Antimicrobial and anti-inflammatory effect of southern African plants against *Propionibacterium acnes*

by

Marco Nuno de Canha

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Abstract

Twenty southern African plants were selected based on traditional use. The ethanol extracts were tested for their antimicrobial activity against *P. acnes* (ATCC 11827) [*Propionibacterium acnes* (Gilchrist) Douglas and Gunter deposited as *Corynebacterium acnes* (Gilchrist) Eberson]. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of the extracts were also determined.

Cytotoxicity on human macrophage cells (U937) was performed using the 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) reduction assay. The cytotoxicity was performed to ensure that the extracts are not toxic to human macrophage cells (U937) and to obtain a non-lethal range of concentrations to be tested in the anti-inflammatory assay. The anti-inflammatory potential was tested using IL-8 as a marker cytokine. This is a pro-inflammatory cytokine secreted when cells are stimulated with heat-killed *P. acnes* cultures.

During the investigation of the antimicrobial activity, four plant extracts were found to have significant inhibitory activity against *P. acnes. Helichrysum odoratissimum* showed the best activity with a minimum inhibitory concentration (MIC) at 7.81µg/ml. *Clausena anisata*, *Rapanea melanophloeos* and *Helichrysum kraussii* were also active with MICs at 31.25µg/ml, 15.63µg/ml and 125µg/ml, respectively. All MICs were selected based on PrestoBlue as the growth indicator. *Helichrysum odoratissimum* showed also showed the best antioxidant activity with an IC_{50} of 3.86 ± 0.24µg/ml. It was also reported to have the best selectivity index (SI) of 2.76 on U937 cells. *Clausena anisata* exhibited good antimicrobial activity and low toxicity on U937 cells with an IC_{50} at 74.46µg/ml and an SI of 2.38.

During the investigation of the synergistic activity of the extracts, the combination of 3.13µg/ml of *Helichrysum odoratissimum* and *Helichrysum* kraussii at 0.78µg/ml showed better antimicrobial activity of either of the plant extracts acting alone against *P. acnes* with a fractional inhibitory index (∑FIC) of 0.42.

Clausena anisata was selected as the extract for the Interleukin-8 (IL-8) inhibition assay as it was shown to be traditionally used for treatment of many inflammatory disorders or symptoms. The inhibition of IL-8 by *C. anisata in vitro* when plant extract was added to stimulated U937 cells was low but there was some inhibition. The IL-8 protein concentration produced by U937 cells treated with 100µg/ml of heat-killed *P. acnes* was 840.52pg/ml. Low levels of IL-8 inhibition were observed when cells stimulated with *P. acnes* were treated with non-lethal concentrations of *C. anisata* extract. Treatment with 50µg/ml, 25µg/ml, 12.5µg/ml

and 6.25µg/ml showed a decrease in IL-8 to 322.48 ± 0.07pg/ml, 365.98 ± 0.24pg/ml, 383.62 ± 0.08pg/ml and 409.52 ± 0.13pg/ml, respectively. The untreated cell control however, seemed to show spontaneous production of IL-8 with quantified as 488.76 ± 0.06pg/ml, making it difficult to analyse effects of other cell stimulants. This spontaneous release was also inhibited with the addition of *C. anisata* extract at 50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml which showed IL-8 levels at 299.24 ± 0.13pg/ml, 357.82 ± 0.07pg/ml, 387.14 ± 0.12pg/ml and 388.74 ± 0.19pg/ml, respectively

The use of many polyherbal formulations is becoming popular practice. Due to the variety of symptoms observed with acne vulgaris it would be beneficial to investigate mixtures of plants showing good antimicrobial, antioxidant, anti-inflammatory and anti-pathogenic activity as potential treatments for acne vulgaris.

This is the first report of the synergistic activity of *Helichrysum odoratissimum* and *Helichrysum kraussii* crude ethanol extracts used in a synergistic combination. Also the production of hyaluronidase by the tester strain *P. acnes* (ATCC 11827). The combination and some other active plants shown in the study should be further investigated as possible novel medicinal agents against acne vulgaris.

Chapter 1

Literature Review

1. Introduction

1.1. Ethnopharmacology in South Africa

The past 20 years have seen an immense increase in worldwide research in the field of ethnopharmacology. One of the major reasons for this peaked interest is the need for discovery of new drugs. South Africa is unique with a large floral diversity estimated to have 30 000 species of higher plants, many of which are endemic. This diversity can be attributed to diverse climatic zones and habitat types. The importance of ethnopharmacology in South Africa can be illustrated by fact that Zulu people (a South African cultural group) use 1032 species of plants as traditional medicines. As a whole, South Africa has recorded 3000 species of plants which are used medicinally, many of which are sold at informal medicinal plant markets (Fig. 1.1), locally known as "muthi" markets (Light *et al*., 2005).

Figure 1.1: A muthi market that can be found in Durban, South Africa (SA Forestry Magazine, 2012)

1.2. The "pros and cons" of traditional medicine in South Africa

Most ethnopharmacological studies are based on ethnobotanical information which makes it an integral part of the research. The major problem with African traditional medicines, when compared to the age-old Ayurvedic and Chinese traditional medicines is that they are not well documented. There are very few documented references to traditional medicines in Africa. An important reference is the published work by Watt and Breyer-Brandwijk, which is a

documented, intensive publication of medicinal plants of southern and eastern Africa and holds large value as a reference work (Light *et al.*, 2005).

The limited documentation of this information can be attributed to the fact that African traditional knowledge is largely based on oral, as opposed to written documentation, which makes it difficult to access. Another problem is that, with an increase in urbanisation a lot of this traditional knowledge is being lost. Apart from this there are other factors such as bioprospecting and intellectual property rights. Conservation of these medicinal plant resources is also a concern as interest in the medicinal resources leads to over-harvesting. The pressure of habitat destruction through agricultural practices and urban and industrial development also has a big impact on medicinal plant conservation (Light *et al*., 2005). Another major problem with traditional medicines in South Africa is that there are common misconceptions associated with the safety of traditional natural products because they are considered to be pure and therefore, harmless. The fact is that not all secondary metabolites from plants are safe to use. This is mainly due to the evolutionary defence mechanisms of plants through the production of these secondary metabolites. Some of these metabolites are used by plants as inhibitors of other species and poisons against predators (Gurib-Fakim, 2006). This misconception has significant consequences as inappropriate collection times; storage and processing may all lead to contamination of collected material and ultimately affect safety. Use of medicinal plants may also be a danger if the use of these resources is not regulated. The regulation of these medicinal plants is important when considering the limited documentation of medicinal plants, which means there is limited documentation of safety, efficacy and quality. The quality of medicinal plants in South Africa may be affected by the use of poor farming practices that lead to contamination by pesticides, microorganisms, heavy metals and toxic substances from dumping sites (Street *et al.*, 2008).

However, efforts are being made to investigate, understand and document some of the traditional medicinal practices in a number of cultural groups in southern Africa and there is considerable progress in the field of ethnopharmacology (and its related fields of study) in South Africa itself (Light *et al*., 2005).

1.3. Traditional healers in South Africa

In South Africa there are three types of traditional healers. These are the traditional doctors locally referred to as the "inyangas" who are generally males who use herbal and other forms of preparations to treat diseases (Fig. 1.2). There are also the "isangomas" (Zulu), "dingakas" (Sotho) and "amgqiras" (Xhosa) who are generally females that who are able to provide links between the ancestors of the patients being treated. Finally, there are the traditional healers referred to as the "umprofethis" or "umthandazis" who incorporate Christian practices together with traditional medicine and are somewhat faith healers (Freeman and Motsei, 1992).

Figure 1.2: An inyanga with herbal and other forms of preparations (Growth and Development Strategy, 2014)

1.3.1. Why is traditional healing in South Africa so important?

One of the major reasons for the increased interest in traditional health practices by government and other organisations is the lack of conventional health resources to meet the needs and demands of those seeking healthcare. In 1992 it was estimated that 70-80% of the population that came from undeveloped countries has little or no access to basic healthcare services. In the 1970s it was realised that the only way to provide healthcare for everyone was to make use of existing resources within communities; in most cases, the traditional healers. In South Africa however, there is not much shortage with regard to healthcare personnel and resources; instead there is a shortcoming in the distribution and approach to modern healthcare (Freeman and Motsei, 1992). Although the government provides western

medical care and practices, the majority of the population still consults with traditional health practitioners for most of their healthcare needs. The use of these traditional medicines commonly found in areas where access to conventional medicines is minimal and where it is just too expensive to consult. However, the main reason for the use of these herbal remedies is the cultural importance (Light *et al*., 2005). For many people in the rural areas and urban townships primary healthcare can be associated with the care provided by traditional healers (Freeman and Motsei, 1992). It is for this reason that even people with Grade 12 and those who have had access to tertiary education still visit traditional health practitioners (Mander, 2007) (Table 1.1). Medicinal plants are commonly used to treat skin diseases.

Table 1.1: Level of education among people who visit traditional healers (Mander, 2007)

Education level	*% of respondents surveyed at healers' practices (n=99)*
No schooling	7.8
Up to Grade 7	31.0
Up to Grade 10	26
Up to Grade 12	26
Tertiary	8.7

1.4. Southern African plants traditionally used for skin care

History has shown the extensive use of natural ingredients such as herbs, roots, essential oils and flowers for skin care (Lall & Kishore, 2014). Some common examples of plant and plant-derived resources that are used in many cosmetic products worldwide are palm oil, sesame seed oil, linseed oil, jojoba oil, sandalwood, tea tree oil and many others (Pandey *et al*., 2010). Plants and plant products cosmetically benefit the skin by providing protection and by influencing the biological function of the skin by supplying essential nutrients required for healthy status (Dureja *et al*., 2005). Some South African plants have shown potential to be used as cosmetics for treating skin disorders.

A review article on South African plants with potential for use in the cosmetic industry has shown that *Helichrysum odorratissimum* L., *Eucalyptis camaldulensis* Dehnh. and

Melianthus major L. are traditionally used to treat pimples, the common name of acne vulgaris lesions. *Aloe ferrox* Mill. is used topically or taken orally to treat acne. It can be found in many cosmetic products where the gel is used as a cleanser, moisturiser and UV protector. There are also records of South African plants used to treat the symptoms of acne vulgaris (Lall & Kishore, 2014).

Becium obovatum E. Mey. ex. Benth., *Curcumis hirsutus* Sond. and *Datura stramonium* L. are a few examples of South African plants whose leaves have been reported to treat inflammation (Lall & Kishore, 2014). The entire plant *Galenia africana* L. is used to treat inflammation when formulated into a lotion (Van der Lugt *et al*., 1992). However, not much information has been reported on the scientific investigation of southern African plants for treating acne vulgaris (Mabona *et al*., 2013).

1.5. The functions of different components of human skin

The skin is the largest human organ that accounts for about 16% of total body weight (Venus *et al*., 2010). This organ is complex and is responsible for numerous functions. For this very reason it contains specialized cells that have evolved to perform a variety of protective functions (Table 1.2). The main components of the skin are the epidermis; a superficial layer and a deeper skin layer, known as the dermis. These are separated by the basement membrane (Powell, 2006) (Fig. 1.3). The epidermis and dermis are each made up of several components.

1.5.1. Epidermis

The epidermis is a layer of closely compacted cells that are produced by the process of cell division (Fig. 1.3). The basal cell layer is a columnar arrangement of cells at the lowest level of the epidermis. Thirty percent of the cells in this basal cell layer are prepared for cell division at any one point. Once the cells have divided, one of the cells remains in this basal layer to allow for reduplication of cells. Reduplicated cells are used for repair or merely adhere to the underlying basement membrane. The basal cell layer consists mainly of keratinocytes and 5-10% of the cell population are melanocyte cells. The second cell resulting from the division then moves supra-basally and differentiates into a keratinocyte cell (Powell, 2006), which is the major cell type found in the epidermis. These cells are

responsible for the production of keratin and cytokines which are secreted in response to injury (Venus *et al.*, 2010). More and more cells are created by the cycle and are pushed up through the granular and spinous layers ultimately reaching the horny layer of the skin, after which they can shed (Fig. 1.3) (Powell, 2006). The horny layer of the skin is the outermost layer of the epidermis and is composed mainly of cells which migrate from the granular layer after division in the basal cell layer. Cell division takes approximately 28 days (Powell, 2006). This time period, however, can be altered with various disease processes (Venus *et al.*, 2010).

Table 1.2: Function of specialized cell structures in the skin

How different components of the skin contribute to overall function	
Structure	**Functions**
Horny layer	Barrier protection against unregulated loss of salt and water and entry of particles (e.g. chemicals, microbes)
Keratinocytes	Adhesion, cytokine production, keratin production, production of vitamin D
Basal cell layer	Reduplication and repair
Langerhans cells	Immunological defence
Melanocytes	Protection against ultraviolet radiation
Basement membrane	Adhesion of epidermis to underlying zone supporting dermis
Dermis and subcutaneous fat	Strength with suppleness, shock absorption, insulation
Blood vessels	Delivery of nutrients and removal of waste; temperature regulation
Lymphatics	Drainage and removal of particulate waste
Eccrine sweat glands	Temperature regulation
Apocrine sweat glands	Production of pheromones
Sebaceous glands	Waterproofing and moisturizing
Fibroblast cells	Synthesis of collagen, elastin, collagenase, fibronectin
Phagocytic cells	Engulf and destroy bacteria
Lymphocytes	Immunological defence
Mast cells	Mediator release under antigenic stimulation, chemotaxis

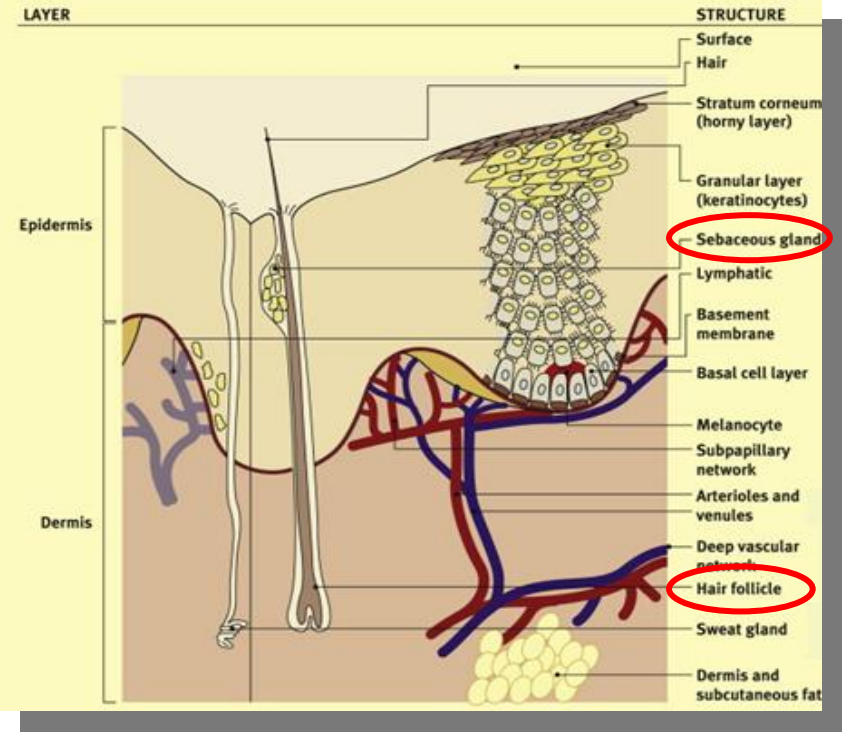

Figure 1.3: Components of the human skin, the body's largest organ (Venus *et al.*, 2010)

1.5.2. Dermis

The dermis is not only closely associated with the epidermis but is also associated internally with the subcutaneous fat layer (Fig. 1.3). This layer of the skin functions to protect the body from any physical or mechanical injury and contains specialized structures. These structures include macromolecules such as collagen and elastin fibres that are synthesized by fibroblast cells (Powell, 2006). The skin acts as the first line of defense against invading entities, with several components being involved in different protective mechanisms.

1.5.3. Mechanisms of protection by the skin

1.5.3.1. Protection as a physical barrier

The epidermis is the outermost layer of skin that provides protection from the environment due to the presence of the horny layer (stratum corneum) (Fig. 1.3 & Fig. 1.4).

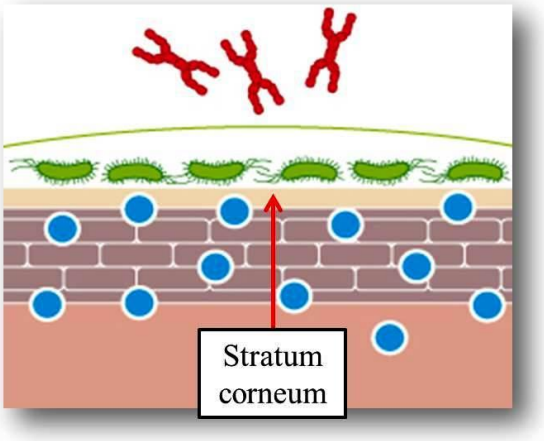

Figure 1.4: Skin as a physical barrier by presence of horny layer (Probioxi, 2010)

The horny layer is able to function as a protective barrier for two main reasons, firstly due to the fact that keratinocyte cells within this layer are arranged in such a way that a scaffold-like lattice is formed and is also reinforced by proteins and secondly due to the occupation of the intracellular spaces by a lipid-rich matrix to ensure that it is impermeable and waterproof. However, it is important to note that the skin is not completely impervious to absorption. The skin acts as a physical barrier by increasing the time taken for particular materials to pass the epidermal layer (Venus *et al.*, 2010). The skin not only acts as a physical barrier but also has some physiologically advanced features that can combat pathogenic entities.

1.5.3.2. Immune functions of the skin

The skin functions as the first line of defense against unknown antigens, whether chemical or organic. The skin is able to protect against microorganisms through the production of antimicrobial peptides, resident Langehans cells and also due to the presence of T-cells. The dryness of the outer layer of the epidermis and its continuous shedding of keratinocytes, help to prevent prolonged growth of organisms on the skin (Venus *et al.*, 2010). Various antimicrobial peptides are produced by the skin.

Antimicrobial peptides are produced by the living cells of the epidermis. These kill Gram-positive bacteria, Gram-negative bacteria, fungi and some viruses. There two major families of anti-microbial peptides include the defensins and the cathelicidins (Fig. 1.5). The defensin family can be further sub-divided into α and β types. The cathelicidins function mainly as

chemo-attractants for a range of immune system cells involved in inflammation (Venus *et al.*, 2010). Specialised cells within the skin are responsible for the immune response.

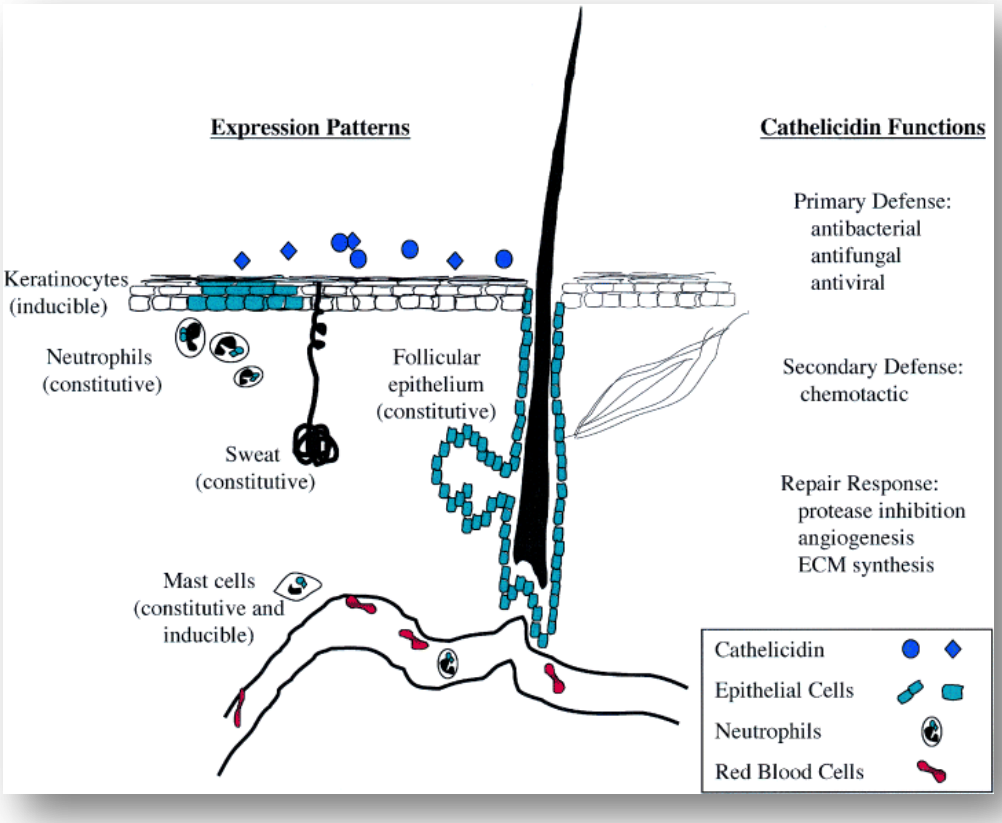

Figure 1.5: Function of cathelicidins (Braff *et al.*, 2005)

Langerhans cells are more commonly known as antigen-presenting cells. Upon injury to the epidermis these cells are altered and leave the epidermis and enter into the dermal lymphatic system. From here they are able to migrate and present antigens on MHC class II complexes to the immune T-cells. This allows the T-cells to blast and return to the site of the original antigen source in the skin (Venus *et al.*, 2010).

T-cells are not only found throughout the epidermal layer and in post-capillary venules of the dermal layer, but also in other skin appendages. Dysfunction of the skin immune system is an important factor that may result in problems related to healing of wounds and in skin disorders such as dermatitis, psoriasis and cutaneous T-cell lymphoma (Venus *et al.*, 2010).

The skin is also able to protect itself from the harmful effects associated with UV radiation (Fig. 1.6).

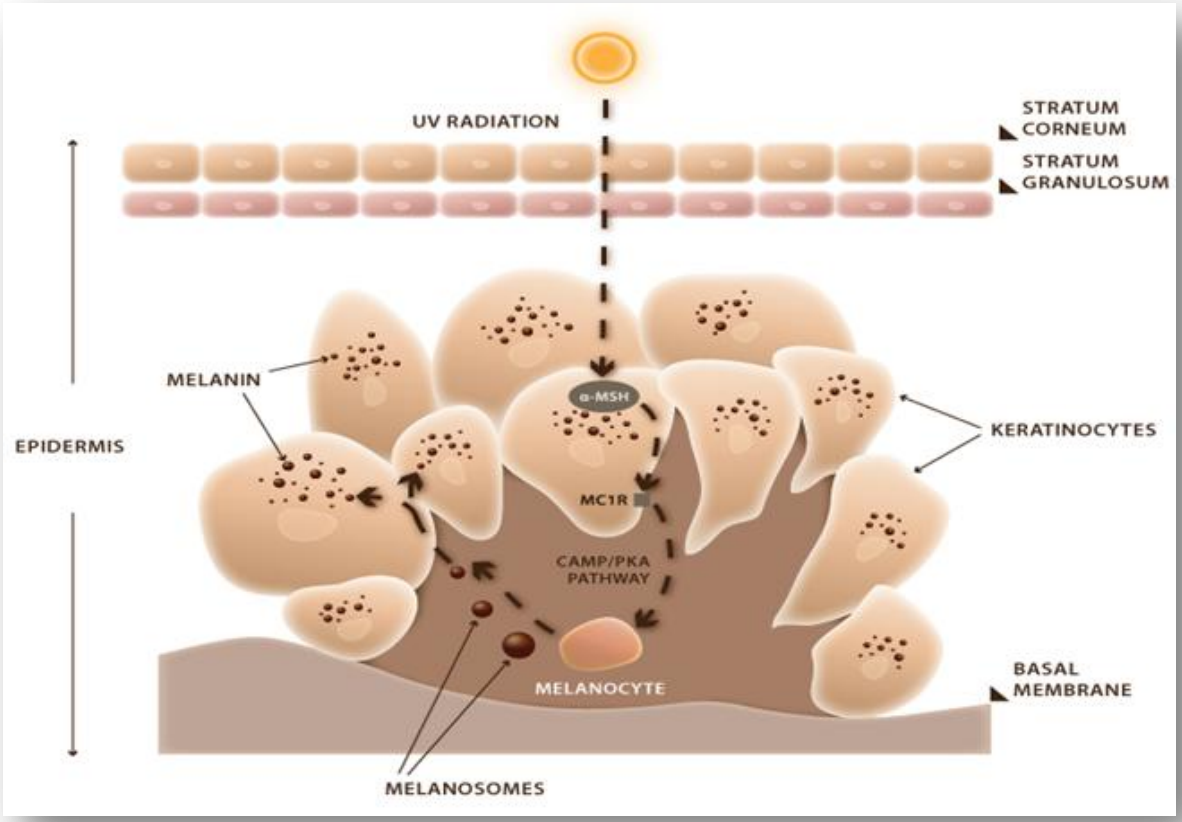

Figure 1.6: Absorption of UV light by production of melanin

Ultraviolet radiation is the electromagnetic energy that has a range of wavelengths from 200-400nm. The UV wavelength spectrum can be divided into three different types that include UVA (400-315nm), UVB (315-290nm) and UVC (290-200nm). UVA is involved in ageing skin and has also been known to act as a co-carcinogen with the UVB type in the induction of sunburn and skin cancer. The carcinogenic behaviour is due to photochemical damage to the epidermal DNA itself, damage to systems involved in DNA repair and also due to the suppression of immunological cell activity. UVC is the most carcinogenic but is almost entirely absorbed by the ozone layer. The skin is able to protect from these types of UV light through two main components, namely, the horny layer that is able to reflect radiation and reduce the amount of exposure to this radiation and the sun induced increase in melanocyte

cells which increase the amount of melanin transfer to keratinocyte cells. Melanin acts by decreasing the absorption of UV radiation by cell constituents and DNA itself (Fig. 1.6) (Venus *et al.*, 2010). Even with all of these features the skin is not completely invulnerable to pathogens and has resident microorganisms that may cause adverse effects.

1.6. *Propionibacterium acnes* (*P. acnes*)

P. acnes is the most common Gram-positive, non-spore forming, anaerobic, rod-shaped bacterium that is found across a number of clinical specimens (Fig. 1.7). The bacterium is typically grown as an obligate anaerobe but some strains are said to be aerotolerant (but still grow optimally in anaerobic conditions). The bacterium has the ability to synthesise propionic acid, catalase, indole and nitrate. *P. acnes* is similar in morphology to *Corynbacterium*, the major difference being that it is non-toxigenic (European Bioinformatics Institute, 2012). *P. acnes* is a member of the resident micro-flora found in the skin (Bojar & Holland, 2004).

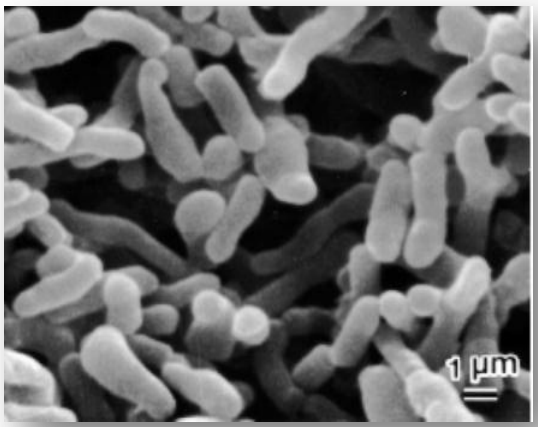

Figure 1.7: *Propionibacterium acnes* by scanning electron microscopy (The Science of Acne, 2012)

It is most abundant beneath the surface of the skin particularly in areas that are rich in sebaceous glands, which include the scalp and face (Fig. 1.8). Other parts of the skin, with less sebaceous glands can be observed to have much lower prevalence of *P. acnes.* Apart from sebaceous gland density there is also age related prevalence of *P. acnes.* This can be

illustrated by the fact that in pre-pubertal teenagers there is little recovery of the microorganism but it begins to appear after the maturation of sebaceous gland function (which occurs during puberty) (Leyden, 2001). Although *P. acnes* is a resident micro-organism it is known to be the causative pathogen of the skin disorder, acne vulgaris, and the pathogenesis is characterised by four main features.

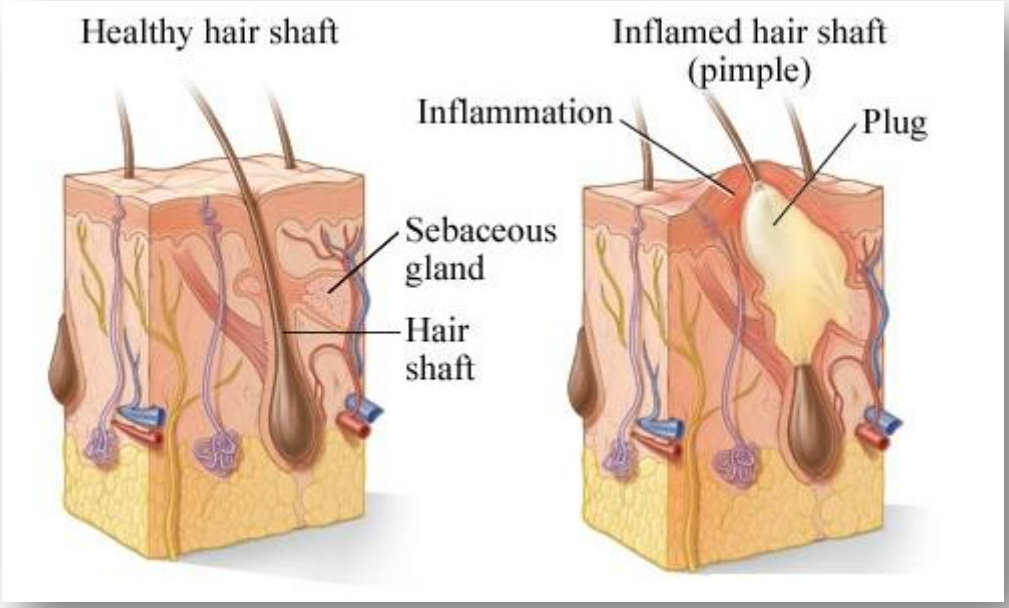

Figure 1.8: A healthy pilosebaceous unit compared with that of a colonized unit (Acne Treatment, 2012)

1.7. The possible mode of pathogenesis by *P. acnes*

1.7.1. Non-inflammatory lesions: the initial step in pathogenesis

Acne vulgaris is one of the most common inflammatory dermatological disorders. It is hypothesised that the inflammatory responses observed with acne vulgaris are a result of a secondary action of some sort, which is preceded by the formation of non-inflammatory lesions. This is the idea conceived from the fact that the emergence of papules and pustules occur after the presence of blackheads (closed comedone) and whiteheads (open comedone) where these micro-comedones are the primary lesions in the acne vulgaris disorder. The production of these comedones is due to the hyper-proliferation of keratinocytes which ultimately results in increased packing of these cells (Fig. 1.9). Accumulation of shed

keratinocyte cells along with increased sebum levels (second step) causes blockage of the follicle in the sebaceous unit and results in comedone formation. Keratinocyte hyper-proliferation is said to be the main cause of comedone formation as some immunohistochemical studies indicated a rise in proliferation of keratinocytes associated with the acne vulgaris disorder (Dessinioti & Katsambas, 2010). The next step in pathogenesis is the colonization of the pilosebaceous unit with *P. acnes* and ultimately inflammatory responses.

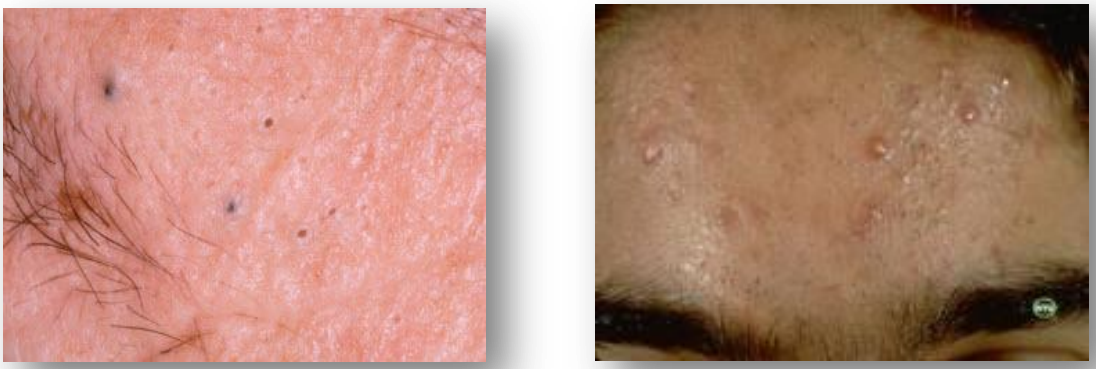

Figure 1. 9: Closed comedones (non-inflammatory lesions) (left) and pustules (inflammatory lesions) (right) (Advice, 2012 and Acne Net, 2002)

1.7.2. Possible causes of inflammation

The *P. acnes* bacterium has been identified as the culprit organism of acne vulgaris for more than 100 years. *P.* acnes has been identified in lesions of acne patients. Other studies have shown that when the *P. acnes* bacterium was injected into sterile keratinous cysts it led to rupture and inflammation. Another indication is that there is a significant increase in the level of *P. acnes* within the pilosebaceous unit. This is however, debatable as a study has shown that there is no difference in the concentrations of *P. acnes* on the skin, in patients with acne when compared with control patients. Also the number of viable *P. acnes* within follicles did not correlate with the severity of inflammation (Dessinioti & Katsambas, 2010).

It is therefore worthwhile to investigate the pathogenesis of *P. acnes*, for the development of therapeutic agents; mainly due to the onset of inflammation that is associated with acne

vulgaris. The major target of therapy is the *P. acnes* bacterium because the colonization of the pilosebaceous follicle ultimately results in inflammation. There are a number of ways in which the *P. acnes* bacterium causes inflammatory reactions.

P. acnes activates both the classical and alternative complement pathways, which results in the production of complement component fragment 5a (C5a) and also the production of chemotactic factors which activate neutrophils (type of white blood cell). Neutrophils ingest the *P. acnes* within the sebaceous follicle resulting in the release of hydrolases which disrupt the follicular walls (Dessinioti & Katsambas, 2010).

Reactive oxygen species (ROS) are also a bi-product of neutrophil action. Within acne comedones, lowered levels of linoleic acid have been recorded. Linoleic acid is able to inhibit superoxide, hydrogen peroxide and hydroxyl radicals. These reactive oxygen species are increased with acne vulgaris and ultimately contribute to inflammation (Portugal *et al*., 2007).

P. acnes also releases lipases, proteases and hyaluronidases which are all enzymes, which once secreted, contribute to tissue injury and spread of the bacterial colonization (Brüggermann, 2010). This suggests that acne treatments could also be investigated for their ability to act as antioxidants and as inhibitors of enzymes involved in the pathogenesis of *P. acnes* as opposed to just targeting the inflammatory modulators.

In the host immune response the production of pro-inflammatory cytokines such as TNF-α (tumor necrosis factor), IL-1α (interleukin) and IL-8 is common. The *P. acnes* bacterium contributes to the inflammatory responses by activating secretion of these pro-inflammatory cytokines by monocyte cells. The human innate immune response is mainly due to the presence of TLR-2 (toll-like receptor). It is the host defence mechanism against bacteria, fungi and parasites. *P. acnes* is said to trigger the TLR to produce κB (nuclear factor) transcription factor, which regulates the expression of many other immune response genes. This is especially applicable to the keratinocytes and sebocytes of the pilosebaceous unit which may be activated by TLRs (Dessinioti & Katsambas, 2010).

Some strains of *P. acnes* are not only able to activate the innate immune response, but also enhance the growth of human keratinocyte cells. *P. acnes* stimulates the induction of mRNA for the expression of β-defensin-2 as well as the expression of IL-8. Studies have shown that anti-TLR-2 antibodies and anti-TLR-4 neutralizing antibodies are able to inhibit the

expression of this mRNA. Increased keratinocyte growth may be indicative of ductural hyper-cornification and inflammatory processes that form part of the pathogenesis in acne vulgaris (Dessinioti & Katsambas, 2010).

In vitro, *P. acnes* has been known to induce the release of cytokines and anti-microbial peptides. Non-viable *P. acnes* however, does not seem to result in the release of pro-inflammatory cytokines in keratinocyte cells (Dessinioti & Katsambas, 2010). The release of pro-inflammatory cytokines contributes to the pathogenesis of *P. acnes*.

1.7.3. Effects of some pro-inflammatory cytokines

IL-1α has been identified as the possible causative cytokine in the formation of comedones by causing hyper-cornification in the pilosebaceous follicles. IL-8 is able to attract neutrophil immune cells to the pilosebaceous unit, where they release lysosome enzymes resulting in the bursting of follicle epithelia and inflammation at the site of action. When IL-12 is secreted it enhances the Th-1 response (type of cellular immunity) that has been known to cause tissue injury in some autoimmune and inflammatory disorders (Dessinioti &Katsambas, 2010). Plants are chemically diverse and are the logical option for exploring new treatments for acne vulgaris and the various immunological effects associated with the disorder.

1.8. Plants with anti-acne activity

A study on 19 medicinal plants which were collected from a number of locations in Thailand, were tested for their antimicrobial activity.

Figure 1.10: Garcinia mangostana (Mangosteen tree) (Public Domain Photos, 2012)

These medicinal plants were tested against *Propionibacterium acnes* and *Staphylococcus epidermidis* (another organism implicated in acne vulgaris). The study indicated various plants with minimum inhibitory concentrations (MICs) ranging from 0.039-2.5mg/ml. *Garcinia mangostana* showed an MIC at 0.039mg/ml against *P. acnes* (Chomnawang *et al.*, 2005) (Fig 1.10) (Table 1.3). In another study, the extract of *Garcinia mangostana* showed an IC_{50} of 6.13µg/ml on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The same species was also able to inhibit 77.84% and 94.59% of TNF-α secretion in Peripheral Blood Monocyte Cells (PBMC) at 5 and 50µg/ml concentrations, respectively. This shows the potential of plants to treat inflammation, a major symptom which is observed throughout the progression of acne vulgaris (Chomnawang *et al.*, 2007).

Table 1.3: The antimicrobial activity of Thai medicinal plants against *P. acnes* (Chomnawang *et al.*, 2005)

Thai medicinal plant	*MIC against P. acnes (mg/ml)*
Andrographis paniculata	0.63
Barleria lupulina	1.25
Eupatorium odoratum	0.63
Garcinia mangostana	0.04
Hibiscus sabdariffa	2.50
Houttuynia cordata	0.04
Lawsonia inermis	2.50
Psidium guajava	2.50
Senna alata	0.63
Senna occidentalis	2.50
Senna siamea	1.25
Tagetes erecta	2.50

South African plants also show the same promise as possible treatments for acne vulgaris. A study on the plants used by people of the Bredasdorp/Elim region in the Western Cape has shown that *Allium cepa*, *Bulbine lagopus* N.E.Br., *Artemisia afra* Jacq. Ex. Willd., *Chirona baccifera* L. and *Eucalyptus spp.* are all used for the treatment of acne directly (Thring & Weitz., 2006). Plants which are also of commercial importance, such as, *Centella asiatica* (Asiatic Pennywort) have also been reported to treat acne (Van Wyk, 2008). These studies, however, are only able to provide ethnobotanical data. This shows the importance of research which not only looks at the antimicrobial efficacy on *P. acnes* but also other factors that contribute towards skin infection and bacterial pathogenesis.

1.9. Problem statement

1.9.1. Acne vulgaris

Acne vulgaris is a chronic inflammatory disorder affecting the pilosebaceous unit and is characterised by inflammatory and non-inflammatory lesions (Dessinioti & Katsambas, 2010) where the causative agent is *P. acnes* but may also be as a result of *S. epidermidis* (Lalla *et al.*, 2001). Acne vulgaris affects 85% of the population, throughout their lives (Virtual Medical Centre, 2012). It is most common in individuals between the ages of 12-24 years (85% prevalence in this age group), 25-34 years (8% prevalence in this age group) and 35-44 years (3% prevalence in this age group) according to a study done on the American population (White, 1998). In adolescents the 95-100% of males and 83-85% females suffer from acne, where females and males generally experience severe acne between the ages of 14-17 years and 16-19 years respectively (Virtual Medical Centre, 2012).

Pustules, papules and nodules are characterised as inflammatory lesions and blackheads (open comedones) and white heads (closed comedones) are characterised as non-inflammatory lesions (Guidance for Industry-Acne vulgaris: Developing Drugs for Treatment, 2005). The blackhead is the most basic acne lesion and is produced by hyperkeratosis (hardening) of the lining of the hair follicles. This often results in keratin formation, sebum secretion and accumulation of *P. acnes*. Scarring of the face, back, neck and trunk are common with inflammatory lesions (Lalla *et al.*, 2001).

Acne vulgaris is differentiated in terms of severity. Acne can be mild, moderate or severe. Each degree can be associated with the presence or absence of lesions. Mild acne is defined

by the presence of open and closed comedones with limited inflammatory lesions. With moderate acne the appearance of papules and pustules is common and are found in increased numbers. Severe acne is defined by an abundance of inflammatory lesions that include nodules and if untreated results in post-inflammatory scarring of the skin (Layton, 2005).

1.9.2. Antibiotic resistance of *Propionibacterium acnes*

Acne treatments include both antibiotic and non-antibiotic, which are aimed at interfering with the growth and metabolism of the microorganism. Antibiotic treatments have been used for over 40 years and are currently still being prescribed. The emergence of resistance to antibiotics was first observed in the 1970s, where in previous years *Propionibacteria* remained susceptible. Studies done in the early 1980s in countries such as the Netherlands, United Kingdom, United States of America, New Zealand and Japan have shown a clear relationship between resistance to clindamycin, tetracyclines, erythromycin and trimethoprim-sulfamethoxazole in *P. acnes* and therapeutic use of these antibiotics (Table 1.4). From 1991 to 1996 there was an increased incidence of antibiotic resistance from 35% to 60% and a maximum of 64% was observed in 1997. Antibiotic resistance is enhanced because acne treatments generally have minimal duration times of several months and normal skin flora is also affected. It is therefore common practice for combinations of antibiotics to be used. It is also common to see alternations between different antibiotics and also between oral and topical applications of these. The major problem with these practices is the resulting multi-resistant strains of *Propionibacteria* (Oprica *et al.*, 2004).

Table 1.4: Indication of emergence of *P. acnes* resistance through history (Leyden, 2001)

Year	***Resistance***
1983	Clinically relevant strains reported
Late 1980s	Erythromycin resistance
Early 1990s	Erythromycin and Tetracycline resistance common
Late 1990s	Resistance to multiple antibiotics is common
2001	Resistant strains found worldwide

1.9.3. Psychological effects of acne on patients

According to evolutionary psychology, for early humans, social status and rank was obtained through threats and aggression between competitors for resources. This has been replaced with the need to "present oneself as attractive" to others. A study of the emotional stress related to different medical conditions showed that patients suffering from acne, experience a greater emotional stress, when compared with patients suffering from other medical disorders. The study reported that patients awaiting acne treatment had significantly high levels of anxiety and depression (Kellet and Gawkrodger., 1999).

The same study was also done for acne patients who were treated using isotretinoin. The study investigated the psychological and dermatological responses during the course of treatment in acne patients. The study showed that there was significant reduction in psychological and dermatological distress over the course of the treatment but in the mid-phase of treatment there was a rise in psychological distress (Kellet and Gawkrodger., 1999).

The study concluded that acne vulgaris causes an emotional burden on patients suffering from the disorder. It also suggested that acne should not only be treated as a skin disorder, but can also cause great damage (long term) to the emotional state of patients (Kellet and Gawkrodger., 1999).

1.9.4. Conventional acne treatments

Topical treatments of acne target mainly the formation of comedones. These applications often take up to several months before maximal results are observed (Shaw and Kennedy, 2007). It is for this reason that topical treatments of acne are often accompanied by an oral treatment as well (Lalla *et al*., 2001).

Retinoids are vitamin A derivatives; they are used to target keratinocytes and are used to treat comedones. Tretinoin and isotretinoin are also commonly used. Some adverse effects associated with these drugs include dryness, redness and irritation of the skin. Some patients find the treatment to be highly uncomfortable (Shaw and Kennedy, 2007).

Isotretinoin is one of the so called "gold standard" treatments for acne. It is commonly found in products such as Roaccutane. It is able to reduce sebum; it affects epithelial follicular differentiation and also has anti-inflammatory effects. The treatment is however, expensive and has to be dosed under the supervision of a licenced dermatologist. Some less common

side effects include muscle aches, pains, tiredness, hyperlipidaemia and hepatitis. It is also a teratogen and specialists often warn that the effects of the acne may worsen during the first week of treatment, when the drug is used (Shaw and Kennedy, 2007).

Benzoyl peroxide is a common active ingredient found in many products that are available, over the counter (i.e. they do not require a prescription by a dermatologist). It can be found in formulations at concentrations ranging from 2.5-10%. This compound treats the inflammation associated with acne by acting as an antimicrobial agent and also affecting inflammatory mechanisms. Dryness of the skin has been observed with this treatment and it has been known to bleach hair and even clothing (Shaw and Kennedy, 2007).

Azelaic acid (Skinoren) is used for its anti-bacterial activity and comedolytic effects. This treatment it used mainly when the benzoyl peroxide and retinoid treatments are not well tolerated by patients. It may cause hypopigmentation in patients, however in dark skin types this may be advantageous due to post-inflammatory hyperpigmentation that is observed in patients with acne (Shaw and Kennedy, 2007).

Common antibiotics used in the treatment of acne are erythromycin and clindamycin. Antibiotic treatments are generally used for their ability to treat the inflammation associated with acne. The main problem with antibiotic treatments is the appearance of increased *P. acnes* resistance and also that they must be used together with topical applications.

Tetracycline and **oxytetracycline** are also used. In the case where there is resistance to tetracycline it may be replaced by minocycline which puts less stress on the appearance of resistant *P. acnes* strains. Minocycline has however, been observed to produce a bluish pigment under the skin, may cause hypersensitivity, photosensitivity and has also been implicated in autoimmune hepatitis (Shaw and Kennedy, 2007).

The use of natural remedies which use medicinal plants dates back thousands of years. A review on the use of medicinal herbs has shown the high potency and potential of these herbs to be used as antimicrobials, anti-inflammatory, antioxidant and anti-androgenic for the development of new anti-acne products. This review also showed that a variety of plant metabolites target different aspects of acne vulgaris. Antimicrobial effects against *P. acnes* was shown when using essential oils, extracts containing alkaloids, glycosides, tannins, flavonoids and many other secondary metabolites (Azimi *et al.*, 2012). Hence it was decided

that in this study twenty plants that have shown potential as antimicrobial, anti-inflammatory and antioxidant agents would be tested for their *in vitro* efficacy against *P. acnes*.

1.10. Objectives

- Identify the antimicrobial activity of 20 southern African plant ethanol extracts against the *P. acnes* bacterium
- Identify synergistic effects of the extracts showing antimicrobial activity
- Identify the potential antioxidant activity of 20 southern African plant ethanol extracts on the DPPH free radical
- Identify the cytotoxicity of the active plant extracts on U937 cells
- Investigate anti-inflammatory effects of active plant extracts monitoring IL-8 cytokine levels of *P. acnes* infected cells
- Investigate the production of hyaluronidase enzyme by *P. acnes* (ATCC 11827) strain

1.11. Methodology

1.11.1. Antibacterial activity of extracts

The plant extracts were tested against *P. acnes* using the micro-dilution broth assay. The *P. acnes* cultures were inoculated in nutrient broth using an inoculation loop. INT (Iodonitrotetrazolium violet) and PrestoBlue were used as growth indicators and the MIC (Minimum Inhibitory Concentration) were determined (Tsai *et al.*, 2010).

1.11.2. Anti-oxidant activity of extracts

The antioxidant activity of the twenty plant extracts, were determined using the DPPH radical scavenging assay (du Toit *et al.*, 2001).

1.11.3. Cytotoxicity of selected extracts

The most plant extracts which showed high antibacterial and antioxidant activity were tested for their cytotoxicity on human monocyte cells (U937). The 2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction assay was used to check the viability of the cells (Tsai *et al.*, 2010). Samples which showed low cytotoxicity and high efficacy were selected for anti-inflammatory activity.

1.11.4. Synergistic studies

Active plant extracts were used in combination to determine the added effect on antimicrobial activity. Combinations were tested according to methods described by Suliman *et al.*, 2010 adapted for *P. acnes* bacterium.

1.11.5. Cytokine analysis of *P. acnes* infected cells

The supernatant of human monocytic cells (ATCC U937) treated with heat killed *P. acnes* bacteria will be tested for IL-8. The times of release will be investigated before treatment with the plant extracts and supernatants will be assessed using the respective cytokine kits (Tsai *et al.*, 2010).

1.11.6. Production of Hyaluronate lyase (Hyaluronidase) by *P. acnes*

Hyaluronate lyase (Hyaluronidase) is an enzyme produced by *P. acnes*. It is involved in the degradation of hyaluronan and hyaluronate (hyaluronic acid), the major polysaccharide of extracellular matrix connective tissue. The breakdown products of this enzyme can be located at sites of inflammation and tissue injury (Brüggermann., 2010). The rapid plate method was used to investigate whether *P. acnes* strain (ATCC 11827) produced the hyaluronidase enzyme (Smith & Willett, 1968).

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Chapter 2

Selected Plant Species for the Present Study

2. Introduction

Compounds derived from plants and other natural sources have been used medicinaly since before the first recording of human history. The impact of natural products on medicine and human health is immense. For our human predecessors the use of these medicinal plants was most likely the only form of treatment for disease and injury (Ji *et al*., 2009).

The use of traditional medicines is common, especially in developing countries of the modern world. In the rural areas of developing countries it is common to find traditional medicine for the treatment of skin infections. Medicinal plants are used against dermatological ailments as they have the ability to stop or reduce bleeding, enhance wound healing, treat burns and alleviate symptoms of skin conditions. In southern Africa, there are many species which have been targeted for commercialisation but have not yet been sufficiently tested for toxicity or pharmacological efficacy (Mabona & Van Vuuren, 2013). Also, medicinal plants have recently gained popularity for their application as cosmetic ingredients (Lall & Kishore, 2014). This can be observed with the launch of African Extracts™, which supply body products containing Rooibos and the Elixir™ skin care product range which contains *Aloe ferox* (Mabona & Van Vuuren, 2013).

Therefore it was decided to investigate the efficacy of 20 southern African plants for their activity against *P. acnes*. Plants were selected based on their traditional use (by South African cultural groups and southern African groups) for the direct treatment of acne vulgaris, any of its associated symptoms (inflammation, blackheads, and redness), other skin disorders, skin wounds or for their antimicrobial activity. Most of the plant species in the current study are indigenous to South Africa, however, those species that are not, were selected due to their widespread occurrence throughout South Africa and in other southern African countries like Zimbabwe.

2.1. *Acacia karroo* Hayne

2.1.1. Description

Acacia karroo Hayne (Fig. 2.1) (now *Vachellia karroo* Hayne) is commonly known as the Sweet Thorn. It can be found in most well-known terrestrial habitats. It is a leguminous shrub to medium-sized tree that is widespread throughout many areas of southern Africa. It is found in grasslands and is one of the most important invaders of the South African biome (Taylor &

Barker, 2012). It belongs to the Fabaceae family and spans from the Western Cape through to neighbouring countries Zambia and Angola (Adedapo *et al.*, 2008) but is indigenous to South Africa. White, narrow spines can be found on the young leaves. Yellow and globose flowers are characteristic of the species (Van Wyk & Van Wyk, 1997). There are various forms of the species which are categorized according to their habitats and phenotypic characteristics (Taylor & Barker, 2012).

Figure 2.1: Aerial parts of the *Acacia karroo* Hayne (Van der Merwe *et al*., 2001)

2.1.2. Traditional usage

The gum of *Acacia karroo* is an important source of food in many rural communities. The bark, leaves and gum of the species have many medicinal uses that range from wound wrappings, eye treatments and influenza (Adedapo *et al*., 2008). It has also been recorded to treat diarrhoea, dysentery, conjunctivitis and haemorrhages (Nyila *et al.*, 2012). The bark is used as an astringent medicine (Hutchings *et al*., 1996). In the Cape region, the gum of *Acacia karroo* is used medicinally together with *Capsicum sp.* in vinegar to draw abscesses, pus-filled wounds caused by inflammatory processes (Grace *et al*., 2003).

2.2. *Buddleja saligna (Willd.)*

2.2.1. Description

Buddleja saligna Willd is commonly known as the False Olive (Fig. 2.2). It occurs mainly in dry hilly areas, edges of forests and in coastal thickets. The species belongs to the Buddlejaceae family and is indigenous to South Africa. The leaves have a shiny, smooth texture. The species is rich in white-cream flowers (Palgrave, 2002) having a characteristic honey scent and appearing from August to January (Aubrey, 2002).

Figure 2.2: The flowers of *Buddleja saligna* (Willd.) (Aubrey, 2002)

2.2.2. Traditional usage

The entire plant has been used by the South African, Zulu community to treat colds and coughs; and the leaf decoction used by the South African, Tswana and Kwena communities for the same purpose. Other South African communities reported that the roots are used as purgatives and root decoctions are used for anasarca (widespread swelling of the skin) (Hutchings *et al*., 1996).

2.3. *Buddleja salviifolia* (L.) Lam.

2.3.1. Description

Buddleja salviifolia L. Lam. is commonly known as the Sagewood (Fig. 2.3). It can be found in evergreen forest margins, mountain slopes and at high altitude waterways. It belongs to the Buddlejaceae family and is indigenous to South Africa. The leaves are velvet when young and gradually darken and wrinkle when matured. Flowers are white-cream, but can appear lilac or purple (Palgrave, 2002).

Figure 2.3: The flowers and leaves of *Buddleja salviifolia* (L.) Lam. (Mutshinyalo, 2001)

2.3.2. Traditional usage

Decoctions made from the roots are used traditionally to treat upset or flatulent stomach and diarrhoea. Leaf decoctions are used as eye washes and to treat colic infants. The flowers of the plant are traditionally used as a wash solution for sores and are also taken as tonics by the Tswana community (Hutchings *et al*., 1996).

2.4. *Cheilanthes viridis* (Forssk.) Swartz var. viridis

2.4.1. Description

Cheilanthes viridis (Forssk.) Swartz var. viridis is a perennial fern that originates from a creeping rhizome (Fig. 2.4). It is commonly found growing in areas exposed to high sunlight

(Hyde *et al*., 2013). Its preferred habitats include woodlands, grasslands, rocky hills and gorges (Vega-Hernandez & Caudales-Cepero, 2008). It belongs to the Pteridaceae family and is indigenous to South Africa. The creeping fronds often have pinnate leaves and species variation exists with differences in shape and size.

Figure 2.4: Leaves and stems of *Cheilanthes viridis* (Forssk.) Swartz var. viridis fern (White, 2009)

2.4.2. Traditional usage

It is traditionally used by the Zulu community to treat sores and other skin disorders. In the Eastern Cape, a South African Province, a paste made from the dried leaves is applied to burns on the skin. In Madagascar, the aerial parts are commonly used as a diuretic agent (Hutchings *et al*., 1996).

2.5. *Clausena anisata* (Willd.) Hook.f. ex Benth.

2.5.1. Description

Clausena anisata (Willd.) Hook.f. ex Benth is commonly known as the Horsewood (Fig. 2.5). It is a shrub or small tree capable of reaching 3-5m. It can be found in evergreen forests, low-altitude woodlands, palm fields and along streams and river banks. It belongs to the Rutaceae family and is indigenous to Zimbabwe. Leaves are imparipinnate. Flowers are small white-yellow appearing from August to November (Palgrave, 2002).

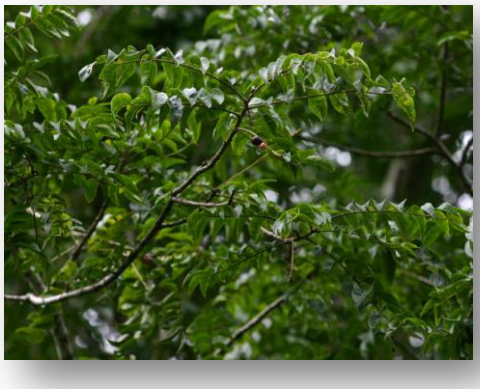

Figure 2.5: Aerial parts of *Clausena anisata* (Willd.) Hook.f. ex Benth. (Hyde *et al.*, 2013)

2.5.2. Traditional usage

This plant is used traditionally by the Western Cape, Rasta community for treating body pains and for strength. Other uses include the treatment of parasites, worms, rheumatic fever, toothache, constipation and can also be burned as a mosquito repellent (Philander, 2011). Various parts of the plant are used to treat eye problems, respiratory problems, heart disorders, high blood pressure, abdominal cramps, gastroenteristis, boils, swollen gums, arthritis and other inflammatory conditions (Kenechukwu *et al.*, 2012).

2.6. *Clematis brachiata* Thunb.

2.6.1. Description

Clematis brachiata Thunb. is commonly referred to as Traveller's Joy (Fig. 2.6).

Figure 2.6: Flowers and leaves of *Clematis brachiata* Thunb. (Viljoen, 2002)

It is a deciduous climber that can reach up to 5m in height. It is commonly found around roadsides, creeping on other bush species, on rocks, fences and grassy areas. It belongs to the Ranunculaceae family and is indigenous to South Africa. It has cream white flowers that appear between February and May (Viljoen, 2002).

2.6.2. Traditional usage

Infusions of the stems and leaves are traditionally used as enemas and abdominal disorders by the Zulu community. Crushed leaves are mixed with red sand and applied to the skin to treat rashes in children. Other South African communities, like the Xhosas, use the stems as an inhalant against colds, however, the leaves and roots are also used for the same purpose. Powdered roots are used as snuff in Zimbabwe and the smoke from burning leaves are inhaled for haemolytic problems or to treat itching sores in Botswana, a neighbouring country (Hutchings *et al*., 1996).

2.7. *Euclea undulata* Thunb.

2.7.1. Description

Euclea undulata Thunb. is commonly known as the Small-leaved Guarri (Fig. 2.7).

Figure 2.7: Aerial parts of *Euclea undulata* Thunb. (Vlok & Schutte-Vlok, 2010)

This is a dense twiggy shrub or tree that grows up to 7m. It can be found in a wide number of habitats including rocky slopes, low hills and watercourses. It belongs to the Ebenaceae family and is indigenous to South Africa. The leaves are opposite, sub-opposite or arranged in 3-4 leaf whorls. Small white flowers appear from December to April (Palgrave, 2002).

2.7.2. Traditional usage

The entire plant is used by people and animals. The entire plant is used to make potent enemas. Unspecified plant parts are used by the Zulu community as *inembe*, which is taken during pregnancy to ensure an easy childbirth. The roots of the plant are used to soothe tooth ache and other pains in the Cape region of South Africa. In the neighbouring country Botswana, root decoctions are used for fevers (Hutchings *et al*., 1996). The roots are powdered and dried and can be used for inflammation as well (Gardening Eden, 2014). Leaf decoctions are prepared as a gargle for swollen tonsils (Voigt, 2013).

2.8. *Faurea saligna* Harv.

2.8.1. Description

Faurea saligna Harv. is commonly known as the Willow Beechwood (Fig 2.8).

Figure 2.8: *Faurea saligna* Harv. tree (Mbambezeli, 2008)

It is a narrow, small to medium sized tree that can grow up to 10m high. It belongs to the Proteaceae family and is indigenous to South Africa. It is common in habitats such as sandy or red loamy soils and also in areas with rocky ridges. The leaves are long and narrow with a characteristic sickle shape. The flowers are a creamy-white and appear from August to February (Palgrave, 2002).

2.8.2. Traditional usage

Medicinally it is used by the Zulu communities of South Africa. The bark is used as a tonic and the roots are boiled and the tea is used for treating diarrhoea and indigestion (Hutchings *et al*., 1996). An ethnobotanical survey has also shown that the bark is boiled and used for treating sores (Mthethwa, 2009).

2.9. *Gunnera perpensa* L.

2.9.1. Description

Gunnera perpensa L. is commonly known as the River Pumpkin (Fig. 2.9). This is a perennial herb that grows up to 1m and is generally found near a water source. It thrives in wetlands or shallow pools along streams. It belongs to the Gunneraceae family and is indigenous to South Africa. The leaves are large, green and kidney-shaped. The reddish-brown flowers can be seen during September to February (Glen, 2005).

Figure 2.9: *Gunnera perpensa* L. leaves (Glen, 2005)

2.9.2. Traditional usage

It is traditionally used in combination with other species for treating several disorders. A decoction made from a mixture of its roots with the bulbs of *umduze* (a *Crinum* species) is used to treat cystitis and stricture of the bladder. It is also used against pain in people suffering from rheumatic fever. Infusions made from the roots are used in the Eastern Cape Province, where they are used topically for treating swelling and cancerous sores. Also in the Cape, the roots are used for stomach ailments and the entire plant is used by local farmers for dressing wounds. In southern Africa the roots and leaves are used for colds and topically or orally administered for psoriasis (Hutchings *et al*., 1996).

2.10. *Gymnosporia buxifolia* (L.) Szyszyl.

2.10.1. Description

Gymnosporia buxifolia (L.) Szyszyl. is commonly referred to as the Spike-thorn (Fig. 2.10). It is an evergreen shrub or small tree that can grow up to 2-3m. It belongs to the Celestraceae family and is indigenous to Zimbabwe. The leaves of this species occur in clusters along dwarfed branchlets with fine, irregular margins. Flowers bloom between September and April but may differ with regard to growth region (Palgrave, 2002).

Figure 2.10: Aerial parts of *Gymnosporia buxifolia* (L.) Szyszyl. (Hyde *et al*., 2013)

2.10.2. Traditional usage

Medicinally it is used to stop vomiting (Shai *et al*., 2010). Bark infusions are administered as emetics or enemas for diarrhoea by the Zulu communities. Other medicinal uses include mixing the plant with snake parts as a remedy for snake bites. Root and thorn decoctions have been taken for colds and coughs by the Tswana, Koba and Subiya communities as well. In West African countries the leaves and roots of this species are used for viral infections and as anti-inflammatory agents (Hutchings *et al*., 1996).

2.11. *Helichrysum aureonitens* Sch. Bip.

2.11.1. Description

Helichrysum aureonitens Sch. Bip. is commonly known as the Golden Everlasting (Fig. 2.11). It is a perennial shrub with thin, erect stems that can reach a height of 300mm. It belongs to the Asteraceae family and is indigenous to South Africa. Much like other species the stems and leaves are abundant with white woolly hairs. The flowers are clusters of many small yellow flowers and can be seen from September to February (Ready, 2007).

Figure 2.11: Aerial parts of *Helichrysum aureonitens* Sch. Bip. (Ready, 2007)

2.11.2. Traditional usage

It is used traditionally as an aromatic smoke which calls upon the goodwill of the ancestors. The leaves and stems are often sold commercially as a decoction, used for children with uncontrollable bladder function (enuresis). Extracts made from this plant are used topically for skin infections against *Herpes zoster* virus and infections that are associated with *Herpes simplex* virus (Lourens *et al*., 2008).

2.12. *Helichrysum kraussii* Sch. Bip.

2.12.1. Description

Helichrysum kraussii Sch. Bip. is commonly named the Curry Bush (Fig. 2.12). This is a small shrub species with the ability to grow up to 1.5m. It belongs to the Asteraceae family and is indigenous to South Africa. The leaves of this plant are linear with flowers appearing from April to September. This species is commonly seen at altitudes between 1000-2000m and in woodland or grassland habitats where soil quality is poor due to over-grazing (Hyde *et al*., 2013).

Figure 2.12: The aerial parts of *Helichrysum kraussii* Sch. Bip. (Hyde *et al*., 2013)

2.12.2. Traditional usage

Certain parts of the plant are known to have medicinal value. The leaf decoction for example is used as a wash for keloid scars on the skin (Lourens *et al*., 2008).

2.13. *Helichrysum odoratissimum* (L.) Sweet

2.13.1. Description

Helichrysum odoratissimum (L.) Sweet is commonly referred to as the Kooigoed (Afrikaans) or Imphepho (Xhosa) (Fig. 2.13). Much like the other *Helichrysum* species it is a perennial herb that can grow up to 50cm. The habitat for this species includes mountains, coastal areas, grassy, rocky slopes and bare roadside paths. It belongs to the Asteraceae family and is indigenous to South Africa. Its leaves appear small and silvery from the presence of white woolly hairs on either side. Flowers bloom throughout the year but in the south western Cape it is common from August to December. In other areas flowering occurs from January to June (Swelankomo, 2004).

Figure 2.13: The aerial parts of *Helichrysum odoratissimum* (L.) Sweet (Swelankomo, 2004)

2.13.2. Traditional usage

The Zulu community uses the leaves and stems as incense to bring the goodwill of the ancestors. In the Eastern Cape the plant is inhaled as a cleanser and as a medicine for colds and coughs. The plant is boiled with fat and used as an ointment for pimples (Hutchings *et al*., 1996). The whole plant is also used to treat abdominal pains, heartburn, coughs, colds, female sterility, eczema and wounds (Prinsloo, 2002).

2.14. *Helichrysum splendidum* (Thunb.) Less.

2.14.1. Description

Helichrysum splendidum (Thunb.) Less. is commonly referred to as the Cape Gold (Fig. 2.14). It is considered as a shrub that can grow into a dense mound. The habitat of this species includes rocky slopes, edges of forests and mountain tops. It is mostly found in areas with a summer rainfall. It belongs to the Asteraceae or Daisy family and is indigenous to South Africa. The leaves overlap as they are arranged in an upward position covering the stem surface. Flowering occurs from November to February with the arrival of bright yellow flowers (Van der Walt, 2003).

Figure 2.14: The leaves and flowers of *Helichrysum splendidum* (Thunb.) Less. (Van der Walt., 2003)

2.14.2. Traditional usage

Traditionally the plant's roots are used to treat rheumatism. The leaves are boiled for the production of an inhalant that is used to induce sweating. Together with some *Senecio* species the plant is known to treat pimples (Lourens *et al*., 2008).

2.15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.

2.15.1. Description

Heteromorpha arborescens (Spreng.) Cham. & Schltdl. is commonly known as the Pasley Tree (Fig. 2.15). It is commonly found in grasslands, bushvelds and along forest margins. It belongs to the Apiaceae family and is indigenous to South Africa (Harris, 2003). It is a woody shrub or small tree that may grow up to 15m. The leaves are simple, trifoliate or imparipinnate. The flowers are greenish-white or yellowish with a strong odour. The flowers appear between December and January (Palgrave, 2002).

Figure 2.15: The aerial parts of *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. (Harris, 2003)

2.15.2. Traditional usage

In Zimbabwe the root infusion is used as a remedy for people with fever (Fowler, 2006). The plant's essential oils are traditionally used for headaches, as inhalants which show potential for use as aromatherapeutic agents (Van Wyk, 2011). The plant has been reported to have

antimicrobial activity against *S. epidermidis*, a microorganism that has been found in some acne lesions (Nkomo & Kambizi, 2009).

2.16. *Leucas martinicensis* (Jacq.) R. Br.

2.16.1. Description

Leucas martinicensis (Jacq.) R. Br. is commonly referred to as the Wild Tea Bush (Fig. 2.16). It is an annual herb that can grow up to 1m in length. It is found in grassy areas and wastelands that are habitable. It is not known whether the plant is indigenous to Africa or introduced but it belongs to the Lamiaceae family. The leaves are opposite and are serrated at the leaf margins. The flowers are small and white (Muhammad *et al*., 2012).

Figure 2.16: Aerial parts of *Leucas martinicensis* R. Br. (Hyde *et al*., 2013)

2.16.2. Traditional usage

This plant is used traditionally by the Zulu communities for the treatment of fevers in children. Inhalation or ingestion of the plant in the form of a hot decoction and is used for colds and gynaecological application in some parts of southern Africa. Zimbabwean natives use leaf infusions of the plant for stimulation of poor appetites. Ointments containing the

fruits of this species are used for rubbing the depressed fontanelles (cranial bone spaces) in new borns. The leaves can also be eaten to prevent vomiting. In West Africa the plant is burnt as a mosquito repellent (Hutchings *et al*., 1996). The leaves are used to make tea which is used to treat colds and flu. In Europe, hot decoctions are also used for colds (Suliman, 2010). It is also used for the treatment of otitis, inflammation of the middle part of the ear (Santos *et al*., 2012).

2.17. *Rapanea melanophloeos* (L.) Mez

2.17.1. Description

Rapanea melanophloeos (L.) Mez is commonly named the Cape-beech (Fig. 2.17). It is a medium sized tree that may grow to 4-10m. It is commonly found in evergreen forests, riverine margins and occasionally drier areas such as coastal or mountain forests. It belongs to the Myrsinaceae family and is indigenous to South Africa. Leaves are crowded at the ends of branches and are shiny, thick and leathery. Flowers tend to appear from May to August and are green or white (Palgrave, 2002).

Figure 2.17: The stems and leaves of *Rapanea melanophloeos* (L.) Mez. (Xaba, 2005)

2.17.2. Traditional usage

Traditionally the Zulu herbalists use the bark of this plant for its ability to strengthen the heart. It is also used for acidity, stomach pains, muscular pains and fevers. Decoctions made from the bark of this species are administered as expectorants, emetics and enemas. In

various parts of the Eastern Cape, the roots and bark are used for treating heart palpitations. In the Cape regions the leaves are used as astringents (Hutchings *et al*., 1996).

2.18. *Scadoxus puniceus* (L.) Friis & Nordal

2.18.1. Description

Scadoxus puniceus (L.) Friis & Nordal is commonly known as the Paintbrush Lily (Fig. 2.18). This species can be found in habitats like coastal bush, ravines and forests. It belongs to the Amarylidaceae family and is indigenous to South Africa. The leaves of this species are glossy, green and are a characteristic wave-like shape at the leaf edge. The flowers appear in the form of an inflorescence which consists of many small scarlet flowers that appear from early spring to early summer (Turner, 2001).

Figure 2.18: The aerial parts of *Scadoxus puniceus* (L.) Friis & Nordal (Turner, 2001)

2.18.2. Traditional usage

The root decoction is traditionally used by the Zulu community for coughs; it is also used as an emetic. The infusion of unspecified plant parts is taken during pregnancy to ensure a safe delivery. In other regions, like the Eastern Cape, warm water emetics are made from the bulbs and the roots which are used for those who are suspected of being poisoned. The roots are also used for headaches and as skin poultices in some parts of southern Africa (Hutchings *et al*., 1996).

2.19. *Tabernaemontana elegans* Stapf.

2.19.1. Description

Tabernaemontana elegans Stapf. is commonly known as the Toad Tree (Fig. 2.19). It is a small tree growing between 3-5m. It is found in evergreen riverines, coastal forests and more prominently in rocky areas. It belongs to the Apocynaceae family and is indigenous to South Africa. The leaves are slightly leathered, glossy and dark green. Flowers are cream-white and appear from October to February (Palgrave, 2002).

Figure 2.19: The leaves and flowers of *Tabernaemontana elegans* Stapf. (Hyde *et al*., 2013)

2.19.2. Traditional usage

Traditionally the plant roots are used by the Zulu community for chest pains. Other medicinal uses include the use of the latex as a styptic (astringent); The Tonga community uses the roots to treat those suffering from pulmonary related problems. Extracts made from the entire plant have strong antimicrobial activity against Gram-positive microorganisms (Hutchings *et al*., 1996).

2.20. *Urtica dioica* (L.)

2.20.1. Description

Urtica dioica (L.) is known to occur abundantly throughout Europe, North Africa and in the temperate regions of the Asian continent (Fig. 2.20). It is grows vegetatively with the use of underground runners. It belongs to the Urticaceae family and is a naturalized exotic species of South Africa (SANBI, 2013). The leaves are in opposite orientation with serrated leaf margins. The aerial shoots can grow up to 1m in length and possess stinging hairs (Olsen, 1921).

Figure 2.20: The aerial parts of *Urtica dioica* (L.)

2.20.2. Traditional usage

Mainly the roots and the leaves of the plant are used. The plant is used both orally and topically. Topical usage has been reported for relief of pinched nerves, skin and joint inflammation and wounds. It is ingested orally for treating fluid retention, problems in urinating, menstrual disorders, asthma, allergic reactions, sinusitis and intestinal inflammation. It has been used traditionally and is also currently used for treating prostrate inflammation (Globinmed, 2010).

2.21. Materials and Methods

2.21.1. Materials

All chemicals used were of analytical grade and purchased from Merck (Pty) Ltd, South Africa.

2.21.2. Methods

2.21.2.1. Plant selection and identification

Various plant parts were collected from the University Of Pretoria Botanical Garden, South African National Biodiversity Institute (SANBI) and also supplied by the Edakeni Community in the KwaZulu-Natal Province. Species were selected based on traditional usage which included their ability to directly treat acne vulgaris, any of its associated symptoms (inflammation, comedones etc.); their application in the treatment of other skin disorders or their reported antimicrobial potential. Plants were allowed to shade dry. Their identities were confirmed and voucher specimen numbers deposited at the H. G. W. J. Schweickerdt Herbarium at the University of Pretoria (Table 2.1). The identified species were then used for extraction. The other extracts (*Acacia karroo*, *Euclea undulata*, *Gunnera perpensa*, *Helichrysum aureonitens*, *Helichrysum splendidum* and *Heteromorpha arborescens*) were donated by Professor Namrita Lall, Associate Professor at the University of Pretoria, Department of Plant Science.

2.21.2.2. Plant extraction

Dried plant material of each species was individually extracted using organic ethanol. Plant material was mixed with solvent and vigorously shaken for 48h using a Labcon 3086U machine at moderate speed. This was done to ensure that the solvent penetrated deep into the plant material so that more compounds could be extracted (Silva *et al*., 1998). The plant material was then filtered using a vacuum system. The filtrate was then concentrated using a Rotavapor (Buchi R-200) and stored in pre-weighed, labelled glass Polytops. Any excess solvent was then evaporated under a steam of air in a fume cupboard at room temperature. Extracts were then stored at 4ºC before use in the subsequent assays.

2.22. Results and Discussion

2.22.1. Plant identification

Plant species were identified at the H. G. W. J. Schweickerdt Herbarium at the University of Pretoria. Dried and pressed material was used to produce a herbarium specimen and a voucher specimen number was obtained (PRU). The plant parts and PRU numbers were as follows:

Table 2.1: List of species included in this study with voucher specimen and plant part information

[a] Identified Species Name	[b] Plant part used	[c] Voucher Specimen Number (PRU)
1. *Acacia karroo* Hayne	Leaves	120017
2. *Buddleja saligna* (Willd.)	Leaves	120009
3. *Buddleja salviifolia* (L.) Lam.	Leaves	120008
4. *Cheilanthes viridis* (Forssk.) Swartz	Leaves and stems	120011
5. *Clausena anisata* (Willd.) Hook.f. ex Benth	Leaves and stems	118954
6. *Clematis branchiata* Thunb.	Roots, leaves and stems	120016
7. *Euclea undulata* Thunb. Var. myrtina	Root bark	95254
8. *Faurea saligna* Harv.	Leaves	118700
9. *Gunnera perpensa* L.	Leaves and stems	120010
10. *Gymnosporia buxifolia* (L.) Szyszyl	Leaves and aerial parts	119357
11. *Helichrysum aureonitens* Sch. Bip.	Leaves	NEPM
12. *Helichrysum kraussii* Sch. Bip.	Flowers, stems and leaves	96694
13. *Helichrysum odoratissimum* (L.) Sweet	Stems and leaves	118963
14. *Helichrysum splendidum* (Thunb.) Less	Leaves and stems	120012
15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	Leaves	120022

[a]	[b]	[c]
16. *Leucas martinicensis* (Jacq.) R. Br.	Seeds and leaves	96690
17. *Rapanea melanophloeos* (L.) Mez	Leaves and stems	118953
18. *Scadoxus puniceus* (L.) Friis & Nordal	Leaves	120023
19. *Tabarnaemontana elegans* Stapf.	Leaves and stems	96692
20. *Urtica dioica* (L.)	Leaves	NEPM

[a] Identified Species Name

[b] Plant Part Used

[c] Voucher Specimen Number

* NEPM (Not Enough Plant Material)

2.22.2. Plant selection

A study by Afolayan *et al* (2014) has shown that the most common plant families used in the Eastern Cape for treating skin disorders include Asteraceae, Solanaceae, Fabaceae, Poaceae and Euphorbiaceae. The most common families encountered in this study were the Asteraceae and Solanaceae, with a number of species belonging to these families.

The *Helichrysum* genus belongs to the Asteraceae family (Fig. 2.21). The genus consists of about 500-600 species. These occur in many parts of the world but about 244-250 of these occur in South Africa and Namibia (Lourens *et al*., 2008). The plant parts that are used include the leaves, stems, roots and flowers separately; sometimes the entire plant is also used. The leaves of *Helichrysum* species are generally used as applications for wounds. This explains why 20% of the selected plant families were from the Asteraceae family. Four of the selected species in this study belong to the Asteraceae family.

This can also be explained by the selection method for the plant species. Based on the traditional usage, 22% are traditionally used to treat wounds, sores and for application to the skin (Fig 2.22). The *Helichrysum spp.* are common in this category. Also *Helichrysum odoratissimum* and *Helichrysum splendidum* are traditionally used to treat acne directly as applications for pimples (Hutchings *et al*., 1996).

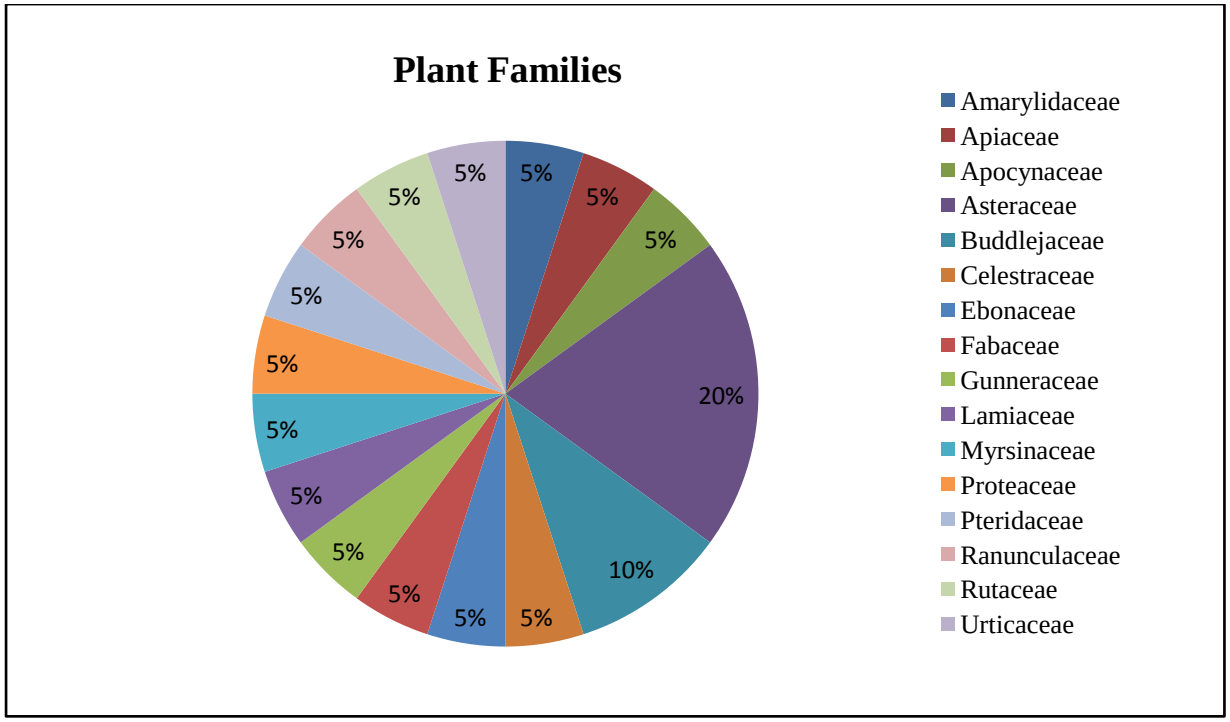

Figure 2.21: Frequency of plant families (%) included in the present study

The selected species were chosen based on their ability to treat the symptoms of acne vulgaris, the most dominant of these being inflammation.

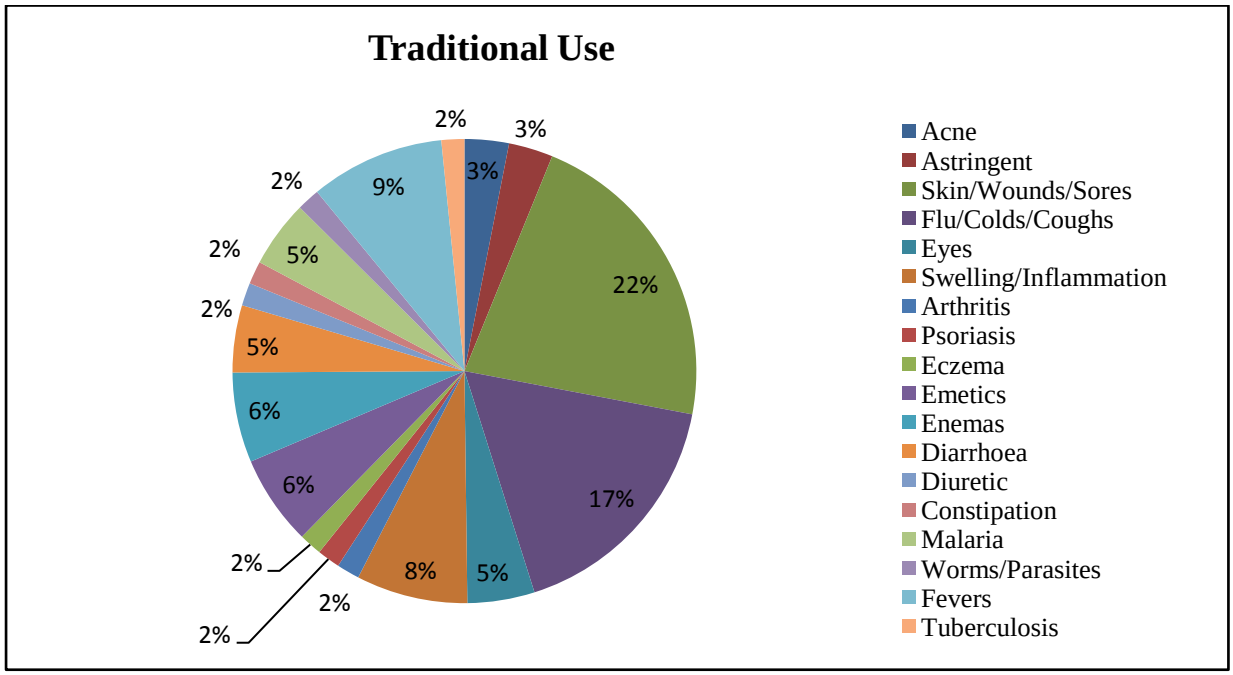

Figure 2.22: Frequency of reported traditional usage (%) of species included in the study for various ailment

The fourth largest percentage of traditional use for various ailments was 8%, which was for the treatment of inflammation or swelling (Fig. 2.22). However, this percentage would increase if we group together the species that were recorded for treating arthritis or fevers which also result from inflammatory processes.

This shows that many of the selected species are in fact chosen based mostly on their ability to treat inflammation, wounds, sores and other skin problems. This was expected as species selection was based on a targeted approach with certain criteria. These criteria included the use of the species to directly target the microorganism, the traditional use for the symptoms of acne vulgaris and also any application to the skin, even for other skin disorders. This showed that the plant species could be applied topically which is generally how acne products are applied.

2.22.3. Plant extraction

The percentage yield for each species was determined using the following formula:

$$\text{Extract percentage yield} = \frac{\text{Crude extract weight (g)}}{\text{Powdered material weight (g)}} \text{ x } 100\%$$

Ethanol was used as the extraction solvent for the preparation of extracts. It is considered to be of medium to high polarity organic solvent that has the ability to extract a variety of secondary metabolites such as tannins, polyphenols, flavonols, terpenoids, sterols, alkaloids, polyacetylenes and propolis (Gupta *et* al., 2012). These compounds have been identified to have antimicrobial activity. For example, terpenoids are known for their ability to disrupt cell membranes of bacterial cells and tannins are able to bind to proteins and enzymes inhibiting their function within bacterial cell systems (Cowan, 1999).

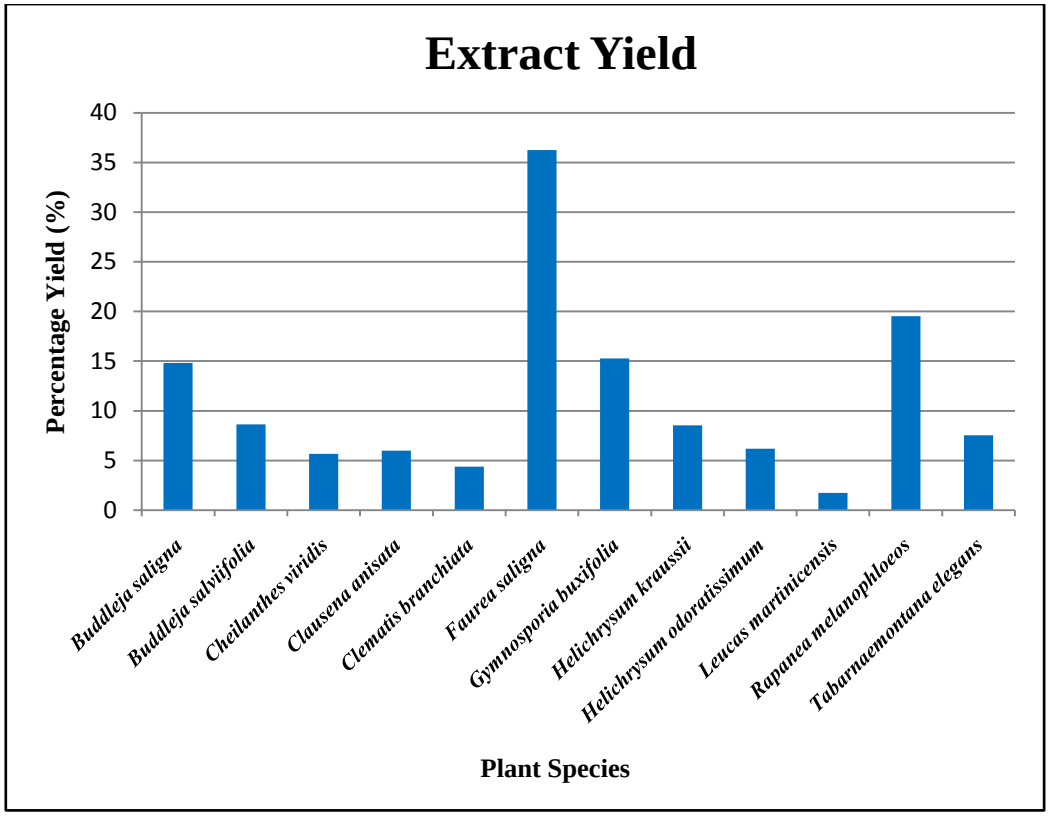

Figure 2.23: The percentage yield (%) from species included in the study

It was found that among the plant extracts that were prepared, *F. saligna* gave the highest yield of 36.27% and *L. martinicensis* gave the lowest with 1.72%. This indicates that leaf extracts of the selected plants had maximum yield, which has been previously reported by Rajeh *et al* (2010) where it was found that the methanol extraction of *Euphorbia hirta* L. leaves, stems, flowers and roots gave extraction yields of 11.1%, 7.3%, 4.7% and 4.1% respectively.

2.23. Conclusion

The selection of plants was successfully achieved through a review on the recorded literature entailing plant's traditional usage. At least one of the selection criteria was met for each of the selected plant species.

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Chapter 3

Antimicrobial Activity of Selected Plants against *Propionibacterium acnes*

3. Introduction

Many plants have been reported to have antimicrobial activity against Gram-negative and Gram-positive bacteria. In terms of dermatologically relevant microorganisms, antimicrobial activity is mostly performed against *Staphylococci* species, *Pseudomonas aerugiosa* and *Candida albicans*. The antimicrobial activity of South African medicinal plants against acne inducing bacteria (*Propionibacterium acnes*) is sometimes overlooked when compared to other geographical ethnobotanical-relevant studies (Mabona *et al*., 2013).

In the present study 20 southern African plants were investigated for their antimicrobial activity and screened against the *P. acnes* microorganism. A synergistic study was also conducted to determine the antimicrobial activity to compare the effect of using the plant extracts alone and in combination. Various methods were employed for the investigation of antimicrobial potential of plant extracts against anaerobic microorganisms, such as *P. acnes*.

3.1. Determining susceptibility of microorganisms

Microbial susceptibility to treatment with antibiotics is just as important as the biological testing of new antimicrobial agents. Natural products occurring in the form of pure compounds or as crude plant extracts are considered important sources of novel drug treatments due to their large chemical complexity. A good method for determining the antimicrobial activity of novel agents should have the following characteristics: simple, fast, reproducible, inexpensive and have the ability to accommodate a large sample number (Klančnik *et al*., 2010). Various methods are employed and even modified for the determination of the minimum inhibitory concentration (MIC) of an agent against a specific microorganism.

The broth-based turbidometric assay (TB assay) is based on the principle that in the presence of an active plant extract there is a decreased optical density (OD). The OD is read at 600nm every 30 minutes which is often tedious. Results are observed as a kinetic growth. The effective concentration (EC_{50}) can be determined for the plant extracts using this method (Othman *et al*., 2011).

The broth microdilution assay is a simple method that allows for the rapid testing of plant extracts as antimicrobial agents. The assay is performed in a 96-well plate making it possible to test a large number of samples. The MIC is then determined with the addition of a growth

indicator which is an indicator of microbial inhibition (Eloff, 1998). This is a good method for visual determination of MIC values, without the need of a spectrophotometer.

It is important to investigate microbial growth when determining the antimicrobial potential of chemical compounds or plant extracts. Microbial growth can be calculated by plate counts, microscopic counting, turbidity, absorbance and bioluminescence. Tests done in 96-well micro-titre plates are advantageous when testing multiple samples in the same test. This type of testing is usually based on an increased absorbance value within the wells of the micro-titre plate. Micro-titre plate based methods are simple and allow for increased data collection from a test. The absorbance type methods and bioluminescence are the most popular ones; however, they are also somewhat flawed. Absorbance methods are relatively simple but can only be tested in certain conditions. Bioluminescence is a sensitive method for determination of bacterial growth but requires the purchase of expensive laboratory equipment in order to quantify growth or inhibition and also requires lengthy preparation before each assay can be carried out. This makes the visual determination of the MIC, the preferred method (Gabrielson *et al*., 2002).

Indicators of bacterial growth are very useful not only when microbial cells are prone to adherence or aggregation but also in cases where supplements in the growth medium affect the colour. Tetrazolium salts and resazurin-based growth indicators have been used since the 1940s. These indicators of growth act by detecting oxidative enzymes and act as electron acceptors to become reduced. Their reduction is generally accompanied with a colour change (Eloff, 1998; Gabrielson *et al*., 2002).

Tetrazolium salts like 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) are used to determine the respiratory activity of microorganisms in their natural environment. This type of growth indicator is reduced by microbial dehydrogenase enzymes of aerobic, facultative and anaerobic microbes. Tetrazolium salts are reduced from their native form to an insoluble formazan dye (Fig. 3.1). This dye is soluble in organic solvents and can be quantified calorimetrically (Hatzinger *et al*., 2003).

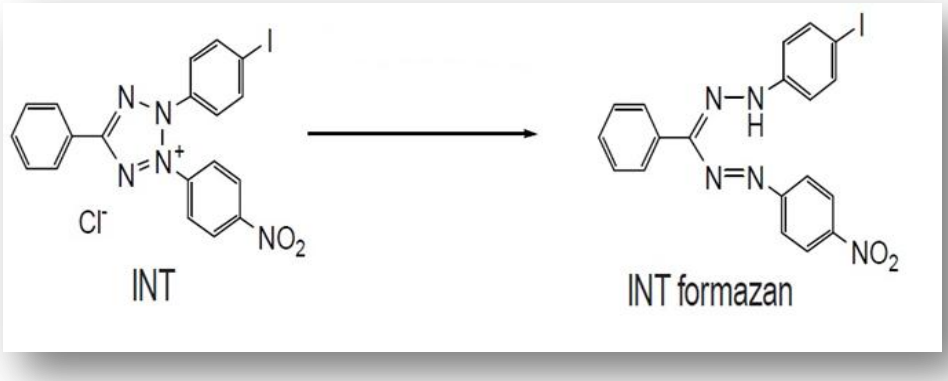

Figure 3.1: The reduction of INT to INT Formazan (Dojindo Molecular Technologies, 2013)

Resazurin is a blue dye in its oxidised form. It is reduced to a pink colour in the presence of viable microbial cells by the reduction to resorufin (Fig. 3.2). This reduction is due to the presence of oxidoreductase enzymes within microbial cells.

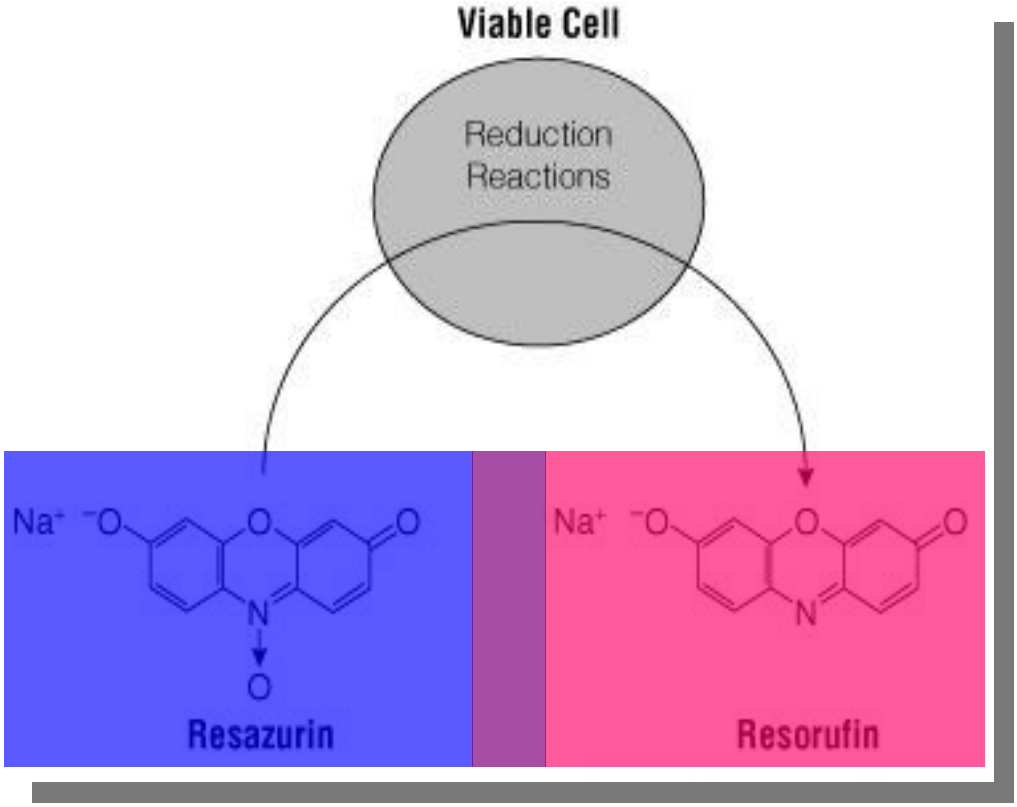

Figure 3.2: Reduction of resazurin to resorufin with gradual colour change (Adapted from Promega Corporation, 2014)

PrestoBlue (PB) has been newly developed for detecting cell-mediated cytotoxicity *in vitro*. It is a resazurin-based compound, similar to Alamar Blue. It is converted to the reduced form by mitochondrial enzymes of viable cells in the tested systems. The reagent exhibits a change in colour and a shift in fluorescence, which can be quantified using fluorometric or spectrophotometric approaches. PB is commercially prepared as a non-toxic, ready-to-use solution and is said to be the fastest live assay for assessing cell viability with an incubation time as short as 10 min. It is also a very sensitive assay, which can detect as few as 12 cells per well. This was adapted for determining microbial growth (Elavarasan *et al*., 2013; Khalifa *et al*., 2013; Lall *et al*., 2013; Sarker *et al*., 2007).

3.2. Materials and Methods

3.2.1. Materials

Chemicals were all of analytical grade and purchased from Merck (Pty) Ltd and Sigma-Aldrich (Johannesburg, SA). Nutrient agar, nutrient broth, anaerocult jars and anaerocult A strips were purchased from Merck (Pty) Ltd. Microbial strain of *P. acnes* (ATCC 11287) was purchased from Anatech Analytical Technology (Johannesburg, SA). PrestoBlue was purchased from Life Technologies (Johannesburg, SA).

3.2.2. Methods

3.2.2.1 Antimicrobial assay

Pure cultures of *Propionibacterium acnes* (ATCC 11827) were maintained on sterile nutrient agar (NA) plates and subcultured 72h before use. Subcultures (72h) were then inoculated in nutrient broth (NB) for the determination of the minimum inhibitory concentration (MIC). The antimicrobial activity of the 20 selected species was performed according to the methods described by Eloff (1998), with minor alterations. Different plant extracts were dissolved in 10% DMSO to a concentration of 2mg/ml. In sterile 96-well micro-titre plates 100µl of each extract was serially diluted 2-fold (in triplicate). *P. acnes* subcultures were inoculated in sterile NB and prepared to a density of 1.5 x 10^8 colony forming units (CFU) per ml (CFU/ml) corresponding with the 0.5 McFarland Standard. Inoculated NB (100µl) was added to the plates. Tetracycline (0.2mg/ml) and 10% DMSO were used as positive and negative control agents, respectively. After 72h of incubation at 37°C in anaerobic jars (containing anaerocult A) 0.2mg/ml INT (40µl) and PrestoBlue (20µl) were added as indicators of

bacterial growth. The MIC was assessed 2h after the addition of the growth indicators, as the lowest concentration that inhibited bacterial growth. The recorded MICs were compared when using PB and INT as growth indicators. Each growth indicator was investigated using three independent experiments.

3.2.2.2. Synergistic assay

The antimicrobial potential of extract combinations was tested after determination of the extract MIC alone. The synergistic assay was performed according to a combination of methods (checkerboard, variable ratio and micro-dilution), with some modifications (de Rapper *et al*., 2012; Eloff, 1998; Orhan *et al*., 2005).

To determine the combined antimicrobial interactions, varying concentrations of two test extracts were mixed; a selection of *Helichrysum odoratissimum* (2mg/ml) and *Helichrysum kraussii* (2mg/ml) extracts were combined in nine ratios (9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9). The extracts of *Clausena anisata* and *Rapanea melanophloeos* were also combined and tested using the same variable ratio method.

The synergistic activity of two extracts was tested because they belonged to the same genus, *Helichrysum odoratissimum* (Plant A) and *Helichrysum kraussii* (Plant B) and another two extracts were selected because they showed similar MIC, *Clausena anisata* (Plant A) and *Rapanea melanophloeos* (Plant B).

In a sterile 96 well micro-titre plate 100µl of Plant A (2mg/ml) was then added in a ratio from 9:1 (90µl Plant A: 10µl Plant B) to 1:9 (10µl Plant A: 90µl Plant B). The extract combination ratios were then serially diluted 2-fold (in triplicate).The *P. acnes* subcultures were inoculated in sterile NB and prepared to a density of 1.5 x 10^8 colony forming units (CFU) per ml (CFU/ml) corresponding with the 0.5 McFarland Standard. Inoculated NB (100µl) was added to the plates. Tetracycline (0.2mg/ml) and 10% DMSO were used as positive and negative control agents, respectively. After 72h of incubation at 37°C in anaerobic jars (containing anaerocult A) 20µl of PB was used as the bacterial growth indicator. The MIC was assessed 2h after the addition of PB, as the lowest concentration that inhibited bacterial growth.

The fractional inhibitory concentration (FIC) is used to determine synergy where the sum of each FIC gives the Fractional Inhibitory Index (Σ FIC) which is calculated with the following formula:

$$\sum \text{FIC} = \frac{\text{MIC (Plant A + Plant B)}}{\text{MIC (Plant A)}} + \frac{\text{MIC (Plant A + Plant B)}}{\text{MIC (Plant B)}}$$

The ∑ FIC can be interpreted in a number of ways. If the ∑ FIC is: ≤ 0.50 the combination is synergistic; > 0.5 – 1.00, the combination is additive; > 1.00 - ≤ 4.00, the combination is non-interactive (indifferent); or > 4.00, the combination is antagonistic.

3.3. Results and Discussion

To determine the antimicrobial activity of 20 southern African plant extracts, two different growth indicators were used. The MICs were initially determined using INT and were recorded (Table 3.1).

Table 3.1: MIC (µg/ml) of the selected species against *P. acnes* determined using INT

[a] Plant extract	[b] MIC (µg/ml) against *P. acnes*
1. *Acacia karroo* Hayne	500
2. *Buddleja saligna* (Willd.)	500
3. *Buddleja salviifolia* (L.) Lam.	500
4. *Cheilanthes viridis* (Forssk.) Swartz	500
5. *Clausena anisata* (Willd.) Hook.f. ex Benth	31.25
6. *Clematis branchiata* Thunb.	500
7. *Euclea undulata* Thunb. Var. myrtina	500
8. *Faurea saligna* Harv.	500
9. *Gunnera perpensa* L.	500
10. *Gymnosporia buxifolia* (L.) Szyszyl	500
11. *Helichrysum aureonitens* Sch. Bip.	500

12. *Helichrysum kraussii* Sch. Bip.	62.50
13. *Helichrysum odoratissimum* (L.) Sweet	< 3.91
14. *Helichrysum splendidum* (Thunb.) Less	500
15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	500
16. *Leucas martinicensis* (Jacq.) R. Br.	500
17. *Rapanea melanophloeos* (L.) Mez	15.63
18. *Scadoxus puniceus* (L.) Friis & Nordal	500
19. *Tabarnaemontana elegans* Stapf.	500
20. *Urtica dioica* (L.)	500
Tetracycline	1.56

[a] Plant extract

[b] Minimum Inhibitory Concentration (MIC) (µg/ml) against *P. acnes*

The MIC values for the tested plant extracts were then determined visually and recorded using INT. The MIC is defined as the lowest concentration of plant extract that is required to inhibit or prevent the growth of viable bacterial cells. Of the twenty plant extracts that were tested, the lowest MIC was reported for *Helichrysum odoratissimum* lower than 3.91µg/ml (Fig. 3.3). *Clausena anisata* and *Rapanea melanophloeos* were also considered active with MICs at 31.25µg/ml and 15.63µg/ml, respectively (Fig. 3.3). *Helichrysum kraussii* was considered moderately active with an MIC at 62.50µg/ml (Fig. 3.3). The other 16 extracts therefore showed activity against *P. acnes* with MICs reported at 500.00µg/ml or higher than 500.00µg/ml. Antimicrobial activity was rated using the system proposed by Holetz *et al* (2002). The tetrazolium salt was a relatively good indicator of growth for *Clausena anisata*, *Rapanea melanophloeos* and *Helichrysum kraussii*. The presence of the reduced INT-formazan indicated that the bacterial cells were unaffected after incubation with plant extracts (Fig. 3.3). These are the concentrations of plant extracts that were unsuccessful in the prevention of bacterial growth. This was an indication that the electron transport system (oxidoreductase enzymes) in the test organism was still functional, indicating viable, metabolically active microbial cells (Dufour & Colon, 1992). The use of INT for the determination of the MIC worked well for *Clausena anisata*, *Rapanea melanophloeos* and *Helichrysum kraussii*.

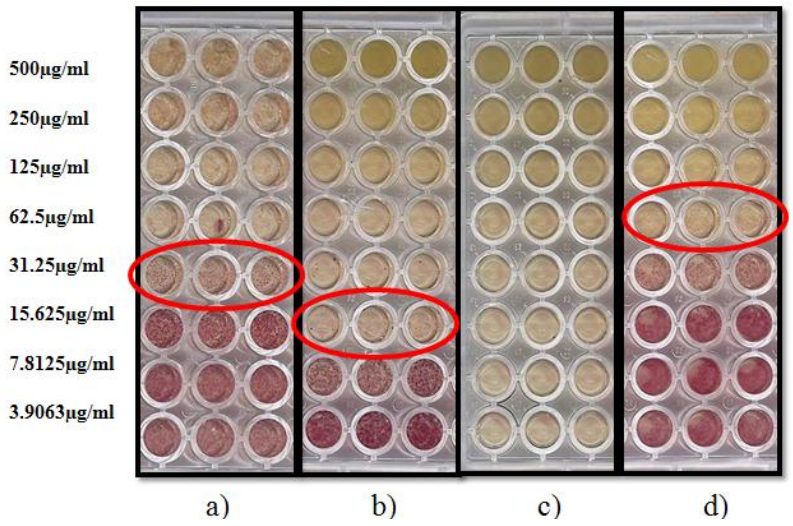

Figure 3.3: MIC for a) *Clausena anisata* b) *Rapanea melanophloeos* c) *Helichrysum odoratissimum* d) *Helichrysum kraussii* using INT

However, for *Helichrysum odoratissimum* it was difficult to visually predict the MIC because even at the lowest concentration tested (3.91µg/ml) the absence of the red INT-formazan dye was clearly observed. The results would suggest that the MIC could be lower than the lowest concentration tested, however when looking closely there was a slight pink cloud observed at the lowest concentration. The results showed that there may be some problems with using INT as a growth indicator. An additional problem is that the formation of the reduced INT-formazan dye is more prominent in wells where bacterial cells became coagulated or formed cell masses. The determination of the MIC was very difficult, especially if there was only slight formation of the reduced INT-formazan dye, as opposed to the prominent red colour.

The results can be explained by the ability of the *P. acnes* microorganism to form biofilms as they grow within the pilosebaceous unit of the skin. The genome sequence of the *P. acnes* showed three gene clusters, that code for polysaccharide biosynthesis and are specific for the formation of the extracellular biofilm matrix. A previous study has reported a number of strains of *P. acnes* and *P. acnes* isolates, which can in fact form biofilms *in vitro*. This could also explain the increased antibiotic resistance and decreased antimicrobial efficacy of certain compounds. *Helichrysum odoratissimum* may have inhibitory effects against the formation of cell aggregation and thus an increased activity (Coenye *et al.*, 2007).

Tetracycline is an antibiotic that is used as a treatment for acne vulgaris. It is known for its antimicrobial activity on follicular *P. acnes* (Williams *et al.*, 2012). This was included in the

study as a positive control. Tetracycline showed good *in vitro* activity against *P. acnes* with an MIC reported lower than 0.78µg/ml (Fig. 3.4). However, the same phenomenon that was observed with *Helichrysum odoratissimum* was apparent. A bacterial control was included in the assay to confirm that the bacterial cells used were viable and that any inhibitory effects were due to the activity of the plant. The formation of the reduced INT-formazan dye showed that the bacterial inoculum consisted of viable, metabolically active *P. acnes* cells (Fig. 3.4). A solvent control consisting of DMSO was added at a final concentration of 5% (Fig. 3.4). This was added to check that the solvent used to dissolve the plant extracts had no antimicrobial activity of its own, ensuring that any observed activity could be attributed to the plant extract.

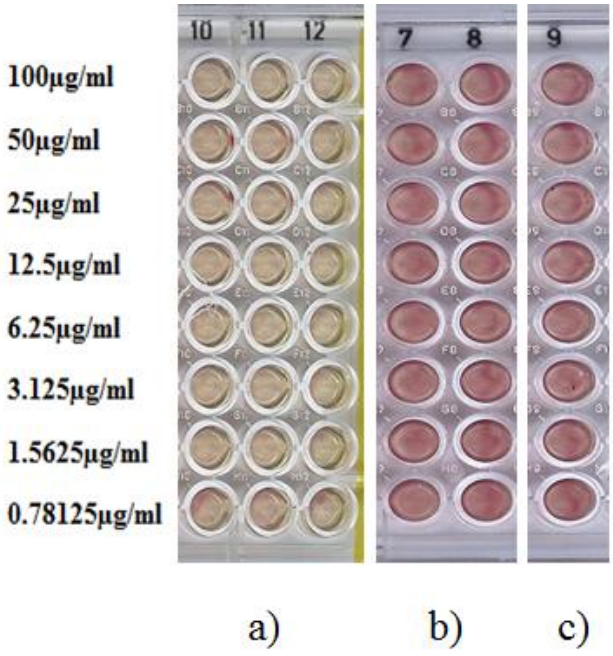

Figure 3.4: Antimicrobial control plate for a) Tetracycline (+) b) Bacterial control (-) c) Solvent control (DMSO)

Due to the problems encountered with the determination of the MIC of some of the tested extracts as well as the positive control, it was decided that PB would be used as an alternative to investigate the growth of *P. acnes* treated with plant extract.

Table 3.2: MIC (µg/ml) of the selected species against *P. acnes* determined using PB

[a] Plant extract	[b] MIC (µg/ml) against *P. acnes*
1. *Acacia karroo* Hayne	500
2. *Buddleja saligna* (Willd.)	500
3. *Buddleja salviifolia* (L.) Lam.	500
4. *Cheilanthes viridis* (Forssk.) Swartz	500
5. *Clausena anisata* (Willd.) Hook.f. ex Benth	31.25
6. *Clematis branchiata* Thunb.	500
7. *Euclea undulata* Thunb. Var. myrtina	500
8. *Faurea saligna* Harv.	500
9. *Gunnera perpensa* L.	500
10. *Gymnosporia buxifolia* (L.) Szyszyl	500
11. *Helichrysum aureonitens* Sch. Bip.	500
12. *Helichrysum kraussii* Sch. Bip.	125
13. *Helichrysum odoratissimum* (L.) Sweet	7.81
14. *Helichrysum splendidum* (Thunb.) Less	500
15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	500
16. *Leucas martinicensis* (Jacq.) R. Br.	500
17. *Rapanea melanophloeos* (L.) Mez	15.63
18. *Scadoxus puniceus* (L.) Friis & Nordal	500
19. *Tabarnaemontana elegans* Stapf.	500
20. *Urtica dioica* (L.)	500
Tetracycline	1.563

[a] Plant extract

[b] Minimum Inhibitory Concentration (MIC) (µg/ml) against *P. acnes*

The MIC values for the tested plant extracts were then determined visually and recorded using PB. Again *Helichrysum odoratissimum* showed the lowest MIC at 7.81µg/ml (Table

3.2). *Clausena anisata* and *Rapanea melanophloeos* were again active and had MICs at 31.25µg/ml and 15.63µg/ml, respectively (Table 3.2). With INT their MICs were at 31.25µg/ml and 15.63µg/ml, respectively, showing that the resulting MICs were similar for *Clausena anisata* and *Rapanea melanophloeos* when using PB. *Helichrysum kraussii* with an MIC at 125.00µg/ml (Table 3.2) showed an MIC one concentration higher than that observed with INT. The other 16 extracts therefore showed activity against *P. acnes* with MICs reported at 500.00µg/ml or higher than 500.00µg/ml.

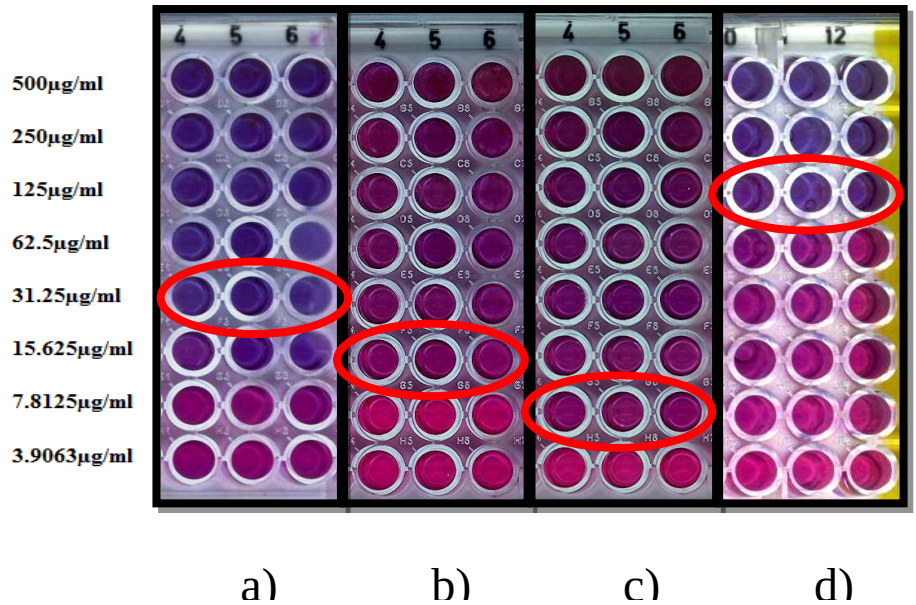

Figure 3.5: The MIC of a) *Clausena anisata* b) *Rapanea melanophloeos* c) *Helichrysum odoratissimum* d) *Helichrysum kraussii* using PB

The main advantage of using PB, however, was that the recorded MIC for *Helichrysum odoratissimum* was clearly observed and confirmed that the slight pink cloudy formation observed on the INT plate, could be defined as actively growing bacterial cells. This is attributed to the ability of PB to produce a colour change in the entire well, even in the presence of low numbers of active cells (Lall *et al*., 2013). The bluish purple colour was indicative of bacterial cells that were inhibited (Fig. 3.5). The appearance of a pink colour however, was indicative of the bacterial cells that were unaffected by the antimicrobial agent and were metabolically active (Fig. 3.5). The reduction of the blue resazurin dye to the pink

resorufin dye was through the activity of oxidoreductase enzymes in the cells of viable *P. acnes* cultures (Sarker *et al*., 2007).

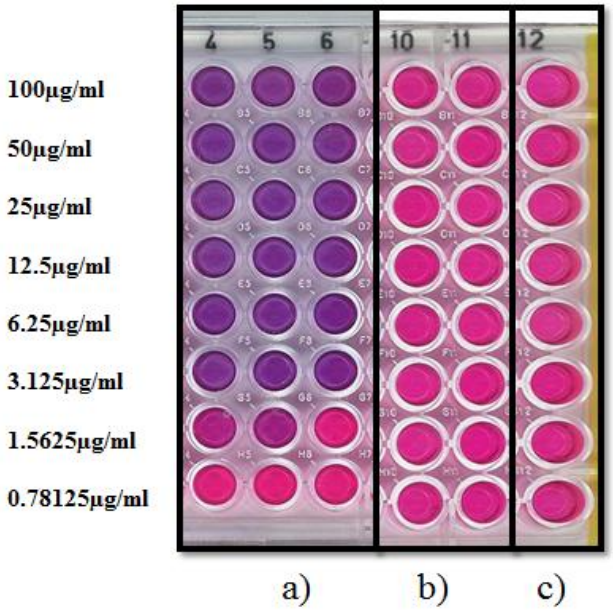

Figure 3.6: Antimicrobial control plate with a) Tetracycline (+) b) Bacterial control (-) c) Solvent control (DMSO)

PrestoBlue was also able to elucidate the MIC of tetracycline, which was not clear when using INT. The MIC of the positive control was determined to be 3.13µg/ml (Fig. 3.6). In another study it was reported that 16 strains of *P. acnes* were susceptible at a concentration of 1.60µg/ml. The results of this study were comparable with published data from Martin *et al* (1972).

Several species have been reported by other researchers to have high antimicrobial activity against *P. acnes*. The 70% ethanol extract of *Orixa japonica* was tested against *Propionibacterium acnes* and *Staphylococcus epidermidis.* This plant is traditionally used for swelling, one of the major symptoms of acne vulgaris. The MIC against each microorganism was 250.00µg/ml and 125µg/ml respectively. This compares well with the results obtained for *Helichrysum kraussii*. In the same study, *Angelica anomala* which is used for itching of the skin, exhibited an MIC at 15.63µg/ml which was similar to the findings with regard to

antimicrobial activity, reported for *Clausena anisata* and *Rapanea melanophloeos* in the current study (Kim *et al*., 2008).

Antimicrobial screening assays on *Helichrysum* spp. showed that acetone extracts of *Helichrysum odoratissimum* showed an MIC of 10µg/ml. It has been labelled a broad-spectrum Gram-positive antimicrobial which would explain the high levels of inhibition against the *P. acnes* microorganism *in vitro* at 7.81µg/ml (Van Vuuren, 2008).

The synergistic antimicrobial potential of two extracts together was investigated to determine whether combinations of plant extracts were more effective. The extracts chosen for the synergistic assay were based on their individual MIC determined using PB.

The MICs of *Helichrysum* spp. alone were 7.81µg/ml and 125.00µg/ml for *Helichrysum odoratissimum* and *Helcihrysum krausii*, respectively. For the synergistic assay the Σ FIC was determined. The ∑ FIC for the combination in well H2 was calculated to be 0.42. This combination was considered synergistic. The combination of 3.13µg/ml of *Helichrysum odoratissimum* and *Helichrysum kraussii* at 0.78µg/ml showed better antimicrobial activity than either of the plant extracts acting alone against *P.* acnes. However, the presence of the pink resorufin in well B6 may suggest some contamination. The pattern suggests that the synergistic combinations may continue throughout concentrations lower than 3.91µg/ml within the ratio range of 8:2, 7:3, 6:4, 5:5, and 4:6 (Fig. 3.7).

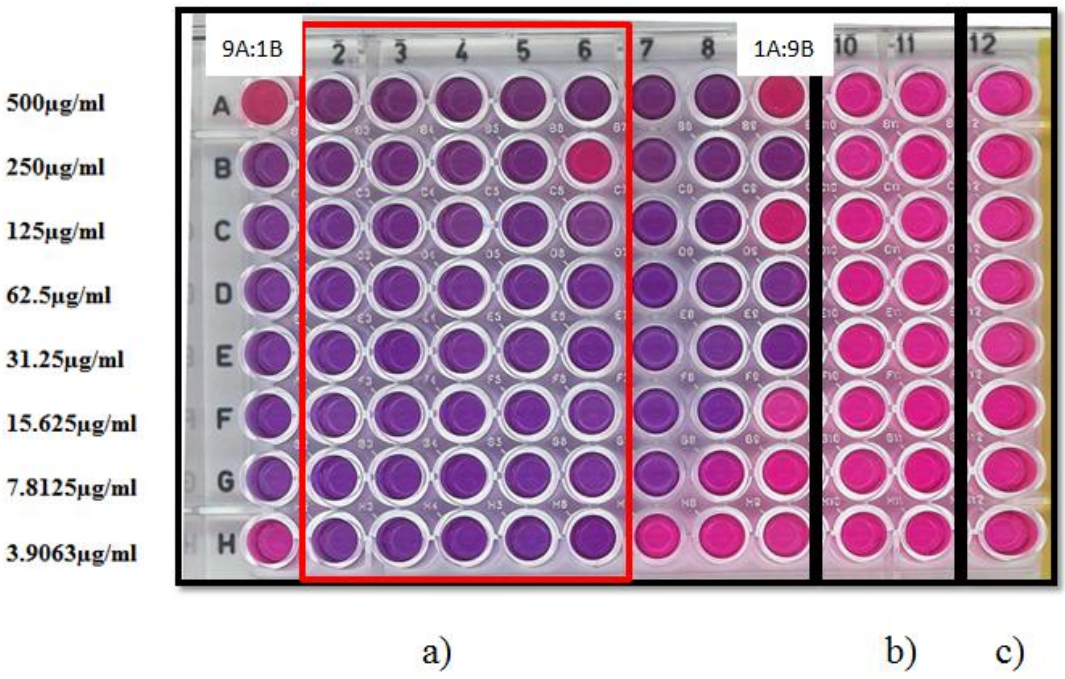

Figure 3.7: MIC of a) *H. odoratissimum* and *H. kraussii* ratios b) Bacterial control (-) c) Solvent control (DMSO) using PB

The MICs for *Clausena anisata* (Plant A) and *Rapanea* melanophloeos (Plant B) alone were at 31.25µg/ml and 15.625µg/ml, respectively.

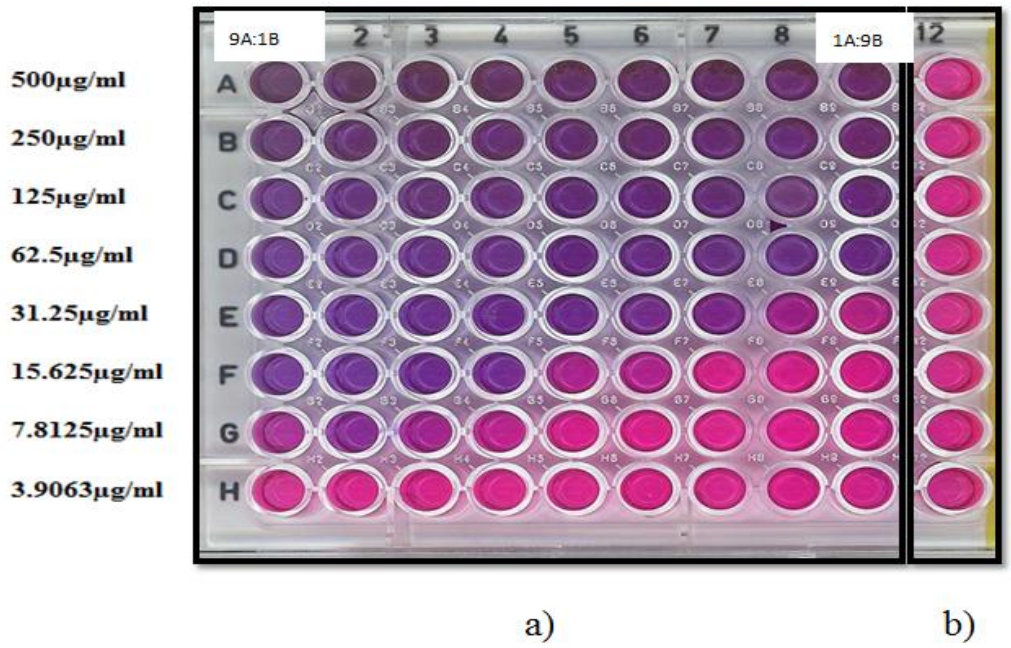

Figure 3.8: MIC of a) *C. anisata* and *R. melanophloeos* ratios b) Solvent control (DMSO)

The only significant combination that showed a potential synergistic effect was G2 as the MIC was recorded at 7.81µg/ml, which is lower than that of *Rapanea melanophloeos* and *Clausena anisata* alone. The ∑ FIC for the combination was calculated as 0.75. This means that the combination is additive. For this combination, the effect is equal to the sum of the effects of the two drugs alone.

Not many synergistic assays have been reported for the use of combinations of species from the same genus. It is more common for plant extracts to be combined with conventional antimicrobial agents. Tetracycline in combination with *Purica granatum*, *Thymus vulgaris* and *Commiphora molmol* showed synergistic activity against *Staphylococcus aureus*, a morphologically similar microorganism to *P. acnes*. Another study has reported the synergistic antimicrobial combination of the essential oils of *Boswellia papyrifera* and *Commiphora guidotti* against *Staphylococcus aureus* which showed a ∑ FIC of 0.5 (de Rapper *et al*., 2012; Hussin & El-Sayed, 2011).

3.4. Conclusion

Helichrysum odoratissimum was the most active antibacterial with a MIC at 7.81µg/ml. This was successfully determined using PB, whereas with INT determination of the MIC was difficult. Other active extracts included *Clausena anisata*, *Rapanea melanophloeos and*

Helichrysum kraussii with MICs at 31.25µg/ml, 15.625µg/ml and 125µg/ml, respectively. The other 16 plant extracts showed little activity at 500µg/ml or no activity (above 500µg/ml), the highest concentration which was tested. The determination of the MICs for the 20 southern African plant species was performed using a tatrazolium salt (INT) and resazurin-based (PB) growth indicators. When comparing the two indicators, there was no major difference between the MIC obtained for each extract using INT and PB. PB did however, have some advantages over INT. The coloration of the entire sample well, made MIC determination easy, when visually examining the test plates. INT was reduced more prominently at the bottom of the test plate and more so when aggregated *P. acnes* cells were observed. Although the incubation time after addition of the growth indicators was the same, the development of reduced products was much faster for PB. The results were reproducible and compared well with published data on the antimicrobial activity of plant extracts against *P. acnes*. The plant extracts with the best antimicrobial activity were selected for the synergistic assay and the evaluation of the cytotoxicity. The synergistic antimicrobial assay showed the combination of *Helichrysum odoratissimum* and *Helichrysum kraussii* in a variable ratio of 8:2 was synergistic against *P. acnes*. None of the combinations of *Clausena anisata* and *Rapanea melanophloeos* were found to have any synergistic activity. The broth micro-dilution assay was found to be a rapid method for determining the MIC for 20 extracts against *P. acnes* especially when used in combination with PB as the growth indicator.

3.5. References

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Chapter 4

Antioxidant Activity of Selected Plant Extracts

4. Introduction

Reactive oxygen species (ROS) are classified as highly reactive molecules which result from the metabolic breakdown of oxygen. They can exist as free radicals in the form of the superoxide anions (O_2^-) and hydroxide anions ($^{\bullet}OH$) and non-free radicals in the form of hydrogen peroxide (H_2O_2), organic peroxide (ROOH), ozone (O_3) and singlet oxygen ($^1O_2$) (Kumar *et al*., 2013; Temraz & El-Tantawy, 2008).

Free radicals cause oxidative stress and damage which is associated with a variety of human diseases. ROS result in detrimental damage to cells and tissues. They have been implicated as one of the major causes of diabetes and some degenerative disorders such as cardiovascular disease, Alzheimer's, Parkinson's, Down syndrome. They also play a role in viral infection, inflammation and ulcer formation (Kumar *et al*., 2013; Temraz & El-Tantawy, 2008; Wei *et al*., 2014). These free radicals are combated by antioxidants.

Antioxidant molecules are defined as those substances which are able to slow down or completely prevent the effects of oxidation and thus of oxidative damage. This protection is imperative for prevention of lipid peroxidation and DNA strand degradation. Synthetic antioxidants are used in foods for their preservation and for extending product shelf-life; however, some of these antioxidants are also the cause of decreased product quality, nutritional loss and changes in flavour profiles. Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been identified as potential health risks when used as food and cosmetic additives as they exhibit toxic and mutagenic effects. It is for this reason that there has been an increase in research for natural antioxidants that are derived from medicinal and nutritional plant sources (Kumar *et al*., 2013; Wei *et* al., 2014). The effects of ROS are combated by the body's natural defences and antioxidant compounds obtained exogenously from the diet.

The human body consists of naturally occurring antioxidant defence mechanisms which are generally in the form of enzymes. Non-enzyme endogenous defence mechanisms also include what are known as low molecular weight antioxidants (Portugal *et al*., 2007; Temraz & El-Tantawy, 2008).

Antioxidant enzymes are important defence mechanisms against ROS as they are responsible for maintaining a balance between oxidants and antioxidants in the body. There are various

types of enzymes which are responsible for protection against specific ROS (Temraz & El-Tantawy, 2008).

The superoxide dismutase enzyme is responsible for defence against the superoxide anion, as they are effective at binding single charge oxygen anions. There are three types of these enzymes within the human body which include the cytosolic, extracellular and the mitochondrial analogs (Matés *et al*., 1999). The enzyme catalyses the following reaction:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (Jacob, 1995)}$$

The catalase enzyme is responsible for the effective removal of hydrogen peroxide. The resulting products of the reaction are molecular oxygen and water (Matés *et al*., 1999). The enzyme is responsible for the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (Jacob, 1995)}$$

The glutathione peroxidise enzyme is responsible for protection against hydroperoxidases using reduced glutathione (GSH) which is ultimately oxidised (GSSG) and recycled. It is considered to be one of the most important protective mechanisms in the human body (Matés *et al*., 1999). The enzyme catalyses the following reaction:

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O \text{ (Jacob, 1995)}$$

Antioxidants that are present in foods are not only important in preserving the food itself but also provide essential antioxidants to the consumer. It has been said that high intake of fruits and vegetables help to decrease the oxidative stress levels. Plant-based food sources are high in antioxidants such as carotenoids, phenolic compounds, flavonoids, proanthocyanidins, stilbenes, coumarins, tocopherols (Vitamin E), tocotrienols, uric acid and ascorbic acid (Vitamin C). These natural antioxidants can also be found in microorganisms, fungi and animal tissues. These are characterised as the low molecular weight antioxidants. They are a larger group than the enzymatic systems and have several advantages. They have the ability to infiltrate and target cell specific regions where oxidative stress is high. They also have various antioxidant capabilities, targeting more than one ROS in some cases. The mechanisms upon which they act are in direct or indirect association with ROS. The direct method is generally through the ability to donate an electron to a single electron radical (Charles, 2013; Portugal *et al*., 2007).

The skin makes up a large part of the human body. It is the largest organ of the human body and is a site of high levels of metabolic reactions. It acts as a physical and biological barrier for protection to internal organs. It is also constantly exposed to environmental hazards such as air pollution, UV irradiation, microbial pathogens, viruses and xenobiotics which act as exogenous ROS. These ROS however, are also formed during normal cell processing such as metabolism and immune responses where they are referred to as endogenous. The skin is also a producer of ROS by aerobic respiration that is observed in keratinocyte cells. The hydrolytic activity of *P. acnes* on increased sebum levels is known to cause release of free fatty acids (FFA). These FFA act as triggers for cells of the immune response causing the disruption of follicular walls of the pilosebaceous unit, culminating in oxidative damage through the release of free radicals. It was decided therefore, that antioxidant activity of the 20 southern African plant extracts would be investigated (Portugal *et al*., 2007, Sharma *et al*., 2013).

There are a number of methods that are used to determine the antioxidant activity of substances. One of the most popular methods however, involves the ability of a substance to scavenge free radicals. This ability is generally observed through the disappearance of free radicals such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) or the 2, 2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) (ABTS). The DPPH radical is generally a better choice as it is considered to be more stable than ABTS.

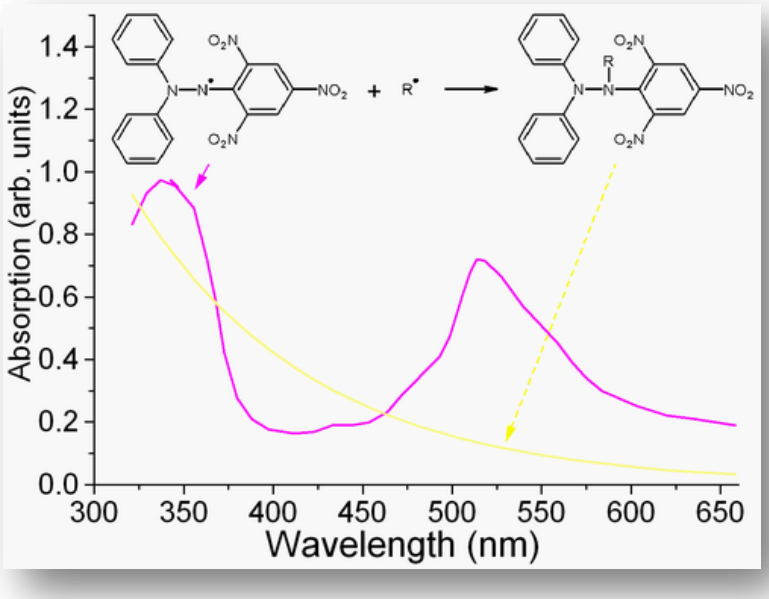

Figure 4.1: The DPPH radical interacts with an antioxidant

DPPH shows a maximum absorbance at 515nm (Fig. 4.1). In the presence of antioxidants, which can successfully donate an electron, a stable compound is formed which is characterised by a colour change from purple to yellow (du Toit *et al*., 2001, Mishra *et al*., 2012).

4.1. Materials and Methods

4.1.1. Materials

DPPH and ascorbic acid (Vitamin C) were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

4.1.2 Methods

The DPPH antioxidant assay was performed using the methods of du Toit *et al*. (2001) with minor modifications. For each extract a dilution series was prepared in a 96-well ELISA plate by adding distilled water as the dilution medium. Final concentrations of the samples ranged from 3.91-500µg/ml. Each concentration was tested in triplicate. Plant extracts which showed potent antioxidant activity during the initial screening were diluted further and tested at final concentrations from 0.78-100.00µg/ml. Vitamin C (1mg/ml) was used as the positive control and ethanol was used as the solvent control. Colour controls for each extract were also used. The DPPH (0.04M) was then added to each test plate. After incubation for 30 minutes at room temperature, the radical scavenging capacity of the extracts was determined using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at 515nm and KC Junior software (Highland Park, Winooski, Vermont, USA). The radical scavenging activity was measured as the amount of antioxidant necessary to decrease the initial absorbance of DPPH by 50%. This is known as the 50% inhibitory concentration (IC_{50}). The IC_{50} for each extract was determined using GraphPad Prism Version 4.0 (San Diego, California, USA). The results were then expressed as Vitamin C equivalents.

4.2. Results and Discussion

Most of the plants seemed to show good antioxidant activity. *Faurea saligna* and *Gunnera perpensa* were the most active radical scavenging extracts, with IC_{50} at 1.17 ± 0.04µg/ml and 1.10 ± 0.01µg/ml, respectively, which were comparable to that of the positive control Vitamin C with an IC_{50} at 1.98 ± 0.01µg/ml (Fig. 4.2). *Faurea saligna* was previously

investigated for the DPPH scavenging activity and was found to have an IC_{50} of 0.02 ± 0.01µg/ml (Masevhe, 2013). Previous investigation of *Gunnera perpensa*, however, showed an IC_{50} of 16µg/ml for DPPH radical scavenging activity (Simelane *et al*., 2010). The least active extracts were *Tabernaemontana elegans* and *Urtica dioica* with IC_{50} at 102.50µg/ml and 109.10µg/ml, respectively. *Urtica dioica* has previously been investigated for its antioxidant potential against DPPH where 60µg/ml of a water extract inhibited 37% of the DPPH free radical (Gülçina *et al*., 2004).

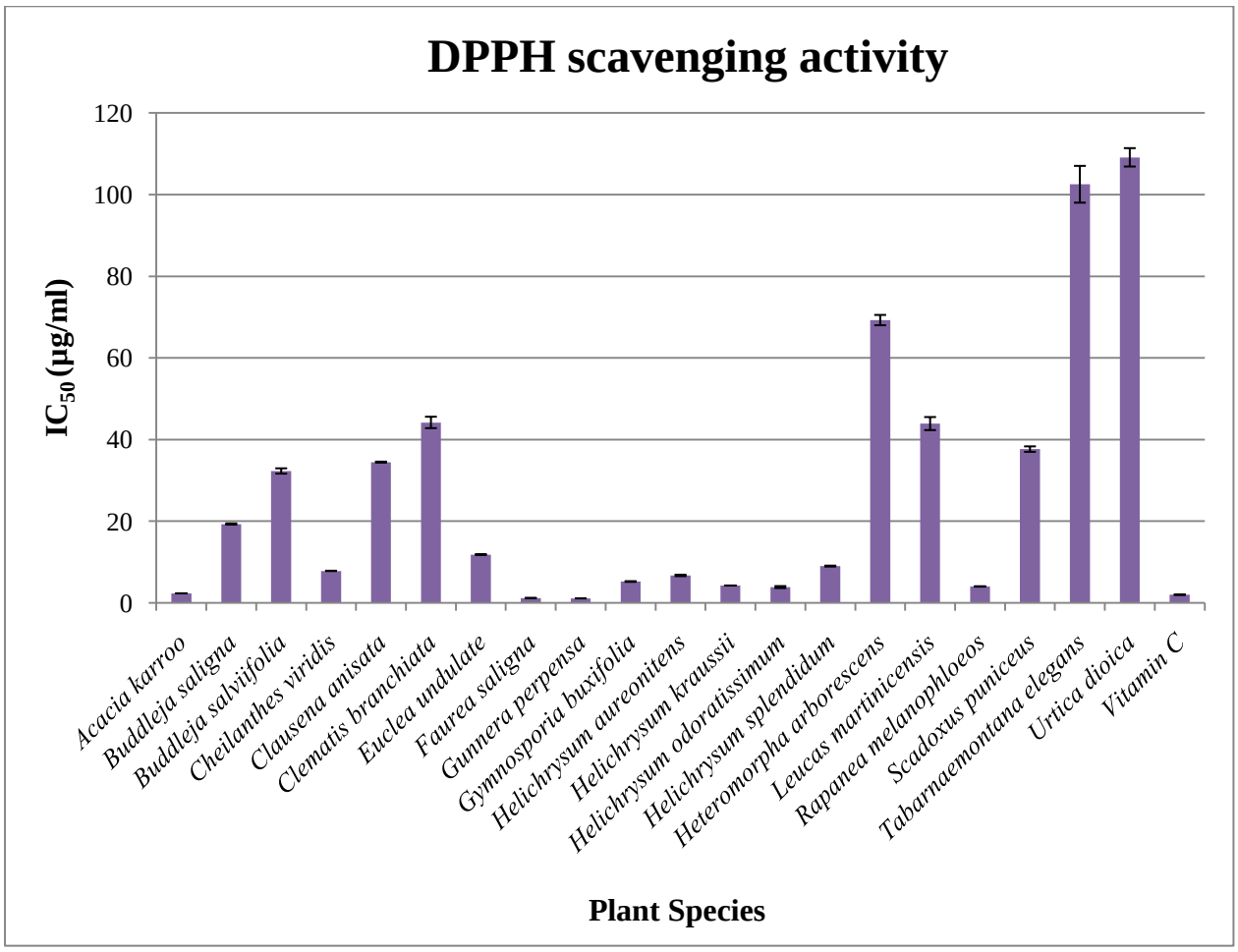

Figure 4.2: The 50% inhibitory concentration (IC_{50}) (µg/ml) for extracts on DPPH scavenging activity

Of the four plant extracts that were active against *P. acnes*, *H. odoratissimum* had the highest radical scavenging activity with an IC_{50} at 3.86 ± 0.24µg/ml. *R. melanophloeos* and *H. kraussii* also showed good DPPH scavenging activity with IC_{50} at 4.01 ± 0.05µg/ml and 4.24 ± 0.01µg/ml, respectively. *C. anisata* was the least active radical scavenger with an IC_{50} at 34.46 ± 0.12µg/ml.

Results for *H. odoratissimum* and *H. kraussii* were difficult to compare with previously recorded data as the extracts were prepared from the flowers or the DPPH scavenging activity was previously tested for the essential oils of the plant rather than the crude extracts (Legoalea *et al*., 2013). The radical scavenging activity was a measurable colorimetric reduction in the purple colour of the DPPH radical in the presence of an antioxidant compound. This is clearly indicated in the test plate (Fig. 4.3).

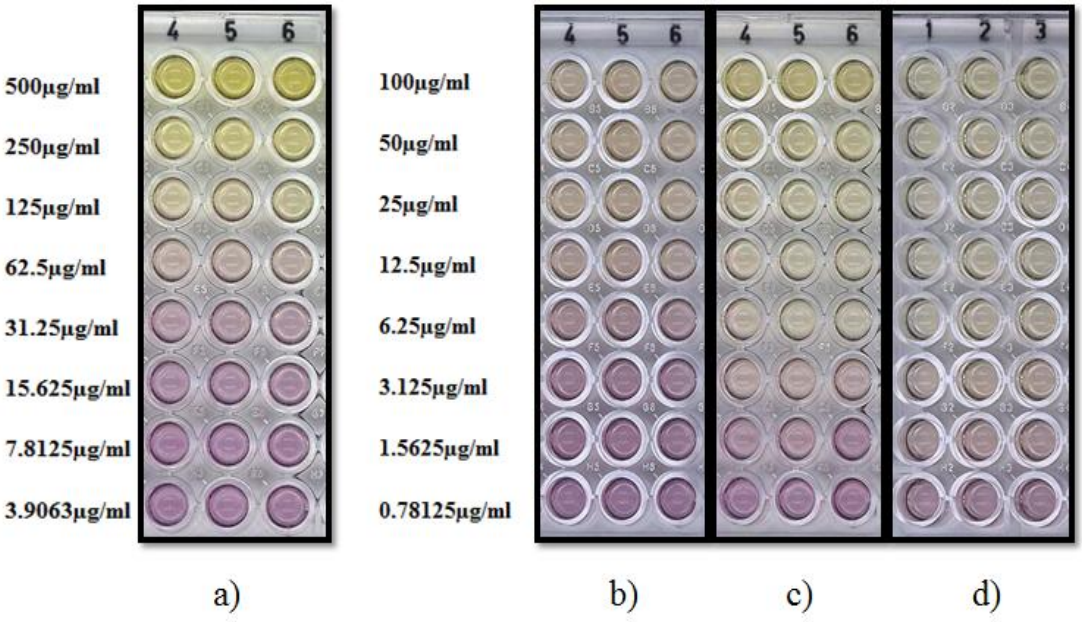

Figure 4.3: The test plate for a) *Clausena anisata* b) *Rapanea melanophloeos* c) *Helichrysum odoratissimum* d) *Helichrysum kraussii*

The results obtained for *R. melanophloeos* were much better than those in a study which looked at the DPPH scavenging ability of the methanol bark extract of *R. melanophloeos*. The IC_{50} was 3.31mg/ml whereas the ethanol leaf extract in the present study was 4.01µg/ml. This suggests that the ethanol extract of the stems and leaves of *R. melanophloeos* which was tested in the current study was more successful at extracting antioxidant compounds when compared with the methanol bark extract (Mosa *et al*., 2011).

The IC_{50} obtained for *C. anisata* was compared with a 50% methanol extract that was made using the same plant parts (leaves and twigs). The IC_{50} of the 50% methanol extract was tested for extracts made from stored plant material and fresh plant material. The extracts

made from stored and fresh plant material exhibited an IC_{50} at 33.20 ± 3.89µg/ml and 26.80 ± 2.06µg/ml, respectively. There was no significant difference between the activity of the fresh and stored plant material on DPPH scavenging activity. The ethanol extract tested in the current study was prepared from fresh material and showed similar results to that of the extracts prepared from the stored plant material. The only difference was 50% methanol was used as the extraction solvent as opposed to ethanol used in the current study (Amoo *et al.*, 2012).

All extracts were initially tested from 500µg/ml (Fig 4.3a). The potent antioxidant activity of *H. odoratissimum*, *H. kraussii* and *R. melanophloeos* were determined using an initial concentration of 100µg/ml (Fig. 4.3b, c, d).

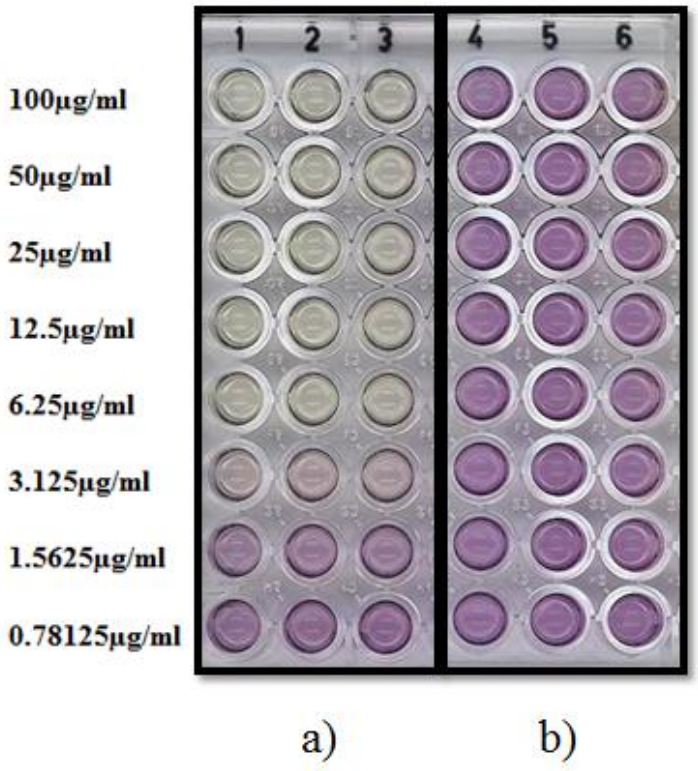

Figure 4.4: Antioxidant control plate with a) Vitamin C b) Blank/Solvent control

The natural, dietary antioxidant Vitamin C was used as the positive control for the assay. Vitamin C exhibited an IC_{50} of 1.98 ± 0.01µg/ml (Fig. 4.4a). It is considered as the most important antioxidant to be found within the extracellular fluids and plays an influential role within cells where it is involved in antioxidant processes (du Toit *et al.*, 2001). It is known for its ability to detoxify free radicals. The IC_{50} value obtained compares well with other

published data which reported an IC_{50} at 2.1 ± 0.05µg/ml for Vitamin C (Amoo *et al*., 2012). Ethanol was used as a solvent control for the assay. There was no significant reduction in the initial absorbance value of the DPPH radical by ethanol (Fig 4.4b). This control was used to ensure that the recorded antioxidant activity was attributed to the plant extracts and not due to the solvent vehicle in which the extracts were dissolved.

A. karroo, C. brachiata, C. viridis, E. *undulata, G. buxifolia, T. elegans, H. arborescens, H. aureonitens, H. splendidum* have not been previously investigated for their DPPH radical scavenging activity. *B. saligna* showed similar results when compared with a study performed by Adepapo *et al*., 2009 where the methanol leaf extract showed 93.8% inhibition at a concentration of 100µg/ml. *B. salviifolia* showed 94.2% inhibition of DPPH at 100µg/m (Amoo *et al*., 2012). The ethanolic extract of the entire *L. martinicensis* plant showed antioxidant activity with an inhibition of 81.48% at a concentration of 250µg/ml (Habila *et al*., 2010). *S. puniceus* showed maximum inhibition below 50% at the highest concentration tested (0.125 mg/ml) (Adewusi & Steenkamp, 2011).

The antioxidant activity of the tested plant extracts can be calculated as Vitamin C equivalents (du Toit *et al*., 2001). This parameter is based on the principle that a single capsule contains 200mg of Vitamin C. The Vitamin C equivalents can be calculated as follows:

$$\text{Vitamin C equivalents} = \text{IC50 (Extract)} \times \frac{200\text{mg}}{\text{IC50 (Vitamin C)}}$$

The Vitamin C equivalents were calculated for each of the tested extracts (Table 4.1).

Table 4.1: Antioxidant activity on DPPH and Vitamin C equivalents

[a] Plant extract	[b] IC_{50} (µg/ml)	[c] Vitamin C equivalents (mg)
1. *Acacia karroo* Hayne	2.325 ± 0.01	235
2. *Buddleja saligna* (Willd.)	19.30 ± 0.15	1949
3. *Buddleja salviifolia* (L.) Lam.	32.29 ± 0.65	3262
4. *Cheilanthes viridis* (Forssk.) Swartz	7.83 ± 0.04	791
5. *Clausena anisata* (Willd.) Hook.f.	34.46 ± 0.12	3481

ex Benth		
6. *Clematis branchiata* Thunb.	44.18 ± 1.395	4463
7. *Euclea undulata* Thunb. Var. myrtina	11.82 ± 0.10	1194
8. *Faurea saligna* Harv.	1.17 ± 0.04	118
9. *Gunnera perpensa* L.	1.10 ± 0.01	111
10. *Gymnosporia buxifolia* (L.) Szyszyl	5.22 ± 0.09	527
11. *Helichrysum aureonitens* Sch. Bip.	6.71 ± 0.18	678
12. *Helichrysum kraussii* Sch. Bip.	4.24 ± 0.01	428
13. *Helichrysum odoratissimum* (L.) Sweet	3.86 ± 0.24	390
14. *Helichrysum splendidum* (Thunb.) Less	8.99 ± 0.11	909
15. *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	69.24 ± 1.27	6994
16. *Leucas martinicensis* (Jacq.) R. Br.	43.90 ± 1.60	4434
17. *Rapanea melanophloeos* (L.) Mez	[a] 4.01 ± 0.05	[b] 405
18. *Scadoxus puniceus* (L.) Friis & Nordal	37.65 ± 0.67	3803
19. *Tabarnaemontana elegans* Stapf.	102.5 ± 4.51	10354
20. *Urtica dioica* (L.)	109.10 ± 2.26	11020
Vitamin C	1.98 ± 0.01	-

[a] Plant extract
[b] 50% inhibitory concentration (IC_{50}) (µg/ml) on DPPH radical
[c] Vitamin C equivalents (mg)

The Vitamin C equivalents (Table 4.1) can be interpreted as the amount of plant extract required to be synonymous to one 200mg capsule of Vitamin C in mg. The two extracts which showed the highest DPPH scavenging activity were the only two extracts that could be taken in lower dosages to obtain the same antioxidant effects as a 200mg Vitamin C capsule; the two extracts are *F. saligna* and *G. perpensa* which exhibited 111mg and 118mg Vitamin C equivalents, respectively.

4.3. Conclusion

The use of natural antioxidants is desired over those which are produced synthetically. The DPPH radical scavenging activity of extracts varied, due to differences in the presence or absence of certain phenolic compounds. However, the antioxidant activity of these phenolic compounds also depends on the number of free hydroxyl groups and polarity (Klančnik *et al*., 2009). It is possible that *G. perpensa* and *F. saligna* could contain phenolic compounds that have an increased number of free hydroxyl groups able to donate hydrogen to the DPPH free radical. *H. odoratissimum*, *H. kraussii* and *R. melanophloeos* showed good antioxidant activity when compared with *C. anisata* which could indicate the presence of increased phenolic content. Phenolic compounds and polyphenols are known to be responsible for antimicrobial activity against *P. acnes*, this could explain the antimicrobial activity of these four extracts (Cowan, 1999)

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Chapter 5

Cytotoxicity of Plant Extracts on human monocyte cells (CRL 1593.2)

5. Introduction

The use of medicinal plants for the treatment of skin disorders is common in southern Africa, especially in rural communities. Skin disorders can persist for long periods of time and can spread from one sufferer to another, affecting people with compromised immune systems. Medicinal plants have become popular due to increased demand for cheaper alternatives for commercial brands of medicines and also for their ability to relieve patients of some symptoms associated with skin conditions (Mabona & Van Vuuren, 2013).

Plants that are used for their therapeutic effects are commonly labelled as safe and free of toxicity but it is important to obtain more scientific information regarding their safety and efficacy (Prozesky *et al*., 2001). The reason for this misconception stems from the fact that medicinal plants have long been used to treat disorders based on prior knowledge which has been shared among generations over a number of centuries. Studies have reported the toxicity, mutagenicity and carcinogenesis of many medicinal plants employed as traditional medicines (Fennell *et al*., 2004).

Toxicity of plant extracts can be investigated in a number of ways, including mutagenicity assays, skin irritation tests or investigating their effects on selected cell lines in direct cytotoxicity assays. Having obtained plant extracts with good antimicrobial and antioxidant activity it was decided that the cytotoxic effects of *H. odoratissimum*, *H. kraussii*, *R. melanophloeos* and *C. anisata* would be investigated using an *in vitro* cytotoxicity assay on the human monocytic cell line U937 (ATCC CRL1593.2).

The cytotoxicity assay can be performed using a cell proliferation kit. This is a non-radioactive, spectrophotometric technique which is able to quantify cell proliferation and cell viability. The assay is advantageous when measuring cell proliferation in the presence of cell stimulants such as growth factors, cytokines, mitogens and supplementary nutrients. The assay also allows for the analysis of cytotoxic compounds which is important for pharmacological drug testing. In this study it has been employed to determine the cytotoxic effect of plant extracts on human monocytic type cell line U937 (ATCC 1593.2).

The assay is based on monitoring the cleavage of the tetrazolium salt 2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) which ultimately produces a soluble, intensely red, formazan salt (Fig. 5.1). This reaction can only take place in the presence of viable cells. Viable cells have functional XTT cleavage enzymes such as

succinate-tetrazolium reductase and mitochondrial dehydrogensases which form part of the respiratory functions within the mitochondria, which are only active in metabolically intact cells. In the assay *N*-methylphenazonium methyl sulphate (PMS) is mixed with XTT as an electron coupling reagent. This reagent increases sensitivity of XTT reduction. The reagent is advantageous as cellular XTT reduction reactions occur at the cell surface, which is detected by the addition of PMS acting as a reactive intermediate.

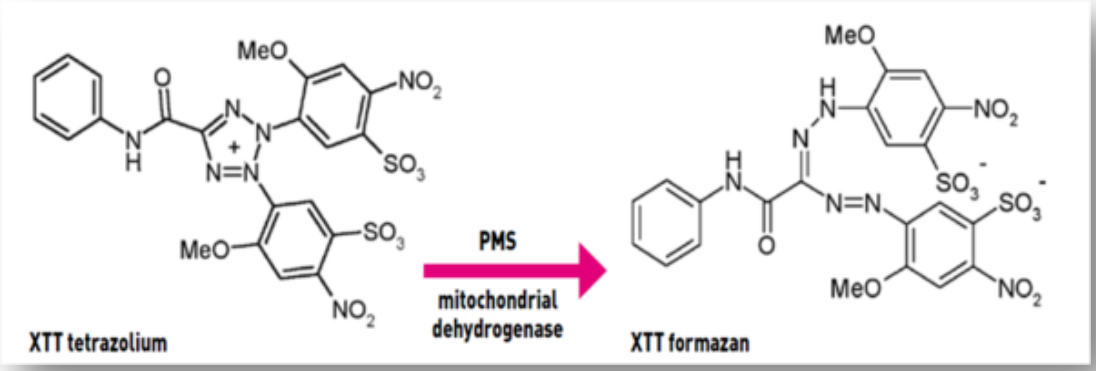

Figure 5.1: Reduction of XTT by dehydrogenase enzymes (AppliChem, 2003)

The U937 cell line is a human cell line that is isolated from the pleural effusion of malignant cells of a patient suffering from histiocytic lymphoma. The cells were first derived by Sundstrom and Nilsson in 1974, from a 37 year old male (ATCC, 2014).

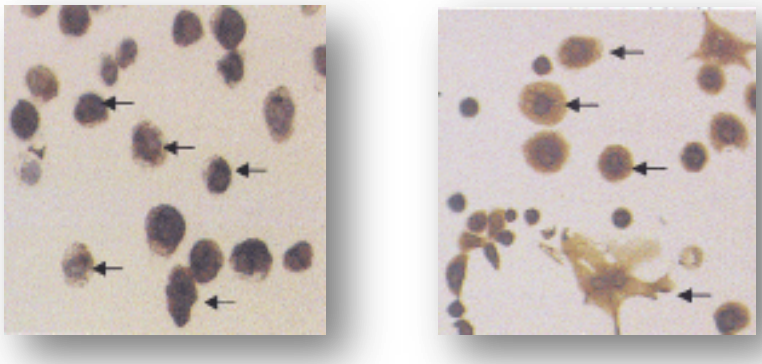

Figure 5.2: Untreated U937 monocyte cells (left) and PMA differentiated macrophages (Right) (Chen *et al.*, 2003)

The genes that are expressed by stimulated cells include tumor necrosis factor alpha (TNF-α) and lysozyme. The morphology of the cells resembles that of monocyte cells. These cells are an important model for differentiation of monocyte cells to macrophages *in vitro,* when treated with phorbol esters such as phorbol-12-myristate 13-acetate (PMA) (Fig. 5.2).

This cell line was selected based on the morphological similarities to human acute monocytic leukaemia cell line (ThP-1) and peripheral blood mononuclear cells (PBMCs) and also their ability to secrete cytokines such as IL-8, IL-1β and TNF-α which have been linked to *Propionibacterium acnes* pathogensis (Vowels *et al*., 1995).

5.1. Materials and Methods

5.1.1. Materials

Cell culture flasks, 96 well micro-titre plates and foetal bovine serum (FBS) were purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, South Africa). The U937 cell line (CRL1593.2) including the Roswell Park Memorial Institute 1640 medium (RPMI 1640), potassium phosphate buffer saline (PBS) and gentamycin (1%) were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, South Africa). Chemical reagents that were used were of analytical grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The Cell Proliferation Kit II (XTT) was purchased from Roche Diagnostics (Pty) Ltd. (Randburg, Johannesburg, South Africa).

5.1.2. Methods

5.1.2.1. Cell culture

The U937 cell line was cultured and maintained in culture flasks containing RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% gentamycin. Cell culture flasks were then incubated at 37°C in a humidified atmosphere set at 5% CO_2. Once cells were confluent they were centrifuged at 980 *g* and washed with PBS. Cells were then split into two flasks with new complete medium and maintained until a concentration of $1x10^5$ cells/ml was attained (Lin *et al*., 2004).

5.1.2.2. Cytotoxicity XTT assay

The methods of Zheng *et al*. (2001) were followed for the XTT assay. Briefly, two samples were tested on a single 96-well plate (Fig. 5.3).

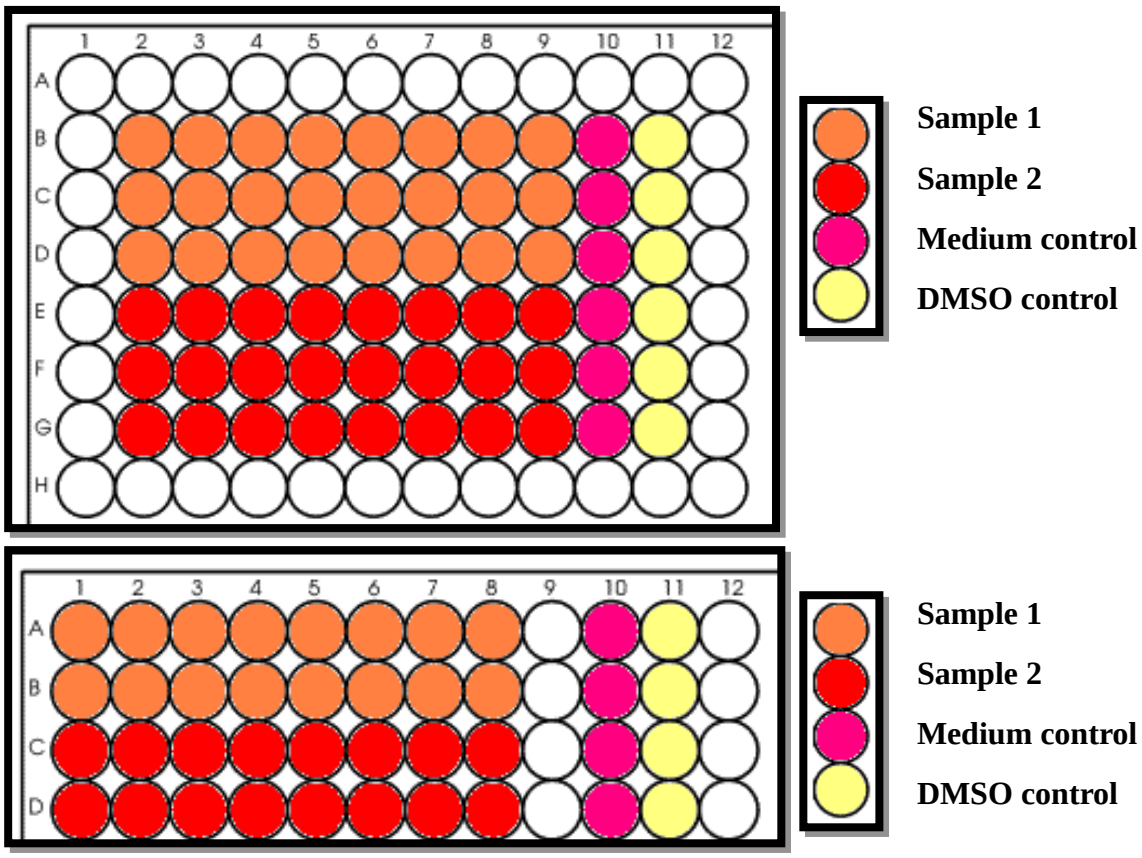

Figure 5.3: Plate layout for the XTT cytotoxicity assay

Sterile 96-well micro-titre plates were used to seed the U937 cells at the required concentration $1x10^5$ cells/ml with an added final concentration of 0.1µg/ml, phorbol-12-myristate-13-acetate (PMA). PMA was used to differentiate the monocyte cells to macrophages. The plates were then incubated for 24h to allow the cells to attach. Plant extracts were diluted in sterile medium then added to the test plate over a range of concentrations (3.13-400.00µg/ml). Pentoxyphylline was used as a positive control. PHA, the medium control and a 2% DMSO solvent control were also included in the assay. Plates were then incubated for a further 72h at 37 °C in 5% CO_2. After 72h, 50µl of XTT reagent (1 mg/ml XTT with 0.383 mg/ml PMS) was added to each test plate. Plates were then incubated for a further 2h. The plates were then read using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at 490nm and a reference wavelength of 690nm. Statistical analysis was done using GraphPad Prism Version 4.0 (San Diego, California, USA) in order to obtain the 50% inhibitory concentration (IC_{50}) for each extract and all the controls. The results were also interpreted using the selectivity index (SI) which was calculated using the following formula (Vicente *et al*., 2009):

$$\text{Selectivity Index (SI)} = \frac{\text{IC 50 (U937 cells)}}{\text{MIC } (P.\,acnes)}$$

5.2. Results and Discussion

The XTT reduction method was used for the determination of the IC_{50} for the best antibacterial plant extracts. The IC_{50} values of the four most active antibacterial extracts and their dose response curves on the viability of U937 cells are depicted in Table 5.1 and in Appendix 1, respectively.

Table 5.1: The 50% inhibitory concentration (IC_{50}) (µg/ml) and Selectivity Index (SI) on U937 cells

[a] Test sample	[b] IC_{50} (µg/ml)	[c] SI (Selectivity Index)
Clausena anisata	74.46 ± 0.42	2.38
Rapanea melanophloeos	25.21 ± 1.23	1.61
Helichrysum odoratissimum	21.54 ± 0.56	2.76
Helichrysum kraussii	51.74 ± 0.52	0.41
Pentoxyphylline	169.5 ± 7.80	-
PHA	317.6 ± 6.40	-

[a] Test sample

[b] 50% inhibitory concentration (IC_{50}) (µg/ml)

[c] Selectivity Index (SI)

R. melanophloeos and *H. odoratissimum* showed toxicity on U937 cells with an IC_{50} at 25.21 ± 1.23µg/ml and 21.54 ± 0.56µg/ml, respectively (Table 5.1). Extracts were considered toxic at concentrations lower than 25µg/ml. A study has reported the cytotoxic effect of 0.1mg/ml chloroform: methanol extract of *H. odoratissimum* on non-cancerous human kidney epithelial cells (Graham cells). At the tested concentrations, the percentage of viable cells treated with *H. odoratissimum* was 17.50%. *H. kraussii* was also tested on the same cells and showed some toxicity with the percentage viability of treated cells at 0.1mg/ml reported as 28.60%

(Lourens *et al*., 2011). Cytotoxicity of other *Helichrysum* species have been reported on Vero African monkey cells (Vero) (Heyman & Meyer, 2009). There are no reported cytotoxicity studies for crude extracts of *R. melanophloeos*, however, a triterpene isolated from this species has been investigated. The compound 3β-Hydroxylanosta-9, 24-dien-21-oic acid was evaluated for its cytotoxicity on human embryonic kidney cells (HEK293) and hepatocellular carcinoma cells (HepG2) but showed no toxicity with IC_{50} observed at 851.50µg/ml and 796.00µg/ml for HEK293 and HepG2 cells, respectively (Gwala, 2011).

C. anisata and *H. kraussii* were found to be moderately toxic to U937 cells with IC_{50} at 74.46 ± 0.42µg/ml and 51.74 ± 0.52µg/ml, respectively. Moderate toxicity was described for extracts showing IC_{50} between 50-100µg/ml. A cytotoxicity assay on Vero monkey kidney cells has reported that the acetone extract of *C. anisata* was the least toxic with an IC_{50} at 0.17mg/ml. It also showed the best antifungal activity against *Aspergillus fumigatus* with an MIC of 0.02mg/ml (Adamu *et al*., 2012).

Pentoxyfilline and PHA were non-toxic to U937 cells with IC_{50} at 169.50 ± 7.80µg/ml and 317.60 ± 6.40µg/ml, respectively. Non-toxic concentrations were described as extracts showing IC_{50} between 200-400µg/ml. The cytotoxicity results obtained for the plant extracts showed a dose response curve. Cell viability was higher at lower concentrations of plant extract. For toxic samples, *R. melanophloeos* and *H. odoratissimu*m, zero viability was observed at concentrations as low as 25-50µg/ml (Appendix 1).

The minimum inhibitory concentration (MIC) against *P. acnes* and the 50% inhibitory concentration (IC_{50}) were used to calculate the selectivity index (SI) for each extract. This can be defined as a ratio showing the selectivity of the extract against *P. acnes* and compared with the selectivity for U937 cells. *H. odoratissimum* and *C. anisata* showed the highest SI values of 2.76 and 2.38, respectively. Larger values indicated the selectivity for the *P. acnes* rather than acting as toxic agents for the U937 cells. Based on these results *C. anisata* and *H. odoratissimum* were considered as extracts for further testing on the inflammatory response of cells treated with *P. acnes*, for the investigation of the inflammatory response using cytokine analysis.

5.3. Conclusion

The XTT assay was a good *in vitro* technique for the determination of the IC_{50} of the selected plant extracts. It was clear that the cells were able to convert the XTT tetrazolium salt to its reduced form, which was an indication of the cell viability. It was easily adapted from drug-type compounds and pharmacological samples to plant extracts. U937 cells were maintained with little difficulty and were suitable for the cytotoxicity assay. The cell viability seemed to follow a dose response curve, which is expected for drug susceptibility testing. *C. anisata* and *H. kraussii* were moderately toxic. *H. odoaratissimum* and *R. melanophloeos* showed some toxicity on U937 cells. However, the cytotoxicity assay was used to obtain a range of concentrations for one of the plant extracts, which would allow for the analysis of cytokine secretion in treated U937 cells, without being detrimental to cell viability levels, ensuring that cytokine secretion would be observed. Pentoxyfilline and PHA were also investigated in the assay as they are used as positive controls for the cytokine assays.

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Chapter 6

Anti-inflammatory Potential of *Clausena anisata* extract

6. Introduction

Acne vulgaris is characterised as a chronic inflammatory disorder. The pathogenic process of *Propionibacterium acnes* has multiple steps involving infection of the pilosebaceous unit (sebaceous gland and hair follicle) and cytokine-mediated inflammatory responses (Popovic *et al*., 2012).

The literature in Chapter 2 revealed that *C. anisata* has been used traditionally for swollen gums, other inflammatory disorders and arthritis. This plant extract exhibited the least toxic effect on U937 cells, showed good antibacterial activity and was therefore selected for investigation of IL-8 cytokine inhibitory activity.

6.1. The immune response to *P. acnes*

The host pilosebaceous unit is constantly exposed to *P. acnes* due to favourable environmental and chemical composition available for optimal growth. Acne vulgaris is characterized by four main events, and each of these has a distinct immune response (Burkhart *et al*., 1999).

The first of these events is the hyper-cornification of keratinocyte cells. This process occurs due to altered lipid composition with a rise in sebum levels. Free fatty acids such as Linoleic acid and squalene have been identified as inducers of comedogenesis. Lipids have been associated with the proliferation and differentiation of keratinocyte cells. *N*-acetylated sphingolipids, such as ceramides, promote keratinocyte differentiation and sphingosine and sphingophosphorylcholine promote keratinocyte proliferation (Wakita *et al.*, 1998). Cytokines have also been shown to contribute to comedone formation through the activity of the Interleukin-1α (IL-1α) cytokine. This cytokine has been found in large quantities in non-inflammatory lesions of acne vulgaris (Koreck *et al.*, 2003). Comedogenesis is generally followed by an increase in *P. acnes* density within the pilosebaceous unit (Tsai *et al*., 2010).

The inflammatory response is the major symptom associated with acne vulgaris. The onset of inflammation is attributed to the immunological responses that occur when the host immune system becomes aware of infiltration by *P. acnes*.

The bacterium stimulates monocyte cells to secrete pro-inflammatory cytokines such as Interleukin-1β (IL-1β), Interleukin-8 (IL-8) and Tumour Necrosis Factor-α (TNF-α) (Tsai *et*

al., 2010). IL-8 is a chemotactic factor for neutrophils which contributes to the onset of the inflammatory response (Sugisaki *et al*., 2009).

The recognition of bacterial infiltration is largely due to the presence of pattern recognition receptors (PRRs). These are responsible for the recognition of pathogen-associated molecular patterns (PAMPs). These PAMPs include bacterial lipopolysaccharide (LPS) and pepttidoglycans (PGN). The recognition of bacterial components by PRRs induces the innate immune response and also activates the adaptive immune response (Grange *et al*., 2009; Pivarcsi *et al*., 2003) (Fig. 6.1).

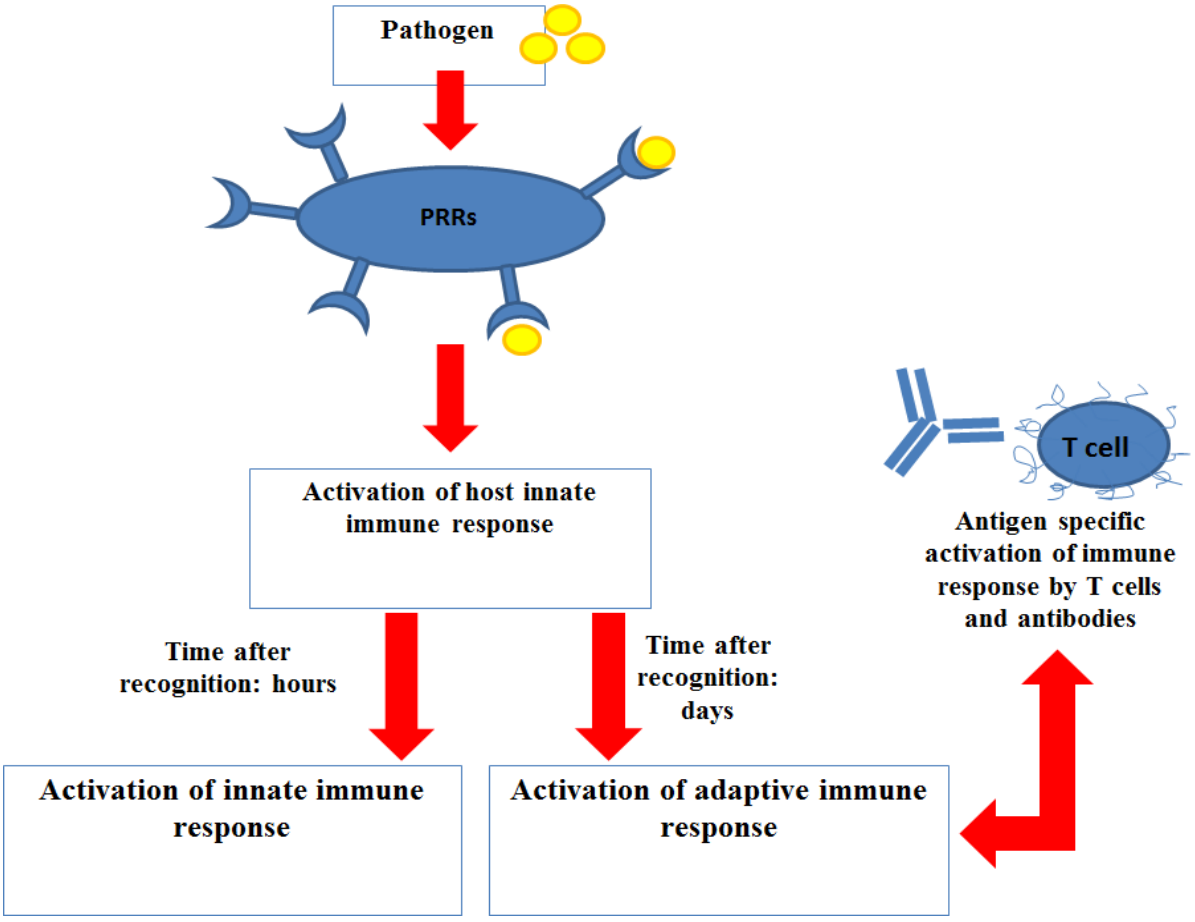

Figure 6.1: Recognition of pathogens through PRRs and the activation of the host immune response (Adapted from Medzhitov, 2007)

Examples of these PRRs are Toll-like receptors (TLRs). These receptor molecules can differentiate between Gram-positive and Gram-negative bacterial ligands. Gram-positive

bacterial ligands are said to activate immune monocyte cells through recognition by TLR-2 or TLR-4 receptors and is the proposed mechanism for *P. acnes* inflammatory response since it is a Gram-positive bacterium (Kim *et al*., 2002). TLR-2 is expressed on the cell surface of macrophage cells which are found around the pilosebaceous follicles within acne lesions, and the secretion of IL-12 and IL-8 is said to be triggered through the TLR-2 activation pathway (Koreck *et al*., 2003). IL-8 is responsible for attracting neutrophils to the pilosebaceous unit where they secrete lysosomal enzymes. These enzymes ultimately lead to the bursting of the follicular epithelium and continued inflammation (Webster *et al*., 1980). IL-12 enhances the Th-1 immune response; the overproduction of this cytokine is said to lead to tissue injury and autoimmune and inflammatory disorders (Kim *et al*., 2002). In keratinocytes, both of these receptors are responsible for the detection of peptidoglycan (by TLR-2) and lipopolysaccharide (by TLR-4) (Koreck *et al*., 2003). *In vivo* experiments have shown that the expression of TLR-2 and TLR-4 in keratinocyte cells increased after 1 h of incubation with bacterial fractions. These experiments also showed an increase in the expression and secretion of metalloproteinase-9 (MMP-9), which is associated with inflammatory processes. These MMPs are endopeptidases produced by a variety of cells and are responsible for the degradation of components of the extracellular matrix. MMPs also result in the rupture of the pilosebaceous follicle, which could account for their link to inflammation (Jugeau *et al*., 2005).

P. acnes also has the ability to activate both the classical and alternative complement pathways which produce C5a, a highly inflammatory peptide. Leukocytes are also implicated in the pathogenesis of inflammatory acne (Norris & Cunliffe, 1988; Scott *et al*., 1979).

Infections by *P. acnes* trigger antimicrobial peptides and cytokine release in human skin. The skin secretes β-defensin-2 (hβD2) upon infection with the bacteria. These hβD2 indirectly contribute to the host immune function. They are able to activate the adaptive immune response through activation of dentritic cell to induce the migration of CD45RO memory T cells and can also act as chemoattractants for neutrophil cells (Nagy *et al*., 2005; Yang *et al*., 1999).

It has been shown that the overproduction of ROS by the immune system may result in the onset of inflammatory responses. Comedogenesis results in a reduction of linoleic acid, a potent scavenger of ROS. The result is an increase in ROS produced by neutrophil cell function. *P. acnes* can generate nitric oxide radicals ($NO^{\bullet}$) through the attraction and

activation of neutrophils (Portugal *et al*., 2007). The aim of the cytokine analysis was to investigate the anti-inflammatory activity of *C. anisata*. *P. acnes* is able to stimulate immune cells to secrete copious amounts of the pro-inflammatory cytokine IL-8. The cytokine is indirectly involved in the pathogenic process of *P. acnes*. The secretion of IL-8 is stimulated by a number of factors mentioned above.

6.2. Materials and Methods

6.2.1. Materials

Reagent set B, BD Falcon ELISA micro-titre plates and human IL-8 ELISA kit were purchased from BD Biosciences (San Diego, CA, USA). All chemical reagents including PHA and pentoxyfilline were of analytical grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

6.2.2. Methods

6.2.2.1. Cytokine ELISA assay

The potential anti-inflammatory effect of the selected plant extract against IL-8 secretion by U937 cells was performed using an ELISA technique. The protocol for the assay was included in the reagent set B and human IL-8 antibody kits (Appendix 2). Log phase culture of *P. acnes* was harvested and washed three times with phosphate buffer saline (PBS). Cultures were then incubated at 80°C for 30 minutes. The heat-killed bacteria were then stored at 4°C until required. U937 cells were seeded in a 24-well microtitre plate at a cell density of 10^5 cells/well. Cells were stimulated with 100µg/ml of heat-killed *P. acnes* alone and together with *C. anisata* extract at non-lethal concentrations. Pentoxyfilline was used as the positive control, 1% DMSO as a solvent control. After 24h the plates were centrifuged and cell-free supernatants collected and aliquoted (Fig. 6.2). Supernatants were stored at -72°C until required. The concentration of IL-8 present at each tested concentration was evaluated using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at 450nm with a reference wavelength of 570nm. Calibration curves were constructed from a serial dilution series of IL-8 standards for calculation of IL-8 protein concentrations. Results were then analysed using an online program ReaderFit.

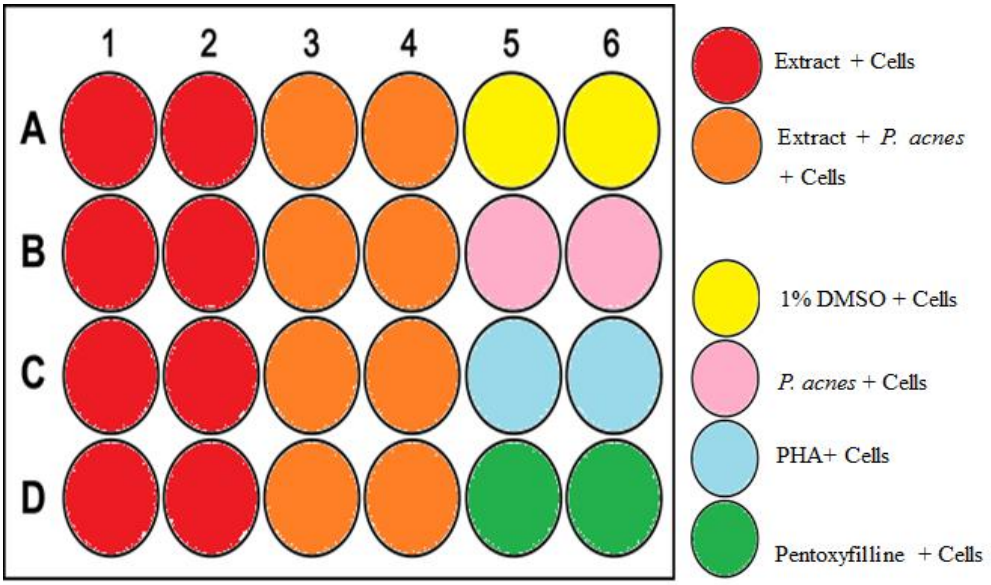

Figure 6.2: Plate layout for collection of supernatant from treated cells

6.3. Results and Discussion

The concentrations of *C. anisata* were selected based on the cytotoxicity results shown in Chapter 5. The concentrations ranged from 50µg/ml to 6.25µg/ml. The viability was investigated after 24h with the various treatments before the ELISA assay was performed (Fig. 6.3).

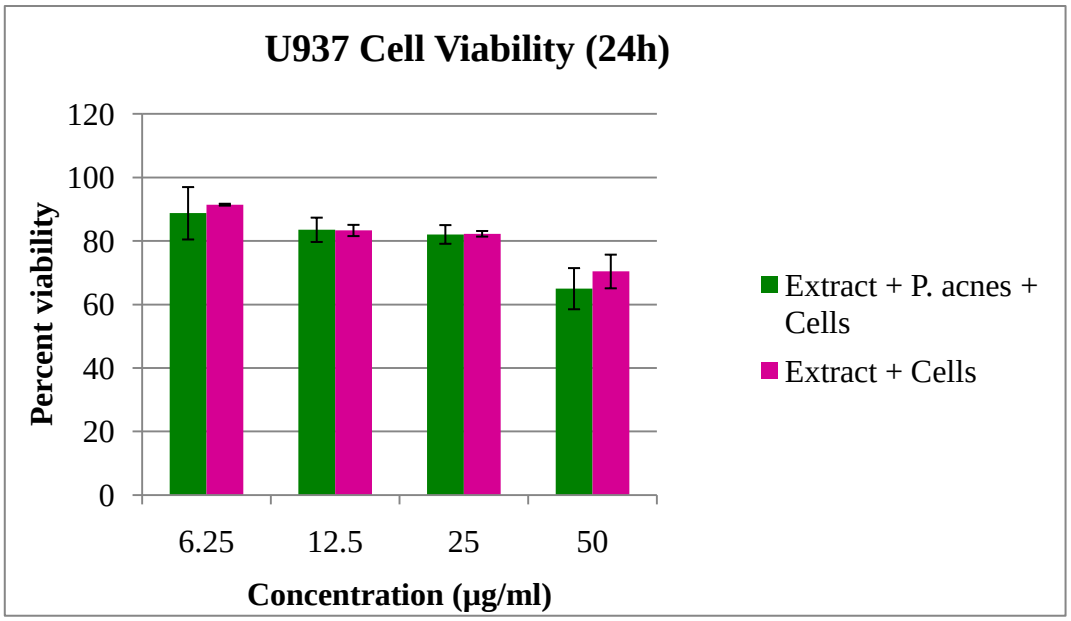

Figure 6.3: Cell viability 24h after plant extract was added

The cell viability after 24h exhibited a dose response. Cell viability at the lowest concentration (6.25µg/ml) was above 80% for both the extract treatment alone and the bacteria-extract combination treatment. The lowest viability observed was for the bacteria-extract treatment at 50µg/ml which was 65%. The viability levels suggest that any IL-8 protein inhibition was due to treatment effects and not due to decreased cell viability.

The results obtained in this study have shown that U937 cells treated with 100µg/ml heat-killed *P. acnes* secreted increased amounts of IL-8 when compared to that of the untreated cell control (Fig 6.4). The IL-8 protein concentration for the *P. acnes* treatment was quantified as 840.52pg/ml, using a standard curve (not shown). It is important however, to note that the IL-8 protein recorded for the untreated cell control was relatively high at 488.76pg/ml, when compared to that of a similar study by Sharma *et al*. (2014) (Fig. 6.4). This phenomenon was previously observed in peripheral blood monocyte cells (PBMCs) where they spontaneously produced IL-8 making it difficult to differentiate between the effects of various cell stimuli (Kasahara *et al*., 1991; Vowels *et al*., 1995)

P. acnes has been reported to induce inflammatory responses through activation of monocyte cells. The monocyte activation results in the secretion of pro-inflammatory cytokines such as Tumour Necrosis Factor alpha (TNF-α), Interleukin 8 (IL-8) and Interleukin 1β (IL-1β). IL-8 is known for its ability to attract neutrophil cells to the pilosebaceous unit (Kim *et al*., 2002). *P. acnes* ingestion by activated neutrophil cells results in the release of hydrolase enzymes. These are known to disrupt the follicular wall of the pilosebaceous unit inducing inflammation. Studies have shown that THP-1 cells stimulated with *P. acnes* at various concentrations show a dose dependant release of IL-8. The U937 cell line is characteristically similar to the THP-1 cells and has also shown a similar pattern for IL-8 secretion (Vowels *et al*., 1995). This explains the involvement of IL-8 in the pathogenic process of *P. acnes* and also the increased amounts of IL-8 protein secreted in U937 cells treated with the bacterium in this study. Due to the limits on the BIO-TEK Power-Wave XS multi-well reader the test samples were diluted 2-fold. U937 cell supernatants were collected and the IL-8 protein levels were quantified (Table 6.1).

Table 6.1: Concentration of IL-8 detected (pg/ml) for each treatment

[a] Test sample	[b] Diluted IL-8 concentration (2x) (pg/ml)	[c] Actual concentration (pg/ml)	[d] Concentration of treatment (µg/ml)
***C. anisata* + *P. acnes* + Cells**	161.24	322.48	50
***C. anisata* + *P. acnes* + Cells**	182.99	365.98	25
***C. anisata* + *P. acnes* + Cells**	191.81	383.62	12.5
***C. anisata* + *P. acnes* + Cells**	204.76	409.52	6.25
***C. anisata* + Cells**	194.37	299.24	50
***C. anisata* + Cells**	193.57	357.82	25
***C. anisata* + Cells**	178.91	387.14	12.5
***C. anisata* + Cells**	149.62	388.74	6.25
Pentoxyfilline (PF)	172.48	344.96	20
Phytohaemagglutinin (PHA)	53.82	107.64	5
Cell control	244.38	488.76	-
***P. acnes* + Cells**	420.46	840.52	100

[a] Test sample
[b] Diluted IL-8 concentration (2x) (pg/ml)
[c] Actual concentration (pg/ml)
[d] Concentration of treatment (µg/ml)

Protein levels of IL-8 detected in U937 cells treated with various (non-lethal) concentrations of *C. anisata* extract without *P. acnes* stimulation were compared with the untreated cell control. Low levels of IL-8 inhibition were observed. Inhibition was dose-dependent with 50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml showing IL-8 levels at 299.24 ± 0.13pg/ml, 357.82 ± 0.07pg/ml, 387.14 ± 0.12pg/ml and 388.74 ± 0.19pg/ml, respectively. This means that *C. anisata* was able to reduce the levels of IL-8 from 488.76 ± 0.06pg/ml.

Protein levels of IL-8 detected in U937 cells treated with various (non-lethal) concentrations of *C. anisata* extract with *P. acnes* stimulation were compared with U937 cells stimulated with *P. acnes* only. The inhibition levels were lower than that of cells treated without *P. acnes* stimulation. Treatment of cells with 50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml of *C. anisata* extract in the presence of heat-killed *P. acnes* showed IL-8 levels at 322.48 ± 0.07pg/ml, 365.98 ± 0.24pg/ml, 383.62 ± 0.08pg/ml and 409.52 ± 0.13pg/ml respectively. This means that the *C. anisata* extract was able to decrease the secretion of IL-8 from 840.52 ± 0.06pg/ml.

When comparing the cells treated with *C. anisata* and *P. acnes* stimulation and those cells without stimulation there was a slight difference. Treated cells with no *P. acnes* stimulation showed a higher IL-8 inhibition than those that also contained the *P. acnes* suspension. This is due to the increased secretion of IL-8 in the presence of *P. acnes* which reduced the efficacy of the *C. anisata* extract (Lyte *et al*., 2009). A study by Sharma *et al*. (2013) showed what levels of inhibition would be required for effective treatment of inflammation in acne vulagaris, with the *Syzygium jambos* extract inhibiting 85%, 65.8% and 23.9% of IL-8 secretion at 100, 50 and 10µg/ml, respectively.

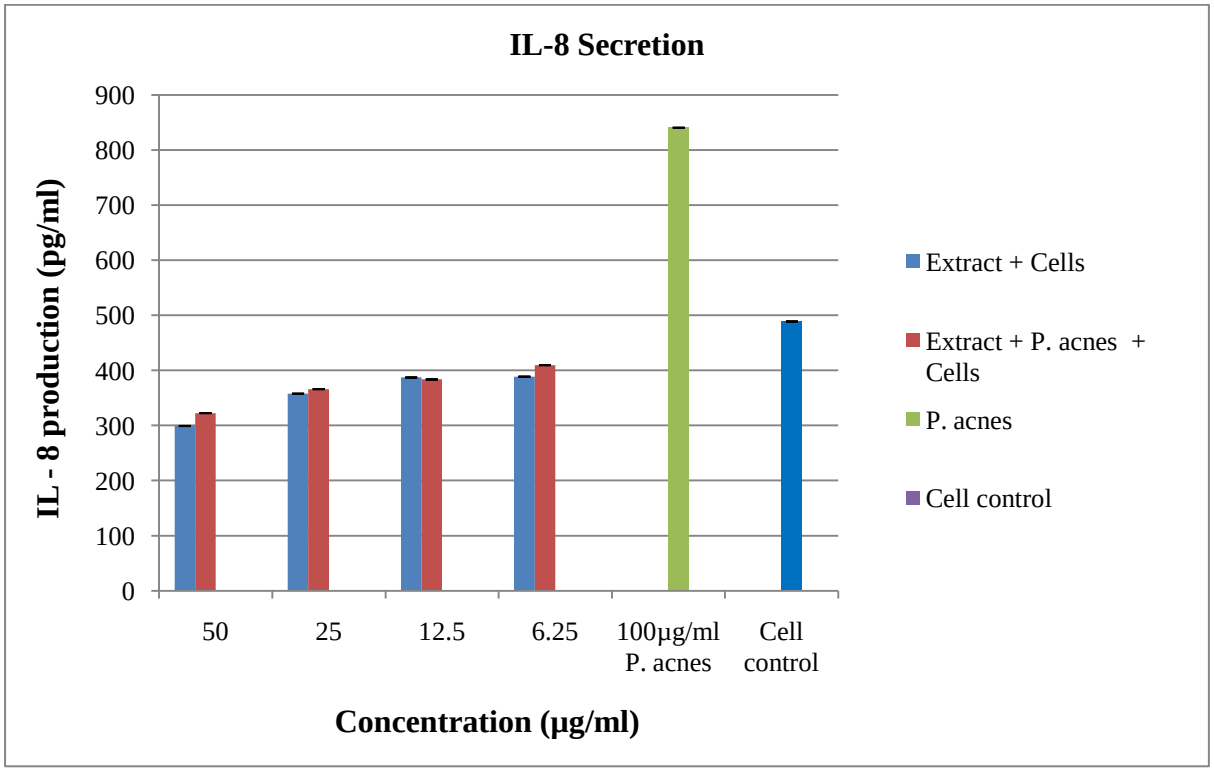

Figure 6.4: IL-8 production in U937 cells with various treatments

Cells treated with pentoxyfilline (20µg/ml) showed low levels of IL-8 inhibition, with 344.96 ± 0.06pg/ml of IL-8 protein detected. Pentoxyfilline is a methylxanthine compound which is known for its ability to inhibit the production of inflammatory cytokines such as TNF-α, IL-1, and IL-6. An experiment by Neuner *et al*. (1994) suggested that the *in vivo* activity of pentoxyfilline was not only applicable to TNF-α inhibition but in particular circumstances to IL-1β, IL-6 and IL-8 inhibition as well. The study evaluated the effects of pentoxyfilline *in vitro.* The results of the study on PBMCs treated with pentoxyfilline showed significant inhibition at 72h and virtually complete inhibition at 96h. There was however, no effect on IL-8 production at 24h and 48h. This could explain the low levels of inhibition by pentoxyfilline observed in the current study which was performed for 24h (Sharma *et al*., 2013). This could explain why similar amounts of IL-8 were secreted with *C. anisata* and pentoxyfilline (a known inhibitor).

Cells treated with Phytohaemagglutinin (PHA) showed an IL-8 production of 107.64 ± 0.05pg/ml. PHA, however did not seem to stimulate the cells to the expected degree. A study performed by Liebler *et al*. (1993) showed that purified adherent monocytes produced IL-8 after stimulation with PHA where detectable antigenic IL-8 was detected at 8h and showed a maximum at 24h. The spontaneous production of IL-8 observed in the untreated cell control could however be interfering with the PHA-induced IL-8 secretion.

6.4. Conclusion

The concentrations of *C. anisata* that were tested showed low levels of inhibition of U937 cells treated with cell stimulants. The untreated cell control showed spontaneous secretion of IL-8, making it difficult to compare the effects of other cell stimulants such as *P. acnes* and PHA. The *C. anisata* extract seemed to show low levels of inhibition on IL-8 secretion to some extent which confirms why the plant is used traditionally to treat other inflammatory diseases. Cells treated with the *P. acnes* suspension showed an increased IL-8 secretion; but it seemed to be reduced slightly with the addition of the plant extract at the tested concentrations Pentoxyfilline also showed low levels of IL-8 inhibition, but could be due to the 24h treatment period. To the best of our knowledge this the first report on the IL-8 inhibition *in vitro* for *C. anisata*.

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Chapter 7

Screening of *P. acnes* (ATCC 11827) for Hyaluronidate Lyase Activity

7. Introduction

The complete genome sequence of a cutaneous type I isolate of *P. acnes* was obtained in 2004 at the Göttingen Genomics Laboratory. This information is important for the understanding of the pathogenic ability of *P. acnes* (with a detailed description of some genes given below). It also gives an indication of the ability of *P. acnes* to colonise the harsh environments found within the skin and sebaceous glands (Brügermann *et al.*, 2004). The results of the genome sequence have revealed the presence of proteins involved in host-tissue degradation (Brügermann, 2005).

The *P. acnes* genome encodes for a number of genes that are responsible for similar functions of proteins which degrade eukaryotic cell surface and tissue structures. The PPA2105 (33kDa) triacylglycerol lipase (GehA) gene and a closely homologous gene (PPA1796) are involved in the degradation of sebum, which acts as a growth medium for *P. acnes* which have colonised sebaceous glands (Miskin *et al.*, 1997).

The PPA680 gene encodes for the protein hyaluronate lyase (hyaluronidase). This enzyme is responsible for the degradation of substrates hyaluronan (hyaluronic acid, HA), hyaluronate (Sodium-HA) and non-sulfur glycosaminoglycan which are the major polysaccharides in the extracellular matrix of connective tissues. The hyaluronan (D-glucuronate-β-1,3-*N*-acetyl-D-glucosamine) is cleaved at β-1,4-glycosidic links by hyaluronidases to 3-(4-deoxy-β-D-gluc-enuronosyl)-*N*-acetyl-D-glucosamine. The products of this reaction have been identified at sites of inflammation and tissue injury (Steiner *et al.*, 1997).

Bacterial hyaluronic acid splitting enzymes function through β-elimination, resulting in unsaturated oligosaccharides of various lengths or disaccharides with double bonds at the 4,5-position on the glucuronyl group (Hertel *et al.*, 2006) (Fig. 7.1). Some of the products of the cleavage reaction are responsible for the regulatory functions. A major function is the suppression of the immigration of immune system cell components (Frost *et al.*, 1996).

The aim of the study was to screen the *P. acnes* strain (ATCC 11827) for the presence of hyaluronidase enzyme, which is involved in the pathogenic process of acne vulgaris. The inhibition of pathogenic *P. acnes* enzymes by plant extracts has been reported but little has been done on hyaluronidase enzymes in this strain.

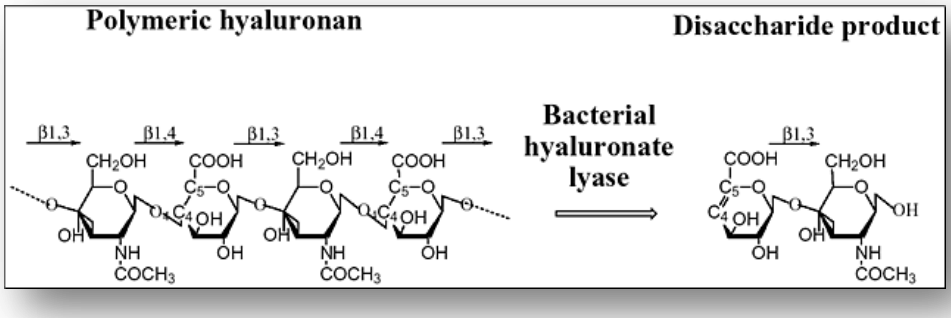

Figure 7.1: Cleavage of Hyaluronic Acid via β-1,4 elimination to release a disaccharide (Jedrzejas, 2002)

7.1. Materials and Methods

7.1.1. Materials

Brain Heart Infusion (BHI) Broth, BHI Agar and Acetic acid were purchased from Merck (Darmstadt, Germany). Reagents were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) including Sodium Hyaluronan (Sodium-HA) and Bovine Serum Albumin (BSA) fraction V which were of analytical grade. The *P. acnes* strain (ATCC 11827) was purchased from Anatech Analytical Technology (Sloane Park, Johannesburg, South Africa).

7.1.2. Methods

Screening for the presence of hyaluronidase in the tested *P. acnes* strain (ATCC) was performed according to Smith & Willett (1968) with some modifications. The basic medium was prepared using 100ml of BHI broth and 5g of BHI agar. The medium was autoclaved at 121°C for 20min and allowed to cool to 50°C. Aqueous solutions of Sodium-HA at 2mg/ml and BSA fraction V at 5% were prepared. The Sodium-HA was added at a final concentration of 400µg/ml and BSA fraction V was added at a final concentration of 1%. The growth medium was then poured into sterile petri plates (8.6cm) to a depth of 4mm. The final pH of the medium was 6.8 ± 0.1. After solidification of the plates they were stored at 4°C until use. Pure *P. acnes* cultures were then swabbed onto the growth plates and incubated at 37°C in an Anaerocult Jar with Anaerocult C for anaerobic conditions. Once the stationary phase was observed plates were flooded with a 2*N* acetic acid solution for 10min.

7.2. Results and Discussion

The control plates were tested to check that the agar had no presence of constitutive hyaluronidase. Both control plates were supplemented with 1% BSA and 400µg/ml Sodium-HA but only one was flooded with 2*N* acetic acid solution. The control plate without acetic acid showed no formation of the Sodium HA-BSA conjugates. The control plate flooded with *2N* acetic acid showed the appearance of the white precipitate indicating the formation of Sodium HA-BSA conjugates in the absence bacterial hyaluronidase (Fig. 7.2).

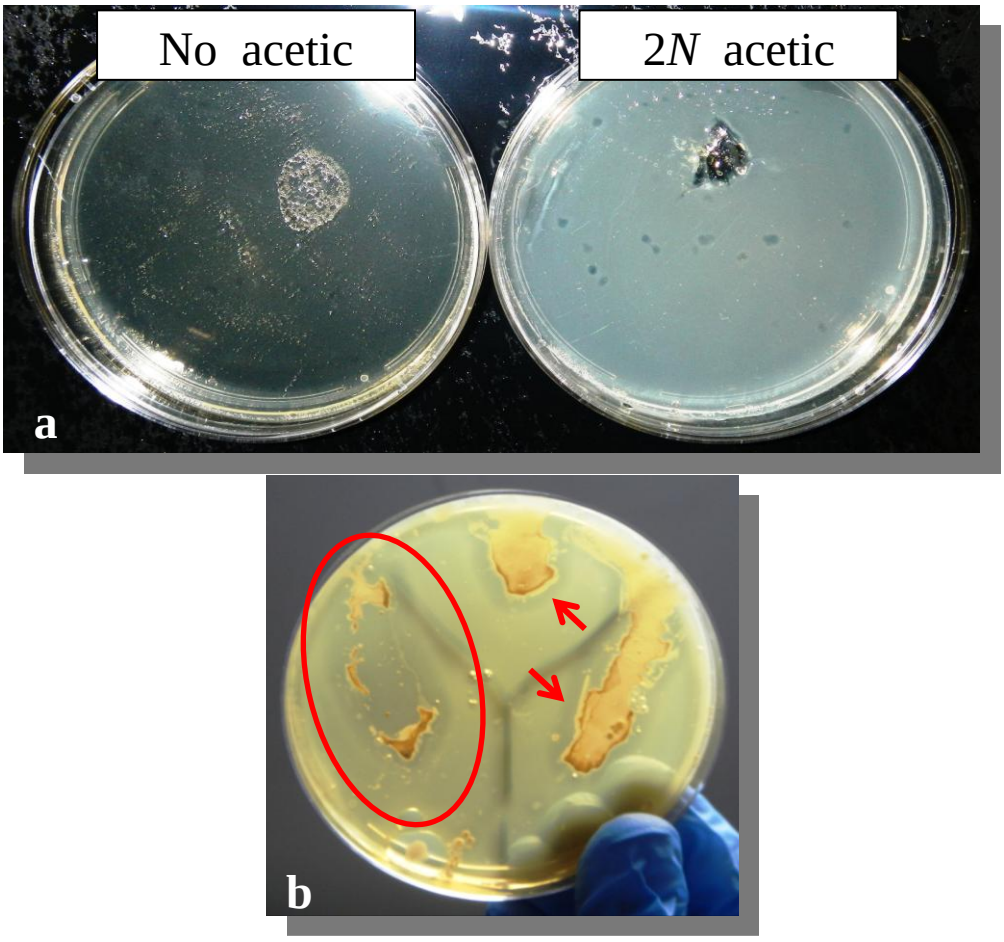

Figure 7. 2: a) Control plates with and without 2N acetic acid b) Test plates with active hyaluronidase producing *P. acnes*

The rapid plate method was employed for the screening of the *P. acnes* 11827 strain. The method is adapted from the sensitive turbidity reducing unit (TRU) method which is used for

detection of muco-polysaccharide degrading enzymes. The principle of the assay is based on the conjugates formed between the undigested substrate (Sodium-HA) and BSA fraction V in the presence of acetic acid. The turbidity formed by the complexes or absence thereof is related to the amount of digested substrate in solution. This technique was adapted to a plate screen method based on the same principle (Smith & Willett, 1968).

The test plates showed a clear zone of inhibition when plates were held towards a light source. These zones are indicated with red arrows (Fig. 7.2). One of the large colonies was removed from the plate using a sterile inoculation loop to observe the effects on the sections of agar underneath the colonies. The sections underneath the *P. acnes* colonies also showed a zone of inhibition. This was an indication that the 11827 strain of *P. acnes* produces the hyaluronidase enzyme that is able to degrade the Sodium-HA substrate which may be involved in the mechanism of pathogenesis for the strain (Hertel *et al*., 2006).

Results are comparable with that of Smith & Willett (1968) who tested the *P. acnes* 11828 strain which was also shown to produce hyaluronidase as well as chrondroitin-sulfatase. The conjugates that were formed in the control plates for the current study, however, were not as clear as reported by Smith & Willett (1968) but this could be due to the use of BHI agar in place of the suggested Noble agar. This was overcome by holding the plates against a darkened background (control plates) and towards the light (test plates).

The inhibitory assay for hyaluronidase was not tested due to time constraints after late delivery of reagents required for the test. The next step would be to test *C. anisata* in an enzyme inhibition assay to check whether it can decrease the *P. acnes* hyaluronidase enzyme activity and therefore reduce pathogenesis of the bacteria as well as reduce the effects of scarring caused by destruction of extracellular hyalruronic acid.

7.3. Conclusion

The *P. acnes* strain used in this study (ATCC 11872) was able to degrade the hyaluronan in the supplemented agar plates. This strain of *P. acnes* could use this enzyme to degrade the hyaluronic acid within the skin extracellular matrix and spread to other cells, forming part of the pathogenic process of the bacterium.

7.4. References

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Conclusions and Recommendations

Conclusions:

- Ethanol extracts of 20 southern African plants were prepared and investigated for their antimicrobial and antioxidant activities
- *Helichrysum odoratissimum* showed the best antimicrobial activity with a MIC at 7.81µg/ml and *Clausena anisata* showed the lowest cytotoxicity with an IC_{50} of 74.46µg/ml on U937 cells
- Combinations of *Helichrysum odoratissimum* and *Helichrysum kraussii* showed synergistic activity on *P. acnes* when the MIC was reduced to 3.90µg/ml
- PrestoBlue was the better of the two growth indicators (PB and INT) used in the study
- *Helichrysum odoratissimum* and *Clausena anisata* also showed the highest SI at 2.76 and 2.38 respectively
- *Clausena anisata* was reported to have some inhibitory activity on IL-8 secretion but not in significant amounts to be used on its own as a treatment
- This is the first report of *P. acnes* (ATCC 11827) producing the bacterial hyaluronidase enzyme
- The ability of the selected plant extracts to act as anti-oxidants (*Helichrysum odoratissimum, Rapanea melanophloeos* and *Helichrysum kraussii*), anti-inflammatory agents (*Clausena anisata*) and as antimicrobial agents (*Helichrysum odoratissimum, Rapanea melanophloeos, Helichrysum kraussii* and *Clausena anisata*) shows that they could be used to control acne vulgaris

Recommendations:

- Phytochemical screening of compounds present in active extracts
- Bioassay guided fractionation to isolate active compounds from plant extracts to determine which compounds are responsible for antimicrobial activity
- Evaluation of other cytokines secreted by cells treated with *P. acnes* suspensions in the presence of potential anti-inflammatory cytokines
- Further dilutions of the lowest concentration of the *Helichrysum spp.* would need to be done before the actual MIC of the combinations can be determined
- Test other anti-inflammatory bioassays to confirm *Clausena anisata*'s anti-inflammatory activity reported by traditional usage

- Look at enzyme inhibition of hyaluronidase by the active plant extracts
- Look at plants that are traditionally used for acne specifically

Publications:

- Lall, N., Heneley-Smith, C.J, **de Canha, M.N**., Oosthuizen, C.B., Berrington, D., 2013. "Viability Reagent, PrestoBlue, in Comparison with Other Available Reagents, Utilized in Cytotoxicity and Antimicrobial Assays", *International Journal of Microbiology*, vol. 2013, pp. 1-5

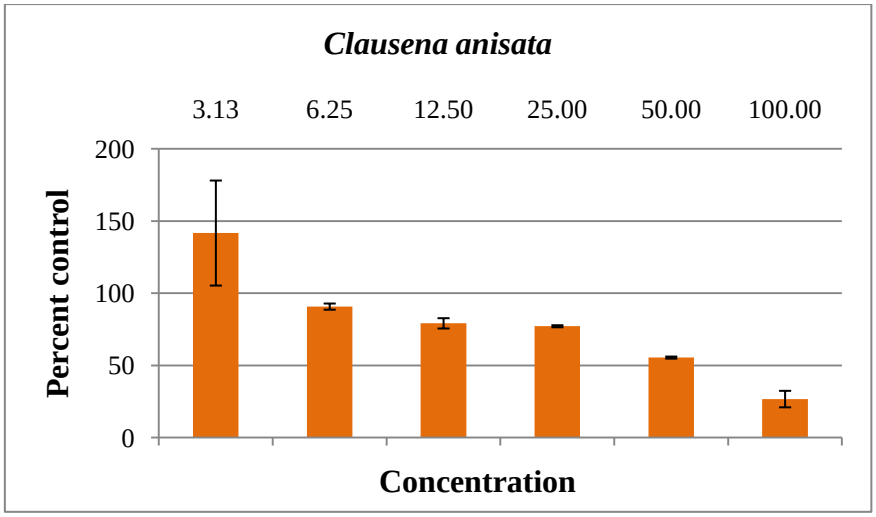
Possible manuscripts:

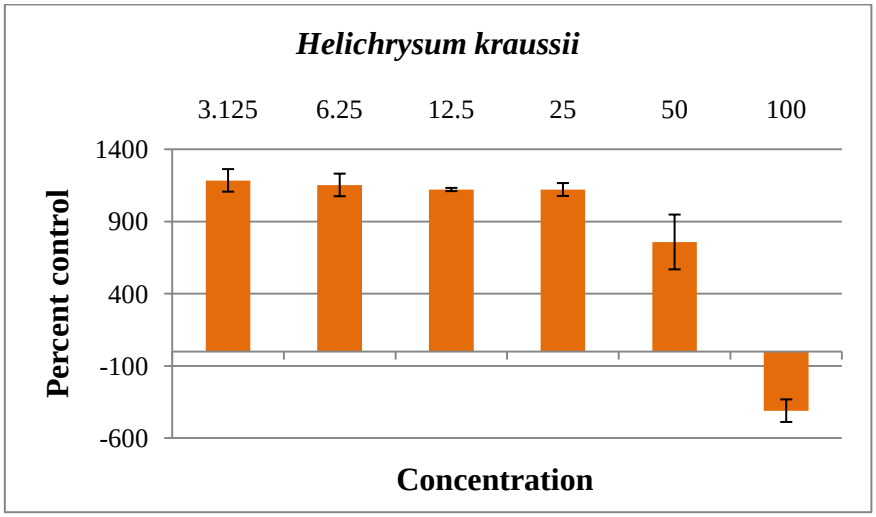
- **de Canha, M.N.,** Lall, N., Antimicrobial and anti-inflammatory effects of some southern African plants against acne vulgaris. *BMC Complementary and Alternative Medicine* (Under preparation)

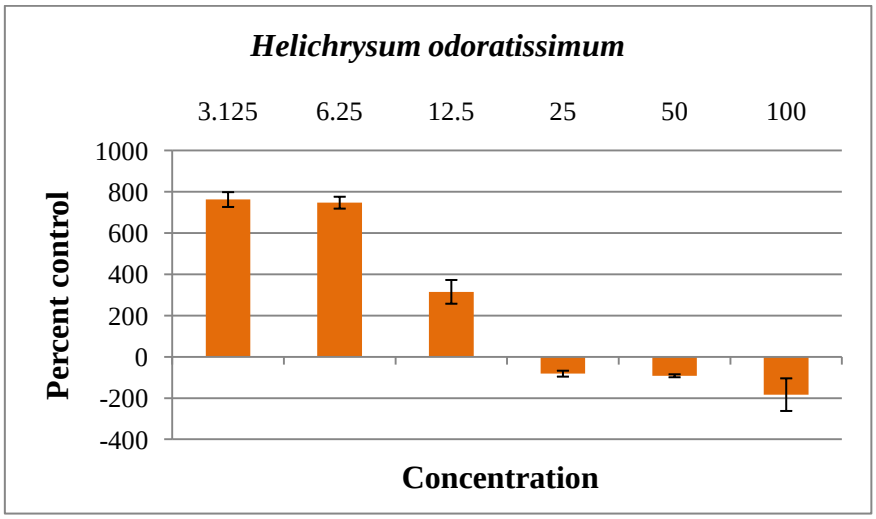
- **de Canha, M.N.,** Lall, N., The synergistic combination of two *Helichrysum spp.* For the treatment of acne vulgaris. *BMC Complementary and Alternative Medicine* (Under preparation)

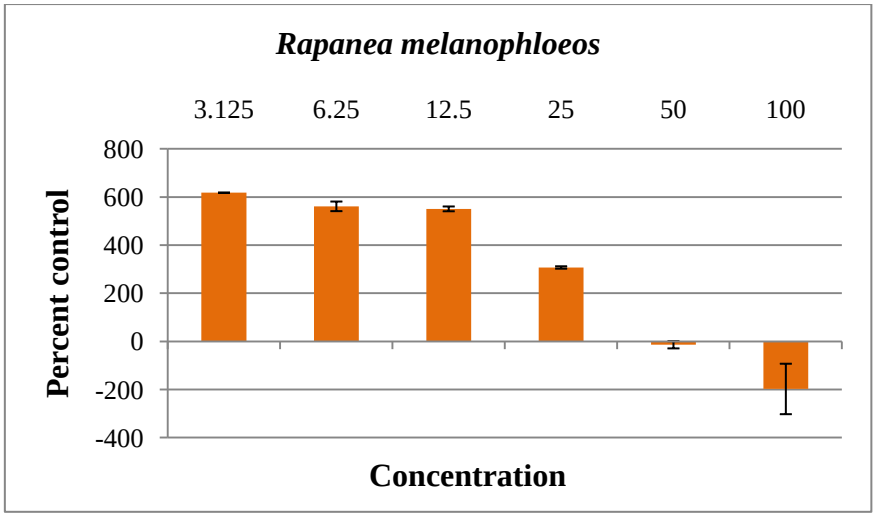
Appendix 1

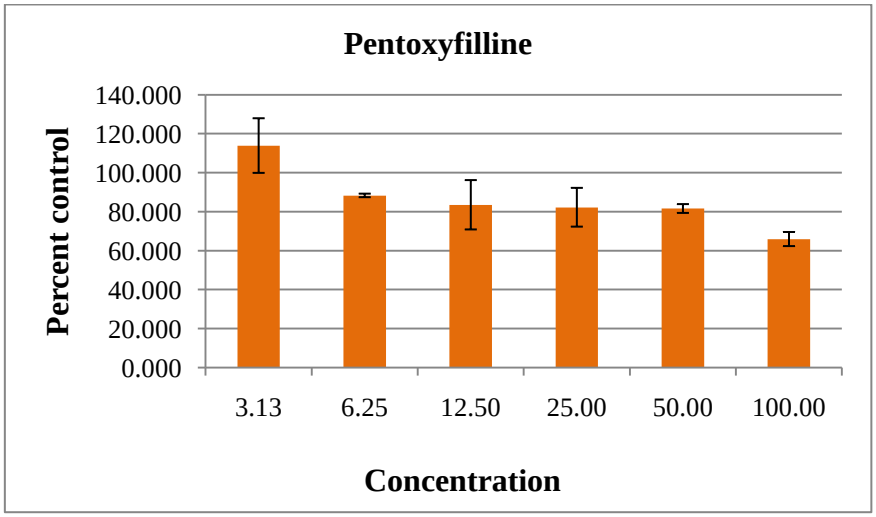
Cytotoxicity IC_{50}

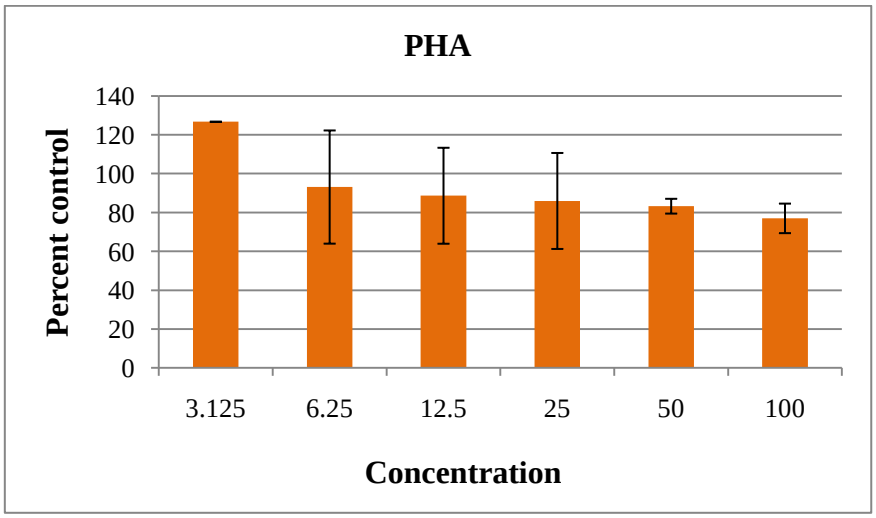












Appendix 2

Cytokine IL-8 ELISA Kit

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance from each.

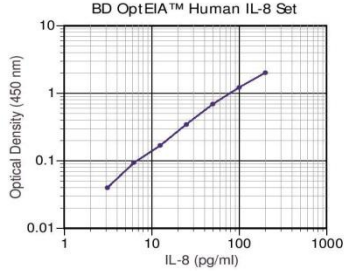
Plot the standard curve on log-log graph paper, with IL-8 concentration on the x-axis and absorbance on the y-axis. Draw the best fit curve through the standard points.

To determine the IL-8 concentration of the unknowns, find the unknown's mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the IL-8 concentration. If samples were diluted, multiply the IL-8 concentration by the dilution factor.

Computer data reduction may also be employed, utilizing log-log regression analysis.

Typical Standard Curve

This standard curve is for demonstration only. A standard curve must be run with each assay.



Specificity

Cross Reactivity: The following factors were tested in the BD OptEIA™ assay at ≥ 10 ng/mL and no cross-reactivity (value ≥ 2 pg/mL) was identified.

Recombinant Human
IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-13, IL-15, G-CSF, GM-CSF, IFN-γ, CD23, Lymphotactin, MIP-1α, MIP-1β, MCP-1, MCP-2, NT-3, PDGF-AA, SCF, TNF, LT-α (TNF-β), VEGF

Recombinant Mouse
IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12 (p70), IL-15, IFN-γ, GM-CSF, MCP-1, TCA3, TNF

Recombinant Rat
IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF

Other:
Viral IL-10 (1 ng/mL), Rabbit TNF

Interfering Substances:
The following substances at levels ≥ 2 mg/ml were added to Standard Diluent spiked with 200 pg/ml IL-8. No effect on assay results was observed: Bilirubin, Human γ-globulin,Human hemoglobin, Human serum albumin, Human transferrin, Triglycerides, Heparin (300 units/mL), Sodium Citrate, EDTA

2654KI-08 04/16/03

Standardization

This immunoassay is calibrated against purified Baculovirus - expressed recombinant human IL-8 produced at BD Biosciences Pharmingen.

The NIBSC/WHO International standard 89/520 (recombinant human IL-8 expressed in *E.Coli*) was evaluated in this set. The conversion factor for NIBSC material is as follows:

1 µg NIBSC 89/520 IL-8 = 1.06 µg BD OptEIA™ IL-8

Assay Optimization

1. BD OptEIA™ Sets allow flexible assay design to fit individual laboratory needs. To design an immunoassay with different sensitivity and dynamic range, the following parameters can be varied: Capture, Detection Antibody titers, Incubation time, Incubation temperature, Assay Diluent formulation, Buffer pH, ionic strength, protein concentration, Type of substrate, Washing technique (i.e., number of wash repetitions and soak times)
2. "Typical Standard Curve" and 20-plate yield were obtained in the BD Biosciences Pharmingen laboratory, using the recommended procedure and manual plate-washing.

Troubleshooting

Poor Precision

Possible Source	*Corrective Action*
• Inadequate washing/ aspiration of wells	• Check function of washing system
• Inadequate mixing of reagents	• Ensure adequate mixing
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Incomplete sealing of plate	• Ensure complete seal on plate

Poor Standard Curve

Possible Source	*Corrective Action*
• Improper standard handling/ dilution	• Ensure correct preparation, storage of standards
• Incomplete washing/ aspiration of wells	• Check function of washing system
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Improper buffer/ diluent used	• Check buffer/diluent preparation, pH

Low Absorbances

Possible Source	*Corrective Action*
• Inadequate reagent volumes added to wells	• Check/ calibrate pipettes
• Incorrect incubation times/ temperature	• Ensure sufficient incubation times/ reagents warmed to RT
• Incorrect antibody titration	• Check Capture Ab and Working Detector preparation
• Improper buffer/diluent used	• Check buffer/diluent preparation, pH
• Overly high wash/aspiration pressure from automated plate-washer	• Utilize manual washing

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BD OptEIA™

Technical Data Sheet

Human IL-8 ELISA Set

Cat. No. 555244

Materials Provided

The BD OptEIA™ Set for human interleukin-8 (IL-8) contains the components necessary to develop enzyme-linked immunosorbent assays (ELISA) for natural or recombinant human IL-8 in serum, plasma, and cell culture supernatants. Sufficient materials are provided to yield approximately 20 plates of 96-wells if the recommended storage, materials, buffer preparation, and assay procedure are followed as specified in this package.

Capture Antibody
Anti-human IL-8 monoclonal antibody

Detection Antibody
Biotinylated anti-human IL-8 monoclonal antibody

Enzyme Reagent
Avidin-horseradish peroxidase conjugate

Standards
Recombinant human IL-8, lyophilized

Instruction/ Analysis Certificate
(lot-specific)

United States
877.232.8995

Canada
888.259.0187

Europe
32.53.720.211

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

BD
BD Biosciences

BD Biosciences Pharmingen
10975 Torreyana Road
San Diego, CA 92121
Customer/Technical Service
Tel 877.232.8995 (US)
Fax 858.812.8888
www.bdbiosciences.com

Recommended buffers, solutions

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

The BD OptEIA™ Reagent Set B (Cat. No. 550534) containing Coating Buffer, Assay Diluent, Substrate Reagents A and B, Stop Solution and 20X Wash Buffer Concentrate is recommended.

1. **Coating Buffer-** 0.1 M Sodium Carbonate, pH 9.5 8.40 g $NaHCO_3$, 3.56 g Na_2CO_3; q.s. to 1.0 L; pH to 9.5. Freshly prepare or use within 7 days of preparation, stored at 2-8°C.
2. **Assay Diluent-** PBS* with 10% FBS#, pH 7.0. The BD Pharmingen™ Assay Diluent (Cat. No. 555213) is recommended.
   *Phosphate-Buffered Saline: 80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0g KCl, q.s. to 10 L; pH to 7.0.
   #Fetal Bovine Serum: Hydone Cat. No. SH30088 (heat-inactivated) recommended.
   Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
3. **Wash Buffer-** PBS* with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
4. **Substrate Solution-** Tetramethylbenzidine (TMB) and Hydrogen Peroxide. The BD Pharmingen™ TMB Substrate Reagent Set (Cat. No. 555214) is recommended.
5. **Stop Solution -** 1 M H_3PO_4 or 2 N H_2SO_4

Additional Materials Required

1. 96-well BD Falcon™ ELISA Plates (Cat. No. 353279) are recommended.
2. Microplate reader capable of measuring absorbance at 450 nm
3. Precision pipettes
4. Graduated cylinder, one liter
5. Deionized or distilled water
6. Wash bottle or automated washer
7. Log-log graph paper or automated data reduction
8. Tubes to prepare standard dilutions
9. Laboratory timer
10. Plate sealers or parafilm

Storage Information

1. Store unopened reagents at 2-8°C. Do not use reagents after expiration date, or if precipitation or turbidity is evident.
2. Before use, bring all reagents to room temperature (18-25°C). Immediately after use, return to proper storage conditions.
3. Lyophilized standards are stable until expiration date. See below for reconstituted standard storage information.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic.

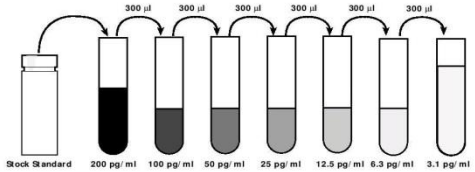
Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤-20°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum and assay immediately or store samples at ≤-20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin as anticoagulant. Centrifuge for 10 minutes at 1000 x g within 30 minutes of collection. Assay immediately or store samples at ≤-20°C. Avoid repeated freeze-thaw cycles.

Standards Preparation and Handling

1. **Reconstitution:** After warming to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized Standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.
2. **Storage/ handling of reconstituted standard:** After reconstitution, immediately** aliquot standard stock in polypropylene vials at 50 µl per vial and freeze at ≤ -70°C for up to 6 months. **Do not leave reconstituted standard at RT.**
   **If necessary, store at 2-8°C for up to 8 hr prior to aliquoting/freezing.
3. **Standards Preparation for Assay:**
   a. Prepare a 200 pg/mL standard from the stock standard. Vortex to mix.
   b. Add 300 µL Assay Diluent to 6 tubes. Label as 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.3 pg/mL, and 3.1 pg/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. Assay Diluent serves as the zero standard (0 pg/mL).



Serial dilutions within the plate may also be performed, by pipetting 100 µL of Assay Diluent into each standard well except the highest (200 pg/mL), then adding 100 µL of the 200 pg/mL standard to both that well and the 100 pg/mL well, mixing the well contents by rinsing the pipette tip, and adding 100 µL of the 100 pg/mL standard to the 50 pg/mL well. Continue these dilutions to the 3.1 pg/mL standard well, out of which the extra 100 µL should be discarded.

Working Detector Preparation

(Note: One-step incubation of Biotin/Avidin reagents.) Add required volume of Detection Antibody to Assay Diluent. Within 15 minutes prior to use, add required quantity of Enzyme Reagent, vortex or mix well. For recommended dilutions, see lot-specific Instruction/ Certificate of Analysis. For a full 96-well plate, prepare 12 mL of Working Detector. Discard any remaining Working Detector after use.

Warnings and Precautions

1. Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
2. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
3. Capture Antibody contains <0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
4. The Enzyme Reagent contains less than 0.02% of Thimerosal, an organic mercury compound. Foreseeable use of this product does not pose a known threat of reproductive toxicity. Mercury and mercury compounds are chemicals known to the State of California to cause reproductive toxicity.

Recommended Assay Procedure

1. Coat wells with 100 µL per well of Capture Antibody diluted in Coating Buffer. For recommended antibody coating dilution, see lot-specific Instruction/ Analysis Certificate. Seal plate and incubate overnight at 4°C.
2. Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.
3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room temperature (RT) for 1 hour.
4. Aspirate/wash as in step 2.
5. Prepare standard and sample dilutions in Assay Diluent. See "Standards Preparation and Handling".
6. Pipette 100 µL of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hours at RT.
7. Aspirate/ wash as in step 2, but with 5 total washes.
8. Add 100 µL of prepared Working Detector (Detection Antibody + Avidin-HRP reagent) to each well. Seal plate and incubate for 1 hour at RT.
9. Aspirate/ wash as in step 2, but with 7 total washes. NOTE: In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash.
10. Add 100 µL of Substrate Solution to each well. Incubate plate (without plate sealer) for 30 minutes RT in the dark.
11. Add 50 µL of Stop Solution to each well.
12. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. Add 100 µL diluted Capture Ab to each well. Incubate overnight at 4°C.
2. Aspirate and wash 3 times.
3. Block plates: 200 µL Assay Diluent to each well. Incubate 1 hr RT.
4. Aspirate and wash 3 times.
5. Add 100 µL standard or sample to each well. Incubate 2 hr RT.
6. Aspirate and wash 5 times.
7. Add 100 µL Working Detector (Detection Ab + Av-HRP) to each well. Incubate 1 hr RT.
8. Aspirate and wash 7 times (with 30 sec to 1 min soaks).
9. Add 100 µL Substrate Solution to each well. Incubate 30 min at RT in dark.
10. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min with λ correction at 570 nm.

Technical Data Sheet

Reagent Set B

Product Information

Material Number:	**550534**
Size:	20 plates
Component:	**51-9003739**
Description:	Wash Concentrate 20X
Size:	1 liter (1 ea)
Component:	**51-2606KZ**
Description:	Substrate Reagent A
Size:	125 ml (1 ea)
Component:	**51-2607KZ**
Description:	Substrate Reagent B
Size:	125 ml (1 ea)
Component:	**51-2608KZ**
Description:	Stop Solution
Size:	125 ml (1 ea)
Component:	**51-2641KC**
Description:	Assay Diluent
Size:	1000 ml (1 ea)
Component:	**51-2713KC**
Description:	Coating Buffer
Size:	250 ml (1 ea)

Description

The BD OptEIA™ Reagent Set B (Cat. No. 550534) is intended for use in BD OptEIA™ ELISA sets utilizing 0.1 M sodium carbonate coating buffer at pH 9.5. Reagents are provided to be sufficient for use in ELISA for 20 plates (96-well). Please refer to the BD OptEIA™ specific Technical Data Sheet and/or the Certificate of Analysis for determination of the correct coating buffer.

When using BD OptEIA™ ELISA Sets utilizing 0.2 M sodium phosphate coating buffer at pH 6.5, investigators are encouraged to use BD OptEIA™ Reagent Set A (Cat. No. 550536).

Substrate Reagent A (51-2606KZ) contains hydrogen peroxide in a buffered solution (should be clear and colorless).
Substrate Reagent B (51-2607KZ) contains 3, 3', 5, 5' tetramethylbenzidine (TMB) in an organic solvent.
Substrate Reagent B should be colorless to a very light amber.
Wash Concentrate (20X) (51-9003739) contains concentrated detergent solution with ProClin™-150 as a preservative (should be clear and colorless). If the Wash Concentrate contains visible crystals or precipitate, warm to room temperature and mix gently until dissolved. Prepare a working concentration of wash buffer (1X) by diluting the required quantity of 20X Wash Concentrate with deionized or distilled water (e.g. to prepare 2.0 L of Wash Buffer, add 100 mL 20X Wash Concentrate to 1900 mL water). At least 500 mL Wash buffer (1X) should be prepared for one 96-well plate.
Stop Solution (51-2608KZ) contains 1M phosphoric acid (should be clear and colorless).
Assay Diluent (51-2641KC) contains animal serum in a buffered solution with ProClin™-150 as a preservative (should be clear and red in color). Assay Diluent is designed to be used for (a) the blocking of ELISA plates, (b) performing serial dilutions of the recombinant standard or the experimental sample, and/or (c) for the preparation of the Working Detector outlined in BD OptEIA™ protocols.
Coating Buffer (51-2713KC) contains 0.1 M sodium carbonate buffer, pH 9.5 (should be clear and colorless).

Preparation and Storage

Before use, bring all reagents to room temperature (18-25°C). Immediately after use, return to proper storage conditions.
Store all components at 2-8°C.
Do not use components past the expiration date. Discard any remaining working solutions after use.
Avoid prolonged exposure to light, contact with metal, air, or extreme temperatures, as color may develop.

BD Biosciences
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877.232.8995	800.979.9408	32.53.720.550	0120.8555.90	65.6861.0633	55.11.5185.9995

For country contact information, visit **bdbiosciences.com/contact**

Application Notes

Application

ELISA	Routinely Tested

Recommended Assay Procedure:

TMB Preparation: Prepare a working concentration of TMB substrate solution within 15 minutes prior to use by mixing equal volumes of Substrate Reagent A and Substrate Reagent B (e.g. for one 96-well plate, a 12 mL TMB substrate working solution can be prepared by mixing 6 mL of Substrate Reagent A with 6 mL of Substrate Reagent B). After mixing, the solution should be colorless to a very faint blue. In the presence of peroxidase-labeled conjugates, the solution should develop a definitive blue color. After the addition of Stop Solution, the color changes from blue to yellow.

Warnings and Precautions

Wash Concentrate (20X) (51-9003739)
Code letter and hazard designation of product: Xi (Irritant)
Risk phrases:
36/37/38 Irritating to eyes, respiratory system and skin.
43 May cause sensitization by skin contact.
Safety phrases:
7 Keep container tightly closed.
23 Do not breathe gas/fumes/vapour/spray.
24 Avoid contact with skin.
26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
37 Wear suitable gloves.
60 This material and its container must be disposed of as hazardous waste.

Substrate Reagent B (51-2607KZ)
Code letter and hazard designation of product: T (Toxic) ; F (Highly flammable)
Risk phrases:
11 Highly flammable.
23/24/25 Toxic by inhalation, in contact with skin and if swallowed.
36/37/38 Irritating to eyes, respiratory system and skin.
39/23/24/25 Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed.
Safety phrases:
4 Keep away from living quarters.
7/9 Keep container tightly closed and in a well-ventilated place.
16 Keep away from sources of ignition - No smoking.
36/37/39 Wear suitable protective clothing, gloves and eye/face protection.
45 In case of accident or if you feel unwell, seek medical advice immediately.
60 This material and its container must be disposed of as hazardous waste.

Stop Solution (51-2608KZ)
Code letter and hazard designation of product: Xi (Irritant)
Risk phrases:
36/38 Irritating to eyes and skin.
Safety phrases:
23 Do not breathe gas/fumes/vapour/spray.
26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
37 Wear suitable gloves.
45 In case of accident or if you feel unwell, seek medical advice immediately.
60 This material and its container must be disposed of as hazardous waste.

Product Notices

1. ProClin is a trademark of Rohm and Haas Company.
2. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
3. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

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