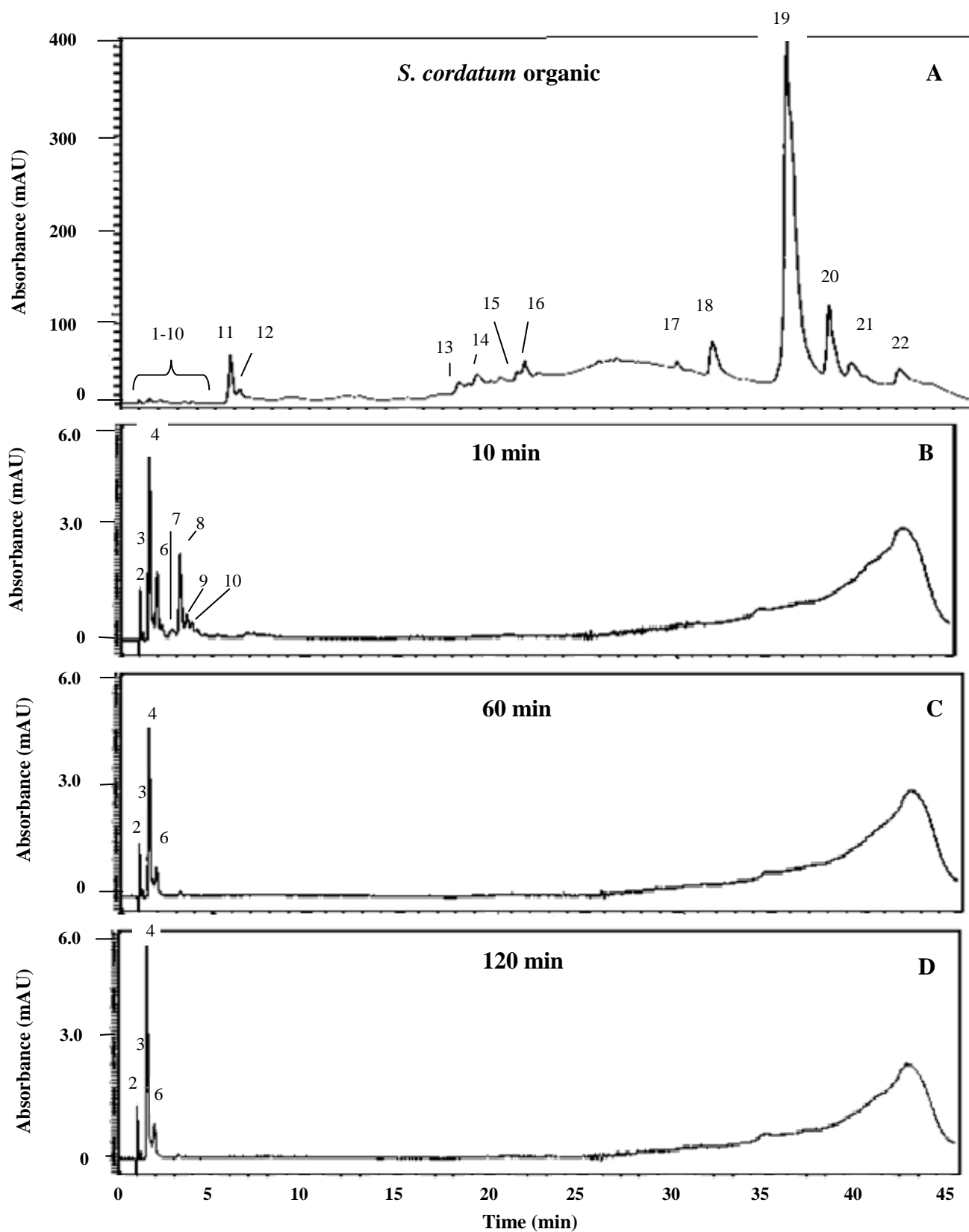


Figure 4.9: Chromatograms of *S. cordatum* organic extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

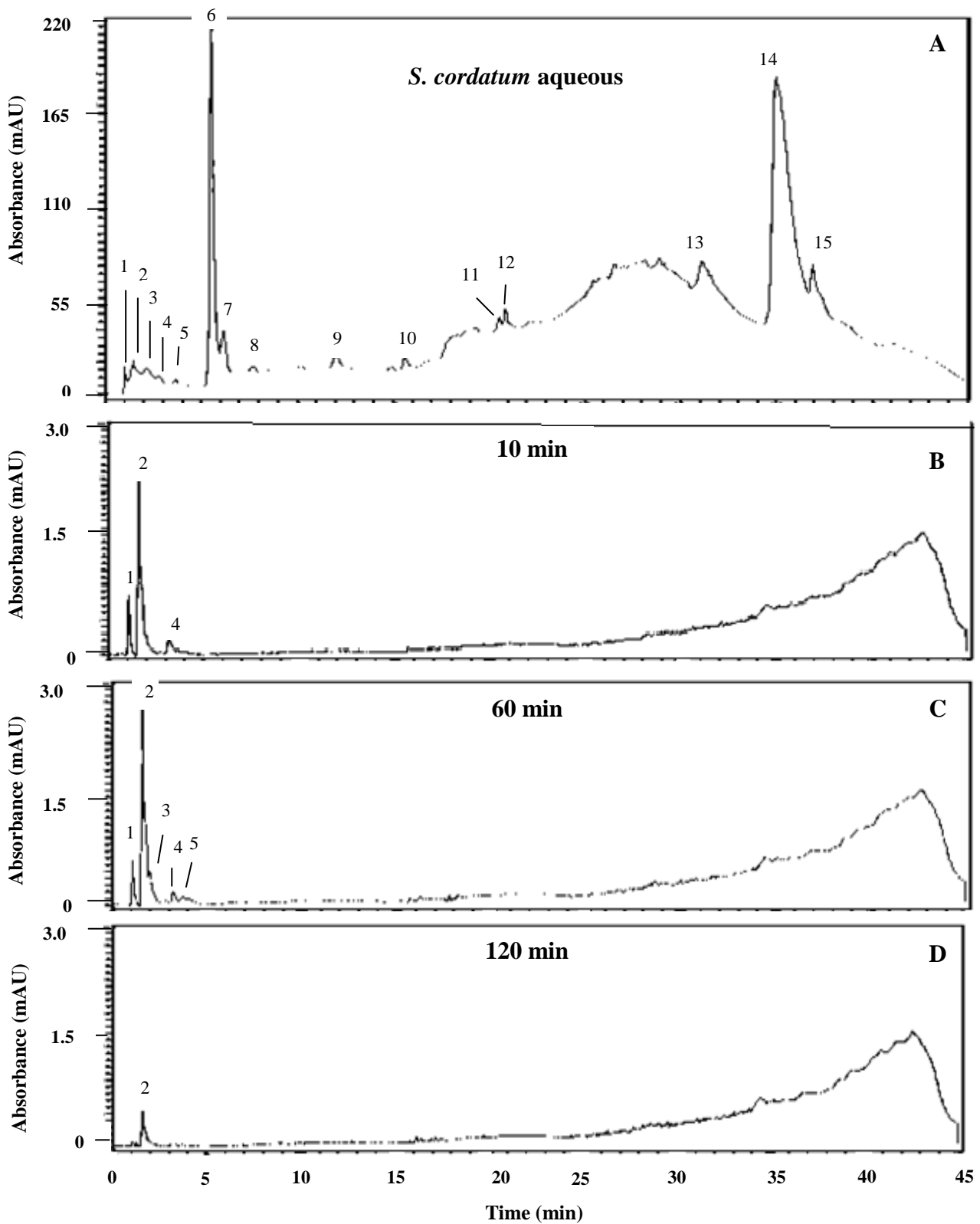


Figure 4.10: Chromatograms of *S. cordatum* aqueous extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

Table 4.7: Major peaks detected in *S. cordatum* organic extract (a) prior and (b) after the permeability assay min) detected after the *in vitro* permeation assay with peak 2-10 detected after 10 min while only peak 2-6 remained after 60 to 120 min.

(a)

Peak number	Retention time
1	0.06
2	0.97
3	1.15
4	1.22
5	1.38
6	1.54
7	2.07
8	2.31
9	3.34
10	3.78
11	5.79
12	6.28
13	17.86
14	18.34
15	20.94
16	21.34
17	29.38
18	31.24
19	35.16
20	37.41
21	38.57
22	41.12

(b)

Peak number	Retention time
2	0.98
3	1.16
4	1.22
6	1.52
7	2.05
8	2.23
9	3.22
10	3.83

Table 4.8: Major peaks detected in *S. cordatum* aqueous extract (a) prior and (b) after the permeability assay with peak 1, 2 and 4 detected after 10 min while peak 1-5 were detected at 60 min and only peak 2 was detected at 120 min.

(a)

Peak number	Retention time
1	0.98
2	1.18

(b)

Peak number	Retention time
1	1.02
2	1.21
3	1.99
4	3.15
5	3.67

Peak number	Retention time
3	2.18
4	3.35
5	3.67
6	5.51
7	6.14
8	7.70
9	12.30
10	15.62
11	20.55
12	20.86
13	31.36
14	34.99
15	36.90

Shaded are the retention times of the group of compounds detected prior and after the *in vitro* permeability assay. The numbers given to the peaks after the permeability experiment correspond to the peak numbers before the permeability experiment.

permeation profile of the compounds from *S. cordatum* organic extract was in congruent with one of its traditional uses (the treatment of sores).

For *G. livingstonei* organic (Figure 4.11A) and aqueous (Figure 4.12A) extracts, few compounds diffused across the skin even though the organic extract of the plant was found to have broad-spectrum antimicrobial activity 0.27 mg/ml when compared to the other extracts. More groups of compounds were also detected from *G. livingstonei* organic extract under phytochemical screening compared to the other extracts. Only four groups of compounds (after 10, 60 and 120 min) from the 34 compounds detected from the crude organic extract permeated the skin. In the aqueous extract, six (10 min), three (60 min) and two (120 min) compounds from the 28 compounds detected in crude extract permeated across the skin. The diffusion of such compounds, however, might correlate with the traditional use of *G. livingstonei* since the plant was reported to treat burns. In most cases, burn wounds usually affect the top epidermal layers of the skin. Third degree burns that affect all layers of the skin are usually rare. *G. livingstonei* was reported to treat superficial burn wounds and associated secondary bacterial infections. In such cases the accumulation of chemical compounds within the skin will be necessary.

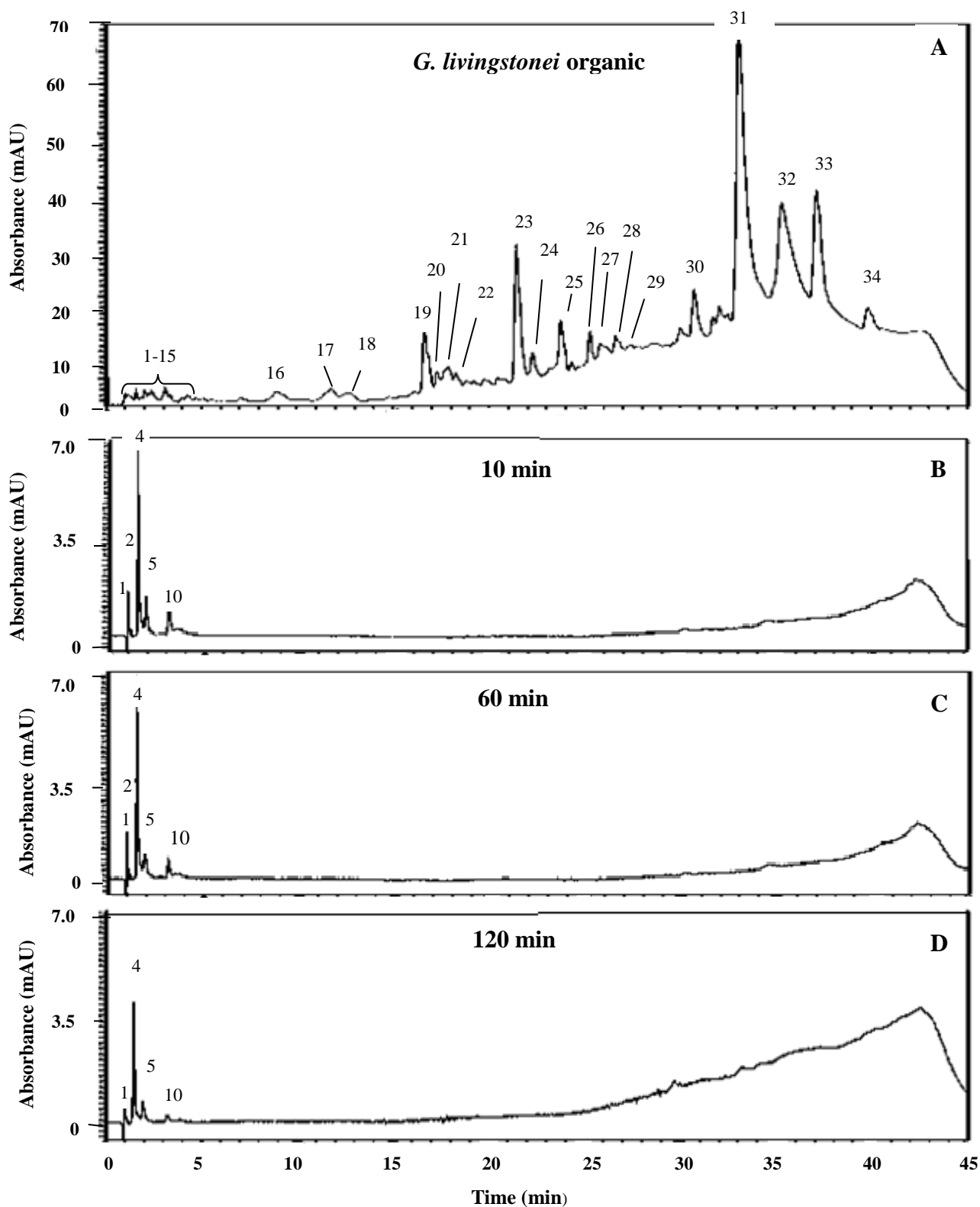


Figure 4.11: Chromatograms of *G. livingstonei* organic extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

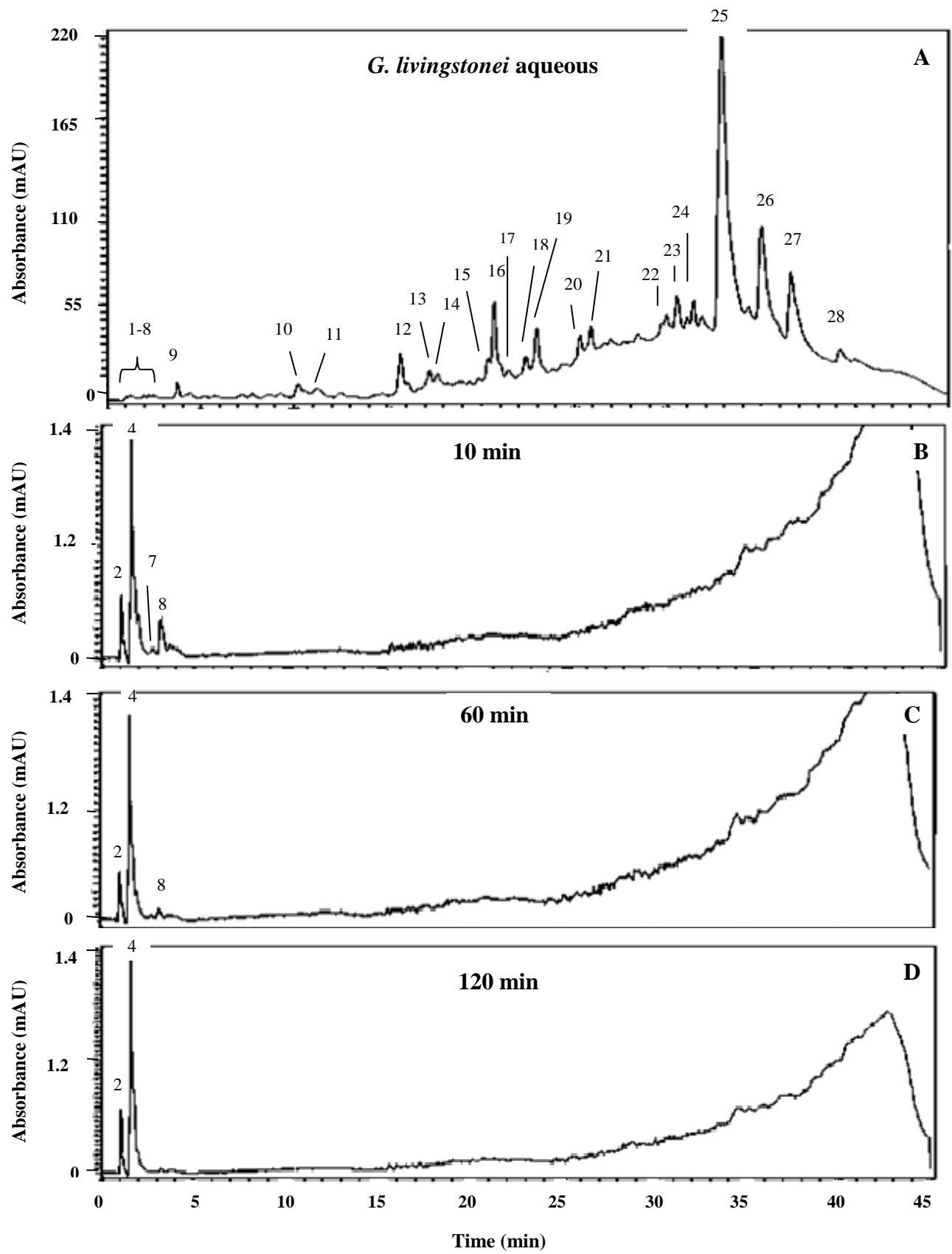


Figure 4.12: Chromatograms of *G. livingstonei* aqueous extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

Table 4.9: Major peaks detected in *G. livingstonei* organic extract (a) prior and (b) after the permeability assay with peak 1,2,4,5 and 10 detected at 10 up to 120 min.

(a)

Peak number	Retention time
1	0.97
2	1.09
3	1.23
4	1.55
5	1.84
6	2.00
7	2.22
8	2.37
9	3.08
10	3.34
11	3.97
12	4.28
13	4.70
14	5.06
15	5.50
16	8.92
17	11.75
18	12.75
19	16.64
20	17.28
21	17.78
22	18.26
23	22.27
24	23.75
25	24.31
26	25.28
27	25.82
28	26.63
29	29.64
30	30.72
31	33.05
32	35.30

(b)

Peak number	Retention time
1	0.98
3	1.17
4	1.49
5	1.87
10	3.36

Peak number	Retention time
33	37.09
34	39.79

Table 4.10: Major peaks detected in *G. livingstonei* aqueous extract (a) prior and (b) after the permeability assay with peaks 2,4,7 and 8 detected after 10 min while peaks 2,4 and 8 remained at 60 min and only peaks 2 and was detected at 120 min.

(a)

Peak number	Retention time
1	0.93
2	1.06
3	1.20
4	1.63
5	1.95
6	2.46
7	2.60
8	3.24
9	3.75
10	10.20
11	11.23
12	15.68
13	17.22
14	17.60
15	20.38
16	20.69
17	21.46
18	22.38
19	22.96
20	24.56
21	25.28
22	29.67
23	30.16
24	31.36
25	32.85
26	34.99

(b)

Peak number	Retention time
2	1.05
4	1.57
7	2.60
8	3.15

Peak number	Retention time
27	36.57
28	39.21

Shaded are the retention times of the group of compounds detected prior and after the *in vitro* permeability assay. The numbers given to the peaks after the permeability experiment correspond to the peak numbers before the permeability experiment.

S. birrea was expected to have more compounds capable of permeating the skin because it was reported to treat deep skin infections (boils and sores) (De Wet et al., 2013). The penetration of compounds across the skin is important so as to reach the target site and exert their effects. For the organic extract, 22 groups of compounds were observed in the crude extract, however, only six and five were detected after 10 and 60 to 120 min of the permeability assay (Figure 4.13A-D). In the aqueous extract (Figure 4.14A-D) where 19 compounds were observed before the permeability experiment only four compounds were detected after 10 to 60 min which then decreased (three peaks) as the duration increased to 120 min. The permeation behavior of compounds from *S. birrea* extracts may indicate that the permeated compounds were relatively small and permeated across the skin easily compared to the larger compounds detected prior the permeation assay. The larger compounds that did not diffuse through the skin (not detected) might accumulate within the skin thus exerting their effect locally within the skin.

Some compounds also permeated rapidly across the skin within 10 min from organic and aqueous extracts of a combination of *S. birrea* and *S. cordatum* (Figure 4.15-4.16). About nine peaks were detected after 10 min from approximately 21 peaks observed from the crude organic extract before the permeation assay (Figure 4.15A-B). The peaks then decreased at 60 min (eight peaks) and increased at 120 min (nine peaks) (Figure 4.15C-D). For the aqueous extract, six peaks diffused across the skin after 10 min [which then decreased at 60 (five peaks) and 120 min (four peaks)] from approximately 17 peaks detected from the crude extract (Figure 4.16A-D). The permeation behavior of the compounds from the combined extracts particularly the combined organic extracts resembled that of *S. cordatum* organic extract. Thus, it might be concluded that *S. cordatum* compounds are capable of permeating to a greater extent across the skin compared to *S. birrea* compounds.

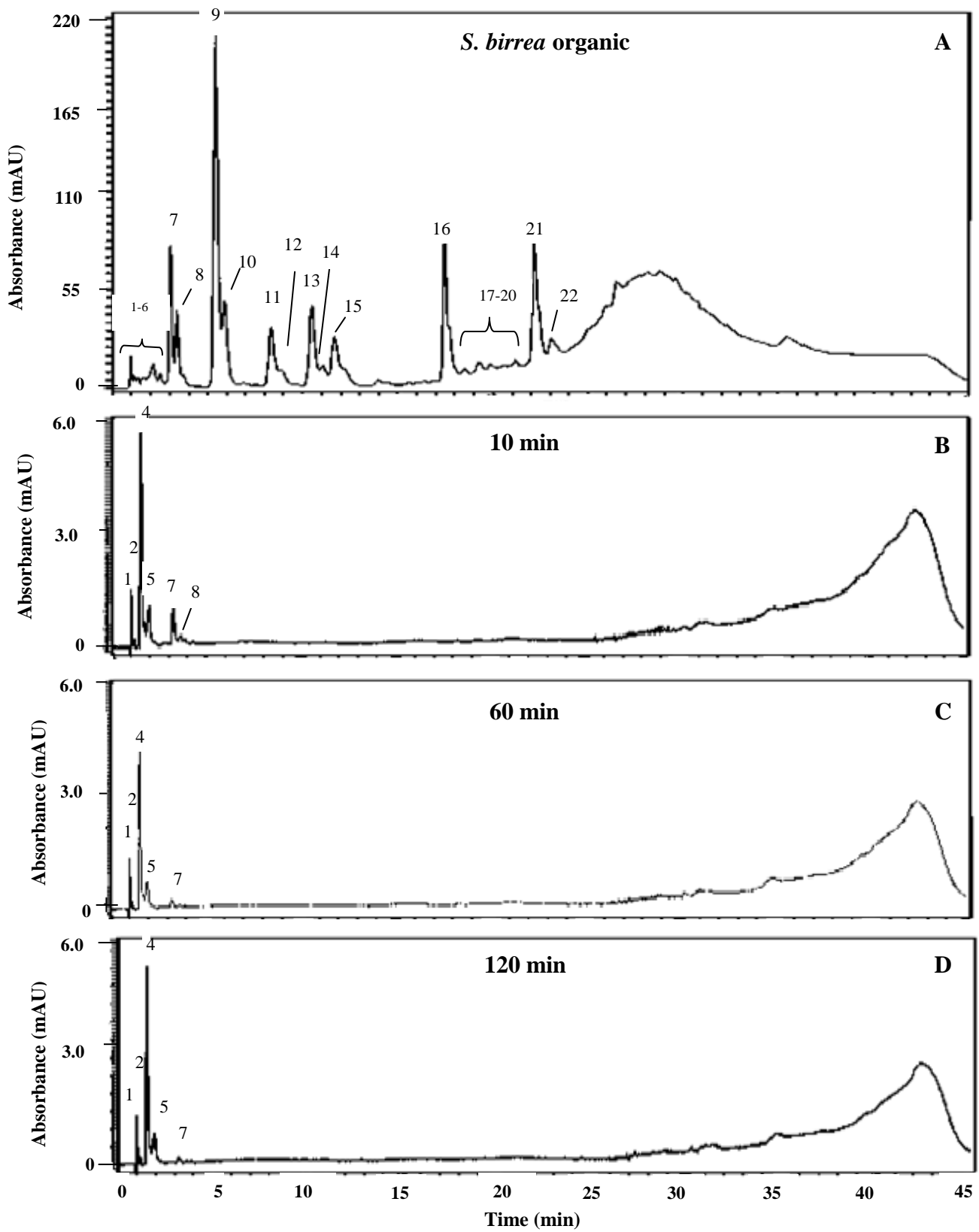


Figure 4.13: Chromatograms of *S. birrea* organic extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

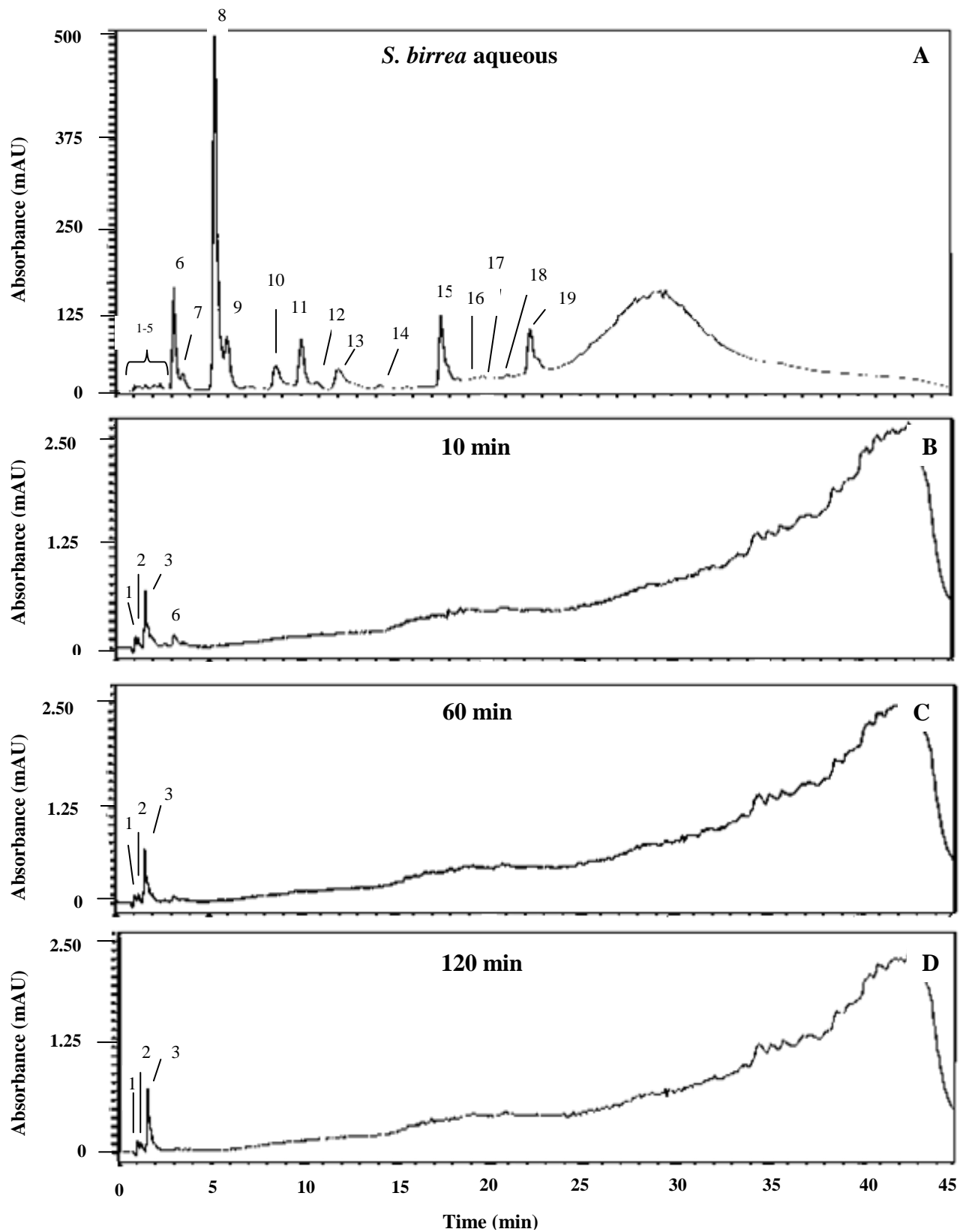


Figure 4.14: Chromatograms of *S. birrea* aqueous extract prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

Table 4.11: Major peaks detected in *S. birrea* organic extract (a) prior and (b) after the permeability assay with peak 1,2,4,5,7 and 8 detected at 10 up to 120 min.

(a)

Peak number	Retention time
1	0.96
2	1.13
3	1.31
4	1.52
5	2.13
6	2.49
7	3.04
8	3.39
9	5.41
10	5.88
11	8.33
12	8.87
13	10.47
14	11.07
15	11.65
16	17.44
17	18.48
18	19.25
19	19.54
20	21.17
21	22.17
22	23.07

(b)

Peak number	Retention time
1	0.99
2	1.17
4	1.52
5	1.95
7	3.22
8	3.63

Table 4.12: Major peaks detected in *S. birrea* aqueous extract (a) prior and (b) after the permeability assay with peaks 1-3 and 6 detected after 10 min while peaks 1-3 remained after 60 to 120 min.

(a)

Peak number	Retention time
1	0.97
2	1.17
3	1.61
4	1.89
5	2.37
6	3.13
7	3.62
8	5.33
9	5.99
10	8.62
11	9.99
12	10.83
13	11.99
14	14.25
15	17.52
16	18.45
17	19.79
18	21.08
19	22.13

(b)

Peak number	Retention time
1	1.02
2	1.20
3	1.56
6	3.11

Shaded are the retention times of the group of compounds detected prior and after the *in vitro* permeability assay. The numbers given to the peaks after the permeability experiment correspond to the peak numbers before the permeability experiment.

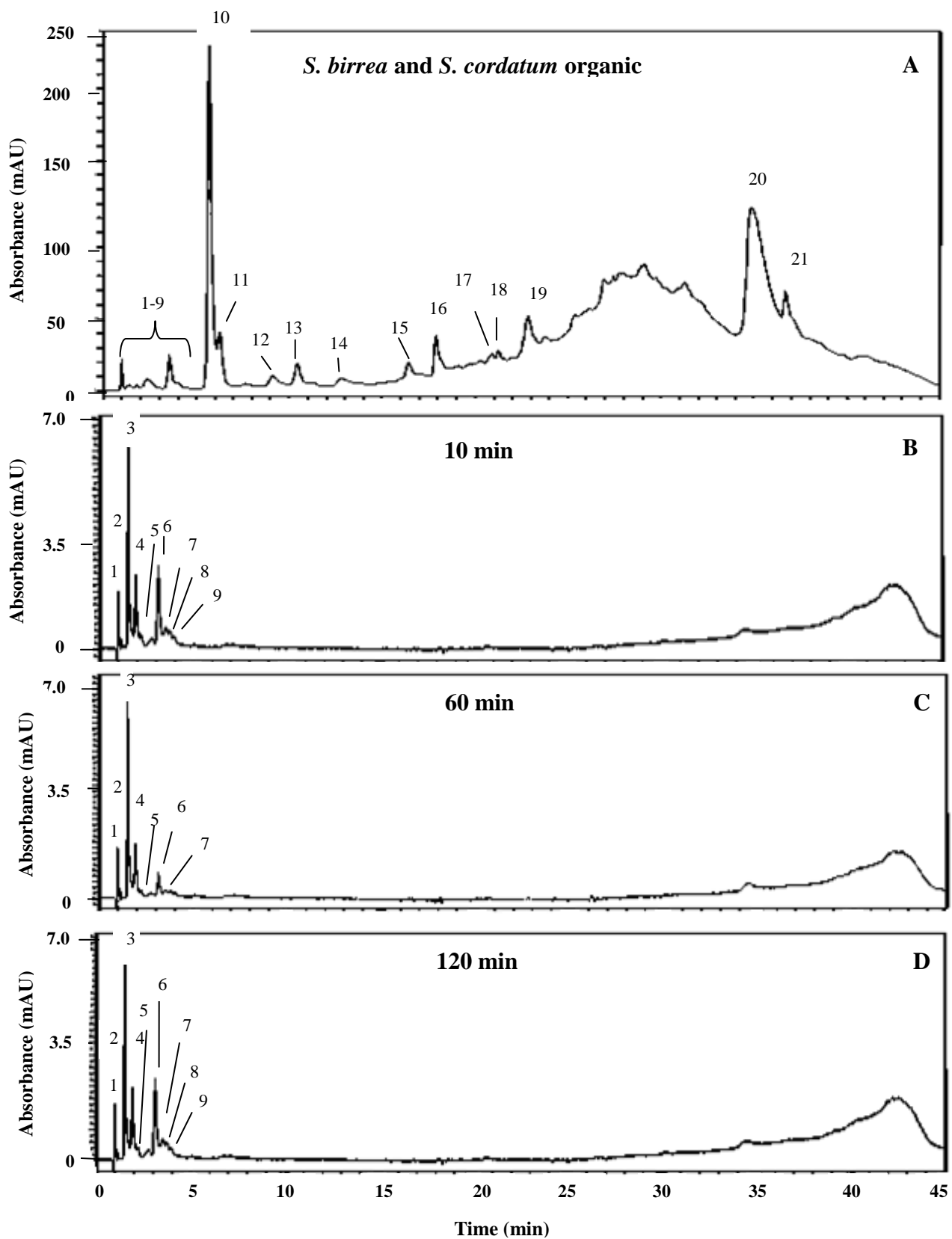


Figure 4.15: Chromatograms of *S. birrea* in combination with *S. cordatum* organic extracts prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

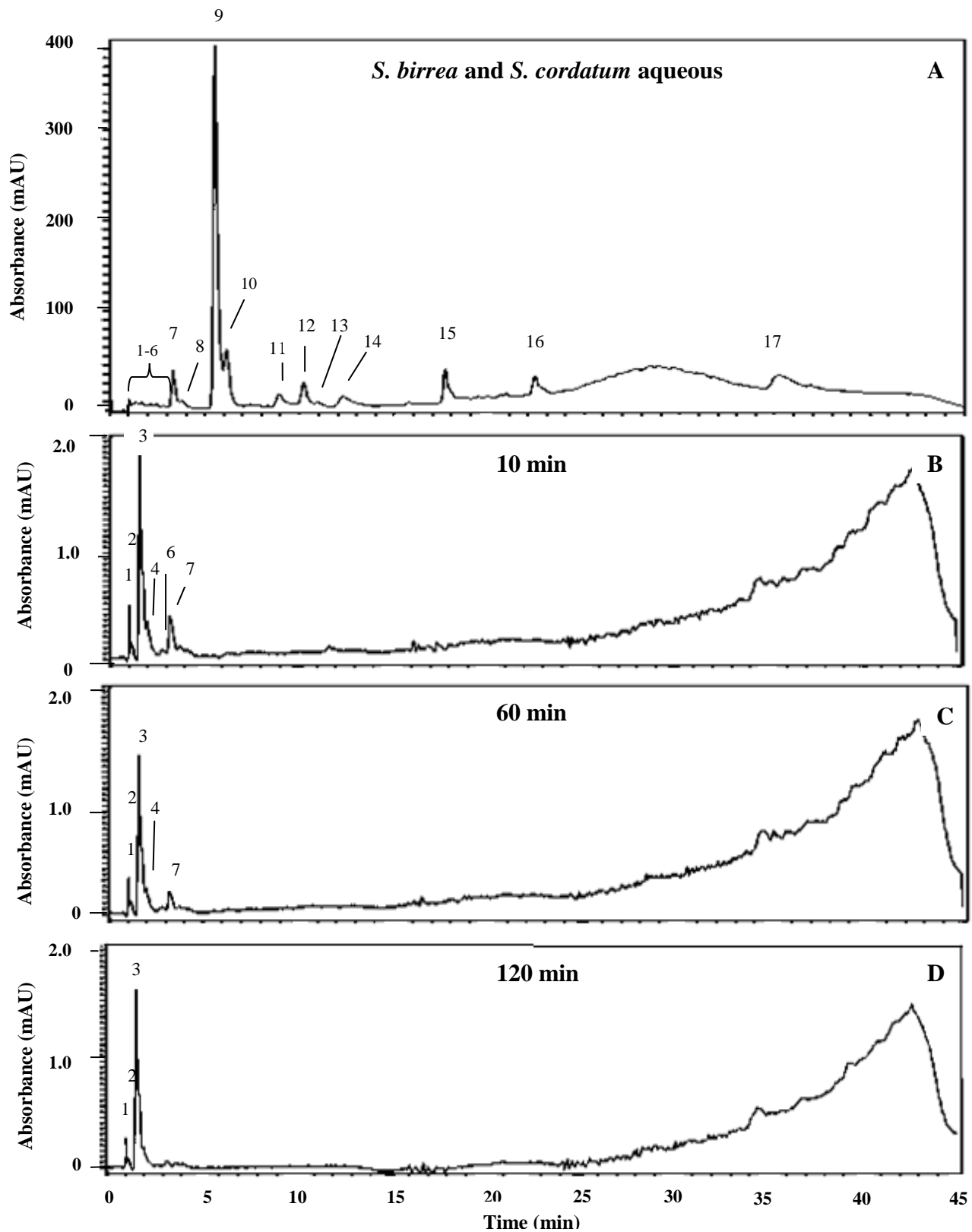


Figure 4.16 Chromatograms of *S. birrea* in combination with *S. cordatum* aqueous extracts prior (A) and after (B-D) the permeability assay at different time intervals (10, 60 and 120 min) of exposure to the skin at 260 nm.

Table 4.13: Major peaks detected in *S. birrea* in combination with *S. cordatum* organic extract (a) prior and (b) after the permeability assay with peaks 1-9 detected at 10 min, peaks 1-7 detected at 60 min while peaks 1-9 were detected at 120 min.

(a)

Peak number	Retention time
1	0.97
2	1.16
3	1.27
4	1.39
5	1.79
6	2.35
7	2.54
8	3.55
9	4.00
10	5.68
11	6.25
12	9.11
13	10.43
14	12.79
15	16.42
16	17.89
17	20.29
18	20.91
19	22.84
20	23.78
21	34.86
22	36.71

(b)

Peak number	Retention time
1	0.98
2	1.18
3	1.30
4	1.50
5	1.92
6	2.22
7	2.76
8	3.51
9	3.76

Table 4.14: Major peaks detected in *S. birrea* in combination with *S. cordatum* aqueous extract (a) prior and (b) after the permeability assay with peaks 1-3,4 and 6-7 detected after 10 min while peaks 1-3,4 and 7 remained at 60 min and only peaks 1-3 were detected at 120 min.

(a)

Peak number	Retention time
1	0.98
2	1.18
3	1.37
4	1.69
5	2.05
6	2.83
7	3.34
8	3.79
9	5.53
10	6.14
11	8.92
12	10.20
13	10.97
14	12.29
15	17.64
16	22.37
17	35.24

(b)

Peak number	Retention time
1	1.06
2	1.20
3	1.57
5	1.97
6	2.75
7	3.19

Shaded are the retention times of the group of compounds detected prior and after the *in vitro* permeability assay. The numbers given to the peaks after the permeability experiment correspond to the peak numbers before the permeability experiment.

All the investigated plant extracts and the combined extracts of *S. birrea* and *S. cordatum* displayed a similar pattern in their permeation behavior through the skin. The chemical fingerprints of all the extracts prior to the permeability experiments were very complex. However, only relatively hydrophilic compounds eluting within 5 min from the RP-HPLC column were detected after a 10 min exposure time of the intact porcine skin to the various extracts. The permeation behaviour of the compounds from all the extracts indicated that they were most probably small, have a balance between hydrophilicity and lipophilicity and were able to diffuse rapidly through the intact stratum corneum. The predominantly lipophilic compounds

observed within the crude extracts most probably accumulated within the intact skin due to their size as well as their affinity for the lipophilic stratum corneum. Usually, lipophilicity is among the physicochemical properties that determine the diffusion rate of drug molecules through the skin. As stated in Section 4.1, the stratum corneum is lipophilic, and drug molecules have to be lipophilic to be able to partition within the lipid lamellae of the corneocytes (Naik et al., 2000). Permeation through the intact skin will depend on a balance between the hydrophilicity and the lipophilicity of the compound diffusing through the skin to give maximum skin penetration. The rate of diffusion of compounds through intact skin also decreases with increasing molecular weight or size (Jakasa et al., 2007).

In intact skin, the stratum corneum is the main barrier to hydrophilic compounds and facilitates the movement of lipophilic compounds. Because these crude extracts are used under conditions where a compromised skin barrier is present e.g. dermatitis, psoriasis, burns, infections and inflammation, permeation behaviour of compounds will be altered (Davies et al., 2015). Previous studies have indicated that diffusion of hydrophilic and amphipathic compounds increases through a compromised barrier of the skin, for example where there are abnormalities in the lipids of the stratum corneum, as is the case with psoriasis, dermatitis and UV irradiation (Tsai et al., 2001). The epidermal and dermal layers of the skin are hydrophilic and it was shown by a previous study that the viable epidermis also provides a rate limiting step to the diffusion of compounds in the event that the stratum corneum becomes diseased, damaged or absent, as is the case with burn wounds (Andrews et al., 2013). When the barrier function of the skin is compromised, the rate of diffusion of most molecules will increase, including compounds of larger molecular size, however, depending on their physicochemical properties such as their lipophilicity. In the case of a compromised barrier function, it can thus be assumed that the compounds that were found diffusing through the intact skin within 10 min from these extracts would diffuse at a much faster rate. The larger and presumably quite lipophilic compounds present in these extracts that did not diffuse through intact skin even after 120 min, would be expected to penetrate the compromised skin to a far larger extent. However, the intact skin surrounding the areas where the skin barrier function is compromised such as burn wounds, could possibly act as a reservoir where active compounds may accumulate after topical application to the area, thus extending and enhancing the pharmacological effect such as the antimicrobial effect at the compromised site.

From the preliminary phytochemical analyses, flavonoids, saponins, anthraquinones and tannins were predominantly present in all the extracts analysed. Thus, the *in vitro* diffusion of these groups of compounds across the intact skin would largely depend on their physicochemical properties. Flavonoids possess numerous hydroxyl groups which render the molecule hydrophilic with low stability and solubility in lipophilic media (Danilova et al., 2012). In addition, the hydroxyl groups in flavonoids are highly ionizable (Herrero-Martinez et al., 2005). The ionization state of a drug molecule also determines its permeation rate through the skin with non-ionized compounds permeating faster than the ionized compounds. In the current study, the permeability experiments were conducted at a physiological pH of 7.4. Flavonoids are weak acids and their pKa values usually vary between 7.3 and 12.5 since they possess phenyl hydroxyl groups (Liang et al., 1997). It has been reported that, flavonoids with a vicinal-trihydroxyl functional group are unstable at pH 7.4 (Yao et al., 2013) while the 3,4,-hydroxyflavonoids are negatively charged at pH 7 (Jovanoic et al., 1994). Furthermore, the presence of keratin in the stratum corneum presents both negatively and positively charged groups that provide an effective barrier against charged molecules (Bartosova and Bajgar, 2012). Thus, it might be concluded that the flavonoids were not amongst the major groups of compounds that permeated rapidly through the skin. Other studies also found flavonoids to permeate the skin at a very low rate with their permeability usually improved using penetration enhancers (Bonina et al., 1996; Saija et al., 1998; Artc et al., 2002; Vicentini et al., 2008). Dvorakova et al. (1999) found a flavonoid, epigallocatechin gallate (EGCG) impermeable across the human skin *in vitro* with low penetration observed in mouse skin. Dal Belo et al. (2009) also found EGCG to accumulate in the stratum corneum of the human skin and quercetin (another flavonoid) to permeate the stratum corneum but remain in the viable epidermis. When the skin barrier function however becomes compromised, the penetration of flavonoids is expected to be much greater and at a faster rate because the stratum corneum, which is the main barrier to compounds in intact skin, in some cases may be absent (such as in burn wounds). In the case of skin infection being present, these agents could act on the microorganisms much more rapidly resulting in an antimicrobial effect.

Saponins contain both lipophilic (sapogenin) and hydrophilic (sugar) properties (Shi et al., 2004). It has been reported that a good candidate drug for topical application must possess both lipophilic and hydrophilic properties to be adequately soluble in both lipophilic (stratum corneum) and hydrophilic (dermis) layers of the skin. Because of their amphipathic nature, it can

possibly be speculated that saponins might have been among the major compounds that permeated the skin as they were also found to be predominant in all the extracts. Pino et al. (2012) investigated the transdermal permeation of the macromolecules avicins which belong to the family of saponins where high permeability was observed. It was then concluded that, both the hydrophilic and hydrophobic portions act synergistically in facilitating the penetration of saponins molecules across the skin lipids. In the case of a compromised skin barrier, these agents are expected to diffuse at a higher rate into the diseased skin tissue exerting a much more rapid pharmacological effect.

The basic chemical structure of all anthraquinones is the merger of three benzene rings (Gessler et al., 2013). Drugs with benzene rings in their structures are usually lipophilic and diffuse easily through the stratum corneum of the skin. Thus, anthraquinones were also assumed to be present in the major compounds that were capable of permeating more rapidly across the skin. The anthraquinones derivatives, danthron and dianthrone were also found to be capable of permeating the skin *in vitro*, even though the permeation rate was higher in a diseased skin than in a normal skin (Wang et al., 1987).

Tannins are polyphenolic compounds with a molecular mass extending from 300 – 3000 (Khanbabaee and Van Ree, 2001; Ashok and Upadhyaya, 2012). These molecules contain a large number of hydroxyl groups rendering them water soluble. Tannins have astringent properties, thus dehydrate the skin, bind and precipitate proteins (Ashok and Upadhyaya, 2012). These compounds are known to form a protective layer over compromised skin and thus as such are not expected to penetrate through intact skin, but rather to accumulate within the skin layers (Ashok and Upadhyaya, 2012). It would thus be assumed that tannins will penetrate further within compromised skin tissue. However, due to the astringent properties and the ability to bind proteins, tannins would have a strong local effect due to the accumulation within compromised skin layers. These agents are then assumed to exert a local effect due to the formation of a reservoir within the skin.

The presence of alkaloids, coumarins, flavones and sterols was also indicated in some of the crude extracts. Alkaloids are a diverse group of chemicals found in plants classified as organic nitrogenous bases (Fester, 2010) of which many show good skin penetration (Chan, 2012).

Alkaloids present in some of the tested extracts are thus also assumed to have diffused rapidly through intact skin. Coumarin is a fragrant compound that occurs naturally in some plants and can be used as an anti-inflammatory or anticancer agent. It penetrates the skin well and is thus also expected to be one of the compounds that penetrated rapidly across intact skin (Beckley-Kartey et al., 1997). Plant sterols are similar to cholesterol in structure and when consumed orally can eventually transfer to the skin where they form part of the surface lipids. These compounds are thus not expected to permeate across the skin but rather to accumulate within the skin layers, hydrating the skin and causing anti-inflammatory effects (Gustav Parmentier GmbH, 2014). The plant sterols are thus not expected to have diffused across the skin after the exposure of the intact skin to the organic extracts in which they were originally detected. Flavones form part of the flavonoids which were described above. As mentioned before, all of these compounds would also have penetrated much more rapidly through compromised skin resulting in a more rapid therapeutic effect.

4.4 Summary

- The preliminary phytochemical screening results revealed the predominant presence of flavonoids, saponins, anthraquinones and saponins from all the tested plant extracts.
- All the extracts gave the first maximum absorption peaks between 212-218 nm under the UV-Vis spectrometry. The second absorption maxima occurred between 250-268 nm from all the extracts. *G. livingstonei* extracts gave another absorption peak at 310 nm for the aqueous extract and at 330 nm for the organic extract. The third absorption maximum peak was also observed from the organic extract of *S. birrea* at 362 nm.
- All the extracts displayed a similar pattern in their permeation behavior across the intact porcine skin with major peaks being eluted within a retention time of 5 min from the HPLC column and other compounds being eluted in small amounts after 15 min retention time. The number of peaks detected in the organic extracts was greater than in the aqueous extracts.
- The extracts of *K. africana* and *S. cordatum* proved to have more compounds capable of permeating the skin compared to than other extracts. The results demonstrated that lipophilicity, molecular weight and particle size largely influence the permeability rate of compounds across the skin.

Chapter five: General conclusions and further recommendations

5.1 Thesis summary

Medicinal plants are abundant in northern Maputaland and are frequently used by the rural inhabitants for the treatment of various ailments. The special focus of this study was to validate the traditional use of plants by the lay people of Maputaland to treat skin diseases. Thirty six plants documented in an ethnobotanical study conducted in four rural communities of Maputaland were considered for investigation in order to support their ethnobotanical claims. The 36 plants extracted with water and dichloromethane: methanol were first investigated for their antimicrobial activity against dermatological relevant pathogens. The individual plants were then combined into eight combinations as used by the lay people of northern Maputaland for the treatment of skin ailments. Six combinations were further investigated for their interactions when combined in various ratios. Four of the most antimicrobial active plants and a combination of *S. birrea* and *S. cordatum* which were reported to be used topically were further investigated for their chemical profiles and the group of chemical compounds that are capable of permeating the skin.

5.1.1 Antimicrobial validation of individual plants

When testing the antimicrobial activity of individual plants, it was found that the dichloromethane: methanol extracts have higher inhibitory effect with 31.7% of the extracts showing broad-spectrum antimicrobial activity (mean MIC < 1.00 mg/ml). Plants that demonstrated broad-spectrum activity included *A. senegalensis*, *E. elephantina*, *G. livingstonei*, *K. africana*, *O. engleri*, *P. capensis* subsp. *capensis*, *S. brachypetala*, *S. birrea*, *S. cordatum*, *T. sericea*, and *Z. capense*. The aqueous extracts displayed less activity when compared to the organic extracts. Only 4.9% displayed broad-spectrum activity with a few (25) displaying pathogen specific activity. When analyzing the efficacy of the frequently used plant species, it was concluded that the commonly used plants do not necessarily give higher antimicrobial efficacy. For instance, *S. serratuloides* which was the most mentioned plant by the lay people of Maputaland was least active in terms of antimicrobial activity. However, more than 80% of the

organic extracts displayed a correlation between antimicrobial efficacy and their traditional use which indicates that some of the plants have a potential in the management of skin infections, as claimed by rural inhabitants of northern Maputaland.

5.1.2 Antimicrobial interactions of combined plants

The aqueous and organic extracts of the eight plant combinations mostly displayed additive interactions when investigated against *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Notable synergistic interaction from the combination of *A. burkei* with *K. africana* (aqueous and organic extracts) against *T. mentagrophytes* gave some validation to the reported traditional use of this combination against ringworm infections. When combined further into various ratios, the effect of the three selected combinations predominantly displayed additive interactions with some of the ratio points giving synergistic interactions. This indicated that the interaction between these plants vary depending on the ratio in which they were mixed. However, the organic combination of *H. hemerocallidea* and *S. rigescens* displayed noteworthy synergistic interactions against *S. epidermidis* irrespective of the ratio in which the two plants were combined.

5.1.3 Chemical and permeability profiles of the most active topically used plants

Results observed from the preliminary phytochemical screening indicated that all the four plants (*G. livingstonei*, *K. africana*, *S. birrea* and *S. cordatum*) tested possess anthraquinones, flavonoids, tannins and saponins. Even though the detected compounds may differ in their modes of action when targeting microbial cells, they all possess antimicrobial activity which may account for the broad-spectrum antimicrobial activity observed from the organic extracts of the tested plants. Based on the UV-Vis spectrometry, all the extracts indicated absorption peaks between 212 and 294 nm. The obtained absorption peaks for the plant extracts were characteristic of the phytochemicals determined in the preliminary phytochemical screening as they possess specific structures and functional groups that absorb at specific regions under UV-Vis spectrometry. The HPLC profiles of the extracts prior to the permeability experiments were very complex indicating the presence of a large number of chemical compounds in the extracts. The permeability pattern of some of the group of compounds across the skin provided some basic scientific insight on the pharmacokinetics (diffusion across porcine skin) of compounds present in medicinal plants intended for topical application.

5.2 Future recommendations

Even though the traditionally used plants are perceived to have less side effects when compared to the conventional drugs (Saikia et al., 2006), some studies have reported toxic effects associated with many plants used as traditional medicine (Fennell et al., 2004). Plants used to treat skin diseases have been associated with allergic reactions, dermatitis and photosensitization to mention a few (Mabona and Van Vuuren, 2013). Even some of the plant species investigated in the current study have been reported to possess cytotoxicity, mutagenic, irritation and even lethal effects (Table 5.1). Of concern is that these plant species are used on regular basis by the rural inhabitants of northern Maputaland and the current study has only provided the *in vitro* evidence of the antimicrobial properties of the documented plants. Thus, toxicity studies should be carried out on plants with unknown toxicity information and those with reported toxicity properties should be used with caution. Furthermore, for combination studies, the plants with toxic effects should be incorporated with non-toxic plants to determine if toxicity will be reduced to a more safe level.

Some of the investigated plants such as *S. panduriforme*, *S. spinosa* and *R. multifidus* were reported to treat warts and shingles by the lay people of Maputaland. These plant species displayed poor activity when investigated against the selected pathogens. Warts and shingles are caused by the human papillomavirus and varicella-zoster virus respectively. These viruses usually affect the immune-compromised individually due to HIV infection. The KwaZulu-Natal province has a high rate of HIV infection and the lay people of Maputaland believe that warts and shingles can only be treated with traditional medicine (De Wet et al., 2013). Thus, a future recommendation is to examine these plants against the associated viral pathogens.

Even though the therapeutic effect of medicinal plants can be identified *in vitro*, it is necessary to further investigate these plants *in vivo* to validate their *in vitro* efficacy. It should be considered that the *in vitro* results may not always correlate with the *in vivo* activity. Thus, both the active and least active plant extracts should be taken to the *in vivo* model as it might happen that the extract which demonstrated weak activity *in vitro* may become active when subjected to an

Table 5.1: Toxicity reports of plants used to treat skin diseases in northern Maputaland.

Plant species	Toxicity reports	References
<i>A. adianthifolia</i>	The aqueous and organic extracts were found to be non-toxic when tested in the MTT (3-[4, 5-dimethyl-2-thiazol-yl]-2, 5-diphenyl-2H-tetrazolium bromide) cellular viability assay.	Naidoo et al., 2013.
<i>A. burkei</i>	No evidence found.	
<i>A. senegalensis</i>	Varying degrees of depression, weakness, droopiness, intestine lesions and death were observed from aqueous stem bark extracts after parenteral administration to chicks.	Nwosu et al., 2011.
	The root bark extracts were found to be safe when orally administered to mice at lower doses; however, caution is warranted when taken at higher doses.	Okoye et al., 2012.
<i>A. vesicolor</i>	Pods tested toxic when investigated in guinea pigs.	Gummow and Erasmus, 1990.
	Severe nervous toxicity effects were observed from the sheep after being drenched with pod-material.	Gummow et al., 1992.
	Clinical signs such as hyperaesthesia, wild running, nystagmus, rapid blinking and death occurred in sheep and goats after ingestion of ripe dry pods.	Soldan et al., 1996.
<i>B. discolor</i>	Non-toxic or had low toxicity with marine C2C12 myoblasts, preadipocytes and human liver cells.	Van de Venter et al., 2008.
<i>C. inerme</i>	No evidence found.	

Plant species	Toxicity reports	References
<i>D. cinerea</i>	Tested non-toxic in Wistar albino mice.	Jayakumari et al., 2011.
	Tested non-toxic in brine shrimp lethality assay, human myelogeneous leukaemia cells and Salmonella Mutagenecity assay.	Hurinanthan, 2009.
	Tested non-toxic in brine shrimp lethality assay.	Nguta and Mbaria, 2013.
	Aqueous root extracts tested toxic against <i>Artemia salina</i> larvae, the brine shrimp larvae.	Nguta et al., 2012.
<i>D. obovata</i>	No evidence found.	
<i>D. schlechteri</i>	No evidence found.	
<i>E. elephantina</i>	Histopathological effects were observed in kidneys, lungs, liver and spleen when given in high doses to rats.	Maphosa et a., 2012.
<i>E. tirucalli</i>	Has been found to possess irritant and tumour promoting compounds.	Chhabra et al., 1990.
	Has been found to be lethal after consumption by humans.	Neuwinger, 1996.
	No toxic effects were observed when boipolymeric fractions were consumed by Wistar rats.	Bani et al., 2007.
	The latex was found to be non-toxic when tested on Wistar rats.	Silva et al., 2007.
	The aqueous extract of <i>E. tirucalli</i> latex was found to have high piscicidal activity when tested on the fresh water fish, <i>Heteropneustes fossilis</i> .	Kumar et al., 2010.
<i>G. livingstonei</i>	The compounds, amentoflavone and 4-methoxy amentoflavone isolated from <i>G. livingstonei</i> leaves displayed low toxicity against Vero monkey kidney cells in MTT assay.	Kaikabo et al., 2009.

Plant species	Toxicity reports	References
<i>H. hemerocallidea</i>	Aqueous corm extracts caused brief hypertension and bradychardia in guinea-pigs and rats. Impairment in kidney function was also observed in rats.	Owira and Ojewole, 2009.
	The corm extract showed minimal toxicity when tested by the MTT cellular viability assay.	Morobe et al., 2012.
<i>H. malabarica</i>	No evidence found.	
<i>H. surratensis</i>	No evidence found.	
<i>K. africana</i>	Aqueous and organic leaf extracts have been found to be toxic when tested against kidney epithelial cells in MTT cellular viability assay.	Naidoo et al., 2013.
<i>L. javanica</i>	The leaf extracts were found to cause detrimental effects when administered at high doses in BALB/c mice.	Madzimure et al., 2011.
	Has been found to possess mutagenic effects.	Elogarashi et al., 2003.
<i>M. balsamina</i>	Reported to be toxic at very high doses on tissues and organs of rats.	Geidam et al., 2007.
	Isolated compounds (triterpenoids and balsaminol) showed weak toxicity towards human breast cancer (Mcf-7) cell line.	Ramalhete et al., 2010.
	Aqueous extracts tested safe in the <i>Salmonella</i> Mutagenicity assay.	Ndhlala et al., 2011.
	A compound, balsaminoside tested toxic at high doses against human hepatoma (Huh-7) cell line.	Ramalhete et al., 2014.
<i>O. engleri</i>	Dichloromethane extracts exhibited high cytotoxicity effects in monkey kidney cell test.	Prozesky et al., 2001.

Plant species	Toxicity reports	References
<i>O. engleri</i>	The aqueous and organic extracts were found to be non-toxic when investigated against kidney epithelial cell line in MTT assay.	Naidoo et al., 2013.
<i>P. afra</i>	No evidence found.	
<i>P. capensis</i>	Compounds isolated from stem extracts were reported to be toxic <i>in vivo</i> .	Uys et al., 2002.
<i>R. multifidus</i>	The aqueous and organic extracts were found to be non-toxic when investigated against kidney epithelial cell line in MTT assay.	Naidoo et al., 2013.
<i>S. birrea</i>	No toxic effects were observed from the Ames and VITOTOX toxicity tests.	Elogarashi et al., 2003.
	Genotoxicity was observed from the micronucleus test.	Fenell et al., 2004.
	No toxic effects were observed in brine shrimp toxicity assay.	McGaw et al., 2007.
	Cytotoxicity was observed from the dichloromethane: methanol extracts on Chang liver, 3T3-L1 adipose and C2C12 muscle cells.	Van de Venter et al., 2008.
	Aqueous and methanol extracts of the stem bark were found to be non-toxic on mice.	Ojewole et al., 2010.
	The aqueous and organic extracts were found to be non-toxic when investigated against kidney epithelial cell line in MTT assay.	Naidoo et al., 2013.
<i>S. brachypetala</i>	Tested positive on the brine shrimp toxicity assay.	McGaw et al., 2007.
<i>S. cordatum</i>	Showed non-toxic effects on the Ames and VITOTOX toxicity tests.	Elogarashi et al., 2003.
	Bark and leaf extracts were found to be non-toxic when tested on cultures of Vero cells.	Samie et al., 2008.
<i>S. madagascariensis</i>	No evidence found.	

Plant species	Toxicity reports	References
<i>S. panduriforme</i>	No evidence found	
<i>S. rigescens</i>	No evidence found.	
<i>S. serratuloides</i>	The aqueous and organic extracts were found to be non-toxic when investigated against kidney epithelial cell line in MTT assay.	Naidoo et al., 2013.
<i>S. spinosa</i>	Tested non-toxic against J774 mammalian cell lines but toxic on L6 cells.	Hoet et al., 2004.
	Tested non-toxic on L-6 and MRC-5 mammalian cell lines.	Zirihi et al., 2005.
	Aqueous extracts exhibited tonic muscle spasms and high mortality rate (83%) when evaluated in BALB/c mice.	Nyahangare et al., 2012.
	Displayed low toxicity in the MTT assay.	Isa et al., 2014.
<i>T. elegans</i>	Tested toxic against resting and PHA- stimulated human lymphocytes.	Pallant and Steenkamp, 2008.
	Tested non-toxic in the MTT assay.	Naidoo et al., 2013.
<i>T. sericea</i>	Showed cytotoxicity effects on the velvet monkey kidney cells with ID value of 24 mg/ml.	Tshikalange et al., 2005.
	No mutagenic effects were observed.	Eldeen et al., 2006.
	Acetone extracts of <i>T. sericea</i> were found to non-toxic when investigated in brine shrimp assay.	Eloff et al., 2008.
<i>W. indica</i>	The roots, stem, leaves and aerial parts of <i>W. indica</i> have been reported to be toxic when investigated on mice. Leaf extract showed minimum toxicity against a miracidia of <i>Shistosoma mansoni</i> . An alkaloid, adouetin isolated from <i>W. indica</i> exhibited a minimal lethal dose of 75 mg/mL in mice.	Zongo et al., 2013.

Plant species	Toxicity reports	References
<i>W. somnifera</i>	The root extract was found to be non-toxic when orally administered to Wistar rats.	Prabu et al., 2012.
<i>X. caffra</i>	The aqueous and organic extracts were found to be non-toxic when investigated against kidney epithelial cell line in MTT assay.	Naidoo et al., 2013.
<i>Z. capense</i>	Aqueous extracts tested safe in the <i>Salmonella</i> Mutagenicity assay.	Ndhlala et al., 2011.
<i>Z. mucronata</i>	The plant extract has been reported to be highly toxic by causing DNA damage and chromosomal aberrations.	Fenell et al., 2004.

animal model, and *vice versa* (York et al. 2012). In addition, some of the plant species were reported to treat wounds and burns (De Wet et al., 2013) which are not necessarily infected. The effect of those plants can only be observed when investigated for their anti-inflammatory activities or in *in vivo* studies where they may promote wound healing by inducing the proliferation of skin cells and not as antimicrobial agents.

Lastly, the antimicrobial screening should always be followed with phytochemical screening to fully comprehend the pharmacological properties of medicinal plants. Phytochemistry usually involves the identification and isolation of the active compounds from the most antimicrobial active plants. In the current study, phytochemical analysis was qualitative which did not provide a full insight of the exact compounds responsible for the observed antimicrobial activity. The permeability studies indicated which compounds observed in the crude extracts are able to diffuse across the skin. Further studies, however, should be performed on compromised skin such as the tape stripped skin for comparative purposes. The permeated compounds also have to be identified as well as those that possibly accumulated within the skin in order to obtain their quantitative permeability profiles. Isolating and analysing the permeated compounds may provide insight for potential future compound efficacy where new compound derivatives could possibly be synthesised. The isolated compounds might also be used directly as potential antimicrobials for skin infections which are more active and safer, since the compounds are usually more effective and pure, compared to the extracts (Rates, 2001). Furthermore, for plants that were reported to treat deep skin infections such as *S. birrea* and *S. cordatum*, isolation of the permeated compounds could be useful in the synthesis of antimicrobials which may have a quick therapeutic effect as the compounds can easily permeate the skin in pure form to the site of action and treat the core root of the infection (Mabona and Van Vuuren, 2013).

5.3 Final conclusion

This study has scientifically supported the ethnobotanical claims of the plants traditionally used to treat skin diseases by the lay people of Maputaland. The extensive use of these herbal remedies was justified by their antimicrobial properties individually and in combinations against the selected dermatological relevant pathogens as well as some compounds that permeated the

skin *in vitro*. These findings serve as the scientific basis for understanding the pharmacological properties of the traditionally used medicinal plants and their use against skin diseases.

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Appendix A: Research Publication.

De Wet et al. *Journal of Ethnobiology and Ethnomedicine* 2013, **9**:51
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RESEARCH

Open Access

Medicinal plants used for the treatment of various skin disorders by a rural community in northern Maputaland, South Africa

Helene De Wet^{1*}, Sibongile Nciki¹ and Sandy F van Vuuren²

Abstract

Background: Skin diseases have been of major concern recently due to their association with the Human Immunodeficiency Virus and Acquired Immunity Deficiency Syndrome (HIV/AIDS). The study area (northern Maputaland) has the highest HIV infection rate in South Africa, which made them more prone to a wide range of skin conditions. Fungal infections due to the hot climate and overcrowding households are common in this area, as well as burn accidents due to the use of wood as the major fuel for cooking. It is known that the lay people in this area depend on medicinal plants for their primary health care. However no survey has been done in northern Maputaland to document the medicinal plants used to treat various skin disorder.

Methods: Interviews were undertaken at 80 homesteads, using structured questionnaires. The focus was on plants used for dermatological conditions and information regarding vernacular plant names, plant parts used, preparation (independently and in various combinations) and application was collected.

Results: A total of 87 lay people, both male (22%) and female (78%) were interviewed on their knowledge of medicinal plants used to treat disorders of the skin. Forty-seven plant species from 35 families were recorded in the present survey for the treatment of 11 different skin disorders including abscesses, acne, burns, boils, incisions, ringworm, rashes, shingles, sores, wounds and warts. When searching the most frequently used scientific databases (ScienceDirect, Scopus and Pubmed), nine plant species (*Acacia burkei*, *Brachylaena discolor*, *Ozoroa engleri*, *Parinari capensis*, subsp. *capensis*, *Portulacaria afra*, *Sida pseudocardifolia*, *Solanum rigescens*, *Strychnos madagascariensis* and *Drimys delagoensis*) were found to be recorded for the first time globally as a treatment for skin disorders. Fourteen plant combinations were used. Surprisingly, the application of enema's was frequently mentioned.

Conclusions: The preference of traditional medicine over allopathic medicine by most of the interviewees strengthens previous studies on the importance that traditional medicine can have in the primary health care system in this rural community. Studies to validate the potential of these plants independently and in their various combinations is underway to provide insight into the anti-infective role of each plant.

Keywords: Medicinal plants, Skin disorders, Lay people, Northern Maputaland, South Africa

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Appendix B: Abstract for publication submitted to the Journal Pharmaceutical Biology.

Abstract

Context: Medicinal plants in Maputaland are used by the rural inhabitants for the treatment of skin diseases. Scientific evidence supporting the ethnobotanical claims of these plants is limited.

Objective: To establish scientific validity for a selection of the plants used in Maputaland to treat skin diseases.

Materials and methods: Antimicrobial screening was performed on 12 dermatological relevant pathogens using the micro-titre plate dilution assay. Since some plants were reported to be effective when used in combinations, their effect was evaluated using the fractional inhibitory concentration (FIC) determination and those with notable interactions were investigated further in varied ratio studies. The most antimicrobially active topically applied plants (organic extracts) were subjected to qualitative chemical analysis using phytochemical screening tests, ultra-violet visible (UV-Vis) spectrometry and reverse-phase High Performance Liquid Chromatography (RP-HPLC). The penetration of compounds from extracts of these topically applied plants was investigated *in vitro* across excised porcine skin using the ILC07 automated system and analysed through RP-HPLC.

Results: When investigated individually, all the organic extracts proved to be antimicrobially active with 31.7% displaying broad-spectrum antimicrobial activity (average MIC values <1.00 mg/mL). The organic extract of *Garcinia livingstonei* was found to be most antimicrobially active displaying a mean MIC of 0.27 mg/mL. The efficacy of the aqueous extracts was mostly moderate with 66% exhibiting pathogen specific noteworthy effects (MIC values <1.00 mg/mL). The combination of *Sclerocarya birrea* and *Syzygium cordatum* displayed notable interactions with synergistic effects observed against *Staphylococcus aureus*, *S. epidermis* and *Pseudomonas aeruginosa* having the Σ FIC values of 0.13 (aqueous extract combination), 0.34 (organic extract combination) and 0.19 (aqueous extract combination) respectively. When combined in various ratios, the effect of the selected combinations predominantly displayed additive interactions with some of the ratios giving synergistic interactions. The four antimicrobial active topically applied

plants phytochemically analysed were found to possess flavonoids and saponins. Two to three absorption peaks were obtained between 210 and 360 nm for the crude extracts using UV-Vis spectrometry. The extracts of *K. africana* and *S. cordatum* were found to have more compounds capable of permeating the skin than the extracts of other plants investigated.

Conclusion: Although most of the aqueous extracts demonstrated only moderate antimicrobial activity, more than 80% of the organic extracts tested displayed a correlation between antimicrobial efficacy and the reported traditional uses of the plants. Interesting antimicrobial effects (synergy) were also noted for selected plant combinations. Furthermore, the traditional use of topically applied plant preparations is validated as some compounds from the active plants are capable of permeating the skin *in vitro*. Thus, the current investigation supported the ethnobotanical claims of some of the plants used for the treatment of skin diseases in northern Maputaland.

Appendix C: Abstract for poster presentation at the Postgraduate Symposium at UJ, 2013 and Research Day at Wits, 2014.

VALIDATING THE TRADITIONAL USE OF MEDICINAL PLANTS IN MAPUTALAND TO TREAT SKIN DISEASES.

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Medicinal plants are widely used in northern Maputaland to treat various ailments including skin diseases. Recently (2013), published information has revealed 47 plant species used by lay people of rural Maputaland to treat various skin diseases. Some plant species has for the first time been reported in northern Maputaland as a treatment for skin diseases. Thus, their anti-infective role needed to be explored. The aim of this study was to establish scientific validity of these plants by investigating their efficacy against skin-associated pathogens. Aqueous and organic (1:1 dichloromethane-methanol) extracts were prepared from plants collected from four rural communities of northern Maputaland. Antimicrobial screening was performed on bacteria and fungi known to cause skin diseases. The investigation was carried out using the micro-titre plate dilution assay with crude extracts at a starting concentration of 32 mg/ml. The most antimicrobially active organic extract was that of *G. livingstonei* with MIC values ranging from 0.06 to 0.50 mg/ml. For aqueous extracts, the antimicrobial efficacy was mostly moderate with *Acacia burkei*, *Annona senegalensis*, *Brachyleana discolor*, *Euphorbia tirucalli*, *Kigelia africana*, *Portulacaria afra*, *Solanum rigescens* and *Strychnos madagascariensis* displaying pathogen specific antimicrobial effects. However, more than 80% of the plants tested displayed a significant correlation between antimicrobial efficacy and pathogens responsible for the diseases reported to be treated. Six (*Acacia burkei*, *Brachyleana discolor*, *Ozoroa engleri*, *Parinari capensis*, *Portulacaria afra*, and *Solanum rigescens*) of the nine plant species documented for the first time in Maputaland also demonstrated positive antimicrobial effects. Thus, the current investigation provided scientific evidence that some plant species have potential in the management of skin infections as claimed by the rural inhabitants of northern Maputaland.

Appendix D: Poster presented at the Postgraduate Symposium at UJ, 2013 and Research Day at Wits, 2014.

Validating the traditional use of medicinal plants in Maputaland to treat skin diseases

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RATIONALE

Medicinal plants are widely used in northern Maputaland to treat various ailments. De Wet et al.⁽¹⁾ documented 47 plant species used by the lay people of rural Maputaland to treat various skin diseases. Some plant species have been reported for the first time in Maputaland as a treatment for skin ailments. Thus, their anti-infective role needed to be explored. The aim of this study was to establish scientific validity for the antimicrobial effects of these plants by investigating their efficacy against skin-associated pathogens.

STUDY AREA

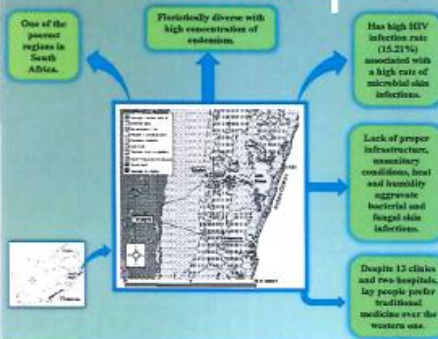


Figure 1: Northern Maputaland, KwaZulu-Natal, South Africa.

EXPERIMENTAL METHODS

Plant collection and preparation

Plants were collected from four regions (Mabibi, Mbazwana, Mseleni and Tshongwe) of northern Maputaland (Figure 1). Collection was undertaken between June and July 2012. Plant parts used were collected based on their availability during the interview process with the lay people. Voucher specimens were prepared on site and are housed at the herbarium of Botany Department at the University of Zululand. Identity of plants was authenticated by Dr. T.C.H. Morstert from University of Zululand as well as Mkhopheni Ngwenya from SANBI, Durban. Collected plant material was air-dried, finely ground and extracted with water and 1:1 dichloromethane: methanol.

Antimicrobial analysis

Micro-titre plate dilution assay was employed to determine the minimum inhibitory concentrations (MICs) of the plant extracts^(2,3). Analysis was performed on bacterial and fungal pathogens known to cause skin infections with crude extracts at starting concentrations of 32 mg/ml. Ciprofloxacin and amphotericin B at starting concentrations of 0.01 mg/ml and 0.10 mg/ml were used as positive controls for bacteria and yeasts respectively. They were included in each assay to confirm antimicrobial susceptibility. Negative controls (sterile media, solvents) were also included in all assays to monitor the antimicrobial susceptibility and sterility. All assays were undertaken in duplicate and where variation was observed, a third replicate was undertaken.

RESULTS AND DISCUSSION

- From the 37 collected plant species, tested against nine pathogens of dermatological relevance, the most prominent results are presented in Table 1.
- The most antimicrobially active organic extract was *G. Irvingstonii*, with MIC values ranging from 0.06 to 0.50 mg/ml.
- For aqueous extracts, the antimicrobial efficacy was mostly moderate with *A. burkei*, *A. senegalensis*, *B. discolora*, *E. tirucalli*, *K. africana*, *P. Afr.*, *S. rigescens* and *S. madagascariensis* displaying pathogen specific antimicrobial effects.
- O. engleri*, documented for the first time as a treatment for skin diseases demonstrated noteworthy activity against seven of the nine tested pathogens.
- Organic and aqueous extracts of extracts *A. burkei* (also documented for the first time to treat ringworm), exhibited noteworthy activity against the causative pathogen *T. Mentagrophytes* with MIC values of 0.25 and 0.50 mg/ml respectively.
- Similarly, *P. capensis* subsp. *capensis* reported to treat sores displayed noteworthy activity against MRSA (0.50 mg/ml), *P. aeruginosa* (0.25 mg/ml) and *S. epidermidis* (0.25 mg/ml).

Table 1: Antimicrobial efficacy of the most active plant species used to treat skin diseases by lay people of northern Maputaland.

Plant species	Part(s) used	Disease(s) treated	Antimicrobial efficacy		
			Test micro-organisms	MIC values (mg/ml)	Aqueous
<i>Albizia burkei</i>	Bark	Sores and ringworm	<i>C. albicans</i>	1.00	0.50
			<i>P. aeruginosa</i>	0.25	0.50
			<i>S. epidermidis</i>	0.25	0.50
<i>Azorella capensis</i>	Roots	Sores	<i>E. coli</i>	0.50	2.00
			MRSA	0.50	NS
			<i>P. aeruginosa</i>	0.50	NS
<i>Brachylaena discolor</i>	Twig	Sores	MRSA	3.00	0.50
			<i>P. aeruginosa</i>	0.75	0.50
			<i>S. epidermidis</i>	0.25	NS
<i>Combretum acrocom</i>	Leaves	AIDS	<i>P. aeruginosa</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	NS
			<i>T. mentagrophytes</i>	0.25	NS
<i>Cymbopogon sp.</i>	Bark	AIDS	<i>P. aeruginosa</i>	0.50	NS
			<i>S. epidermidis</i>	0.50	NS
			<i>T. mentagrophytes</i>	0.50	NS
<i>Dialium ebense</i>	Leaves	Burns	<i>P. aeruginosa</i>	0.50	NS
			<i>T. mentagrophytes</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	NS
<i>Dialium rotundifolium</i>	Bark	Burns	<i>P. aeruginosa</i>	0.50	4.00
			<i>S. epidermidis</i>	0.75	3.00
			<i>T. mentagrophytes</i>	0.12	4.00
<i>Diphysa senegalensis</i>	Bark	Sores	<i>C. albicans</i>	0.12	2.00
			<i>E. coli</i>	0.25	2.00
			<i>P. aeruginosa</i>	0.12	2.00
<i>Euphorbia umbellata</i>	Modified stems	Sores	MRSA	3.00	0.50
			MRSA	0.50	0.50
			<i>P. aeruginosa</i>	0.75	NS
<i>Garcinia Irvingstonii</i>	Bark	Burns	<i>P. aeruginosa</i>	0.12	NS
			MRSA	0.12	NS
			MRSA	0.25	1.20
<i>Hybanthus leucostachyus</i>	Roots	Bark, ringworm and sores	<i>C. albicans</i>	0.50	NS
			<i>P. aeruginosa</i>	0.50	NS
			<i>S. epidermidis</i>	0.50	4.00
<i>Ignatia spinescens</i>	Bark	Infections and ringworm	<i>P. aeruginosa</i>	0.25	NS
			<i>S. epidermidis</i>	0.50	NS
			<i>T. mentagrophytes</i>	0.12	NS
<i>Ignatia spinescens</i>	Fruit	Ringworm	<i>C. albicans</i>	0.50	4.00
			<i>P. aeruginosa</i>	0.25	0.12
			<i>S. epidermidis</i>	0.50	0.25
<i>Lippia prostrata</i>	Twig	Sores	<i>C. albicans</i>	0.25	NS
			<i>E. coli</i>	0.50	4.00
			<i>P. aeruginosa</i>	0.50	0.50
<i>Mimosa pudica</i>	Leaves	Sores and ringworm	<i>P. aeruginosa</i>	0.25	1.00
			<i>S. epidermidis</i>	0.50	NS
			<i>T. mentagrophytes</i>	0.12	NS
<i>Guzmania engleri</i>	Bark	Sores	<i>E. coli</i>	0.50	1.00
			<i>P. aeruginosa</i>	0.25	0.50
			<i>S. epidermidis</i>	0.25	1.00
<i>Ocotea engleri</i>	Leaves	Sores	<i>C. albicans</i>	0.50	2.00
			<i>P. aeruginosa</i>	0.50	2.00
			<i>S. epidermidis</i>	0.50	2.00
<i>Panicum capense subsp. capense</i>	Bark	Sores	MRSA	0.50	0.06
			<i>P. aeruginosa</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	0.06
<i>Portulaca olerace</i>	Leaves	Bark and chronic sores	MRSA	0.50	0.50
			<i>P. aeruginosa</i>	0.12	0.06
			<i>T. mentagrophytes</i>	0.25	0.06
<i>Albizia umbellata</i>	Whole plants	Sores	<i>C. albicans</i>	0.25	NS
			<i>S. epidermidis</i>	0.75	NS
			<i>T. mentagrophytes</i>	0.50	NS
<i>Delonix brachyloba</i>	Bark	Sores	<i>E. coli</i>	0.50	4.00
			<i>P. aeruginosa</i>	0.25	1.50
			<i>S. epidermidis</i>	0.25	NS
<i>Ziziphus birniei</i>	Bark	Bark, herbs and sores	<i>E. coli</i>	0.50	3.00
			<i>P. aeruginosa</i>	0.02	0.50
			<i>S. epidermidis</i>	0.12	2.00
<i>Delonix reginae</i>	Fruit	Bark	<i>C. albicans</i>	0.50	NS
			<i>P. aeruginosa</i>	0.50	NS
			<i>S. epidermidis</i>	0.25	NS
<i>Zuluzium panderifolium</i>	Fruit	Warts	<i>S. epidermidis</i>	0.05	NS
			<i>T. mentagrophytes</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	NS
<i>Strychnos madagascariensis</i>	Leaves	Sores	<i>C. albicans</i>	0.25	NS
			<i>P. aeruginosa</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	NS
<i>Albizia umbellata</i>	Bark	Sores	<i>P. aeruginosa</i>	0.25	0.50
			<i>S. epidermidis</i>	0.12	NS
			<i>T. mentagrophytes</i>	0.12	NS
<i>Strychnos capensis</i>	Fruit	Warts	<i>S. epidermidis</i>	0.25	NS
			<i>T. mentagrophytes</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	NS
<i>Ignatia umbellata</i>	Bark	Burns and sores	<i>P. aeruginosa</i>	0.25	4.00
			<i>S. epidermidis</i>	0.50	0.06
			<i>T. mentagrophytes</i>	0.25	0.06
<i>Tekomomomomoe elegans</i>	Leaves	Ringworm	<i>P. aeruginosa</i>	0.25	NS
			<i>S. epidermidis</i>	0.50	2.00
			<i>T. mentagrophytes</i>	0.50	1.00
<i>Tekomomomomoe elegans</i>	Fruit	Ringworm	<i>S. epidermidis</i>	0.50	2.00
			<i>T. mentagrophytes</i>	0.50	1.00
			<i>S. epidermidis</i>	0.50	1.00
<i>Tekomomomomoe elegans</i>	Leaves	Bark	<i>P. aeruginosa</i>	0.25	NS
			<i>S. epidermidis</i>	0.25	NS
			<i>T. mentagrophytes</i>	0.25	NS
<i>Walteria indica</i>	Roots	Burns and sores	<i>P. aeruginosa</i>	0.75	4.00
			<i>S. epidermidis</i>	0.25	NS
			<i>T. mentagrophytes</i>	0.25	NS
<i>Walteria senegalensis</i>	Roots	Shingles	<i>P. aeruginosa</i>	0.25	0.06
			<i>S. epidermidis</i>	0.25	1.00
			<i>T. mentagrophytes</i>	0.25	0.06
<i>Ziziphus birniei</i>	Twig	Sores	<i>P. aeruginosa</i>	0.50	1.00
			<i>S. epidermidis</i>	0.50	4.00
			<i>T. mentagrophytes</i>	0.25	0.06
<i>Ziziphus capensis</i>	Bark	Burns	<i>P. aeruginosa</i>	0.25	0.06
			<i>S. epidermidis</i>	0.25	0.06
			<i>T. mentagrophytes</i>	0.25	NS

The pathogen(s) most likely to be responsible for the specific disease to be treated are given in MIC values < 1.00 mg/ml are considered noteworthy.

¹Dickerson, M. M., *Journal of Ethnopharmacology* 103:1-10 (2005). ²Van Vuuren, S.F., *Journal of Ethnopharmacology* 103:1-10 (2005). ³Van Vuuren, S.F., *Journal of Ethnopharmacology* 103:1-10 (2005).



CONCLUSIONS

- Although most of the aqueous extracts demonstrated only moderate antimicrobial activity, more than 80% of the organic extracts displayed a significant correlation between antimicrobial efficacy and the pathogens most likely to be responsible for the specific diseases reported to be treated.
- Six of the nine plant species (*A. burkei*, *B. discolora*, *O. engleri*, *P. capensis*, *P. Afr.* and *S. rigescens*) documented for the first time in Maputaland as a treatment for skin infections, also demonstrated positive antimicrobial effects.
- This *in vitro* investigation provides scientific evidence that some plant species have a potential in the management of skin infections, as claimed by rural inhabitants of northern Maputaland.

References:
1. De Wet, H., Nelis, S., Van Vuuren, S.F. *Journal of Ethnopharmacology and Ethnomedicine* 9:51
2. CLSI, Clinical and Laboratory Standards Institute 2012. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Information Supplement.
3. Bluff, J.N., 1998. *Planta Medica* 64, 711-713

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Financial assistance from National Research Foundation and National Student Financial Assistance.
Lay people of northern Maputaland for their hospitality and willingness to share their ethnobotanical knowledge.

**Appendix E: Abstract for oral presentation at the IPUF national conference,
2014.**

**PHARMACOLOGICAL EVALUATION OF PLANTS TRADITIONALLY USED TO
TREAT SKIN DISEASES IN NORTHERN MAPUTALAND, SOUTH AFRICA.**

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Medicinal plants are widely used in Maputaland to treat various ailments including skin diseases. Recently (2013), published information revealed 47 plants used by lay people of rural Maputaland to treat various skin diseases. Some plants were reported for the first time in Maputaland, as a treatment for skin diseases. Thus, their anti-infective role needed to be explored. The aim of this study was to establish scientific validity of these plants by investigating their efficacy against skin-associated pathogens. The study also determined the possible group of chemical compounds that are capable of permeating the skin from the most antimicrobial active plants. Aqueous and organic (1:1 dichloromethane-methanol) extracts were prepared from plants collected from four rural communities of Maputaland. Antimicrobial screening was performed on dermatologically relevant pathogens using the micro-titre plate dilution assay with crude extracts at a starting concentration of 32 mg/ml. Since other plants were reported to be effective when used in combinations, their effect was evaluated using the fractional inhibitory concentration (Σ FIC) assay. Qualitative chemical analysis of the most active plants (aqueous and organic extracts) was performed using phytochemical screening tests, UV-Vis spectrometry and a reverse-phase High Performance Liquid Chromatography (RP-HPLC). The penetration of compounds was investigated *in vitro* on porcine skin using the ILC07 automated system and qualitatively analysed through RP-HPLC. The most antimicrobially active organic extract was that of *Garcinia livingstonei* with MIC values ranging from 0.06 to 0.50 mg/ml. For aqueous extracts, the antimicrobial efficacy was mostly moderate. The combination of *Sclerocarya birrea*

and *Syzygium cordatum* displayed notable interaction with synergistic effects observed against *Staphylococcus aureus*, *Staphylococcus epidermis* and *Pseudomonas aeruginosa* having Σ FIC values of 0.125, 0.38 and 0.19 respectively. All the active plants phytochemically analysed were found to possess flavonoids and saponins with most of their compounds absorbed between 210 and 360 nm under UV-VS spectrometry. *Kigelia africana* had more groups of compounds capable of permeating the skin than other extracts. With more than 80% of plants tested displaying a correlation between antimicrobial efficacy and the diseases they were reported to treat, the current investigation provided scientific evidence that some plants have potential in the management of skin infections as claimed by the rural inhabitants of Maputaland.

Appendix F: Abstract for poster presentation at the Pharmacology and Toxicology Congress at Wits University.

IN VITRO DIFFUSION CHARACTERISTICS OF FOUR CRUDE MEDICINAL PLANT EXTRACTS ACROSS INTACT PORCINE SKIN.

A.D. van Eyk^{1,2}, S. Nciki², S. van Vuuren², H. de Wet³

¹Division of Pharmacology, ²Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa

³Department of Botany, University of Zululand, KwaDlangezwa, South Africa

Background: Medicinal plants are used topically by rural communities in South Africa for the treatment of various skin afflictions. It is imperative to know whether any of the compounds present within the crude extracts have the ability of diffusing rapidly across intact skin and as such exert their therapeutic effects.

Methods: Plant material was collected in the rural areas of Maputaland, from four plants, *Garcinia livingstonei*, *Kigelia africana*, *Sclerocarya birrea* and *Syzygium cordatum* both aqueous and organic (dichloromethane: methanol) extracts were prepared. After drying, the plant material was solubilised in phosphate buffered saline (PBS) at a concentration of 32mg/ml. Preliminary phytochemical screening was performed on both types of extracts using standard procedures. The HPLC chemical fingerprint of each plant extract was determined using a RP-HPLC column (C18, 100 x 4.6 mm, 5µm, 260nm, 25°C) at 1 ml/min, and using methanol (A): H₂O (0.1% formic acid) (B) gradient elution (1%-60%, 45 min). *In vitro* diffusion studies were performed after exposure of intact porcine skin to the extracts for 120 min using a PermeGear flow-through diffusion system with PBS as elution medium (1.5ml/h, 37°C). Fractions collected at 10 min time periods were analysed using the RP-HPLC column.

Results: Preliminary phytochemical analysis indicated the presence of tannins, anthraquinones, flavonoids and saponins in all the extracts. Some extracts additionally indicated the presence of sterols and alkaloids. HPLC chemical fingerprints indicated the presence of a large number of compounds within each extract. Similar HPLC profiles were obtained after skin exposure,

indicating the ability of a limited number of compounds capable of diffusing across the skin. Most of the compounds may have accumulated within the skin exerting a local therapeutic effect.

Conclusion: A number of compounds diffused across intact skin within 10 min, further studies are required to identify these compounds and those that accumulated within the skin.

Appendix G: Ethics clearance certificate for using microbial cultures.

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265



Ref: W-CJ-131026-2

26/10/2013

TO WHOM IT MAY CONCERN:

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

Investigator: Prof Sandy van Vuuren, Sibongile Nciki (Student No. 712730).

Project title: Validating the traditional use of medicinal plants in Maputaland to treat skin diseases.

Reason: This is a laboratory study using microbial cultures including: *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus* (MRSA), Gentamycin and methicillin resistant *Staphylococcus aureus* (GMRSA), *Staphylococcus epidermidis*, *Propionibacterium acnes*, *Brevibacterium agri*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Trichophyton mentagrophytes*, *Microsporum canis* and *Candida albicans*. There are no human participants

A handwritten signature in black ink, appearing to read 'Peter Cleaton-Jones'.



Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.

Appendix H: HPLC data

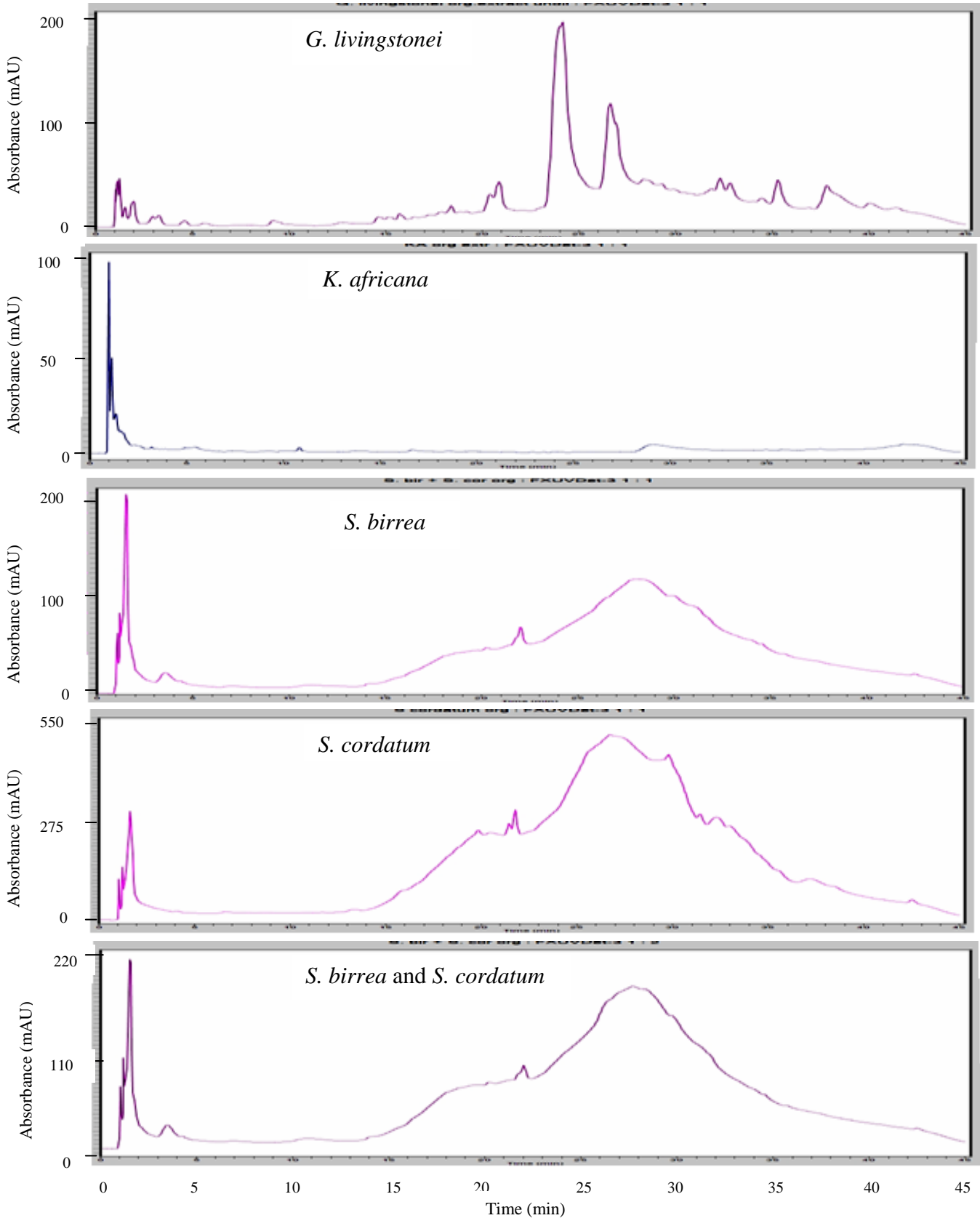


Figure H.1: Chromatograms obtained at 260 nm when using methanol: water as a mobile phase 172

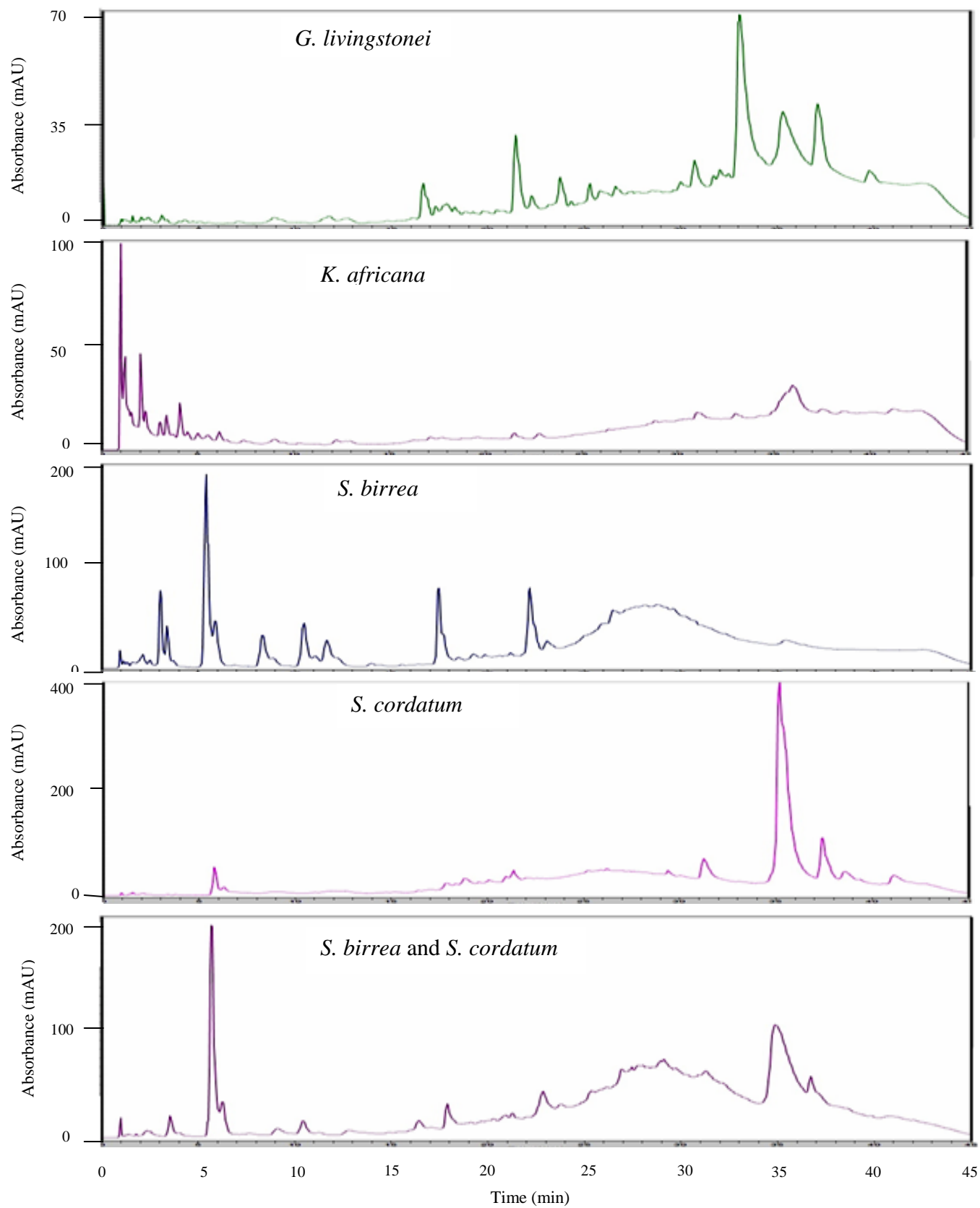


Figure H.2: Chromatograms obtained at 260 nm when using methanol: water with 0.1% formic acid as a mobile phase.

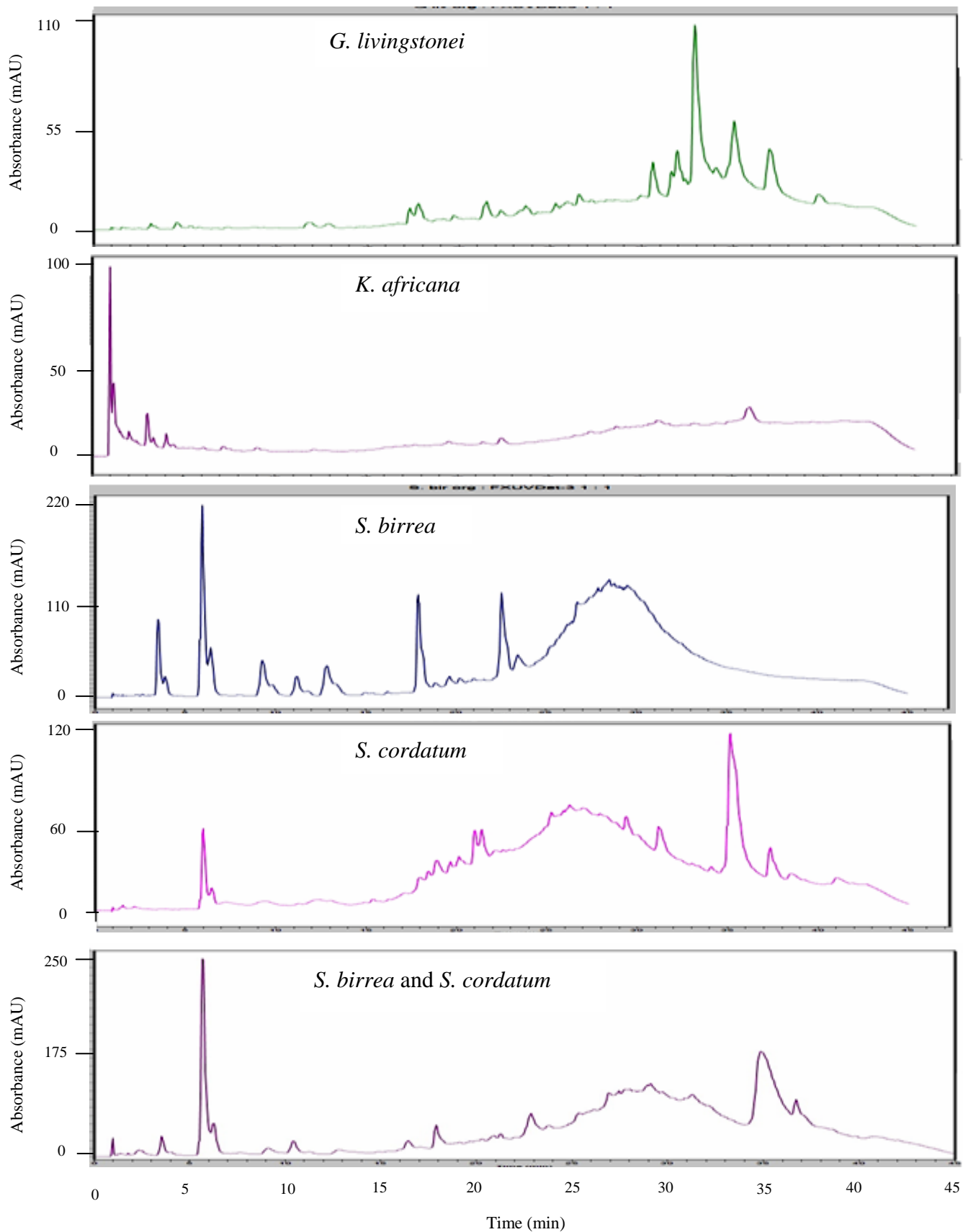


Figure H.3: Chromatograms obtained at 360 nm using methanol: water with 0.1% formic acid as a mobile phase.

Appendix I: Ethics clearance certificate for using porcine skin tissue samples.



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University
of the
Witwatersrand,



June 14, 2013

To: Medical Research Council

Approval of the use of animal tissue samples collected for other purposes

This letter is to confirm that Prof Sandy van Vuuren does not require animal ethics clearance to collect pig skin tissue.

The grant applicant, Ass. Prof Sandy van Vuuren and collaborator, Dr Armorel van Eyk will be using pig skin tissue from already euthanized animals, hence full animals ethics clearance is not required, as these animals have been euthanized for other purposes. The collected animal tissue (skin) from the pig for in vitro diffusion studies will be collected with permission from the Central Animal Service Unit. A request for permission to the Animal ethics committee has been initiated.

If you would like any further or more specific information in this regard, I can be reached on +27 11 7176438 or at graham.alexander@wits.ac.za.

Yours sincerely

Graham Alexander
Associate Professor
Animal, Plant and Environmental Sciences
University of the Witwatersrand