The stimulating effect of extracts of South African plants on melanin production and their antibacterial activity against *Cutibacterium acnes*

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Declaration

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Abstract

Pigmentary disorders are a global problem. Although most hypopigmented diseases are not harmful and do not result in physical pain, the emotional and psychological effects of these conditions can be devastating to the affected patient. Progressive macular hypomelanosis (PMH) is a skin disorder which results in reduced amount of pigment and the PMH-lesion areas contain a high-density of *Cutibacterium acnes*. The current treatments include the use of ultra-violet radiation in combination with antibiotics, however side effects from antibiotic include severe rashes, blistering and dryness, whereas excessive UV radiation leads to premature ageing and susceptibility to skin cancer development. Hence in the present study, eleven medicinal plants, prepared into thirty-three extracts using water, ethanol and dichloromethane were investigated for their antibacterial activity against *C. acnes* and their stimulatory activity towards tyrosinase and melanin production as possible treatments for PMH.

Of the eleven plants, two showed significant results, the ethanolic *Sideroxylon inerme* extract had a minimum inhibitory concentration (MIC) of 125 µg/mL against *C. acnes* (ATCC 6919) and when combined with tetracycline, had an additive effect. The ethanolic *Bulbine frutescens* extract increased the monophenolase activity of tyrosinase and increased melanin production by 31.44 ± 1.41% and 8.55 ± 1.66%, respectively, at 200 µg/mL. The α-melanocyte stimulating hormone increased melanin production by 13.39 ± 1.44% at 100 µM. Both ethanolic *Sideroxylon inerme* and *Bulbine frutescens* showed to have no antiproliferative activity towards the human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cell lines at 400 µg/mL.

The ethanolic leaf and stem extracts of *Sideroxylon inerme* and *Bulbine frutescens* could potentially be used as an alternative treatment for PMH dur to their antibacterial and melanin production activity. Further investigation into the cosmetic safety of the extracts as a topical treatment by evaluating the irritancy potential of the extracts and conducting *in vivo* hypopigmentation trials to determine their efficacy.

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Research Outputs

1. Manuscript under preparation
   Title: The stimulating effect of extracts of South African plants on melanin production and their antibacterial activity against Cutibacterium acnes
   Authors: Aimee Steyn, Danielle Twilley, Analike Blom van Staden, Thobeka Kentane and Namrita Lall
   Journal: Evidence-Based Complementary and Alternative Medicine/Special Issue/ Medicinal Plants and Natural Products in Health Promotion and Disease Prevention
2. Clinical studies
   Evaluation of *Bulbine frutescens* cosmetic safety through irritancy potential analysis. Conducting *in vivo* hypopigmentation trials to determine its efficacy.

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Chapter 1: General introduction

1. Background and motivation of the study

The purpose of the study was to evaluate the bioactivity of certain South African plants species in relation to the disease, "Progressive Macular Hypomelanosis" (PMH). This research is not only relevant to South Africa but has the potential to be of great importance and significance to the rest of the world as it is estimated that 1% of the world population suffer from this disease. PMH affects both teenagers and adults worldwide, and while it is more noticeable in people of colour, it does however, affect persons of all skin colours, and women more than men (Duarte *et al.*, 2010).

Throughout time, from as far back as the Egyptian and Mesopotamian civilisations (2900 BCE) natural products from herbal plants have had significant use for the treatment of various diseases and illnesses (Dias *et al.*, 2012). Their use has been well-documented and has continued to this day within the areas of modern medicine where clinical, pharmacological and chemical studies have often successfully followed traditional usage with quinine and morphine being amongst the most well-known. This is not to say that traditional usage has been discontinued, in fact the World Health Organisation (WHO) indicates that the primary healthcare needs of nearly 80% of the global population are still met through traditional medicine with many countries being almost totally reliant on herbal plants for their healthcare (Farnsworth *et al.*, 1985; Lall & Kishore 2014). In many parts of the world herbal medicine is both the main source of medicine and the most easily accessible. As a consequence of this and as the western world turns to alternate solutions there has been a noticeable rise in the demand for natural products on the market.

It is estimated that there are nearly 300 000 species of plants globally and in many parts of Africa, herbal medication is often the only available health resource for the community (Mahomoodally 2013). In terms of species richness South Africa has an exceedingly rich biodiversity of plants with over 3 689 species of its plants being utilised in traditional medicines (Cherry 2005; de Wet *et al.*, 2013). This however, represents only 20% of South Africa's plants. Furthermore, South Africa houses 20 500 vascular plant taxa, which contribute to nearly 6% of the world's plant diversity (Klopper 2010). Within the actual confines of South Africa, a large percentage of its rural area dwellers are still dependant on herbal plants for medication including any skin treatment. There is therefore, a dire need to explore South Africa's diverse

flora to establish their pharmacologically active natural products and determine any potential medicinal capabilities towards skin treatment.

Some skin diseases can affect the skin pigmentation in that they either increase (hyperpigmentation) or decrease (hypopigmentation) the natural pigmentation of the skin. According to Fistarol and Itin (2010) there are numerous dermatological disorders associated with pigmentation defects. Progressive macular hypomelanosis is a skin disorder that causes hypopigmentation of the skin. It is characterized by hypopigmented macules that are ill defined and symmetrically located (Relyveld *et al*., 2010). It has been postulated by Westerhof *et al*., (2004) that PMH is caused by an increase of *Cutibacterium acnes* which affects the melanogenesis pathway. *Cutibacterium acnes* forms part of the natural flora of the skin, however as an opportunistic pathogen, *C. acnes* is the main attributer to acne vulgaris (Handa 2019).

Current treatments include the use of phototherapy in combination with antibiotics, topical treatments containing benzoyl peroxide and oral treatment that includes doxycycline (Duarte *et al.*, 2010). However, these antibiotics have been known to cause severe side effects and the treatment is not always long lasting. It is obvious that there is a need for more effective and less aggressive treatments alongside a desire to move to natural rather than a chemical or synthetic solutions. Thus, an investigation into herbal medicine for any potential solutions is understandable. PMH along with many other skin pigmentation disorders, can have multiple consequences on the quality of life for those affected. As a result, noting that many of the current treatments are not satisfactory, the cosmetic and pharmaceutical industries continuously pursue new solutions (Ebanks *et al.*, 2009).

Whilst the majority of hypopigmented diseases are not harmful and do not result in any physical pain (Eleftheriadou *et al.*, 2011), it is the emotional and psychological effects of these conditions that can be devastating to the affected patient. Halder and Rodney (2012) and Noh *et al.* (2013) further elaborate on this factor by pointing out that the skin as the largest organ of the body, is also the most noticeable and observable area of the human body. As a result, any defect or blemish to the skin can affect a person's quality of life and impair their social interaction, especially in darker skinned patients where lesions and discoloration are more evident and visible. In India, for example, people with hypopigmented diseases, in particular women, are frequently discriminated against. Further evidence of these psychological effects can be found in the research of Noh *et al.* (2013) and Picardi *et al.* (2001) who observed that

patients became depressed and showed signs of psychiatric morbidity caused by worry, feelings of shame and general societal stigmatization.

2. Objectives of the study

The present study's objective was to establish if any plant extracts were capable of inhibiting the growth of *C. acnes* and further, were able to induce melanin production. Currently the only way to increase melanin in hypopigmented lesions is through the utilisation of ultraviolet (UV) mechanisms which ultimately are considered harmful treatments with alternatives being preferred. The goal is to understand the melanogenesis pathway and the mechanisms involved in this process, and as a result develop solutions to incorporate the active plant components to regulate these mechanisms. At present the number of herbal treatments or plant extracts utilised for PMH symptoms is small, and it is hoped that the studies conducted in this dissertation will offer the opportunity to establish South African plants with antibacterial activity and those which could stimulate melanin production.

The specific objectives of the study were to:

Extracts-Preparation of the selected plant species

The leaves and non-woody stems of selected plant material were shade dried and powdered. They were blended separately in dichloromethanol (DCM) and ethanol extractants. The succulent plant material was blended immediately upon harvesting in both DCM and ethanol extractants, separately. The aqueous extracts were prepared to mimic the traditional use of the plant; thus, water was heated, and a decoction was made.

Investigation of phytochemical groups

The ethanolic extracts were investigated for the presence of major phytochemical groups tannins, alkaloids, saponins, terpenes, flavonoids and phenolics (Mushtaq *et al.,* 2014).

Investigation of the selected plant extract's ability to increase tyrosinase activity

The plant extracts were all investigated for their effect on mushroom tyrosinase. The focus was on the extracts that increased the activity of the tyrosinase enzyme.

Investigation of the antibacterial activity against *C. acnes* of the selected plant extracts

The plant extracts were all investigated for their ability to inhibit the growth of *C. acnes.* The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

of the plant extracts was determined. Significant samples were then used in a combinational study with the antibiotic tetracycline.

Investigation of the cytotoxicity of selected plant extracts

The best course of action in treating PMH is through a topical application on the hypopigmented areas. In order for any of the plant extracts to be used as a potential topical treatment, their cytotoxicity on skin needs to be established. Extracts that showed good antibacterial activity and tyrosinase activity were tested for their cytotoxicity on human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cell lines.

Investigation of the selected plant extracts and their effect on melanin production

Only the plant ethanolic extract that showed significant tyrosinase activation and no cytotoxicity towards the UCT-Mel 1 and HaCaT cells; were tested for their effect on melanin production. The amount of melanin produced by UCT-Mel-1 cells, after the treatment with different plant extracts was determined by the Fontana Masson assay. The extracts were compared with the activity of the α-melanocyte stimulating hormone (αMSH), which promotes the production of melanin in humans.

3. Structure of this thesis

Chapter 2 This chapter provides a review on the melanogenic pathway, the hypopigmentation disorders Progressive macular, their current available treatments and the proposed cause for Progressive macular hypomelanosis.

Chapter 3 This chapter discusses the background on the selected plants, including their traditional uses, Phytochemical compounds and their pharmacological activities.

Chapter 4 The presence of phytochemical compounds in the selected plants. The effect of all the extracts on the activation of mushroom tyrosinase was identified. The antibacterial activity of all the samples were investigated to obtain the minimum inhibitory concentration (MIC) against *C. acnes*. The cytotoxic activity of selected plant extracts through the use of the cell viability assay on human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cell lines. The selected extracts were analysed for their effect on melanin production in UCT- Mel-1 cells. The general discussion of the results is an attempt to synthesize a more coherent picture of the results of this study.

Chapter 5 The general conclusion gives a perspective of future studies.

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Chapter 2: Literature review and research overview

1. Introduction

Throughout history herbal plants have had significant uses, with many countries being reliant on herbal plants for healthcare. According to the World Health Organisation (WHO) approximately 80% of the global population still depend on herbal medicine for their primary healthcare needs (Lall & Kishore 2014). Within the confines of South Africa, a large percentage of its rural area dwellers are still dependant on herbal plants for skin treatment. South Africa boasts more than 24 000 plant species and more than 3 000 of those have been recorded as part of the various cultural groups' medicinal materials (De Wet & van Vuuren 2013).

In addition to healthcare uses, the utilisation of herbal medicine in modern cosmetics is increasing primarily due to the lack of recorded side effects (Joshi & Pawar 2015). Herbal extracts constitute the major portion of pharmaceutical cosmetics found in the market today, which can be attributed to the high consumer demand driven by the current vogue for natural products. Beneficial skin biological effects as well the provision of various proteins, antioxidant, tannins, terpenoids, essential oils and alkaloids have been noted from Herbal products (Kapoor *et al.*, 2015). Maintenance of skin integrity is important not only for youthful looks but for many important biological functions such as thermoregulation, prevention of excess water loss; and protection against hostile external/foreign bodies of the environment e.g., bacteria, viruses, fungi, ultraviolet radiation. To produce a wider range of novel skin care cosmeceuticals, it is necessary to make greater effort with the association of modern cosmetology with bioactive ingredients that are based on the traditional system of medicine (Sonka 2017).

Amongst the studies that have focused on the reduction of skin pigmentation with particular respect to aging spots and ultra violet radiation damage are Ichihashi *et al.*, (2003) and Svobodová *et al.*, (2003). The reduction of melanogenesis is important as it prevents the formation of skin cancer and reduces post inflammatory hyperpigmentation (Riley *et al.*, 1997; Briganti *et al.*, 2003). However, it is not only important to investigate the reduction of melanogenesis, but also to research the chemicals or compounds that induce melanin

production. Skin pigmentation functions as a broadband UV absorbent and is the most important photoprotective factor (Brenner & Hearing 2008). The aforementioned claim was supported by a study conducted by Chiang *et al.* (2011), indicating that the naringenin content in citrus plant extracts stimulated cellular melanogenesis and initiated an up-regulation in tyrosinase activity. Their method has a preventative approach and can be used to determine plant extracts for "melanin-promoting suntan lotions" instead of just focusing on UV-light damage recovery. Other hypopigmented disorders such as leucoderma and vitiligo could also benefit from plants that induce melanogenesis.

2. Hypopigmentation disorders

Hypopigmented macules are lesions associated with decreased pigmentation or melanin when compared to the surrounding unaffected skin. This is not the same as depigmentation which refers to a complete lack of melanin due to a loss of melanocytes. There are a variety of hypopigmentary disorders, which fall into 2 categories, namely genetic or acquired (Sood & Tomecki 2017). Piebaldism and Waardenburg syndrome are genetic hypopigmentary disorders whilst progressive macular hypomelanosis and vitiligo fall under acquired hypopigmentation disorders (Orlow 1994). Hypopigmentation is rarely associated with malignancy or systemic diseases and subsequent conditions related to it are benign (Madireddy & Crane 2020). The focus of the present study has been on acquired hypopigmentation disorder, specifically progressive macular hypomelanosis. Acquired hypopigmentation disorders also include post-inflammatory hypopigmentation, vitiligo, chemical leukoderma and pityriasis alba, which are further discussed in Appendix A. Hypopigmentation disorders are of particular concern to darker skin types (Halder 2012; Sharquie *et al.*, 2013).

2.1. Progressive macular hypomelanosis

Progressive macular hypomelanosis (PMH) is not a very common skin disorder, however it is a global one. Its clinical characteristics are hypopigmented macules that are symmetrical and ill-defined and are non-scaly. They are found primarily on the trunk, namely the back and chest, with a confluence in and around the midline of the trunk (Figure 2.1). However, in some cases they have been found to occur on the neck and face (Hassan *et al.*, 2014). It was first reported by Guillet in 1988 following a study in the French West Indies (Guillet *et al.*, 1988). It is often misdiagnosed as other hypopigmentation diseases such as vitiligo, post-inflammatory hypopigmentation and pityriasis alba. These hypopigmented areas can occur without any history of inflammation or trauma and the skin is reported to have normal sensations (Selim *et*

al., 2011; Martínez-Martínez *et al.*, 2012). The hypopigmented areas are usually distributed in areas with a high level of sebaceous glands (Relyveld *et al.*, 2007).

This is a stable disease with slow progression, sometimes over decades (Selim *et al.*, 2011). PMH is identified with the use of the Wood's lamp and Confocal Laser Scanning Microscopy in order to determine the lesions' features as it reveals a specific follicular pattern of fluorescence (Wu *et al.*, 2010; Leonard *et al.*, 2020). There have been reports that PMH spontaneously regresses up to three to four years after its first appearance, however in some cases it regresses after mid-life, it has never been observed in the elderly (Guillet *et al.*, 1988, Selim *et al.*, 2011). Less than 50 % of PMH cases have been reported to be linked with a family history of the disease (de Morais Cavalcanti *et al.*, 2011a).

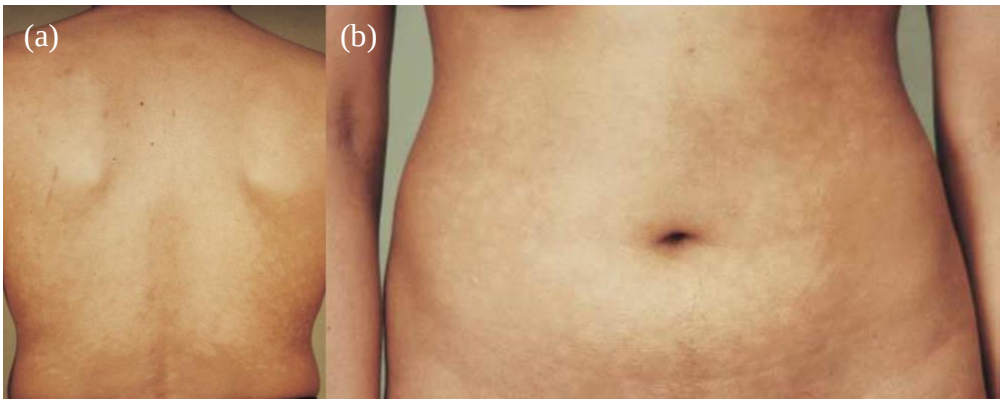

Figure 2.1: A (a) male and (b) female patient of skin type V with progressive macular hypomelanosis (Westerhof et al., 2004).

The pathogenesis of PMH is poorly understood, however the melanosomes in the melanocytes produce reduced amounts of pigment as they were undersized and under-developed (Marks & Seabra 2001; Martínez-Martínez *et al.*, 2012). The melanocytes themselves were not damaged or degraded and it was reported that the number of melanocytes in lesional skin compared to normal (non-lesional) skin was the same (Selim *et al.*, 2011). Both types of skin contained melanosomes, which are the primary components required to synthesize melanin, however these melanosomes were significantly undersized and less melanised in the lesional skin compared to the non-lesional skin (Marks & Seabra 2001; Martínez-Martínez *et al.*, 2012). Westerhof *et al.*, (2004) was the first to suggest that *Cutibacterium acnes*, previously *Propionibacterium acnes*, was the cause of PMH (Figure 2.2). This was due to the high-density presence of *C. acnes* localised in the lesion areas (Hassan *et al.*, 2014). Relyveld *et al.*, (2007) suggested that since PMH was only first reported in the 1980, it could be due to a mutation of *C. acnes* or that it was not recognised as a separate disease and was rather misdiagnosed as one

of the other hypopigmented disorders. It is further postulated that, as the lesions are mainly distributed in areas with high levels of sebaceous glands, these glands provide a greater growth medium for the bacteria and thus the hypopigmentary factors that the bacteria produce are able to diffuse into the surrounding skin (Relyveld *et al.*, 2007). The fact that PMH has never been observed in the elderly could be explained by the decrease in sebum production in the elderly and thus a lower number of *C. acnes* bacteria in the glands (Kim *et al.*, 2010).

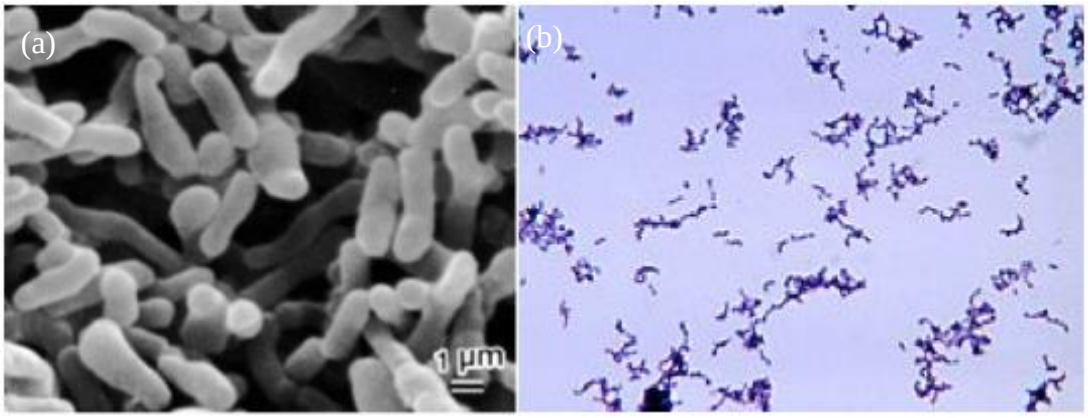

Figure 2.2: *Cutibacterium acnes*, (a) gram-positive rod-shaped bacteria and (b) the bacterium stained with crystal violet (Abate 2013).

A further three hypotheses are suggested by Westerhof *et al.*, (2004) for the development of PMH hypopigmentation. They hypothesis that the *C. acnes* produced a melanogenesis inhibitory factor, tyrosinase inhibitory factor or interferes with the melanin transfer process. This was further substantiated by Relyveld *et al.*, (2007), through electron microscopy analysed lesional and non-lesional skin of a patient with PMH. The lesional skin of the patient showed small clustered, less dense melanosomes in membrane groups in the keratinocytes. Whereas the non-lesional keratinocytes of the patient showed numerous large single melanosomes that were intensely melanocytic. The morphology of the melanosome granules could be due to a factor produced by *C. acnes* that either inhibits tyrosinase, alters tyrosinase or influences the keratinocyte's acceptance of the type of melanosome and the subsequent distribution pattern.

3. Available treatments for hypopigmentation disorders and their side effects

There are several treatments available for hypo-pigmentation disorders which include surgical based therapies, phototherapy and steroidal therapies (Nicolaidou & Katsambas 2014). However, each of these treatments has their own side effects, which are discussed in Appendix A (Lahiri 2009; Mysore & Salim 2009). Further research is aimed at non-medical treatments

for hypo-pigmentation with a specific focus on cosmetics and the use of plant extracts. Sunscreen is used as a protective measure for hypopigmentation as these areas are more susceptible to sun damage (Kaur *et al.*, 2012). Relyveld *et al.*, (2007) have reported that topical and systemic antifungal agents and topical corticosteroids treatments were unsuccessful. They also tested psoralen plus UVA (PUVA) therapy which was reported to be successful while the treatment was ongoing, however the exact induced pigmentation disappeared after cessation of the treatment. One of the more commonly used treatments for hypopigmentation disorders is phototherapy which has shown success in treating PMH when combined with antimicrobial treatments.

3.1. Antibiotic and phototherapy combinations

With the hypothesis that *C. acnes* is the main cause of PMH, attempts at treating the disease have led to the combinational use of antibiotics and phototherapy. Phototherapy involves the use of light and laser therapy that requires the patient to be exposed to controlled amounts of non-ionising ultraviolet (UV) light, such as UVA, UVB and sunlight (Menter *et al.*, 1994; Halder 2012). Relyveld *et al.*, (2006) performed a clinical study in which 45 patients, that suffered from PMH, were treated with 5% benzoyl peroxide gel at night, and 1% clindamycin cream in the morning, in combination with 20 minutes UVA irradiation three times weekly, over a period of 26 weeks. It was found that after 14 weeks, 62% of the patients and dermatologists thought their skin was completely re-pigmented. The authors state that the antibacterial treatment removed the cause of the disease and the UVA irradiation sped up the re-pigmentation process (Relyveld *et al.*, 2006). Isotretinoin in combination with narrow band UVB irradiation has also shown success in the treatment of PMH (Kim *et al.*, 2012a). Antibiotics alone without the use of phototherapy have also proved to be successful in a few cases (Thng *et al.*, 2016; Damevska *et al.*, 2017). However, antibiotics in combination with narrowband UVB treatment have proved to be the most successful treatment of PMH (Kim *et al.*, 2012b; Leonard *et al.*, 2020).

Narrow band UVB (NBUVB) has shown promising results in the treatment of PMH however it has not shown to be permanently effective. A clinical study compared the use of NBUVB irradiation as opposed to the combinational use of narrow band UVB irradiation and benzoyl peroxide gel and clindamycin cream. The study found no significant difference between the two treatments (Sim *et al.*, 2011). Because NBUVB has been reported to be a successful treatment of acne (Sim *et al.*, 2011; Zeichner 2011) this could explain the antimicrobial effect NBUVB had on *C. acnes*. In other studies, NBUVB has shown some success in the treatment

of PMH, with more than 50% of the patients experiencing re-pigmentation of the affected areas (Kwah *et al.*, 2010; Sim *et al.*, 2011). Treatment with solely NBUVB has shown to have high relapse occurrence after two years while treatment with antimicrobial agents showed no relapse (Thng *et al.*, 2016). As of yet there is no standard treatment for PMH.

The antibacterial treatments used in these studies have been based on acne studies, as *C. acnes* is the main cause of acne vulgaris. Antibacterials that have been shown to aid in the re-pigmentation of PMH lesions include doxycycline, minocycline, benzoyl peroxide in combination with clindamycin and benzoyl peroxide with lymecycline (Perman *et al.*, 2008; Alexeyev *et al.*, 2009; de Morais Cavalcanti *et al.*, 2011b). Side effect experienced due to benzoyl peroxide and clindamycin treatment included dry skin, itching, scaling, erythema, burning sensation and edema (Relyveld *et al.*, 2006). Side effects of doxycycline and minocycline can include mild symptoms such as vomiting, mild diarrhoea, hair loss, skin rash and darkening of the skin. More severe side effects include such as throat irritation, chest pain, irregular heart rhythm, shortness of breath, and it may even affect the psyche, cause the development of thyroid cancer and the possibility of death (Durbin 2020a; 2020b; Cunha 2021).

The main concern surrounding treatments that include NBUVB are that they require long term and continuous treatments of up to 18 months, and there is a high likelihood that re-pigmentation will be lost after completion of treatment (Halder 2012). Some of the consequences reported are premature aging of the skin, inflammation due to damaged keratinocytes, DNA breakage, susceptibility to skin cancer and the depletion of antioxidants in the cell or the production of reactive oxygen species (Madireddy & Crane 2020).

The selective pressure employed by antibiotics have steered the path towards drug resistance. Forty percent of *C. acnes* strains have presented resistance to erythromycin, clindamycin and tetracycline. This level of resistance increases the potential of treatment failure (Dreno *et al.*, 2014). Thus, an alternative melanin production stimulatory treatment is needed to replace UV irradiation, as well as an antibacterial agent that causes decreased skin irritation.

3.2. Natural products with melanin stimulating activity

A search of literature indicates that globally, several plants are used for hypopigmented diseases both commercially and traditionally. These are plants known to stimulate melanogenesis and/or up-regulate tyrosinase activity in the melanocytes and are used for vitiligo treatment (Scott *et al.*, 1976). Fahmy and Abu-Shady (1947) initially identified the use

of psoralen-containing plants for the treatment of vitiligo patients in 1947 when they began promotion of the use of 'phototherapy' (Fahmy & Abu-Shady 1948; Roelandts 2002). Their proposed initial phototherapy treatment involved the use of 8-methoxypsoralen and 5-methoxypsoralen in conjunction with exposure to the sun; this UV-A radiation (PUVA) along with psoralens is still employed in the treatment of PMH (Westerhof *et al.,* 2004).

Since their initial research, the use of psoralens has increased within the furacoumarins phytochemical group, with the *Psoralea corylifolia* plant providing a rich source for the main ingredient of 'Anti-vitiligo®' (Song & Tapley 1979; Li *et al.,* 2001). Ethanol extracts of *P. corylifolia* upregulates the activity of tyrosinase which results in an increase in melanin content in the affected epidermal skin area. This traditional herbal formulation aids in the re-pigmentation in the hypopigmented skin patches of vitiligo patients. 'Anti-vitiligo®' is used topically or taken orally (Tahir *et al.,* 2010). Several traditional Chinese medicines used in the treatment of vitiligo come from the plants *Paeonia veitchii, Ligusticum chuanxiong, Cuscuta chinensis* and *Tribulus terrestris* all of which contain psoralens, (Li *et al.,* 2001).

Piper nigrum or the black pepper fruit as well as other members of the *Piperaceae* family originating from the tropical regions of India and Sri Lanka contain the alkaloid piperine (Tiwari *et al.,* 2020). Piperine was reported to stimulate the growth of melanocytes as well as dendrite formation. It however does not promote the melanogenic pathway therefore it is suggested that it is used in conjunction with UV radiation (Soumyanath *et al*., 2006; Gutierrez & Writer 2014). An ingredient of 'Kalawalla®', an acknowledged and commercialized herbal treatment for vitiligo, is a synthetic derivative of piperine. This synthetic compound when exposed to UV radiation stimulates pigmentation in the melanocytes and after six weeks of treatment can result in a light brown colour (Tahir *et al.,* 2010).

Studies on leukoderma treatments cite the use of *Psoralea corylifolia,* the tubers of *Gloriosa superba* as well as the roots of *Angelica archangelica* and *Olea europea* (Vaidya 2006; Chandak *et al.,* 2009; Khandel *et al.,* 2012). The latex from other Indian plants, such as *Telosma pallida* and *Launaea asplenifolia* and their fruit paste is generally applied topically to the leukoderma affected areas. The best results reported by Singh & Narain, (2010) were that, after repeated applications for a period of six months, the affected areas on the patient's skin were reduced by almost 90%. The paste is at its most effective when used as a treatment in the early stages of the disease and when used in conjunction with UV radiation. Upadhyay *et al.,* (2007) and Khare (2008) have also documented the use of pastes rendered from the leaves of

Xanthium strumarium and those of *Citrullus colocynthis*, from a mix of seeds from *Raphanus sativus* and *Achyranthes aspera* seeds and also from the bark of *Tecomella undulate* branches.

This overview clearly illustrates that there are a variety of plants utilised in the treatment of hypopigmented diseases. As many plants still remain untested further investigations of the efficacy of medicinal plants for hypopigmentation diseases should continue. It becomes apparent that this even more imperative with regard to PMH as there have been limited studies towards its treatment through the use of medicinal plants.

3.3. Natural products with antibacterial activity

Propolis and other bee products have a long history of usage as natural remedies because of their antimicrobial, antioxidant, antiviral, and antifungal properties. This has led researchers to hypothesize about its efficacy in the treatment of facial acne (Ali *et al.* 2015). Currently, results are very promising with the outcome that both propolis and other honey-based creams are readily available as commercial products in the market place.

There has been a rapidly increasing demand for natural, herbal and botanical based product solutions in preference over such as antibiotic treatments for the treatment of acne. This has happened for a variety of reasons which include growing antibiotic resistance, irritation side-effects from synthetic products leading to poor patient compliance combined with the perception that the natural product is safer, more effective, and cheaper (Mazarello *et al.*, 2018). Other commercial products with similar claims to success include tea-tree oil and *Aloe vera* either individually or in conjunction with each other and with propolis.

Less well known but freely available oil for skin treatment is clove essential oil (*Syzygium aromaticum*) (Fu 2009). In traditional Chinese medicine the versatile clove is praised for its antifungal and antibacterial abilities. Used over centuries for the treatment of toothache it has now been established that clove oil has broad-spectrum antimicrobial activity and significant anti-inflammatory activity (Sharma *et al.*, 2013; Tsai *et al.*, 2017). Following the increase in the number of resistant bacterial strains, Sadiq and Azeem (2017) discussed the benefits of the antibacterial and anti-inflammatory properties of Neem (*Azadirachta indica*) and Coriander leaves (*Coriandrum sativum*) when used topically for *C. acnes.* Both plants have had long usage in Ayurvedic and folk medicines.

Yet another natural product used for the inhibition of *C. acnes* from the Ayurvedic medicine chest is turmeric (*Curcuma longa*) (Ornelas *et al.*, 2013). While more locally Kalahari Tsamma Melon (*Citrullus lanatus*) oil and *Helichrysum italicum* oil are South African traditional

treatments. Both of these South African products have anti-inflammatory and antimicrobial properties suitable for treating acne. *Helichrysum italicum* being one of the most widely used medicinal plants due to its high level of terpenoids which have been shown to exhibit high levels of antimicrobial activity (Mastelic *et al.*, 2005). *Helichrysum odoratissimum*, another South African *Helichrysum* species, has shown high specificity towards *C. acnes* (De Canha *et al.*, 2019).

4. Melanin production

Melanogenesis is the synthesis of melanin and it occurs in the melanocytes, which are specialised pigment-producing cells in the epidermal of the skin. Melanocytes are located in the basal layer of the epidermis and the hair follicles (DermaMedics 2018). One melanocyte is surrounded by a large number of keratinocytes (Figure 2.3). The molecular structure of melanin is designed so that it is well suited for the absorption of UV radiation along with visible light. Melanin serves as a protection barrier against UV radiation functioning as an antioxidant and a radical scavenger (D'Mello *et al.*, 2016).

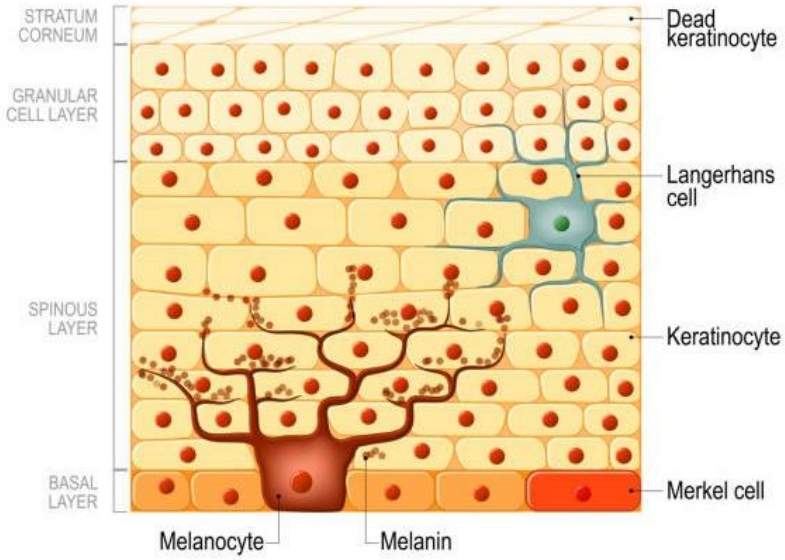

Figure 2.3: The layers of the epidermis, from stratum corneum down to the basal layer, indicating the relationship between the melanocytes and the keratinocytes. The dendritic melanocyte is located in the basal layer and reaches into the spinous layer where it is surrounded by keratinocytes. The melanocyte produces melanin and transports the melanin through the dendrites to the keratinocytes. Keratinocytes progressively move from the basement membrane towards the stratum corneum (iStock 2021).

Melanosomes are subcellular lysosome-related organelles that are found in melanocytes. Melanin is generated as melanosomes that form a mosaic of eumelanin (brown-black pigments) and pheomelanin (yellow-red pigments) (dos Santos Videira *et al.*, 2013). Eumelanin and pheomelanin are synthesised by the substrate L-tyrosine being converted to L-DOPA

(dihydroxyphenylalanine), which is then converted to dopaquinone (Figure 2.4). The conversion of L-tyrosine to L-DOPA is catalysed by tyrosine hydroxylase and the conversion of L-DOPA to dopaquinone is catalysed by DOPA-oxidase (D'Mello *et al.*, 2016). The tyrosine hydroxylase and DOPA-oxidase enzymes are collectively known as tyrosinase (Olivares *et al.*, 2001). The above-mentioned transition is the first step in the triphasic process of melanin production and is fundamental to melanogenesis (Halaban *et al.*, 2002). Phenylalanine, which can be obtained from the cystol, can be converted to tyrosine by phenylalanine hydroxylase (PAH) which can then serve as a substrate for tyrosinase (D'Mello *et al.*, 2016).

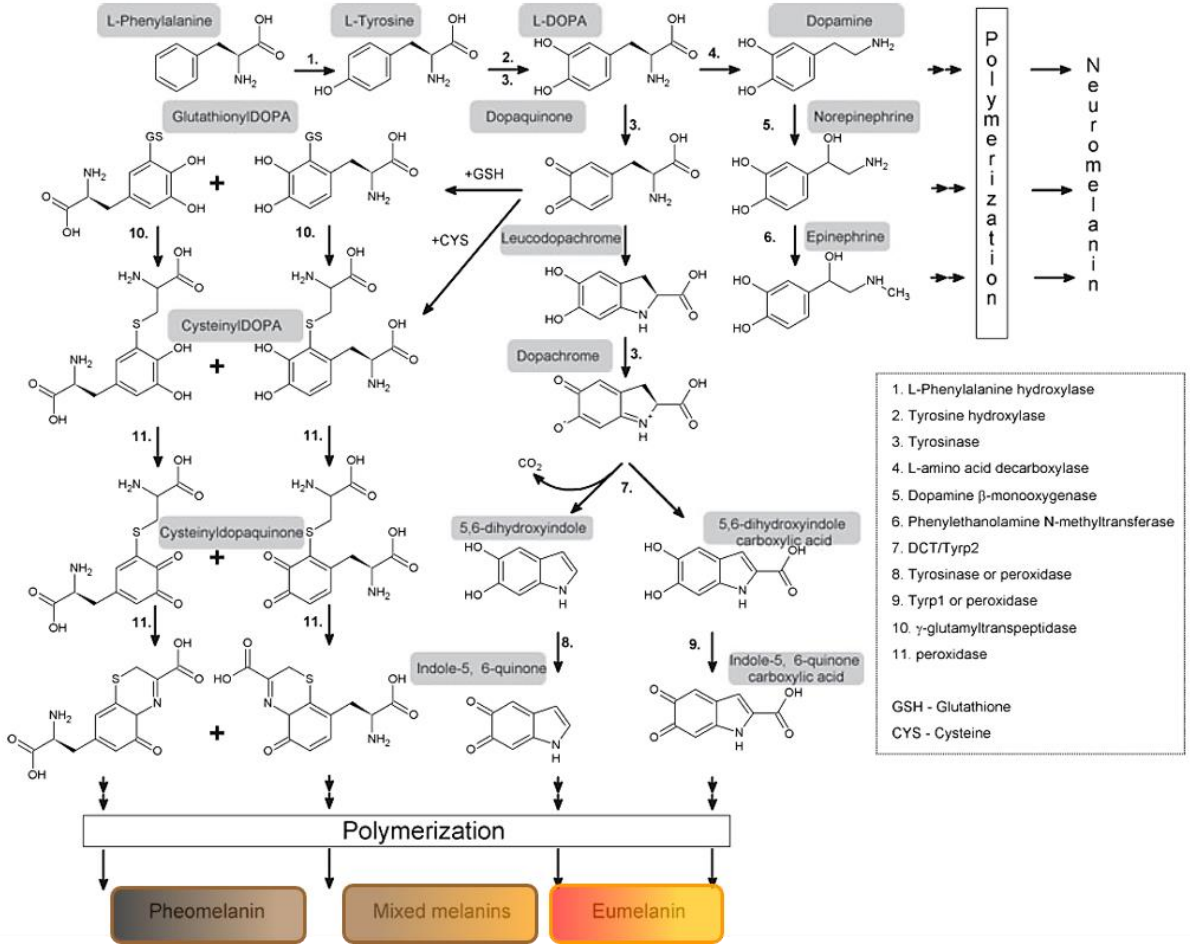

Figure 2.4: The enzymatic steps of the eumelanin and pheomelanin biosynthesis pathway (Slominski et al., 2011).

Pheomelanin synthesis is dependent on the presence of the sulfhydryl compounds glutathione and cysteine. It involves the condensation of DOPA quinone and L-cysteine which results in the production cysteine DOPA. L-Tyrosine is the starting material for the biosynthesis of melanin, it stimulates melanosome synthesis, changes the subcellular distribution of the tyrosinase enzyme and it increases tyrosinase activity. The consecutive product, L-DOPA, upregulates melanin synthesis by primarily activating the tyrosinase activity and regulating the

type of melanogenesis that occurs, either eumelanin or pheomelanin (Slominski *et al.*, 1988; 1989). Other important metalloproteins are tyrosinase related protein 1 (TRP1) and tyrosinase related protein 2 (TRP2), which are, as the name suggests, highly similar to tyrosinase. They play an important role in the catalysation of eumelanin. Tyrosinase related protein 1 has a peroxidase effect that protects against oxidative stress. It also increases the eumelanin to pheomelanin ratio (Slominski *et al.*, 2011).

Tyrosinase is responsible for the rate-limiting step of melanogenesis, the hydroxylation of L-DOPA, and can be activated by two pathways through phosphorylation (D'Mello *et al.*, 2016). The two pathways are intrinsic and extrinsic regulation and are discussed in detail below with the regards to their ability in activating tyrosinase.

4.1. Intrinsic regulation of melanogenesis

Melanogenesis is regulated by 125 known distinct genes that are responsible for the control of pigmentation production and melanosomes creation and function. Melanogenesis is initiated and regulated by signalling systems and transcription factors. This includes the keratinocyte-produced-stem cell factor (SCF) and its binding to the tyrosine kinase KIT receptor and the melanocortin 1 receptor (MC1R) (Tsatmali *et al.*, 2002; dos Santos Videira *et al.*, 2013; Slominski *et al.*, 2013; D'Mello *et al.*, 2016).

Melanocortin 1 receptor, which is the main leading factor in melanin production, is part of the family of G-protein-coupled receptors (D'Mello *et al.*, 2016). Eumelanin synthesis is stimulated via melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH). Pheomelanin is stimulated via agouti signal protein (ASP). Agouti signal protein is another antagonist of MC1R that competes with α-MSH (dos Santos Videira *et al.*, 2013). Melanocortin 1 receptor is controlled by the two secreted ligands α-MSH and ASP (Le Pape *et al.*, 2008). The SCF-KIT receptor tyrosine kinase pathway is responsible for the activation of the MITF transcription factors. These are responsible for the regulation of melanocyte pigmentation, such as the induction of tyrosine (TYR) and TRP1. Ultra violet radiation (UVR) acts as a stimulatory factor towards the expression of the pro-opiomelanocortin (POMC) gene (dos Santos Videira *et al.*, 2013; D'Mello *et al.*, 2016). Slominski *et al.*, (2013) suggest that the activation of the POMC gene might be a response due to oxidative stress caused by UVR exposure.

The hormones, α-MSH and ACTH, are derived from a precursor protein, POMC, that is synthesised in the pituitary gland and epidermal keratinocytes. The melanotrophic activity of

α-MSH and ACTH come from the shared tetrapeptide His-Phe-Arg-Trp (dos Santos Videira *et al.*, 2013). The α-MSH or ACTH bind on the cell surface to the MC1R, thus activating the adenylate cyclase enzyme by phosphorylation, resulting in an increase in cAMP (Figure 2.5). The increase in cAMP causes the activation of protein kinase A (PKA), which in turn activates the cAMP response element (CREB) (Park *et al.*, 2009).

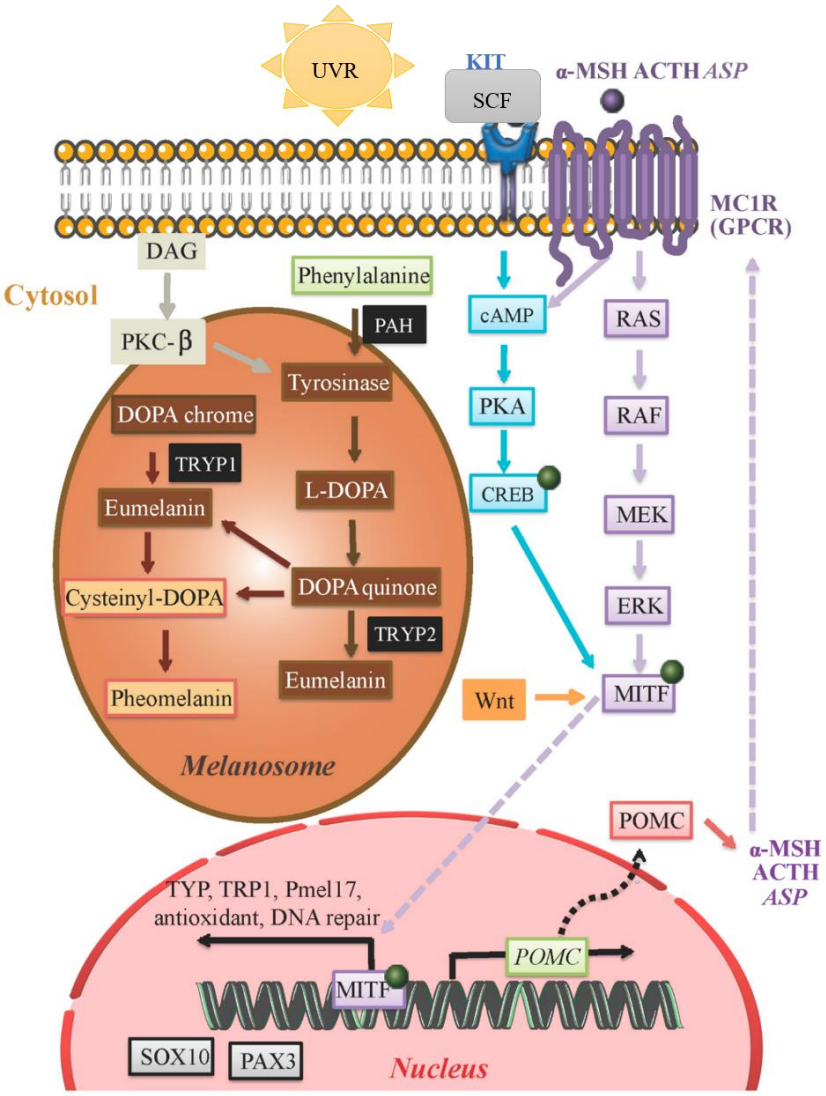

Figure 2.5: The melanogenesis process and the regulating enzymes involves in the signalling pathway that takes place in the melanocyte. Pheomelanin and eumelanin are synthesized in the melanosomes by a series of reaction catalysed by melanogenic enzymes. It is stimulated by either the stem cell factor produced by the keratinocytes, alpha melanin stimulating hormone (αMSH) / adrenocorticotropic hormone (ACTH) or Ultra Violet radiation (D'Mello et al., 2016).

The cAMP response element acts as a transcription factor by involving the microthalmia-associated transcription factor (MITF). In its phosphorylated state MITF regulates the expression of melanogenic enzymes such as TYR, TRP1 and TRP2, and is responsible for the promoting eumelanogenis (Park *et al.*, 2009; dos Santos Videira *et al.*, 2013). Microthalmia-

associated transcription factor is activated through phosphorylation, which is dependent on mitogen-activated protein kinases (MAPK) (Tsatmali *et al.,* 2002). The SCF-KIT receptor tyrosine kinase pathway stimulates the activity of the MAP kinases (dos Santos Videira *et al.,* 2013; D'Mello *et al.,* 2016). The MITF protein expression is dependent on CREB and is regulated by transcription factors produced by keratinocytes (Yamaguchi & Hearing 2009).

There are still some uncertainties regarding the transcriptional and post-transcriptional mechanisms of the molecular pathway that govern melanogenesis. However, it is known that the activation of MITF via cAMP stimulates the transcription of several melanogenic genes. MITF phosphorylation can be induced by upstream MAPK-ERK signalling and downstream by ribosomal S6 kinase (dos Santos Videira *et al.,* 2013; D'Mello *et al.,* 2016). Signalling downstream requires the involvement of the CREB transcription factor and the activation of cAMP. The CREB transcription factor induces the expression of MITF. The wingless-related integration site (WNT) pathway has also been shown to contribute in the expression of MITF (D'Mello *et al.,* 2016).

Microthalmia-associated transcription factor targets specific sequences of the TYR gene which results in the upregulation of tyrosinase. Forskolin, a CAMP elevating agent, has shown to upregulate the protein levels of MITF without the accompaniment of TYR mRNA. The activity of forskolin suggests that there are post transcriptional mechanisms that regulate the protein production of MITF. It was noted that the level of tyrosinase is significantly lower when α-MSH is not involved, which indicates that the hormone plays some form of upregulation in the tyrosinase formation and possibly an increase in its activity (D'Mello *et al.,* 2016).

Ultra violet radiation exposure activates diacylglycerol (DAG), which in turn activates protein kinase C-β (PKC-β) (dos Santos Videira *et al.,* 2013). The phosphorylation of PKC-β is vital as it gives rise to activation of tyrosinase. Ultra violet radiation causes keratinocytes to produce several factors that may inhibit or stimulate melanogenesis as a response to the UVR, this is known as extrinsic regulation (Tsatmali *et al.,* 2002; dos Santos Videira *et al.,* 2013; D'Mello *et al.,* 2016).

4.2. Extrinsic regulation of melanogenesis

Ultra violet radiation is the most prominent extrinsic factor in the regulation of melanogenesis as it is responsible for an increase in the abundance and recruitment of melanocytes (Lin & Fisher 2007). Diacylglycerol (DAG) activates PKC-β and sequentially tyrosinase, after exposure to UVR. UVR is the main inducer for acquired pigmentation or 'tanning' and can

result in DNA damage (dos Santos Videira *et al.*, 2013). The tanning response is affected by the tumour-suppressor protein p53 responsible for regulating the α-MSH production in keratinocytes. Protein p53 regulates the transcription of the tyrosinase transcription factor and hepatocyte nuclear factor 1α in melanocytes (Khlgatian *et al.*, 2002). An increase in oestrogen, recognised by the α- and β-oestrogen receptors can cause an increase in pigment production. Other extrinsic factors that have an effect on the melanogenesis pathway include skin-whitening products that can interfere with the core signalling pathway, can have an inhibitory effect directly on tyrosinase, competitively inhibit tyrosinase or they can interfere with the transfer of melanosome to the keratinocytes (Parvez *et al.*, 2006; D'Mello *et al.*, 2016). The next step in the melanogenesis process is the transfer of the melanosomes from the melanocytes to the keratinocytes.

4.3. Melanin transfer

Following the synthesis of melanin, the melanosomes move toward the tips of melanocyte's dendrites that are in contact with up to 40 keratinocytes (Delevoye 2014). The melanosomes are then transferred from the tips to the adjacent keratinocytes (Cardinali *et al.*, 2005). The transfer of melanocytes to the keratinocytes requires microfibrils and particular receptors within the melanocyte dendrites. The biological processes involved in melanin transfer are not completely defined, there are however three identified mechanisms of melanin transfer, direct injection of the melanosome in the keratinocyte via a filopodia tube, cytophagocytosis and exocytosis (Singh *et al.*, 2010; Delevoye 2014; D'Mello *et al.*, 2016;). Therefore, hypopigmentation occurs with a decrease in the rate of melanosome transfer. Treatments for hypopigmentation are aimed at either increasing the synthesis or/and the rate of transfer.

4.3.1. Cytophagocytosis mediated melanin transfer

This transport mechanism involves the phagocytosis (cellular engulfment) of a portion of the melanocyte by the keratinocyte (Figure 2.6). The melanocyte dendrite tip within the keratinocytes, was first detected by electron microscopy (EM) (Okazaki *et al.*, 1976). Keratinocytes are classified as 'non-professional' or 'amateur phagocytes' and their phagocytic activity occurs due to the proteinase-activated receptor-2 (PAR-2). This process is broken into four stages. The first step is the extension of the melanocyte dendrite and contact with the surrounding keratinocytes. The keratinocyte reacts to the contact with the ruffling of the membrane and the engulfment of the melanocyte dendrite through the use of villus-like cytoplasmic projection. In the second step the melanocyte tip is pinched off, which forms a cytoplasmic vesicle filled with melanocytes. The third step includes the fusion of the lysosomes

and the newly formed melanokerasomes to form the phagolysosome. The formation of the phagolysosome leads to the degradation of the cytoplasmic constituents and the internal melanocyte membranes. In the fourth step, the melanized phagolysosome fragments into smaller vesicles containing aggregates or single melanin granules dispersed in the cytoplasm (Sharlow *et al.*, 2000; Van Den Bossche *et al.*, 2006).

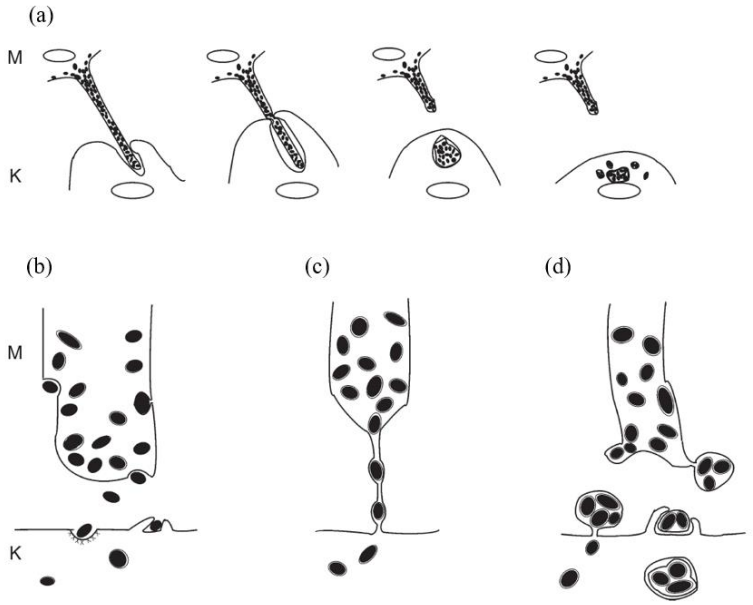

Figure 2.6: The different melanin transfer modes. (a) Cytophagocytosis: melanin granules are dispersed throughout the cytoplasm via a melanocytic dendrite being severed and phagocytosed into the keratinocyte. (b) Exocytosis: the fusion of the melanosomal membrane with the plasma membrane externalises the melanin which is then taken up by endocytosis or phagocytosis. (c) Membrane fusion: the melanocyte and keratinocyte plasma membranes merge allowing melanosome to pass through a channel. (d) Shed membrane vesicle: vesicles containing shed melanosomes are either ingested by phagocytosis or fused with the keratinocytes plasma membrane. M: melanocyte; K: keratinocyte (Van Den Bossche et al., 2006).

4.3.2. Exocytosis mediated melanin transfer

This mode of transport of the melanosome involves the fusion of the melanocyte plasma membrane with the melanosome membrane (Figure 2.6). The melanosome core is then released as extracellular melanin, which is then taken up via phagocytosis by the neighbouring keratinocyte. This model of transport was first observed in human hair follicles and skin in 1964 (Swift 1964). Electron microscopy observed extracellular or 'naked' melanin in the intracellular space between the melanocytes and keratinocytes (Yamamoto & Bhawan 1994). The melanin was lacking a membrane in the extracellular space (Hurbain *et al.*, 2018). When taken up by the keratinocytes, the melanin was encased in a single membrane that was lacking the melanosome membrane protein tyrosinase-related protein (TYRP)-1 (Tarafder *et al.*, 2014). The melanin was proposed to be internalised by the keratinocytes as melanin clusters or as individual granules. When stimulated with α-MSH, naked melanin was identified in the media

of co-cultures of human melanocytes and keratinocytes. The α-MSH and endothelin-1 (ET-1) have been reported to induce melanosome secretion from melanocytes (Virador *et al.*, 2002; Van Den Bossche *et al.*, 2006).

4.3.3. Membrane fusion mediated melanin transfer

In this transport mode, the melanocyte and keratinocyte plasma membrane fuse to form a channel that connect the two cells (Figure 2.6). The cytoplasm of both cells are connected and allows for the transfer of the melanosomes as single membrane-bound organelles (Scott *et al.*, 2002). Melanocyte filopodia have been documented through transmission electron microscopy to attach to the surface of keratinocytes. It was found that Rab17 was involved in the melanocyte filopodia formation and its reduction results in a build-up of melanin in the cell periphery (Singh *et al.*, 2010; Beaumont *et al.*, 2011). Another factor found to be involved in this mode of transport was the Myosin X and N-methyl-D-aspartate (NMDA) receptor. When inhibited, the filopodia formation and melanin transfer to the keratinocytes was impaired (Singh *et al.*, 2010; Ni *et al.*, 2016). It was found, with regards to this mode of transport, that E-cadherin was essential for filopodia formation and melanin transfer after UVR stimulation (Singh *et al.*, 2017).

4.3.4. Shed membrane vesicle mediated melanin transfer

This mechanism of melanin transfer involves the shedding of melanosome-loaded vesicles that are released from the melanocyte into the extracellular space and are then either phagocytosed by the keratinocytes or fusion takes place between the two plasma membranes (Figure 2.6). Cerdan *et al.*, (1992) first observed this phenomenon in human melanoma cells and later in *Xenopus laevis* (Aspengren *et al.*, 2006), initiating a new melanin transfer model. There are four sequential steps that make up the proposed mechanism: the packaging of multiple melanosomes in a single vesicle; shedding of these vesicles enclosed by the melanocyte plasma membrane; internalization by keratinocytes of these vesicles through phagocytosis; and release of the individual melanosomes into the cytoplasm of keratinocytes (Nicol 2005; Lai-Cheong & McGrath 2013).

In vivo evidence of the occurrence of melanosome shedding in samples of chicken embryonic skin was recently provided which, according to Tadokoro *et al.*, (2016), implicates the Rho small GTPase family in melanocyte membrane remodelling prior to vesicle release. A vesicles population thought to be the shed melanosome-loaded vesicles was revealed by flow cytometry analysis of human melanoma cells (Wäster *et al.*, 2016). Multiple melanosomes loaded vesicles

were found in the melanoma cells culture medium, something inconsistent with the cytophagocytosis and membrane fusion models (Ando *et al.*, 2011). Moreover, EM studies showed that keratinocytes incorporate aggregates of melanosomes enclosed by a double membrane (Ando *et al.*, 2011; 2012; Tadokoro *et al.*, 2016). The transfer of these vesicles was furthermore, reported to increase through protease-activated receptor (PAR)-2 stimulation (Cardinali *et al.*, 2005). Importantly, melanin uptake in human keratinocytes *in vivo* and *in vitro* has been shown to be mediated by PAR-2's activation which stimulates melanin transfer through increased phagocytosis in keratinocytes (Sharlow *et al.*, 2000; Correia *et al.*, 2018). Ultimately, gradual degradation of the membranes within the enveloped melanosome occurs upon internalization (Tadokoro *et al.*, 2016).

5. *Cutibacterium acnes*

Cutibacterium acnes, formerly *Propionibacterium acnes*, belongs to the family *Propionibactetriaceae*. *Cutibacterium acnes* is a non-motile, gram-positive bacterium that is non-spore forming. It is pleomorphic in shape, though usually takes on a rod-shape appearance with a slight curve (Mayslich *et al.*, 2021). It is facultatively anaerobic and forms part of the natural flora of the skin and is known for being an opportunistic pathogen of low virulence (de Morais Cavalcanti *et al.*, 2011a; Peterson *et al.*, 2015). It is also found in the conjunctiva, mucosa of the anterior of the nose, the mouth, large intestine and in the upper respiratory tract (Nagy *et al.*, 2013; Platsidaki & Dessinioti 2018).

The cutaneous microbiome of healthy skin exists in a delicate balance where any disturbance or alteration in its equilibrium can lead to a variety of inflammatory skin diseases and infections (Platsidaki & Dessinioti 2018). *Cutibacterium acnes* forms part of the skin's natural microbiota along with *Corynebacteria* and *Staphylococci*. Apart from being associated with PMH, *C. acnes* has been associated with cerebral-spinal fluid infection, prosthetic device infections, postoperative infections, endophthalmitis, bacteremia, primary biliary cirrhosis granulomas, sarcoidosis, infective endocarditis and is the main attributor to acne vulgaris (de Morais Cavalcanti *et al.*, 2011b; Davidsson *et al.*, 2016; Yamamoto *et al.*, 2018).

Cutibacterium acnes was previously known as *P. acnes*; however, it was reclassified due to its genomic adaptive changes and to distinguish it from other *Propionibacteria* species. Specifically, *C. acnes* has unique lipase genes for triacylglycerol lipase and lysophospholipase. The lipases give this pathogen the ability to degrade sebum lipids (Platsidaki & Dessinioti 2018). This ability to degrade and feed upon sebaceous matter means that the bacteria inhabit

the pilosebaceous ducts and hair follicles. This accounts for the distribution pattern of PMH around dense sebaceous gland areas (Leonard 2020). These sebaceous ducts are predominantly found on the torso and studies aimed at assessing the *C. acnes* distribution pattern have found a correlation between the specific *C. acnes* associated with PMH and their habitation of these pilosebaceous ducts (Figure 2.7a). Through the use of quantitative real-time PCR (qPCR) and culturing, the bacterium in lesional skin was found to be much higher when compared to non-lesional skin in patients suffering from PMH (de Morais Cavalcanti *et al.*, 2011a; Barnard *et al.*, 2015; 2016;). When viewed under the Wood's lamp *C. acnes* appears as a coral-red follicular fluorescence due to the presence of porphyrins produced by the bacteria (Peterson *et al.*, 2015; Barnard *et al.*, 2016).

The genome of *C. acnes* has been completely sequenced and is 2.5 Mb in size. These genes encode the metabolic enzymes that allow the bacterium to survive in microaerophilic conditions as well as its specialized lipases (Platsidaki & Dessinioti 2018). Through the use of multi and single locus sequence typing (MLST and SLST), *C. acnes* has been classified into three phylotypes, namely, phylotype I, II and III and subtypes within those phylotypes. This was based on the gene sequences and lipase activity (Peterson *et al.*, 2015). Type III strains differ gnomically to the other strains and 20% of their identified genes are unique to this phylotype (Peterson *et al.*, 2015). Type III was only discovered in 2008 and thus far has been significantly associated with PMH (de Morais Cavalcanti *et al.*, 2011b; Barnard *et al.*, 2016). Type I strains are associated with acne vulgaris (Barnard *et al.*, 2016).

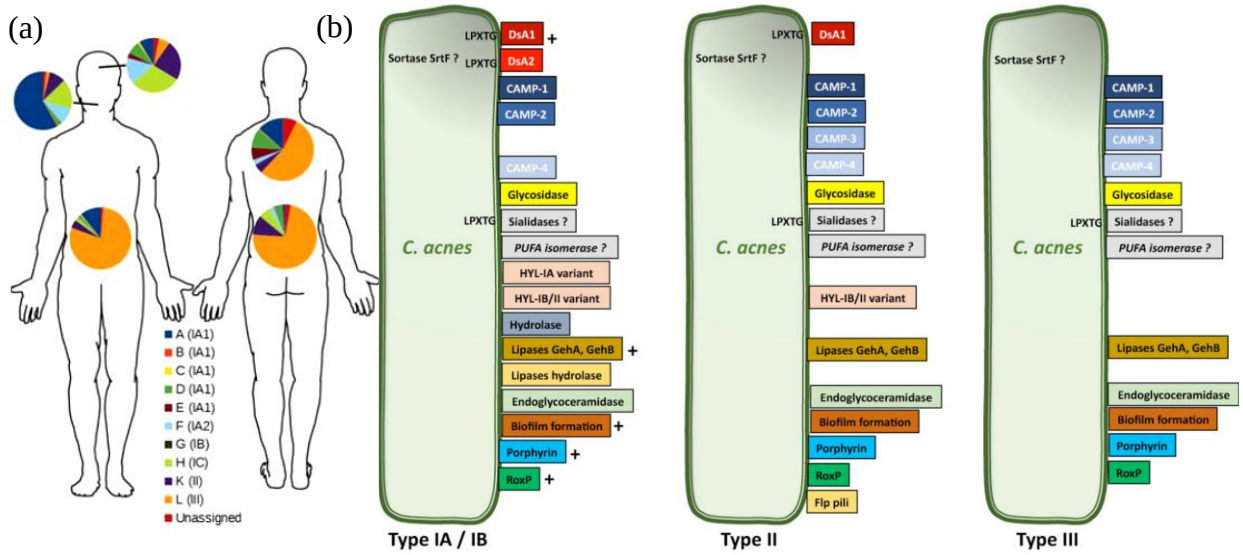

Figure 2.7: The Cutibacterium acnes types and subtypes (a) distribution on the anterior (left) and posterior (right) aspects of the body isolated from patients with PMH. Orange represents *Cutibacterium acnes* type III which is closely associated with PMH. Type III appears in significantly higher levels in PMH lesional skin when compared

to non-lesional skin (Peterson et al., 2017). (b) A comparison of the known virulence factors produces by the different sub-types of *Cutibacterium acnes* (Mayslich et al., 2021).

Type III has shown to have both morphological and genomic differences. Through microscopy, it was seen that the bacterium had a long slender filamentous morphology, which formed tangled aggregates (McDowell *et al.*, 2008). Two subtypes of type III have been isolated from PMH lesional skin from the lower back of a patient in Aalborg, Denmark, namely, *C. acnes* PMH5 and PMH7 (Peterson *et al.*, 2015; Barnard *et al.*, 2016). Four subtypes of type III have had their genomes sequenced, HL201PA1, JCM18909, PMH5 and PMH7, which includes the two isolated from PMH lesional skin. These subtypes have been compared to the genomes from type I and type II (Figure 2.7b). The process of how type III influences the development of PMH is still unknown.

In order to identify an effective antibacterial natural product, the pathophysiology process of *C. acnes* needs to be understood. *Cutibacterium acnes* over proliferation causes *C. acnes* target cells to produce pro-inflammatory responses (Figure 2.8). These target cells are cell types that make up or come into contact with the pilosebaceous units and include fibroblasts, keratinocytes, monocytes and sebocytes (Zouboulis 2004). The innate immune system is involved in recognizing *C. acnes* in the skin through the use of pattern recognition receptors (PRRs). These PRRs include AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), protease-activated receptors (PARs), RIG-I-like receptors (RLRs), Toll-like receptors (TLRs) and intracellular DNA sensors (cGAS-STING) (Kim & Ewbank 2018; Mayslich *et al.*, 2021). Toll-like receptors are transmembrane proteins and contain two domains which include an extracellular domain containing a leucine-rich repeat (LRR) and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domain (Köllisch *et al.*, 2005; Mayslich *et al.*, 2021;)

The LRR domain is responsible for the damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). The DAMPs and PAMPs are cell wall components of the pathogen and include lipoproteins, lipids, proteins, and nucleic acids (Kim & Ewbank 2018; Roh & Sohn 2018). While the TIR domain is responsible for transmitting signals that trigger the production of pro-inflammatory molecules. Toll-like receptors 1, TLR2, TLR4, TLR5 and TLR6 are responsible for recognizing gram-positive bacteria and fungi. They recognize endogenous ligands, flagellin, hyaluronic acid, lipids, lipoproteins, lipoarabinomannan, lipoteichoic acid (LTA), oxidized proteins, peptidoglycan (PGN) and zymosan. TLR3, TLR7, TLR8 and TLR9 are responsible for the recognition of nucleic acids

from damaged cells, bacteria and viruses. When the target cell PRRs are activated by the PAMPs and DAMPs, the innate immune response is triggered. This results in the production of antimicrobial peptides (AMPs), chemokines and cytokines (Köllisch *et al.*, 2005; Lee *et al.*, 2019; Mayslich *et al.*, 2021).

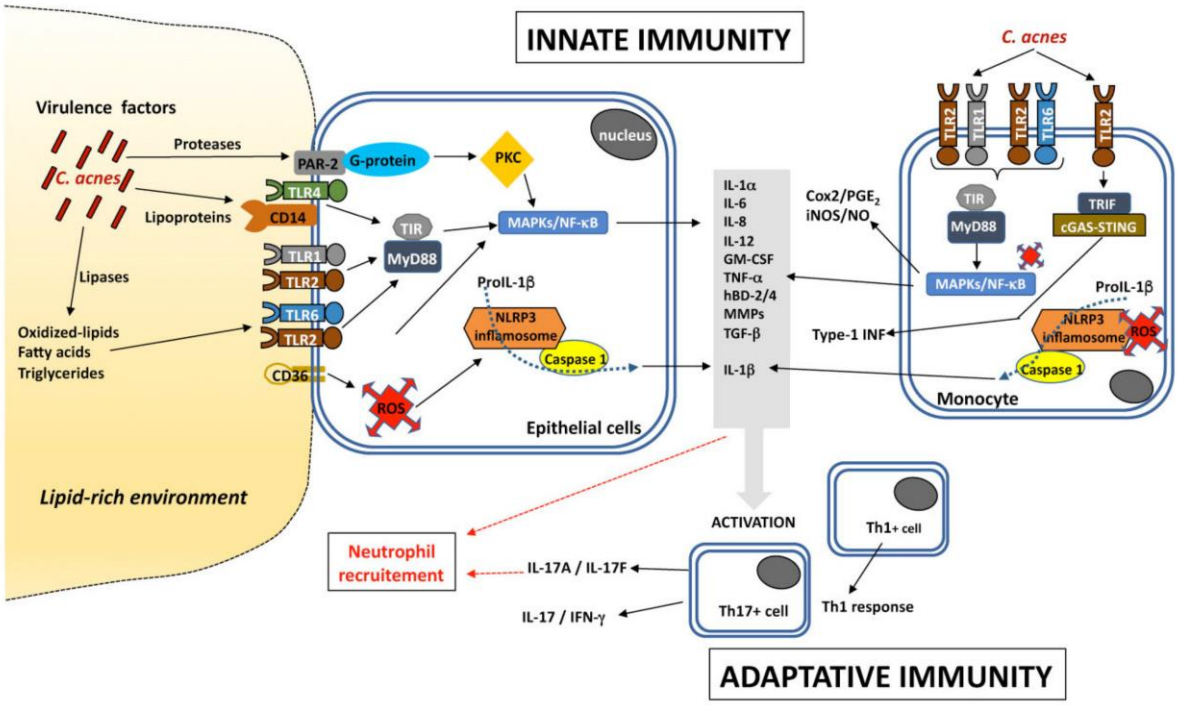

Figure 2.8: The adaptive and innate immune systems' repose to *Cutibacterium acnes* resulting in the inflammatory pathway (Mayslich et al., 2021).

Cutibacterium acnes metabolizes sebum triglycerides into free fatty acids, triglycerides and oxidised lipids, which induces a cytotoxic inflammatory reaction. This is worsened by the presence of *C. acnes* and causes polynuclear neutrophils to move to the site of inflammation. The neutrophils release of lysosomal hydrolytic enzymes causes a follicular destruction by digesting the follicular wall. The lytic enzymes released are hyaluronidase, lipase, metalloprotease and protease which elicit an immune response (Fabbrocini *et al.*, 2010; Lee *et al.*, 2010). TLR2 and TLR4 recognise *C. acnes* and activate the NF-κB and MAPK signal pathways along with the NLRP3 inflammasome pathway. The monocytes, keratinocytes and sebocytes produce IL-1α, IL-1β, IL-6, IL-8 and IL-12 together with TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), matrix metalloproteases (MMPs) and β-defensin-2 (hBD-2) (Jugeau *et al.*, 2005; Zouboulis *et al.*, 2016).

Cutibacterium acnes release extracellular proteases that are recognised by proteinase-activated receptor-2 (PAR-2) which amplify the inflammatory response by the release of IL-1, IL-8,

TNF-α, MMP and hBD-2 (Lee *et al.*, 2010). Another amplifier of the inflammatory response is the keratinocytes production of reactive oxygen species (ROS) as a reaction to *C. acnes.* The ROS are recognised by the scavenger receptor CD36 which amplifies the inflammatory reaction as it stimulates the release of IL-8, separate to the TLR2 signal pathway (Méndez-Lara *et al.*, 2020). The production of ROS leads to the release of superoxide anions, which are converted into H_2O_2 and then into water by the GSH/Gpx system. The release of ROS also stimulates the NF-κB and MAPK signal pathways which in turn leads to the macrophage production of iNOS/NO and Cox2/PGE2 (Tsai *et al.*, 2013; Mayslich *et al.*, 2021). *Cutibacterium acnes* also induces type 1 interferon (IFN-1) synthesis through the macrophage cGAS-STING pathway (Mayslich *et al.*, 2021).

Cutibacterium acnes is involved in the maintenance of healthy skin. For this reason, it is important to not only find antibacterial compounds that can lead to the reduction of excessive proliferation of the bacterium but to also achieve it without provoking a negative reaction from the skin. It is imperative that the skin's microbiota balance is maintained.

6. Psychological effect of skin diseases

Although most hypopigmented diseases are not harmful and do not result in any physical pain (Eleftheriadou *et al.*, 2011), the emotional and psychological effects of these conditions can be devastating to the affected patient. Halder and Rodney (2012) and Noh *et al.*, (2013) further expanded on this notion by noting the fact that the skin is the most noticeable and visible area of the human body, which means any defect or blemish to the skin can affect a person's quality of life and impair social interaction, especially in patients with darker skin where lesions and discoloration become more evident and visible. No studies have been performed on the quality of life of persons with PMH, however, it is assumed that it is similar to those that have vitiligo (Relyveld *et al.*, 2007). Comparing it to that of vitiligo has shown that the quality of life can be impaired from a mild to moderate state. It can affect the choice of clothing, cause feelings of embarrassment and has even been reported to result in problems within the workplace and with friendships (Anaba *et al.*, 2019).

Some people have found PMH to be cosmetically disturbing while others do not notice they even suffer from a hypopigmentation disease. In parts of the world where leprosy is endemic, PMH is often confused with the disease (Relyveld *et al.*, 2007). In India, for example, people with hypopigmented diseases, in particular women; are often discriminated against. According to Kaur *et al.*, (2012), in some cases the development of hypopigmented lesions after marriage

has been known to lead to divorce. Studies by Kent and Al'Abadie (1996) and Sampogna *et al.* (2008) have shown that people with hypopigmentation diseases have displayed signs of anger and frustration and in particular people with vitiligo have been shown to exhibit anxious behaviour. Further evidence of these psychological effects can be found in the work of Picardo *et al.* (2001) and Noh *et al.* (2013) who observed that patients became depressed and show signs of psychiatric morbidity caused by worry, feelings of shame and general stigmatization by society.

7. References

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Chapter 3: Selected Plants

1. Current significance of selected plants

The aim of the study was to identify plants that could potentially inhibit the growth of *Cutibacterium acnes* and induce melanin production in melanocytes. This chapter discusses the background of the plants that were investigated and the basis upon which those specific plants were selected. Eleven plants were selected based on their traditional use in treatment for bacterial infection and skin diseases such as acne and hypopigmentation disorders (Table 3.1 and 3.2).

All the selected plants can be found in the Cape Floristic Region (CFR). Located at the South-western tip of South Africa is the biogeographic region known as the CFR, this extends from 31° and 34°30'S latitude (Manning & Goldblatt 2012). Approximately 9 000 species of vascular plants are native to this rich and diverse botanical area. In comparison to other floras of the African region, the CFR, which comprises a land area of 90 000 km^2, is exceptionally species rich, three of the seven largest plant families in the CFR can be found here. The largest families, *Asteraceae* and *Fabaceae,* constitute 20% of the total combined species in this arid region (Manning & Goldblatt 2012).

As the smallest of the six recognised floral kingdoms of the world, the CFR is an area of extremely high and unique diversity and endemism. The CFR is home to 69% endemic vascular plant species from amongst more than 9 000 species (Odendaal *et al.,* 2008). Approximately 45% of the total traditional medicine trade in the Cape comes from the ecologically unique CFR (Petersen 2014) for instance the African potato *(Hypoxis hemerocallidea)*, which is sold for use as a traditional tonic, cosmetics; and wild sage (*Salvia africana-caerulea)* used for respiratory and digestive ailments (van Wyk 2008). However, many of these plants' usages are yet to be confirmed through scientific studies. The remarkable diversity and confined area of the CFR provides an excellent model system for scientific studies (Schnitzler *et al.,* 2011). Cosmetic scientific research criteria in the CFR has also received little attention and there is an evident need for more rigorous investigations (Sonka 2017).

Table 3.1: Medicinal plants that are endemic to South Africa

Plants	**Herbarium number**	**Pigmentation**	**Antibacterial activity**	**Other pharmacological activity reported**	**References**
Hypoestes aristata (Vahl) Roem. & Schult.	PRU 125925	Not tested	Isolated compounds have strong antibacterial activity towards gram-positive and gram-negative bacteria	Compounds have strong antioxidant activity, strong anti-inflammatory activity, weak anti-plasmodial activity, moderate antiviral activity, strong trypanocidal activity	Da Silva *et al.*, 2005; Abrantes *et al.*, 2008; Esperandim *et al.*, 2013; Saeed *et al.*, 2016; Ramabulana *et al.*, 2020
Pelargonium citronellum J.J.A. Van der Walt	PRU 127869	Not tested	Moderate antibacterial activity	Moderate anti-inflammatory activity, moderate antioxidant activity, strong antimalarial activity	Lalli 2005

Table 3.2: Selected plants that are not endemic to South Africa

Plants	**Herbarium number**	**Pigmentation**	**Antibacterial activity**	**Other pharmacological activity reported**	**References**
Barleria obtusa Nees	PRU 125926	Not tested	Not tested	Not tested	Isaacs 2001

Bulbine frutescens (L.) Willd.	PRU 125216	Not tested	Low to no antibacterial activity towards gram-positive and gram-negative bacteria	Strong anti-oxidant activity, weak anti-inflammatory activity, strong wound healing activity, antidiabetic activity	Mocktar 2000; Huyssteen *et al.*, 2011; van Huyssteen *et al.'s* 2011; Ghuman *et al.*, 2016;
Carpobrotus dimidiatus (Haw.) L. Bolus	PRU 125927	Not tested	Weak to no antibacterial activity towards gram-positive and gram-negative bacteria	Weak acetylcholinesterase enzyme inhibition, strong antidiabetic activity, moderate anti-inflammatory activity, moderate anti-oxidant activity, strong mosquito repellent, immunostimulating activity	Hurinanthan 2009; Fawole *et al.*, 2010; Chalannavar *et al.*, 2013; Mulaudzi *et al.*, 2019; Singh *et al.*, 2021
Cotyledon orbiculata L.	PRU 128848	Not tested	MIC value of >500 µg/mL against *C. acnes* (ATCC 11827)	Moderate anthelmintic activity, anticonvulsant activity, high COX-1 and COX-2 inhibition moderate antioxidant activity, antinociceptive activity, immunomodulating activity	Amabeoku *et al.*, 2007; Maja 2009; Aremu *et al.*, 2010; Sharma and Lall 2014; Ondue *et al.*, 2019; Tyavambiza *et al.*, 2021
Cussonia spicata Thunb.	PRU 128851	Not tested	Good to no antimicrobial activity	Good acetylcholinesterase enzyme inhibition, weak anti-inflammatory activity, moderate antioxidant activity, strong antileishmanial, strong anti-malarial activity	Tetyana *et al.* 2002; De Villiers *et al.* 2010; Amoo *et al.*, 2012; Bapela *et al.* 2017

Hypoestes forskaolii (Vahl) R.Br	PRU 127863	Not tested	No antibacterial activity, Weak antifungal activity	Strong antileishmanial activity, strong antitrypanosomal activity, strong antiplasmodial activity towards, moderate anti-cancer activity, moderate antioxidant activity	Mothana *et al.*, 2011; Almehdar *et al.*, 2012; Al Musayeib, *et al.*, 2014
Pelargonium graveolens L'Hér	PRU 128847	Not tested	Moderate antibacterial activity towards gram-positive and gram-negative bacteria	No LOX-5 inhibitory activity, moderate antioxidant activity, strong antimalarial activity	Dorman and Deans 2000; Shin and Lim, 2004; Lalli 2005; Ghannadi *et al.*, 2012
Portulacaria afra Jacq.	PRU 128849	Not tested	MIC value of 8000 µg/mL against *C. acnes* (ATCC 11827)	Strong antioxidant activity, moderate glucose utilisation activity	Sharma and Lall 2014; Nciki *et al.* 2016; Olaokun *et al.*, 2017; Khanyile *et al.*, 2021
Sideroxylon inerme L.	PRU 128850	Moderate tyrosinase inhibition	MIC values of 250 µg/mL against *C. acnes* (ATCC 11827)	Strong to moderate antioxidant activity,	Momtaz *et al.*, 2008; Sharma and Lall's 2014; Shelembe 2014

2. *Barleria obtusa*

2.1.General Description on Selected plants

2.1.1. Botanical nomenclature

Barleria obtusa Nees.

2.1.2. Botanical Family

Acanthaceae (Isaacs 2001).

2.1.3. Vernacular

English: Bush violet, Barleria (Isaacs 2001).

Afrikaans: Bosviooltjie (Isaacs 2001).

Zulu: Idololenkonyane (Isaacs 2001).

Figure 3.1: *Barleria obtusa* (a) shrub, (b) flowers, leaves and (c) flower buds (Ebedes 2021a). (d) The distribution of *Barleria obtusa* in South Africa (GBF Secretariat 2021a).

2.2. Botanical Description

The *B. obtusa* is a multi-stemmed shrublet, with branches that have a decumbent habit giving rise to variable growth size dependent on its different growing environments. In open regions,

it can grow as low as one meter while taking on a bushier appearance, whilst in shady areas such as those at the forest margin, it can reach a height of two meters with long sprawling branches (Isaacs 2001).

When mature and after the seed capsule has become woody, seeds are scattered through a released explosion (Isaacs 2001). Flowers are blue or pink with 0-30 mm wide petals occurring on the upper section of the branch. Each flower consists of a style and two stamens covered with violet hued pollen. The plant's sage green upturned leaves are placed oppositely, are soft with fine translucent hairs on their margins (Figure 3.1).

2.3. Distribution

Barleria obtusa is not endemic to South Africa (Kamundi 2006a). It can be found throughout several South African regions including the Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, and the North West (Figure 3.1). It is also found in Zimbabwe. It favours hillsides and the subtropical regions found along the forest margins (Isaacs 2001).

2.4. Ethnobotanical usage

Barleria obtusa leaves have been reported to be used topically by Xhosa communities for the treatment of rashes and burns. It is used in the form of a paste that is applied to the affected area (Afolayan *et al.*, 2014).

2.5. Phytochemical constituents

Barleria obtusa is the most understudied and undocumented plant in the *Barleria* family. Literature shows a lacuna regarding information on this plant.

2.6.Pharmacological properties

Based on the current available literature, there is a marked absence of recorded experiments carried out on this plant.

2.6.1. Antibacterial activity

Unknown.

2.6.2. Melanogenic activity

Unknown.

2.6.3. Other

Unkown.

2.7. Additional information

There is a dearth of evidence available for *B. obtusa.*

2.7.1. Therapeutic (proposed) usage

Unknown.

2.7.2. Safety data

Unknown.

2.7.3. Trade information

Barleria obtusa is not threatened or endangered, it is abundant and is of least concern (Kamundi 2006a).

2.7.4. Dosage

Unknown.

3. *Bulbine frutescens*

3.1. General Description on Selected plants

3.1.1. Botanical nomenclature

Bulbine frutescens (L.) Willd.

Synonyms include: *Anthericum frutescens* L., *Anthericum fruticosum* Salisb., *Anthericum incurvum* Thunb., *Anthericum rostratum* Jacq., *Bulbine caulescens* L., *Bulbine rostrata* Willd. (Foden & Potter 2005d).

3.1.2. Botanical Family

Asphodelaceae (Harris 2003).

3.1.3. Vernacular names

English: Stalked bulbine, Snake flower, Cat's tail and Burn jelly plant (Harris 2003).

Afrikaans: Balsem kopieva and Geelkatstert (Harris 2003).

Zulu: Ibhucu (Harris 2003).

3.2. Botanical Description

Bulbine frutescens is a hardy fast-growing perennial succulent. It has branched soft green fleshy linear leaves that grow in opposite rows with greyish stems that are clasped at the base of each leaf (Figure 3.2). This plant can be variable in its height and leaf length. It is a vigorous plant that forms clumps that often spread with its adventitious roots. During spring and

summer, the small yellow to orange flowers are star-shaped with 6 petals that grow in an upright cluster formation, known as a spreading raceme. The flowers have a bi-coloured appearance with stamens that are fluffy and yellow. The seeds are easily wind dispersed; they are found in the fruits which are rounded small dry capsules (Harris 2003).

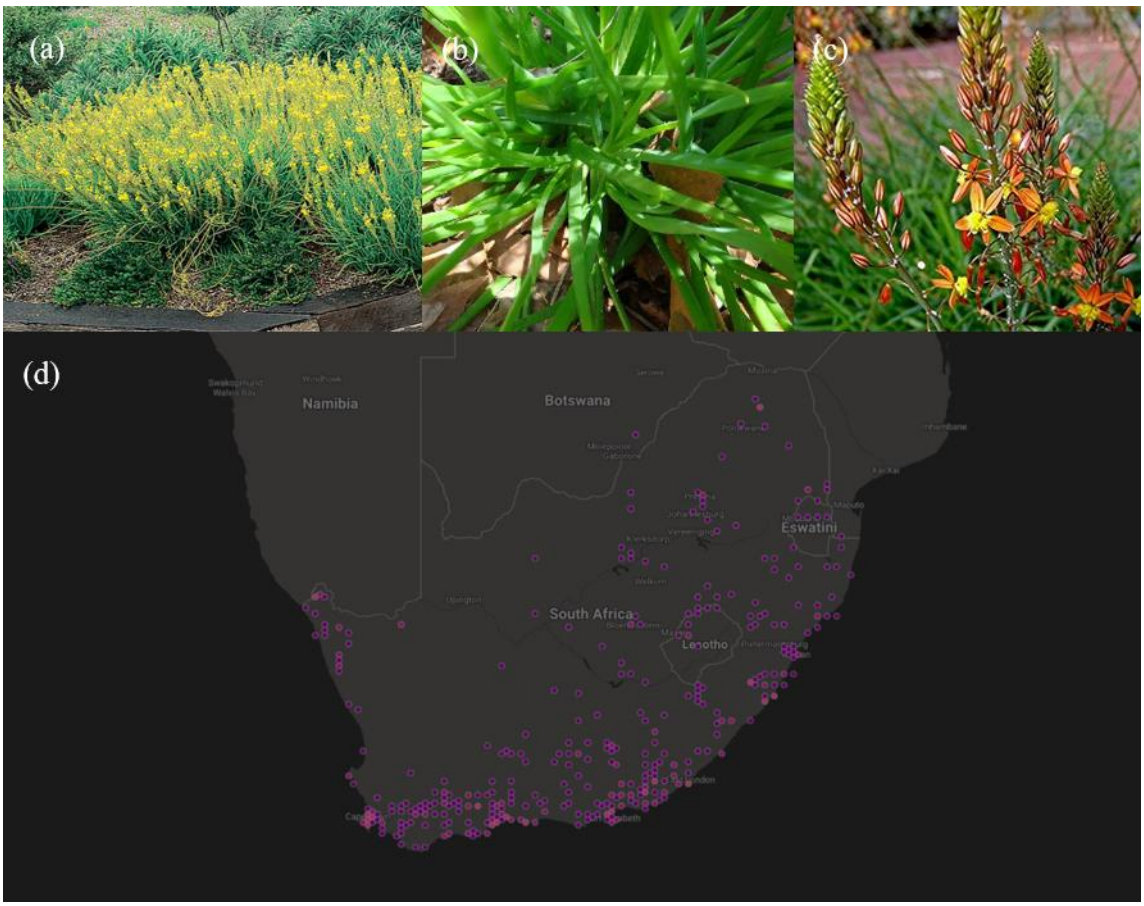

Figure 3.2: *Bulbine frutescens* (a) shrub, (b) fleshy leaves and (c) flowers (Plantinfo 2021). (d) The distribution of *Bulbine frutescens* in South Africa (GBIF Secretariat 2021b).

3.3.Distribution

Although distributed throughout South Africa *B. frutescens* is not endemic to South Africa (Foden & Potter 2005d). Whilst concentrated in the succulent rich Eastern Cape dry-valleys, it is also prolific throughout the Eastern and Western Cape and less abundantly, however still present, throughout the Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape and North West (Harris 2003; Foden & Potter 2005d) (Figure 3.2).

3.4. Ethnobotanical usage

Bulbine frutescens is applied topically for a variety of skin ailments (Mabona & Van Vuuren 2013). Among the Xhosa people of the Western Cape, the leaves are made into a paste and used topically for burns (Afolayan *et al.*, 2014). As is suggested by one of its common names,

the burn jelly plant, the fresh leaf gel is used topically for burns, in addition to its other wound healing uses, including cuts, sores, grazes, itches, rashes, cracked lips, blisters, insect bites, eczema, mouth ulcers and ringworm (Sagbo & Mbeng 2018; Brendler *et al.*, 2010; Pather *et al.*, 2011). It is occasionally used to treat sciatica and herpes (Brendler *et al.*, 2010; SANBI 2010).

A survey performed among Eastern Cape indigenous people established that traditional healers and herbalists make use of the whole plant and dried leaves in a decoction for the treatment of diarrhoea as well as its other topical uses (Stafford 2009; Brendler *et al.*, 2010; Coopoosamy 2011).

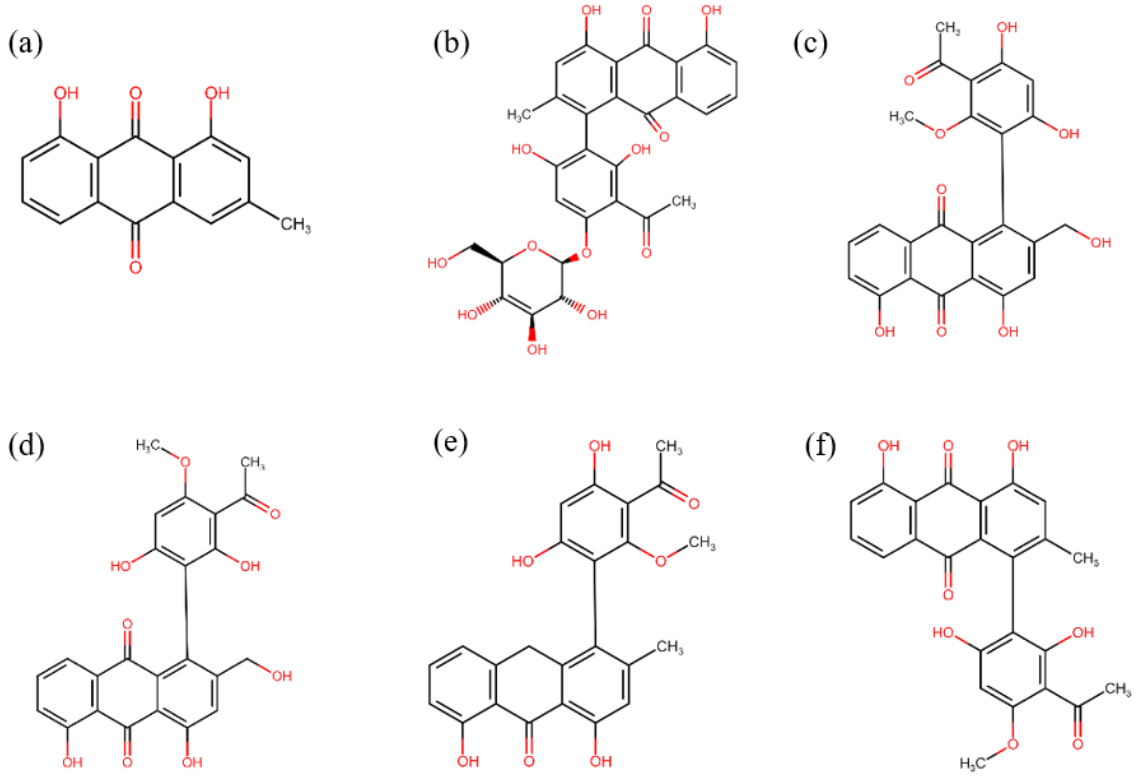

Figure 3.3: The phytochemical structures that have been isolated from Bulbine frutescens (a) chrysophanol, (b) bulbineloneside D, (c) gaboroquinone A, (d) gaboroquinone B, (e) isoknipholone anthrone and (f) knipholone

3.5. Phytochemical constituents

Rachuonyo *et al.*, (2016) performed phytochemical screening and reported that *B. frutescens* contained alkaloids, flavonoids, saponins and tannins. Some agglutinins (lectin-like proteins) have been isolated, however only a partial purification was performed and the precise group-specificity was not obtained for the particular agglutinins (Gaidamashvili & Van Staden 2002). Six compounds (Figure 3.3), namely knipholone, isoknipholone, chrysophanol, gaboroquinones A, gaboroquinones B and bulbineloneside D, have been isolated from the *B.*

frutescens roots (van Wyk *et al.*, 1995; Abegaz *et al.*, 2002). The latter three compounds have been isolated specifically from the roots of *B. frutescens* growing in Botswana (Abegaz *et al.*, 2002).

3.6.Pharmacological properties

3.6.1. Antibacterial activity

Bulnine frutescens was tested against gram-positive bacterium *Actinomycetes brasielensis, B. subtilis, Micrococcus kristinae, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pneumoni*a, and gram-negative bacteria *Enterobacter aerogenes, E. coli, K. pneumonia, Proteus mirabilis, Proteus vulgaris, P. aeruginosa, Shigella sonnei* and *Streptococcus pyogenes*, using the cup-plate method and the disc diffusion method (Coopoosamy, 2011; Ghuman *et al.*, 2016). Coopoosamy (2011) discovered that *B. frutescens* aqueous, acetone and ethyl-acetate extracts of the leaf, root and rhizome showed moderate activity, with MIC values ranging between 2 - 5 mg/mL, against the gram-positive bacterium *B. subtilis, M. kristinae* and *S. aureus*. None of the extracts showed activity against the gram-negative bacterium. The plant material was collected from traditional healers and rural dwellers of the Eastern Cape.

Ghuman *et al.*, (2016) used chloroform, methanol, hexane, dichloromethane (DCM) and acetone for the *B. frutescens* extracts. The leaves, stems and roots used were collected from the Durban area in 2013 and the extracts were made with 1 g ground dry plant matter in 20 mL of extractant. Neomycin was used as the control in the disc diffusion method and had a MIC value range of 0.08 - 0.16 mg/mL. The leaf chloroform extract showed an MIC of 0.63 mg/mL against *B. subtilis, S. pneumonia, S. aureus, S. pyogenes, P. vulgaris, P. aeruginosa* and *P. mirabilis.* The bulb chloroform extracts had an MIC of 0.63 mg/mL against *S. epidermidis, A. brasielensis, P. mirabilis* and *S. sonnei*. The methanolic extracts of the leaves had an MIC of 0.63 mg/mL against *S. aureus* and *P. vulgaris*. Ghuman *et al.*, (2016) defined good activity having a MIC value of < 1 mg/mL. *Bulbine frutescens* was further analysed for its anti-fungal activity against *Trichophyton rubrum, Trichophyton mentagrophytes, C. albicans* and *Candida tropicalis* using the chloroform extract of the leaves. Amphotericin was used as a control and had a MIC value of 0.93 mg/mL. The MIC values were 0.63 mg/mL against *T. mentagrophytes, C. albican*s and *C. tropicalis* and 1.25 mg/mL against *T. rubrum.*

Ghuman *et al.*, (2016) determined methanolic *B. frutescens* had a MIC of 12.5 mg/mL which was not considered prominent against *E. coli.* The plant material used in this study came from

Kenyatta University, Nairobi. It should be noted that these MIC values are considered less than moderate (Van Vuuren & Holl 2017). This was further confirmed in Mocktar (2000) where *B. frutescens*, leaves, roots and stem extracts were tested against the gram-positive bacterium *S. aureus, Enterococcus faecalis, S. epidermidis* and *B. subtilis*, and the gram-negative bacterium *E. coli; Klebsiella oxytoca, K. pneumoniae, P. mirabilis, E. aerogenes, P. aeruginosa, Citrobacter freundii, Morganella morganii, Salmonella typhi* and *Shigella flexneri.* The plant material was collected from the University of Kwa-Zulu Natal, Westville and the extracts were made using a 1:1 methanol: dichloromethane (DCM) solvent. Both the disc diffusion method and the bore well methods were used to determine the antibacterial activity. *Bulbine frutescens* displayed some activity against *C. freundii, E. coli, E. faecalis, K. pneumoniae, M. luteus, M. morganii, P. mirabilis, S. aureus, S. epidermidis, S. typhi* and *S. flexneri.* Mocktar (2000) also tested *B. frutescens* against the fungi *C. albicans, Aspergillus* species and *Penicillium* species using the disc diffusion and the bore well methods for *C. albicans* and a TLC bioassay to test the *Aspergillus* species and *Penicillium* species. The root extract inhibited *C. albicans* and some of the components of the stem extract of *B. frutescens* inhibited the *Aspergillus* species, whilst none of the extracts inhibited the *Penicillium* species

3.6.2. Melanogenic activity

Unknown.

3.6.3. Other

Anti-oxidant and anti-inflammatory activity

Mocktar (2000) determined the anti-inflammatory activity of *B. frutescence* using plant material collected from Kwa-Zulu Natal province. The extracts were made using 1 g of powdered plant material in 20 mL of 50% aqueous methanol. It was reported that *B. frutescens* aqueous methanol leaf extract had a significant anti-inflammatory activity with an IC_{50} of 26.32 µg/mL towards NO inhibition, while quercetin, which was the control, had an of IC_{50} 6.30 µg/mL. *Bulbine frutescens* did not however have a good bioactivity for the LOX inhibition assay as the leaf and root extract had an IC_{50} of > 100 and 81.54 µg/mL, respectively. The anti-oxidant activity of *B. frutescens* leaves proved to have a strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity compared to the roots with and EC_{50} 0.053 and 1.31 µg/mL respectively, while ascorbic acid had an EC_{50} of 0.07 µg/mL. The lower the EC_{50} value indicates the quicker the DPPH radical was lightened, thus indicating its antioxidant potential (Ghuman *et al.*, 2019).

Bulibine frutescens was also assessed for its ability to delay the oxidation of β-carotene in the β-Carotene linoleic acid model system (Ghuman *et al.*, 2019). The leaves and roots had an antioxidant activity of 91 and 78%, respectively, compared to the 76% of butylated hydroxytolulene (BHT). The leaves, roots and BHT had an oxidation reduction rate (ORR) value of 0.09, 0.22 and 0.23, respectively. Thus, it was concluded that *B. frutescens* has a strong antioxidant activity and was significant for the β-Carotene linoleic acid model system. Ghuman *et al.*, (2019) also tested *B. frutescens* for its ferric-reducing antioxidant power (FRAP) which yielded, at 1.0 mg/mL, an absorbance value in the range of 0.24 to 0.57. This is important for its wound healing capabilities as plants with higher anti-oxidant and anti-inflammatory activity are more effective in the healing process.

Wound healing

In Ghuman *et al.,'s* (2019) paper it was reported that *B. frutescens* aqueous methanolic leaf extract was tested using the protein precipitation/binding assay to establish its effectiveness as a wound healing agent. The plant material was collected from the Kwa-Zulu Natal province. The extracts were made using 1 g of powdered plant material in 20 mL of 50% aqueous methanol. *Bulbine frutescens* showed to have a 32% protein-precipitating capacity which was considered moderate. This is important as the wound healing process is extremely intricate. Its many overlapping phases include inhibition microbial infection, reduction in inflammation, the reformation of granular tissues, re-epithelialisation of the wound and remodelling or correction of damaged and wounded areas (Perini *et al.*, 2015).

Pather *et al.*, (2011) tested *B. frutescens* leaf gel on sow skin for its wound healing capabilities. The plant material used was from the Walter Sisulu Botanical Gardens in Roodepoort, Johannesburg. The rate of contracture and period taken for re-epithelialisation was determined, the tensile strength of the treated incisions was measured and the treated tissue was analysed for its collagen, hexosamine, protein and DNA levels. It was found that the wounds treated with *B. frutescens* were completely re-epithelialised by day 4 and by day 10 the treated wounds were indistinguishable from the surrounding normal skin compared to the untreated skin where it occurred by day 16. The tensile strength of the healed incisions was tested and *B. frutescens* had a significantly higher tensile strength at $p = 0.002$ than compared to the untreated wounds. A constituent of collagen is hydroxyproline which was measured to determine the collagen levels. The collagen levels of the treated wounds had a significant difference when compared to the untreated wounds ($p = 0.018$). Throughout the 16-day measuring time period, the treated

wounds had a higher collagen level when compared to the untreated wounds, with the highest being 2.13 times greater on day 10. Hexosamine is used to determine the collagen concentration increases as its levels will decrease when there is an accumulation of collagen. The *B. frutescens* treated wound hexosamine content was 1.07 times higher than the untreated wounds. Over the 16-day period the protein concentration of the *B. frutescens* treated wounds was significantly increased when compared to the untreated wounds ($p = 0.04$) with a mean ratio of 2.02. The final experiment was to measure the DNA content in the treated wounds. The treated wounds were significantly different ($p = 0.04$) when compared to the untreated wounds and had a mean ratio of 1.77. An indication of collagen synthesis can be determined by the ration between the collagen content and the DNA content. There was however found to be no significant difference between untreated and treated wounds with regards to this ration ($p = 0.37$). The study by Pather *et al.*, (2011) determined that *B. frutescens* had a beneficial effect on the synthesis collagen which in turn shows a beneficial effect on wound contraction and faster healing. It was also found that there was an increase in the myofibroblast presence which indicates a promotion of cell proliferation along with the collagen deposition.

Cytotoxicity

Bulbine frutescens cytotoxic activity was tested on Chang liver cells in van Huyssteen *et al.*, (2011). The plant material was collected from the Nelson Mandela Metropolitan area and the fresh material was macerated separately in water and 99% ethanol. The ethanolic extract had a 33.3% growth inhibition of Chang liver cells at a concentration of 62.5 µg/mL.

Kushwaha *et al.*, (2019) investigated *B. frutescens'* ability to be effective against breast cancer due to the presence of flavonoids and terpenoids. The plant material was collected from Farm Vredelus in Namibia. Fresh bulbs were used and were macerated in the extractants hexane and methanol, separately. The methanol and hexane extracts were assessed for their potential to inhibit the hormone dependent breast cancer cell lines (T47D and MDA-MB-231) using the MTT assay. The methanolic *B. frutescens* inhibited the T47D and the MDA-MB-231 cell lines by 53% at 10 µg/mL and had IC_{50} values of 6.35 and 4.88 µg/mL, respectively. The hexane *B. frutescens* inhibited the T47D and the MDA-MB-231 cell lines by 63 and 75%, respectively, and had IC_{50} values of 12.08 µg/mL and 13.12 µg/mL, respectively.

Anti-diabetic

In van Huyssteen *et al.,'s* (2011) paper, the whole plant aqueous and ethanolic extracts of *B. frutescens* was tested for its capability to aid in the glucose utilisation in C2C12 muscle cells

and Chang liver cells. The plant material was collected from the Nelson Mandela Metropolitan area and the fresh material was macerated separately in water and 99% ethanol. Insulin was used as the control. With regards to the C2C12 cells, both aqueous extracts, at 0.5 and 50 µg/mL, increased glucose utilisation more than insulin at 130.1 and 121.3%, respectively. The ethanolic extracts at the same concentrations showed a 128 and a 123%, increase, respectively. It was however not concentration dependent. At a concentration of 50 µg/mL the ethanolic extract was significantly active when combined with insulin which suggested a possible additive or synergistic effect when combined with insulin. With regards to the Chang liver cells, *B. frutescens* aqueous extract at 0.5 µg/mL significantly increased the glucose uptake into the cells at 143.5%. The aqueous extracts also showed a significant concentration independent response, $p < 0.0001$. The ethanolic extracts did not have an uptake of glucose more than insulin at the concentrations tested.

3.7. Additional information

3.7.1. Therapeutic (proposed) usage

Antimicrobial, anti-inflammatory, antioxidant and as wound healing.

3.7.2. Safety data

No official safety data is available however Mocktar (2000) used a brine shrimp to assay to determine the toxicity of *B. frutescens* with the leaves and stems showing a degree of toxicity, whilst the roots showed no toxicity.

3.7.3. Trade information

B. frutescens is not threatened, not endangered, abundant and is of least concern (Foden & Potter 2005d).

3.7.4. Dosage

No specific concentration has yet been determined for the use of *B. frutescens*.

4. *Carpobrotus dimidiatus*

4.1. General Description on Selected plants

4.1.1. Botanical nomenclature

Carpobrotus dimidiatus (Haw.) L. Bolus (Nonjinge 2007).

Synonyms: *Carpobrotus juritzii* (L.Bolus) L.Bolus, *Mesembryanthemum dimidiatum* Haw., *Mesembryanthemum juritzii* L.Bolus.

4.1.2. Botanical Family

Aizoaceae (Nonjinge 2007).

4.1.3. Vernacular names

English: Natal sour fig (Nonjinge 2007).

Afrikaans: Natalse suurvy/strandvy (Nonjinge 2007).

Zulu: Ikhambi lamabulawo (Nonjinge 2007).

4.2. Botanical Description

Carpobrotus dimidiatus is a robust, trailing plant with fleshy, green, three-sided leaves that spreads over large areas creating a tough ground cover that is resistant to drought and salt spray. It bears single vygie (mesembryanthemums) flowers that are large, shiny, and mauve on relatively erect flowering branches (Figure 3.4). Each flower has a base that is sharply 2-ridged.

The fleshy walls of the top-shaped fruit become dry and tough and do not open. To reach the slimy, sourish pulp and small, brown, shiny seeds the fruit has to be bitten or cut open. The seeds are dispersed by animals after consuming the fruit (Nonjinge 2007).

Carpobrotus dimidiatus does not grow particularly high, only averaging between 15 and 30 centimetres, but spreads outwards providing a dense cover over a large ground area. The evergreen succulent leaves are sword-shaped and not particularly long while the flower has thin, needle-like, pink to purple-pink petals (SA Venues 2021).

4.3. Distribution

Whilst not endemic to South Africa, this plant can now be found on the east coast sand-dunes ranging northwards from the Eastern Cape through KwaZulu-Natal as far as Mozambique (Nonjinge 2007). In addition to being found in Kwa-Zulu Natal it covers the sand dunes of the Eastern Cape and up into Mozambique (Burgoyne 2006; SA Venues 2021) and can be found contributing a unique scrubby type of beauty to the biodiversity of coastlines (Figure 3.4).

4.4.Ethnobotanical usage

Although sourish to taste, the fruit can be eaten raw in its natural state or dried, preserved or even made into a jam. As a syrup it is reputed to have a laxative effect. In all its forms it is considered a good source of catechol tannin (Watt & Breyer-Brandwijk 1962).

Amongst its myriad remedies for a range of ailments, the strained juice extracted from the pounded leaf is utilised as a sore throat or thrush gargle, for diarrhoea and dysentery and for

digestive troubles and also toothache (Fawole *et al.*, 2010). The leaf extract and pulp are generally used with only the fruit and stem being occasionally used (Malan & Notten 2006). The astringent and mildly antiseptic leaf juice is mixed with water and ingested as a treatment for diarrhoea, dysentery and stomach cramps and used as a gargle for laryngitis, sore throat and infections. Malan and Notten (2006) note that it is also used for diabetes control and diphtheria in the Eastern Cape region.

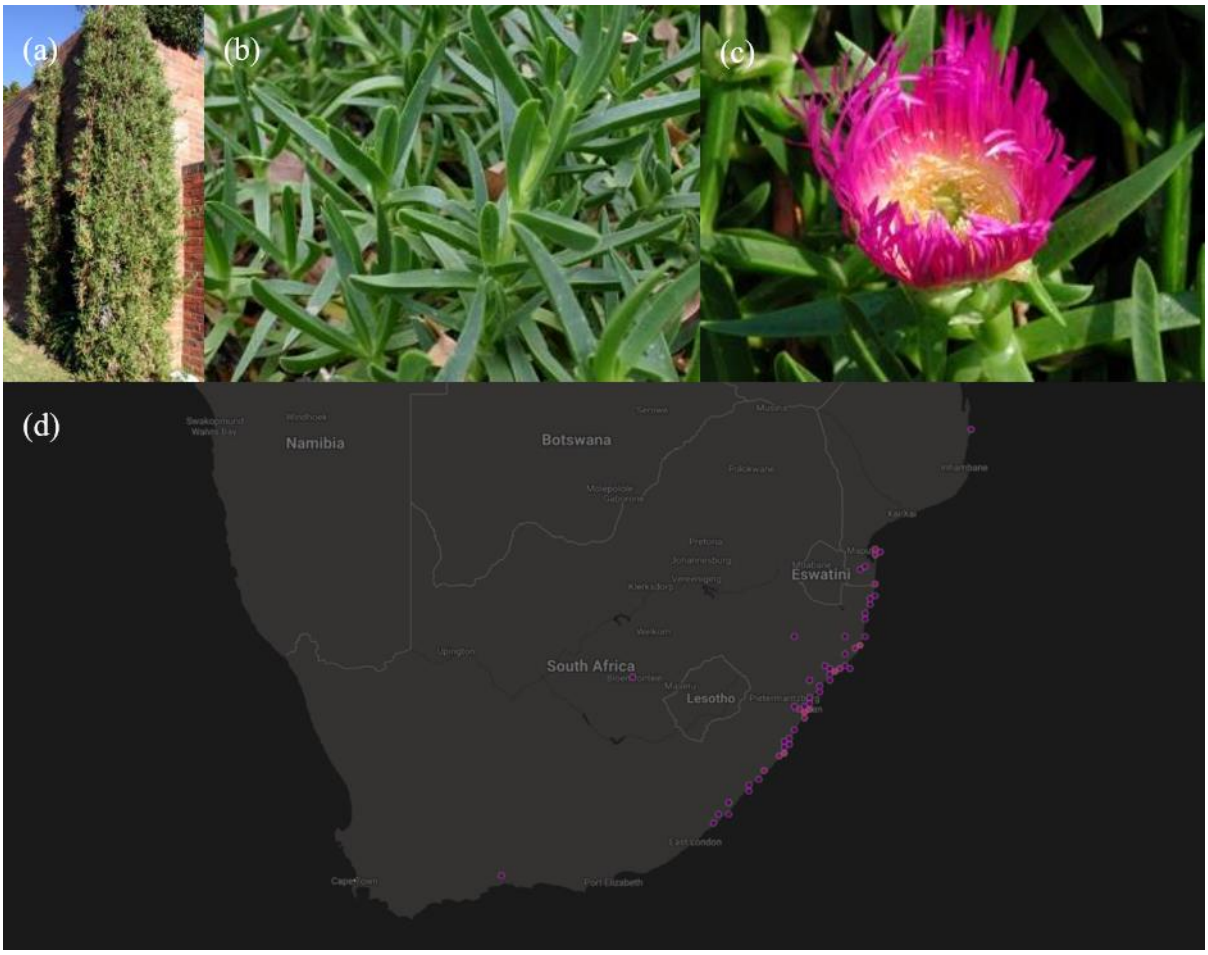

Figure 3.4: *Carpobrotus dimidiatus* (a) dangling down a wall, (b) fleshy leaves and (c) flower (Nonjinge 2007). (d) The distribution of *Carpobrotus dimidiatus* in South Africa (GBIF Secretariat 2021c).

The juice of the leaf is rendered as a lotion and applied to burns bruises, scrapes, cuts, grazes and sunburn, ringworm, eczema, dermatitis, herpes, nappy rash, thrush, cold sores, cracked lips and chafing skin allergic reactions or is melted to make an ointment for the treatment of wounds and burns. The KwaZulu-Natal National Botanical Garden distributes 10-15 bags of this plant on a monthly basis to various organisations, for example CINDI (Children in Distress Network), Lifeline and PADCA (Pietermaritzburg & District Association for the Care of the Aged) to assist with skin problems, sores and rashes (Watt & Breyer-Brandwijk 1962). Watt

and Breyer-Brandwijk (1962) attribute its effective soothing effect on blue-bottle stings to its malic acid, citric acid and other calcium salts content.

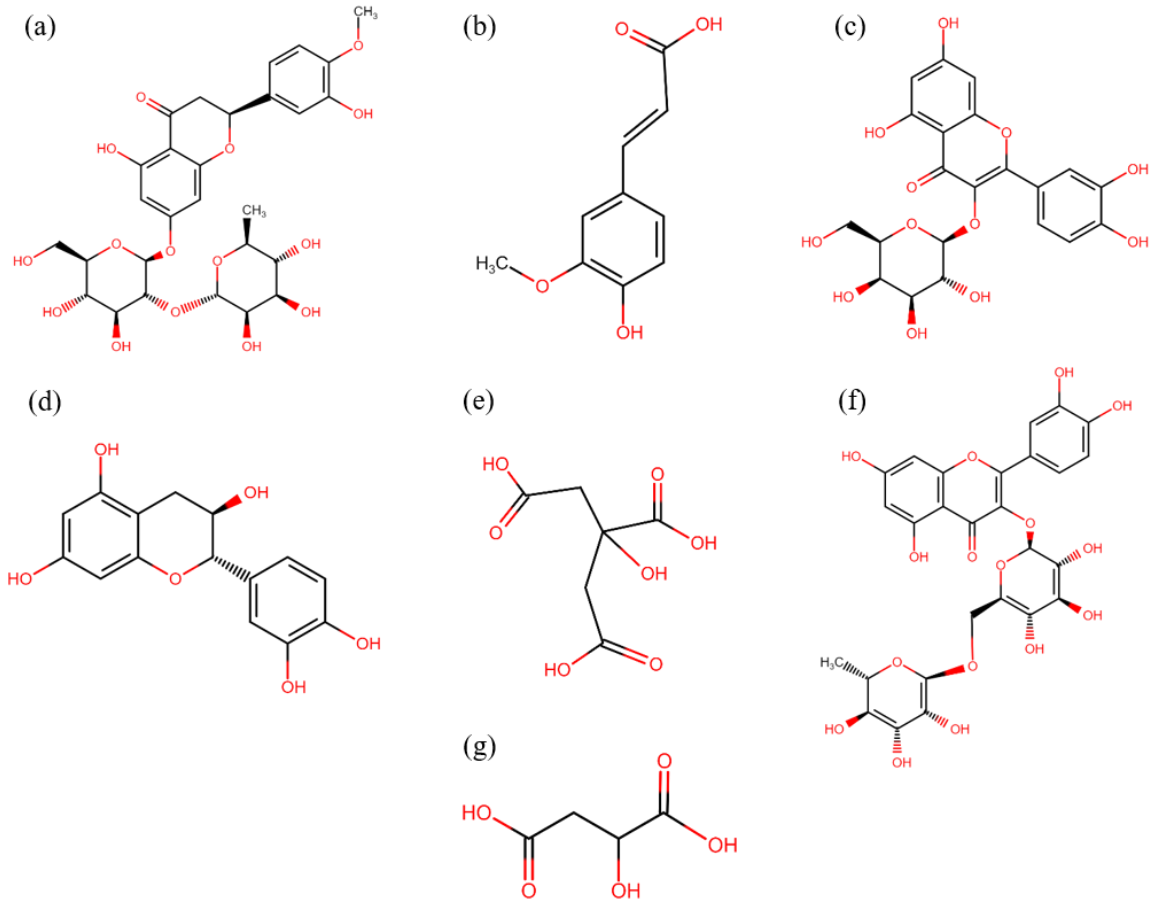

Figure 3.5: The phytochemical structures that have been isolated from *Carpobrotus dimidiatus* (a) catechin, (b) hyperoside, (c) ferulic acid, (d) neohesperidin, (e) malic acid, (f) rutin and (g) citric acid.

4.5. Phytochemical constituents

Low levels of phenols (< 20 mg GAE/g dry matter), flavonoids (> 10 mg CE/g dry weight) and condensed tannins (< 6% DW in LUE) were found in *C. dimidiatus* (Mulaudzi *et al.*, 2019). Seven compounds have been isolated in *C. dimidiatus* (Figure 3.5); catechin, hyperoside, ferulic acid, neohesperidin, malic acid, rutin and citric acid (Springfield *et al.*, 2003; Hurinanthan 2009; Singh 2016).

4.6.Pharmacological properties

4.6.1. Antibacterial activity

Carpobrotus dimidiatus was tested for its inhibitory activity against *Mycobacterial* strains; *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* H37Rv, clinical isolate multidrug-resistant (MDR) *M. tuberculosis* (ATCC 25177) and clinical isolate extensively drug-resistant

(XDR) *M. tuberculosis* (Singh *et al.*, 2021). The plant material was collected from the University of KwaZulu-Natal, Westville. The dried leaves were extracted separately in water and methanol. Using the micro-dilution assay, it was shown that the aqueous extracts had no effect on the *Mycobacterial* strains, whereas the methanolic extracts had an MIC of 0.13 mg/mL against both *M. smegmatis* and *M. tuberculosis* H37Rv, and an MIC of 0.50 mg/mL against MDR- *M. tuberculosis* and XDR- *M. tuberculosis.* The controls used were Rifampicin and Isoniazid, against which both MDR- *M. tuberculosis* and XDR- *M. tuberculosis* are resistant.

Carpobrotus dimidiatus aqueous and methanolic extracts were tested, using the disc diffusion method, against gram-positive bacterium *Bacillus cereus, S. epidermidis, S. aureus, Bacillus stearothermophilus* and *Micrococcus* spp, and gram-negative bacterium *E. coli, P. aeruginosa, K. oxytoca, S. typhimurium* and *Serratia marcescens.* The plant material was collected from the Durban area of KwaZulu Natal and the extracts were made using 1 g of powdered plant material to 4 mL of 80% methanol and water, separately. The control used was Ciprofloxacin. *Carpobrotus dimidiatus* showed no activity against *B. stearothermophilus, S. aureus, S. epidermis, S. marcescens* and *Micrococcus sp*. at 1000 µg/mL. The methanolic and aqueous extracts at 1000 µg/mL showed activity against gram-positive bacterium *B. cereus* and gram-negative bacterium *E. coli, P. aeruginosa, K. oxytoca and S. typhimurium* (Hurinanthan 2009).

In Hurinanthan (2009), *C. dimidiatus* aqueous and methanolic extracts were also tested against *Aspergillus niger*, *Cladosporium* sp, *Fusarium* sp., *Geotrium* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp., and against the yeast *Saccharomyces cerevisiae* and *C. albicans*. Amphotericin B served as the control in the paper disc method used. The extracts only showed antifungal activity against *Trichoderma* sp. at 1000 µg/mL.

4.6.2. Melanogenic activity

Unknown.

4.6.3. Other

Acetylcholinesterase enzyme inhibition

In Fawole *et al.*, (2010), *C. dimidiatus* showed some level of inhibitory activity against the acetylcholinesterase enzyme (AChE), however it was not considered good inhibition (> 50%), as they were all below 40%. The plants used were from Kwa-Zulu Natal Botanical Gardens in Pietermaritzburg. The extracts were made using 1 g grounded dry plant material in 20 mL sequentially 50% methanol, petroleum ether, dichloromethane (DCM) and ethanol.

Galanthamine was used as the control. The DCM extract of *C. dimidiatus* did not show a dose-dependent activity. This was supported in Mulaudzi *et al.*, (2019) who also reported a low AChE inhibitory activity (< 10%).

Antidiabetic

Carpobrotus dimidiatus was tested for its alpha glucose inhibitory activity using aqueous-methanolic, acetone and aqueous extracts. The aqueous and aqueous-methanolic extracts had IC_{50} values of 0.652 and 0.144 mg/mL, respectively. When compared to acarbose, the positive control, (IC_{50} 0.429 mg/mL), suggests that the aqueous-methanolic extract showed strong activity as a possible alpha glucose inhibitor (Mulaudzi *et al.*, 2019). In this study the plants were collected from the Research Station, Pretoria and the Botanical Garden of KwaZulu-Natal, Pietermaritzburg.

Anti-inflammatory and anti-oxidant

The anti-inflammatory activity of *C. dimidiatus* was determined by assessing its lipoxygenase (LOX) enzyme inhibition. The 15-LOX model was used to determine the inhibition activity. The plants were collected from the Research Station, Pretoria and the Botanical Garden of KwaZulu-Natal, Pietermaritzburg. The aqueous-methanolic, acetone and aqueous extracts of *C. dimidiatus* showed a potential to manage inflammatory conditions, however they were not significant against 15-LOX (Mulaudzi *et al.*, 2019). In Fawole *et al.*, (2010) it was found that aqueous-methanolic, petroleum ether, dichloromethane (DCM) and ethanolic extract showed poor COX-1 inhibition, < 50% inhibition activity, whilst the aqueous-methanolic extract showed a good COX-2 inhibition at 65%, where a minimum of 50% is considered as good activity.

The β-Carotene–linoleic acid model assay was used to assess the antioxidant activity, where by the acetone and aqueous extracts used of *C. dimidiatus* showed a high lipid peroxidase activity of > 70% (Mulaudzi *et al.*, 2019). DPPH radical scavenging assay determined that the aqueous-methanolic extract of *C. dimidiatus* had an EC_{50} of 9.2 µg/mL. In Fawole *et al.*, (2010) the aqueous-methanolic extract had an EC_{50} of 7.63 µg/mL in the DPPH radical scavenging assay. The positive control ascorbic acid had an EC_{50} of 5.06 µg/mL. *Carpobrotus dimidiatus* was also assessed for its ferric-reducing power, it was shown to have a dose-dependent reducing activity, however it did not have an absorbance higher than ascorbic acid (Fawole *et al.*, 2010).

Anti-mosquito

In studies performed by Chalannavar *et al.*, (2013) and Hurinanthan (2009), *C. dimidiatus* was examined for its insecticidal activity against *Anopheles arabiensis,* which transmit malaria via their bite. The plant material was collected from the Durban area of KwaZulu Natal. The extracts were made using 1 g of powdered plant material to 4 mL of 80% methanol and water, separately. In a 7-day trial, *C. dimidiatus* extracts showed no larvicidal activity on the *A. arabiensis* larvae and all larvae developed to the pupae stage and then the adult mosquito stage normally. An organophosphate, Mostop, was used as the control. None of the extracts displayed inhibition or mutagenic activity. *Mastomys coucha*, a rodent, was used to determine the repellency of the extracts when applied topically. The aqueous and methanolic extracts showed to have a repellence of 97 and 87%, respectively, however no knockdown was seen in the two minutes and mortality was seen in the 24-hour period. Knockdown refers to a paralysis that can be rapidly reversed. In the insecticidal assay, the knockdown percentage was measured during the first 30 minutes and the first 1 hour after being exposed to the extract. K-Othrine was used as the control. In the first 30 minutes, the aqueous and methanolic extracts showed a 12 and 15% knockdown, respectively. In the first 1 hour, the aqueous and methanolic extracts showed a 43 and 15% knockdown, respectively. After 24 hours, a mortality rate of 42 and 33% was observed for the aqueous and methanolic extracts, respectively. The results obtained from Hurinanthan (2009) suggests that the leaves of *C. dimidiatus* could hopefully be used as a potential mosquito repellent against *A. arabiensis.*

Cytotoxicity

The cytotoxicity of *C. dimidiatus* was determined against three cell lines RAW 264.7 murine macrophage, Vero (African green monkey kidney) and HepG2. The results were analysed using the MTT reduction assay. The aqueous-methanolic extract had an LC_{50} of > 1000 µg/mL towards the RAW264.7 and the Vero kidney cell line and an LC_{50} of 826.15 µg/mL on the HepG2 liver cells. The acetone extracts had an LC_{50} of 244.70, 176.93 and 160.86 µg/mL towards the RAW264.7, Vero kidney and HepG2 liver cell lines, respectively, and aqueous extracts had an LC_{50} 420.57, >1000 and 69.58 µg/mL, respectively. Overall, the extracts had very low cytotoxic activity, except the aqueous extract against the HepG2 liver cell line, which was considered moderate (Mulaudzi *et al.*, 2019).

Carpobrotus dimidiatus was tested for its cytotoxicity against human peripheral blood mononuclear and mouse BALB/C monocyte macrophage cell lines. The plant material was

collected from the University of KwaZulu-Natal, Westville and a methanolic extract was prepared from fired leaves. The methanolic extract was not toxic towards the mouse BALB/C monocyte macrophage cell lines and stimulated the growth of the human peripheral blood mononuclear cell line at the highest concentration tested, 1000 µg/mL (Singh, 2016).

When tested against the Human erythroleukemia cell line (K562), the extract showed a stimulatory effect in a concentration dose dependent manner from 10 to 1000 µg/mL. The plant material was collected from the Durban area of KwaZulu Natal. The extracts were made using 1 g of powdered plant material to 4 mL of 80% methanol and water, separately (Hurinanthan 2009).

Immunomodulation

The immune system can be altered by either immunostimulating or immuno-suppressing agents. Immunomodulating agents from plants and animals can increase the body's immune responsiveness against pathogens via the activation of the nonspecific immune system. Thus, by investigating phytochemical constituents, specific stimulatory, suppressive or regulator effects can be induced on the immune system. Hurinanthan (2009) examined the lymphocyte activation when exposed to *C. dimidiatus*. An MTT assay was used to determine lymphocyte viability, it was determined that the *C. dimidiatus* aqueous and methanolic extracts stimulated the proliferation of the human peripheral blood mononuclear cells in a dose dependent manner. At a concentration of 250 µg/mL, both the extracts showed a higher level of proliferation than compared to the untreated cells, with the methanolic extract having a slightly higher activity than the aqueous extract. *Carpobrotus dimidiatus* was also shown to stimulate lymphocytes, at 250 µg/mL, to the same degree of the positive control Concanavalin A (20 µg/mL), which is known for its activation of lymphocytes. At the optimum concentration of 250 µg/mL, it was also determined that the extracts were found to stimulate the secretion of Interferon-ɣ (INF-ɣ) and Interleukin-10 (IL-10). *Carpobrotus dimidiatus* results indicate that the lymphocytes were directly enhanced with a significant increase in the IL-10 secretion and a slight elevation in the secretion of cytokine INF-ɣ. The methanolic extract had an IL-10 secretion level of 540 pg/mL at 250 µg/mL, which was similar to those of the controls Concanavalin A with 545 pg/mL at 20 µg/mL and Phytohaemagglutinin with 545 pg/mL at 250 µg/mL. Upon further investigation it was found that *C. dimidiatus* had a significant and direct elevation of the activated peripheral blood mononuclear cells, T cells, activated T cells, $CD4^+$ cells, $CD8^+$ cells and the total B cells, whereas there was a decrease in the NK cells (Hurinanthan 2009).

4.7. Additional information

4.7.1. Therapeutic (proposed) usage

Antidiabetic, mosquito repellent, anti-inflammatory and as an immune system altering agent.

4.7.2. Safety data

No official safety data is available however, in Singh, *et al.*, (2021), *C. dimidiatus* had an average of > 20% brine shrimp death in the brine shrimp lethality assay; however, this is considered minimal to non-existent. In Hurinanthan (2009), the plant showed no toxic effect on the brine shrimp. *Carpobrotus dimidiatus* was tested for its mutagenic potential against the *S. typhimurium* TA 98 and TA 100 strains using the Ames assay, towards which *C. dimidiatus* showed no mutagenicity up to 1000 µg/mL.

4.7.3. Trade information

Carpobrotus dimidiatus is not threatened, not endangered, is abundant and of minimal concern (Burgoyne 2006).

4.7.4. Dosage

No specific concentration has yet been determined for the use of *C. dimidiatus.*

5. *Cotyledon orbiculata*

5.1. General Description on Selected plants

5.1.1. Botanical nomenclature

Cotyledon orbiculata L.

5.1.2. Botanical Family

Crassulaceae.

5.1.3. Vernacular names

English: Pig's ears, Cotyledon (Harris 2004).

Afrikaans: Plakkie, Platjies, Varkoorblare, Varkoor, Kouterie (Harris 2004).

Xhosa: Imphewula (Harris 2004).

Zulu: Ipewula (Harris 2004).

5.2. Botanical Description

Cotyledon orbiculata is a succulent with thick leaves which vary in colour from green to grey with a red line around their margin (Figure 3.6). Variances in leaf and flower shape differentiate

the five varieties of *C. orbiculata.* These differences between leaf size, shape and colour are in their turn influenced by the plant's immediate environment. In the Western Cape, which has winter rainfall areas, the plant flowers in midsummer, whereas in other regions this occurs mostly between June and August, winter. The plant has predominantly orange-red, with some yellow, hanging, tubular/bell-shaped flowers that are clustered at the ends of an elongated flower stalk (Harris 2004).

Figure 3.6: *Cotyledon orbiculata* (a) clusters, (b) fleshy leaves and (c) flowers (Ebedes 2021b). (d) The distribution of *Cotyledon orbiculata* in South Africa (GBIF Secretariat 2021d).

5.3. Distribution

Cotyledon orbiculata is endemic to South Africa (Foden & Potter 2005c). Whilst mostly confined to rocky outcrops in grassland, fynbos and in the Karoo regions, *C. orbiculata* is still widespread throughout South Africa. Although the plant is tolerant of moderate frosts, those planted in unprotected areas can have their flowers damaged by black frost (Figure 3.6) (Harris 2004).

5.4.Ethnobotanical usage

The leaves of this plant are readily available in the marketplace or can be easily collected as needed and is widely used for a variety of medicinal purposes. It is recognisable by its bright green to grey-green leaf with characteristic red margin, its fleshy texture and faint odour.

The fleshy part of the peeled leaf can be applied to plantar warts, warts and corns where it is held in place with adhesive plaster or may be used warm as a poultice. This results in the hard tissue softening allowing it to be easily removed after treatment of a few days. The same can be applied to boils, abscesses and skin eruptions. Fresh warm leaf juice may be used as drops for earache, applied to the skin as an acne lotion or used locally on a painful tooth. Leaf juice is taken orally to treat epilepsy and as a decoction as an enema for syphilis (Hutchings *et al.*, 1996; van Wyk *et al.*, 1997; SANBI 2009).

5.5. Phytochemical constituents

The total phenolic content found in the methanolic extracts leaves and stems was < 10 mg GAE/g dry matter and > 10 mg GAE/g dry matter, respectively. The gallotannin concentration in the methanolic extracts leaves and stems was < 5 µg GAE/g dry matter and < 10 µg GAE/g dry matter, respectively. The condensed tannin content found in methanolic extracts leaves and stems was < 0.1% LCE/g dry matter. The flavonoid concentration in methanolic extracts leaves and stems was < 0.1 µg CTE/g dry matter and < 0.4 0.1 µg CTE/g dry matter, respectively (Aremu *et al.*, 2010). Four phytochemical constituents have been isolated in *C. orbiculata*, namely orbicusides A, D and C, and tyledoside D (Figure 3.7) (Steyn *et al.*, 1986).

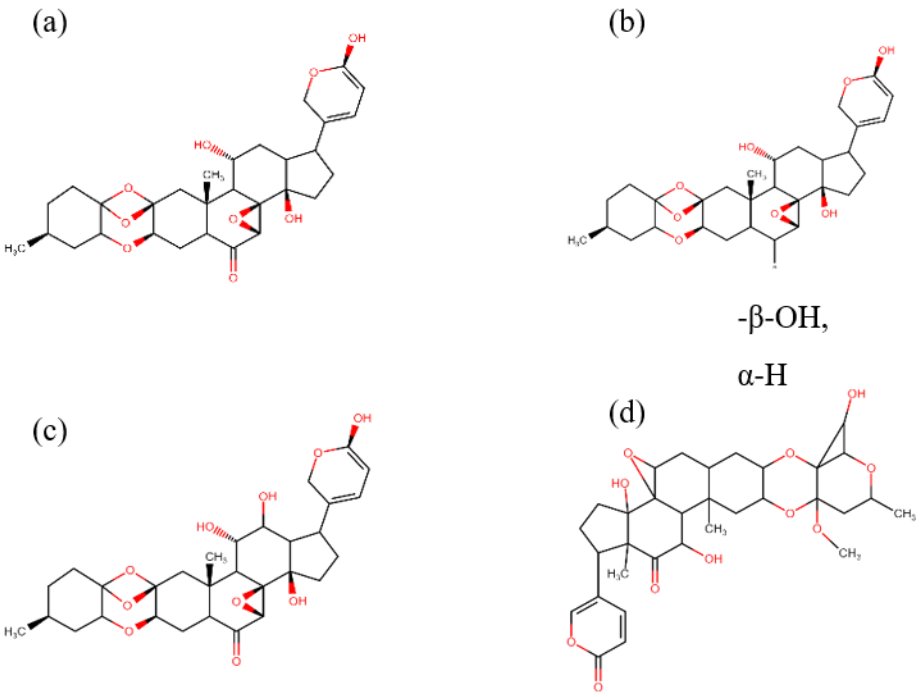

Figure 3.7: The phytochemical structures that have been isolated from *Cotyledon orbiculata* (a) orbicuside A, (b) orbicuside B, (c) orbicuside C and (d) tyledoside D.

5.6. Pharmacological properties

5.6.1. Antibacterial activity

In Sharma and Lall (2014) *C. orbiculata* was tested for its antibacterial effect using the antibacterial microdilution assay against *C. acnes* (ATCC 11827). The plant material was collected from the Botanical Garden of the University of Pretoria. One gram of powdered plant material was placed in 3.75 mL ethanol extractant. The ethanolic extract of *C. orbiculata* leaves had an MIC >500 µg/mL. The positive drug control, tetracycline, had an MIC of 3.1 µg/mL.

Kumari *et al.*, (2016) tested the various parts of the plant throughout different stages of propagation and growing in differing media. The plant material was collected from the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg. These extracts were tested on the gram-positive bacterium *E. faecalis*, *Micrococcus luteus* and *S. aureus*, and gram-negative bacterium *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa*. The leaf extracts of the mother plant (which was not subject to altered enhanced growth media) was not active against any of the bacterium.

Maja (2009) tested *C. orbiculata* against the gram-negative bacterium *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivitis* and *Tannerella forsythensis* using the micro-dilution assay. The plant material was collected from the Tip Top nursery in Pretoria North, and the extracts were made using 1 g powdered plant material in 100 mL ethanol. The recorded MIC values were 0.1 mg/mL against *A. actinomycetemcomitans* and 10 mg/mL against both *P. gingivitis* and *T. forsythensis.* The control, chlorohexidine gluconate, had an MIC of 2.5 x10^{-3} mg/mL against the three bacteria.

Tyavambiza *et al.*, (2021) analysed the antimicrobial activity of *C. orbiculata*-AgNPs and aqueous extracts against the gram-positive bacteria *S. aureus*, Methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis*, the gram-negative bacteria *P. aeruginosa* and the yeast *C. albicans.* The plant material was collected from Van Der Berg Garden Village nursery in Stellenbosch, Cape Town. The fresh leaves were macerated in water. The micro-dilution assay was used and the controls used were ampicillin and fluconazole. The aqueous extracts did not show any antimicrobial activity against the selected pathogens. The *C. orbiculata*-AgNPs had MIC of 20 µg/mL towards *S. aureus* and *S. epidermidis*, 40 µg/mL towards MRSA, 5 µg/mL and 80 µg/mL *C. albicans.* The difference in antimicrobial activity observed with the *C. orbiculata*-AgNPs can be due to the large surface area of the AgNPs, which allows for greater

contact with the micro-organism cell walls (Tyavambiza *et al.*, 2021). Aremu *et al.*, (2010) also found *C. orbiculate* leaf and stem extracts to not have any significant antimicrobial activity.

5.6.2. Melanogenic activity

Unknown.

5.6.3. Other

Anthelmintic activity

Aremu *et al.*, (2010) analysed the effect of *C. orbiculata* on the viability of the nematode larvae *aenorhabditis elegans*. The plant material was collected from the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg. The extracts were made using 1 g ground plant material in 10 mL petroleum ether, ethanolic and dichloromethane (DCM), separately. This study used an *in vitro* colourmetric assay with levamisole as the control. The petroleum ether, ethanolic and DCM leaf extracts had a minimum lethal concentration (MLC) of 0.26 mg/mL, 0.26 and 1.04 mg/mL, respectively. An MLC of < 1mg/mL is considered as having a high anthelmintic activity and between 1 mg/mL and 4 mg/mL is considered moderate. The stem extracts showed to have low lethality against *C. elegans.*

Anticonvulsant activity

Amabeoku *et al.*, (2007) conducted a study to determine the anticonvulsant activity of *C. orbiculata*. The plant material was collected from the Kirstenbosch National Botanical Garden, Cape Town. Aqueous and methanolic extracts were investigated on male albino mice for seizures induced by bicuculline, *N*-methyl-$_{DL}$-aspartic (NMDLA), pentylenetetrazole (PTZ) and picrotoxin. The mice were first observed for 30 minutes period where seizures were induced with bicuculline (40 mg/kg, i.p.), NMDLA (400 mg/kg, i.p.), picrotoxin (12 mg/kg, i.p.) and PTZ (95 mg/kg, i.p.). The mice were pre-treated with *C. orbiculata* and then administered with the convulsant agents and the plant extract's ability to delay or prevent the seizures was measured. For seizures induced by PTZ, the aqueous and methanolic extracts significantly prolonged the onset (50–400 mg/kg, i.p., protected 25% of the mice and 100–400 mg/kg, i.p., protected 62.5% of the mice). For seizures induced by bicuculline the aqueous and methanolic extract significantly delayed the onset of convulsions (100-200 mg/kg, i.p., protected 37.5% of the mice and 100-200 mg/kg, i.p., protected 75%). For seizures induced by picrotoxin the aqueous extract significantly prolonged the onset of convulsions (100-200 mg/kg, i.p., protected 50% of the mice and 100-200 mg/kg, i.p., protected 25% of the mice). For seizures induced by NMDLA the aqueous extract significantly delayed the onset of

convulsions (100-200 mg/kg, i.p., protected 0% of the mice and 100-200 mg/kg, i.p., protected 0% of the mice). Amabeoku *et al.*, (2007) suggested that *C. orbiculata* could be affecting the GABAergic and glutaminergic mechanism to exert its anticonvulsant and delaying activity.

Anti-inflammatory and antioxidant

Cotyledon orbiculata was tested for its inhibition against the COX-1 and COX-2 enzymes, at 250µg/mL, by Aremu *et al.*, (2010). COX-1 and COX-2 enzyme assays was used and indomethacin was used as the control. It was noted that > 70% is considered high inhibition, 70 - 40% is considered moderate inhibition, 40 - 20% is considered low inhibition and < 20% is considered insignificant inhibition. The petroleum ether, DCM, ethanolic and aqueous leaf extracts had 97, 93, 67 and 15% inhibition against COX-1, respectively, and the stem stem extracts had 0, 62, 97 and 42% inhibition, respectively. The petroleum ether, DCM, ethanolic and aqueous leaf extracts had 85, 80, 66 and 0% inhibition against COX-2, respectivelyand the stem extracts had 32, 46, 71 and 25% inhibition, respectively (Aremu *et al.*, 2010). The LOX-15 inhibition assay showed that *C. orbiculata* acetone extract had an IC_{50} of 18.10 µg/mL, compared to the control Quercetin which had an IC_{50} of 24.60 µg/mL (Ondua *et al.*, 2019).

Amabeoku and Kabatende (2012) tested the anti-inflammatory activity of *C. orbiculata* on carrageenam-induced paw oedema. The plant material was collected from the Kirstenbosch National Botanical Garden, Cape Town. Over a four-hour period, 100–400 mg/kg, i.p. of *C. orbiculata* significantly reduced the oedema with a paw volume of 0.19 mL compared to the mice injected with phycological saline with a paw volume of 0.69 mL.

Ondua *et al.*, (2019), determined the anti-oxidant activity of *C. orbiculata.* The plant material was collected in Countryview, Midrand. The extracts were made using 1 g powdered dried leaves in 5 mL hexane, acetone, ethanol, methanol, and water, separately. In the DPPH and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays, the methanolic extract had an IC_{50} of 3.76 and 3.35 µg/mL, respectively, and the ethanolic extract had an IC_{50} of 16.2 and 1.96 µg/mL, respectively. Ascorbic acid and Trolox were used as the controls. *Cotyledon orbiculata* acetone extract exhibited a good nitric oxide (NO) inhibition with 99% at 100 µg/mL and an LPS-stimulated RAW 264.7 macrophage cell viability of 92%, at 100 and 12.5 µg/mL.

Antinociceptive activity

Amabeoku and Kabatende (2012) conducted a study to determine the antinociceptive activity of *C. orbiculata*. Methanolic extracts were investigated on male albino mice using the acetic acid-induced writhing test. The mice were given injections of 3% acetic acid solution, which is a known irritant and induces writhing, contractions of the abdominal muscles and elongations of the limbs. Over a 20-minute period, the writhing movements were counted. In a dose-dependent manner (100–400 mg/kg, i.p.), *C. orbiculata* significantly reduced the number of writhing movements by up to 76%. The hot plate test was also used to determine the antinociceptive activity of *C. orbiculata*. The mice were exposed to a hot plate set at 50 - 55°C, where the pain threshold was measured. The lifting and licking of paws or the attempt to jump out of the beakers was determined to be the pain threshold. The pain threshold time is the response or reaction time. The methanolic leaf extracts of *C. orbiculata* significantly delayed the response time (400 mg/kg, i.p.).

Cytotoxicity

Cotyledon orbiculata was assessed for its cytotoxicity towards the human periodontal ligament fibroblasts and human gingival fibroblasts cell lines using MTT analysis. The plant material was collected from the Tip Top nursery in Pretoria North, and the extracts were made using 1g powdered plant material in 100 mL ethanol. Towards the gingival fibroblasts, *C. orbiculata* showed inhibition at the highest concentration (10 mg/mL) and towards the ligament fibroblasts, *C. orbiculata* showed inhibition up to 0.31 mg/mL (Maja 2009).

Immunomodulating activity

Tyavambiza *et al.*, (2021) determined the immunomodulating activity of *C. orbiculata*-AgNPs. At 10 µg/mL, *C. orbiculata*-AgNPs had no toxic effect on the human monocytes THP-1 macrophage cells and there was an 84% viability of the THP-1 cells at 5 µg/mL. The cytokine production in the differentiated human monocytes THP-1 macrophage cells was determined using ELISA. *Cotyledon orbiculata*-AgNPs reduced the levels of the pro-inflammatory cytokines: tumour necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β), when compared to the levels stimulated by lipopolysaccharides. *Cotyledon orbiculata*-AgNPs decreased the TNF-α cytokines 3.5-fold, the IL-6 cytokines 7-fold and the IL-1β cytokines 10.5-fold. This implies that *C. orbiculata*-AgNPs has an anti-inflammatory activity as it decreased the pro-inflammatory cytokines in the human monocytes THP-1 macrophage cells that were stimulated by the lipopolysaccharides (Tyavambiza *et al.*, 2021).

5.7. Additional information

5.7.1. Therapeutic (proposed) usage

Anthelmintic, anticonvulsant, anti-inflammatory, antioxidant, antinociceptive and immunomodulating activity.

5.7.2. Safety data

No official safety data is available however, Amabeoku and Kabatende (2012) tested the toxicity of *C. orbiculata*, that had been orally administered to mice. At 400 mg/kg, p.o. there were no signs of acute toxicity nor deaths. It was mentioned that the lethal dose for 50% of the population (LD_{50}) is most likely greater than 400 mg/kg, p.o. in mice.

5.7.3. Trade information

Cotyledon orbiculata is not threatened, not endangered, is abundant and of least concern (Foden & Potter 2005f).

5.7.4. Dosage

No specific concentration has yet been determined for the use of *C. orbiculata.*

6. *Cussonia spicata*

6.1. General Description on Selected plants

6.1.1. Botanical nomenclature

Cussonia spicata Thunb. (Hankey 2004).

Synonyms: *Cussonia kraussii* Hochst. (Foden & Potter 2005b).

6.1.2. Botanical Family

Araliaceae (Hankey 2004).

6.1.3. Vernacular names

Afrikaans: Cabbage-tree, Common cabbage tree; Kiepersol (Hankey 2004).

Sotho, Zulu and Xhosa: Umsenge (Hankey 2004).

Northern Sotho: Motshetshe (Hankey 2004).

6.2. Botanical Description

Cussonia spicata can reach a height of 15m and is considered to be a fast-growing and relatively long-lived tree. Over-all, *Cussonias* tend to produce attractive leaves in large round

heads at the ends of thick branches (Figure 3.8). Although older specimens may develop additional heads, in the main, these trees typically have between 1 - 15 heads (Hankey 2004).

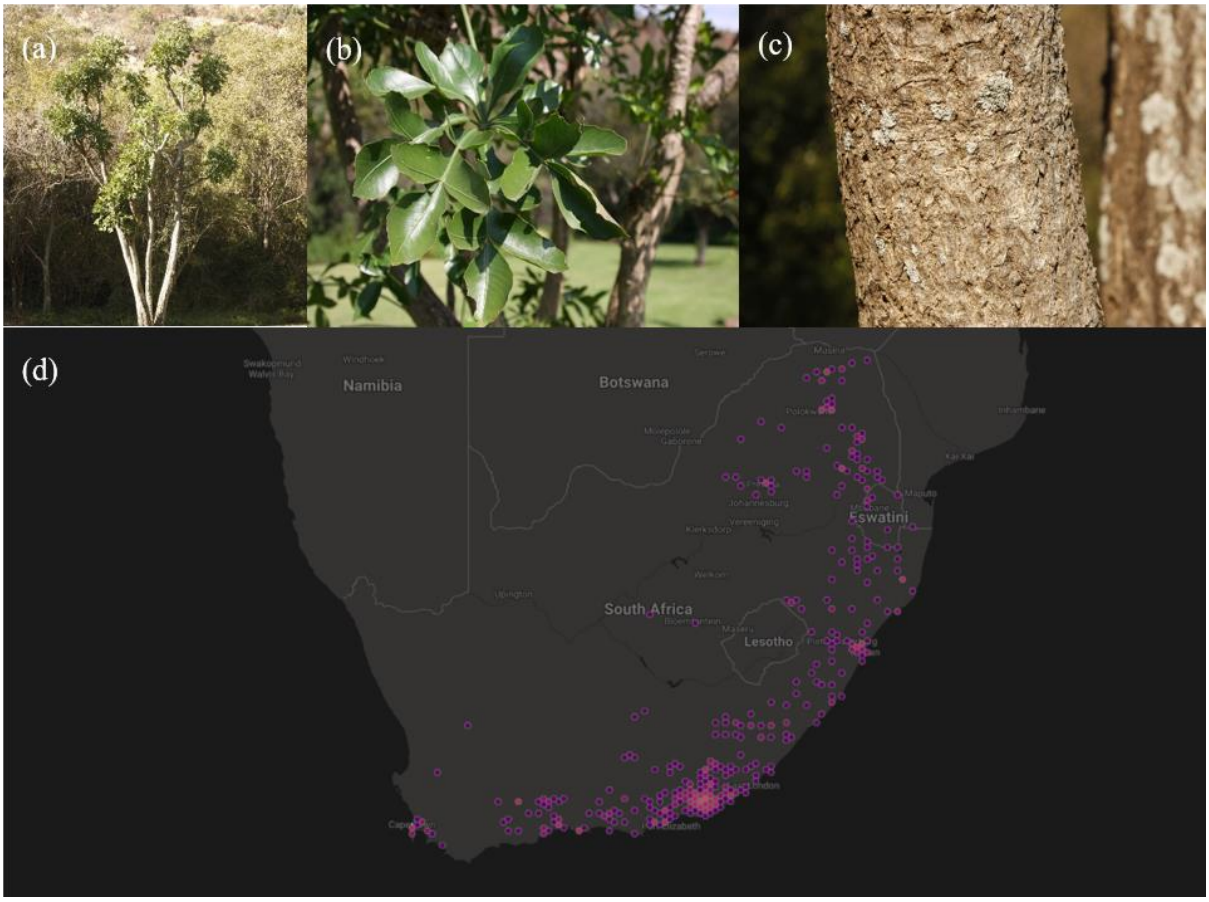

Figure 3.8: *Cussonia spicata* (a) tress, (b) leaves and (c) bark (Ebedes 2021c). (d) The distribution of *Cussonia spicata* in South Africa (GBIF Secretariat 2021e).

6.3.Distribution

Although not endemic to South Africa, *C. spicata* has an expansive natural distribution range throughout the wetter areas of southern Africa, ranging from the eastern parts of the country and southern Cape, extending as far as tropical Africa by way of Mozambique, Zimbabwe, and Zambia (Figure 3.8) (Foden & Potter 2005b). The *C. spicata* flourishes where this is ample water, in full sun or semi shade, particularly when young. Although the more resistant forms of species can tolerate a moderate degree of frost, the species is variable depending on origin and requires protection when young. Those originating from sub-tropical areas such as KwaZulu-Natal and the Eastern Cape coast are intolerant of any frost (Hankey 2004).

6.4.Ethnobotanical usage

All components of *C. spicata* including the bark, flowers, flower stalks, fruits, leaves, roots, and stems are used for herbal remedies against 43 known human diseases as well as in

ethnoveterinary medicine. South Africa, Kenya, Tanzania, Zimbabwe and Swaziland use medicinal applications of *C. spicata* for antifebrile, fever, appetite reduction, emetic, nausea, vomiting, gonorrhoea, venereal diseases, malaria, Human immunodeficiency virus (HIV) and mental illness (Chhabra *et al.*, 1987; Hutchings *et al.*, 1996; McGaw *et al.*, 2000; Shangali *et al.*, 2008; Verschaeve & Van Staden 2008; Bapela 2016;). The roots, flowers, leaves, bark and stems of *C. spicata* have been used to treat a plethora of ailments including abdominal pain, amenorrhea, dysmenorrhea, biliousness, constipation, indigestion, stomach complaints, convulsions, epilepsy, measles, pimples, shingles, skin irritation, muscular spasm, camps, painful legs, and uterine pain (Chhabra *et al.*, 1984; Tetyana *et al.*, 2002; Olajuyigbe & Afolayan, 2012; Hughes *et al.*, 2015; Kigen *et al.*, 2017; Mhlongo & van Wyk 2019; Thakur *et al.*, 2019).

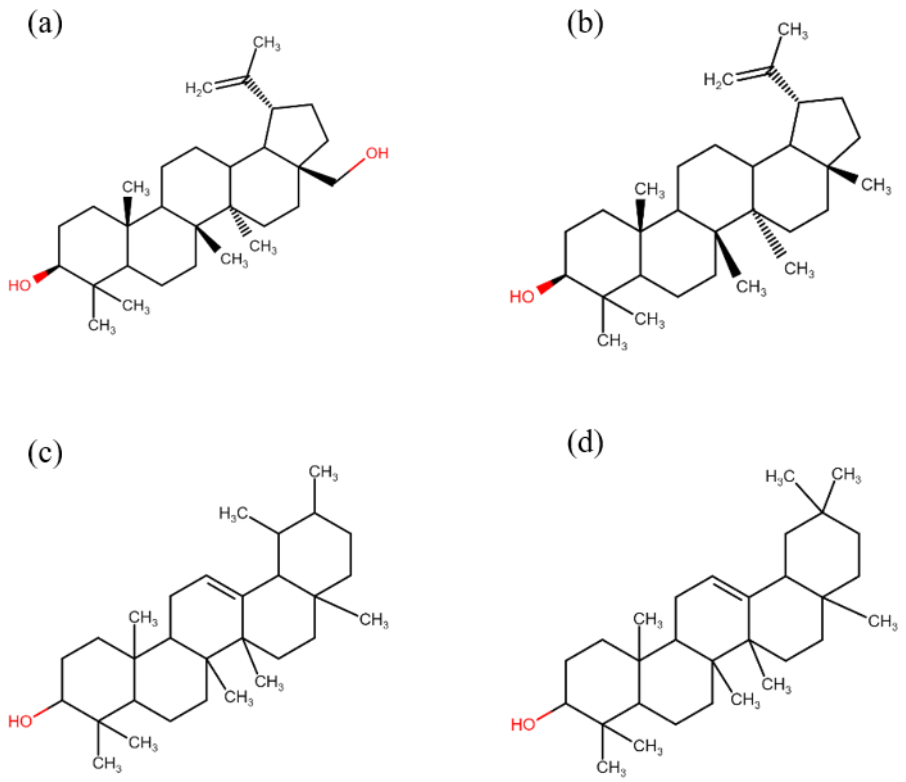

Figure 3.9: The phytochemical structures that have been isolated from *Cussonia spicata* (a) betulin (b) lupeol, (c) α- amyrin and (d) β- amyrin

6.5. Phytochemical constituents

The total phenolic content found in the methanolic extracts was 7.6 mg GAE/g dry matter. The iridoids concentration in the methanolic extracts was 3.88 µg HE/g dry matter. The flavonoid concentration in the methanolic extracts was 3.4 mg CE/g dry matter (Amoo *et al.*, 2012). There have been a variety of identified phytochemicals from the roots, bark, stems and leaves

of *C. spicata*. They include alkaloids, anthocyanins, anthracene glycosides, betulin, condensed tannins, free gallic acid, gallotannins, pentacyclic triterpenoids (specifically α-amyrin, β-amyrin and lupeol), saponins, steroids, tannins, triterpenoids, and volatile oils. Four phytochemical constituents have been isolated from *C. spicata*, namely, botulin, lupeol, α-amyrin and β- amyrin (Chhabra *et al.*, 1984; Wollenweber *et al.*, 1999)

6.6. Pharmacological properties

6.6.1. Antibacterial activity

Cussonia spicata was tested in Tetyana *et al.*, (2002), for its activity against gram-positive bacteria *B. subtilis, M. luteus, S. aureus* and *S. epidermidis,* and gram-negative bacteria *E. coli, K. pneumoniae* and *P. aeruginosa.* The plant material used was collected from the University of Natal Botanic Garden, Pietermaritzburg. The extracts were made using 1g powdered plant material in 10 mL ethanol and ethyl acetate, separately, and the disc diffusion method was used to determine the zones of inhibition. Aqueous, ethanolic and ethyl acetate extracts were made of the bark and roots of *C. spicata*. The aqueous extracts showed minimal to no inhibition. The most significant ethanolic and ethyl acetate extracts were those made from the roots which had a MIC of 0.5 mg/mL against *S. aureus*.

McGaw *et al.*, (2000) assessed *C. spicata* against the gram-positive bacteria *B. subtilis* and *S. aureus,* and the gram-negative bacteria *E. coli* and *K. pneumoniae*. The plant material used was collected from the KwaZulu Natal region. The extracts were made using 1 g of powdered dried plant material in 10 mL water, ethanol and hexane, separately. Neomycin was used as the control. Aqueous, hexane and ethanolic extracts were tested using the disc-diffusion method. The hexane extract had a MIC of 6.25 mg/mL against *E. coli* and the ethanolic extract had a MIC of 3.13 mg/mL against both *B. subtilis* and *E. coli*. In McGaw *et al.*, (2007), *C. spicata* was tested, using the microdilution assay, against the gram-positive bacteria *E. faecalis* and *S. aureus*, and the gram-negative bacteria *E. coli* and *P. aeruginosa.* Aqueous, hexane and methanolic root extracts extracts exhibited some activities with a range of MIC values of 6.3 mg/mL to >12.5 mg/mL.

De Villiers *et al.*, (2010) used the microplate assay against the gram-positive bacteria *E. faecalis*, and *S. aureus*, the gram-negative bacteria *E. coli, P. aeruginosa* and *Neisseria gonorrhoea* and the parasites *Trichomonas vaginalis.* The plant material used was collected from Weltevreden Park, Johannesburg and the Research Station, Stellenbosch, Western Cape. Extracts were prepared using 1 g powdered dry leaf material in 25 mL water and methanol,

separately. The control used was ciprofloxacin. *Cussonia spicata* methanolic extracts showed good activity against *P. aeruginosa, T. vaginalis* and *N. gonorrhoea* with MIC values of 1.4, 1.3 and 0.4 mg/mL, respectively. The aqueous extract had a MIC of 0.3 mg/mL against *N. gonorrhoea*.

6.6.2. Melanogenic activity

Unknown.

6.6.3. Other

Acetylcholinesterase

In Amoo *et al.*, (2012) the AChE inhibitory activity of *C. spicata* was determined. This study compared the activity of fresh plant material and long term (12 years) stored plant material. The fresh material was collected from the same locality and in the same season as the older samples. The extracts were made using 1 g powdered dried plant material in 100 mL 50% aqueous methanol. The fresh material and stored plant material at 1 mg/mL had an 86.5 and 72.1% AChE inhibition. Galanthamine was used as the control and had an 84% inhibition at 20 µM.

Anti-inflammatory and anti-oxidant activity

In Tetyana *et al.*, (2002), it was determined that *C. spicata* had anti-inflammatory activity against COX-1. At 200 µg/mL the ethanolic leaf extract had a 56% inhibition and the aqueous stem extract had a 51% inhibition of the COX-1 enzyme. Indomethacin was used as the control and had a 78% inhibition.

Amoo *et al.*, (2012) determined the β-Carotene-linoleic acid model system and DPPH free radical scavenging activity and of *C. spicata*. At 100 µg/mL fresh *C. spicata* had a 61% scavenging activity, at 200 µg/mL it had a 41% anti-oxidant activity, with an EC_{50} of 43.6 µg/mL and an anti-oxidant index of 0.5. At 100 µg/mL stored *C. spicata* had a 93% scavenging activity, at 200 µg/mL it had a 55% anti-oxidant activity, with an EC_{50} of 14.3 µg/mL and an anti-oxidant index of 1.4. The control used for the β-carotene bleaching model was butylated hydroxytoluene which had a 94% antioxidant activity at 200 µg/mL. The control for the DPPH radical scavenging assay was ascorbic acid and butylated hydroxytoluene which had a 96 and 93% radical scavenging activity, respectively.

Antileishmanial

Bapela *et al.*, (2017) analysed the effectiveness of *C. spicata* against *donovani*. *Leishmania donovani* is one of the protozoan species responsible for the tropical disease Leishmaniasis which leads to, depending on the *Leishmania* spp., cutaneous, mucocutaneous and visceral leishmaniasis. The most fatal is caused by *L. donovani* which causes visceral leishmaniasis. The plant material was collected from the Mutale Municipality, Limpopo. The extracts were made using ground plant material in a DCM: methanol mixture to yield two layers of differing polarities. The DCM and methanolic polarities extracts had an IC_{50} of 8.15 and > 100 µg/mL, respectively. Miltefosine, which was the positive control, had an IC_{50} of 0.191 µg/mL.

Anti-malarial activity

Tetyana *et al.*, (2002) assessed *C. spicata* bark for its activity against a PfUP1 strain of chloroquine resistant strain of *Plasmodium falciparum* using the parasite lactate dehydrogenase assay. It was determined that at a concentration of 200 µg/mL, the aqueous and ethanolic extracts of the bark showed 20 and 35% inhibition, respectively. De Villiers *et al.*, (2010) determined the chloroquine-sensitive *P. falciparum* (3D7) sensitivity to *C. spicata.* The methanolic extract had an IC_{50} of 28.20 µg/mL and the aqueous extract had an IC_{50} > 50 µg/mL. Quinine was used as the control.

Clarkson *et al.*, (2004) also tested *C. spicata* against the D10 strain of chloroquine-sensitive *P. falciparum*, but with DCM, DCM/methanol, methanolic and aqueous extracts. The plant material used was collected throughout South Africa. The extracts were made using dried powdered plant material which was sequentially extracted with dichloromethane (DCM), DCM: methanol, methanol and water. In this study, the [^{3}H] hypoxanthine incorporation assay was used and the control used was chloroquine diphosphate. The lowest IC_{50} was the DCM/methanol leaf extract with an IC_{50} of 13 µg/mL. Promising and high activity were classified as having IC_{50} values of < 10 and < 5 µg/mL, respectively. Kraft *et al.*, (2003) tested the leaves and roots against both PoW strain of chloroquine-sensitive *P. falciparum* and Dd2 strain of chloroquine resistant *P. falciparum*. The plant material used in this study was collected from different locations in Zimbabwe. Extracts were made using 1 g dried plant material in 7.5 mL in water: methanol. *Cussonia spicata* did not have promising results with IC_{50} values of 45.1 and 47.5 µg/mL for the leaves, and IC_{50} values of > 50 µg/mL and 67.9 µg/mL for the chloroquine-sensitive *P. falciparum* and chloroquine resistant *P. falciparum*, respectively. Artemisinim and chloroquine $2H_3PO_4$ were used as the controls.

However, in Bapela *et al.*, (2014) and Bapela *et al.*, (2019) it was found that *C. spicata* DCM extracts had an IC_{50} of 3.25 µg/mL against the NF54 strain of chloroquine-sensitive *P. falciparum* with a selectivity index of 14.7. The [^{3}H] hypoxanthine incorporation assay was also used and the controls used was chloroquine and podophyllotoxin. It is explained that plants with an IC_{50} value of < 5 µg/mL and a selectivity index > 10 is considered a potential drug discovery. *Cussonia spicata* DCM root extract showed considerably significant in vitro antiplasmodial activity and selectivity towards the malarial parasite. The difference in results could be due the parasite strain, geographical location of the used plants, parts of the plant harvested or the extract solvent (Bapela 2016).

Cytotoxicity

Cussonia spicata was tested for its cytotoxicity against the human T-cell leukaemia (Jurkat) cancer line in De Villiers *et al.*, (2010). It was determined that the aqueous and methanolic extracts had IC_{50} values of 27.69 and > 50 µg/mL, compared to camptothecin, the control, with an IC_{50} of 0.07 µg/mL. Bapela *et al.*, (2017) determined the cytotoxicity of DCM and methanolic *C. spicata* on rat skeletal myoblast L6 cells which had IC_{50} values of 47.8 and 69.1 µg/mL, respectively. The control used was podophyllotoxin which had an IC_{50} of 7 x10^{-3} µg/mL.

6.7. Additional information

6.7.1. Therapeutic (proposed) usage

Anti-inflammatory, anti-oxidant, antileishmanial and anti-malarial.

6.7.2. Safety data

No official safety data is available however, *C. spicata* has shown some, although not significant, toxicity against the human T-cell leukaemia (Jurkat) cancer line and rat skeletal myoblast L6 cells.

6.7.3. Trade information

Cussonia spicata is not threatened, not endangered, abundant and is of least concern (Foden & Potter 2005b).

6.7.4. Dosage

No specific concentration has yet been determined for the use of *C. spicata.*

7. *Hypoestes aristata*

7.1. General Description on Selected plants

7.1.1. Botanical nomenclature

Hypoestes aristata (Vahl) Sol. ex Roem. & Schult. (Mtsweni 2010).

7.1.2. Botanical Family

Acanthaceae (Mtsweni 2010).

7.1.3. Vernacular names

English: Ribbon bush (Mtsweni 2010).

Afrikaans: Seeroogblommetjie (Mtsweni 2010).

Zulu: Uhladlwana olukhulu, Uhlonyane, Uhlalwane (Mtsweni 2010).

Figure 3.10: *Hypoestes aristata* (a) bush, (b) flowers and (c) leaves (Ebedes 2021d). (d) The distribution of *Hypoestes aristata* in South Africa (GBIF Secretariat 2021f).

7.2. Botanical Description

Hypoestes aristata is a hardy evergreen that appears as a medium rounded shrub. It produces soft, hairy, dark green oval leaves with lighter veins that give a variegated appearance (Figure 3.10). It bears attractive lilac pink or white flowers in spike-like inflorescences on a fast-

growing shrub that can grow to a height of 1.5 metres. The flowering period is late Autumn through winter into early spring (Mtsweni 2010).

7.3.Distribution

Hypoestes aristata is not endemic to South Africa. It can usually be found in dry thicket, forest and damp places throughout the Eastern Cape to tropical Africa in the north (Figure 3.10) (Kamundi 2006c; Mtsweni 2010).

7.4.Ethnobotanical usage

Differing parts of the *H. aristata* are used in the treatment of varying illnesses such as gonorrhoea, hepatitis, high blood pressure, worm infestations, and fungal, skin, chest, and heart disease by differing ethnic groups (Pettit *et al.*, 1984; Mothana *et al.*, 2009). *Hypoestes aristata* is also considered effective by some for eye sores, breast diseases, respiratory infections and malaria. Major diseases such as cancer, arthritis, and tuberculosis as well as bone fractures are treated with this plant by Xhosa healers (Saeed *et al.*, 2016).

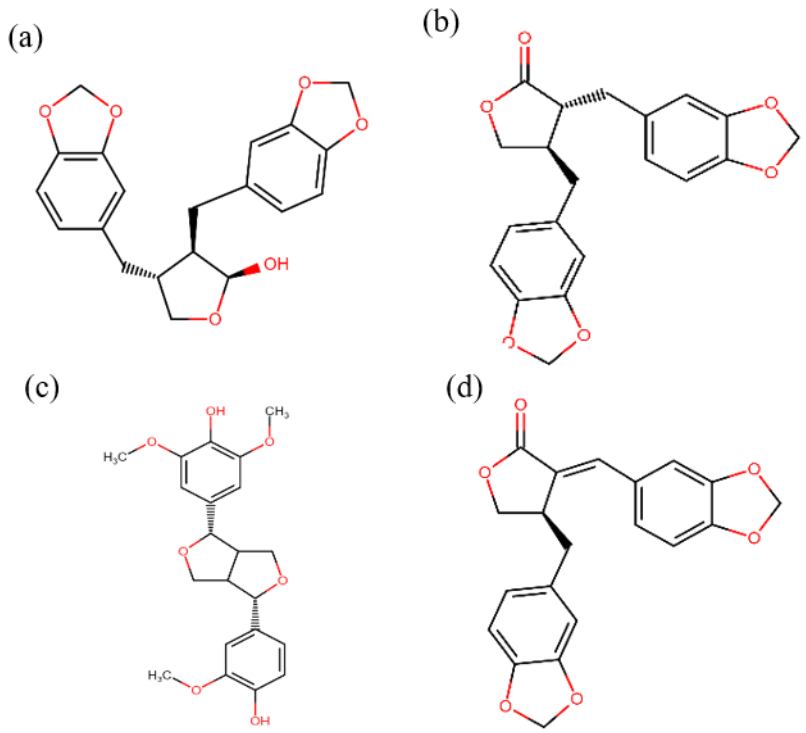

Figure 3.11: The phytochemical structures that have been isolated from *Hypoestes aristata* (a) cubebin, (b) hinokinin, (c) medioresinol and (d) savinin.

7.5.Phytochemical constituents

The *Hypoestes* genus has had had some phytochemical investigation and a variety of secondary metabolites have been isolated from other plants in this genus, including alkaloids,

diterpenoids, lignans and triterpenoids (Pettit *et al.*, 1984; Muhammad *et al.*, 1997; Muhammad *et al.*, 1998; Al Musayeib *et al.*, 2014; Wu *et al.*, 2016; Al-Haidari 2018). In the study by Ramabulana *et al.*, (2020), nine lignans were isolated from *H. aristata*: hinokinin, medioresinol, savinin, α- and β-cubebin, three new butyrolactol lignans [(7R,8S,9R,7′R,8′R)-7,7′- diacetoxy-α-cubebin, (7R,8S,9R,7′R,8′R)-7,7′- diacetoxy-β-cubebin, (7S,8R,9S,7′R,8′R)-7,7′-diacetoxycubebin] and three new butyrolactone lignans [(7S,8S,7′S,8′R)-7′-acetoxy-7-hydroxyhinokinin, (7S,8S,7′S,8′R)-7-acetoxy-7′-hydroxyhinokinin and (7S,8S,7′S,8′R)-7,7′-diacetoxyhinokinin] (Figure 3.11).

7.6.Pharmacological properties

7.6.1. Antibacterial activity

Silva *et al.*, (2007) assessed cubebin and hinokinin for its antimicrobial activity against oral pathogens. These include the gram-positive bacteria *E. faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *Streptococcus mitis* (ATCC 49456), *Streptococcus mutans* (ATCC 25275), *Streptococcus sanguinis* (ATCC 10556) and *Streptococcus sobrinus* (ATCC 33478), and the yeast *C. albicans* (ATCC 28366). The MIC values for the two compounds ranged from 0.25 mM to 0.38 mM, thus showing a discrete antimicrobial activity. The positive control used was chlorhexidine which had MIC values ranging from 1.5 - 7.9 mM.

Cubebin and hinokinin were further tested in Silva *et al.*, (2009) against *Mycobacteria. Mycobacterium avium* (ATCC 15769), *Mycobacterium kansasii* (ATCC 12478) and *M. tuberculosis* H37Rv (ATCC 27294). This study used the microdilution assay and Rifampicin was used as the control drug. Cubebin showed no activity against the bacteria. Hinokinin had moderate activity against *M. tuberculosis* with a MIC value of 62.25 µg/mL, however it showed no activity against *M. avium* and *M. kansasii*.

7.6.2. Melanogenic activity

Unknown.

7.6.3. Other

Anti-oxidant and anti-inflammatory activity

Chen *et al.*, (2009) tested hinokinin as an anti-inflammatory activity with regards to its inhibition of the human neutrophil superoxide generation and elastase release by the neutrophils. It was found that hinokinin was an efficacious inhibitor with an IC_{50} of 0.06 µg/mL

against the superoxide radical anion generation and a 24.7% inhibition of the elastase release at 10 µg/mL. Diphenyleneiodonium was used as the control against the superoxide radical anion generation and had an IC_{50} value 0.54 µg/mL, while phenylmethylsulfonyl fluoride was used as the control for the inhibition of the elastase release and had a 35.24 % inhibition. Lee *et al.,* (2013) assessed hinokinin for its ability to inhibit LPS-induced nitric oxide generation in RAW 264.7 macrophage cells. It showed a moderate inhibition with an IC_{50} value of 21.56 µM. Aminoguanidine was used as the control with an IC_{50} value of 6.51 µM.

Da Silva *et al.,* (2005) assessed hinokinin and cubebin for their anti-inflammatory activity using the rat paw oedema reduction assay and their analgesic activity using the acetic acid-induced writhing test in mice. At 30 mg/kg cubebin and hinokinin inhibited the oedema formation in a dose dependent manner by 53 and 63%, respectively. Hinokinin was more active than cubebin with regards to the writhing test with an inhibition of 97%.

Ramos *et al.,* (2006) established hinokinin to have an immunosuppressive activity. The compound was tested using the lipopolysaccharide (LPS) induced cytokine production assay, specifically for IL-10, IL-12, and TNF-α. At 10µg/mL hinokinin had cytokine production ratios of 0.36, 0.44 and 0.37 against IL-10, IL-12, and TNF-α, respectively. Prednisolone was used as the drug control and had cytokine production ratios of 0.6, 0.2 and 0.41 against IL-10, IL-12 and TNF-α, respectively. It was reported that these results indicate that hinokinin is not selective and is an inhibitor against the whole immune system. Desai *et al.,* (2014) evaluated hinokinin for its anti-inflammatory effect on IL-6 and TNF-α with IC_{50} values 20.5 and 77.5 µM, respectively. It was discovered that the anti-inflammatory effect of hinokinin is exerted via an NFκB-dependent mechanism.

Anti-plasmodial activity

In Abrantes *et al.,* (2008) hinokinin was tested for its anti-plasmodial activity. The 2 strains of *P. falciparum* used were the Dd2-chloroquine resistant strain and the 3D7-chloroquine sensitive stain. Hinokinin had IC_{50} values of 54.4 and 90.7 µg/mL, respectively. Chloroquine had an IC_{50} value of 0.09 µg/mL, showing that hinokinin did not possess a noteworthy antimalarial activity against either strain.

Antiviral activity

Ramabulana *et al.,* (2020) along with isolating compounds from *H. aristata* also assessed their inhibitory activity against the HIV-1 protease enzyme. At concentrations of 100 µM, all the

isolates showed significant inhibition, at a concentration of < 60 µM, hinokinin and savinin had moderate inhibition.

Huang *et al.,* (2003) tested hinokinin for its antiviral activity against human hepatitis B virus (HBV). Through the use of an enzyme-linked immunosorbent assay (ELIZA), the HBsAg and HBeAg inhibition was determined. The positive controls used in this study were α-Interferon and osthole. Hinokinin showed significant HBsAg and HBeAg inhibition. The highest inhibition percentages obtained from hinokinin were, at 50 µM, 68.1 and 52.3 % against HBsAg and HBeAg, respectively.

Wen *et al.,* (2007) tested hinokinin and savinin against the severe acute respiratory syndrome coronavirus (SARS-CoV). The compounds were tested using the cytopathogenic effect (CPE) assay on Vero E6 cells. Hinokinin at 20, 10 and 1 µM had a CPE reduction of < 25, 25 - 50 and 50 - 70, respectively. Savinin at 20 and 10 µM had a CPE reduction of < 25%. Both compounds were tested for their cytotoxicity on Vero E6 cells using the MTT colorimetric method. They were both considered biologically safe as they had CC_{50} values of > 250 µM. Niclosamide was used as the control and had a CC_{50} value of 22.1 µM. The compounds were tested for their inhibition of viral replication of SARS-CoV in Vero E6 infected cells. Hinokinin and savinin had EC_{50} values of > 10 and 1.13 µM, respectively. It was determined that compounds such as savinin that had high activity against CPE also had significant inhibitory effect on SARS-CoV replication. Savinin had a higher SI value (66.7) than the positive control valinomycin (41.4). With regards to the SARS-CoV 3CL protease inhibition assay, only savinin showed significant results, which is thought to be due to its stabler structure in comparison to hinokinin. Savinin and niclosamide had an IC_{50} values of 25 and 40 µM, respectively. It was further determined that savinin had a competitive inhibition.

Cytotoxicity

Saeed *et al.,* (2016) assessed *H. aristata* for its cytotoxicity against leukaemia cells. The plant material was collected in Pretoria and was extracted using 1 g fresh leaves in 3 mL methanol extractant. The leukaemia cell lines used were the human drug sensitive T-lymphoblastoid (CCRF-CEM) cells and the multidrug-resistant T-lymphoblastoid (CEM/ADR 5000) cells. The resazurin reduction assay was used and the methanolic extracts were tested at a fixed concentration of 10 µg/mL. *Hypoestes aristata* had a cell viability percentage of < 40%, therefore it exerted a strong inhibitory effect on the human drug sensitive leukaemia and the multidrug-resistant leukaemia cell lines. A dose response curve was performed to determine

that *H. aristata* had IC_{50} values of 2.28 and 3.8 µg/mL against the human drug sensitive leukaemia cells and the multidrug-resistant leukaemia cells, respectively, with a degree of resistance of 1.67.

Zhang *et al.*, (2010) assessed cubebin and hinokinin for their cytotoxicity against the human cancer cell line BEL-7402/5-FU, HeLa and mouse fibroblast cell line L929 using the MTT colorimetric method. Cubebin had IC_{50} values of > 200, 180.55 and 170.37 µg/mL against BEL-7402/5-FU, HeLa and L929, respectively, and hinokinin had IC_{50} values of 110.82, 140.28 and 134.44 µg/mL, respectively. Esperandim *et al.*, (2013) tested the compounds cubebin and hinokinin for their cytotoxicity on rhesus monkey kidney epithelial ($LLC\text{-}MK_2$) fibroblast cells using the MTT assay. Cubebin and hinokinin had IC_{50} values of 328.2 and 318.4 µM, respectively.

Savinin and hinokinin have been tested for their cytotoxicity against lung carcinoma epithelial cells (A549), human breast cancer (MCF-7) and human colorectal adenocarcinoma (HT-29) cell lines. Savinin had ED_{50} values of 0.4, 0.5 and 0.3 µg/mL, respectively. Hinokinin had ED_{50} values of 26.1, 13.8 and 11.4 µg/mL, respectively. Andriamycin was used as the drug control with ED_{50} values of 0.02, 0.1 and 0.1 µg/mL, respectively (Chang *et al.*, 2000). Hinokinin also showed activity against murine metastatic melanoma (B16F10), human cervical cancer (HeLa), murine gastric adenocarcinoma (MK-1) and murine lymphocytic leukaemia (P-388), with ED_{50} values of 2.72, 2.58, 1.67 and 1.54 µg/mL, respectively (Lin *et al.*, 2004). Huang *et al.*, (2002) tested hinokinin for its toxicity in the brine shrimp lethality assay. It was found that hinokinin was highly toxic with an LD_{50} of 24.3 µg/mL.

Trypanocidal activity

Esperandim *et al.*, (2013) found there to be significant parasitemia reduction from animals treated with cubebin and hinokinin. In this study male BALB/c mice were infected via an intraperitoneal injection with blood trypomastigote forms of the CL Brener clone strain. The mice were treated orally with either cubebin or hinokinin for 20 days and the number of parasites were quantified. At 50 mg/kg cubebin and hinokinin showed 43 and 34.2%, respectively, reduction of the number of circulating parasitic forms at peak parasitemia when compared to the infected untreated mice ($p < 0.01$). At 20 mg/kg cubebin and hinokinin showed a reduction of the number of circulating forms at peak parasitemia by 80.7 and 51.9%, respectively, when compared to the infected untreated mice ($p < 0.01$). The administrations of cubebin and hinokinin were able to increase the survival time of the mice compared to the

untreated infected mice. These results were also considered more satisfactory than those obtained from the mice treated with Benznidazole, the positive control. Esperandim *et al.*, (2013) suggests that the non-linear behaviour between the two doses was due to a possible immunomodulatory response that hinokinin exerts.

Cubebin and hinokinin were further tested by Esperandim *et al.*, (2013) as a treatment for tissue infection of *Trypanosoma cruzi*. The compounds were tested on male BALB/c mice. The spleen, liver and heart were harvested and from slides prepared from the organs, the nuclear parameters were determined. Infected untreated mice showed to have increased cell nuclei compared to uninfected mice. Those treated with cubebin and hinokinin had nuclear diameters similar to those of the uninfected mice. The untreated infected mice had a nuclear perimeter value of 11.76 and 18.54 µm for the spleen and liver, respectively. Cubebin at 50 mg/kg had nuclear perimeter values of 10.85 and 19.06 µm for the spleen and liver, respectively. Hinokinin had a nuclear perimeter value of 10.90 µm at 20 mg/kg for the spleen and 18.61 µm at 50 mg/kg for the liver. It was noted that the oral administration was more effective for all the evaluated organs than intraperitoneal administration.

7.7. Additional information

7.7.1. Therapeutic (proposed) usage

Antiviral activity, antibacterial activity, trypanocidal activity, anti-cancer activity, anti-oxidant, anti-inflammatory activity and anti-plasmodial activity.

7.7.2. Safety data

Hypoestes aristata showed a strong inhibitory effect on leukaemia cells with as cell viability percentage of <40% (Saeed *et al.*, 2016). Savinin and hinokinin had strong toxicity towards lung carcinoma epithelial cells (A549), human breast cancer (MCF-7) and human colorectal adenocarcinoma (HT-29) cell lines (Zhang *et al.*, 2010). Hinokinin also showed to have strong activity towards murine metastatic melanoma (B16F10), murine gastric adenocarcinoma (MK-1) and murine lymphocytic leukaemia (P-388) (Lin *et al.*, 2004). Hinokinin was highly toxic in the brine shrimp lethality assay (Huang *et al.*, 2002).

7.7.3. Trade information

Hypoestes aristata is not threatened, not endangered, abundant and is of least concern (Kamundi 2006c).

7.7.4. Dosage

No specific concentration has yet been determined for the use of *H. aristata.*

8. *Hypoestes forskaolii*

8.1. General Description on Selected plants

8.1.1. Botanical nomenclature

Hypoestes forskaolii (Vahl) R.Br.

Synonyms: *Hypoestes depauperata* Lindau, *Hypoestes verticillaris* of other authors, not of (L.f.) Sol. ex Roem. & Schult., *Justicia forskaolii* Vahl.

8.1.2. Botanical Family

Acanthaceae.

8.1.3. Vernacular names

English: White ribbon bush (Hyde *et al.*, 2016).

Figure 3.12: *Hypoestes forskaolii* (a) bush, (b) leaves and (c) flower (Royal Botanic Gardens, Kew 2012a). (d) The distribution of *Hypoestes forskaolii* in South Africa (GBIF Secretariat 2021g).

8.2. Botanical Description

This perennial herb can grow up to 1 metre tall. often sprawling. The leaves of this often-sprawling herb are opposite, petiolate, ovate, 2-8 cm long and covered in short hairs (Figure

3.12). The white to pale pinkish flowers are produced in terminal and axillary clusters. The corolla 2-lipped dark lilac marked flowers are held in glandular hairy bracts (Hyde *et al.*, 2016).

8.3.Distribution

Whilst not endemic to South Africa (Kamundi 2006b), *H. forskaolii* is widespread in tropical and Southern Africa and can be found as far away as the Saharan Highlands, Arabia and Madagascar (Hyde *et al.*, 2016). Within South Africa it can be found in all the provinces, Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape, North West, Western Cape (Figure 3.12) (Kamundi 2006b).

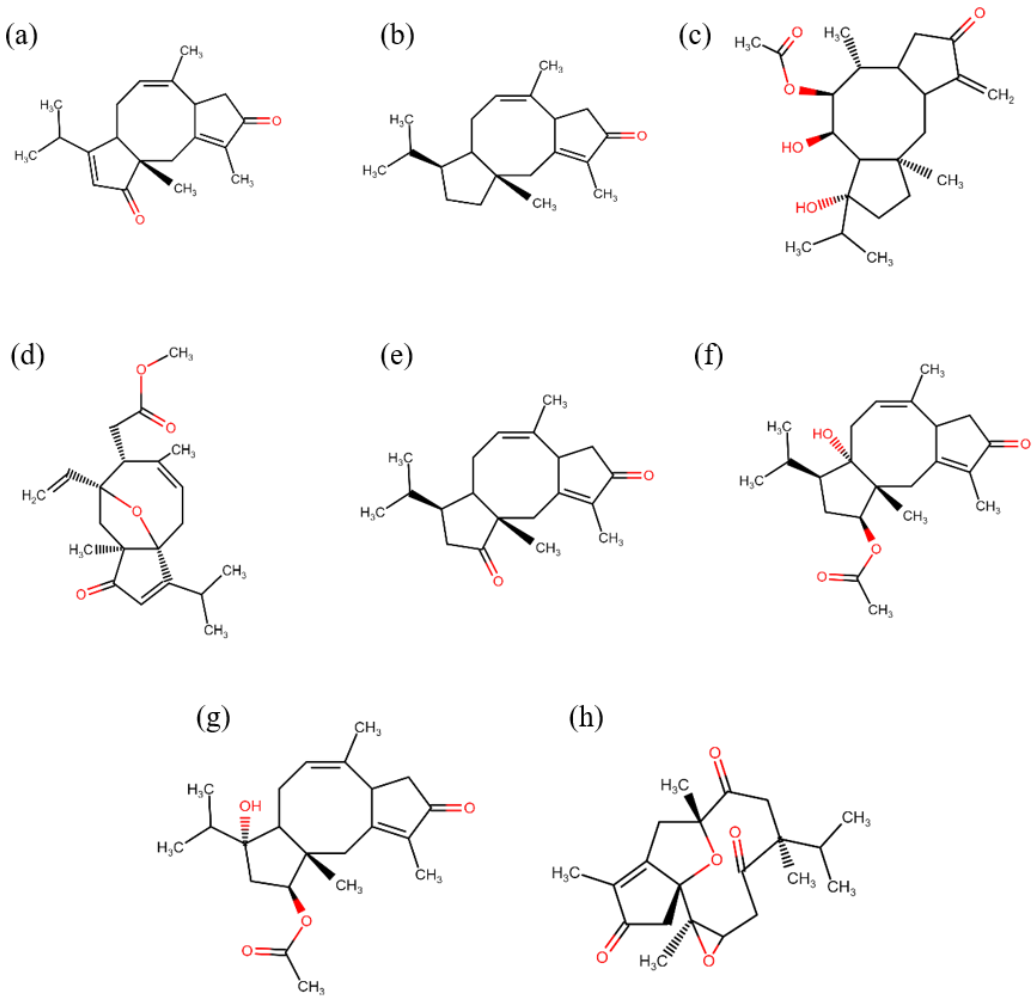

Figure 3.13: The phytochemical structures that have been isolated from *Hypoestes forskaolii* (a) dehydrohypoestenone, (b) deoxyhypoestenone, (c) fusicoplagin D, (d) hypoestene, (e) hypoestenone, (f) hypoestenonol A, (g) hypoestenonol B and (h) verticillarone

8.4.Ethnobotanical usage

Hypoestes forskaolii is utilized for a variety of ailments in differing countries. In East African folk medicine, it is used for treating vomiting, headache, nausea, heartburn, and nightmares (Al

Musayeib *et al.*, 2014) in Cameroon, the macerated plant is used topically for skin infections (Fongod *et al.*, 2013). In Ethiopia, the plant is mixed with honey and oil once crushed and ingested for treatment of a tick bite infection, babesia, and when macerated and mixed with the seeds of *Lepidium sativum*, and roots of *Solanum incanum* is used as a drink for anthrax (Teklay *et al.*, 2013). Saudi and Yemeni traditional medicines utilize the plant when treating fungal skin diseases, scabies, and itching (Mothana *et al.*, 2009; Mothana *et al.*, 2011; Mothana *et al.*, 2014). Additionally, Saudi Arabia and Madagascar use *H. Forskaolii* to treat heart disease and hypertension (Wu *et al.*, 2016) and amongst the Marakwet, it is used as a pesticide (Kipkore *et al.*, 2014).

8.5.Phytochemical constituents

Ten compounds have been isolated from the ariel parts of *H. forskaolii*: dehydrohypoestenone, deoxyhypoestenone, epoxydeoxyhypoestenone, epoxyhypoestenone, fusicoplagin D, hypoestene, hypoestenone, hypoestenonol A, hypoestenonol B, verticillarone (Figure 3.13) (Muhammad *et al.*, 1997; Muhammad *et al.*, 1998; Al Musayeib *et al.*, 2014).

8.6.Pharmacological properties

8.6.1. Antibacterial activity

Mothana *et al.*, (2011) tested *H. forskaolii* for its antimicrobial activity against the gram-positive bacteria *B. subtilis* (ATCC 6059), *Micrococcus flavus* (SBUG 16), *S. aureus* (ATCC 6538), the gram-negative bacteria *E. coli* (ATCC 11229) and *P. aeruginosa* (ATCC 27853), the multidrug resistant bacteria *S. aureus* north German epidemic strain, *S. epidermidis* 847 and *S. haemolyticus* 535 and the yeast *Candida maltosa* (SBUG). The plant material used was obtained from Yemen. Aqueous and methanolic extracts were made using 1 g of dried powdered plant material in 40 mL water and methanol, separately. The disc diffusion assay was used to determine the antimicrobial activity of the extracts. *Hypoestes forskaolii* methanolic extract showed activity only towards *C. maltose* with a zone of inhibition of 18 mm at 4 mg/disc compared to the positive drug control amphotericin with a zone of inhibition of 11 mm at 10 µg/mL.

8.6.2. Melanogenic activity

Unknown.

8.6.3. Other

Antiprotozoal activity

Al Musayeib, *et al.*, (2014) investigated *H. forskaolii* and some of its isolated compounds hypoestenonols A, verticillarone and hypoestenone for their antiprotozoal activity against *Leishmania infantum*, chloroquine-resistant *P. falciparum*, *Trypanosoma brucei* and *T. cruzi*. The plant material was collected from the South of Saudi Arabia and an extract was made by macerating 1 g of dried powdered aerial parts in 7mL 70% aqueous methanol. The methanolic extract, hypoestenonols A, verticillarone and hypoestenone exhibited an antiplasmodial activity against the chloroquine-resistant *P. falciparum* with IC_{50} values of 8.8 µg/mL, 18.9 µM, 25.1 µM and 16.7 µM, respectively, compared to the positive control chloroquine with an IC_{50} value of 0.3 µM. The aqueous methanolic extract had an antileishmanial effect towards *L. infantum* with an IC_{50} value of 8.1 µg/mL, compared to the positive control fungizone (IC_{50} 1.5 µM). The isolated compounds showed no activity with IC_{50} values of > 65 µM. With regards to their antitrypanosomal activity against *T. cruzi* and *T. brucei*, the methanolic extract had IC_{50} values of 9.1 and 8.1 µg/mL, respectively. Hypoestenone had an IC_{50} value of 41.7 µM against *T. cruzi* and showed no activity against *T. brucei.* The compounds hypoestenonols A and verticillarone showed no antitrypanosomal activity. The positive controls suramine (IC_{50} 0.03 µM) and benznidazole (IC_{50} 2.4 µM) were used for *T. brucei* and *T. cruzi*, respectively. Mothana *et al.*, (2014) obtained similar results with their methanolic extract of.

Mothana *et al.*, (2014) tested the methanolic extract of *H. forskaolii* for its antileishmanial activity towards *L. infantum*, its antiplasmodial activity towards chloroquine-sensitive *P. falciparum* K1-strain and its antitrypanosomal activity towards *T. brucei* and *T. cruzi*. *Hypoestes forskaolii* had an IC_{50} value of 8.1 µg/mL against *L. infantum* compared to the positive drug control miltefosine which had an IC_{50} value of 3.32 µg/mL. The methanolic extract had IC_{50} values of 8.1 and 9.1 µg/mL against *T. brucei* and *T. cruzi*, respectively. The positive drug controls were suramin, with an IC_{50} value of 0.03 µg/mL against *T. brucei* and benznidazole which had an IC_{50} value of 2.2 µg/mL against *T. cruzi*. *Hypoestes forskaolii* had an IC_{50} value of 8.8 µg/mL against *P. falciparum* compared to the positive drug control chloroquine which had an IC_{50} value of 0.3 µg/mL. Muthaura *et al.*, (2015), also assessed the methanol extract of *H. forskaolii* for its antimalarial activity against *P. falciparum* (D6 clone) using the 3H-hypoxanthine incorporation assay and reported it to have an IC_{50} value of 5.6 µg/mL

Cytotoxicity

Al Musayeib, *et al.*, (2014) investigated hypoestenonols A, verticillarone and hypoestenone for their cytotoxicity on human lung fibroblasts (MRC-5) cells. The extract and selected compounds showed no cytotoxic effect on the MRC-5 cells with IC_{50} values all >64 µg/mL and >64 µM, in the fluorimetrical assay. The positive control used was tamoxifen with an IC_{50} of 11.3 µM. Mothana *et al.*, (2014) reported the methanolic extract of *H. forskaolii* to have a cytotoxicity IC_{50} value of 11 µg/mL on MRC-5 cells.

Almehdar *et al.*, (2012) assessed the methanolic extracts of *H. forskaolii* against human breast cancer (MCF7), human hepatocellular carcinoma (HEPG2), human cervix cancer (HELA) and human normal melanocyte (HFB4) cell lines. The plant material used was collected from Westerns and Southern parts of Saudi Arabia. The extracts were made using 1 g dried powdered plant material in 3 mL methanol The sulforhodamine B colorimetric assay was used to determine the cytotoxicity of the extract and the positive control utilised was doxorubicin (DOX) which had IC_{50} values of 4.5, 3.96, 4.9 and 3.64 µg/mL against MCF7, HFB4, HEPG2 and HELA, respectively. The total methanolic extract had IC_{50} values of >62, 4.18 µg/mL, 29.9 and 16.3 µg/mL, respectively. Thus, the methanolic extract showed no activity against the MCH7 cells. From the total methanolic extract, a petroleum ether, a chloroform and a n-butanol fractions was made along with a mother liquor. The chloroform fraction, IC_{50} values of 4.17, 5.59, 4.98, and 3.56 µg/mL, and the n-butanol fraction, IC_{50} values of 4.17 µg/mL, 5.59 µg/mL, 4.98 µg/mL and 3.56 µg/mL, showed good activity against the cell lines MCF7, HFB4, HEPG2 and HELA, respectively. The petroleum ether fraction showed activity against the MCF7, HFB4, HEPG2 and HELA cell lines with IC_{50} values of 10.3 µg/mL, 20.6 µg/mL, 13.3 µg/mL and 10.3 µg/mL, respectively. The mother liquor had moderate activity with IC_{50} values of 15.8, 22.3, 20.6 and 12.1 µg/mL, respectively against the four cell lines.

Mothana *et al.*, (2011) tested *H. forskaolii* for its cytotoxic effect on the human urinary bladder carcinoma (5637) and human breast cancer (MCF-7) cells. The methanolic extract had IC_{50} values of 14.3 and 32.1 µg/mL for the 5637 and MCF-7 cells, respectively. The aqueous extract had an IC_{50} value of >500 µg/mL. The positive drug control used was etoposide with IC_{50} values of 2.27 and 5.62 µg/mL, respectively. Mothana *et al.*, (2014) also tested the methanolic extract (IC_{50} 11 µg/mL) for its toxicity towards human lung fibroblast cell lines (MRC-5) using the resazurin fluorescent assay.

Anti-oxidant

Mothana *et al.*, (2011) tested *H. forskaolii* for its anti-oxidant activity using the DPPH free radical scavenging assay. At 500 µg/mL the ethanolic extract had a 96% radical scavenging activity compare to the positive drug control, ascorbic acid, which had a 97% radical scavenging activity at 50 µg/mL.

8.7. Additional information

8.7.1. Therapeutic (proposed) usage

Anti-fungal activity, antileishmanial activity, antiplasmodial activity, antitrypanosomal activity and anti-oxidant activity.

8.7.2. Safety data

Hypoestes forskaolii showed to have a toxic effect towards human breast cancer (MCF7), human hepatocellular carcinoma (HEPG2), human cervix cancer (HELA) and human normal melanocyte (HFB4) cell lines (Almehdar *et al.*, 2012). It also was reported to have some toxicity towards human urinary bladder carcinoma (5637), lung fibroblast cell lines (MRC-5) and human breast cancer (MCF-7) cells (Mothana *et al.*, 2011; Mothana *et al.*, 2014).

8.7.3. Trade information

H. forskaolii is not threatened, not endangered, abundant and is of least concern (Kamundi 2006b).

8.7.4. Dosage

No specific concentration has yet been determined for the use of *H. forskaolii.*

9. *Pelargonium citronellum*

9.1. General Description on Selected plants

9.1.1. Botanical nomenclature

Pelargonium citronellum J.J.A. van der Walt (Raimondo & Helme 2007).

9.1.2. Botanical Family

Geraniaceae (Mjuleni 2007).

9.1.3. Vernacular names

English: citronella pelargonium, lemon-scented pelargonium (Mjuleni 2007).

Afrikaans: malva (Mjuleni 2007).

9.2.Botanical Description

Pelargonium citronellum is a strongly lemon-scented evergreen bush that can grow up to 2m high with a spread of 1m. When young the shrub is herbaceous and woody at the base. The leaves are simple, alternately arranged and are sparsely covered in small glandular hairs as are the stems (Figure 3.14). The leaf is palmately shaped with sharply pointed lobes with conspicuous veins at the back. The pink-purple flowers consist of two larger upper petals and three smaller lower petals, with clear dark marking on the upper petals. The flowering season occurs during spring and summer, August to January, with its peak being early summer, between September and October (Mjuleni 2007).

9.3.Distribution

Endemic to South Africa, *P. citronellum* grows well near streams in well-drained soil (Mjuleni 2007; Raimondo & Helme 2007) and is common on the northern foothills of the Langeberg Mountains, from Miskraal to Herbertsdale. It can also be found in a small area in the south-eastern Western Cape close to Ladysmith (Figure 3.14).

Figure 3.14: *Pelargonium citronellum* (a) plant, (b) leaves and (c) flowers (Mjuleni 2007). (d) The distribution of *Pelargonium citronellum* in South Africa (GBIF Secretariat 2021h).

9.4.Ethnobotanical usage

Although no traditional use specifically for *P. citronellum* was found through literature research, the Pelargonium species have been used to treat coughs related to bronchitis and pharyngitis.

The local communities use the plant widely as a traditional medicine for the treatment of various ailments, including diarrhoea, colic, gastritis, tuberculosis, cough, hepatic disorders, menstrual complaints and gonorrhoea (Brendler & van Wyk 2008; Colling *et al.*, 2010). Instila, a stomach ailment in infants is treated with a remedy from the roots (Hutchings *et al.*, 1996). An indication that *Pelargonium sidoides* may exhibit antibacterial properties is shown through the use of a facial cream in the traditional treatment of skin pimples made from powdered plant materials soaked in water (Lewu *et al.*, 2007).

9.5.Phytochemical constituents

Studies have shown that *P. citronellum* essential oils contain a range of compounds. Only 6 of those are present in high levels: geranial, geraniol, geranic acid, linalool and neral (Figure 3.15) (Lis-Balchin 2002; Lalli 2006).

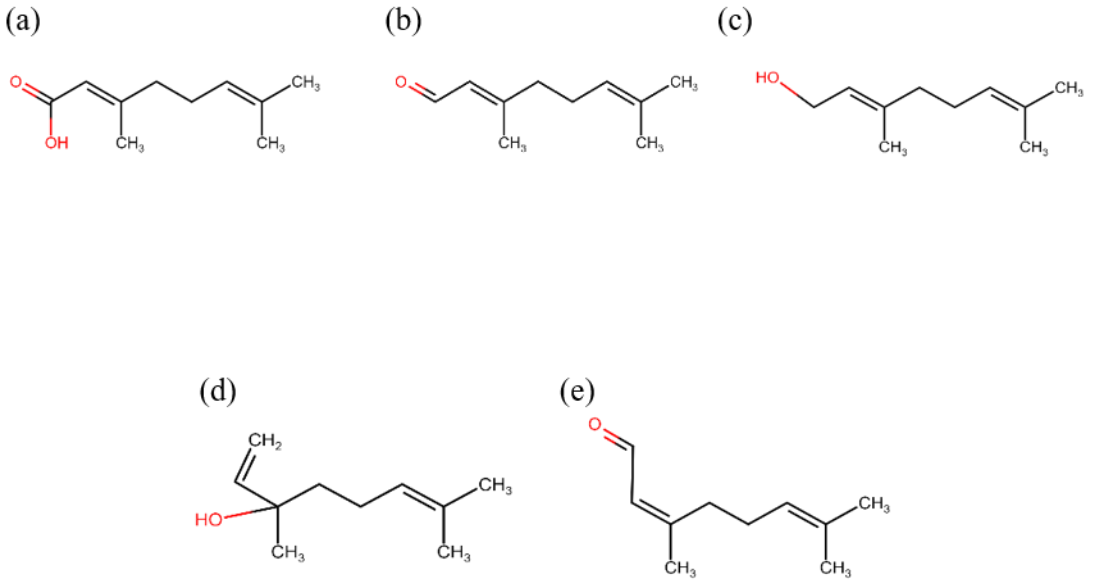

Figure 3.15: The phytochemical structures that have been isolated from *Pelargonium citronellum (a)* geranic acid, (b) geranial, (c) geraniol, (d) linalool and (e) neral.

9.6.Pharmacological properties

9.6.1. Antibacterial activity

Lalli (2006) investigated *P. citronellum* against the gram-positive bacteria *B. cereus* (ATCC 11778) and *S. aureus* (ATCC 12600), the gram-negative bacterium, *K. pneumoniae* (NCTC 1633) and the yeast *C. albicans* (ATCC 10231). The plant material was collected from the Stellenbosch Botanical Gardens and Kirstenbosch National Botanical Garden. The dilution assay was used to determine the antibacterial activity. The acetone extract had MIC values of 3, 0.25-0.41, 0.16 and 0.5 - 1 mg/mL against *K. pneumoniae, B. cereus, S. aureus* and *C. albicans*, respectively. The *P. citronellum* essential oil had MIC values of 16 mg/mL against *K. pneumoniae* and 4 mg/mL for the remaining 3 microbes. The positive controls for the bacteria and fungi were ciprofloxacin (MIC $2.5x10^{-3}$ mg/mL) and amphotericin B (MIC $1.25x10^{-3}$ mg/mL), respectively. Although *P. citronellum* essential oil displayed weak activity towards *K. pneumoniae,* the bacteria were not resistant to the oil.

Dorman and Deans (2000) assessed the compounds geraniol, linalool and neral against 25 bacterial strains. Geraniol showed moderate activity with zones of growth inhibition ranging between 5.2 - 12.9 mm. While linalool and neral showed moderate to low activity with zones of growth inhibition ranging between 7.5 - 27.5 and 7.1 - 19.1 mm, respectively.

Shin and Lim, (2004) tested geraniol against six *Trichophyton* spp., namely *T. erinacei* (KCCM 60411), *T. mentagrophytes* (KCCM 11950), *T. rubrum* (ATCC 6345), *T. schoenleinii* (KCCM 60477), *T. soudanense* (KCCM 60448) and *T. tonsurans* (KCCM 11866). The MFC was determined through the use of the dilution assay. Geraniol had MFC values of 1 mg/mL *T. mentagrophytes, T. rubrum, T. schoenleinii* and *T. tonsurans,* 2 mg/mL against *T. erinaceid* and 0.5 mg/mL against *T. soudanense*

9.6.2. Melanogenic activity

Unknown.

9.6.3. Other

Anti-oxidant activity and anti-inflammatory

Lalli (2006) investigated *P. citronellum* acetone extract in the DPPH radical-scavenging assay. The acetone extract from the Kirstenbosch National Botanical Garden had a DPPH IC_{50} value of 23.70 µg/mL and the acetone extract from the Stellenbosch Botanical Gardens had an IC_{50} value of 84.01 µg/mL. The drug control vitamin C had an IC_{50} of 4.72 µg/mL. *Pelargonium citronellum* showed limited anti-oxidant activity. *Pelargonium citronellum* essential oil was

investigated for its anti-inflammatory activity using the 5-lipoxygenase assay. The essential oil had an IC_{50} value of 50.04 µg/mL, when compared to nordihydroguaiaretic the positive control with an IC_{50} value of 5 µg/mL. It was reported that *P. citronellum* inhibited the activity of 5-LOX in a co-dependant manner and displayed moderate inhibitory activity.

Anti-malarial activity

Lalli (2006) assessed *P. citronellum* for its antiplasmodial activity against *P. falciparum* by using a semi-automated microdilution assay. This measured the inhibition of [^{3}H]-hypoxanthine into the malaria parasite by the acetone extract. The drug controls chloroquine and quinine had IC_{50} values of 0.06 and 0.03 µg/mL, respectively. The acetone extract from the Kirstenbosch National Botanical Garden had an IC_{50} value of 1.58 µg/mL and the acetone extract from the Stellenbosch Botanical Gardens had an IC_{50} value of 1.74 µg/mL. *Pelargonium citronellum* showed considerable selectivity for *P. falciparum* and thus has good antimalarial activity.

Cytotoxicity

The *P. citronellum* acetone extract and essential oils tested for its toxicity on transformed human kidney (Graham) cells using the microculture tetrazolium (MTT) colourimetry assay in Lalli (2006). The acetone extract from the Stellenbosch Botanical Gardens had an IC_{50} value of 59.94 µg/mL, the acetone extract from the Kirstenbosch National Botanical Garden had an IC_{50} value of 19.14 µg/mL and the essential oil had an IC_{50} value of 22.4 µg/mL (Lalli 2006).

9.7. Additional information

9.7.1. Therapeutic (proposed) usage

Anti-oxidant, anti-inflammatory and anti-malarial activity.

9.7.2. Safety data

Pelargonium citronellum had shown to have some cytotoxic towards transformed human kidney (Graham) cells (Lalli 2006).

9.7.3. Trade information

Pelargonium citronellum is rare however, it is not threatened, not endangered, abundant and is of least concern (Raimondo & Helme 2007).

9.7.4. Dosage

No specific concentration has yet been determined for the use of *P. citronellum.*

10. *Pelargonium graveolens*

10.1. General Description on Selected plants

10.1.1. Botanical nomenclature

Pelargonium graveolens L'Heritier (Lawrence 2002).

Synonyms: *Pelargonium intermedium* R. Knuth (Foden & Potter 2000e).

10.1.2. Botanical Family

Geraniaceae (Lawrence 2002).

10.1.3. Vernacular names

English: Rose-scented pelargonium, wildemalva (Lawrence 2002).

Figure 3.16: *Pelargonium graveolens* shrub (A), leaves (B) and flowers (C) (Royal Botanic Gardens, Kew 2012b). The distribution of *Pelargonium graveolens* in South Africa (GBIF Secretariat 2021i).

10.2. Botanical Description

This shrub can reach a height of up to 1.3 m and a spread of 1 metre. The stems of the erect, many-branched *P. graveolens* are herbaceous when young, becoming woody with age. The deeply incised leaves which are strongly rose-scented can feel soft to the touch and velvety due

to its large number of glandular hairs on both the leaves and the stems (Figure 3.16). Showy white to pinkish flowers appear in an umbel-like inflorescence during its flowering season of late winter to summer, August – January, but are at their best during spring, September - October (Lawrence 2002).

10.3. Distribution

While not endemic to South Africa (Foden & Potter 2000e), *P. graveolens* can be found in two distinct regions in southern Africa and has also been recorded in Zimbabwe and Mozambique. While both South African regions have hot summers and mild winters, it receives only summer rain in Limpopo, and rain throughout the year in the south-eastern part of the Western Cape (Figure 3.16). The plant grows in relatively moist habitats in sheltered positions such as kloofs on the mountains (Lawrence 2002).

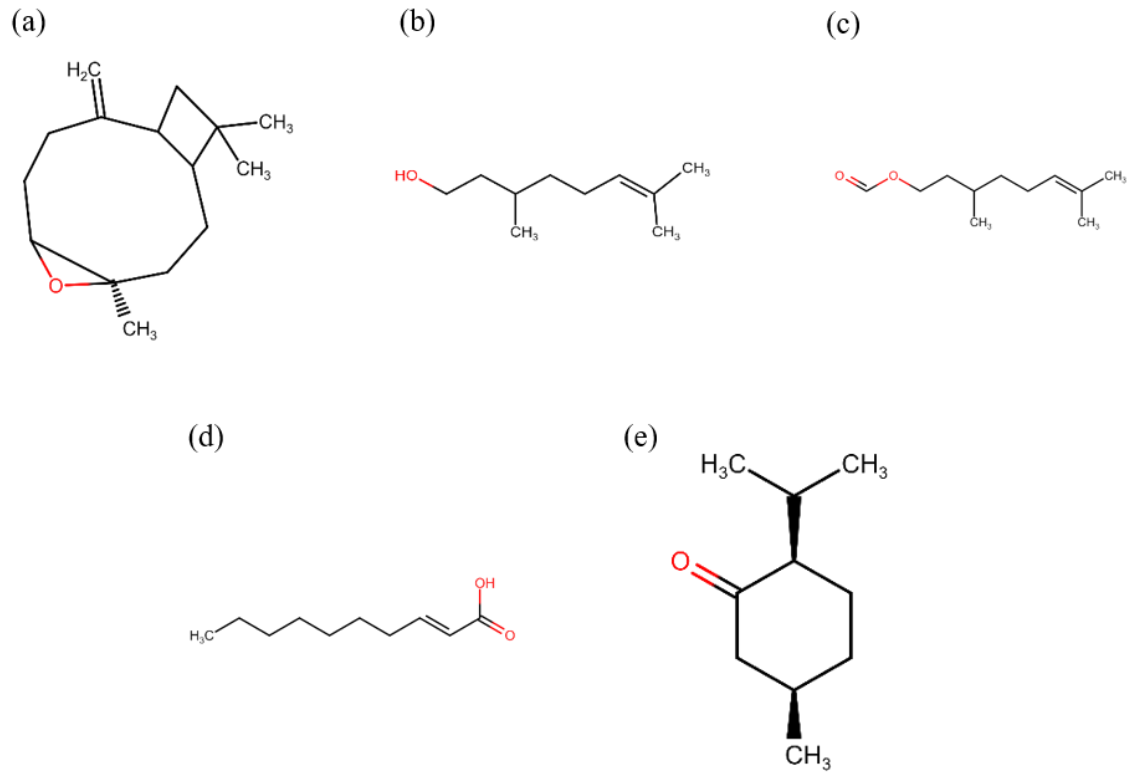

Figure 3.17: The phytochemical structures that have been isolated from *Pelargonium graveolens* (a) caryophyllene oxide, (b) citronellol, (c) citronellyl formate, (d) decenoic acid and (e) isomenthone.

10.4. Ethnobotanical usage

Although no traditional use specifically for *P. graveolens* was found through literature research, the Pelargonium species have been used to treat coughs related to bronchitis and pharyngitis. Further this indigenous South Africa plant species is widely used by Zulu, Basuto, Xhosa, and Mfengi traditional healers to treat dysentery, diarrhoea, hepatic complaints,

wounds, colds, fatigue, fevers, generalized weakness, and infections of the respiratory tract including tuberculosis (Kolodziej 2000). Major Charles Stevens was treated by a tribal healer with a root extract of Pelargonium species in 1897 establishing Western use of *P. sidoides* and *P. reniforme* in the treatment of tuberculosis (Kolodziej & Berlin 1998). Its introduction in Europe in the late 1890s is probably the most compelling ethnobotanical use of *Pelargonium sidoides* (Brendler & van Wyk 2008).

10.5. Phytochemical constituents

Studies have shown that *P. graveolens* essential oils contain a wide range of compounds. Only 6 of those are present in high levels: caryophyllene oxide, citronellol, citronellyl formate, decenoic acid and isomenthone (Figure 3.17). It also has common compounds with *P. citronellum* such as geraniol and neral (Lalli 2006; Ghannadi, *et al.*, 2012; Sharopov *et al.*, 2014;). *Pelargonium graveolens* contains ellagitannins, free ellagic acid and proanthocyanidins (Williams *et al.*, 2000).

10.6. Pharmacological properties

10.6.1. Antibacterial activity

Lalli (2006) assessed *P. graveolens* against the gram-positive bacteria *B. cereus* (ATCC 11778) and *S. aureus* (ATCC 12600), the gram-negative bacterium, *K. pneumoniae* (NCTC 1633) and the yeast *C. albicans* (ATCC 10231). The plant material was collected from the Walter Sisulu Botanical Garden and Stellenbosch Botanical Gardens. The dilution assay was used to determine the antibacterial activity. The positive controls for the bacteria and fungi were ciprofloxacin (MIC $2.5x10^{-3}$ mg/mL) and amphotericin B (MIC $1.25x10^{-3}$ mg/mL), respectively. The acetone extract had MIC values of 2 mg/mL, against *K. pneumoniae* and *B. cereus*, respectively. The MIC value against *S. aureus* was 4 mg/mL and against *C. albicans* was 3.33 mg/mL. *K. pneumoniae* was resistant to the *P. graveolens* essential oil. For the remaining bacteria, the essential oil had a MIC of 8 mg/mL.

Dorman and Deans (2000) tested the volatile oils of *P. graveolens* against 25 bacterial strains. They showed moderate activity with zones of growth inhibition ranging between 6.9 - 30.9 mm. Against 9 of the bacterial strains the volatile oils showed no activity.

In Shin and Lim, (2004) *P. graveolens* essential oil was tested against six *Trichophyton* spp., namely *T. erinacei* (KCCM 60411), *T. mentagrophytes* (KCCM 11950), *T. rubrum* (ATCC 6345), *T. schoenleinii* (KCCM 60477), *T. soudanense* (KCCM 60448) and *T. tonsurans* (KCCM 11866). The MFC was determined through the use of the dilution assay. The essential

oil had MFC values of 2 mg/mL against *T. erinaceid*, 1 mg/mL against *T. mentagrophytes* and *T. tonsurans*, 0.5 mg/mL against *T. rubrum*, *T. schoenleinii* and *T. soudanense*.

Ghannadi *et al.*, (2012) tested *P. graveolens* essential oil against the gram-positive bacteria *S. aureus* (PTCC 1112), *B. subtilis* (PTCC 1023) and *Listeria monocytogenes* (PTCC 1297) and the gram-negative bacteria *E. coli* (PTCC 1330), *P. aeruginosa (*PTCC 1074) and *Salmonella enteritidis* (PTCC 1091). The plant material used was obtained in Mazandaran province, Iran. The disc diffusion method was used to determine the zone of inhibition. The standard antibiotics uses were amoxicillin and chloramphenicol. The pure essential oil zones of inhibition were 9.5 mm, 25.3 mm, 42 mm, 16.5 mm and 9 mm against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis* and *S. enteritidis*, respectively, while it showed no activity against *L. monocytogenes.*

10.6.2. Melanogenic activity

Unknown

10.6.3. Other

Anti-oxidant activity and anti-inflammatory

Lalli (2006) investigated *P. graveolens* acetone extract in the DPPH radical-scavenging assay. The acetone extract from the Walter Sisulu Botanical Garden had a DPPH IC_{50} value of 26.81 µg/mL and the acetone extract from the Stellenbosch Botanical Gardens had an IC_{50} value of 14.49 µg/mL. The drug control vitamin C had an IC_{50} of 4.72 µg/mL. *Pelargonium graveolens* essential oil was investigated for its anti-inflammatory activity using the 5-lipoxygenase assay. The positive control nordihydroguaiaretic had an IC_{50} value of 5 µg/mL. The essential oil had an IC_{50} value of > 100 µg/mL thus showing no inhibitory activity against LOX-5.

Anti-malarial activity

Pelargonium graveolens was assessed for its antiplasmodial activity against *P. falciparum* by using a semi-automated microdilution assay (Lalli 2006). This measured the inhibition of [3H]-hypoxanthine into the malaria parasite by the acetone extract. The drug controls chloroquine and quinine had IC_{50} values of 0.06 and 0.03 µg/mL, respectively. The acetone extracts from the Walter Sisulu Botanical Garden had an IC_{50} value of 9.48 µg/mL and from the Stellenbosch Botanical Gardens had an IC_{50} value of 22.46 µg/mL

Cytotoxicity

The *P. graveolens* acetone extract and essential oils tested for its toxicity on transformed human kidney (Graham) cells using the microculture tetrazolium (MTT) colourimetry assay in Lalli (2006). The acetone extract from the Stellenbosch Botanical Gardens had an IC_{50} value of 83.31 µg/mL and the acetone extract from the Walter Sisulu Botanical Garden had an IC_{50} value of 80.48 µg/mL. The essential oil had an IC_{50} value of < 0.10 µg/mL (Lalli 2006).

10.7. Additional information

10.7.1. Therapeutic (proposed) usage

Anti-oxidant and anti-fungal activity.

10.7.2. Safety data

Pelargonium graveolens had shown to have some cytotoxic towards transformed human kidney (Graham) cells (Lalli 2006).

10.7.3. Trade information

Pelargonium graveolens is not threatened, not endangered, abundant and is of least concern (Foden & Potter 2005e).

10.7.4. Dosage

No specific concentration has yet been determined for the use of *P. graveolens.*

11. Portulacaria afra

11.1. General Description on Selected plants

11.1.1. Botanical nomenclature

Portulacaria afra Jacq. (Hankey 2002).

Synonyms: *Claytonia portulacaria* (L.) L., *Crassula arborea* L., *Crassula portulacaria* L., *Portulaca fruticosa* Thunb. (later homonym) (Von Staden 2015).

11.1.2. Botanical Family

Portulacaceae (Hankey 2002).

11.1.3. Vernacular names

English: Porkbush, Elephant's Food (Hankey 2002).

Afrikaans: Spekboom. Olifantskos (Hankey 2002).

isiZulu: iNtelezi, isiDondwane, isAmbilane, iNdibili, isiCococo (Hankey 2002).

isiXhosa: iGqwanitsha (Hankey 2002).

11.2. Botanical Description

With the ability to reach 2 - 5 m in height naturally and 1.5 - 2 m domestically in garden situations, *P. afra* is an attractive, evergreen succulent shrub or small tree (Figure 3.18). During late winter to spring the shrub bears small star-shaped pink flowers with small round succulent leaves and red stems. Due to the variety and number of insects attracted to its rich nectar source, *P. afra* appeals to insectivorous birds (Hankey 2002).

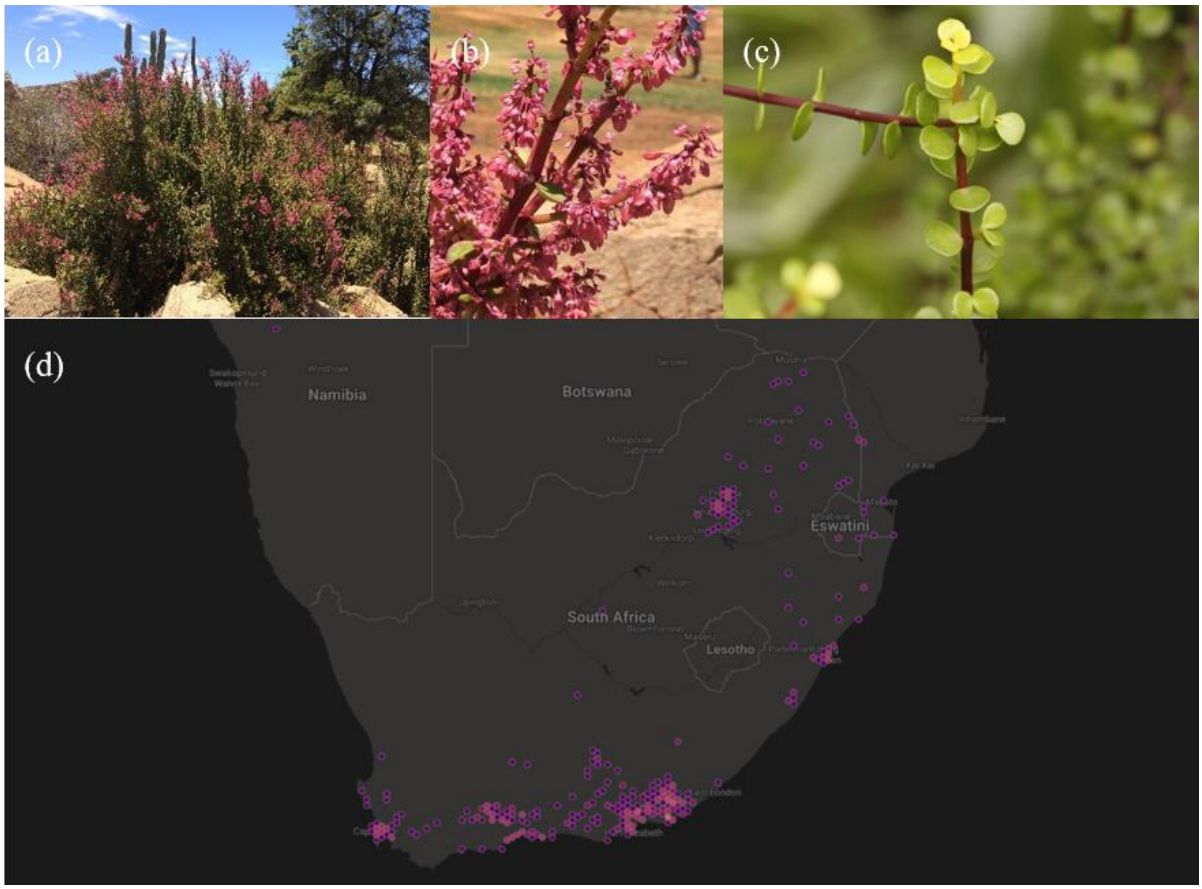

Figure 3.18: *Portulacaria afra* (a) shrub, (b) flowers and (c) fleshy leaves (Ebedes 2021e). (d) The distribution of *Portulacaria afra* in South Africa (GBIF Secretariat 2021j).

11.3. Distribution

Portulacaria afra is not endemic to South Africa and can be found growing in warm positions on rocky slopes across the eastern parts of South Africa from the Eastern Cape into KwaZulu-Natal, and northwards into Swaziland, Mpumalanga and the Limpopo Province (Figure 3.18). The shrub can also be found as far as Mozambique favouring succulent karoo scrub, thicket, bushveld and dry river valleys (Hankey 2002; Von Staden 2015).

11.4. Ethnobotanical usage

Portulacaria afra`s leaves are used traditionally for various disorders and diseases. It is used as an antiseptic to soothe skin ailments, rashes and chronic sores as well as other skin infections

(De Wet *et al.*, 2013; Olaokun *et al.*, 2017; Van Vuuren & Holl, 2017). Traditionally, it has been known to be administered for respiratory infections, diarrhoea, inflammatory disorders, obesity and as well as for cancer treatment (De Wet *et al.*, 2013; Khanyile *et al.*, 2021).

11.5. Phytochemical constituents

Khanyile *et al.*, (2021) performed a phytochemical analysis on *P. afra* and found that it contains alkaloids, glycoside, saponins and tannin. It was reported that it does not contain terpenoids. Two flavonoids have been isolated from the plant, kaempferol and quercetin (Figure 3.19) (Nyananyo & Olowokudejo 1986).

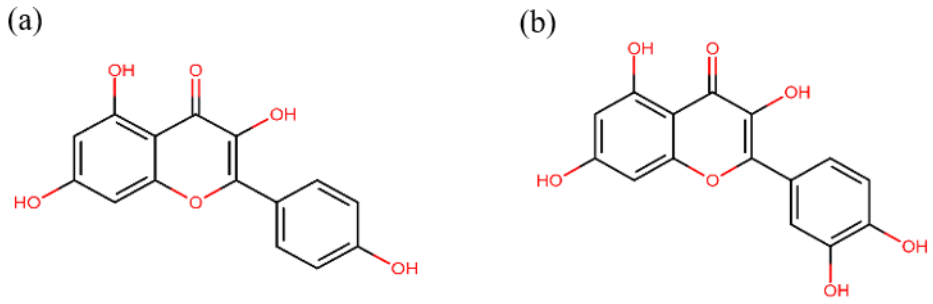

Figure 3.19: The phytochemical structures that have been isolated from *Portulacaria afra* (a) kaempferol and (b) quercetin

11.6. Pharmacological properties

11.6.1. Antibacterial activity

The plant leaves of *P. afra* used by Nciki *et al.*, (2016) were collected from northern Maputaland, KwaZulu-Natal. These were then tested against 12 pathogens to determine its antibacterial activity against skin pathogens. The aqueous and DCM: methanol extracts were prepared with 1 g dried ground plant material in 3.3 mL of extractant. The dilution assay was used to obtain the MIC values. The positive controls were ciprofloxacin and amphotericin B for the bacteria and fungi, respectively. *Portulacaria afra* showed weak activity against the bacterium *C. acnes* (ATCC 11827) with an MIC value of 8000 and > 8000 µg/mL for the DCM: methanol and aqueous extract, respectively. The DCM: methanol extract did show activity against methicillin-resistant *S. aureus* (ATCC 43300), *P. aeruginosa* (ATCC 27853), *M. canis* (ATCC 36299) and *T. mentagrophytes* (ATCC 9533) with MIC values of 380, 500, 190 and 250 µg/mL, respectively.

Khanyile *et al.*, (2021) tested *P. afra* against two bacteria, *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923). The *P. afra* leaves were obtained from Empangeni in KwaZulu-

Natal. 1 g of dry powdered leaves were extracted with 10 mL of methanol. The dilution assay was used to determine the MIC and MBC values. Ciproflaxacin was used as the control, with MIC and MBC values of 25 mg/mL and 50 mg/mL, respectively, against *S. aureus* and *P. aeruginosa*. The methanolic extract had MIC and MBC values of 6.25 and > 50 mg/mL, respectively, against *S. aureus* and values of 12.5 and > 50 mg/mL, respectively, against *P. aeruginosa*.

Portulacaria afra was assessed against *violaceum* (ATCC 12472) for its anti-quorum sensing activity by inhibiting the production of violacein, a purple pigment produced by the bacteria. *Chromobacterium violaceum* produces violacein in the presence of N-hexanoyl homoserine lactone. The methanolic extract showed significant violacein inhibition at 12.5 mg/mL with 93% activity. At 0.2 mg/mL the extract showed moderate, 65%, activity. *P. afra* has a potential use against resistant pathogens through its strong anti-quorum sensing potential (Khanyile *et al.*, 2021).

11.6.2. Melanogenic activity

Unknown.

11.6.3. Other

Antioxidant and anti-inflammatory activity

Anti-oxidant activity of *P. afra* was determined by Khanyile *et al.*, (2021). The DPPH and ABTS scavenging assays were used. The positive controls used were ascorbic acid and butylated hydroxyl anisole. *Portulacaria afra* displayed a 70 and 90% DPPH and ABTS scavenging activity at 0.08 mg/mL. The extract had IC_{50} values of 0.26 and 0.25 mg/mL for its DPPS and ABTS radical scavenging activity, respectively.

Olaokun *et al.*, (2017), tested *P. afra* for its inhibitory activity towards 5-lipoxygenase enzyme. The plant material used was collected from the SANBI Pretoria National Botanical Garden. The extracts were made using 1 g of dried powdered plant material in 10 mL acetone extractant. The positive drug control used was quercetin, with an IC_{50} 9.02 µg/mL. *Portulacaria afra* was shown to have weak LOX-5 inhibition with an IC_{50} of 107.26 µg/mL. The acetone extract was also tested for its DPPH radical scavenging ability and had an IC_{50} value of 32.05 µg/mL, which was considered weak. The positive control used was ascorbic acid and Trolox with IC_{50} values of 0.21 and 0.47 µg/mL, respectively.

Glucose utilisation activity

Portulacaria afra was tested for its glucose utilisation by Olaokun *et al.*, (2017). The acetone extract was first tested for its effect on the glucose utilisation of C2C12 muscle myotubules. In a dose-response manner, *P. afra* enhanced the glucose utilization activity of the C2C12 muscle cells by 64.17% at 500 µg/mL. Insulin was used as the positive drug control and at 1 µM, it had an enhancement of 71.73%. The plant was then tested for its glucose utilization activity of 3T3-L1 adipocytes. The acetone extract and insulin enhanced the glucose utilization activity of 3T3-L1 adipocytes by > 60% at 500 µg/mL and 70.45% at 1 µM, respectively.

Cytotoxicity

Khanyile *et al.*, (2021) assessed *P. afra* for its cytotoxicity towards the human hepatocellular carcinoma (HepG2) cell-line. The MTT colorimetric assay was used to determine the IC_{50} values. *Portulacaria afra* was reported to have an IC_{50} value of 4010 µg/mL. At 500 µg/mL the extract had a cell viability of 120% and at 2500 µg/mL it decreased to 100%. The authors suggest that *P. afra* is only safe to use at 2500 µg/mL due to its decrease in cell viability.

11.7. Additional information

11.7.1. Therapeutic (proposed) usage

Antibacterial activity, glucose utilisation activity, anti-oxidant and anti-inflammatory activity.

11.7.2. Safety data

Portulacaria afra has shown some toxicity towards human hepatocellular carcinoma cells.

11.7.3. Trade information

Portulacaria afra is not threatened, not endangered, abundant and is of least concern (von Staden 2015).

11.7.4. Dosage

No specific concentration has yet been determined for the use of *P. afra.*

12. Sideroxylon inerme

12.1. General Description on Selected plants

12.1.1. Botanical nomenclature

Sideroxylon inerme L. subsp. (Bosman, 2006).

Synonyms: *Calvaria inermis* (L.) Dubard, *Sideroxylon atrovirens* Lam., *Sideroxylon inerme* L. var. schlechteri Engl. (Foden & Potter 2005a).

12.1.2. Botanical Family

Sapotaceae (Bosman, 2006).

12.1.3. Vernacular names

English: white milkwood (Bosman, 2006).

Afrikaans: witmelkhout, melkhoutboom, melkbessie (Bosman, 2006).

Zulu: aMasethole-amhlope, uMakhwela-fingqane (Bosman, 2006).

Xhosa: aMasethole, umQwashu (Bosman, 2006).

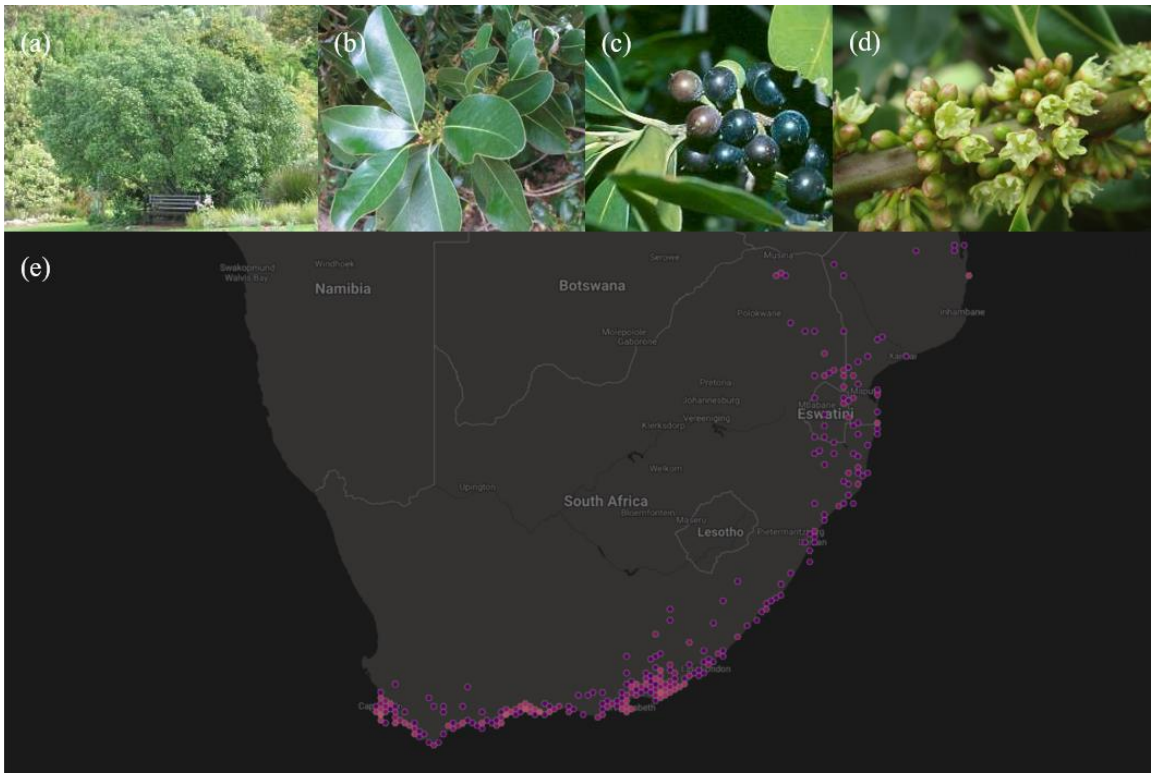

Figure 3.20: *Sideroxylon inerme* (a) tree, (b) leaves, (c) fruit and (d) flowers (Bosman 2006; Ebedes 2021f). (e) The distribution of *Sideroxylon inerme* in South Africa (GBIF Secretariat 2021k).

12.2. Botanical Description

The bark of this small to medium evergreen tree is normally grey-brown to black. It has a sturdy trunk of 600mm in diameter and can grow to a height of 10-15 m developing a large, dense, rounded crown. The dark green leaves are dull on the underside, leathery and spirally arranged (Figure 3.20). Fine hairs are found on young leaves and branches. The tree produces small greenish white flowers with a strong, unpleasant smell during summer and autumn (November to April). Purplish black, small, round and fleshy fruits present from late summer to spring (February to September) containing a milky latex as do the leaves (Bosman, 2006).

12.3. Distribution

While not endemic to South Africa this plant is native to East Indies and Malaysia and can also be found in India, Sri Lanka, Africa and Pacific Islands (Sharma & Lall 2014). In South Africa the tree can be commonly found in dune forests, almost always in coastal woodlands and also in littoral forests (Figure 3.20). It also occurs further inland in Zimbabwe and Gauteng (Foden & Potter 2005a; Bosman, 2006).

12.4. Ethnobotanical usage

The Xhosa of the Western Cape use a decoction from the leaves of *S. inerme* for bathing in order to remove body odour (Afolayan *et al.*, 2014) and also for stomach ache (van Wyk *et al.*, 1997). Both the Zulu and Xhosa have a variety of medicinal uses for the bark, while the stem, bark and roots are used to treat fevers and broken bones (Watt & Breyer-Brandwijk 1962).

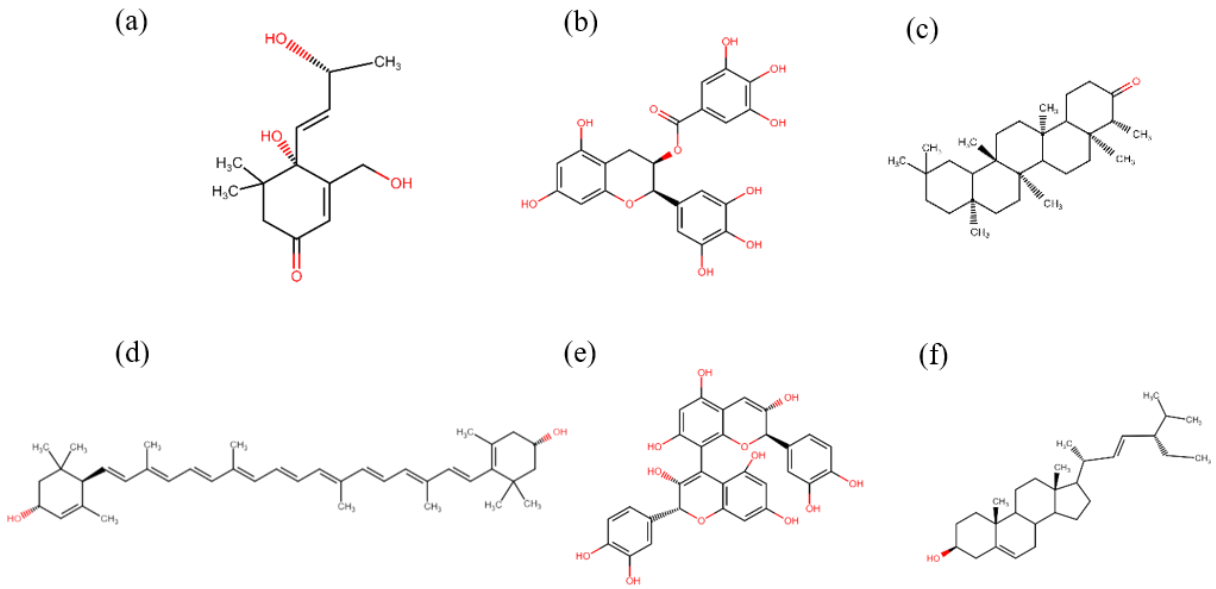

Figure 3.21: The phytochemical structures that have been isolated from *Sideroxylon inerme* (a) apocynol B, (b) epigallocatechin gallate, (c) friedelin, (d) lutein, (e) procyanidin B1 and (f) stigmasterol

12.5. Phytochemical constituents

Eight compounds have been isolated from *S. inerme*: α-amyrin, β-amyrin, apocynol B, epigallocatechin gallate, friedelin lutein, procyanidin B1 and stigmasterol (Figure 3.21) (Momtaz *et al.*, 2008; Shelembe 2014). α-Amyrin and β-amyrin have also been found in *C. spicata* (Figure 3.9).

12.6. Pharmacological properties

12.6.1. Antibacterial activity

In Sharma and Lall's (2014) study *S. inerme* was assessed for its antibacterial activity against *C. acnes* (ATCC 11827). The plant material used was obtained from the Botanical Garden of the University of Pretoria. The extract was made using 1 g of dried powdered plant material in 3.75 mL of ethanol extractant. The micro-dilution assay was used, the positive drug control, tetracycline, and *S. inerme* had MIC values of 3.12 and 250 µg/mL, respectively.

12.6.2. Melanogenic activity

Momtaz *et al.*, (2008) assessed *S. inerme* and its phytochemical constituents for their effect on mushroom tyrosinase. The bark of the plant was obtained from Venda. The extract was made using 1 g dried powdered bark in 5 mL acetone, DCM and methanol, separately. The colorimetric tyrosinase inhibition and tyrosinase inhibition bioautography assays were used. Kojic acid was used as the positive drug controls, with IC_{50} values of 1.13 µg/mL for the L-tyrosine inhibition and 50.51 µg/mL for the L-DOPA inhibition. At 200 µg/mL, the acetone, methanol and DCM extracts had IC_{50} values of 63, 82.1 and > 400 µg/mL for the L-tyrosine inhibition, respectively, and for the L-DOPA inhibition the IC_{50} values were > 400 µg/mL. The acetone and methanolic extracts showed significant inhibition ($p < 0.01$). Two isolated compounds, epigallocatechin gallate (IC_{50} 30 and > 200 µg/mL) and procyanidin B1 (IC_{50} 200 and > 200 µg/mL) were also tested for L-tyrosine and L-DOPA inhibition, respectively.

Sideroxylon inerme methanolic extract and epigallocatechin gallate were further tested for their inhibition of melanin production on the mouse melanocyte (B16F10) cell line. It was reported that the extract inhibited melanin production by 37% at 6.2 µg/mL and had a low level of cytotoxicity as it had a cell viability of 80%. Epigallocatechin gallate showed no significant melanogenesis inhibition. Thus, epigallocatechin gallate has a minor role in the inhibition of melanogenesis (Momtaz *et al.*, 2008). The study went on to further determine if *S. inerme* inhibited tyrosinase at a transcriptional level using B16F10 cells treated with the methanolic extract and semi-quantitative RT-PCR. The GAPDH gene served as the housekeeping gene. At 25 µg/mL, *S. inerme* acted as a potent tyrosinase inhibitor at the transcriptional level when compared to the untreated cells and the cells treated with Kojic acid.

12.6.3. Other

Anti-oxidant activity

Momtaz *et al.*, (2008) assessed the methanolic extract of *S. inerme* and two isolated compounds epigallocatechin gallate and procyanidin B1 for their anti-oxidant activity using the DPPH radical scavenging assay. The positive drug control used was ascorbic acid which had an EC_{50} value of 3.34 µg/mL. The methanolic extract, epigallocatechin gallate and procyanidin B1 had EC_{50} values of 1.54, 1.33 and 1.68 µg/mL, respectively. *Sideroxylon inerme* and its two isolated compounds had a two-fold increase in radical scavenging activity when compared to that of ascorbic acid. The authors report that epigallocatechin gallate and procyanidin B1 are likely responsible for the methanolic extract's antioxidant activity.

Shelembe (2014) also looked at the anti-oxidant activity of *S. inerme*. The plant material used was collected from KwaZulu-Natal. Separate extracts of the roots, leaves, bark and fruit were made using dried powdered material aqueous methanol extractant. The DPPH radical scavenging assay was used and the positive drug control was ascorbic acid. The methanolic root, bark, leaf and fruit extracts had IC_{50} values of 191.1, 41.1, 178.3 and 815.4 µg/mL, respectively. Ascorbic acid had an IC_{50} values of 41 µg/mL. The isolated compound apocynol B was also tested and had an IC_{50} value of 71.4 µg/mL. The author suggests that the higher antioxidant activity of the bark extract could be attributed to the presence of apocynol B.

12.7. Additional information

12.7.1. Therapeutic (proposed) usage

Anti-bacterial, anti-oxidant and tyrosinase inhibition.

12.7.2. Safety data

Sideroxylon inerme showed to have cytotoxic activity towards mouse melanocyte cells (Momtaz *et al.*, 2008).

12.7.3. Trade information

Sideroxylon inerme is not threatened, not endangered, abundant and is of least concern (Foden & Potter 2005a).

12.7.4. Dosage

No specific concentration has yet been determined for the use of *S. inerme.*

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Chapter 4: The stimulating effect of extracts of South African plants on melanin production and their antibacterial activity against *Cutibacterium acnes*

The stimulating effect of extracts of South African plants on melanin production and their antibacterial activity against *Cutibacterium acnes*[1]

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Abstract

Progressive macular hypomelanosis (PMH) is a poorly understood hypopigmented skin disorder that has an indefinite treatment. It is caused by the bacteria, *Cutibacterium acnes* which decreases melanogenesis in the affected areas resulting in hypopigmented macules. Current treatments include a combination of ultra-violet radiation and antibiotics, however, these antibiotics cause side effects such as severe rashes, blistering and dryness, whereas UV radiation leads to increased oxidative stress which can cause premature ageing and susceptibility to skin cancer development. Therefore, in this study, thirty three extracts, prepared using ethanol, water and dichloromethane from the leaves and twigs of eleven South African medicinal plants, were investigated for their antibacterial activity as well as tyrosinase and melanin stimulatory activity as possible targets for the treatment of PMH.

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The *Sideroxylon inerme* ethanolic extract showed a noteworthy minimum inhibitory concentration (MIC) of 125 µg/mL against *C. acnes* (ATCC 6919) and had an additive effect when combined with the positive control, tetracycline. The *Bulbine frutescens* ethanolic extract (at 200 µg/mL) increased the monophenolase activity of tyrosinase by 31.44 ± 1.41 % and increased melanin production by 8.55 ± 1.66 % (at 100 µM) compared to the α-melanocyte stimulating hormone (13.39 ± 1.44 %). Furthermore, *Bulbine frutescens* and *S. inerme* ethanolic extracts showed no antiproliferative activity at the highest tested concentration of 400 µg/mL against human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cells.

Consequently, the ethanolic leaf and stem extract of *B. frutescence* and *S. inerme* show potential use for the treatment of hypopigmentation due to their melanin production and antibacterial activity. Further investigation would include evaluating the irritancy potential of these extracts to determine cosmetic safety as topical treatments and to conduct *in vivo* hypopigmentation trials to determine their efficacy.

Key words

Cutibacterium acnes, progressive macular hypomelanosis, antibacterial activity, tyrosinase, melanin production

1. Introduction

Several studies have focused on the reduction of skin pigmentation with respect to age spots and ultra-violet (UV) radiation damage [1-3]. Age spots and UV induced skin damage is primarily caused by the over-production of melanin. However, it is not only important to investigate the potential of natural products or medicinal plants to reduce the production of melanin but also the potential to stimulate melanin production. Pigmentation of the skin functions as a broadband UV absorbent and is the most important photoprotective factor of the skin [4]. Chiang et al [5] reported that naringenin, found in citrus plant extracts, stimulated cellular melanogenesis through the increase of the microthalmia-associated transcription factor which upregulates tyrosinase expression in mouse B16 melanoma cells. The cells were treated with hydrolysate of *Citrus paradisi* and *Citrus grandis*, at 20 mg/mL for 72 hrs and increased melanin synthesis by 147 and 157 %, respectively. The focus of their study was to determine the potential of plant extracts to increase melanin production without the use of UV radiation.

There are numerous skin pigmentation disorders such as progressive macular hypomelanosis, post-inflammatory hypopigmentation, vitiligo, chemical leukoderma and *pityriasis alba*. Progressive macular hypomelanosis (PMH) is identified by hypopigmented macules that are symmetrical, ill-defined and found primarily on the back and chest, and can also been found to extend towards the face [6]. The melanocytes in the lesions produce significantly less pigment as the melanosomes are undersized and under-developed [7–9]. Westerhof et al [9] was the first to suggest that *Cutibacterium acnes*, previously known as *Propionibacterium acnes*, is the causative bacteria for the skin disorder, due to the high-density presence of the bacteria localised in the lesions. Three hypotheses have been suggested for the development of PMH; *C. acnes* produces inhibitory factors that either inhibit melanogenesis, inhibit or alter tyrosinase, or interfere with melanin transfer [6, 9].

With the hypothesis that *C. acnes* is the main cause of PMH, attempts at treating the disease have led to the combinational use of antibiotics and phototherapy. Phototherapy involves the use of light and laser therapy that requires the patient to be exposed to controlled amounts of non-ionising ultraviolet (UV) light, such as UVA, UVB and sunlight [10]. Relyveld et al [11] performed a clinical study in which 45 patients, that suffered from PMH, were treated with 5 % benzoyl peroxide gel, at night, and 1 % clindamycin cream, in the morning, in combination with 20 minutes UVA irradiation, three times weekly, over 26 weeks. It was found that after 14 weeks, 62 % of the patients and dermatologists thought their skin was completely re-pigmented, through visual comparison before and after treatment. The authors concluded that the antibacterial treatment together with the UVA radiation enhanced the re-pigmentation process, by inhibiting the causative bacteria and stimulating the melanogenesis process [11]. Isotretinoin in combination with narrow band UVB radiation has also shown success in the treatment of PMH [12]. Antibiotics, without the use of phototherapy, have proven to be successful in a few cases [13, 14]. However, the most successful treatment has been the use of

antibiotics together with narrowband UVB treatment, however this does not result in permanent re-pigmentation [12, 15]. The main concern with the current treatments is that they are long term and continuous, up to 18 months, and there is a high likelihood of pigmentation loss after the treatment has been completed [10].

The aim of this study was, therefore, to determine if the selected plant extracts were able to inhibit the growth of *C. acnes* or induce the melanogenesis process via an increase of the monophenolase activity of tyrosinase and melanin production within melanocytes, and therefore mitigate the use of UV radiation and antibiotics as a treatment option for PMH.

2. Materials and methods

2.1. Materials

Kojic acid (purity ≥ 98.5%), mushroom tyrosinase, L-tyrosine substrate, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (purity ≥ 99%), tetracycline (purity ≥ 98%), α-melanocyte stimulating hormone, hemotoxylin, actinomycin D (purity ≥ 95%) and all analytical grade reagents were supplied by Sigma Aldrich (Johannesburg, South Africa). Dulbecco's Modified Eagle medium, fetal bovine serum, PrestoBlue™ cell viability reagent, phosphate-buffered saline and phenol red trypsin-EDTA (0.25%) was supplied by Thermo Fisher Scientific, (Johannesburg, South Africa). *Cutibacterium acnes* (ATCC 6919), brain heart infusion (BHI) agar and broth was supplied by Anatech Instruments (Pty) Ltd (Randburg, South Africa). The human keratinocyte (HaCat) and the University of Cape Town-Melanoma 1 (UCT-Mel-1) cell lines were donated by Dr. Lester Davids (Department of Human Biology, University of Cape Town).

2.2. Methods

2.2.1. Preparation of plant extracts

The aerial parts (leaves and twigs) of eleven plants were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria. A herbarium specimen of each plant was deposited at the H.G.W.J. Schweickerdt Herbarium (Table 1). Plant species names were validated using The Plant List [16] and Kew Royal Botanical Gardens [17].

Extracts were prepared as described by Sharma and Lall [18] with minor changes. The dochloromethane (DCM) and ethanolic extracts for the non-succulent plants (*Barleria obtusa, Cussonia spicata, Hypoestes aristata, Hypoestes forskaolii, Pelargonium citronellum, Pelargonium graveolens* and *Sideroxylon inerme*), were prepared using air-dried powdered plant material which was blended in the extractant and shaken for 48 hrs. The menstruum was

filtered using a Buchner funnel (Whatman 1.0 filter), followed by rotary evaporation (Buchi-R-200) (Heidolph, Hei-Vap value digital HB/G3B, Germany), freeze drying (Alpha 1-2 LDplus) (Christ, Osterode am Harz, Germany) and storage at -20ºC until further use. The succulent plants (*Bulbine frutescens, Carpobrotus dimidiatus, Cotyledon orbiculata* and *Portulacaria afra)* were harvested and immediately macerated in a blender in DCM and ethanol, respectively shaken for 48 hrs, filtered, followed by rotary evaporation, freeze drying and storage at -20ºC.

The aqueous plant extracts were prepared by making a decoction in hot water, which was then left to cool and placed in the fridge overnight. The aqueous decoction was filtered, frozen in the -20ºC freezer, freeze dried and stored at -20ºC. The aqueous extract of the succulent plants followed a similar procedure except they were blended in the hot water on the same day of harvesting.

2.2.2. Phytochemical screening

The major phytochemical groups present in the ethanolic extracts, were determined as described by Lall et al [19], with minor modifications. Ethanol is of medium polarity and able to extract both polar and no-polar compounds and therefore these were the only extracts tested. Stock concentrations of the extracts were prepared at 15 mg/mL (in dH_2O) to determine the presence of tannins, saponins and terpenes. The presence of tannins was observed after the addition of 2 mL 5% ferric chloride ($FeCl_3$) in dH_2O by the development of a yellow-brown precipitate. The presence of saponins was observed by the formation of froth after being shaken vigorously. Terpenes were observed by the formation of a reddish-brown colour on the interface after the addition of 5 mL chloroform, 2 mL glacial acetic acid and several drops of concentrated H_2SO_4 (85%).

Stock concentrations of the extracts were prepared at 15 mg/mL (in methanol) for the determination of alkaloids, flavonoids and phenolics. Alkaloids were detected by adding1.5 mL of 1% HCl, heating the solution in a water bath at 90 ºC and then adding a few drops of Dragendroff's reagent to form an orange precipitate. Flavonoids were detected through the immediate appearance of a pink-red colour after 2 drops of hydrochloric acid (HCl) and 0.5 g magnesium turnings were added to the stock solution. Phenolics were detected after 1 mL 1% ferric chloride was added and a colour change to blue or green was observed.

2.2.3. Antibacterial activity – minimum inhibitory concentration

The antibacterial activity of the extracts was determined through a broth microdilution method as described by Lall et al [20]. *Cutibacterium acnes* (ATCC 6919) was cultured for 72 hrs at 37ºC under anaerobic conditions. Colonies were then inoculated into cow BHI broth at a concentration of 1.5 x 10^8 colony forming unit (CFU) per mL (CFU/mL) (OD_{600} = 0.132). The extracts and the positive control, tetracycline, were prepared in 10% DMSO at a stock concentration of 2 and 0.2 mg/mL, respectively. The samples were serially diluted in a 96-well plate in BHI broth and bacterial suspension (100 µL) was added to obtain a final concentration range of 3.9 - 500 and 0.3 - 50 µg/mL, respectively. Controls included the DMSO (2.5% v/v) as the vehicle control, growth media control (0%) and untreated bacterial cells (100%). The plates were incubated in an anaerobic environment for 72 hrs at 37 ºC after which, 20 µL of PrestoBlue™ was added to visually determine the minimum inhibitory concentration (MIC) after 2 hrs incubation. The threshold for antibacterial activity of extracts was used from Kuete and Efferth [21] and determined as significant (MIC < 100 µg/mL), moderate (100 < MIC ≤ 625 µg/mL) or weak (MIC > 625 µg/mL).

2.2.4. Antibacterial activity – minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined using an adapted method of Afroz et al [22]. Prior to the addition of the PrestoBlue™ to the above wells for determination of MIC, half the wells' content (100 µL) was transferred to new nutrient broth (100 µL) in a new 96-well plate. The 96-well plates were again incubated under anaerobic conditions at 37 ºC for 72 hrs, after which, 20 µL of PrestoBlue™ was added and the minimum bactericidal concentration (MBC) was then visually determined.

2.2.5. Antibacterial activity – combination study

The combined antibacterial activity of the ethanolic *S. inerme* extract, which showed the lowest MIC, and tetracycline was evaluated. The assay was performed as described by Gibango et al [23]. A stock concentration of the extract and tetracycline was prepared at 2 and 0.2 mg/mL (in 10% DMSO), respectively. The extract and tetracycline were added in nine different ratios from *S. inerme*: tetracycline (9:1) to *S. inerme*: tetracycline (1:9). In a sterile 96-well plate, 100 µL of BHI broth was added to each well, followed by 100 µL of the combined ratios, which were serially diluted. Thereafter, 100 µL of bacterial suspension, as described in section 2.2.3, was added to all the wells. Controls include ethanolic *S. inerme* (10:0) (3.91 – 500 µg/mL), tetracycline (0:10) (0.39 – 50 µg/mL), DMSO (2.5% v/v) as the vehicle control, growth media control (0%) and untreated bacterial cells (100%). The plates were incubated for 72 hrs at 37

ºC in anaerobic conditions, where after 20 µL of PrestoBlue™ was added to determine the MIC. The fractional inhibitory concentration (FIC) was calculated using the following formula:

$$\Sigma \text{ FIC } = \frac{\text{MIC (Sample A + Sample B)}}{\text{MIC (Sample A)}} + \frac{\text{MIC (Sample A + Sample B)}}{\text{MIC (Sample B)}}$$

Where an FIC < 0.5 = synergistic effect, 0.5 < FIC < 4 = additive effect and FIC > 4 = antagonistic effect [24].

2.2.6. Tyrosinase activity

The extracts were evaluated for modulatory activity of the mushroom tyrosinase enzyme using a method described by Blom van Staden and Lall [25]. The extracts and the positive control, kojic acid, were prepared to a stock concentration of 2 mg/mL (in 10% DMSO). The extracts and kojic acid were added to a 96-well plate and serially diluted to obtain final concentration ranges of 3.12-200 and 0.19-12.5 µg/mL, respectively. Controls included the DMSO (2%) as the vehicle control and an untreated enzyme control. To each well, 90 µL of 50 mM potassium phosphate buffer (pH 6.5) and 20 µL of monophenolase mushroom tyrosinase (333 Units/mL in phosphate buffer) were added and incubated at room temperature for 5 min. Thereafter, 70 µL of L-tyrosine substrate (2 mM) was added. The absorbance was kinetically measured at 492 nm for 30 min with a BIOTEK Power Wave multi-well plate reader (A.D.P., Weltevreden Park, South Africa). The 50% inhibitory concentrations (IC_{50}) were determined using GraphPad Prism 7 software.

2.2.7. Cell culture

The human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cell lines were prepared as described by Lall et al [26], with a few modifications. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% amphotericin B (250 µg/mL), 10% heat in-activated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were cultured in a humidified incubator set at 5 % CO_2 and 37 ºC. The cells were grown until a confluent monolayer formed in a T75 cell culture flask and were then sub-cultured using 0.25% trypsin-EDTA.

2.2.8. Antiproliferative activity

Ethanolic extracts of *B. frutescens, C. spicata, P. afra* and *S. inerme* were tested for their antiproliferative activity against human keratinocyte (HaCaT) and human melanoma (UCT-Mel-1) cells as previously described [20]. The extracts which increased tyrosinase activity or showed noteworthy antibacterial activity were evaluated. The UCT-Mel-1 and HaCaT cells

were seeded (100 µL) into a 96-well plate (1 x 10^5 cells/ well) and incubated in 5 % CO_2 at 37 ºC for 24 hrs. The extracts and the positive control, actinomycin D were prepared at stock concentrations of 20 and 1 mg/mL (in DMSO) and serially diluted in a 24-well plate in DMEM at concentration ranges of 6.26 – 800 and 7.81 x 10^{-4} – 0.1 µg/mL. Samples dilutions (100 µL) were transferred, in triplicate, to the 96-well plates containing the cells to obtain final concentration ranges of 3.13 - 400 and 3.9 x 10^{-4} – 0.05 µg/mL, respectively. Controls included untreated cells (100%), 1% DMSO treated cells as the vehicle control and a 0% media control. The cells were incubated for 72 hrs, where after, 20 µL of PrestoBlue™ reagent was added to all the wells and incubated for an additional 2 hrs. The fluorescence was measured at an excitation of 530 nm and emission of 625 nm using the Victor Nivo Multimode Microplate plate (PerkinElmer, Midrand, South Africa) reader. Graph Pad Prism 7 software was used to determine the IC_{50} values of the samples. The threshold for antiproliferative activity of the extracts was determined as significant activity (IC_{50} < 100 µg/mL), moderate activity (100 µg/mL < IC_{50} < 300 µg/mL), low activity (300 µg/mL < IC_{50} < 1000 µg/mL) and no activity (IC_{50} > 1000 µg/mL) [27].

2.2.9. Melanin production

The amount of melanin produced by UCT-Mel-1 cells, after the treatment with the ethanolic extract of *B. frutescens*, was determined using the Fontana-Masson assay as described by Kloepper et al [28], with minor changes. The ethanolic *B. frutescens* extract showed an increase in tyrosinase activity and no antiproliferative activity (IC_{50} >400µg/mL), therefore it was further evaluated for its effect on melanin production. The UCT-MEL-1 cells were seeded (1 mL) into 24-well plates at 5 x 10^4 cells/well and incubated for 24 hrs at 5% CO_2 and 37 ºC. The extracts and the positive control, α-melanocyte stimulating hormone, were prepared at stock concentrations of 400 µg/mL and 200 µM (in 1% DMSO), respectively, where after 1 mL was added to the cells to obtain final concentrations of 200 µg/mL and 100 µM, respectively. Controls included untreated cells and 1% DMSO treated cells as the vehicle control. The cells were incubated for another 24 hrs in 5 % CO_2 at 37 °C

After 24 hrs, the cells were washed with tris buffered saline and fixed using ethanol: acetic acid (2:1). The prepared silver nitrate ($AgNO_3$) working solution (0.15 M), was added to the cells and incubated for 40 min in the dark at 56 ºC. Following incubation, sodium thiosulphate ($Na_2S_2O_3$) in water (0.33 M) was added, enough to cover the cells, for 1 min. The cells were then counter stained with hemotoxylin (7 g/L) for 3 min and washed with dH_2O. Thereafter, the cells were dehydrated with 70, 90 and 100% ethanol and visualised with a Zeiss Primo Vert

light microscope (Carl Zeiss (Pty) Limited, Randburg, South Africa) and analysed through Image J.

All images were processed using the following developed macro: (1) the image was converted to 8-bit, (2) and then inverted (3), a 1000 x 1000 square selection was made (4). Image J was used to calculate the percentage pigment granule pixels within the square selection and provided a mean, minimum and maximum pigmentation score. The percentage pigmentation activity was then calculated as follows:

$$\%\,Melanin = \left(\frac{Melanin\ content\ of\ sample}{Melanin\ content\ of\ control}\ x\ 100\right)$$

2.2.10. Statistical analysis

Experiments were performed in triplicate using three independent experiments. The results are represented as the mean ± SD (n = 3). GraphPad Prism version 7 was used for statistical analysis to obtain the effective concentrations resulting in 50% of the activity (IC_{50}), which were derived from a sigmoidal dose response curve. A one-way analysis of variance (ANOVA) together with Dunnett's multiple comparison test was used to determine whether the difference between the controls and the treatments were significant, $^{**}p < 0.01$.

3. Results and discussion

3.1. Extraction of plants and phytochemical analysis

Although not exclusive, alkaloids, terpenes, flavonoids and phenolics are commonly found in plants that have antibacterial activity, which could attribute to moderate antibacterial activity observed from the ethanolic *S. inerme* extract [29-31]. Seven of the selected plants *B. obtusa, C. spicata, H. aristata, H. forskaolii, P. citronellum, P. graveolens* and *S. inerme* contain the above 4 mentioned phytochemical groups (Table 1). To date there have been no reports documenting the phytochemical groups found in *B. obtusa*, therefore the results are presented here for the first time. Alkaloids, flavonoids and phenolics were found in each extract except for *C. orbiculata*. Several flavonoids (naringenin, hesperetin, isosakuranetin), terpenes (geniposide, glycyrrhizin, lupenone) and phenolics (rosmarinic acid) have been found to induce melanin synthesis, increase tyrosinase activity, enhancing melanin production and gene expression [32]. The increased tyrosinase activity observed in this present study by *B. frutescens, C. spicata* and *P. afra* could be attributed to the flavonoids and phenolics that were detected in the three ethanolic extracts.

Table 3

Phytochemical groups detected in ethanolic crude extracts

Plant samples	**Herbarium number**	**Phytochemicals**					
		Tannins	**Alkaloids**	**Saponins**	**Terpenes**	**Flavonoids**	**Phenolics**
Barleria obtusa Nees	PRU 125926	+	+	-	+	+	+
Bulbine frutescens (L.) Willd.	PRU 125216	-	+	+	-	+	+
Carpobrotus dimidiatus (Haw.) L. Bolus	PRU 125927	+	+	+	-	+	+
Cotyledon orbiculata L.	PRU 128848	+	-	+	+	-	-
Cussonia spicata Thunb.	PRU 128851	+	+	+	+	+	+
Hypoestes aristata (Vahl) Roem. & Schult.	PRU 125925	-	+	-	+	+	+
Hypoestes forskaolii (Vahl) R.Br.	PRU 127863	-	+	-	+	+	+
Pelargonium citronellum J.J.A. Van der Walt	PRU 127869	+	+	-	+	+	+

Pelargonium graveolens L'Hér	PRU 128847	+	+	-	+	+	+
Portulacaria afra Jacq.	PRU 128849	+	+	+	-	+	+
Sideroxylon inerme L.	PRU 128850	+	+	-	+	+	+

'+' indicates presence; '-' indicates absence

3.2. Antibacterial activity

Cutibacterium acnes has been associated with PMH due to the high density (73.9 %) of the bacterium found in PMH lesions on the skin compared to non-lesional skin (14.2 %) [29, 33]. Currently one of the PMH treatments is through the use of antibiotics and narrow band ultraviolet (UV) radiation therapy [11, 15]. However, some antibiotics, such as isotretinoin, clindamycin and benzoyl peroxide cause severe side effects (migraines, alteration in eyesight, gastrointestinal bleeding, joint swelling and pain) and have proven to cause skin reactions such as sun sensitivity, severe rashes, peeling, blistering and dryness [15, 34-37]. Due to the selective pressure applied by antibiotics, resistant strains are becoming increasingly common. Thus, the use of combinational antibiotics are used to reduce the further development of drug resistant *C. acnes* strains [11].

Of the 33 extracts tested, nine showed antibacterial activity against *C. acnes*. *Barleria obtusa* ethanolic, *H. aristata* ethanolic and *H. forskaolii* ethanolic extracts showed MIC values of 250 µg/mL, whereas *H. aristata* DCM, *P. citronellum* ethanolic, *P. graveolens* ethanolic and *S. inerme* aqueous and DCM extracts showed MIC values of 500 µg/mL. The *Sideroxylon inerme* ethanolic extract showed the most noteworthy antibacterial activity with an MIC of 125 µg/mL. In previous reports, both a methanolic and aqueous leaf extract of *H. forskaolii* showed MIC values > 1000 µg/mL, against *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* [38].

An acetone extract prepared from the aerial parts of *Pelargonium citronellum* has been reported to have weak activity towards *S. aureus* and *B. subtilis* (MIC >1600 µg/mL) [39]. In a study conducted by Sharma and Lall [18], a similar result was obtained as in the present study for where an ethanolic *S. inerme* bark extract displayed an MIC of 250 µg/mL against *C. acnes*. *Barleria obtusa* (aqueous and DCM), *B. frutescens* (aqueous, ethanolic and DCM), *C.*

dimidiatus (aqueous, ethanolic and DCM), *C. orbiculata* (aqueous, ethanolic and DCM), *C. spicata* (aqueous, ethanolic and DCM), *H. aristata* (aqueous), *H. forskaolii* (aqueous and DCM), *P. citronellum* (aqueous and DCM), *P. graveolens* (aqueous and DCM) and *P. afra* (aqueous, ethanolic and DCM) displayed MIC values > 500 µg/mL.

Extracts which showed moderate MIC values were evaluated for their bactericidal activity. *Barleria obtusa* (ethanolic), *H. aristata* (ethanolic and DCM), *H. forskaolii* (ethanolic), *P. citronellum* (ethanolic), *P. graveolens* (ethanolic) and *S. inerme* (aqueous and DCM) extracts showed MBC values >500 µg/mL, whereas the *S. inerme* ethanolic extract showed and MBC of 500 µg/mL. The positive control, tetracycline, which is used in the treatment of acne vulgaris, specifically follicular *C. acnes*, showed an MIC and MBC of 0.78 and 5.25 µg/mL, respectively [40].

The use of antibiotics in combination is often used for the treatment of bacterial infections as they may show enhanced efficacy, a reduction in the normally required dose of antibiotics and possibly lead to a decrease of bacteria developing resistance (synergism) [41]. Therefore, the combination effect of ethanolic *S. inerme* and tetracycline was evaluated (Table 2).

Table 4

Combined effect of ethanolic Sideroxylon inerme extract and tetracycline against Cutibacterium acnes (ATCC 6919)

Ratio	**Ethanolic *Sideroxylon inerme* MIC (µg/mL)**	**Tetracycline MIC (µg/mL)**	**FIC value[b]**
Sideroxylon inerme ethanolic extract	125	-	-
9:1	56.25	0.63	1.25
8:2	50	1.25	2
7:3	43.75	1.88	2.75
6:4	37.5	2.5	3.5
5:5	15.63	1.56	2.13
4:6	12.5	1.88	2.5
3:7	9.38	2.19	2.88

2:8	6.25	2.5	3.25
1:9	3.13	2.81	3.63
Tetracycline	-	0.78	-

[a] Minimum inhibitory concentration; [b] Fractional Inhibitory Index; Note: the MIC reported is the MIC of the serial diluted ratio for ethanolic *S. inerme* and tetracycline.

Only the *S. inerme* ethanolic extract was tested for its combinational activity with tetracycline as it showed the highest antibacterial activity with a MIC value of 125 µg/mL. The interaction between the ethanolic *S. inerme* extract and tetracycline was found to be additive at each of ratios, the most effective being 9:1 with an FIC value of 1.25. For *S. inerme* and tetracycline the MIC value decreased from 125 to 56.25 µg/mL and 0.78 to 0.63 µg/mL, respectively.

3.3. Melanin production

Tyrosinase plays a role in the production of eumelanin and pheomelanin [42]. Six extracts had an effect on the activity of the tyrosinase enzyme. Four extracts increased tyrosinase activity, *B. frutescens* (ethanolic), *C. spicata* (ethanolic and DCM) and *P. afra* (ethanolic), whereas two extracts decreased the activity, *P. citronellum* (ethanolic and DCM) activity (Figure 1).

The ethanolic, aqueous and DCM extracts of *B. obtusa, C. dimidiatus, C. orbiculata, H. aristata, H. forskaolii*, *P. graveolens* and *S. inerme* did not have an effect on the activity of the tyrosinase enzyme. The ethanolic and DCM *P. citronellum* extracts inhibited the tyrosinase enzyme with IC_{50} values of 12.33 ± 0.83 and 8.28 ± 7.25 µg/mL, respectively. These were compared to kojic acid which showed an IC_{50} values of 7.49 $x10^{-1}$ ±1.21 $x10^{-2}$ µg/mL. The ethanolic *P. afra,* ethanolic and DCM *B. frutescens* and the ethanolic and DCM *C. spicata* showed a significant increase ($p < 0.01$) in tyrosinase activity when compared to the vehicle

control, by 25.76 ± 1.22, 31.44 ± 1.41, 11.07 ± 2.44, 11.47 ± 1.10 and 10.21 ± 1.09%, respectively (Figure 1).

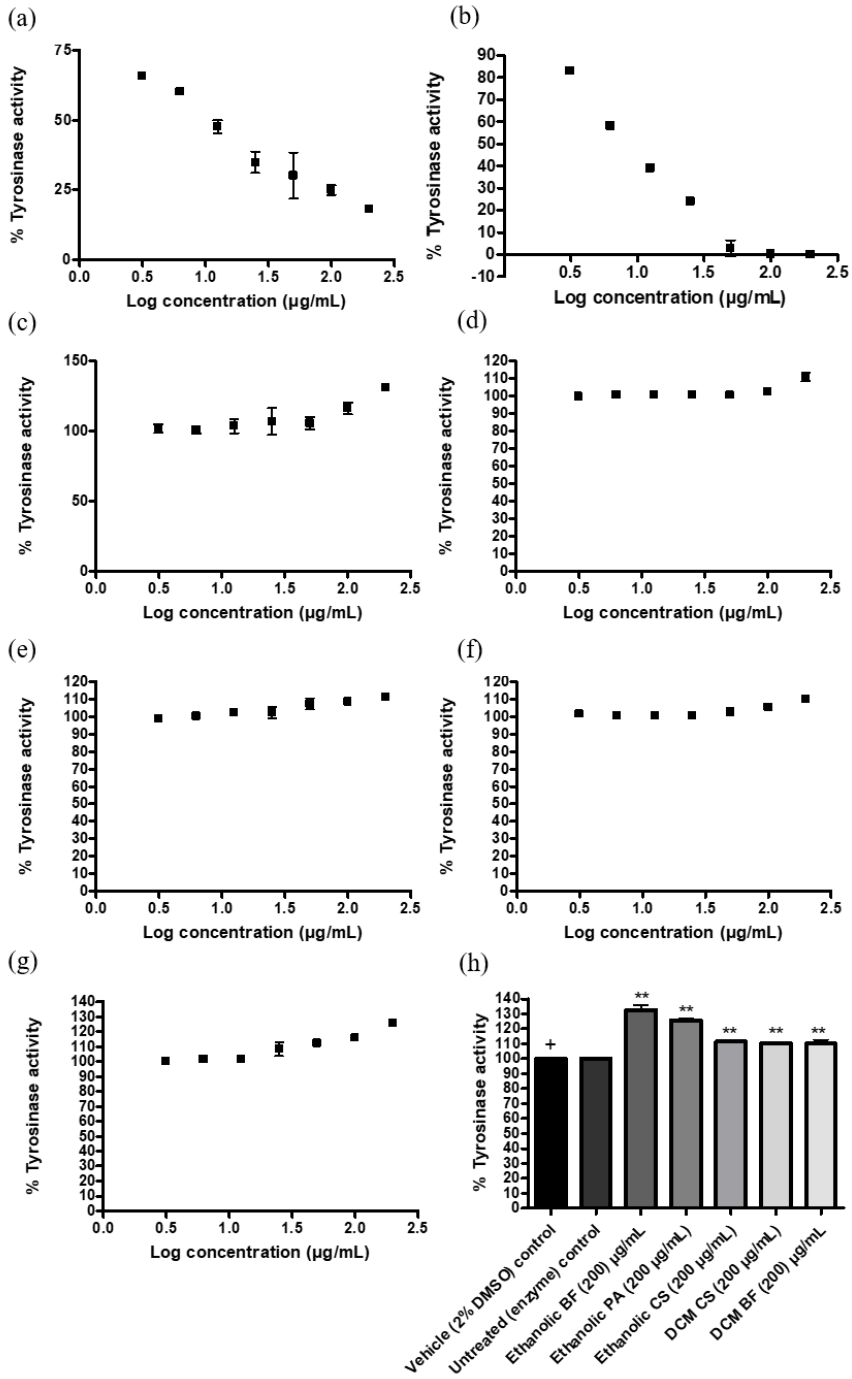

Figure 22

The tyrosinase modulatory activity of (a) ethanolic Pelargonium citronellum, (b) dichloromethane Pelargonium citronellum (c) ethanolic Bulbine frutescens, (d) dichloromethane Bulbine frutescens (e) ethanolic Cussonia spicata, (f) dichloromethane Cussonia spicata and (g) ethanolic Portulacaria afra extracts at concentrations ranging from 3.12 - 200 µg/mL. Data is depicted as the log concentration (µg/mL) versus the tyrosinase activity as mean ± SD (n=3). (h) One-way ANOVA together followed by Dunnett's multiple comparison test was used to determine statistical significance ($p < 0.01^{**}$) compared to the vehicle (2 % DMSO) control (+).

The ethanolic *B. frutescens, C. spicata*, *P. afra* and *S. inerme* extracts were tested for their antiproliferative activity towards UCT-Mel 1 and HaCaT cells (Figure 2). Three of the ethanolic extracts, *B. frutescens*, *P. afra* and *S. inerme*, displayed IC_{50} values > 400 µg/mL against both cell lines, whereas the ethanolic *C. spicata* extract displayed IC_{50} values of 201 ± 4.21 and 172.60 ± 5.73 µg/mL towards UCT-Mel 1 and HaCaT cells, respectively. The ethanolic *B. frutescens* and *P. afra* showed a decrease in UCT-Mel 1 cell viability by 3.48 ± 2.21 and 21.25 ± 10.90%, respectively at 200 µg/mL. These were compared to actinomycin D which showed IC_{50} values of $0.018 \pm 2.0 \times 10^{-3}$ and $0.026 \pm 1.2 \times 10^{-3}$ µg/mL against UCT-Mel 1 and HaCaT cells, respectively.

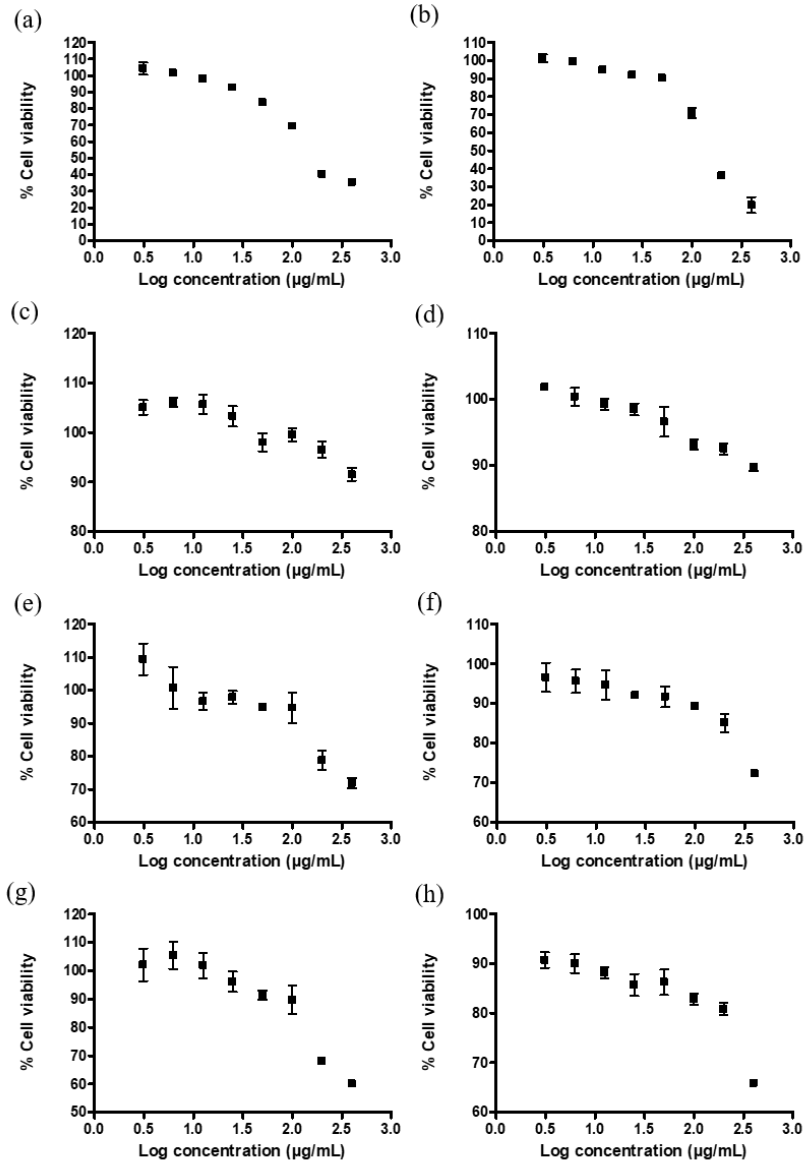

Figure 23

Cell viability of ethanolic Cussonia spicata treated (a) human melanoma (UCT-Mel-1) and (b) human keratinocytes (HaCaT), ethanolic Bulbine frutescens treated (c) UCT-Mel-1 and (d) HaCaT cells, ethanolic Portulacaria afra treated (e) UCT-Mel-1 and (f) HaCaT cells, ethanolic Sideroxylon inerme treated (g) UCT-Mel-1 and (h) HaCaT cells after 72 hrs at concentrations ranging from 3.15 – 400 µg/mL. Data is represented as the log concentration (µg/mL) versus the % cell viability as mean ± SD (n=3).

The methanolic bark extract of *S. inerme* has been reported to show toxicity towards mouse melanocyte cells with an IC_{50} > 100 µg/mL [43].A methanolic leaf extract of *C. spicata* has been reported to be cytotoxic towards T-cell leukaemia (Jurkat) cancer cells with an IC_{50} value of 27.69 ± 1.09 µg/mL [44]. In a previous report by Pather [45], the fresh leaf gel of *B. frutescens* showed less than 20 % decrease in cell viability on HaCaT cells at 1000 µg/mL (IC_{50} > 1000 µg/mL). Khanyile et al [46] reported that a *P. afra* methanolic leaf extract had an IC_{50} value of 4010 µg/mL towards human hepatocellular carcinoma (HepG2) cells.

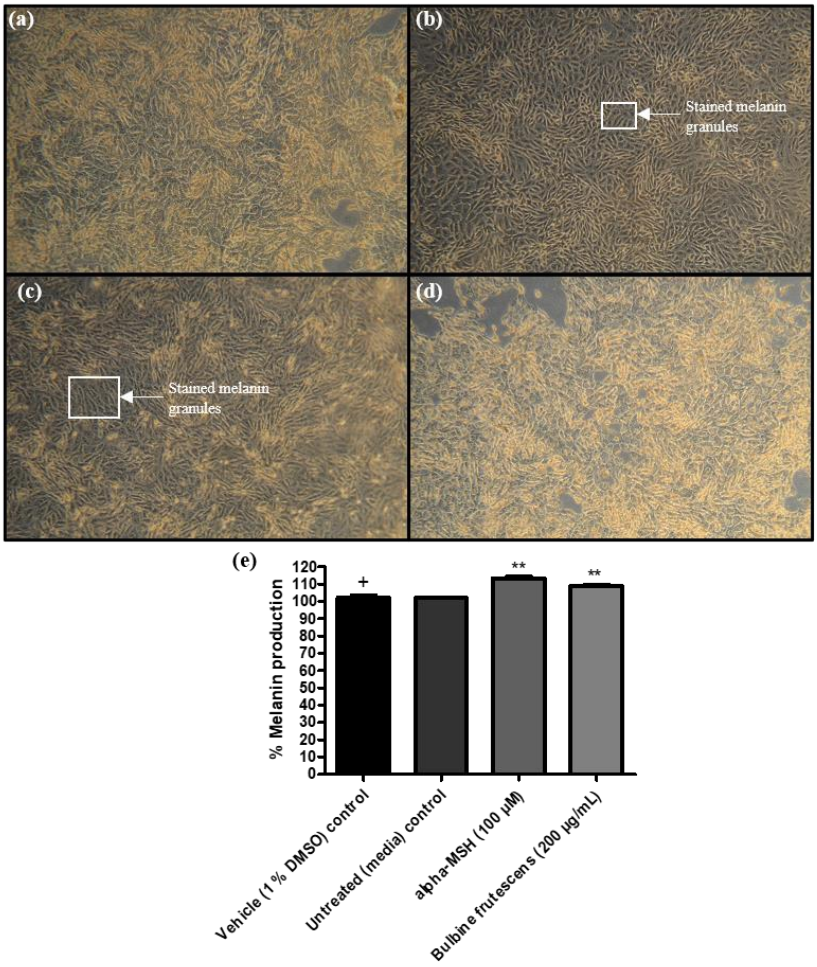

Figure 24

The Fontana-Masson staining of UCT-Mel 1 cells with (a) untreated cells; (b) α-melanocyte stimulation hormone at 100 µM; (c) *Bulbine frutescens* ethanolic extract at 200 µg/mL; (d) the DMSO (1 %) vehicle control after 24 hrs of exposure (e) One-way ANOVA followed by Dunnett's multiple comparison test was used to determine statistical significance ($p < 0.01^{**}$) compared to the vehicle (1% DMSO) control (+).

Only the ethanolic extract of *B. frutescens* was evaluated for its effect on melanin production as it increased tyrosinase activity and showed negligible antiproliferative activity against UCT-Mel 1 and HaCat cells (IC_{50} > 400 µg/mL). The potential of *B. frutescens* ethanolic extract to enhance melanin production was compared to α-melanocyte stimulating hormone (α-MSH), which promotes the production of melanin in humans. The extract (at 200 µg/mL) and α-MSH (at 100 µM) showed an increase in melanin production by 8.55 ± 1.66 and 13.39 ± 1.44%, respectively (Figure 3). The silver nitrate oxidises in the presence of melanin in the melanocytes resulting in a brownish-black colour. An increase in melanin macules was observed in the cells treated with the ethanolic *B. frutescens* and α-MSH when compared to the vehicle control cells ($p < 0.01$). This is the first report on the effect of *B. frutescens* on melanogenesis. In a similar study, an ethanolic *Capparis spinosa* leaf extract has been reported to increase melanin content in B16 murine melanoma cells by 12 and 60 % at a concentration of 0.005 and 0.05 % (w/v), respectively. It further increased the tyrosinase level by 20-fold after 48 hrs after exposure to the extract at a concentration of 0.03% (w/v) [32, 47].

4. Conclusion

The *S. inerme* ethanolic extract showed noteworthy antibacterial activity against *C. acnes*, whereas the ethanolic *B. frutescens* extract displayed a significant increase in the activity of tyrosinase enzyme and melanin production. Both ethanolic extracts had low antiproliferative activity towards both UCT-Mel 1 and HaCat cells. No reports on the activity of *B. frutescens* on melanin production have been published to date. Furthermore, there have been no reports documenting the antibacterial activity of the *B. obtusa*, in particular its antibacterial activity towards *C. acnes* (ATCC 6919) and the tyrosinase activity of *B. obtusa, B. frutescens, C. dimidiatus, C. orbiculata, C. spicata, H. aristata, H. forskaolii*, *P. citronellum P. graveolens* and *P. afra* extracts therefore the results are reported here for the first time.

The ethanolic extracts of *S. inerme* and *B. frutescens* have the potential to be used in the treatment for PMH, as an antibacterial and as a melanin increasing topical treatment, respectively. Further research will investigate the irritancy potential of the two extracts to determine their cosmetic safety as topical treatments and the *in vivo* hypopimentation effects of the *B. frutescens* ethanolic extract.

Data Availability

The data that supports the findings of this study are available from the corresponding author upon reasonable request

Conflicts of interest

The authors declare that there is no conflict of interest

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Chapter 5: Conclusion and future considerations

Cutibacteria acnes was identified as the causative bacteria of progressive macular hypomelanosis (PMH). The current antibiotics that are used for PMH provide short term solutions, but through the investigation of antibacterial plants, an alternative solution may be identified that provides a long-term solution.

The aim of the study was firstly to find a plant extract which could inhibit the bacterial activity, which was achieved by testing the extracts against the ATCC 6919 strain. Of the eleven plants, only *C. orbiculata, P. afra* and *S. inerme* have previously been tested against *C. acnes* (ATCC 11827). Therefore, the results obtained for *B. obtusa, B. frutescens, C. dimidiatus, C. orbiculata, C. spicata, H. aristata, H. forskaolii*, *P. citronellum P. graveolens, P. afra* and *S. inerme* extracts (ethanolic, aqueous and DCM) are reported here for the first time with regards to *C. acnes* (ATCC 6919). *S. inerme* showed the most significant antibacterial activity and had an additive effect when combined with tetracycline. It also showed to have low anti-proliferative activity against the human melanoma (UCT-Mel-1) and human keratinocytes (HaCaT) cell lines.

The second aim was to find a plant extract, which could activate the monophenolase activity of tyrosinase and ultimately, induce melanin production. Of the eleven plants selected, only S. *inerme* had previously been reported to have been tested for its activity towards tyrosinase. Therefore, the results obtained for *B. obtusa, B. frutescens, C. dimidiatus, C. orbiculata, C. spicata, H. aristata, H. forskaolii*, *P. citronellum P. graveolens* and *P. afra* extracts (ethanolic, aqueous and DCM) are reported here for the first time. Of the thirty-three extracts tested, seven showed activity towards the tyrosinase enzyme, namely ethanolic and DCM *P. citronellum, P. afra, B. frutescens* and ethanolic *C. spicata*. However only ethanolic *B. frutescens* showed no anti-proliferative activity against UCT-Mel-1 and HaCaT cell lines.

Ethanolic *B. frutescens* stimulated the activity of tyrosinase and melanin production in human melanocytes. No reports on the activity of *B. frutescens* on melanin production have been published to date.

The ethanolic extracts of *S. inerme* and *B. frutescens* have the potential to be used in the treatment for PMH, as an antibacterial and as a melanin increasing topical treatment,

respectively. Further research will investigate the irritancy potential of the two extracts to determine their cosmetic safety as topical treatments and the *in vivo* hypopigmentation effects of the *B. frutescens* ethanolic extract. Further research will also investigate the effect of the two extracts in combination on melanin production. To the author's knowledge, no pure compound from a plant has been identified that can stimulate melanin transfer; therefore, isolation could possibly be an opportunity to find a novel compound. The mechanism by which *B. frutescens* stimulates melanin production could also be investigated by means of PCR analysis and Western blotting.

Appendix A: Hypopigmentation diseases and available treatments

A.1. Hypopigmentation Disorders

There are a variety of hypopigmentary disorders, which fall into 2 categories, namely genetic or acquired (Sood & Tomecki 2017). Piebaldism and Waardenburg syndrome are part of the genetic hypopigmentary disorders whilst progressive macular hypomelanosis and vitiligo fall under acquired hypopigmentation disorders (Orlow 1994). The focus of the present study will be on acquired hypopigmentation disorder, which includes progressive macular hypomelanosis, post-inflammatory hypopigmentation, vitiligo, chemical leukoderma and pityriasis alba. Hypopigmentation disorders are of particular concern to darker skin types (Halder 2012; Sharquie *et al.*, 2013.). The different types of hypopigmentation disorders are as follow:

A.1.1. Post-inflammatory Hypopigmentation

Post-inflammatory hypopigmentation appears on the skin as partial or totally depigmented areas that have an ill-defined outline (Knott 2016). Post-inflammatory hypopigmentation is an expected result from most inflammatory dermatitis diseases, specifically found in the top layer of the skin. The aftermath of the inflammation results in reduced melanin production leading to hypopigmented areas (Figure A.1) (Morelli & Norris 1993). Hypopigmented lesions may occur as a result of injury, dermatological treatment (Vachiramon & Thadanipon 2011), acne vulgaris or even skin irritation caused by topical therapies such as topical retinoids (Halder & Rodney 2012).

A.1.2. Vitiligo

Vitiligo appears as hypopigmented patches on the face, hands and body (Figure A.1) (Halder & Rodney 2013). It is suspected that vitiligo results from an autoimmune response, however there are other theories as this is a multifactorial disease with both genetic and environmental factors (Nicolaidou & Katsambas 2014). The autoimmune response leads to the destruction of melanocytes, which is not only restricted to the skin layer but also occurs in the mucous membranes, hairs, eyes and in the ears (van den Boorn *et al.*, 2009). Vitiligo affects 0.5% to

2% of the general population with a 25% to 30% onset in childhood (Nicolaidou & Katsambas 2014; Schaffer & Bolognia 2003).

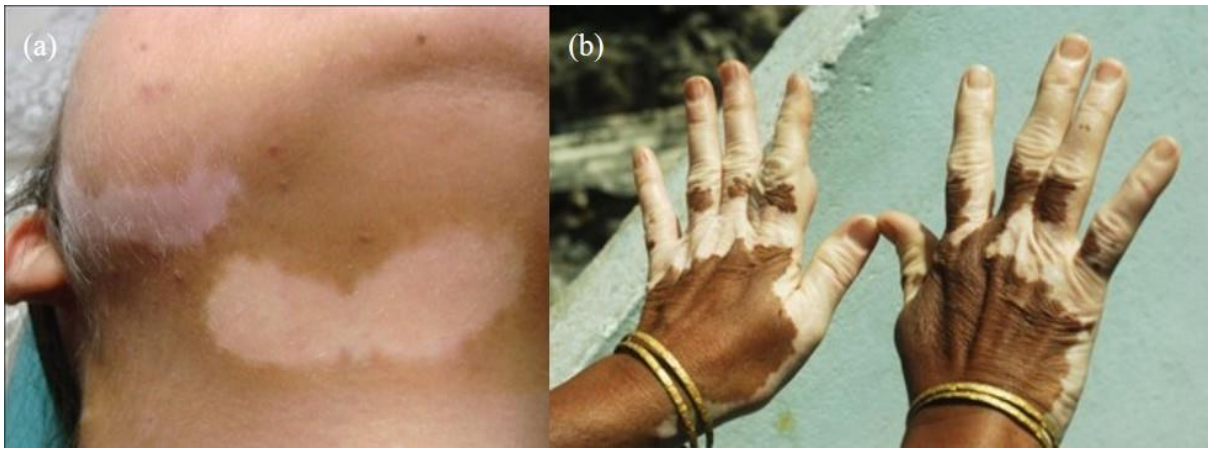

Figure A.1: (a) Post-inflammatory Hypopigmentation (Majid 2013) and (b) vitiligo (Nordqvist 2017).

A.1.3. Chemical Leukoderma

Chemical leukoderma is the appearance of whitened skin (Figure A.2), after contact with chemicals that have destroyed the epidermal melanocytes (Dyall-Smith 2014; O'Reilly *et al.*, 2011). The chemicals are usually those found in the work place but can also be expanded to include cosmetics (Dyall-Smith 2014). Chemical leukoderma is also known as contact leukoderma or occupational leukoderma (Ghosh 2010). It may occur in patients that have pre-existing idiopathic vitiligo, thus suggesting a genetic pre-disposition, which may explain why some people are affected by certain chemicals and others are not (Dyall-Smith 2014).

A.1.4. Pityriasis Alba

Pityriasis alba appears as hypopigmented spots that are predominantly limited to the face and neck (Figure A.2), but may appear on the shoulders (Sharquie *et al.*, 2013). It affects preadolescent children and predominantly darker skin types, and is found to be more prevalent in African, Hispanic, Asian and Egyptian children (Sharquie *et al.*, 2013). Pityriasis has been characterised as a mild form of atopic dermatitis that is usually related to an allergic response which leaves the hypopigmented areas after the inflammation has passed (Bassaly *et al.*, 1963; Martín *et al.*, 1990; Sharquie *et al.*, 2013). It is suggested that the microorganisms such as *Pityrosporum, Streptococcus, Aspergillus* and *Staphylococcus* are the causal factors along with a variety of other contributory factors, such as relative air humidity, temperature variations, altitude and excessive sunlight exposure (Sharquie *et al.*, 2013; Pugliarello *et al.*, 2010). According to Zaynoun *et al.*, (1983) the hypopigmented lesions occur due to reduced melanin

transfer and damaged melanocytes. All the aforementioned hypopigmentation disorders occur due to malfunctions in the melanin synthesis and/ or transfer processes.

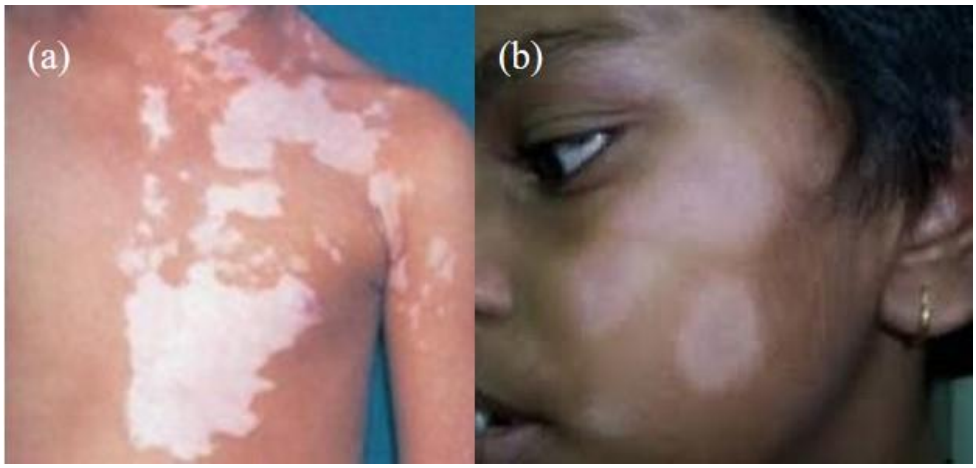

Figure A.2: (a) Chemical Leukoderma (Medical Encyclopaedia 2014) and (b) *Pityriasis Alba* (Health Saline 2016).

A.2. Available treatments for hypopigmentation disorders and their side effects

There are several treatments available for hypo-pigmentation disorders such as surgical based therapies, phototherapy and steroidal therapies (Nicolaidou & Katsambas 2014). However, each of these treatments has their own side effects (Lahiri 2009; Mysore & Salim 2009). Further research is aimed at non-medical treatments for hypo-pigmentation with a specific focus on cosmetics and the use of plant extracts. Sunscreen is used as a protective measure for hypopigmentation as these areas are more susceptible to sun damage (Kaur *et al.*, 2012). Some of the more commonly used treatments for the hypopigmentation disorders are as follows:

A.2.1. Topical steroids

Topical corticosteroids are frequently used as anti-inflammatories for dermatalogical conditions (Dhossche *et al.*, 2017; van Heugten *et al.*, 2018). They are produced in the forms of aerosols, creams, gels, lotions, and solutions (Drugs.com 2018). The topical steroids used to treat hypopigmentation diseases are those of different potency such as betamethasone, desonide, triamcinolone, fluocinolone and fluticasone propionate (Kaur *et al.*, 2012; Dhossche *et al.*, 2017; Drugs.com 2018). Prolonged and incorrect use of topical steroids has serious local, systemic, and psychological side effects and may lead to atrophy, telangiectasia, striae, glaucoma and hyperglycaemia among others (Schaffer & Bolognia 2003; Hengge *et al.*, 2006; Coondoo *et al.*, 2014; Dhossche *et al.*, 2017). The most frequent side effects include striae,

atrophy, perioral dermatitis, rosacea, purpura, acne, delayed wound healing and pigmentation alterations (Hengge *et al.*, 2006; Coondoo *et al.*, 2014; Dhossche *et al.*, 2017). Whilst short term treatment with corticosteroids result in only mild side effects, long term treatment can lead to more severe side effects, such as skin atrophy and telangiectasia (Rigopoulos *et al.*, 2004). The side effects of short-term usage include headaches, viral infection, pancreatitis, hypertension and skin atrophy (Buchman 2001; Bruner *et al.*, 2003; Coondoo *et al.*, 2014). The side effects of long-term usage can result in osteoporosis, growth suppression, aseptic joint necrosis and possible congenital malformations (Buchman 2001).

There are some advantages of topical corticosteroid therapy, which include relatively low cost, ease of application, and ability to use at home (Schaffer & Bolognia 2003; Berti & Lotti 2008). Topical corticosteroids are a treatment option for children of all ages unlike phototherapy (Schaffer & Bolognia 2003; Berti & Lotti 2008).

A.2.2. Photochemotherapy

Photochemotherapy involves the use of light and laser therapy requiring the patient to be exposed to controlled amounts of non-ionising UV light, such as UVA, UVB and sunlight (Menter *et al.*, 1994; Halder 2012). This therapy is used for many cutaneous conditions and is the frontrunner in therapy for hypopigmented disorders. However, it requires long term and continuous treatments, using either phototherapy or topical corticosteroids, as re-pigmentation is lost once therapy is discontinued (Gupta & Anderson 1987; Menke *et al.*, 1997; Kwok, *et al.*, 2002; Parsad *et al.*, 2006). The short-term side effects include nausea, headaches and vomiting, and the long-term side effects include photosensitivity, phototoxic reactions, accelerated skin aging and damage, hepatotoxicity, eye problems and long-term carcinogenic risks, which may give rise to malignant melanoma and squamous cell cancers (Gupta & Anderson 1987).

There are several methods used, such as PUVA (Psoralen + UVA) therapy, targeted phototherapy, narrowband UVB and psoralen ultra violet light or sunlight (PUVASOL) (Halder 2012; Halder & Rodney 2013). PUVA (Psoralen + UVA) therapy is a combination of topically applied psoralen which is responsive to UV light, and UV therapy (Halder 2012). The psoralen is applied to the affected skin and exposed to UV light, which allows for the light treatment to specifically stimulate only the affected areas of the skin (Halder 2012; Halder & Rodney 2013). PUVA stimulates the production of pigmentation by activating the melanocytes through the outer layer of the hair follicles. This activation occurs due to the keratinocytes

releasing immune cytokines and inflammatory facilitators (Arrunategui *et al.*, 1994, Berakha & Lefkovits 1985; Halder *et al.*, 1983, Scherschun, *et al.*, 2001). It is only used when 20% or less of the skin surface is affected with hypopigmentation (Halder 2012). This method is very time consuming and has to be administered every week for 6 months and the patients will only see a 50% improvement in the hypopigmented skin after four to six months (Halder 2012). It also requires maintenance after the therapy with either topical corticosteroids or further PUVA treatment (Halder 2012).

Targeted phototherapy uses laser radiation at the UVB range on the hypopigmented skin (Ranaweera 2013; Halder 2012). This treatment has many advantages, it is quick, the treatment sessions are short, it requires less frequent sessions, the treatment is easily administered to children and treatment can be applied to difficult areas such as the nose or scalp (Ranaweera 2013; Halder 2012). It also has a high improvement rate of 70% (Halder 2012). This treatment is, however, highly expensive and is not used for extensive areas, it is only utilised if the affected area is 10% or less (Ranaweera 2013).

Narrow band UVB therapy is used for extensive hypopigmented areas where the affected area is greater than 20% of the skin and it is the most commonly used phototherapy (Halder 2012; Oakley 2015). After 12 to 18 months, it has shown to have a 60% improvement on the affected areas of the skin (Halder 2012). Photochemotherapy and skin grafting are both used in conjunction with topical steroids.

A.2.3. Skin grafting and melanocyte transplants

Another approach used in an attempt to treat hypopigmentation is the surgical techniques of skin grafts and melanin transplants (Khunger *et al.*, 2009). Skin grafts transplant melanocyte rich tissue and cell grafting transplants melanocyte cells that are useful as they replenish the decreased number of melanocytes that occur in genetic hypopigmentation diseases such as vitiligo and piebaldism (Khunger *et al.*, 2009).

The skin grafting technique involves transplanting healthy skin, also known as donor skin, onto the damaged or unhealthy skin, in this case the hypopigmented skin (Halder 2012). The new skin that has fully functioning melanocytes starts producing pigment in the previously depigmented areas (Halder 2012). Vitiligo patients who undergo this surgery are only eligible to have this procedure if they have stable vitiligo or if their vitiligo spots have not changed in six months. Skin grafting for hypopigmentation is only used for limited areas and different grafting sites result in varying success rates (Hadi *et al.*, 2004; Halder 2012).

There are several skin grafting techniques such as cultured epidermis grafts (Falabella *et al.*,1992), miniature punch grafting (Chinniah 2014), ultrathin epidermal sheets and basal cell layer suspension (Olsson & Juhlin 2012), hair transplants (Malakar & Dhar 1999), suction blister grafting (Chinniah 2014), transplantation of autologous cultured melanocytes (Olsson & Juhlin, 1993), cultured melanocyte grafting (Lerner, 1988), split thickness skin grafting (Chinniah 2014) and mini grafting and NB-UVB (Lahiri *et al.*, 2006). Many of these procedures have side effects. Discussed below are miniature pinch grafting, split thickness grafting and suction blister grafting (Chinniah 2014; Khunger *et al.*, 2009).

Miniature punch grafting is a technique where 2mm sections of full thickness skin grafts are punched out of the donor site, usually from the buttock, thigh or arm (Chinniah 2014; Kahn & Cohen 1995). The graft tissues of unaffected skin are then transplanted into punched out holes in the recipient's hypopigmented sites (Chinniah 2014). Patients are exposed to PUVA or NB-UVB post procedure (Lahiri 2009). This technique is one of the most commonly used due to its simplicity and efficacy and can be done in one sitting (Chinniah 2014). This technique has several risks involved such as infection, loss of the graft tissue, imperfect colour matching, hyperpigmentation, peripheral depigmentation, which results in a halo effect and graft rejection (Chinniah 2014; Falabella *et al.*, 1995; Savant, 1992).

Suction blister grafting is a procedure where negative pressure is applied to the donor site to induce blistering (Chinniah 2014; Khunger *et al.*, 2009). Blistering is formed through the use of syringes, suction cups or suction pumps depending of the size of required donor material (Khunger *et al.*, 2009, Chinniah 2014). Through the process of blistering, the basal cells are cleaved from the basal lamina allowing for the pigmented epidermis, which is the roof of the blister, to be surgically removed (Khunger *et al.*, 2009, Chinniah 2014). The grafts are cut according to the required size and then transplanted to the recipient site (Khunger *et al.*, 2009). Suction blister grafting has good success rates, is easy to perform, inexpensive, is generally safe and the cosmetic results are good with 75% repigmentation and minimal scarring (Chinniah 2014). However, the blistering is very time consuming as it can take 2 to 3 hours for the blisters to form; the procedure can only be performed on small sections of skin and can be very painful (Chinniah 2014; Njoo *et al.*, 1998). Other complications include graft rejection, hyperpigmentation and depigmentation around the graft (Chinniah 2014).

Split thickness grafting is a technique that removes a layer of skin, which includes the dermis and the epidermis, from the designated donor site that is then transplanted onto the recipient

site (Stang 2017). This technique is used for larger areas; thus, the skin is usually harvested from large areas such as the thigh, the back or the abdomen (Stang 2017). Split thickness skin grafting has resulted in 78-91% regimentation making it one of the most effective techniques (Njoo *et al.*, 1998). This high success rate is due to the fact that there are more melanocytes present in the harvested skin because the size of the graft is so large (Pandya *et al.*, 2005). Complications that may result from this technique include milia formation, regimentation, graft rejection and peripheral depigmentation (Chinniah 2014).

Melanocyte transplants involve the transplantation of melanocyte and keratinocyte cells from a donor site, which are incubated and then transferred to a recipient site (Halder 2012). This method has a high success rate of 95% (Halder 2012). There are two methods currently used, cultured melanocyte suspensions and autologous non-cultured epidermal cell suspensions (Chinniah 2014).

Cultured melanocyte suspension involves harvesting a small tissue sample from the donor site and incubating it in trypsin, which is used to separate the dermis, the epidermis, the keratinocytes and the melanocytes (Chinniah 2014). Once the keratinocytes and the melanocytes have been separated, they are incubated in a medium containing growth factors. Following incubation, the culture suspension is transplanted into the recipient site (Chinniah 2014). This method allows for a large recipient area to be transplanted in a single session; however, it has a large donor to recipient ratio and requires special laboratories and the technique produces excellent cosmetic results and colour matching (Chinniah 2014).

Autologous non-cultured epidermal cell suspension is very similar to cultured melanocyte suspension: the donor tissue is harvested and incubated in trypsin to allow for separation of the cells, however only the melanocytes are separated and made into a suspension which is then transplanted into the recipient tissue site (Chinniah 2014). This method allows for large areas to be treated; however, it has a much smaller donor to recipient ratio than cultured melanocyte suspension (Chinniah 2014). This method has provided excellent results; however, it is complex, expensive and it requires more than one session (Chinniah 2014; Mulekar 2004).

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Appendix B: The effect of plant extracts on *Cutibacterium acnes*

Cutibacterium acnes (ATCC 6919) was treated with tetracycline and different plant extracts for three days. Presto Blue was added to determine the bacterial cell viability. The bacterial cell viability can be observed in Figures B.1 to B.3.

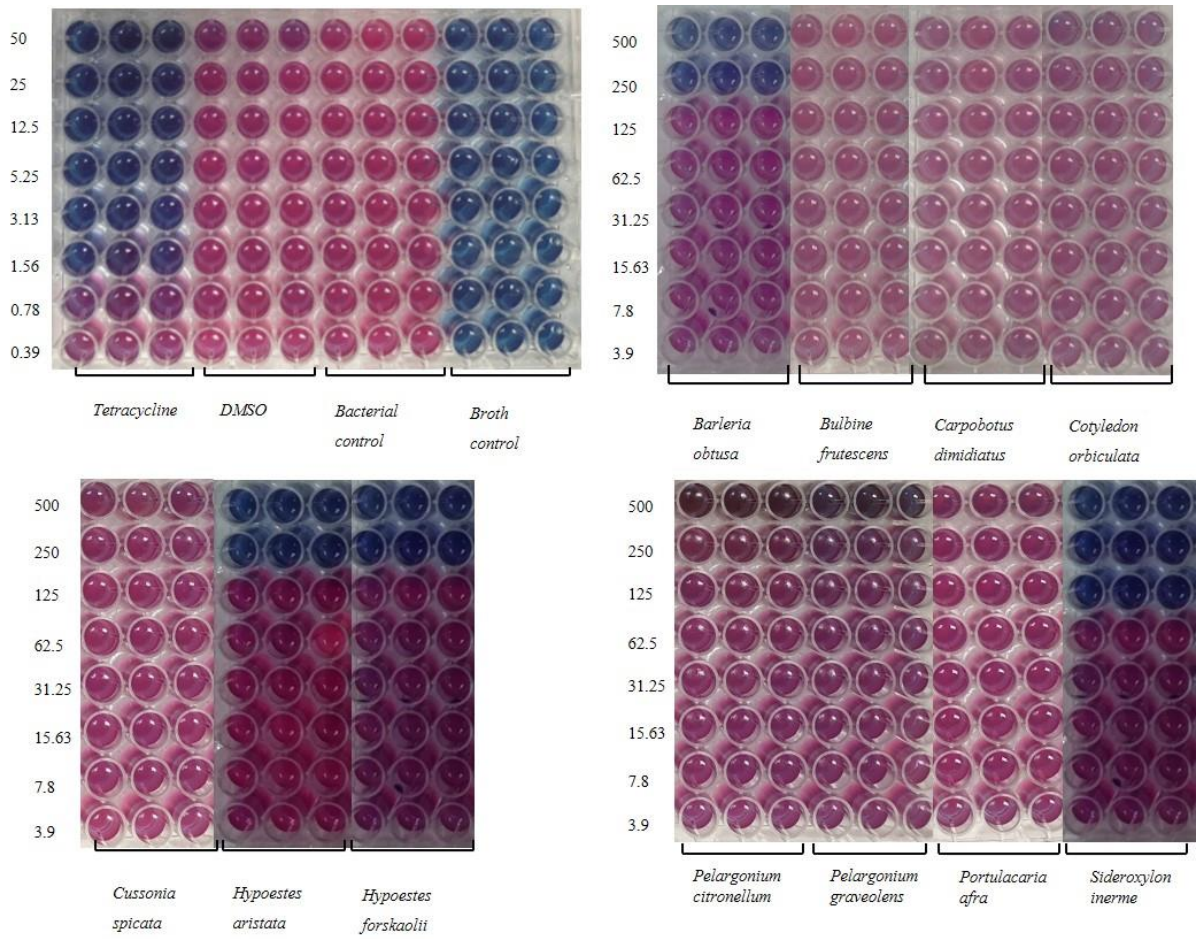

Figure B.1: *Cutibacterium acnes* (ATCC 6919) cell viability after the addition of Presto Blue three days after the treatment with tetracycline (positive control), DMSO, negative control (untreated *C. acnes*), media control and the ethanolic plant extracts; the blue colour indicated bacterial cell death and the purple/pink colour indicates bacterial cell growth

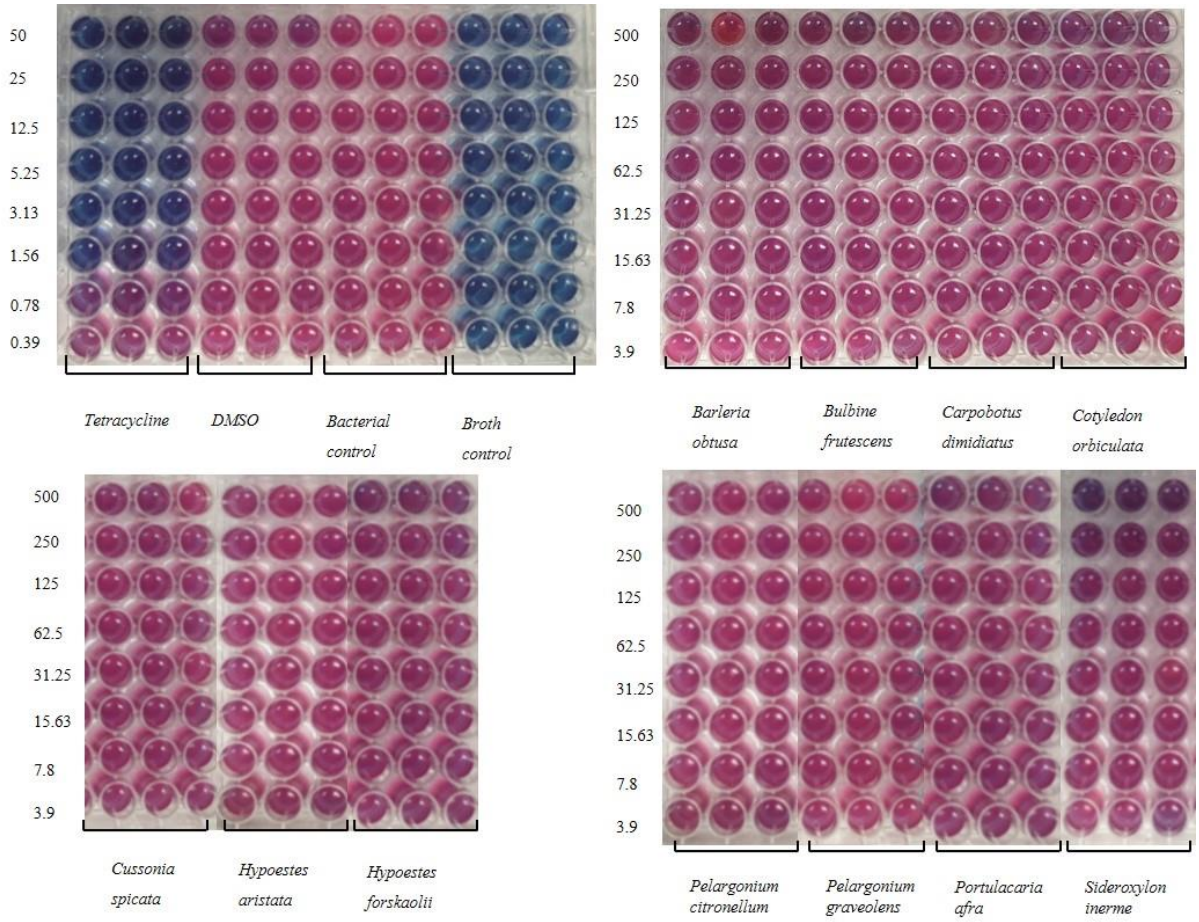

Figure B.2: *Cutibacterium acnes* (ATCC 6919) cell viability after the addition of Presto Blue three days after the treatment with tetracycline (positive control), DMSO, negative control (untreated *C. acnes*), media control and the aqueous plant extracts; the blue colour indicated bacterial cell death and the purple/pink colour indicates bacterial cell growth

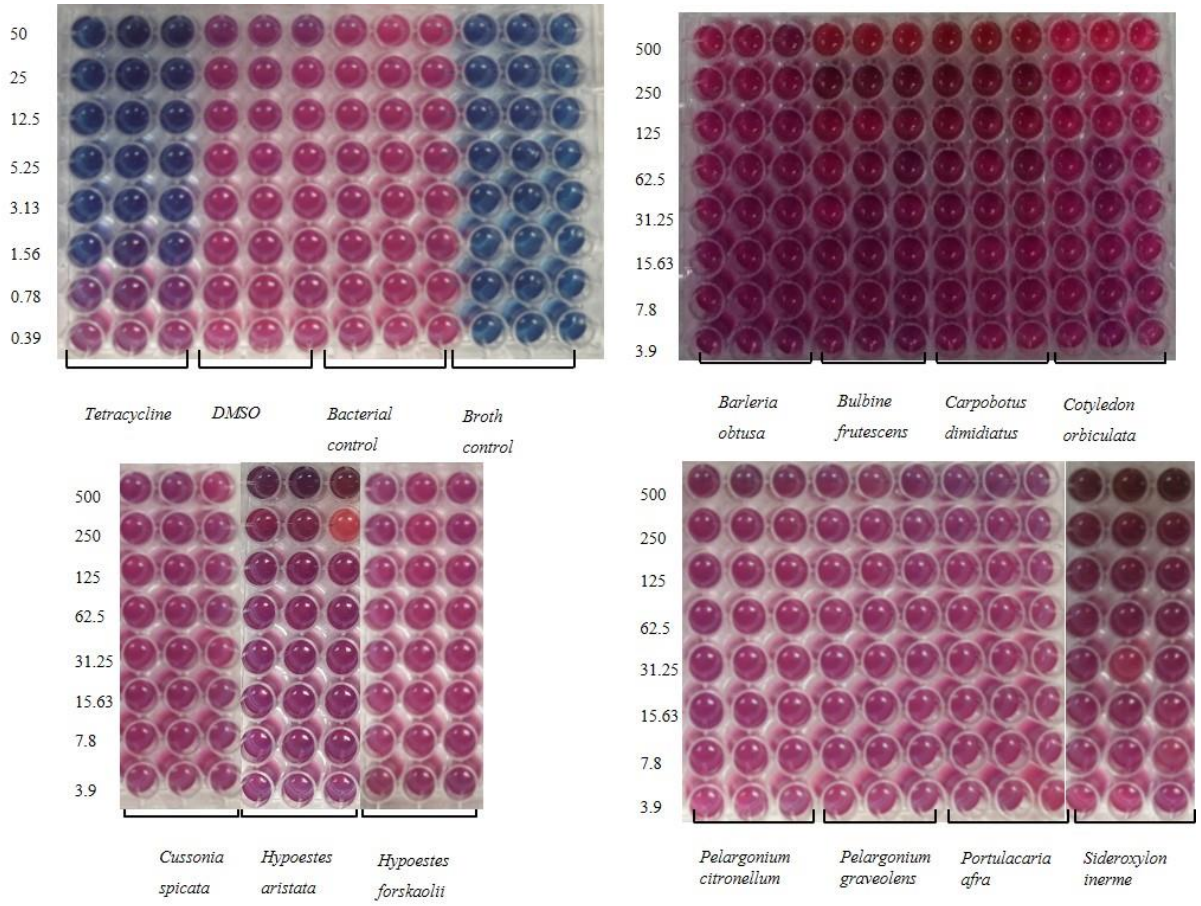

Figure B.3: *Cutibacterium acnes* (ATCC 6919) cell viability after the addition of Presto Blue three days after the treatment with tetracycline (positive control), DMSO, negative control (untreated *C. acnes*), media control and the DCM plant extracts; the blue colour indicated bacterial cell death and the purple/pink colour indicates bacterial cell growth

Appendix C: The effect of plant extracts on mushroom tyrosinase enzyme

The mushroom tyrosinase enzyme was treated with the plnat extrcats. The absorbance was kinetically measured and the 50% inhibitory concentrations (IC_{50}) were determined (Figure C.1-C.3)

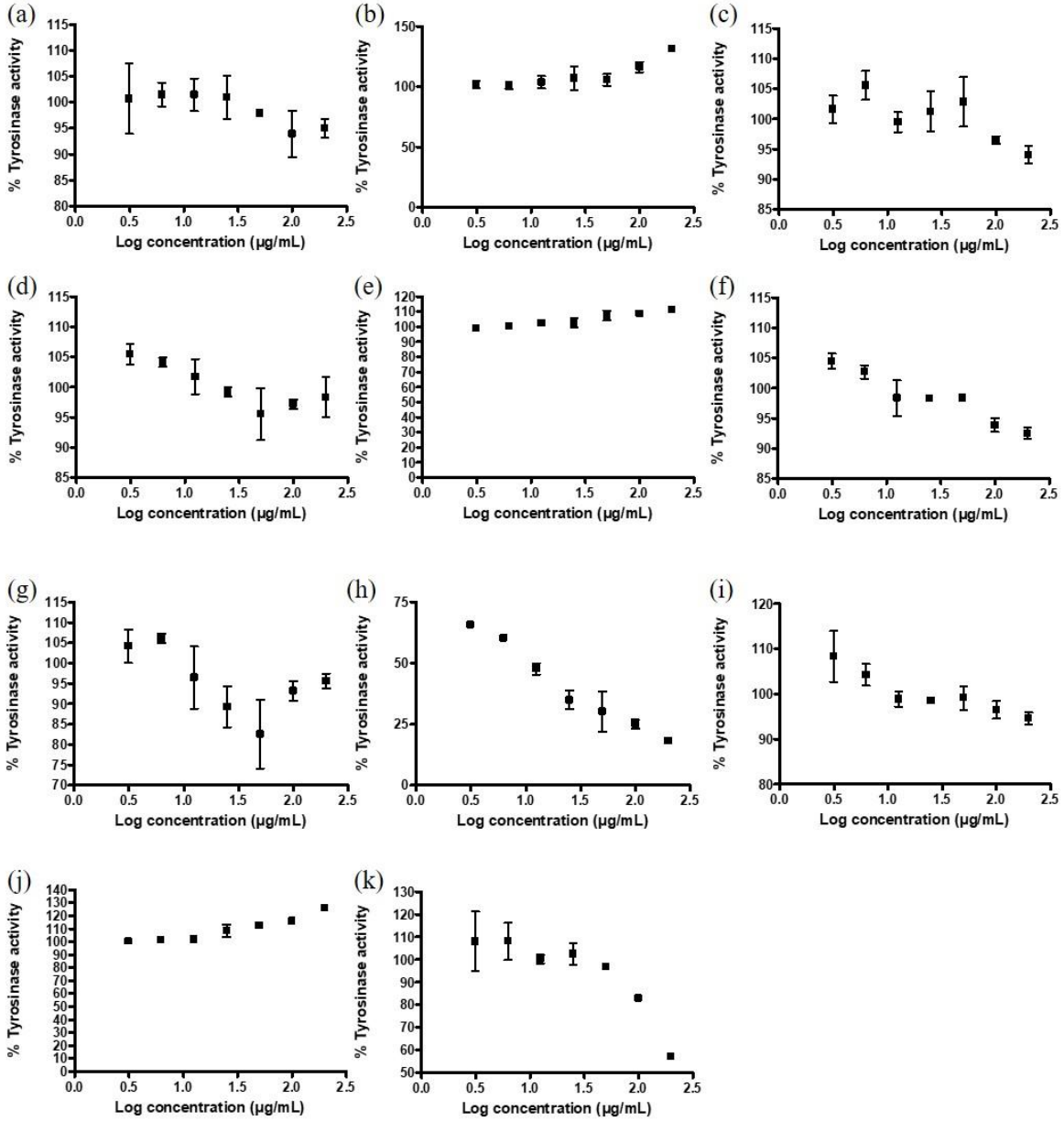

Figure C.1: The tyrosinase modulatory activity of ethanolic (a) *Barleria obtusa*, (b) *Bulbine frutescens*, (c) *Carpobrotus dimidiatus*, (d) *Cotyledon orbiculata*, (e) *Cussonia spicata*, (f) *Hypoestes aristata*, (g) *Hypoestes*

forskaolii, (h) *Pelargonium citronellum*, (i) *Pelargonium graveolens*, (j) *Portulacaria afra* and (k) *Sideroxylon inerme*. Data is depicted as the log concentration (µg/mL) versus the tyrosinase activity as mean ± SD (n=3).

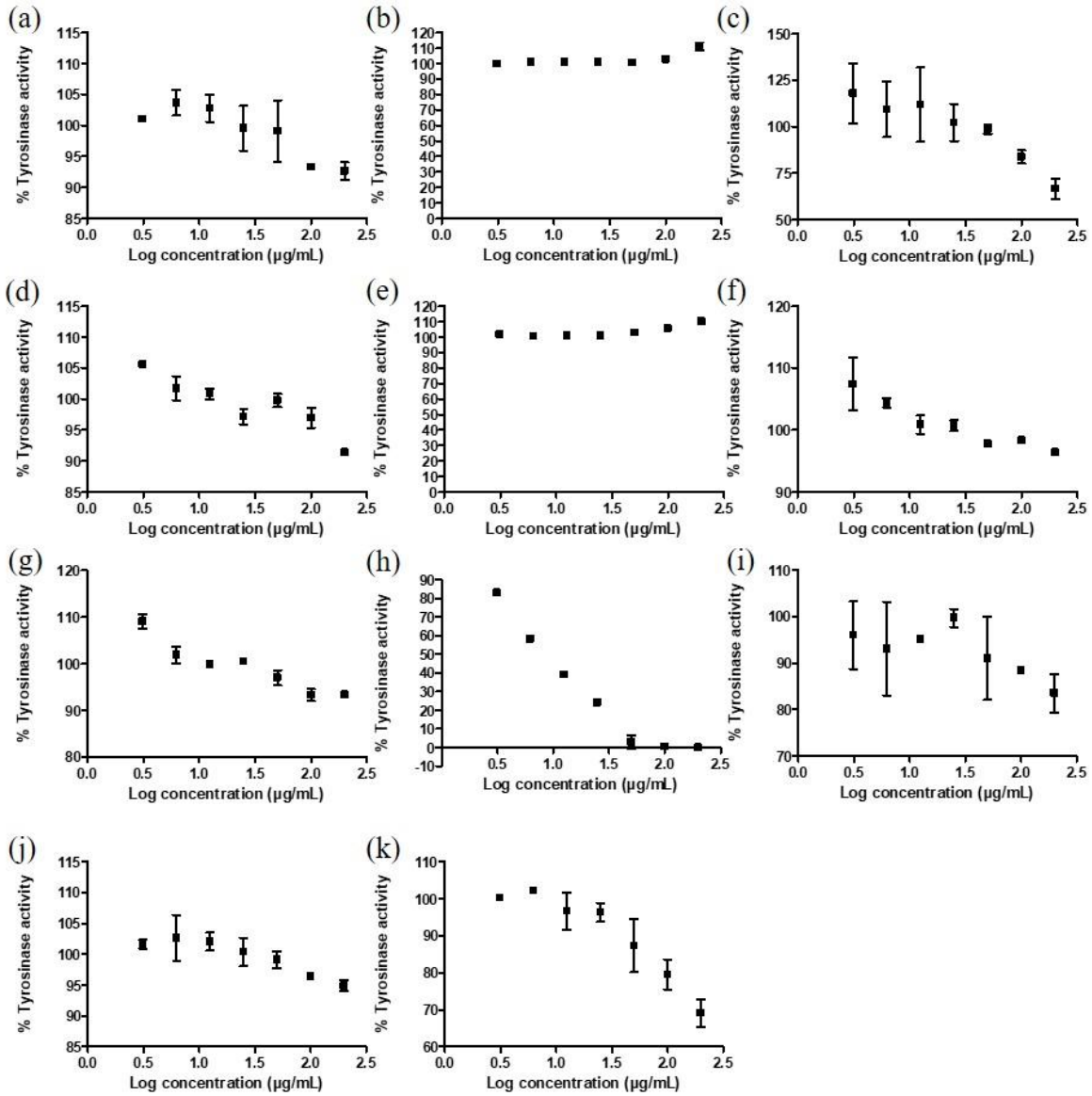

Figure C.2: The tyrosinase modulatory activity of DCM (a) *Barleria obtusa*, (b) *Bulbine frutescens*, (c) *Carpobrotus dimidiatus*, (d) *Cotyledon orbiculata*, (e) *Cussonia spicata*, (f) *Hypoestes aristata*, (g) *Hypoestes forskaolii*, (h) *Pelargonium citronellum*, (i) *Pelargonium graveolens*, (j) *Portulacaria afra* and (k) *Sideroxylon inerme*. Data is depicted as the log concentration (µg/mL) versus the tyrosinase activity as mean ± SD (n=3).

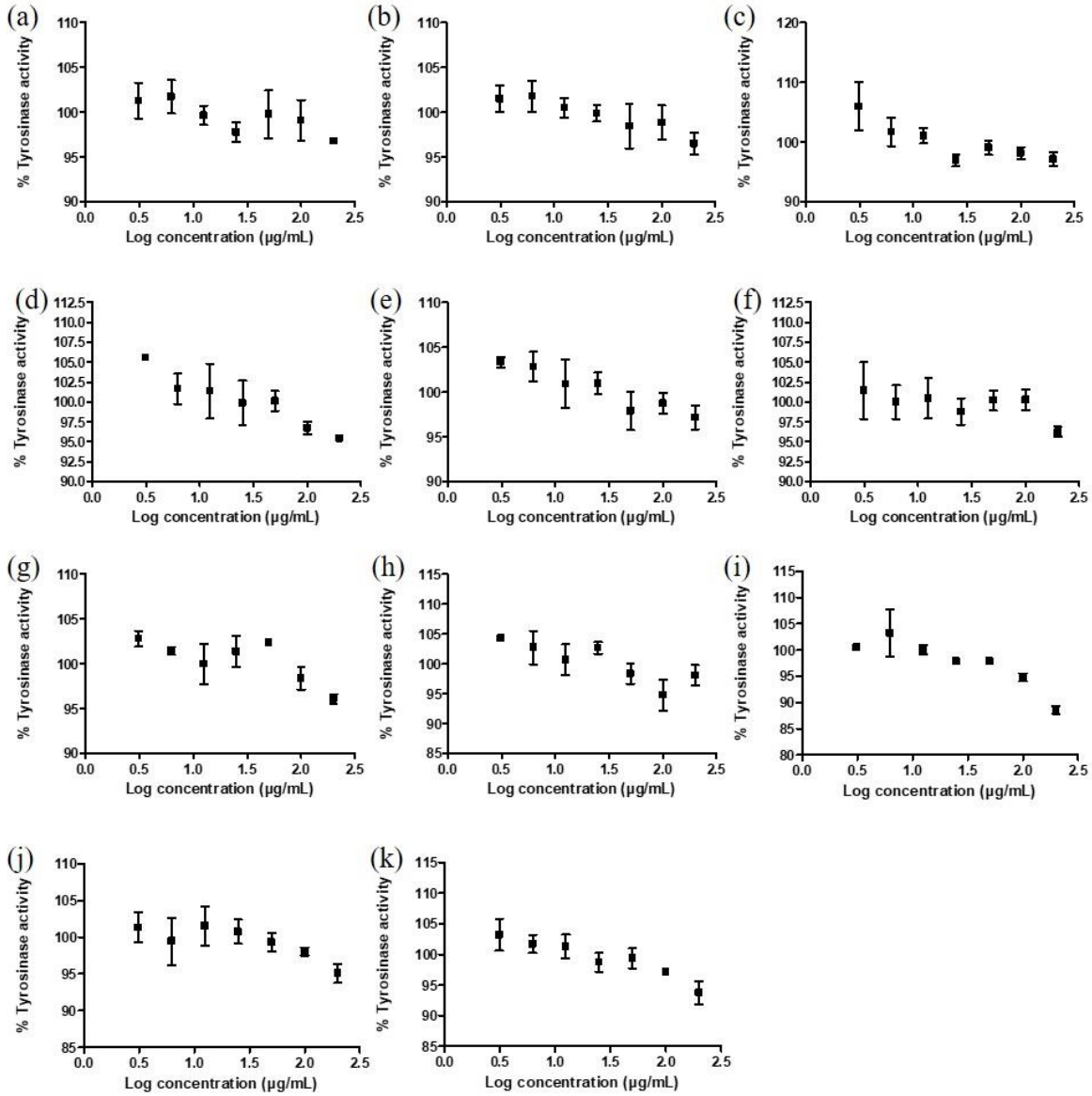

Figure C.3: The tyrosinase modulatory activity of aqueous (a) *Barleria obtusa*, (b) *Bulbine frutescens*, (c) *Carpobrotus dimidiatus*, (d) *Cotyledon orbiculata*, (e) *Cussonia spicata*, (f) *Hypoestes aristata*, (g) *Hypoestes forskaolii*, (h) *Pelargonium citronellum*, (i) *Pelargonium graveolens*, (j) *Portulacaria afra* and (k) *Sideroxylon inerme*. Data is depicted as the log concentration (µg/mL) versus the tyrosinase activity as mean ± SD (n=3).

Table C.1: Dunnetts's multiple comparison test was used to determine statistical significance of the untreated enzyme control, ethanolic *Bulbine frutescens*, dichloromethane *Bulbine frutescens*, ethanolic *Cussonia spicata*, dichloromethane *Cussonia spicata* and ethanolic *Portulacaria afra* compared to the vehicle (2% DMSO) control

Dunnett's Multiple Comparison Test	***p* value**	***p* value summary**	**Are means signif. different? ($p < 0.05$)**
Vehicle (2% DMSO) control vs Untreated (enzyme) control	$p > 0.05$		No
Vehicle (2% DMSO) control vs Ethanolic BF (200) µg/mL	$p < 0.01$	**	Yes
Vehicle (2% DMSO) control vs DCM BF (200) µg/mL	$p < 0.01$	**	Yes
Vehicle (2% DMSO) control vs Ethanolic PA (200 µg/mL)	$p < 0.01$	**	Yes
Vehicle (2% DMSO) control vs Ethanolic CS (200 µg/mL)	$p < 0.01$	**	Yes
Vehicle (2% DMSO) control vs DCM CS (200 µg/mL)	$p < 0.01$	**	Yes