

# **Stability and clinical efficacy of honeybush extracts in cosmeceutical products**

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*maculata* cream

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## ABSTRACT

The progression of skin ageing in individuals is multifaceted and provoked by various aspects, including hereditary and a variety of environmental causes, for instance UV (ultra violet) radiation, resulting in the morphological modifications in the dermal layer of the skin (Makrantonaki & Zouboulis, 2007:40). Transformations caused by ageing skin, in which degenerative alterations exceed regenerative alterations are recognised by the thinning and wrinkling of the epidermis in conjunction with the appearance of lines, creases, crevices and furrows, particularly emphasised in lines of facial expressions (Aburjai & Natsheh, 2003:990).

For human beings to continue to exist in a terrestrial atmosphere, the loss of water from the skin must be cautiously synchronised by the epidermis, a task dependent on the multifaceted character of the stratum corneum (Rawlings & Harding, 2004:43). The stratum corneum (SC) is responsible for the main resistance to the penetration of most compounds; nevertheless the skin represents as an appropriate target for delivery. The target site for anti-ageing treatment includes the epidermal and dermal layers of the skin. Therefore, the need to apply fatty materials to the skin is practically intuitive and may perhaps be as old as man's existence itself (Lodén, 2005:672). Natural therapies have been used for several decades for taking care of skin illnesses and a wide variety of dermatological disorders, such as inflammation, phototoxicity, atopic dermatitis and alopecia areata (Aburjai & Natsheh, 2003:988).

Using the skin as an alternative route for the administration of honeybush extracts for the treatment of ageing skin may be beneficial. Tea contains more than 500 chemical compounds, including, tannins, flavonoids, amino acids, vitamins, caffeine and polysaccharides. Tea polyphenols (flavonoids) have proven anti-inflammatory, antioxidant, antiallergic, antibacterial and antiviral effects (Aburjai & Natsheh, 2003:990). Unfortunately using the skin as an alternative route for administering drugs (transdermal drug delivery) has numerous limitations. One of these limitations is the barrier function of the skin (Naik *et al.*, 2000:319). Because of the skin's outstanding ability to protect the body against unwanted substances from its surroundings, it is necessary to use methods to enhance drug penetration through the skin.

The aim of this study was to formulate two 2% semisolid formulations containing two different honeybush extracts as the active ingredient, and to determine which of the formulations deliver mangiferin and hesperidin best to the target site (dermis). Cosmetic formulations of a natural origin, is designed to protect the skin against exogenous or endogenous harmful agents, as well as to balance the dermal homeostatis lipids altered by dermatosis and ageing (Aburjai & Natsheh, 2003:988).

Stability tests over a three month period were also performed on the different formulations. To determine the stability of the different semi-solid formulations, the formulated products were stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH. HPLC analysis was used to determine the concentrations of the ingredients in all the formulated products. Other stability tests included appearance, pH, viscosity, mass loss, zeta potential and particle size determination. Unfortunately a change in colour, viscosity, zeta potential, mass loss, particle size and concentration of the ingredients in both the formulations, indicated that the products were unstable from the first month of stability testing.

A 2% *Cyclopia maculata* cream as well as a 2% *Cyclopia genistoides* cream was formulated. Franz cell diffusion studies as well as membrane release studies were performed over a 12 h period, followed by tape stripping experiments to determine which semi-solid formulation delivered mangiferin and hesperidin the best to the dermal layer of the skin. The results of the different formulations were compared. Unfortunately there was no significant penetration by any of the honeybush extracts. Results were inconclusive and unquantifiable due to unconvincing penetration results.

The antioxidant properties of both the extracts and the active ingredients were calculated. Antioxidant studies by the use of the TBA-assay method were done to determine whether the honeybush extracts, mangiferin and hesperidin as well as their semisolid formulations had any antioxidant activities. Both the honeybush extracts and the semisolid formulations showed promising results. Mangiferin and hesperidin did not show any antioxidant activity on their own, therefore the assumption can be confirmed that plants do function synergistically.

A clinical study was also conducted to see whether honeybush extracts have the potential to hydrate the skin, counteracting the symptoms and signs of skin ageing. Clinical efficacy studies were done to determine whether the honeybush formulations had any skin hydrating effects in the treatment against skin ageing. The results were statistically inconclusive and variations between the subjects were very high due to skin variations at different skin sites. There was however a trend that *Cyclopia genistoides* performed the best.

Keywords: *Cyclopia maculata*, *Cyclopia genistoides*, honeybush, transdermal diffusion, formulation, stability testing, antioxidant, clinical efficacy

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## UITTREKSEL

Die progressie van velveroudering by individue is veelvlakkig en word veroorsaak deur verskeie aspekte, insluitende erflike en 'n verskeidenheid van omgewingsfaktore, byvoorbeeld ultravioletbestraling wat morfologiese modifikasies in die dermale laag van die vel tot gevolg het (Makrantonaki & Zouboulis, 2007:40). Transformasies van die verouderende vel waarin degeneratiewe veranderings die regeneratiewe veranderings oortref, is sigbaar in die verdunning en plooiing van die epidermis. Dit gaan gepaard met die verskyning van lyne, plooië, skeure en vore, veral beklemtoon in die lyne van gesigsuitdrukkinge (Aburjai & Natsheh, 2003:990).

Vir mense om voort te bestaan in 'n aardse atmosfeer moet die waterverlies van die vel versigtig gesinchroniseer word deur die epidermis. Hierdie taak is afhanklik van die veelvlakkige karakter van die stratum corneum (Rawlings & Harding, 2004:43). Die stratum corneum (SC) is verantwoordelik vir die hoofweerstand teen penetrasie van die meeste verbindings. Nogtans bied die vel 'n geskikte teiken vir oordrag. Die teikenlokaliteit vir die behandeling teen veroudering sluit die epidermiese en dermale lae van die vel in. Dus is die behoefte om vetterige stowwe op die vel aan te wend prakties intuïtief en dit kan miskien so oud wees soos die mens self (Lodén, 2005:672). Natuurlike terapieë is al vir verskeie dekades lank gebruik vir die behandeling van velsiektes en 'n groot verskeidenheid van dermatologiese ongesteldhede, soos inflammasie, fototoksiteit, atopiese dermatitis en alopecia areata (Aburjai & Natsheh, 2003:988).

Om die vel te gebruik as 'n alternatiewe roete vir die toediening van heuningbosekstrakte vir die behandeling van verouderende vel, kan voordelig wees. Tee bevat meer as 500 chemiese verbindings insluitende tannien, flavonoïede, aminosure, vitamien, kaffeïen en polisakkariede. Daar is bewys dat teepolifenole (flavonoïede) anti-inflammatoriese, antioksidant-, anti-allergiese, antibakteriële en antivirale eienskappe het (Aburjai & Natsheh, 2003:990). Ongelukkig het die gebruik van die vel as 'n alternatiewe roete vir die toediening van geneesmiddels (transdermale geneesmiddeloordrag) baie beperkings. Een van hierdie beperkings is die versperringsfunksie van die vel (Naik *et al.*, 2000:319). Vanweë die vel se uitstaande vermoë om die liggaam te beskerm teen ongewenste stowwe uit die omgewing, is dit nodig om metodes te gebruik wat die geneesmiddelpenetrasie deur die vel verhoog.

Die doel van hierdie studie was om twee 2% semisoliede formuleringe te formuleer wat twee verskillende heuningbosekstrakte as aktiewe bestanddeel bevat en om vas te stel watter een van die formuleringe mangiferien en hesperidien die beste oordra op die teikenlokaliteit (dermis). Kosmetiese formuleringe met 'n natuurlike oorsprong word ontwerp om die vel te

beskerm teen eksogene of endogene skadelike middels asook om die dermale homeostatiese lipiede wat verander is deur dermatose en veroudering, te balanseer.

Stabiliteitstoetse op die verskillende formuleringe is ook oor 'n tydperk van drie maande uitgevoer. Om die stabiliteit van die verskillende semisoliede formuleringe vas te stel, is die geformuleerde produkte gestoor teen 25° C/60% RH (relatiewe humiditeit), 30 C/60% RH en 40 C/75% RH. HPLC-analise is gebruik om die konsentrasies van die bestanddele in al die geformuleerde produkte vas te stel. Ander stabiliteitstoetse het voorkoms, pH, viskositeit, massaverlies, zetapotensiaal en deeltjiegroottebepaling ingesluit. Ongelukkig het 'n verandering in kleur, viskositeit, zetapotensiaal, massaverlies, deeltjiegrootte en konsentrasie van die bestanddele in albei formuleringe aangedui dat die produkte onstabiel was vanaf die eerste maand van die stabiliteitstoetsing.

'n 2% *Cyclopia maculata*-room sowel as 'n 2% *Cyclopia genistoides*-room is geformuleer. Franz sel-diffusiestudies sowel as membraanvrystellingstudie is uitgevoer oor 'n periode van 12 uur, gevolg deur "tape stripping"-eksperimente om vas te stel watter semisoliede formulering mangiferien en hesperidien die beste oordrag verskaf aan die dermale laag van die vel. Die resultate van die verskillende formuleringe is vergelyk. Ongelukkig was daar geen beduidende penetrasie deur enige van die heuningbosekstrakte nie. Die resultate was nie-deurslaggewend en onkwantifiseerbaar te wyte aan onoortuigende penetrasieresultate.

Die anti-oksidadant-eienskappe van albei die ekstrate en die aktiewe bestanddele is bereken. Anti-oksidadantstudie deur toepassing van die TBA-onderzoekmetode is gedoen om vas te stel of die heuningbosekstrakte, mangiferien en hesperidien sowel as hulle semisoliede formuleringe enige anti-oksidadantaktiwiteit gehad het. Sowel die heuningbosekstrakte as die semisoliede formuleringe het belowende resultate getoon. Mangiferien en hesperidien het geen anti-oksidadantaktiwiteit op hulle eie getoon nie, dus kan die aanname bevestig word dat plante sinergisties funksioneer.

'n Kliniese studie is ook uitgevoer om te kyk of heuningbosekstrakte die potensiaal het om die vel te hidrateer en sodoende die simptome en tekens van velveroudering teë te werk. Kliniese doeltreffendheidstudie is gedoen om vas te stel of die heuningbosformuleringe enige velhidrateringseffekte gehad het tydens die behandeling teen velveroudering. Die resultate was statisties nie-deurslaggewend en variasies tussen die proefpersone was baie groot weens die verskille in die vel op verskillende vellokaliteite. Daar was egter die neiging dat *Cyclopia genistoides* die beste vertoon het.

Sleutelwoorde: *Cyclopia maculata*; *Cyclopia genistoides*, heuningbos, transdermale diffusie, formulering, stabiliteitstoetsing, anti-oksidadant, kliniese doeltreffendheid.

## Verwysings

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## FOREWORD

In this study we aimed at investigating the transdermal delivery of mangiferin and hesperidin – bioactive flavonoids present in various honeybush extracts and species. Honeybush extracts within a cream was formulated. The formulations were stored under different conditions, and stability tests were performed over a three months period. Antioxidant properties and its clinical efficacy on human subjects were also tested.

This dissertation is presented in the so-called article format, which includes introductory chapters and a full length article for publication in a pharmaceutical journal (Chapter 3). The data procured during the studies are attached in the appendices. The article in this dissertation is to be submitted for publication in *Skin Pharmacology and Physiology* of which the complete guide for authors is included in the Appendix G.

## CHAPTER 1

### INTRODUCTION AND STATEMENT OF THE PROBLEM

Skin ageing is the end result of a constant corrosion process as a result of the injury to cellular deoxyribonucleic acid (DNA) and proteins. The age progression can be divided into two very diverse types, i.e. “sequential skin ageing” (intrinsic) and “photo-ageing” (extrinsic). Intrinsic skin ageing is a common and expected process characterised by physiological modifications in the skin function. Keratinocytes are incapable to form a purposeful stratum corneum and the tempo of the arrangement of neutral lipids slows down dramatically, resulting in dry, pale skin with fine wrinkles. Extrinsic ageing is caused by overexposure to UV (ultraviolet) radiation from sunlight. It can be characterised by dry, pale and shallow skin, displaying fine wrinkles as well as deep furrows caused by the disorganisation of epidermal and dermal components associated with elastosis and heliodermatitis (Ahshawat *et al.*, 2008:184).

With the earliest mention of honeybush in botanical literature in 1705 (Kies, 1951:161), it was soon recognised as a plant with various medicinal properties (ASNAPP, 2010:1). The term honeybush applies to several different species of *Cyclopia*. These are all woody, fynbos shrubs restricted to the mountains near the Cape Peninsula (ASNAPP, 2010:4). Phenolic compounds found in these shrubs are known to be mangiferin, hesperidin, hesperitin and isosakuranentin (De Nysschen *et al.*, 1996:243). Honeybush was originally used as a restorative and an expectorant in chronic catarrh and pulmonary tuberculosis but was later on also know for its anti-inflammatory, antioxidant, antimutagenic, phyto-oestrogenic and antimicrobial effects with a relative low toxicity (Joubert *et al.*, 2008:376).

The use of plants, such as honeybush, were once the main source and foundation of all cosmetics, before methods were discovered of synthesising substances with similar properties (Aburjai & Natsheh, 2003:987). These herbal extracts for topical application deserves to be considered as a cosmeceutical because of their use of treating skin conditions and a wide variety of dermatological disorders for centuries (Aburjai & Natsheh, 2003:988). It can be designed to protect the skin against exogenous and endogenous agents, balancing dermal homeostasis lipids altered by dermatosis and ageing. Plants with a high level of flavonoids such as honeybush, have the potential to reduce skin inflammation and to scavenge free radicals (Aburjai & Natsheh, 2003:990), penetrating the dermal and epidermal layers while counteracting the ageing of the human skin.

Using the skin as an alternative route for the administration of drugs has become very popular over the last few decades and using intact skin as the site of administration for dermatological preparations to elicit pharmacological action in the skin tissue has been well recognised (Barr, 1962:395). The human skin forms a remarkable protective barrier against the external environment, regulating temperature and water balance. This barrier also keeps out harmful microbes and chemicals (Aburjai & Natsheh, 2003:988). Unfortunately, using the skin as an alternative route for administering drugs also has numerous limitations. One of these limitations is the barrier function of the skin (Naik *et al.*, 2000:319). This “horny layer”, which comes in direct contact with the environment, is a collection of dead cells, but is also a very complex organism and forms an integral part in the homeostatic system of the human body (Aburjai & Natsheh, 2003:988).

Although the stratum corneum gives the body outstanding protection against unwanted substances from its surroundings, it is possible for drugs to be administered transdermally. Three possible pathways for transport of drugs through the skin exist, namely intercellular diffusion through the lipid lamellae, transcellular diffusion through the keratinocytes and lipid lamellae and diffusion through hair follicles and sweat ducts (Ho, 2003:50). Regrettably it is simply appropriate for a restricted quantity of drugs and substances that have the proper physiochemical features to allow them to cross the stratum corneum (Harrison *et al.*, 1996:283). There are however several factors that also may affect permeation of drugs through the skin and a few of these factors are skin age, skin condition, skin site, skin metabolism, skin hydration, temperature, pH and the presence of penetration enhancers (Dayan, 2007:31). Therefore, the lipophilic stratum corneum is responsible for the primary barrier function of the skin and present a wide-ranging challenge to scientists in their pursuit to extend the range of drugs suitable for transdermal delivery (Pefile & Smith, 1997:147).

The aim of this study was to investigate the transdermal delivery of mangiferin and hesperidin – bioactive flavonoids present in various honeybush extracts and species, as well as the antioxidant properties and its clinical efficacy on human subjects.

In order to achieve this goal, the following objectives were set:

- Developing and validating a HPLC (high performance liquid chromatography) method to quantitatively determine concentrations of the different active ingredients (mangiferin and hesperidin) in the formulations

- Formulation of two different 2% cream formulations with two different honeybush extracts (*Cyclopia maculata* and *Cyclopia genistoides*) containing mangiferin and hesperidin
- Stability tests on the different formulations, stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH, were conducted. HPLC analysis was used to determine the concentrations of the ingredients in all the formulated products. Other stability tests included appearance, pH, viscosity, mass loss, zeta potential and particle size determination.
- Determining whether mangiferin and hesperidin were released from the formulations by using membrane release studies
- Determining whether mangiferin and hesperidin diffused through the skin after different formulations were applied to the skin by making use of Franz cell diffusion studies
- Determining whether mangiferin and hesperidin were present in the target site (dermal layer) by using tape stripping technique after the different formulations were applied to the skin
- Determination of the antioxidant activity of honeybush extracts, mangiferin, hesperidin and their semisolid formulations
- Determining whether various honeybush extract formulations have any anti-ageing clinical effects on human subjects

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## CHAPTER 2

# TRANSDERMAL DELIVERY OF HONEYBUSH EXTRACTS FOR THE TREATMENT OF SKIN AGEING

### 2.1 Introduction

Ever since the word “cosmeceutical” was used more than twenty years ago, the number of cosmeceutical products that declare to fight dermal ageing has increased significantly. The effectiveness of numerous chemical substances for the prevention and management of dermal ageing is well recognised and the ageing of the baby boomer population has given rise to amplified user curiosity in preserving a younger-looking exterior right through middle age (Bruce, 2008:S17).

The use of flora in herbal remedies is as ancient as mankind. It was previously used as the most important resource of all cosmetics, prior to methods synthesising ingredients with comparable properties. Original “bioactive” components are derived from the ocean, the earth and the plant kingdom. Accepted constituents consist of Chinese herbs, vitamins, minerals, antioxidants, enzymes, hormones and a large amount of “natural ingredients”. Natural products such as honeybush extracts in cosmetic preparations can be used in skin care products to treat conditions such as skin dryness, eczema, acne, free radical scavenging, anti-inflammatory and skin ageing (Aburjai & Natsheh, 2003:987).

Herbal cosmetic creams could have the potential to improve skin visco-elasticity and skin hydration. In this study the focus will be on the clinical and transdermal effects of honeybush extracts on the ageing skin.

### 2.2 Skin ageing

Since the end of the 1800's the subject of skin ageing has been discussed and debated by dermatologists all over the world (Baumann, 2007:241). Not only is the skin the human body's largest organ but also our only organ responsible for sensory touch, temperature control and the symbol of beauty, alluring man since the earliest of times. Ageing can be defined as the process where structural integrity and physiological functions are weakened by both intrinsic and extrinsic factors. These factors include various physiological and environmental influences that cause this degradation of human skin ageing and starts getting noticeable at approximately

the age of 25. After World War II the so-called “baby boomers” generation with a birth date between 1945 and 1965 created a culture of leisure, conservation, youthful looks and overall well-being. This is where the development of anti-ageing products really emerged onto global markets – one of the most financially rewarding industries in the world today (Farage *et al.*, 2008:88).

## **2.2.1 Characteristics of ageing skin**

Ageing skin can be characterised by even and unblemished skin with normal geometric patterns and a variety of overstated expression lines. Looking at skin histologically, these signs are evident in the epidermal and dermal layers of the skin, flattening the epidermal ridges and reducing the amount of fibroblasts and mast cells in the skin layers. Wrinkling, sagging and increased fragility are all associated with skin ageing, usually more noticeable in exposed areas such as the face, chest and extensor surfaces of the arms (Baumann, 2007:242).

### **2.2.1.1 Ageing of the epidermis**

With ageing certain integral changes take place in the epidermal layer of the human skin. Studies done, according to Baumann (2007:243), proved that the epidermis becomes thinner with ageing skin, while the stratum corneum remains the same throughout life. Unexposed epidermal tissue has an overall thinning of 10 to 50% between the age of 30 and 80 years, as stated by Wulf *et al.* (2004:186). The most influential factor causing this damage to human skin is sun exposure. In a histopathological study of 83 biopsies from volunteers aged between 6 and 84 years, they found that overall thickness of skin was greater in older volunteers with longer sun-exposure time (Baumann, 2007:242).

Decreased cell turnover is also a major factor in the age-related changes in the epidermal layer of the skin. According to Robert *et al.* (2009:337) this can be attributed to two dissimilar processes: a slow-down of cell partitioning due to telomere loss and secondly, the departure of cells from the mitotic pool mediated by anti-oncogenes through a “switch mechanism” enabling cells to relinquish the mitotic pool and enter the senescent phenotype while escaping from malignancy. Transit time in the stratum corneum in adolescents is approximately 20 days, whereas older adults have a transit time of more or less 30 days, implicating that cell cycle expansion coincides with a prolonged stratum corneum replacement rate. The stratum corneum and stratum granulosum seem to be unaffected by ageing, while epidermal atrophy seems to affect mostly the spinous cell layer of the epidermis (Wulf *et al.*, 2004:186). Therefore, the skin will take longer periods to heal itself with less efficient desquamation. The appearance of the

skin surface will become rough, uneven and dull due to the increase of corneocytes – created from this cascade of decelerated cell turnover. , It is believed by cosmetic dermatologists that hydroxy acids and retinoid aids counteracting this phenomenon by faster cell turnover (Baumann, 2007:244).

#### **2.2.1.2 Ageing of the dermis**

According to Baumann (2007:244) approximately 20% of dermal width disappears as skin grows old. This aged dermis becomes acellular and avascular during structural inspection. The three main structural components responsible for maintaining a healthy dermis are collagen, elastin and glycosaminoglycans, which also undergo degradation during the ageing process.

Collagen, the most important structural component of the dermis and the most prolific protein found in humans, offers strength and support to human skin. Dry skin mass, comprising of 70% collagen will become thickened fibrils forming prearranged rope-like bundles and emerging in total disorder as the skin ages. Reproduction of collagen will diminish *in vivo* and *in vitro*, and the ratio of collagen types will change dramatically. In adolescent skin collagen-I comprises 80% and collagen-III comprises about 15% of total skin collagen. When the skin ages, the ratio of type III to type I collagen increases due to the loss of collagen-I, with an overall collagen per unit skin surface reduction of more or less 1% per annum (Baumann, 2007:244).

Secondly, modifications in elastic fibres lead to the gathering of amorphous elastin material in the skin due to the harmful properties of UV (ultraviolet) skin damage, also known as “elastosis”. During chronic UV exposure, elastic fibres coil and thicken in the papillary and reticular dermis. This reduces the number of microfibrils in the dermis. Although it is not yet fully understood how elastin changes with age, it is believed that matrix metalloproteinases (MMP-2) plays a major role in the degradation of elastin. The level of exposure to the sun will result in a greater amount of elastin tissue in the skin. However, with ageing skin a secondary reaction to photo-damaged skin will result in a reduction of elastic fibres and a remarkable decline in skin elasticity and resilience, usually characterised by modifications in the regular pattern of undeveloped elastic fibres (oxytalan), positioned in the papillary dermis. Oxytalan, an arrangement of fibres in youthful skin rising vertically from the highest section of the papillary dermis to just below the basement membrane, progressively disappears with age, resulting in loss of skin elasticity and thus in sagging skin (Baumann, 2007:245).

Finally, glycosaminoglycans or GAG’s form the third and final structural component in dermal skin and are responsible for presenting the external appearance of the skin. Hyaluronic acid,

dermatan sulphate and chondroitin sulphate are but three of the members of the GAG family. Consisting of polysaccharide chains repeated with disaccharide units attached to a core protein, these GAG molecules have the capability to attach to water up to 1000 times their own volume. With this great hydration potential, regular skin appears plump, supple and hydrated. It is also understood that GAG molecules aid in maintaining a balanced salt:water ratio within the skin. With age, hyaluronic acid connections fade away leaving collagen and elastin disassociated. This effect leads to skin looking dry and wrinkled, with a reduction in elasticity and turgidity, as well as a decrease in the supportive ability of microvasculature of the skin (Baumann, 2007:245).

### 2.2.1.3 Changes in skin appearance

Singh (2009:447) described aged skin as thin, moderately flattened, dehydrated and unblemished with loss of elasticity and age-related loss of architectural regularity. Several contributing factors of skin ageing include the constant effect that gravity has on soft tissue, resulting in sagging skin over the facial skeleton. Other influential factors are chronic sun exposure, hormonal changes (menopause), a decrease in skin blood circulation, weight gain due to slower metabolism and fat “depots” in certain body regions, facial and ligament laxity, skeletal resorption and the decline of glandular tissue (Singh, 2009:448).



**Figure 2.1:** Changes in skin appearance due to skin ageing (Adapted from Anti Aging Links.com, 2012).

In the elderly the skin is usually dry and scaly according to Baumann (2007:246). With the degradation and loss of the skin barrier function and increased transepidermal water loss (TEWL), recovery of aged skin slows down, leaving the stratum corneum more susceptible to become dry and resulting in greater susceptibility to develop these so-called symptoms. Fine

wrinkles, thin and transparent skin becomes more apparent with loss of underlying facial fat leading to hollowed cheeks and eye sockets (Singh, 2009:448). This can be attributed to a multifactorial progression due to lower lipid levels in lamellar bodies and a decrease in epidermal filaggrin. Dehydrated skin can thus be characterised by an uneven skin surface, wrinkling, skin paleness, hyper- or hypopigmentations, laxity, itching, vulnerability, easy bruising and increased risk of benign or malignant neoplasms. Benign neoplasms can be recognised by acrochordons (skin tags), cherry angiomas, seborrheic keratoses, lentigos (sun spots) and sebaceous hyperplasias (Baumann, 2007:246).

## **2.2.2 Factors causing ageing of the human skin**

According to several authors, there are two types of ageing determinants namely intrinsic ageing and extrinsic ageing responsible for progressive loss of structural integrity and physiological functions of human skin (Singh, 2009:448; Farage *et al.*, 2008:87).

### **2.2.2.1 Intrinsic age determinants**

Intrinsic ageing, defined by Farage *et al.* (2008:88), is the ageing of human skin as an ordinary outcome of physiological changes over a period of time at unpredictable yet inalterable genetically determined rates. In short, it is the slow but gradual biological ageing process from within the human body. Factors that play a major role in intrinsic ageing include ethnicity, anatomical variations and hormonal influences. These factors can differ from populations, individuals as well as different anatomical sites and can be radically subjected to personal and environmental factors, particularly the total amount of sun exposure during an individual's life span (Singh, 2009:448). At a cellular level specialised structures such as telomeres, found at the end of eukaryotic chromosomes, are alleged to play a vital role in the intrinsic ageing process. Telomere length shortens with age, known as telomeric erosion, and serves as a method of measuring age. This forms the foundation for one of the preferred theories on ageing. Researchers determined that telomere shortening associated with cellular ageing of human skin can be characterised by tissue-specific loss rates (Baumann, 2007:242). According to Jenkins (2002:802), intrinsic ageing is the consequence of an assortment of various events such as decreased proliferative capability of skin-derived cells, decreased matrix synthesis in the dermis and increased expression of enzymes that degrade the collagenous matrix.

Looking at ethnicity, pigmentation differences perform an essential function in ageing of human skin. High levels of pigmentation are protective against the effects of photo-ageing. A typical example of defensive pigmentation can be seen in African-Americans, who show modest

cutaneous variation between exposed and unexposed skin. Basal cells carcinoma and squamous cell carcinoma occur almost solely on photo-damaged Caucasian skin, proving that African-American pigmentation provides up to 500-fold more protection against UV radiation, compared to light-skinned individuals. This can be attributed to the fact that African-Americans' skins are more compacted and contain a higher amount of intercellular lipid content, thus resisting the ageing process (Farage *et al.*, 2008:89).

Gigantic anatomic variations have been observed in some skin parameters regarding different body sites. Skin width ranges from less than 0.5 mm on the eyelids to more than 6 mm on the soles of the feet. A decrease in epidermal width was found to be slighter at the temple than at the volar forearm due to the cumulative effect of photo-ageing. In the stratum corneum lipid composition plays an integral role in regional variation in both the content and compositional profile. When the palmoplantar stratum corneum is compared to extensor surfaces such as extremities, abdominal and facial stratum corneum, there is a much higher proportion of sphingolipids and cholesterol present in the palmoplantar stratum corneum. Therefore, it can be understood that there is an inverse correlation between the lipid weight percentage of a particular body site and its permeability. The rigidity of skin is also much higher at the forehead than at the cheeks in post-menopausal women. High blood flow in areas such as the nose tip, lip, finger and forehead decreases with age when compared to areas with baseline low blood flow. Interesting enough, no difference was observed in terms of skin thickness, however sensory sensitivity decreases more profoundly in the nasolabial fold and cheek, than in the chin and forehead (Farage *et al.*, 2008:89).

When oestrogen levels in the skin of menopausal women decrease, the following changes usually take place: vaginal epithelium atrophies, a decrease in collagen and water content, poor wound healing, a decrease in skin thickness and alterations in epidermal lipid synthesis. These symptoms all have an effect on the ageing skin (Singh, 2009:448).

Taking these three determinants into account, the common signs of intrinsic skin ageing, according to Singh (2009:448), are fine wrinkles, dry skin with pruritus, the inability to sweat sufficiently to cool the skin, the greying of hair, bone shrinkage away from the skin resulting in the sagging of skin and bone loss and lastly the loss of underlying fat leading to hollowed cheeks and eye sockets. All this is due to epidermal and dermal atrophy and the reduced amount of fibroblast and mast cells, once again leading to an increase in collagen fibrils and the collagen-III to collagen-I ratio. Overall signs of intrinsic ageing skin are also a smooth, unblemished skin, fading skin colour with the diminishment of pigment. The skin surface markings maintain its youthful geometric patterns with the loss of elasticity (Farage *et al.*,

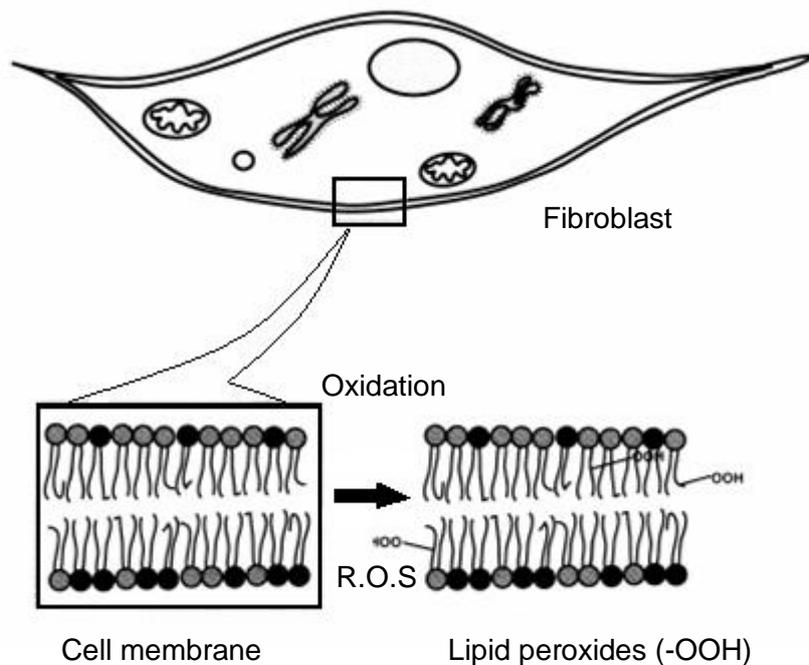
2008:93). It is very important to notice that the effects of intrinsic ageing, which occurs between the age of 50 and 60 years, and extrinsic ageing have totally different characteristics as well as dissimilar effects on the skin.

### **2.2.2.2 Extrinsic age determinants**

Extrinsic factors are controllable and preventable. Exposure to sunlight, greenhouse gases or smoking is all components leading to premature ageing. Repetitive muscle movements, especially frowning and squinting, and overall well-being (diet and sleep) are all contributing factors (Baumann, 2007:243). Farage *et al.* (2008:90-93) clearly state that extrinsic age can start as early as the late teenage years and the two greatest exogenous factors are cigarette smoking and UV light.

Exposure to sunlight and UV radiation is the main contributor to the ruining of human skin. The effects of solar exposure on human skin are so intense, it is estimated to be responsible for up to 90% of observable skin damage, particularly in skin lacking natural protection and with low levels of melanocytes. Sunlight exposure initiates an outbreak of several molecular and cellular responses that end with a fast dynamic disorder. Sunlight consists of three dissimilar types of radiation, namely UVB (290 – 320 nm), UVC (100 – 290 nm) and UVA. UVC is mainly blocked by the ozone layer with almost no effect on human skin. UVB-light causes erythema during acute sunburn and only penetrates the epidermis. It was initially believed that it required 1000-fold elevated levels of UVA radiation to have the similar burning effect of UVB on skin and was considered to have no effect on skin ageing. However, it was soon realised that UVA penetrates as deep as the dermis and is responsible for chronic skin damage and photo-ageing, not immediately visible, but as time passes. The mechanism of this damage is due to a molecular chain reaction with the upregulation of matrix metalloproteinases in both the dermis and epidermis. This results in the inhibition of the production of collagenase, gelatinase A and stromelysin-1 in fibroblasts and keratinocytes (Farage *et al.*, 2008:91). According to Singh (2009:449) photo-aged skin can be classified according to the Glogau score and by the degree of wrinkling observed. Mild skin damage, usually between the age of 28 and 35 years, shows a small number of wrinkles with no keratoses. Moderate photo-aged skin (between 35 and 50 years of age); demonstrates early wrinkling with pale complexion and early actinic keratoses. Advanced skin damage (50 – 60 years), has persistent wrinkling with discolouration of the skin and telangiectases and actinic keratoses. Lastly, severe skin damage between the age of 65 and 70 years, has severe wrinkling and photo-ageing. Actinic keratoses are present with or without skin cancer, with gravitational and dynamic forces affecting the elasticity of the skin.

Absorption of above-mentioned UV light by skin molecules, also produces damaging compounds or cells, also known as reactive oxygen species (ROS), which damage cellular components in the skin. These damaged components include cell walls, lipid membranes, DNA and the mitochondria. With the formation of these sunburn cells, thymine and myrimidine dimmers, molecular pathways are put into disarray, while UVB-light suppresses the immune system leading to the formation of active molecules called tumour necrosis factor (TNF)-alpha and *cis*-urocanic acid (Singh, 2009:449; Baumann, 2007:243). This causes the dejection of delayed hypersensitivity, the suppression of T-lymphocytes and the commencement of cutaneous herpes simplex infections (Singh, 2009:449). Other mechanisms responsible for premature skin ageing include the formation of excessive mast cells and macrophages, the formation of UV-induced apoptotic cells and the presence of infiltrating mononuclear cells. As a result, it can be understood that a smaller number of sunburn cells present in the skin will determine lower skin damage levels due to UV exposure (Baumann, 2007:243).



**Figure 2.2:** Schematic representation of the oxidation of phospholipids in the membrane by ROS (Nicolaj & Paillet, 2002:269).

The overall clinical signs of cutaneous photo-ageing include the change in visible colour with irregular pigmentation. The skin appears nodular, rubbery and blemished. Atrophy of the epidermis appears in the final stages of photo-ageing with a higher than normal proliferation rate. Vitamin A is destroyed by sunlight (Farage *et al.*, 2008:93).

The influence of everyday life is an additional extrinsic skin-ageing factor. Temperature and humidity play a fundamental part in the ambient conditions of the skin. An increase of skin temperature of approximately 7 – 8 °C doubles the evaporative water loss, while lower temperatures reduce the evaporative water loss and stiffens the skin. This occurs even in elevated humidity air due to the fact that structural proteins and lipids in the skin are entirely dependent on the temperature for proper conformation. Certain medications such as hypocholesterolemic drugs may possibly bring on irregular desquamation increase (Farage *et al.*, 2008:90).

Effects of tobacco smoking and nicotine on skin can be directly associated with elastosis in both sexes and red spots on skin, formally known as telangiectasia. Not only does it have a serious effect on internal organs, but it also affects the appearance of the skin and alters body weight and body shape. A smoker's skin usually appears to be worn out and appears greyish due to a decrease in capillary blood flow to the skin. All this leads to an oxygen and nutrient deficiency in the cutaneous tissue with lower concentrations of collagen and elastin fibres in the dermis. The skin becomes loose, toughened and shapeless (Singh, 2009:449). This degradation of human skin can be attributed to the induction of matrix metalloproteases, a large family of degradative enzymes, which can fully degrade skin collagen and components of the elastic arrangement. These enzymes consist of the joint activities of collagenase (MMP-1), gelatinase (MMP-2) and stromelysin-I (MMP-3) (Jenkins, 2002:805).

Finally, cigarette smoke also causes harm to collagen and elastin in lung tissue and with the constriction of capillary blood flow by nicotine, it may contribute to wrinkles on the skin. According to Farage *et al.* (2008:90) it has been proved that a comprehensible dose-response relationship has been identified between wrinkling and smoking, concluding that smoking has an even higher facial wrinkling effect than that of sun exposure. Wrinkle scores were three times larger in smokers than in non-smokers. Another side effect of smoking is the increase in free radical development which aggravates cutaneous squamous cell carcinoma.

### **2.2.3 Prevention and treatment of skin ageing**

Changes in the contour of the face can be related to changes in the skin quality and appearance as well as structural changes. This usually implies that the skin starts thinning with reduced elasticity and wrinkling due to extrinsic and intrinsic factors. Structural changes of facial skin show a loss of soft-tissue volume and the decrease of subcutaneous fat. Products are consequently designed to overturn these signs of skin ageing by addressing dermal and structural changes (Sadick *et al.*, 2009:S4).

The prevention of ageing skin is probably one of the most important steps we can take to overturn the symptoms and signs of skin ageing. Skin protection and basic skin care programmes are the two main preventative steps. Firstly, photo-protection through the application of protecting sunscreens and a decrease in the exposure to sun radiation can progress or even overturn the unpleasant alterations in skin appearance and quality (Sadick *et al.*, 2009:S4). Sunscreens can be defined, according to Singh (2009:449), as agents that defend the skin from UV harm and the defence against sunburn, wrinkles, and pigmentary changes. Using broad-spectrum sunscreens that offer protection from UVA and UVB rays, it can reduce uneven skin texture, fine wrinkling and pigmentation while recovering the skin's general appearance (Sadick *et al.*, 2009:S4).

Caring for the skin on a daily basis is an additional vital step in recovering the appearance and quality of ageing skin. Gentle everyday skincare can improve the look and feel of the skin by diminishing TEWL. This will make the skin appear smooth, but will also cure, reinstate and maintain the integrity of an already compromised skin barrier due to the effects of sun damage and regular contact with irritants.

Several studies have shown that the stratum corneum is the main influence on water flux and retention in the skin, therefore, controlling the level of moisturisation (Rawlings *et al.*, 2004:49). Moisturising products target the signs of photo-damaged skin, which consist of lines and wrinkles, failure of firmness, drooping skin and increased or irregular pigmentation (Rawlings *et al.*, 2004:55).

Skin care therapies such as topical retinoids can help in the treatment of ageing due to sun damage. They can also improve the appearance of fine wrinkling, spotted hyperpigmentation and hypopigmentation and facial lentigines. These effects can be attributed to the upregulation in the synthesis of collagen, resulting in improved skin strength, elasticity and increasing water preservation in the epidermis (Sadick *et al.*, 2009:S4).

The use of topical antioxidants also helps to reduce UV-induced oxygen free radicals in the skin. These products reduce skin damage from UV-radiation and include substances such as vitamin C (L-ascorbic acid), ferulic acid,  $\alpha$ -lipoic acid, coenzyme Q10, idebenone, kinetin, coffee berry and various tea extracts (Sadick *et al.*, 2009:S4). Antioxidants have long since been studied as tools to prevent skin ageing by avoiding major damages to cell lipids due to their capability to scavenge superoxide ions or hydroxyl radicals and to catalyse the breakdown of peroxides. Singlet oxygen, a reactive oxygen type, is presently receiving a great deal of interest in connection with sun defence. Singlet oxygen may be the most profuse reactive oxygen group

created by solar radiation in the skin with its capability to react rapidly with bordering molecules and create chaos in the arranged structures of cells and the extracellular matrix. Therefore, new cosmeceuticals must be capable of scavenging singlet oxygen. Human cells are able to use catalase and superoxide dismutase to cope with peroxides and superoxides, but there is no endogenous enzymatic protection against this singlet oxygen (Giacomoni, 2008:365).

Continual use of topical applications of  $\alpha$ -hydroxy acids such as ascorbic acid, lactic acid, citric acid, glycolic acid and malic acid, has been reported to improve wrinkling, unevenness and skin staining in ageing skins. They may also enhance the epidermal width due to the increase in collagen production and perfusion advancement in the dermis. Preservation of moisture in the epidermal layer of the skin brings about improved skin elasticity and enhances the skin's appearance. The effectiveness of these compounds can drastically increase with higher concentrations of these  $\alpha$ -hydroxy acids with a lower pH. Commercial cosmeceuticals presently available contain only reasonable concentrations of  $\alpha$ -hydroxy acids with pH values greater than 3.5, consequently restraining their action and effectiveness (Sadick *et al.*, 2009:S4).

Nutritional calorie restrictions extend the lifespan and ageing of the skin due to the decrease of oxidative harm, modulation of glycemia and hormesis. Hormonal substitution has also resulted in the postponement of ageing. Growth hormones recover the muscle:fat ratio as well as lipolysis, bone mass loss and increase exercise capability. Melatonin, also an anti-ageing agent, serves as an anti-stream agent and has proved to be successful against early ageing. The substitution of estradiol, testosterone, growth hormones and reduction in cortisol levels are all strategies in maintaining insulin sensitivity, thus preventing premature ageing of skin (Singh, 2009:449).

Other cosmetic procedures available to correct age-related changes are chemical peels that use acids applied directly onto skin damaging the layers of the epidermis and dermis, enabling skin to renew. Nonablative skin renewal, a non-invasive substitute to conventional laser resurfacing or surgery, improves skin quality, discoloration and scarring by using devices that deliver light of thermal energy. Addressing structural changes in the ageing skin, dermatologists use botulinum toxin type-A injections which cause temporary muscle denervation, resulting in a relaxation of hyperfunctional facial muscles and smoothing of the skin overlying these muscles. Dermal fillers are used to repair soft-tissue quantity and to address the problems with motionless wrinkles, furrows and skin folds. Other aesthetic injections include collagen, bovine collagen fillers, human collagen fillers, porcine collagen fillers and polymethylmethacrylate injections (Sadick *et al.*, 2009:S5-S12).

Finally, according to Singh (2009:450), taking anti-ageing supplements such as vitamin A, vitamin C, beta-carotene, selenium, coenzyme Q10, estrogen, testosterone, silymarin, pycnogenol and procyanidins, can help in the prevention of early ageing. Once again, their actions are all attributed to their antioxidant activities, previously discussed.

To conclude, it can be said that good health, that is keeping a healthy weight, avoiding cigarette smoking, constantly using sun protection, regular exercising, following a proper nutritional diet and having a regular skin care regime are all factors that can prevent and treat ageing (Singh, 2009:450).

### **2.3 Honeybush**

The name honeybush applies to approximately 24 species of *Cyclopia* (*Fabaceae* family, *Podalyriaceae* tribe) which have been conventionally used as a tea in South Africa and enjoyed commercial success as a herbal tea. The earliest mention of honeybush in western botanical literature was in 1705 (Kies, 1951:161-176). The use for this herbal tea was uncertain in those days, but the therapeutic properties were soon realised by inhabitants. It was produced on a small scale by these inhabitants, who liked the sweet taste and flowery fragrance of this tea. Many people in those days thought that honeybush was helpful for sleeplessness, digestive problems, curing of skin rashes, promoting lactation, constipation and as an expectorant in the treatment of pulmonary diseases (Joubert *et al.*, 2008:378; McKay & Blumberg., 2007:7).

Honeybush species are all woody legumes which can grow to more than a meter in height. The stems are green and golden yellow, with hairless leaves separated into three smaller leaflets. The first species recognised was *Cyclopia genistoides* according to Kies (1951:161) and grows in the coastal regions and mountain areas of the Western and Eastern Cape Province. The most popular species are currently known as *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides* (Bond & Goldblatt, 1984:285).

Honeybush – as a herbal tea - has gained popularity over the past few years. With the focus nowadays on “food as medicine”, honeybush’s focal point has shifted from what was a folklore medicinal drink and a non-medicinal beverage, back to an organic herbal tea with various medicinal properties (Joubert *et al.*, 2008:377). In this study, however, we will focus on the potential advantageous properties honeybush could have on the human skin. The number of obtainable toiletries and cosmetic products of this totally organic plant at this stage lags behind other herbal products such as rooibos. According to McKay and Blumberg (2007:10), there is at

this time no clinical trial information or results of human studies available to prove that honeybush and its active ingredients have any health promoting effects on the human skin.

Honeybush is a rich source of bioactive polyphenolic compounds with the major ones being mangiferin (a xanthone c-glycoside), hesperidin and to a lesser extent, hesperitin (both being flavanones). Honeybush is a promising product with potential health benefits such as anti-fungal, anti-microbial, antioxidant and anti-carcinogenic activities. Using these characteristics we can formulate a topical product using two species, *Cyclopia genistoides* and *Cyclopia maculata*, specifically for transdermal studies to test whether it has the potential for wider acceptance into larger regional and global marketplaces once consumers and traders become familiar with this indigenous and under-recognised herbal product. Since this is also a totally organic product with no harmful effects on nature, this can also serve as a product going “green”, a market which is growing day by day (Shahidi & Ho, 2005:127).

### **2.3.1 History**

During the travels of several explorers such as Carl Thunberg, a botanist, and numerous early settlers, honeybush tea was recorded to be used as a herbal tea, not only for its enjoyment of floral scents and sweet tastes, but also as a herbal remedy used for various illnesses. *Cyclopia genistoides* was among the first species to be used as a remedy in the region of the Cape. It was believed that honeybush increases the appetite and it was praised by colonists as being a wholesome tea used as a stomachic that aids in weak digestion without any heart stimulating effects. It was also believed that it alleviates heartburn and nausea, while stimulating milk production in breastfeeding women and treating colic in babies (Joubert *et al.*, 2008:377).

### **2.3.2 Botany and geographical distribution**

Honeybush plants are all woody, fynbos shrubs found in the Western and Eastern Province of South Africa. The young twigs are green and golden yellow with each leaf comprising three separate leaflets with fragrant yellow flowers and flat, brown seed pods. It is believed that the original honeybush tea was made from *C. genistoides* (coastal tea), a small shrub with distinctive narrow leaflets, restricted to the mountains near the Cape Peninsula (ASNAPP, 2010:3; Van Wyk, 2008:348). This species is no longer the main source for commercial production. Currently *C. intermedia* (mountain tea) is the most popular species for commercial use, followed by *C. subternata*, *C. sessiliflora* (Heidelberg tea) and *C. maculata* (Genadendal tea), the latter being restricted to the Genadendal district in South Africa (Joubert *et al.*, 2008:386).

Harvesting usually takes place between the months of May and June, followed by fermentation. During fermentation the plant material changes colour from green to dark brown as the phenolic compounds are oxidized (McKay & Bloomberg, 2007:7). It is only in recent years that organic, unfermented (“green”) honeybush has made its appearance on commercial markets, due to higher polyphenolic content present in “green” honeybush than in fermented honeybush. The active ingredients of this plant are produced from the aerial parts (leaves, flowers and stems) and are used to make herbal teas, constituents to herbal tea blends, medicinal teas, nutritional enhancers and are used as food ingredients for the extraction of active compounds for production of medicinal and cosmeceutical items (ASNAPP, 2010:4).



**Figure 2.3:** Flowers and fine leaves of honeybush (Adapted from ITM Online.org, 2012).

Currently Japan, Germany and Switzerland are our largest export clients, where health and natural medicinal products are greatly sought after (ASNAPP, 2010:4; Joubert *et al.*, 2008:386).

### **2.3.3 Physicochemical characteristics of honeybush**

Superior plants manufacture metabolites such as carbohydrates, proteins, amino acids and lipids, which are vital for cell endurance. A large assortment of secondary metabolites, not concerned in direct principal metabolic processes of growth and development, appear to function mainly in the protection against predators and viral, mycoplasma, bacterial and fungal pathogens. They also defend plants against herbivores, pests, mammals, plant opponents and abiotic stresses like UV radiation, ozone and herbicides. Minor metabolites are derived from primary metabolites, mostly amino acids and carbohydrates, through the processes of methylation, hydroxylation and glycosylation. Structurally secondary metabolites belong to

three main classes, the terpenes (isoprenoids, terpenoids), the phenylpropanoids and their derivatives (flavonoids, tannins, glycosides and lignins), and lastly the nitrogen-containing compounds (alkaloids and heterocyclic aromatics) (Korkina *et al.*, 2008:710).

The phenylpropanoids and their derivatives form the main group of plant phenols and are created through the shikimic acid pathway. The universal preliminary action, the deamination of phenylalanine to cinnamic acid by phenylalanine ammonia lyases (PAL) is frequently affected by light, wounding disease, gamma emission, germination and the function of certain macromolecules. Flavanoids, isoflavanoids, coumarines and lignans are all derivatives of the chief phenylpropanoid molecule (Korkina *et al.*, 2008:710).

Flavonoids develop from a universal biosynthetic shikimic acid pathway, forming cinnamic and then hydroxycinnamic acid. Afterwards, chalcone synthase uses three cinnamoyl radicals to create a flavonoid core. These structures lead to possible antifungal defence (Korkina *et al.*, 2008:711).

In superior plants plant polyphenols such as hydroxycinnamic acid, cinnamoyl esters, flavones, flavanols and anthocyanins offer UVA and UVB screening. Both the soluble and insoluble phenols absorb well in the range of 304 – 350 nm and 352 – 385 nm, respectively. Flavonoids consist of two maximum absorption ranges, 240 – 285 nm (band II) and 300 – 550 nm (band I) with the precise positions and relative intensities of these bands providing valuable information on the nature of the flavonoid and its oxygenation pattern. The absorption spectra and extinction coefficients of plant polyphenols are subjective to their electron-accepting and electron-donating substituents in the benzene ring(s), their intra- and intermolecular hydrogen-bonding, their steric properties and their structural differences (Korkina *et al.*, 2008:711).

Plant polyphenols also play an elemental role in the non-cellular “immune system”, defending plants against a variety of biological invaders. This can be attributed to the commencement of polyphenol metabolism through PAL catalyses. Branch pathways lead to the synthesis of complexes that have various self-protective roles in the plant such as cell wall reinforcement, wound restoration, antimicrobial activity and signalling roles (Korkina *et al.*, 2008:711).

The key chemical components present in *Cyclopia* plants are these afore-mentioned polyphenols, represented by xanthenes, flavanones, flavones, flavanols, isoflavones and coumestans (Kokotkiewicz & Luczkiewicz, 2009:534). Honeybush is therefore a rich resource of bioactive ingredient which can have health-promoting effects advantageous to mankind.

The first study completed in 1996 by De Nysschen *et al.* (1996:244), found that there were three main phenolic compounds present in nearly all *Cyclopia* species extracted from the foliage of a variety of honeybush plants. These leaves consisted of mangiferin, a xanthone c-glycoside and two flavanone-O-glycosides, identified as hesperitin (also known as hesperetin) and isosakuranetin. Additional studies were conducted by Ferreira *et al.* (1998:3406-3410) on *C. intermedia* and *C. subternata* and they described the total phenolic complexity in a bit more detail. However, the xanthenes, mangiferin and isomangiferin, and the flavanone, hesperidin, are present in all species analysed to date according to Joubert *et al.* (2008:393).

Stated in more detail, xanthenes such as 2- $\beta$ -D-glucopyranosyl-1,3,6,7-tetrahydroxy-(mangiferin) and 4- $\beta$ -D-glucopyranosyl-1,3,6,7-tetrahydroxy-(isomangiferin) were recognised in honeybush tea, whereas four flavanones such as, 5,7,4'-trihydroxyflavanone (naringenin), 5,7,3',4'-tetrahydroxyflavanone (Eriodictyol), 5,7,3'-trihydroxy-4'-methoxyflavanone (hesperitin), 5,3'-dihydroxy-4'-methoxy-7-O-rutinoxyflavanone (hesperidin) were reported to be isolated and identified from *Cyclopia intermedia* by comparing of <sup>1</sup>H-NMR data of their O-acetyl derivatives with literature in 1998 (Wang *et al.*, 2005:121). McKay and Bloomberg (2007:7), clearly state that it is always important to keep in mind that relative quantities of these phenolics may vary among dissimilar types of *Cyclopia* species as well as within a geographical area.

Honeybush also consists of more than fifteen aromatic volatiles with  $\alpha$ -terpineol as the most important volatile constituent and the monoterpenes which are responsible for the sugary, fruity and flowery notes of the tea. Containing zero caffeine, the highest levels of major ingredients for this particular study were mangiferin (3.61 g/100 g) and hesperidin (1.74 g/100 g), which were primarily found in *C. genistoides* and *C. intermedia* species (Wang *et al.*, 2005:120). According to Juliani *et al.* (2009:281), not only does honeybush contain bioactive ingredients, but also essential minerals such as iron (Fe), zinc (Zn), potassium (K), calcium (Ca), copper (Cu), magnesium (Mg), manganese (Mn) and sodium (Na).

In this study we will focus on only two of the various components present in honeybush. They are mangiferin and hesperidin. Mangiferin (C<sub>19</sub>H<sub>18</sub>O<sub>11</sub>) is a light yellow powder with a molecular weight of 422.34 g/mol. It is soluble in alcohol and ether and the chemical name for mangiferin will be 9H-xanthen-9-one,2- $\beta$ -D-glucopyranosyl-1,3,6,7-tetrahydroxy-(mangiferin) (Lookchem, 2010a).

The second chemical component we will focus on is hesperidin (C<sub>28</sub>H<sub>34</sub>O<sub>15</sub>). This compound is a light brown powder with a molecular weight of 610.57 g/mol. Hesperidin is soluble in pyridine,

sodium hydroxide and dimethylformamide and slightly soluble in methanol and hot glacial acetic acid. It has a low solubility in ether, acetone, chloroform and benzene (Lookchem, 2010b).

#### **2.3.4 Function in the human body**

A few *in vitro*, *in vivo* and *ex vivo* biological studies conducted by mainly South African researchers have been published over the years. As mentioned earlier, honeybush has been used as a household product, with no scientific evidence that its medicinal effects were valid. Recent interest in honeybush made it possible for scientists to investigate these assumptions (Juliani *et al.*, 2009:277).

Taking into consideration that honeybush consists of several concentrations of dissimilar polyphenols and more in particular flavanoids, it is imperative to know the effects of these substances. To take a broad view of these compounds, flavanols are known for their anticancer, antioxidant, anti-tumour, anti-allergic, antitoxic and antihistamine activities. Flavanols also inhibit the manufacture of lipid peroxidation and oxyradical production, implying that conditions such as ageing, atherosclerosis, inflammation, hepatotoxicity and iron toxicity could be reduced. Flavanols also have the ability to inhibit platelet aggregation, adhesion and secretions. Flavanones, a different kind of polyphenol, have anti-microbial, anti-viral and anti-inflammatory effects. The isoflavanones, acting as a phyto-alexin in the resistance in opposition to fungal infections in plants also have anti-cancer, estrogenic and anti-microbial activities. Another substance, flavones, can be recognised by its antioxidant, diuretic and anti-spasmodic properties. Coumestans are well known for its phytoestrogenic qualities (Kamara *et al.*, 2003:3877).

Mangiferin has the potential to be a vigorous antioxidant and anti-viral agent. It is known that this substance aids in the management of melancholia, but even more recent reports have shown that mangiferin has a powerful radical scavenging effect. It is also expected to have the potential to treat diabetes mellitus and to have the capability to lower body weight (Kamara *et al.*, 2004:5394).

Hesperidin, on the other hand, has a considerable potential as a remedial agent for an extensive range of illnesses. For instance, it has effects on the vascular system, anti-inflammatory effects, antimicrobial activity, anticarcinogenic activity, UV protecting activity, analgesic and antipyretic activity, antioxidant activity and wound healing effects (Garg *et al.*, 2001:665). The flavone, luteolin and its glycosides have the ability to induce antihypertensive activity and are therefore known to have anti-spasmodic and anti-oxidant properties. Kamara *et*

*al.* (2004:5393) stated that the highly insoluble hesperidin is known for its vitamin C-like activity, anti-inflammatory, anti-microbial and anti-viral properties. An analgesic effect was also detected with a mild anti-pyresis effect. It has also been reported that hesperidin reduces the aggregation of blood cells (erythrocytes) and irregular capillary permeability, for that reason protecting the host against various traumas and stress.

These properties need to be effectively considered at the clinical level, so as to establish definitely the effectiveness of these flavonoids in the healing or prevention of disease in humans (Garg *et al.*, 2001:665).

### **2.3.5 Clinical uses on human skin**

There is an increasing awareness these days of the utilisation of natural products to prevent human ailments. Plant polyphenols are some of the most talked about substances with possible anti-inflammatory, cardio-protective, cancer-preventive and anti-bacterial properties. Polyphenols such as mangiferin and hesperidin are generally used in Western and conventional folk remedies to take care of numerous skin ailments such as psoriasis and vitiligo, to increase skin wound healing and diminish skin inflammation. When applied topically it is alleged that these polyphenols shield the skin from the harmful effects of solar rays, ozone and air-borne pollutants, thus avoiding early skin ageing and environment-related skin imperfections (Korkina *et al.*, 2008:710).

Skin penetration and transdermal deliverance of plant polyphenols applied topically, may possibly be significantly improved by including them into liposomes or lipogels by electrically supportive techniques for example electroporation and iontophoresis, or by the use of skin permeation enhancers. When transported deeply into the skin, polyphenols may possibly be circulated in a uniform way through all the layers of the skin or be concentrated in an exact region, depending on the character of both the polyphenol used and its delivery system (Korkina *et al.*, 2008:714). Regrettably, investigational statistics are very inadequate. However, the action of tea and tea polyphenols on the inhibition of skin tumorigenesis has been extensively considered. With the topical application of unprocessed and processed honeybush tea extracts, a reduction in skin tumorigenesis was found in the skins of ICR (imprinting control region) mice. With the application of DMBA (7,12-dimethylbenz[ $\alpha$ ]anthracene) and TPA (12-O-tetradecanoylphorbol-13-acetate), both tumour promoters, the tumours were initiated and promoted. An application of honeybush extracts were applied 30 min before the application of these tumour promoting agents, with the whole process repeated twice weekly for 20 weeks. A significant decrease in tumour occurrence was detected. Unprocessed honeybush extract

displayed the maximum reduction in the fraction of tumour-bearing mice (90%); followed by extracts of processed honeybush (84.2%); processed rooibos (75%) and unprocessed rooibos (60%) (McKay & Blumberg, 2007:9-10).

It is fairly evident that UV absorbance, antioxidant, free radical scavenging, metal chelating and antibiotic properties of plant polyphenols are comparable in plants, animals and humans. This makes polyphenols viable as a competent natural skin defender against UV-induced harm as well as antibiotics to fight skin fungal and bacterial diseases. When applied to human skin, it could effortlessly penetrate the metabolic pathways by replacing or competing with endogenous phenylalanine metabolites. This offers a concrete justification for anti-age, anti-acne, anti-hair loss, whitening and anti-hirsutism polyphenol-based skin products (Korkina *et al.*, 2008:722).

The antioxidant activities, free radical scavenging and the metal chelating properties of these polyphenols contribute only partially to the relevant and complex anti-inflammatory effects, leading to accelerated wound healing. The major impact relies on the interaction of plant polyphenols with resident (keratinocytes) and recruited (granulocytes, lymphocytes and dendritic cells) inflammatory cells in the skin. Inhibition of both the expression and the activity of multiple pro-inflammatory enzymes takes place. They react with specific surface receptors, modulating the signal transduction pathways and resulting in the involvement of genes in the inflammatory response. This makes plant polyphenols generally the most capable anti-inflammatory and wound-healing constituent of prospective topical drugs (Korkina *et al.*, 2008:722).

### **2.3.6 Adverse reactions and toxicity**

Current and earlier periods of use of honeybush as an infusion for everyday consumption have led to a common theory of its safety and no information on toxicity. There are no toxicological studies presented, even though a number of studies have addressed characteristics of the safety and toxicity in rooibos. Bearing in mind that the serum biological data presented by Marnewick *et al.* (2003:8113-8119) with constant consumption of aqueous extracts of unfermented and fermented rooibos and honeybush by rats over a period of 10 weeks, did not cause any unfavourable effects in the liver and kidneys (Joubert *et al.*, 2008:407). In general, bioflavonoids, such as hesperidin and mangiferin appear to be extremely safe and without side effects even during pregnancy (Pizzorno Jr & Murray, 1999:79).

Microbial contaminants such as *Rhizomucor pusillus* were present during the fermentation and drying of honeybush tea and could possibly have adverse effects on humans. Two

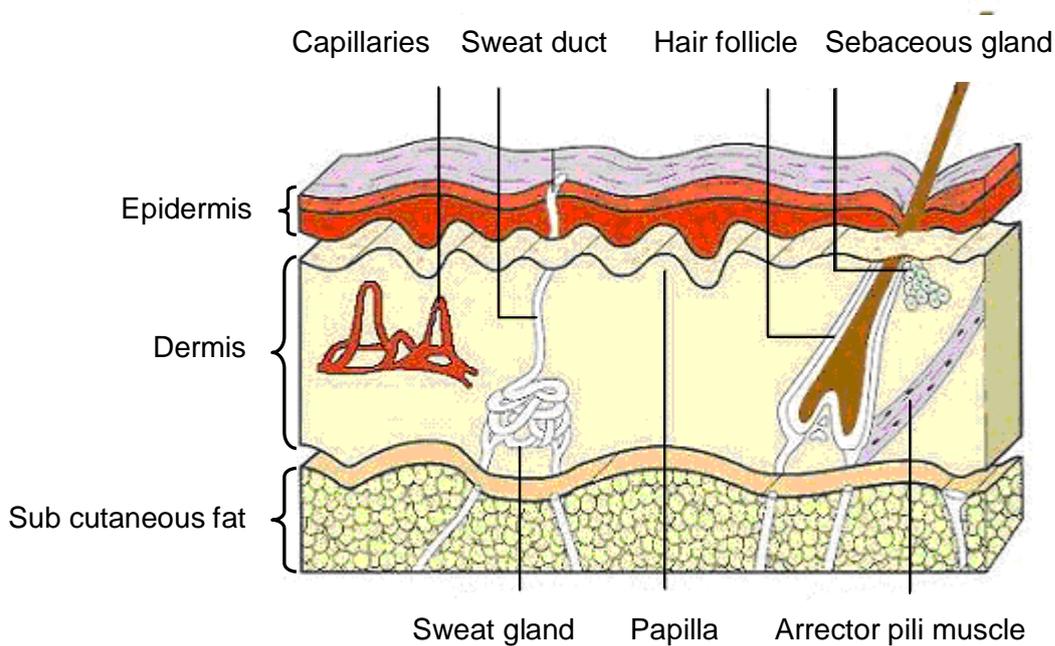
thermophilic moulds, *Humicola grisea* var *thermoida* en *H. lanuginosa*, and five endospore-forming *Bacillus* species, *B. brevis*, *B.adius*, *B. stearothermophilus*, *B. subtilis* and *B. pumilus*, were also isolated. Elimination of these microbial contaminants can be achieved by processing the plant materials at a temperature higher than 60 °C and by controlled curing conditions (Du Toit *et al.*, 1999:2044).

Herb-drug relations play a significant part during clinical studies. The effect on pharmacokinetics of a drug might differ when co-administered in the presence of a specific herb. In order to encourage the health benefits of honeybush and other herbal teas, it must be determined whether any drug-herb interactions take place that may perhaps adversely affect drug metabolism (Joubert *et al.*, 2008:407-408). However, some information has been published on interactions among the aglycone hesperitin (Mitsunaga *et al.*, 2000:193), hesperidin (Melzig *et al.*, 1997:793) and conservative drugs (Garg *et al.*, 2001:658).

Numerous polyphenols created by plants are intended for self-preservation against herbivores, including human beings. For that reason, it could cause toxic, allergenic, irritating or inflammatory effects by the skin and other epithelial surfaces (Korkina *et al.*, 2008:712). Formulation and the clinical effects of plant-derived polyphenols face a variety of difficulties. Chemical instability, interaction with other components of topical preparations, low penetration and fast skin metabolism are all factors that prevent the successful use of polyphenols in skin preparations. Other factors such as the reactions with molecular oxygen, transition metals, UV light and other environmental oxidants frequently lead to the redox cycling of plant polyphenols, thus aggravating oxidative damage and increasing undesirable effects on the skin (Korkina *et al.*, 2008:722).

## 2.4 Anatomy and functions of human skin

### 2.4.1 Structure of the skin



**Figure 2.4:** Anatomy of the skin (Adapted from Yale Medical Group, 2012).

The skin is the largest human organ in the body and covers approximately 1.5 to 2.0 m<sup>2</sup> of an average human body surface with the ratio of the total capillary flow to the corresponding skin surface area at approximately 0.93 cm/h (WHO, 2010:10). This admirable organ system in all its intricacy has no less than five diverse cell types contributing to its construction, including the stratum corneum, viable epidermis, dermis and subcutaneous tissue. Other cell types from circulatory and immune systems are also transitory inhabitants of the skin, but most importantly, the skin consists of two distinct layers, known as the overlying avascular epidermis and the dermis, forming the bulk of the skin (Menon, 2002:S4). Until the commencement of the 20<sup>th</sup> century the skin was considered to be totally lifeless and resistant to chemicals that otherwise penetrate the body. Numerous chemicals do enter the skin, either deliberately or by accident, and cutaneous metabolism does take place. Due to its large surface area, the skin may be a main route of entrance into the body (WHO, 2010:10).

#### 2.4.1.1 The epidermis

The exterior layer of human skin, the multilayered epidermis, serves as a barrier to radiation, micro-organisms and diffusing permeants. This overlying epidermis is primarily composed of

approximately 95% keratinocytes, anchored to the basement membrane by means of hemidesmosomes (Menon, 2002:S4). The stratified epidermal layer, with a thickness of 100 to 150  $\mu\text{m}$ , is further layered with an outermost layer, the stratum corneum, which is composed of lifeless cells, providing a prime diffusional barrier between the human body and its surroundings. The epidermis comprises about 5% of full-thickness skin (WHO, 2010:12). Three other layers, known as the stratum basale, stratum spinosum and the stratum granulosum also form an integral part in the layering of the epidermis (Menon, 2002:S4; Whang *et al.*, 1990:381). The epidermis varies in thickness, ranging from about 0.800 mm on the palms and soles to 0.006 mm on the eyelids (Barry, 2002:502).

#### **2.4.1.1.1 Stratum basale**

The stratum basale is a sole layer of columnar basal cells fabricated of epidermal stem cells and rapidly amplifying cells derived from them. The stratum basale remains attached to the basement membrane via hemidesmosomes, showing a high nucleo-cytoplasmic ratio, cell organelles such as mitochondria and keratin filaments, named tonofilaments that are inserted into these hemidesmosomes. Adjacent and overlaying cells are connected via hemidesmosomes, while two keratins, K14 and K5, are expressed in the basal cells (Menon, 2002:S5).

#### **2.4.1.1.2 Stratum spinosum**

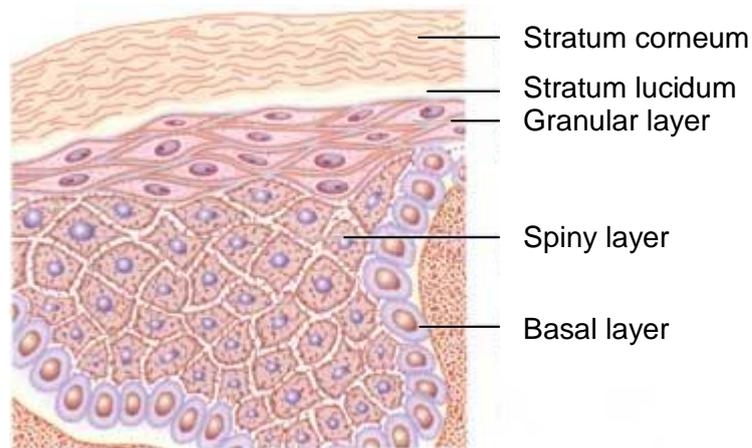
When we look at the stratum spinosum with a large quantity of desmosomes, this layer has a thorny outward show of its cells in histological sections. Not only does the stratum spinosum have typical cell organelles in the basal layer, but it also shows the presence of lipid-enriched lamellar bodies, also known as Odland bodies, together with keratinosomes, membrane-coating granules which usually appear first in this particular layer. These lamellar bodies are 0.2 to 0.5  $\mu\text{m}$  in diameter with parallel loads of lipid-enriched disks covered by a trilaminar membrane (Menon, 2002:S5).

#### **2.4.1.1.3 Stratum granulosum**

This layer can be characterised in histological preparations as distinctive, dark staining keratohyalin granules. Keratohyalin granules consist of profillaggrin, loricrin, a cysteine-rich protein and keratins K1 and K10. They all become increasingly outsized in the upper granuloocytes and this reflects the quantitative raise in keratin synthesis. These topmost cells in the stratum granulosum exhibit a distinctive structural and efficient organisation of the lamellar bodies, during which the lamellar bodies are secreted to the extracellular domain. This entire

cascade of the commencement of the formation of a cornified covering, the large scale secretion of lamellar bodies, the dissolution of the cellular organelles, the condensation of keratin filaments, etc. that lead to this permanent progression of cornification, rely on many signals, the nature of which is still being revealed (Menon, 2002:S5).

#### 2.4.1.1.4 Stratum corneum



**Figure 2.5:** The different layers of the epidermis (Adapted from P & G, 2012).

Natural functions of the human skin are to defend the body against the loss of endogenous substances such as water and to protect the skin from undesired influences from the environment caused by exogenous substances. The skin acts as an obstruction against the diffusion of unwanted substances through all the underlying tissue. This diffusional barrier against the majority of substances is found in the superior layer of the skin, the stratum corneum (Bouwstra, 1997:403). The last step in epidermal differentiation is the formation of the stratum corneum, consisting of a matrix of keratinized cells (corneocytes) and surrounded by lipids (Mukhtar, 1991:16). This combination of corneocytes, also known as terminally differentiated keratinocytes, and the secreted contents of lamellar bodies, gives a brick-and-mortar organisation. The composition of lipids changes noticeably during apical migration through consecutive epidermal layers. When this differentiation procedure is accomplished in the stratum corneum, the lipid composition changes distinctly with the phospholipids degraded enzymatically into glycerol and free fatty acids and glucosylceramides into ceramides (Bouwstra, 1997:403).

In human skin the stratum corneum normally has approximately 18 to 21 cell layers, with individual corneocytes (flattened dead cells) between 20 and 40  $\mu\text{m}$  in diameter. These corneocytes can differ in thickness, packaging of keratin filaments and the number of

desmosomes depending on the body site and their location in the stratum corneum layer (Menon, 2002:S7). The stratum corneum therefore has the ability to sense ambient changes and is truly the body's interface with the rest of the world. This is the organ of touch, where signals are received, signals are sent, emotions are expressed and where dysfunctions are diagnosed. One of these responses is the barrier repair phenomenon, where dramatic increase in TEWL is followed by the removal of lipids or corneocytes or both, which sets in motion a series of signals that initiate cellular and metabolic responses in the underlying nucleated epidermis that leads to the replenishment of barrier lipids and the restoration of the permeability barrier (Menon, 2002:S10).

#### **2.4.1.2 The dermis**

The dermis comprises the most important part of the skin and is accountable for providing its structural strength. This layer of the skin ranges from 3 to 5 mm in width (Barry, 2002:502), consisting of mainly connective tissue such as collagen, elastin and reticulum, entrenched in an unstructured colloidal ground matter (WHO, 2010:16). The dermis also offers an ideal atmosphere for nerve function, vascular networks and appendages safeguarding the epidermis. The most important cell types of the dermis are fibroblasts, macrophages, mast cells. The fibroblasts have the prime purpose of synthesis and remodelling of connective tissue proteins by secreting collagenase and gelatinase. Macrophages start off as predecessor cells in the bone marrow, mature into monocytes in the blood and terminally differentiate to macrophages in the human tissue while processing and presenting antigens to immunocompetent lymphoid cells. Mast cells are present in all layers of the dermis concentrating around blood vessels and producing granules that are vasoactive or chemoattractants for neutrophils and eosinophils. These granules are responsive to light, cold, acute trauma, vibration, pressure and chemical and immunologic stimuli by releasing their contents and initiating chemotaxis or vasodilatation (Mukhtar, 1991:4-7).

Directly underlying the epidermis is the papillary dermis which originates below the basal lamina. The fibroblasts are the major cell type present in the dermal layer and acquire a fairly high synthetic and proliferative ability. Collagen type-III is one of the major synthetic products in the papillary dermis although collagen type-I is also present. Collagenase activity is primarily localised in the papillary layer (Mukhtar, 1991:7).

Underlying the papillary dermis is the reticular dermis which is superficial to the hypodermis. Composed mainly of collagen type-I and organised in large fibrillar bundles, this layer contains

large and fully matured elastic bundles extending between the collagen fibre bundles. These elastic fibres and collagen bundles increase in size toward the hypodermis (Mukhtar, 1991:7).

#### **2.4.1.3 The hypodermis**

The hypodermis is a layer of mesenchymally derived adipose cells that adjoin the connective tissue layer of the reticular dermis. The subcutaneous fat offers a mechanical cushion and a thermal barrier (Barry, 2002:502). This is the deepest layer of skin and it functions to provide a barrier between the external skin layers and the internal structures such as bone and muscle. It is also an energy reserve, allowing for skin mobility, moulding body contours and insulating the body (Mukhtar, 1991:7).

#### **2.4.2 Functions of the skin**

There are numerous functions of the human skin. Only a few aspects of its containment and protective roles will be considered.

- The dermis offers mechanical structure in the skin, with the epidermis only playing an insignificant part (Barry, 2002:502).
- Skin provides a powerful, elastic and self-repairing barrier to external surroundings (WHO, 2010:10).
- Averts loss of endogenous water and nutrients (WHO, 2010:10).
- Manages body temperature by synchronised sweating and arteriovenous thermoregulation (Barry, 2002:503).
- Photo-protects against UV radiation (WHO, 2010:11).
- Defends against microbiological, chemical and electrical threats (Barry, 2002:503).

## 2.5 Transdermal drug delivery

Transdermal drug delivery represents the most successful non-oral systemic drug delivery system. By all accounts transdermal drug delivery is not suitable to all drugs, nor is it acceptable for all therapies. This drug delivery system needs to be carefully identified, given that the skin offers an excellent barrier to molecular transport. There are several instances where the most convenient method of drug intake is in not feasible and where alternative routes must be considered (Naik *et al.*, 2000:318-319). Human skin is an effective and highly selective barrier to chemical permeation with the stratum corneum acting as the major control element. Diffusion of small water-soluble non-electrolytes would be a thousand times more rapid if this horny layer was absent according to Barry (2001:101). Therefore to take full advantage of drug flux, efforts are made to decrease this barrier's hindrance by the integration of drug molecules into vehicles or devices that transport these molecules to the active site at a controlled tempo and concentration (Barry, 2002:507; Barry, 2001:101). The interest in these devices can be attributed to the many advantages of the transdermal drug delivery route. There are also a few limitations to consider.

### 2.5.1 Advantages and limitations of transdermal drug delivery

It is customary to compare the percutaneous route with oral drug delivery since the latter provides the most popular way of delivering drugs.

#### 2.5.1.1 Advantages

Advantages of transdermal drug delivery include the following:

- This drug delivery route steers clear of the unpredictable effect the gastro-intestinal tract has on the absorption of medication, such as pH, enzymatic activity and drug-food interactions (El-Kattan *et al.*, 2000:426).
- Therapeutic benefits such as sustained delivery of drugs to provide a steady plasma profile, predominantly for drugs with short half-lives with reduced systemic side effects (Thomas & Finnin, 2004:697).
- There are no first-pass effects due to drug inactivation by digestive and liver enzymes (El-Kattan *et al.*, 2000:426).
- The skin presents a relatively large and readily accessible surface area (1 – 2 m<sup>2</sup>) for absorption (Naik *et al.*, 2000:319).

- Multiday treatment is achievable with a single application (El-Kattan *et al.*, 2000:426).
- It is a suitable, patient-friendly substitute for drug delivery with the prospect of flexibility, naturally allowing dose changes according to the patients need (Thomas & Finnin, 2004:697-698).
- Drug effects can be terminated rapidly (El-Kattan *et al.*, 2000:426).
- Overcoming needle phobia (Barry, 2001:105).

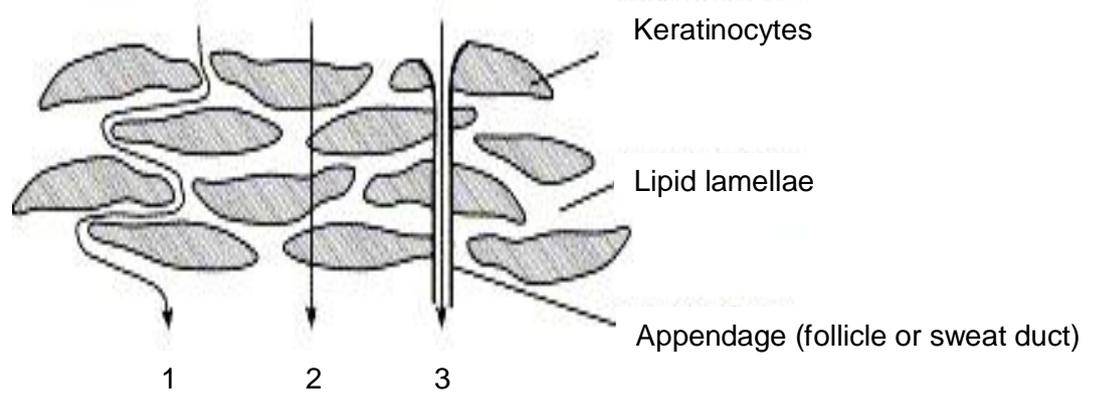
### **2.5.1.2 Limitations**

Limitations of transdermal drug delivery through the skin include the following:

- The accuracy of dosing is mostly a concern with creams and gels, particularly as it has been confirmed that changing the dose applied per surface area influences the systemic delivery profile (Thomas & Finnin, 2004:700).
- Inconsistency of dosing is also a concern and can be accredited to the dissimilar levels of skin hydration found at a variety of anatomical sites, perhaps due to local microcirculation changes in the skin. Unpredictability can also be observed among diverse skin types, skin age and ailing skin (Thomas & Finnin, 2004:700).
- Low skin permeability (Lavon & Kost, 2004:671).
- Cannot deliver large molecules (> 500 Da) (Lavon & Kost, 2004:671).
- Significant lag time (Lavon & Kost, 2004:671).
- Skin irritations and/or sensitisation (Lavon & Kost, 2004:671).

### **2.5.2 Pathways of transdermal penetration**

The transportation of chemical materials through the skin is a multifaceted procedure. The skin is an intricate organ but also a living membrane with continuous interaction with the surrounding atmosphere. There are three key mechanisms by which skin absorption can take place, namely intercellular diffusion, transcellular diffusion and appendageal diffusion (WHO, 2010:17). The skin permeation routes are shown in Figure 2.5.



**Figure 2.6:** Skin permeation routes: (1) intercellular diffusion through the lipid lamellae; (2) transcellular diffusion through both the keratinocytes and lipid lamellae; and (3) diffusion through appendages (Adapted from Ho, 2003:50).

### 2.5.2.1 Intercellular diffusion through the lipid lamellae

There has been a good deal of deliberation over the past 10 years concerning the route of penetration of drug particles all the way through the human skin, although investigational proof proposes that under ordinary conditions, the main route is through the intercellular spaces. The diffusional pathway length is consequently a great deal longer than the plain thickness of the stratum corneum (20  $\mu\text{m}$ ) and has been estimated to be as lengthy as 500  $\mu\text{m}$ . The intercellular spaces enclose prearranged lipids and a dispersing particle has to cross a wide range of lipophilic and hydrophilic areas, prior to reaching the intersection among the stratum corneum and the viable epidermis. The character of the barrier is therefore extremely heterogeneous and it is conceivably astonishing that diffusion through it can be illustrated by a simple equation such as Fick's laws of diffusion (Hadgraft, 2004:292).

### 2.5.2.2 Transcellular diffusion through both the keratinocytes and lipid lamellae

Initially it was believed that transcellular diffusion methods had control over the intercellular and transappendageal pathways all through the passageway of solutes through the stratum corneum. However, according to Roberts *et al.* (2002:94), transport by the transcellular route would engage the repetitive partitioning of the dispersed particle among the lipophilic and hydrophilic sections, as well as the almost impassable corneocyte intracellular matrix of keratin and keratohyaline.

### **2.5.2.3 Diffusion through appendages (hair follicles and sweat ducts)**

Skin appendages start off in the subpapillary dermis and consist of eccrine sweat glands, apocrine sweat glands, sebaceous glands and hair follicles. A typical human skin surface contains 40 – 70 hair follicles and 200 – 250 sweat ducts per square centimetre (WHO, 2010:16). Skin appendages have a small fractional area (about 1%) presented for absorption and does play a decisive role in the absorption of various chemicals. These chemicals sidestep the corneocytes, entering shunts made available by the hair follicle, sweat glands and sebaceous glands (WHO, 2010:17). This route may be significant for ions and large polar molecules that cross an unbroken stratum corneum with difficulty. These appendages could behave as shunts which play an imperative role at short times preceding steady-state diffusion (Barry, 2002:508).

### **2.5.3 Factors influencing permeation across the skin**

#### **2.5.3.1 Biological factors**

Various biological factors may possibly have an effect on penetration across the skin. These factors range from skin age, skin condition, regional skin sites, skin metabolism, circulatory effects and variation in different species.

##### **2.5.3.1.1 Skin age**

Topical formulations must be adapted to suit the physiological conditions under which they are used. The younger the skin, the more underdeveloped the stratum corneum will be (Barry, 2002:510). With ageing skin, the elasticity and permeability of the skin decrease. Alterations in stratum corneum dryness, a decrease in sebaceous gland action, the destruction of the dermal-epidermal junction and the deterioration of the skin capillary network are all signs and symptoms of ageing skin (WHO, 2010:18). Administering topical preparations onto young skin with increased permeability, one runs the risk of overdosing paediatric patients or underdosing geriatric patients. The concentration of active ingredients plays an integral part in the optimal transdermal treatment for patients with different ages (Pefile & Smith, 1997:148).

##### **2.5.3.1.2 Skin condition**

Ailments frequently modify skin conditions (Barry, 2002:509). The state of human skin has a major impact on the diffusion and penetration of chemicals, particularly when the barrier function is disturbed. Physical (weather, sunlight, occlusion), chemical (solvents, detergents, acid, alkalis) and pathological (mechanical damage, disease state) factors play an essential part in

the permeability of the skin (WHO, 2010:19). Broken, damaged or inflamed skin increase permeability, while calluses and corns reduce permeability (Pefile & Smith, 1997:148).

#### **2.5.3.1.3 Skin hydration**

The stratum corneum generally contains 5 to 20% water. Hydrating compounds of the skin cause the skin to swell and amplify the permeability, increasing water content in the stratum corneum to approximately 50% (WHO, 2010:20; Barry, 2002:511). Lipids present in ointments and water-in-oil emulsions prevent water loss and keep the skin hydrated. Various topical formulations cause increased skin hydration, reducing evaporation by means of the formation of an occlusive layer. When choosing a topical base, it is important to select bases that will influence the degree of hydration so as to obtain the desired skin conditions (Pefile & Smith, 1997:149).

#### **2.5.3.1.4 Temperature**

Elevating surface temperature of the skin speeds up the penetration rate of compounds through human skin. A rise in surface temperature enhances the kinetic action of the skin, resulting in improved drug permeability across the stratum corneum (Pefile & Smith, 1997:149). The penetration rate of materials through human skin can change tenfold in large temperature variations (Barry, 2002:511). The motivation for all this can be attributed to the fact that with temperature increase, the lipid layer of the stratum corneum becomes less viscous, consequently decreasing the activation energy for diffusion (Pefile & Smith, 1997:149).

#### **2.5.3.1.5 Regional skin sites**

Transdermal absorption is also subjective to the region onto which the preparation is applied. Size in cells and bilayer lipid composition in the stratum corneum will certainly have an effect on the degree of transdermal drug release. Skin thickness of the eyelids is roughly 0.05 mm while that of the palm and sole are approximately 0.40 mm. A substantial inconsistency arises at a given site, within and between individuals (WHO, 2010:19). Therefore, it can be assumed that the diffusion of a material across the skin is inversely comparable to the width of the stratum corneum. The thinner the epidermal layer, such as on the abdomen, the better the permeability of the drug through the skin's exterior (Pefile & Smith, 1997:148).

#### **2.5.3.1.6 Skin metabolism**

Skin, a metabolic dynamic organ, has the capacity to metabolise numerous drugs, reducing their therapeutic effectiveness and absorption. Underneath the stratum corneum lies the viable

epidermis, the most metabolically dynamic layer in the skin. Percutaneous metabolism in this viable epidermis may decrease the pharmacological potential of the active ingredient through cutaneous first-pass effects and the degree of percutaneous absorption will conclude the deposition of the substance in other parts of the skin such as the release to the capillaries in the dermis (Pefile & Smith, 1997:149).

#### **2.5.3.1.7 Circulatory effects**

Good circulation in the dermis improves drug absorption from the dermis into the systemic circulation. Increased blood flow at the absorption location enhances the concentration gradient to the dermis through the invariable elimination of the drug from the site. Various drugs have a vasoconstrictive effect on the skin which will lessen their own clearance from the skin due to the decreased blood flow at the absorption site (Pefile & Smith, 1997:149).

#### **2.5.3.1.8 Species differences**

Human skin is extraordinary in numerous respects compared to most other living mammals. Significant differences between the dermal absorption in laboratory animals and in that of human skin have been observed (WHO, 2010:17). Mammalian skins vary in characteristics such as horny layer width, sweat glands, hair follicle densities and pelt conditions (Barry, 2002:510). The most noticeable dissimilarity among human and animal models is pelage density, a thick cover of hair that offers a significant measure of defence against the entrance of xenobiotics and the exposure to radiation. Consequently, the stratum corneum of rodents and lagomorphs is in general more porous and considerably more delicate than “naked” species such as pig and human skin (Chilcott, 2008:3). The skin of weanling pigs and monkeys appears to be the most predictive model for human percutaneous penetration (WHO, 2010:18).

#### **2.5.3.2 Physicochemical factors**

Drug diffusion across membranes is multifaceted and based on many probable physicochemical relations. The most important physicochemical determinants consist of partitioning, the molecular weight and size of the drug particle, the solubility, the ionisation form as well as the hydrogen-bonding capability. Other factors incorporate the pH and the diffusion across the skin (Malan *et al.*, 2002:385). According to Pefile & Smit (1997:149), when formulating a pharmaceutical semi-solid used for transdermal drug delivery, the following physicochemical properties have to be taken into account:

- Chemical and physical drug stability

- Excipient or vehicle stability
- Rheological properties of the preparation
- pH effects
- Volatility and loss of water effects
- Stability of the vehicle in terms of phase separation and homogeneity
- Particle size and the distribution of the vehicle
- Particulate and microbial contaminants

Physicochemical factors influencing skin permeation will now be discussed in the following section.

#### **2.5.3.2.1 pH**

Essential knowledge about pH and the skin consists of the following two theories. Firstly, the evident pH of the stratum corneum is about 4.8 – 6.0 and secondly, the permeability of the barrier does not adjust when subjected to pH in the range of 3.5 – 8.5 (Sznitowska *et al.*, 2001:327). The passing of unionised molecules across lipid membranes is more profound than for ionised molecules. This can be attributed to the unionised drugs that are more soluble in lipids whereas ionised molecules are more soluble in aqueous mediums. Ionisation of a drug is dependent upon the pH of the excipient or vehicle in which the drug has been formulated and will conclude both the activity and discharge of a drug from its delivery system. Most drugs are either weak acids or weak bases of which the solubility in a particular vehicle is established by the pH of the medium. Altering the pH of a formulation will manipulate the stability of the drug as well as the stability of the preparation. It can therefore be considered that the pH for optimal solubility is not always the ideal pH for optimal stability for the drug. A balance must be determined for the best possible solubility and the highest stability of the drug (Pefile & Smith, 1997:148).

#### **2.5.3.2.2 Molecular size and weight**

According to Malan *et al.* (2002:387), there is a noticeable correlation among the diffusion coefficient ( $D$ ) and molecular weight ( $M_r$ ). Absorption is in fact inversely associated to molecular weight, meaning that small molecules penetrate the skin faster than large molecules (Barry, 2002:513). The rate-determining aspect in the permeation of undersized molecules through mucosal membranes is the molecular charge of the particle. With the expansion in molecular

size, the “sieving effect” of the pores becomes progressively more selective and even more significant than electrical field interactions. The molecular size is considered to play a key role in the permeation process of drug molecules. Outsized molecules diffuse progressively poorer since they necessitate more space to be created in the medium and can lead to reduced permeability (Malan *et al.*, 2002:387). To some extent, poor penetration can be attributed to active molecules being larger than 500 Dalton (Da) which cannot penetrate the corneal layer of the skin, while smaller molecules are able to pass through the corneal layer, surpassing transcutaneously (Bos & Meinardi, 2000:165). Mangiferin with a molecular weight of 422.34 Da (Wauthoz *et al.*, 2007:114) can cross the skin to some extent, but hesperidin with a molecular weight of 610.56 Da (Garg *et al.*, 2001:656) might show great effort in crossing the skin according to the “500 Dalton rule” (Bos & Meinardi, 2000:165).

#### **2.5.3.2.3 Aqueous solubility**

Ideally a drug must possess a reasonable solubility in both water and oils for proper skin penetration. It also has to have an aqueous solubility of more than 1 mg/ml (Hadgraft, 1996:165). The active ingredient, mangiferin, has very poor solubility in water (Majumbar & Srirangam, 2009:1221), i.e. mangiferin has an aqueous solubility of approximately 0.111 mg/ml, while hesperidin with an aqueous solubility of approximately 4.95 mg/ml could have the potential for sufficient skin penetration.

#### **2.5.3.2.4 Partition coefficient**

The partition coefficient (K) is significant in creating the flux of a chemical substance through the stratum corneum. When the membrane offers the single or main basis of diffusional resistance, the extent of the partition coefficient is particularly significant, for the reason that it can make a difference by the factor of  $10^8$  (Barry, 2002:512).

Mangiferin has a log P value of 2.73 (Wauthoz *et al.*, 2007:115), and hesperidin a log P value of 1.78 (Srirangam & Majumbar, 2010:60). A prediction can therefore be made that permeation of mangiferin in adequate concentrations might not be optimal for skin diffusion purposes, while hesperidin could have a better chance due to more optimal log P values between 1 and 2 according to (Hadgraft, 1996:165).

#### **2.5.3.2.5 Diffusion coefficient**

The characteristics of the drug, the diffusion medium and the interactions amid these factors establish the diffusion coefficient of a drug, either in a topical vehicle or in the skin. Diffusion

lessens as the viscosity of the vehicle increases. The active ingredient present in a solution could experience physical interactions in the skin as well as in the solution itself (Pefile & Smith, 1997:148). In the skin the diffusivities plummet progressively and reach their smallest possible values within the compacted stratum corneum matrix (Barry, 2002:512). If the interactions among the solvent and the drug are profound, the diffusion coefficient will be low and the release of the active ingredient to the stratum corneum will decline. Consequently it is fundamental to acquire maximum bioavailability by having the relevant information describing the transdermal kinetics of the drug concerned prior to its formulation (Pefile & Smith, 1997:148).

### **2.5.3.3 Mathematical approach to drug permeation**

#### **2.5.3.3.1 Fick's law of diffusion**

The diffusion of compounds across a membrane can mathematically be described by Fick's Law whenever the chemical or physical nature of the membrane controls the rate of diffusion (Barry, 2002:506). In passive diffusion material moves from one area of a system to a different, following random molecular motions. Fick's first law states that the flux of a compound at a given time and position is proportional to the differential change in concentration over a differential distance (WHO, 2010:23). The second law by Fick was formulated for the frequent experimental circumstances in which diffusion is unidirectional where the concentration gradient is only along the x-axis (Barry, 2002:506).

Fick's law of diffusion can be written as follows (Rieger, 1993:39):

$$J = \frac{K \cdot D}{l} \Delta C$$

**Equation 2.1**

Where:

- J = flux ( $\mu\text{g}/\text{cm}^2 \cdot \text{h}$ )
- K = partition coefficient
- D = diffusion coefficient ( $\text{cm}^2/\text{h}$ )
- $\Delta C$  = concentration difference ( $\mu\text{g}/\text{cm}^3$ )
- l = membrane thickness (cm)

## 2.6 Conclusion

The efficacy of several chemical agents for the prevention and treatment of dermal ageing is well established and the ageing of the baby boomer population has given rise to increased consumer interest in maintaining a youthful appearance throughout middle age (Bruce, 2008:S17).

Using honeybush extracts with their “bioactive” components in cosmetic preparations to treat conditions such as dryness, eczema, acne, free radical scavenging, anti-inflammatory and skin ageing can create an ideal market for consumers who want to use all natural products. When transported deeply into the skin, polyphenols present in honeybush may possibly be circulated in a uniform way through all the layers of the skin or be concentrated in an exact region, depending on the character of both the polyphenol used and its delivery system.

This makes plant polyphenols viable as competent natural skin defenders against UV-induced harm as well as antibiotics to fight fungal and bacterial skin diseases. When applied to human skin, it could effortlessly penetrate the metabolic pathways by replacing or competing with endogenous phenylalanine metabolites. This offers a concrete justification for anti-age, anti-acne, anti-hair loss, whitening and anti-hirsutism polyphenol-based skin products.

Although most drugs are administered orally, the transdermal route of administration can serve as an attractive alternative. The transdermal route has exceptional advantages, but also a few limitations such as the skin barrier function. The stratum corneum, an excellent protective layer to unwanted substances, can make it very difficult for molecules to pass through the skin. The three possible pathways for drug administration through the skin can be accomplished via intercellular, transcellular and appendageal diffusion. Penetration of these molecules can also be enhanced by chemical and physical penetration enhancers, giving the skin the potential as an alternative route for the successful administration of drugs.

In this study the objectives were to formulate two different creams containing either a 2% *Cyclopia maculata* or a 2% *Cyclopia genistoides* extract for examining the hydrating and smoothing effect in the treatment of skin ageing; to develop and validate a HPLC method to determine quantitatively concentrations of the different ingredients in the formulations; to determine whether honeybush extracts diffuse through the skin or into the skin; to test whether honeybush extracts have potential antioxidant effects; to determine whether honeybush extracts have a clinically moisturising effect on human subjects and to do stability tests on the different formulations.

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## **CHAPTER 3**

# **ARTICLE FOR PUBLICATION IN *SKIN PHARMACOLOGY AND PHYSIOLOGY***

Chapter 3 is written in an article format for the purpose of publication in *Skin Pharmacology and Physiology*. The complete guide for authors of this journal is given in Appendix G. No formatting was used during the writing of this article, other than advised by the guide for authors. However, paragraphs were justified to ease reading and improve neatness. This Chapter differs from the rest of the dissertation as it is written in US English and not UK English.

# **Stability and clinical efficacy of honeybush extracts in cosmeceutical products**

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### 3 Abstract

**Background:** Honeybush extracts, with flavonoids such as mangiferin and hesperidin can be designed to protect the skin against exogenous and endogenous agents, balancing dermal homeostasis lipids altered by dermatosis and aging and have the potential to reduce skin inflammation and to scavenge free radicals, penetrating the dermal and epidermal layers while counteracting the aging of the human skin. **Aim and Methods:** The aim of this study was the formulation of two 2% semi-solid formulations containing two different honeybush extracts (e.g. *Cyclopia maculata* and *Cyclopia genistoides*) and performing accelerated stability tests, membrane release, Franz cell diffusion studies, and tape-stripping techniques. The antioxidant potential and clinical efficacy of the honeybush extracts were also examined. **Results and Conclusion:** During stability testing the change in color, viscosity and concentration of the active ingredients in the formulations indicated that the products were unstable over the three months period. Data obtained during Franz cell diffusion studies, showed that both mangiferin and hesperidin had inconclusive results due to extremely low concentrations present in the dermal and epidermal layers of the skin during membrane release studies, transdermal diffusion and tape--stripping after 12 h. The lipid peroxidation inhibition abilities of the honeybush extracts as well as the semisolid formulations showed promising results, while mangiferin and hesperidin did not show any antioxidant activity on their own. During the clinical efficacy trial, the results were statistically inconclusive and variations between the subjects were very high due to skin variations at different skin sites. There was, however, a trend that *Cyclopia genistoides* performed the best.

#### 3.1 Introduction

Skin aging is the end result of a constant corrosion process as a result of the injury to cellular deoxyribonucleic acid (DNA) and proteins. The aging progression can be divided into two very diverse types, i.e. “sequential skin aging” (intrinsic) and “photo-aging” (extrinsic). Intrinsic skin aging is a common and expected process characterized by physiological modifications in the skin function. Keratinocytes are incapable of forming a purposeful stratum corneum and the tempo of the arrangement of neutral lipids slows down dramatically, resulting in dry, pale skin with fine wrinkles. Extrinsic aging is caused by overexposure to UV (ultraviolet) radiation from sunlight. It can be characterized by dry, pale and shallow skin, displaying fine wrinkles as well as deep furrows caused by the disorganization of epidermal and dermal components associated with elastosis and heliodermatitis [2].

After the earliest mention of honeybush in botanical literature in 1705 [21], it was soon recognized as a plant with various medicinal properties [3]. The term honeybush applies to several different species of *Cyclopia*. These are all woody, fynbos shrubs restricted to the mountains near the Cape Peninsula [3]. Phenolic compounds found in these shrubs are known to be mangiferin, hesperidin, hesperitin and isosakuranetin [12]. Honeybush was originally used as a restorative and an expectorant in chronic catarrh and pulmonary tuberculosis but was later on also known for its anti-inflammatory, antioxidant, antimutagenic, phyto-oestrogenic and antimicrobial effects with a relative low toxicity [20].

The use of plants, such as honeybush, was once the main source and foundation of all cosmetics, before methods were discovered of synthesizing substances with similar properties [1]. These herbal extracts for topical application deserve to be considered as cosmeceuticals because of their use for treating skin conditions and a wide variety of dermatological disorders for centuries [1]. It can be designed to protect the skin against exogenous and endogenous agents, balancing dermal homeostasis lipids altered by dermatosis and aging. Plants with a high level of flavonoids such as honeybush, have the potential to reduce skin inflammation and to scavenge free radicals [1] penetrating the dermal and epidermal layers while counteracting the aging of the human skin.

Using the skin as an alternative route for the administration of drugs has become very popular over the last few decades and using intact skin as the site of administration for dermatological preparations to elicit pharmacological action in the skin tissue has been well recognized [6]. The human skin forms a remarkable protective barrier against the external environment, regulating temperature and water balance. This barrier also keeps out harmful microbes and chemicals [1]. Unfortunately, using the skin as an alternative route for administering drugs also has numerous limitations. One of these limitations is the barrier function of the skin [28]. This “horny layer”, which comes into direct contact with the environment, is a collection of dead cells, but is also a very complex organism and forms an integral part in the homeostatic system of the human body [1].

Although the stratum corneum gives the body outstanding protection against unwanted substances from its surroundings, it is possible for drugs to be administered transdermally. There are three possible pathways for transport of drugs through the skin, namely intercellular diffusion through the lipid lamellae, transcellular diffusion through the keratinocytes and lipid lamellae and diffusion through hair follicles and sweat ducts [16]. Regrettably it is simply appropriate for a restricted quantity of drugs and substances that have the proper physico-chemical features to allow them to cross the stratum corneum [15]. There are however several factors that may also affect permeation of drugs through the skin and a few of these factors are

skin age, skin condition, skin site, skin metabolism, skin hydration, temperature, pH and the presence of penetration enhancers [11]. Therefore, the lipophilic stratum corneum is responsible for the primary barrier function of the skin and presents a wide-ranging challenge to scientists in their pursuit to extend the range of drugs suitable for transdermal delivery [29].

The aim of this study was to investigate the transdermal delivery of mangiferin and hesperidin – bioactive flavonoids present in various honeybush extracts and species, as well as the antioxidant properties and its clinical efficacy on human subjects.

## **3.2 Materials and methods**

### **3.2.1 Materials**

The active ingredients, mangiferin and hesperidin were obtained from Sigma-Aldrich Corporation (Johannesburg, South Africa). The *Cyclopia maculata* hot water extracts were sponsored by ARC Infrutec-Nietvoorbij (Stellenbosch, South Africa). The *Cyclopia genistoides* (Novel-T<sup>®</sup> Organic Honeybush PE) powder was obtained from Afriplex (Pty) Ltd (Johannesburg, South Africa). The other ingredients used in the formulation of the semi-solid products were obtained as follows: liquid paraffin, methyl paraben, propyl paraben, propylene glycol and cetylstearyl alcohol were obtained from Merck Laboratory Supplies (Midrand, South Africa). Cremophor<sup>®</sup> A 6 and Cremophor<sup>®</sup> A 25 were obtained from BASF Chemicals (Johannesburg, South Africa). Potassium dihydrogen orthophosphate and sodium hydroxide used for the preparation of phosphate buffered solution (PBS) were supplied by Merck Laboratory Supplies (Midrand, South Africa). Phosphoric acid and HPLC (high performance liquid chromatography) analytical grade methanol were obtained from Merck Laboratory Supplies (Midrand, South Africa). Ethanol was obtained from Merck Laboratory Supplies (Midrand, South Africa). The dermatome was purchased from Zimmer Electronics (Warsaw, IN, USA). Ascorbic acid, dimethyl sulfoxide (DMSO) and iron(III)chloride were obtained from Merck Chemicals (Wadeville, South Africa). 1,1,3,3-Tetramethoxypropane (TEP), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA) and Trolox<sup>®</sup> were obtained from Sigma-Aldrich Corporation (Steinheim, Germany). Hydrogen peroxide was purchased from a local pharmacy. The Corneometer<sup>®</sup> CM 825, Visioscan<sup>®</sup> VC 98 and Mexameter<sup>®</sup> MX 18 were purchased from Courage-Khazaka Electronics (Cologne, Germany). Deionized HPLC grade water prepared with a Milli-Q<sup>®</sup> water purification system (Millipore, Milford, USA) was used throughout the entire study.

### **3.2.2 Methods**

#### **3.2.2.1 HPLC analysis of mangiferin and hesperidin**

An HPLC method was developed and validated in conjunction with Prof. Jan du Preez from the Analytical Technology Laboratory at the North-West University, Potchefstroom Campus, South Africa. An Agilent® 1200 Series HPLC equipped with an Agilent® 1200 pump, autosampler injection mechanism and UV-detector was used (Agilent Technologies, Palo Alto, CA). The apparatus was interfaced with Chemstation Rev. A.06.02 data acquisition and analysis software. High performance silica-based, reversed phase Agela® Venusil XBP C<sub>18</sub> (2) column, (150 mm × 4.6 mm) with a 5 µm particle size was used (Agela® Technologies, Newark, DE). The two mobile phases consisted of a mixture of 1 ml phosphoric acid in 1000 ml of HPLC water and acetonitrile. Elution was achieved by means of a gradient method, starting at 5 % acetonitrile and increasing linearly to 85 % acetonitrile after 10 minutes, and holding until 15 minutes whereafter the system was re-equilibrated at the starting conditions for 5 minutes. The operating flow rate was 1.0 ml/min and the injection volume was 20 µl. The UV-detector was set at 210 nm for the detection of mangiferin and hesperidin. The retention time of mangiferin was 4.8 min and that of hesperidin was 5.9 min. The total running time was 20 min. Analysis was performed in a controlled laboratory environment at 25 °C. The solvent used was PBS.

##### **3.2.2.1.1 Standard preparation**

The following ingredients were weighed and dissolved in a 100 ml volumetric flask: honeybush extract (50.0 mg); methyl paraben (2.0 mg); propyl paraben (0.4 mg); BHT (butylated hydroxytoluene) (2.0 mg). It was then made up to volume with methanol/HPLC water and injected into the HPLC in triplicate.

##### **3.2.2.2 Formulation of a cosmeceutical cream with honeybush extracts as the active ingredient**

Two 2% semi-solid honeybush extract (*Cyclopia maculata* or *Cyclopia genistoides*) products were formulated with mangiferin and hesperidin (bioactive flavonoids present in honeybush extracts) as the active ingredients for the treatment of skin aging. The components of the oil phase were mineral oil (12.0%), cetyl stearyl alcohol (7.0%), Cremophor® A 6 (1.5%), Cremophor® A 25 (1.5%) and preservatives. The water phase contained deionized water (67.56%). The third phase contained the active honeybush extracts (2.0%) dissolved in propylene glycol (8.0%).

### **3.2.2.3 Stability testing**

The two different semi-solid formulations that were formulated were stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH. The following stability tests were done after 0, 1, 2 and 3 months: concentration assay, pH, viscosity, zeta potential, particle size, visual appearance and mass loss.

#### **3.2.2.3.1 Sample preparation**

2 g of each honeybush extract formulation at each storage condition was weighed in 100 ml volumetric flasks in duplicate. The cream samples were made up to volume with methanol. The solutions were filtered and injected into the HPLC in duplicate for concentration assays.

#### **3.2.2.3.2 Concentration assay**

In order to ensure the accuracy of the test results, all tests were done under Good Laboratory Practice (GLP) conditions. The concentrations of the following ingredients in the different formulations were determined with HPLC analysis: mangiferin, hesperidin, methyl paraben, propyl paraben and BHT. The validation of the HPLC analysis, as well as the chromatographic conditions, is discussed in Section 2.2.1.

#### **3.2.2.3.3 pH**

The pH of the formulations was measured with a Mettler Toledo pH meter (Schwerzenbach, Switzerland). A glass Mettler Toledo Inlab<sup>®</sup> 410 electrode was used and the apparatus was calibrated with Mettler Toledo buffer solutions each time before use. The pH of each honeybush extract formulation at each storage condition was measured in triplicate.

#### **3.2.2.3.4 Viscosity**

A Brookfield Viscometer (Stoughton, Massachusetts, USA) was used to determine viscosity. The formulation was placed in the water bath to reach a temperature of 25 °C. A specific selected spindle (Stoughton, MA) was placed in the formulation and the rate was specified. The viscosity reading was measured every 10 sec for 5 min. Approximately 32 readings were obtained and the average viscosity was determined.

#### **3.2.2.3.5 Zeta potential**

Samples were prepared by weighing 1.0 g of each formulation under each condition in a 100 ml volumetric flask. The cream samples were made up to volume with methanol and the zeta potential of each sample was determined by injecting the prepared samples into a Malvern

Zetasizer 2000 (Worcestershire, United Kingdom). The zeta potential of each formulation at each condition was measured in triplicate.

#### **3.2.2.3.6 Particle size**

Approximately 0.5 g of each formulation under each condition was mixed with approximately 3 ml HPLC water to form a uniform wet dispersion. Dispersions were made up with approximately 4.5 ml HPLC water, mixed and injected in duplicate into a Malvern Mastersizer 2000 (Worcestershire, United Kingdom) using the wet cell, Hydro 2000 SM, as the interface between the sample dispersion and the optical unit.

#### **3.2.2.3.7 Visual appearance**

The visual appearance of each formulation was assessed. Photos were taken with a camera (Pentax® Optio E40) and compared to each other.

#### **3.2.2.3.8 Mass loss**

A Mettler Toledo (Schwarzenbach, Switzerland) balance was used to determine the mass loss of each formulation. The mass of each honeybush extract formulation at each storage condition was determined in triplicate. After the indicated time intervals the mass of each formulation was determined and was subtracted from the original mass to determine the mass loss.

#### **3.2.2.4 Diffusion experiments**

##### **3.2.2.4.1 Preparation of phosphate buffer solution for the receptor phase**

The phosphate buffer solution (PBS, pH 7.4) was prepared according to the British Pharmacopoeia [10]. It was prepared by mixing 13.62 g potassium dihydrogen orthophosphate ( $\text{KH}_2\text{PO}_4$ ) with 500.0 ml HPLC water. Another solution of 3.15 g sodium hydroxide (NaOH) was weighed and dissolved in 786.8 ml HPLC water. The two solutions were mixed and the pH was set to 7.4 with 10% orthophosphoric acid ( $\text{H}_3\text{PO}_4$ ). The solution was filtered and degassed.

##### **3.2.2.4.2 Skin preparation**

Abdominal skin of Caucasian female patients was obtained after cosmetic abdominoplastic surgery. Ethical approval for obtaining and preparing the skin was provided by the Research Ethics Committee of the North-West University under the title “*In vitro* transdermal delivery of drugs through human skin” (NWU-00114-11-A5). The date of issue of the ethical approval was 25 August 2011 and it expires on 24 August 2016. Fresh skin was sponged down with deionized water and dried with tissue paper. The exterior of the skin was wiped once with an

ethanol-moistened cotton swab to eliminate possible fat residual from the subcutaneous fat layer and surface sebaceous lipids. A skin layer of a thickness of approximately 400 µm with a width of 2.5 cm was prepared using an electrical Zimmer dermatome (Warsaw, IN, USA). The skin was positioned dermal side down on filter paper and stored in aluminum foil at -20°C until used. One hour preceding the diffusion experiments, the skin was thawed at room temperature and cut into circular pieces (approximately 15 mm in diameter).

#### **3.2.2.4.3 Membrane permeation**

The aim of the membrane studies was to determine whether mangiferin and hesperidin are released from the formulations. The entire content of the receptor phases was withdrawn and replaced with fresh PBS (pH 7.4) on an hourly basis (hours 1, 2, 3, 4, 5 and 6). Due to extremely low, unquantifiable concentrations depicted during HPLC analysis, a second membrane diffusion study was executed where only a single withdrawal of the entire content of the receptor phases for the formulations was performed after 6 h.

#### **3.2.2.4.4 Franz cell transdermal diffusion**

Vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and a diffusion area of 1.075 cm<sup>2</sup> were used in this study. A total of twelve Franz cells were used for each experiment. Franz cells 1 to 10 contained the active formulation, namely 2% *C. maculata* or 2% *C. genistoides* creams. Franz cells 11 and 12 contained a placebo formulation of the cream used during the experiment. Another diffusion study was performed consisting of twelve Franz cells that contained a 0.003% *C. genistoides* formulation commercially available on South African markets. All Franz cells consisted of a donor (top) and receptor (bottom) compartment. The donor phase (semisolid formulations) was placed in the donor compartment and the receptor phase (PBS, pH 7.4) was placed in the receptor compartment.

Miniature magnetic stirring bars were placed in the receptor compartments of each Franz cell to continue stirring throughout the experiment. The diffusion cells were placed in a tray on a Variomag<sup>®</sup> stirrer plate. The skin circles or cellulose acetate membranes were mounted between the receptor and donor compartment with the stratum corneum facing upwards (towards the donor compartment). Dow Corning<sup>®</sup> high vacuum grease was used to seal the cells to prevent any leakages. The donor and receptor compartments were secured with a metal horseshoe clamp. Thereafter the receptor compartments were filled with PBS (pH 7.4) while care was taken to avoid the entrapment of air bubbles under the surface.

The donor compartments were filled with approximately 1 ml of the semi-solid formulation to keep the skin saturated. It was then covered with Parafilm<sup>®</sup> to avoid evaporation. The Franz

cells were placed in a 37 °C Grant® water bath in order to accomplish a skin temperature of 32 °C.

The complete content of the receptor phases was withdrawn at exact time intervals and substituted with fresh PBS (pH 7.4) that was pre-heated to 37 °C. HPLC vials were then filled with the withdrawn PBS (receptor phase). The concentration of mangiferin and hesperidin was immediately analyzed by HPLC.

#### **3.2.2.4.5 Skin diffusion**

The complete content of the receptor phases was withdrawn and substituted with fresh PBS (pH 7.4) after 20, 40, 60, 80, and 100 min, as well as 2, 4, 6, 8, 10 and 12 h. Due to extremely low, unquantifiable concentrations noticed during HPLC analysis, a second diffusion study was executed where only a single withdrawal of the entire content of the receptor phases for the formulations was performed after 12 h. Tape-stripping was performed after the diffusion studies of 12 h had been completed.

#### **3.2.2.4.6 Tape-stripping**

After topical application and penetration of formulations, the cell layers of the stratum corneum are removed one after another from the same skin area using adhesive films. The tape strips contained the amount of penetrated formulation, which can be analyzed by chemical methods [23]. At the end of the diffusion study the diffusion cells were carefully taken apart and the pieces of skin were pinned onto a piece of Parafilm®, stapled to a solid surface. The diffused area could clearly be seen. The pieces of skin were dabbed dry with tissue. Pieces of 3M Scotch® Magic™ Tape were cut into sizes big enough to cover the diffused area, and small enough not to overlap the areas outside the diffused area. The first tape strip was discarded, as it is seen as part of the cleaning procedure. The next 15 strips (epidermis) were placed in a vial filled with enough PBS (pH 7.4) to cover the strips. An indication of the complete removal of the stratum corneum is when the viable epidermal layer glistens. The vials were kept overnight at 4 °C. The remaining skin was cut into pieces to enlarge the surface area. It was placed in vials filled with enough PBS (pH 7.4) to cover the skin pieces and were kept overnight at 4 °C. The tape samples were filtered and analyzed by HPLC. The skin samples were homogenized and filtered in turn to be analyzed by HPLC.

### **3.2.2.5 Antioxidant experiments**

#### **3.2.2.5.1 Preparation of honeybush extracts**

Four different concentrations of both *Cyclopia maculata* and *Cyclopia genistoides* extracts were prepared. Extracts with each of the following concentrations of 0.3125 mg/ml, 0.6250 mg/ml, 1.2500 mg/ml and 2.5000 mg/ml were dissolved in a water/methanol mixture of equal parts. The actives in the honeybush extracts were made up to represent the approximate concentrations present in the above-mentioned concentrations. For mangiferin, approximately four concentrations of 0.0169 mg/ml, 0.0338 mg/ml, 0.0676 mg/ml and 0.1352 mg/ml were dissolved in a water/methanol mixture of equal parts. Hesperidin concentrations of approximately 0.0046 mg/ml, 0.0092 mg/ml, 0.0184 mg/ml and 0.0368 mg/ml were dissolved in a water/methanol mixture. Lastly, 5 ml of each of the two different semi-solid formulations were dissolved in 5 ml water/methanol mixture.

#### **3.2.2.5.2 Preparation of PBS (pH 7.4) buffer for antioxidant activity determination**

The phosphate buffer solution (PBS) consisted of 137 mM NaCl (sodium chloride), 2.7 mM KCl (potassium chloride), 10 mM Na<sub>2</sub>HPO<sub>4</sub> (disodium hydrogen orthophosphate anhydrous) and 2 mM KH<sub>2</sub>PO<sub>4</sub> (potassium dihydrogen orthophosphate) in 1000 ml Milli-Q water. The pH of the solution was ascertained to be 7.4 and the solution was stored in the refrigerator.

#### **3.2.2.5.3 Test Animals**

*In vitro* experiments were performed on whole rat brain homogenates from adult male Sprague-Dawley albino rats weighing between 200 to 250 g. The animals were housed in a windowless, well-ventilated constant environment (CER) room under a diurnal lighting cycle: 12 h light; 12 h darkness. Ambient temperature of the animal room was maintained at 21 ± 1 °C, with a humidity of 55 ± 5%. The animals received standard laboratory chow and water *ad libitum* and the induction of stress was minimized at all times. The North-West University (Potchefstroom Campus) Animal Ethics Committee approved the experimental assay performed under ethical code 05D05 and it conforms to the University's Regulations Act concerning animal experiments.

#### **3.2.2.5.4 Preparation of the standard**

TEP/MDA was used as a standard. A series of reaction tubes, each containing appropriate aliquots of water and standard solution was prepared with Milli-Q water to a final volume of 1 ml. A calibration curve was generated by measuring the absorbance at 10 nmole/ml intervals, in the range of 0 – 50 nmole/ml at a detection wavelength of 532 nm using an UV-visible

spectrophotometer. The absorbance of the TBA/MDA-complex was plotted against the known concentration of MDA.

#### **3.2.2.5.5 Tissue preparation**

Rats were sacrificed by decapitation and the whole brain of each rat was rapidly excised. The whole rat brain was homogenized in 0.1 M PBS (pH 7.4) to give an ultimate concentration of roughly 10% (w/v). PBS buffer was used as it has been shown not to scavenge free radicals [5].

#### **3.2.2.5.6 TBA-assay**

To establish the potential antioxidant activity of extracts, 1 ml rat brain homogenate containing toxin combination and varying concentrations of extracts, active ingredients and semi-solids was incubated in an oscillating water bath for 60 min at 37 °C, in order to induce lipid peroxidation. After incubation the content was centrifuged at 2000 x g for 20 min, removing all insoluble proteins. The supernatant was removed from each tube and the termination of the incubation period was followed by the addition of 0.5 ml methanolic BHT (0.5 mg/ml), 1 ml TCA (10%) and 0.5 ml TBA to this fraction. Amplification of lipid peroxidation during the assay was prevented by adding the chain-breaking antioxidant BHT to the sample, TCA to start the acid-heating hydrolysis reaction (acid-catalyzed nucleophilic addition reaction) and to precipitate proteins and TBA to bind to the formed MDA and form the pink chromogen. The tubes were sealed (marbles) and the mixtures heated to 60 °C in an oscillating water bath for 60 min, to release the protein-bound MDA through hydrolysis. Following the incubation the samples were cooled on crushed ice until it reached room temperature and the TBA-MDA complexes were extracted with 2 ml butanol and centrifuged at 2000 x g for 10 min. The absorbance was read at 532 nm.

#### **3.2.2.6 Clinical efficacy of semisolid formulations containing honeybush extracts**

##### **3.2.2.6.1 Non-invasive skin measurements**

###### **3.2.2.6.1.1 Skin hydration**

The Corneometer<sup>®</sup> CM 825 measurement is based on capacitance measurement of a dielectric medium, in this case the skin. Changes in water content of the stratum corneum are converted to arbitrary units (AU). The measurement time is at only 1 sec minimizing occlusion effects. The depth of the measurement is 10 – 20 µm, analyzing the stratum corneum and ensuring that deeper skin layers do not influence the measurements [24].

#### **3.2.2.6.1.2 Skin topography**

The Visioscan<sup>®</sup> VC 98 (Courage-Khazaka Electronic, Cologne, Germany) provides the possibility to analyze skin topography. An image of skin area (6 mm x 8 mm) is taken with a built-in CCD camera. The connection of the Visioscan<sup>®</sup> VC 98 to the personal computer is possible by an image digitalization unit which configures the image in 256 gray level pixel by pixel, where 0 resembles the color black and 255 resembles the color white. The topography of the captured skin image can be analyzed by utilizing the surface evaluation of living sels (SELS) software that generates parameters such as skin entropy, scaliness and roughness.

#### **3.2.2.6.1.3 Melanin and hemoglobin content of skin**

The Mexameter<sup>®</sup> MX 18 (Courage-Khazaka Electronic, Cologne, Germany) measures the content of melanin and hemoglobin (erythema) in the skin. The measurement is based on the absorption principle. The special probe of the Mexameter<sup>®</sup> MX 18 emits light of three defined wavelengths. A receiver measures the light reflected by the skin. The positions of the emitter and receiver guarantee that only diffused and scattered light is measured. As the quantity of the emitted light is defined, the quantity of light absorbed by the skin can be calculated. The melanin is measured by two wavelengths. These two wavelengths have been chosen in order to achieve different absorption rates by the melanin pigments. For the erythema measurement, two different wavelengths are used to measure the absorption capacity of the skin. One of these wavelengths corresponds to the spectral absorption peak of hemoglobin. The other wavelengths have been chosen to avoid other color influences. The results achieved are shown as indices on the screen on a scale from 0 – 999.

#### **3.2.2.6.1.4 Human subjects**

The study has been carried out according to the Helsinki declaration (Ethical principles of medical research involving human subjects), under the project title “(In vivo) Cosmetic efficacy studies” (NWU-00097-10-A5). A group of eighteen healthy female subjects between 40 and 65 years of age participated in a two-week treatment phase. A seven day washout period took place before testing started, where participants follow their normal skin cleansing routines but may only use Dove<sup>®</sup> soap that was supplied. All participants complied with both the inclusion and exclusion criteria. All subjects signed an informed consent form and participants could discontinue their participation at any time during the study [24].

#### **3.2.2.6.1.5 Treatment protocol**

The treatment protocol was conducted according to a comparatively similar study performed by [24] The treatment sites were on both (left and right) volar forearms. Subjects were instructed

to follow normal skin cleansing routines and to refrain from any use of lotions or soaps other than Dove® soap on the volar forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory schedule. Three sites of 3 x 2 cm on each arm were marked with a Codman® surgical marker. The three measurements ( $T_0$ ) are taken with the three instruments on the six different areas before product application and are the baseline values for each area.

During the next two weeks (14 days) each site was treated with the assigned cream. The placebo, *C. maculata* cream or *C. genistoides* cream was applied on the correct marked square twice daily, according to the randomized double blind placebo-controlled study guidelines. Each subject received a treatment program and had to use the placebo, as well as the two active formulations in the morning and evening. The amount of product put on the marked areas is 1 – 3  $\mu\text{l}/\text{cm}^2$  (1 – 3  $\text{mg}/\text{cm}^2$ ). The subjects also received a timetable to document the time they applied the cream. Creams should be applied between 06:00 to 08:00 in the mornings and between 18:00 to 20:00 in the evenings. For measurement days subjects refrained from applying the treatment in the morning. They did, however, apply treatment the evening prior to the measurements. The final measurements were made after the two-week period passed ( $T_1$ ).

#### **3.2.2.6.1.6 Environmental conditions**

All measurements were conducted under controlled temperature and humidity conditions ( $22 \pm 2$  °C and  $50 \pm 10\%$  RH) according to the guidelines for standardized hydration measurement. The subjects acclimated to the room conditions for at least 30 min before any measurements were made.

#### **3.2.2.7 Data analysis**

##### **3.2.2.7.1 Data analysis for release and skin diffusion studies**

For the membrane studies the average percentage released was determined as well as the average concentration ( $\mu\text{g}/\text{cm}^2$ ; amount per area) of actives that was released after 6 h.

For the diffusion studies the average percentage diffused as well as the average concentration ( $\mu\text{g}/\text{cm}^2$ ; amount per area) of the actives that penetrated the skin after 12 h was calculated.

##### **3.2.2.7.2 Data analysis for antioxidant experiments**

The absorbance values obtained were converted to MDA levels (nmole MDA) from the calibration curve generated with TEP. Results and the extent to which lipid peroxidation occurred were expressed as nmole MDA/mg tissue.

### 3.2.2.7.3 Data analysis for clinical efficacy experiments

All the parameters were treated as follows:

The difference in the various skin measurements at ( $T_1$ ) relative to the initial conditions ( $T_0$ ) was taken as a percentage change between the measurements calculated for the placebo, *C. maculata* and *C. genistoides* treatments, respectively.

$$\%Change = \frac{T_1 - T_0}{T_0} \times 100$$

The parameters were further subjected to appropriate statistical analysis to determine any significant differences between the normalized parameters for the various treatments.

### 3.2.2.8 Statistical analysis

#### 3.2.2.8.1 Statistical analysis for antioxidant experiments

GraphPad InStat 3 software was used for the statistical analysis of data. Results are given as the mean  $\pm$  S.E.M (standard error of the mean) of 5 repeats ( $n = 5$ ). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman Keuls test for multiple comparisons. The difference between groups was considered to be significant when  $p < 0.05$  when compared to the toxin (#). When  $p < 0.001$  it is considered extremely significant (\*\*\*). When  $p < 0.01$  it is considered fairly significant (\*\*), while  $p < 0.05$  is considered as significant (\*). A  $p > 0.05$  is considered to be not significant (ns).

#### 3.2.2.8.2 Statistical analysis for clinical efficacy experiments

Results are given as the mean  $\pm$  S.E.M. of 5 repeats ( $n = 5$ ). Data were analyzed by one-way analysis of variance (ANOVA) followed by the T-test (paired two sample for means). The difference between groups was considered to be significant when  $p < 0.05$ .

## 3.3 Results and discussion

### 3.3.1 Formulation of a cosmeceutical cream with honeybush extracts as the active ingredient

Two different honeybush extracts (e.g. 2% *Cyclopia maculata* cream and 2% *Cyclopia genistoides* cream) were formulated into two semi-solid formulations: The cream applied easily and was not too oily. It had a homogeneous caramel texture and a sweet odor. The final formulations were prepared in adequate amounts for stability testing. All the formulations were

inspected for appearance and texture. Both formulations were found suitable prior to storage at the three different temperatures used throughout stability testing.

### **3.3.2 Stability testing**

#### **3.3.2.1 Concentration assay**

The assay concentrations of mangiferin and hesperidin in both the formulations showed significant change and did not remain within the acceptable limits (5% change of initial value). This could indicate the active bioflavonoids present in honeybush extracts lack sufficient stability in semi-solid formulations or that interaction with the different ingredients in the formulation could have occurred. The only active ingredient (mangiferin) that did stay within acceptable limits is the *C. maculata* cream stored 25 °C/60% RH. Future formulations containing honeybush extracts should be stored at room temperature in a cool and dry environment for optimal preservation. Preservatives in both formulations did not remain within the acceptable limits. Propyl paraben in the *C. maculata* cream and methyl paraben in the *C. genistoides* cream did not remain within the limits of 5% according to the ICH [18]. Degradation of the active ingredients can be due to insufficient protection by the preservatives. BHT, the antioxidant, showed little change in both formulations. Honeybush extracts are known to emit their own anti-oxidant activity and a saturated supply of antioxidants was present in the formulation.

#### **3.3.2.2 pH**

The pH of neither of the two creams remained stable over the 3-month period. The pH of the formulation could influence the stability of the actives. Human skin is very sensitive to extreme pH ranges, while the stratum corneum can be extremely resistant to changes in pH, tolerating a pH range of 3 to 9 [7]. The *C. maculata* formulation proved to be the more stable formulation with a maximum pH change of 20% stored at 40 °C/75% RH, while the *C. genistoides* formulation with a maximum pH change of 31% was stored at 40 °C/75% RH. This could indicate that the vast variations in pH are a clear indication of the instability of mangiferin and hesperidin.

#### **3.3.2.3 Zeta potential**

The zeta potential can be directly affected by several influencing factors, one being the abovementioned pH. The general distinction between stable and unstable suspensions is usually 25 to 30 mV (positive or negative) as stated by Malvern [26] as well as Kirby & Hasselbrink [22]. Particles with zeta potentials more positive than + 25 mV or more negative than - 25 mV are usually considered stable due to the repelling forces between the particles,

forcing the formulation into suspension. Very low zeta potentials were present during stability testing, indicating incipient instability. Particles within the formulations may adhere to one another and form aggregates of successively increasing size, which may settle on the bottom under the influence of gravity. This may possibly lead to coagulation or flocculation of particles and later lead to total phase separation of the formulation. The drop in pH in both formulations gave the zeta potentials a more negative charge due to higher concentrations of hydronium ions released over time, but these values still remained too low, ensuring incipient instability when storage times were increased. However, in relative terms, the *C. genistoides* formulation proved to be the more “stable” formulation, with the highest increase in zeta potential although incipient instability was present in both formulations.

#### **3.3.2.4 Particle size**

Both formulations had an average particle size increase over the three months. Given their low zeta potential values, it could be predicted that there will be a very low repellent force between the particles in dispersion to prevent flocculation and sedimentation as time passed. In relative terms, it could be concluded that the *C. maculata* formulation had the lowest increase in average particle size, also considering that both formulations had very low zeta potentials. Both creams showed imminent flocculation, sedimentation and possible total phase separation as time progressed.

#### **3.3.2.5 Viscosity**

The viscosity of both creams changed radically over the 3-month period. The *C. genistoides* formulation showed an immense decrease in overall viscosity. Possible explanations for this tendency can be the low zeta potentials detected during this study, indicating imminent flocculation, sedimentation and possible phase separation. Interaction between the different ingredients within the formulations, evident in the breakdown of the actives could also have an effect on the decrease in viscosity. Broken Van der Waals forces between the molecules could also have occurred during the previous viscosity measurements on the same cream, also leading to a decrease in viscosity [4]. An increase in viscosity as seen in the *C. maculata* cream may be due to loss of moisture during stability testing over the period of 3 months, giving the cream a higher viscosity value. Low zeta potentials were detected for the *C. maculata* formulations and predicted that imminent instability was present. The *C. maculata* formulation appeared to be the more stable formulation, due to the imminent phase separation present in the *C. genistoides* cream. Moisture loss was the most attributable factor responsible for the increase in viscosity in the *C. maculata* cream.

### **3.3.2.6 Visual appearance**

The visual appearance and color of both creams showed a radical change over the 3 month-period. The *C. maculata* formulation started with a glossy, light caramel-colored cream and ended as matt, dark clayed paste-like textured cream, supporting the data that a decrease in viscosity was present. The *C. genistoides* formulation changed from a glossy, light caramel-colored formulation to a glossier, burned dark toffee-colored formulation with a tea-like fluid present on the top surface of the cream. The massive decrease in viscosity supports this appearance, with imminent phase separation clearly evident. In this instance, the *C. maculata* cream proved to be the more stable formulation due to the lack in phase separation evident in the *C. genistoides* cream.

### **3.3.2.7 Mass loss**

The mass of neither of the two creams remained stable over the 3-month period. The *C. maculata* formulation showed the highest degree of moisture loss between the two formulations with the highest decrease in mass loss in the formulations stored at 25 °C/60% RH. This could be due to the loss of moisture due to the conveying of moisture from high moisture content within the cream to an atmosphere lower in moisture content. Containers not sealing as desired, could also increase the loss of mass due to the escape of moisture to the surrounding atmosphere caused by the possible porosity of the storage containers. These results are also supported in the data retrieved during the estimation of change in the viscosity as well as in the visual appearance after 3 months. The *C. maculata* cream showed a massive decrease in viscosity and becoming a paste-like formulation after 3 months.

The lowest decrease in mass was present in the formulations stored at 30 °C/60% RH in both extracts. This could be due to the fact that equilibrium between the moisture content within the cream and the surrounding atmosphere was reached faster due to the favorable temperature relative to the humidity. The *C. genistoides* formulation proved to be the more stable formulation, with the lower average decrease in mass loss during the 3-month storage period. In the future, sealable amber glass containers will be more effective to use.

## **3.3.3 Diffusion experiments**

### **3.3.3.1 Membrane release studies**

In both formulations extremely low concentrations were detected in the receptor phase of the Franz cell after penetration through the cellulose acetate membranes. This could be an indication that the active ingredients have insufficient physico-chemical properties for

satisfactory release of the actives and that the active ingredient concentrations within the formulations are too low for accurate detection during HPLC analysis.

In the *C. maculata* formulation, only three of the ten diffusion cells showed release and therefore did not justify a full skin diffusion study. The HPLC method was barely capable of detecting these minuscule concentrations. In the *C. genistoides* cream however, a maximum of six of the ten diffusion cells showed release and therefore a full skin diffusion study was performed. Both mangiferin and hesperidin in the *C. genistoides* cream had better release than that of the *C. maculata* formulation. When examining the average concentration ( $\mu\text{g}/\text{cm}^2$ ) after 6 h, *C. genistoides* cream released mangiferin and hesperidin approximately fourteen and seven times better than the *C. maculata* cream. The *C. genistoides* cream also had extremely low concentrations and the HPLC detection was difficult since the peaks were so small that they had to be integrated by hand. Therefore, it is difficult to draw any accurate conclusions on these observations.

### 3.3.3.2 Diffusion studies

Two diffusion studies were conducted on the 2% *C. genistoides* formulation. The first diffusion study consisted of extractions every 20 min up to 2 h and thereafter 2 hourly up to 12 h. Due to unmeasurable concentrations that diffused in the above-mentioned study, a second diffusion study was initiated with only one extraction after 12 h. Results from the second diffusion study were analyzed and only  $0.250 \mu\text{g}/\text{cm}^2$  mangiferin diffused through the skin. With concentrations too low for accurate quantification, the results were considered inconclusive. Two diffusion studies were also conducted on the honeybush formulation (0.003% *C. genistoides* cream) currently available on South African markets. In one diffusion study extractions were made every 20 min up to 2 h and thereafter 2 hourly up to 12 h and the other after 12 h. In both studies, no mangiferin or hesperidin was detected.

This poor performance by these formulations can be attributed to several factors. Firstly, the human skin has exceptional properties of which functioning as a physico-chemical obstruction is one of its key features [9]. Skin is a complex organ that serves to protect the underlying tissues against external physical, chemical, immunological and pathogenic intrusion and free radical attacks, while retaining moisture and providing thermal regulation [16]. The extent of skin diffusion is mainly reliant on physiological factors of the skin as well as the physico-chemical characteristics [19]. To some extent, poor penetration can be attributed to active molecules being larger than 500 Dalton (Da) which cannot penetrate the corneal layer of the skin, while smaller molecules are able to pass through the corneal layer, surpassing transcutaneously [9]. Mangiferin, with a molecular weight of 422.34 Da [31] can cross the skin to some extent, but hesperidin with a molecular weight of 610.56 Da [13] will not easily cross the skin according to

the “500 Dalton rule” [9]. As seen from the results shown above this statement seems to be true. Although mangiferin did pass the corneal layer, it was in almost unquantifiable amounts.

Secondly, poor penetration can also be due to the physico-chemical considerations for passive transdermal delivery of a formulation such as drug lipophilicity. The stratum corneum is lipophilic [28]. A drug molecule must first be released from the formulation and partition into the uppermost stratum corneum layer, before diffusion through the entire thickness of the skin can occur [28]. Ideally, a drug must possess a reasonable solubility in both water and oils for proper skin penetration. It also has to have an aqueous solubility of more than 1 mg/ml and a log P (octanol-water partition coefficient) in the range of 1 to 2 [14]. The active ingredients, mangiferin and hesperidin, have very poor solubility in water [25], i.e. mangiferin has an aqueous solubility of approximately 0.111 mg/ml and a log P value of 2.73 [31], while hesperidin with an aqueous solubility of approximately 4.95 mg/ml and a log P value of 1.78 [30]. Hesperidin has an ideal log P value for the diffusion through the stratum corneum, but its big particle size might be hindering successful diffusion.

According to [7] particles between 3  $\mu\text{m}$  and 10  $\mu\text{m}$  can concentrate in the hair follicles, while particles less than 3  $\mu\text{m}$  penetrate follicles and the stratum corneum alike. The *C. maculata* formulation had an average particle size of 22.25  $\mu\text{m}$  and the *C. genistoides* formulation an average of 14.09  $\mu\text{m}$ . Thus, it can be agreed that particles larger than 10  $\mu\text{m}$  stay on the skin surface or have extreme difficulty penetrating the skin due to its bigger particle size.

However, the real reason for this poor performance by both the formulations could be attributed to the fact that extremely low concentrations of mangiferin and hesperidin are present in both the 2% and 0.003% *Cyclopia* formulations. Extremely low mangiferin (20.302 mg) and hesperidin (29.544 mg) concentrations within the 2% *Cyclopia genistoides* formulation were released from both the formulations, resulting in inaccurate HPLC quantifications.

### **3.3.3.3 Tape-stripping**

During HPLC analysis, mangiferin (2% *C. genistoides* cream) was the only active ingredient after application to cross the skin in extremely low concentrations. There were also very low concentrations detected in the stratum corneum-epidermis as well as the epidermis-dermis, indicating that very poor skin penetration took place due to the very low concentrations present in the semisolid formulation. Hesperidin was not able to cross the stratum corneum, epidermis or dermis.

No detectable concentrations of mangiferin and hesperidin were present in the stratum corneum-epidermis and epidermis-dermis in the honeybush product currently available on South African markets.

These skin diffusion studies can be compared to a similar study done by Huang *et al.*, [17] that investigated the transport of aspalathin, a unique flavonoid constituent of rooibos tea, across the human skin. These vertical Franz cell diffusion studies were conducted for both pure aspalathin solutions and extracts from unfermented (green) rooibos (*Aspalathus linearis*) aerial plant material across the human abdominal skin. The results obtained showed that only a portion of 0.07% of the initial aspalathin dose penetrated the different layers of the skin for the green rooibos extract solution and 0.08% for the pure aspalathin solution [17].

The incorporation of rooibos extracts as well as honeybush extracts in topical cosmetic formulations, has become a trend in cosmeceuticals to target the skin directly as the target site of action to fight against UV radiation damage and photo-aging through the antioxidant properties of both extracts. The *in vitro* release of the bioactive flavonoids such as mangiferin and hesperidin can be compared to that of aspalathin. Permeation across the skin with the highly resistant stratum corneum from both extracts was relatively low and should be taken into consideration in the future preparation and formulation of cosmeceutical products containing honeybush extracts that aim to provide anti-aging and protective effects in the skin.

### **3.3.4 Antioxidant activity**

#### **3.3.4.1 Antioxidant properties of *Cyclopia maculata* extracts**

When comparing the *Cyclopia maculata* extracts with the toxin it is evident that extract concentrations 0.3125 mg/ml and 0.6250 mg/ml did not demonstrate sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.920 \pm 1.065$  nmole/mg), in turn showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. Comparing the *Cyclopia maculata* extracts with the toxin it is apparent that extract concentrations 1.250 mg/ml and 2.500 mg/ml showed potential antioxidant activity by means of decreasing the MDA formation, which in turn leads to a lower degree of lipid peroxidation and higher OH<sup>•</sup> radical scavenging abilities of the extracts.

After the comparison of *Cyclopia maculata* extracts with trolox ( $3.716 \pm 0.320$  nmole/mg) it is clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations were below trolox.

When comparing the extract concentrations to both toxin and trolox, it is observed that concentrations 1.250 and 2.500 mg/ml were the only two concentrations that fell between the negative (toxin) and positive (trolox) control. Therefore, optimum synergistic activity could be predicted between the concentration range of 1.250 mg/ml and 2.500 mg/ml. Hence, the 1.250 mg/ml concentration had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidizing lipid system.

Focusing on the statistical analysis of the *Cyclopia maculata* extracts it is observed that concentrations 0.3125, 1.250 and 2.500 mg/ml were statistically extremely significant with a p-value of < 0.001. Concentration 0.625 mg/ml was considered statistically fairly significant with a p-value of < 0.01.

#### **3.3.4.2 Antioxidant properties of *Cyclopia genistoides* extracts**

When comparing the *Cyclopia genistoides* extracts with the toxin it is evident that extract concentration 0.3125 mg/ml did not demonstrate sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.920 \pm 1.065$  nmole/mg), sequentially showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. On the contrary, when comparing the *Cyclopia genistoides* extracts with the toxin it is apparent that extract concentrations 0.625, 1.250 and 2.500 mg/ml showed potential antioxidant activity by means of decreasing the MDA formation, which in turn leads to a lower degree of lipid peroxidation and higher OH<sup>•</sup> radical scavenging abilities of the extracts.

After the comparison of *Cyclopia genistoides* extracts with trolox ( $3.716 \pm 0.320$  nmole/mg) it is clear that concentration 2.500 mg/ml of the extract concentrations emitted its own antioxidant activity, as this concentration had an MDA value below that of the trolox.

When comparing the extract concentrations to both toxin and trolox, it is observed that concentrations 0.625 and 1.250 mg/ml were the only two concentrations that fell between the negative- (toxin) and positive (trolox) control, while 2.500 mg/ml was below both negative- and positive controls. Therefore, optimum synergistic activity could be predicted between the concentration range of 0.625 mg/ml and 2.500 mg/ml. Hence, the 2.500 mg/ml concentration had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidizing lipid system.

Focusing on the statistical analysis of the *Cyclopia genistoides* extracts it is observed that concentration 1.250 mg/ml was statistically extremely significant with a p-value of < 0.001. Concentration 2.500 mg/ml was considered statistically fairly significant with a p-value of < 0.01,

while concentrations 0.3125 and 0.625 mg/ml were considered to be not significant with a p-value of  $> 0.05$ .

#### **3.3.4.3 Antioxidant properties of *Cyclopia* semisolid formulations**

When comparing the *Cyclopia* semisolid formulations with the toxin it is evident that both the *Cyclopia genistoides* and the *Cyclopia maculata* semisolid formulation showed potential antioxidant activity by means of decreasing the MDA formation, which in turn leads to a lower degree of lipid peroxidation and higher OH<sup>•</sup> radical scavenging abilities of the formulations.

After the comparison of the *Cyclopia* semisolid formulations with trolox ( $8.497 \pm 0.254$  nmole/mg) it is clear that both the semisolid formulations emitted their own antioxidant activity, as both these formulations had an MDA value below that of the trolox.

When comparing both the semisolid formulations to both toxin and trolox, it is observed that both the *Cyclopia genistoides* and *Cyclopia maculata* formulations fell below the negative (toxin) and positive (trolox) control. Hence, the *Cyclopia genistoides* semisolid formulation had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidizing lipid system.

Focusing on the statistical analysis of the *Cyclopia* semisolid formulations it is observed that both formulations were statistically extremely significant with a p-value of  $< 0.001$ .

#### **3.3.4.4 Antioxidant properties of mangiferin standard**

When comparing the mangiferin standard concentrations with the toxin it is evident that extract concentrations 0.0169, 0.0338, 0.0676 and 0.1352 mg/ml did not demonstrate sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.840 \pm 0.239$  nmole/mg), in turn showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. When comparing the mangiferin standard concentrations with the toxin it is apparent that none of the standard concentrations showed any potential antioxidant activity by means of decreasing the MDA formation.

After the comparison of mangiferin standard concentrations with trolox ( $2.771 \pm 0.305$  nmole/mg) it is clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations was below trolox.

When comparing the standard concentrations to both toxin and trolox, it is observed that all four concentrations fell above the negative (toxin) and positive (trolox) control. This clearly indicate

a very low potential for possible antioxidant activity as well as a low level of lipid peroxidation inhibition and OH<sup>•</sup> radical scavenging abilities of the all the standards.

Focusing on the statistical analysis of the mangiferin standard concentrations, it is observed that concentration 0.0338 mg/ml was considered statistically fairly significant with a p-value of < 0.01, while the standard concentrations 0.0169, 0.067 and 0.1352 mg/ml was considered statistically not significant with a p-value of p > 0.05.

#### **3.3.4.5 Antioxidant properties of hesperidin standard**

When comparing the hesperidin standard concentrations with the toxin it is evident that extract concentrations 0.0046, 0.0092, 0.0184 and 0.0368 mg/ml did not exhibit sufficient *in vitro* antioxidant activity when compared to that of the toxin (13.840 ± 0.239 nmole/mg), in turn showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. When comparing the hesperidin standard concentrations with the toxin it is apparent that none of the standard concentrations showed any potential antioxidant activity by means of decreasing the MDA formation.

After the comparison of mangiferin standard concentrations with trolox (2.771 ± 0.305 nmole/mg) it is clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations was below trolox.

When comparing the standard concentrations to both toxin and trolox, it is observed that all four concentrations fell above the negative (toxin) and positive (trolox) control. This clearly indicate a very low potential for possible antioxidant activity as well as a low level of lipid peroxidation inhibition and OH<sup>•</sup> radical scavenging abilities of all the standards.

Focusing on the statistical analysis of the hesperidin standard concentrations, it is observed that concentrations 0.0046 and 0.0184 mg/ml were considered statistically extremely significant with a p-value of < 0.001, while the standard concentrations 0.0092 and 0.0368 mg/ml were considered statistically fairly significant with a p-value of p < 0.01.

### **3.3.5 Clinical efficacy**

#### **3.3.5.1 Skin hydration**

By using the Corneometer<sup>®</sup> CM 825, it was possible to detect if any change in skin hydration took place during the two week trial period. With the focus on skin aging, one of the best preventative actions one can take is by the proper hydration of the skin. It is important to note

that the subjects in this particular study all had moderately dry skin before testing started. Focusing on all that data, there was no significant statistical change in the skin after two weeks when formulations were compared to each other. There was, however, significant statistical differences present when creams were compared to their own performance from when the study started ( $T_0$ ) until the study was completed ( $T_1$ ), with *C. maculata* ( $p = 0.001$ ), *C. genistoides* ( $p = 0.0004$ ) and the placebo ( $p = 0.0006$ ).

The *C. genistoides* ( $31.8 \pm 27.7\%$ ) cream performed better than the placebo ( $25.5 \pm 20.4\%$ ) and the *C. maculata* cream ( $25.0 \pm 24.4\%$ ). The *C. maculata* cream as well as the placebo had a similar degree of skin hydration after the two weeks of skin treatment. The *C. genistoides* cream compared to the placebo had a slightly higher value, but had the highest variation in accuracy. Of the three formulations the *C. genistoides* will be the cream considered to have the best skin hydration effects.

### 3.3.5.2 Skin entropy

By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin entropy took place in the two-week trial period. Focusing on all the data presented during the trial, there were no significant statistical differences in the skin entropy after two weeks when formulations were compared to one another. *Cyclopia genistoides* had a p-value of 0.459, *Cyclopia maculata* had a p-value of 0.251 and the placebo had a p-value of 0.134.

The results also indicate that the placebo showed an increase in skin entropy with a percentage of  $0.7 \pm 1.5\%$ . This gives the skin a higher level of order, indicating the increase in the appearance of skin smoothness. Both *Cyclopia genistoides* ( $-0.7 \pm 3.1\%$ ) and *Cyclopia maculata* ( $-0.5 \pm 1.6\%$ ) showed a decrease in skin entropy, which in turn indicates that honeybush does not have the ability to improve the appearance of a smooth skin complexion.

### 3.3.5.3 Skin scaliness

By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin scaliness took place in the two week trial period. There was a significant statistical difference in skin scaliness in subjects using the *Cyclopia maculata* formulation with a p-value of 0.002, but no significant statistical differences in skin scaliness when *Cyclopia genistoides* ( $p = 0.279$ ) and the placebo ( $p = 0.075$ ) formulations were applied.

All three formulations showed an improvement in skin scaliness after two weeks of treatment by reducing the skin's appearance of dryness. *Cyclopia maculata* ( $-36.8 \pm 27.7\%$ ) achieved the best results in improving skin scaliness, followed by the placebo ( $-16.0 \pm 41.5\%$ ) and lastly

*Cyclopia genistoides* ( $-10.3 \pm 32.52\%$ ). It is very important to note that variations between the subjects were very great, which may interfere with truthful conclusions.

#### **3.3.5.4 Skin roughness**

By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin roughness took place in the two week trial period. Skin roughness indicates the degree of unevenness visible during analysis. There was a significant statistical difference in skin roughness in subjects using the *Cyclopia maculata* ( $p = 0.03$ ), but no significant statistical change in skin roughness when *Cyclopia genistoides* ( $p = 0.247$ ) and the placebo ( $p = 0.350$ ) were used.

Both *Cyclopia maculata* ( $9.6 \pm 12.7\%$ ) and *Cyclopia genistoides* ( $7.1 \pm 18.0\%$ ) increased roughness in the skin. The placebo ( $-1.8 \pm 6.5\%$ ) did however perform the best in reducing the skin roughness and giving the skin a more even texture.

#### **3.3.5.5 Skin erythema**

By using the Mexameter<sup>®</sup> MX 18, it was possible to detect if any change in skin erythema took place in the two-week trial period and to establish whether any skin irritations had occurred during the study period by measuring the degree of skin erythema within the skin. Focusing on all the data presented during the trial, there were no significant statistical differences in the skin erythema after two weeks when formulations (*Cyclopia genistoides* ( $p = 0.464$ ), *Cyclopia maculata* ( $p = 0.557$ ) and the placebo ( $p = 0.785$ )) were compared to one another.

*Cyclopia genistoides* ( $5.6 \pm 17.2\%$ ), *Cyclopia maculata* ( $9.2 \pm 13.9\%$ ) and the placebo ( $8.0 \pm 9.7\%$ ), did increase the degree of erythema in the skin slightly. It is very important to note that variations between the subjects were very great, which may interfere with truthful conclusions.

### **3.4 Conclusion**

Both formulations were found suitable prior to storage at the three altered temperatures used throughout stability testing.

Unfortunately the change in pH, zeta potential, particle size, color, viscosity and concentration of the active ingredients in the formulations showed incipient instability of the products over the 3 month-period. None of the formulations completely met the ICH and MCC's criteria for stability [18, 27].

There were no profound concentrations of mangiferin ( $0.003 \mu\text{g/ml}$ ) and hesperidin ( $0.000 \mu\text{g/ml}$ ) present in the stratum corneum-epidermis or the epidermis-dermis. When comparing the results of the 2% *C. genistoides* to that of the commercial product ( $0.000 \mu\text{g/ml}$ ), it can be seen

that the *C. genistoides* cream performed 677 times better, even though the concentrations of mangiferin detected were extremely low. This is however, a clear indication that honeybush extract concentrations above 2% are needed for better and more conclusive skin diffusion results in the future. The only problem with higher concentrations of honeybush extracts is that the aesthetics of the cosmeceutical cream will be diminished due to the change from an already inconsistent caramel-colored cream to a brown toffee-colored cream. This will make the commercial buyer skeptical due to potential discoloring of the skin.

Both semisolid formulations showed promising results when compared to the toxin, an oxidant. Both formulations showed potential in emitting their own antioxidant activity when compared to trolox, an antioxidant. The *Cyclopia genistoides* formulation had the lowest value in *in vitro* MDA formation in rat brain tissue and attenuated lipid peroxidation better than that of the *Cyclopia maculata* formulation, pointing towards the better in hydroxyl scavenging ability.

The standard concentrations of mangiferin and hesperidin did not show any promising *in vitro* antioxidant activity during the TBA-assay. The different standards showed an increase or equal concentrations in MDA formation in rat brain tissue *in vitro* when compared to the toxin, the oxidant. This increase in 2TBA-MDA complex formation indicates higher levels of lipid peroxidation and the OH<sup>•</sup> radical scavenging inability of the standards. None of the standards could be compared to the results obtained for trolox, an antioxidant with strong free radical scavenging abilities. Honeybush extracts contain various bioactive flavonoids that have a synergistic antioxidant effect on one another. Mangiferin and hesperidin as single entities could not emit antioxidant activity on their own. Combinations of various actives within honeybush extracts in various concentrations are of absolute importance for potential antioxidant activity.

The clinical efficacy results showed that the *C. genistoides* cream performed better than the placebo and the *C. maculata* cream, while the *C. maculata* cream as well as the placebo had a similar degree of skin hydration after the two weeks of skin treatment. Of the three formulations the *C. genistoides* will be the cream considered to have the best skin hydration effects.

During skin entropy analysis the results also indicated that the placebo showed an increase in skin entropy. This gives the skin a higher level of order, indicating the increase in the appearance of skin smoothness. Both *Cyclopia genistoides* and *Cyclopia maculata* showed a decrease in skin entropy, which in turn indicates that honeybush does not have the ability to improve the appearance of a smooth skin complexion.

All three formulations showed an improvement in skin scaliness after two weeks of treatment by reducing the skin's appearance of dryness. *Cyclopia maculata* achieved the best results in improving skin scaliness, followed by placebo and lastly *Cyclopia genistoides*.

Both *Cyclopia maculata* and *Cyclopia genistoides* increased during the analysis in the roughness in the skin. The placebo did however perform the best in reducing the skin roughness and giving the skin a more even texture.

*Cyclopia genistoides*, *Cyclopia maculata* and the placebo, did increase the degree of erythema in the skin slightly.

Neither honeybush extract formulation had any statistical value when data were compared to the *Cyclopia maculata*, *Cyclopia genistoides* and the placebo formulations. Numerous reasons can be given for these indecisive results during the clinical efficacy studies. Three categories or sources of variation including instrumental, environmental and individual (person-linked factors) variables may interfere with measurements [8].

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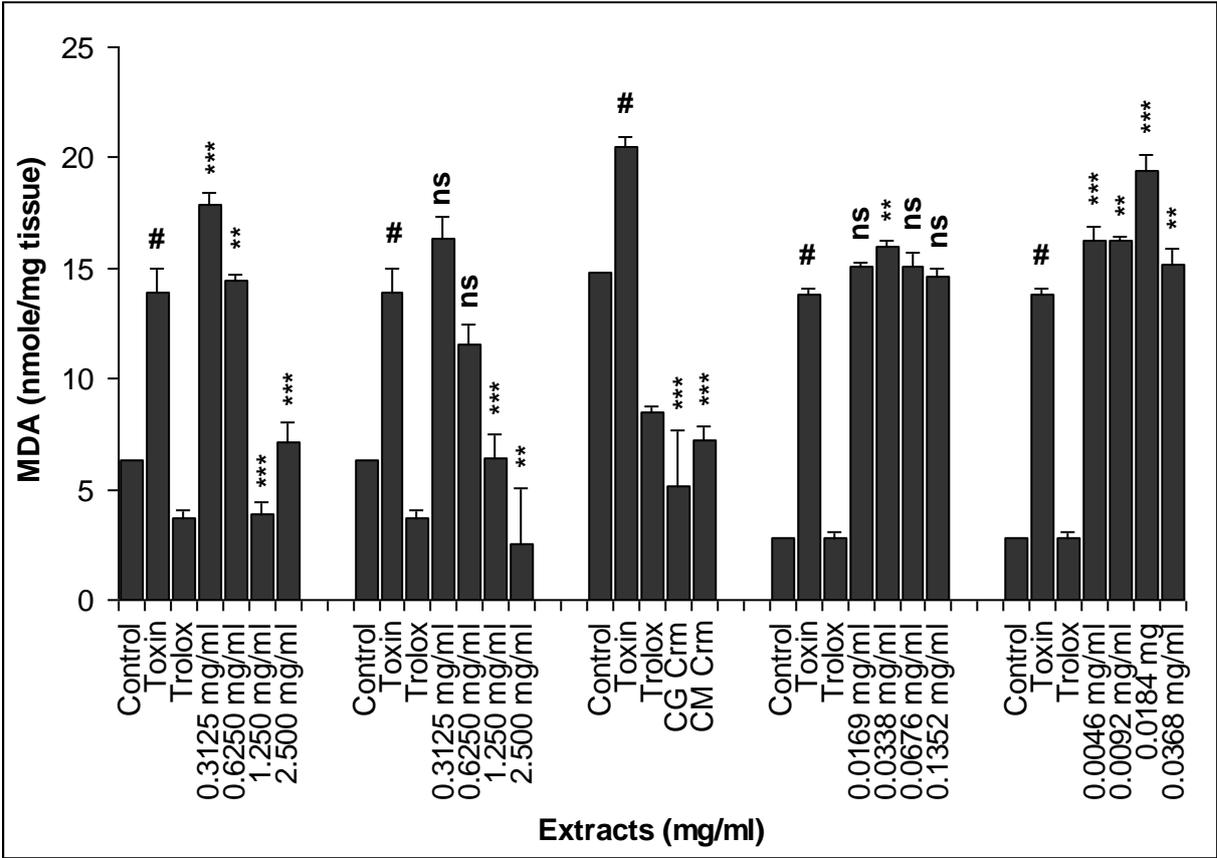
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### 3.7 Figure legends

**Figure 1:** The attenuation of lipid peroxidation by different concentrations of *C. maculata*- and *C. genistoides* extracts, *C. maculata* cream (CM Crm) and *C. genistoides* cream (CG Crm), as well as different concentrations of mangiferin and hesperidin in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). \*\*p < 0.01; \*\*\*p < 0.001 vs. toxin (#), ns = not significant

Figures



**Figure 1:** The attenuation of lipid peroxidation by different concentrations of *C. maculata*- and *C. genistoides* extracts, *C. maculata* cream (CM Crm) and *C. genistoides* cream (CG Crm), as well as different concentrations of mangiferin and hesperidin in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). \*\*p < 0.01; \*\*\*p < 0.001 vs. toxin (#), ns = not significant

## CHAPTER 4

### FINAL CONCLUSIONS AND FUTURE PROSPECTS

As a general rule, attractive skin is professed as being blemish-free, a feature frequently related with youthful skin. Throughout ageing, a progressive deterioration in the functions in the skin gives rise to the emergence of visible heterogeneity of the skin, including redness, blemishes, blotches, wrinkles and rough spots. Pro-inflammatory messengers provoke local redness and greater sensitivity of the blood vessels to environmental or behavioural stress. Similarly, oxidative stress and pro-inflammatory messengers stimulate pigmentation, while the dendricity of melanocytes increases. Phagocyte activity of keratinocytes on melanosomes is also augmented (Mondon *et al.*, 2011:5). Nevertheless, scientific and medicinal progress have lead to a substantial increase in life expectancy with the number of people living to an ever-increasing relatively old age (Giacomoni, 2005:S45).

As the largest organ of the body, one of the skin's major functions is to provide humans with an almost completely impermeable barrier to the external environment (Black, 1993:145). Delaying of the skin ageing process will consequently not only help us to keep a more youthful appearance but will most likely have advantageous effects for the whole organism (Giacomoni, 2005:S46).

The ageing process of the skin can be divided into intrinsic and extrinsic (photo-) ageing (Chung, 2001:1218). When designing products for maximum anti-ageing benefits, formulators should consider both intrinsic and extrinsic ageing factors. Intrinsic ageing is due to the passage of time; whereas extrinsic ageing can be attributed to repeated exposure to UV radiation. These two types can be seen as distinct entities rather than analogous skin ageing processes (Rhie *et al.*, 2001:1212). Varani *et al.* (2000:480) found that photo-aged skin and naturally aged, sun-protected skin share important molecular characteristics, which include reduced collagen production, elevated MMP levels and connective tissue damage. Both also lead to the production of ROS which ultimately leads to the impaired function of the skin (Rittié & Fisher, 2002:709). During the ageing process the epidermal and dermal layers of the skin are affected, making it the target site for topical drug delivery (Vioux-Chagnoleau *et al.*, 2006:S2).

Products must therefore reduce the signs of ageing, focussing on intrinsic ageing by slowing cell proliferation rate to preserve cells in their younger stage. The second approach should be aimed at extrinsic factors to protect the skin from photo- and oxidative damage (Von Oppen-Bezalel, 2011:57).

The aim of this study was to investigate the transdermal delivery of mangiferin and hesperidin – bioactive flavonoids present in various honeybush extracts and species, as well as the antioxidant properties and its clinical efficacy on human subjects.

In order to achieve this goal, the following objectives were set:

- Developing and validating a HPLC (high performance liquid chromatography) method to determine quantitatively concentrations of the different active ingredients (mangiferin and hesperidin) in the formulations
- Formulating two different 2% cream formulations with two different honeybush extracts (*Cyclopia maculata* and *Cyclopia genistoides*) containing mangiferin and hesperidin
- Conducting stability tests on the different formulations, stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH. HPLC analysis was used to determine the concentrations of the ingredients in all the formulated products. Other stability tests included appearance, pH, viscosity, mass loss, zeta potential and particle size determination.
- Determining whether mangiferin and hesperidin were released from the formulations by using membrane release studies
- Determining whether mangiferin and hesperidin diffused through the skin after different formulations were applied to the skin by making use of Franz cell diffusion studies
- Determining whether mangiferin and hesperidin were present in the target site (dermal layer) by using tape stripping technique after the different formulations were applied to the skin
- Determining the antioxidant activity of honeybush extracts, mangiferin, hesperidin and their semisolid formulations
- Determining whether various honeybush extract formulations have any anti-ageing clinical effects on human subjects

Two 2% creams containing either *Cyclopia maculata* or *Cyclopia genistoides* extracts were formulated. Stability tests were done on the different formulations over a 3 month period. The formulations were stored under different conditions namely, 25 °C/60% RH, 30 °C/60% RH and 40 °C/70% RH. The pH, viscosity, zeta potential, particle size, mass loss, physical appearance and concentration were determined on months 0, 1, 2 and 3. Unfortunately the change in colour, concentrations, particle size, zeta potential, viscosity and concentration of the active ingredients in the formulations, indicated that the products were unstable from the first month of stability testing. The assay concentrations of mangiferin and hesperidin in both the formulations

showed significant change and did not remain within the acceptable limits (5% change of initial value) with concentrations decreasing from the first stability testing month. The pH of neither the creams remained stable over the 3-month period. The *C. maculata* formulation proved to be the more stable formulation with a maximum pH change of 20% stored at 40 °C/75% RH, while the *C. genistoides* formulation had a maximum pH change of 31% stored at 40 °C/75% RH. This could indicate that the vast variations in pH are a clear indication of in the instability of mangiferin and hesperidin. Very low zeta potentials were present, indicating incipient instability. Both formulations had an average particle size increase over the three months. In the *C. maculata* and the *C. genistoides* cream the average particle size increased after the 3-month period in all three stability controlled conditions. All the formulations showed a definite change in colour and texture appearance with the *C. maculata* cream becoming almost a paste-like formulation, while the *C. genistoides* cream became a fluid-like, diluted cream after the 3-month period expired making this specific formulation unsuitable for future use due to possible imminent phase separation.

Membrane release studies were done to determine whether the bioactives mangiferin and hesperidin were released from the different formulations and the results confirmed that mangiferin and hesperidin were not released from all the formulations. In both formulations extremely low concentrations were detected in the receptor phase of the Franz cell after penetration through the cellulose acetate membranes. This could be an indication that the active ingredients have insufficient physico-chemical properties for satisfactory release of the actives and that the active concentrations within the formulations are too low for accurate detection during HPLC analysis.

Skin diffusion studies were done to determine the concentration mangiferin and hesperidin in the *Cyclopia genistoides* formulation permeated through the skin. Both mangiferin and hesperidin had inconclusive results due to unquantifiable low concentrations present during transdermal diffusion after 12 h. Only 0.250 µg/cm<sup>2</sup> mangiferin diffused through the skin. The *Cyclopia genistoides* cream performed better than its counterpart, *Cyclopia maculata*. Possible reasons for these results can be attributed to the human skin which has exceptional properties functioning as a physico-chemical obstruction (Bos & Meinardi, 2000:165) while a drug must possess a reasonable solubility in both water and oils for proper skin penetration. It also has to have an aqueous solubility of more than 1 mg/ml and a log P (octanol-water partition coefficient) in the range of 1 to 2 (Hadgraft, 1996:165). However, the real reason for this poor performance by both the formulations could be attributed to the fact that extremely low concentrations of mangiferin and hesperidin are present in both the 2% and 0.003% *Cyclopia* formulations. Extremely low mangiferin (20.302 mg) and hesperidin (29.544 mg) concentrations within the 2%

*Cyclopia genistoides* formulation was released from both the formulations, resulting in inaccurate HPLC quantifications.

Tapestripping experiments were done to determine the concentration of mangiferin and hesperidin that accumulated in the stratum corneum-epidermis and epidermis-dermis. Mangiferin (2% *C. genistoides* cream) was the only active compound after application to cross the skin in extremely low concentrations. Unquantifiable low concentrations were present in the stratum corneum-epidermis and epidermis-dermis. Hesperidin was not able to cross the stratum corneum, dermis or epidermis.

Antioxidant studies by the use of the TBA-assay method were done to determine whether the honeybush extracts, mangiferin and hesperidin as well as their semisolid formulations had any antioxidant activities. Both the honeybush extracts and the semisolid formulations showed promising results and should get further attention. It is of utmost importance to keep in mind that antioxidant capacity is not accurately rated during lipid peroxidation studies. The assay is strictly based on chemical reactions and bears no resemblance to biological systems. The legitimacy of the data is restricted to a stringent chemical sense with context analysis. Any claims concerning bioactivity of a sample based exclusively on this assay would be overstated, unscientific and in isolation (Huang *et al.*, 2005:1842). Mangiferin and hesperidin did not show any antioxidant activity on their own.

Clinical efficacy studies were done to determine whether the honeybush formulations had any moisturising effects in the treatment against skin ageing. The results were statistically inconclusive and variations between the subjects were very high due to skin variations at different skin sites. There was however a trend that *Cyclopia genistoides* performed the best.

Future prospects for further investigation include the following:

- The reason for the significant change in viscosity in the formulated products should be determined. Sufficient resting time after formulation should be applied before viscosity determinations are measured.
- The use of proper storage containers such as sealable amber glass containers during stability testing.
- Preservative testing of formulated products.
- Enhancement of skin penetration by the use of various skin penetration enhancers.
- Further attention needs to be given to establish the real antioxidant properties.

- Further attention needs to be given to lower the deviation in the results during clinical efficacy testing by using only one volar forearm, and by not doing the test near the wrist area.

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## APPENDIX A

# VALIDATION OF THE HPLC ANALYTICAL METHOD FOR ASSAY ANALYSIS

### A.1 Purpose of validation

The purpose of validation of the HPLC method was to ensure that the analytical method was responsive, sensitive and consistent in the determination of the amount of active ingredients in a semi-solid formulation during an intermediate stability test over a period of 12 weeks. Analysing honeybush extracts may cause potential interference in the determination of accurate results due to decomposition of products, contaminants present in the sample and other exogenous factors, such as poor homogeneity, poor solvability and human errors (FDA, 2001:5). It may be important to consider the variability of the sample due to the physiological nature of the sample (FDA, 2001:8). According to FDA (2001:10), it may be useful to adjust the acceptable limits of validation, due to the sample variation of honeybush extracts. The mean value should be within  $\pm 15\%$  of the theoretical value, except that the lower limit of quantification should not deviate by more than  $\pm 20\%$ . Most importantly, confidence interval criteria should be based on the study requirements to successfully analyse the analytes (FDA, 2001:12).

The active ingredients include: mangiferin, hesperidin, methyl paraben, propyl paraben and butylated hydroxytoluene (BHT).

### A.2 Chromatographic conditions

**Analytical instrument:** An Agilent® 1200 Series HPLC system was used for the analysis (Agilent Technologies, Palo Alto, CA). The instrument is designed with an Agilent® 1200 pump, diode array detector, autosampler injection mechanism and Chemstation Rev. A.06.02 software for data acquisition and analysis. Analysis was performed in a controlled laboratory environment at 25 °C.

**Column:** A high performance silica based, reversed phase Agela® Venusil XBP C18 (2) column, (150 mm × 4.6 mm) with a 5 µm particle size was used (Agela Technologies®, Newark, DE).

**Mobile phase A:** A mixture of 1 ml phosphoric acid in 1000 ml of HPLC water.

**Mobile phase B:** Acetonitrile

<b>Gradient table:</b>	The gradient elution was employed starting at 85% phosphoric acid solution and 15% acetonitrile for the first minute, followed by a linear increase to 95% acetonitrile after 10 min. The composition was kept at 95% acetonitrile until 15 min elapsed; thereafter the system was re-equilibrated at starting conditions for 5 min.
<b>Solvent:</b>	Methanol and HPLC water were used in the preparation of the standard solutions. For the preparation of samples methanol was used for the preparation of cream samples.
<b>Flow rate:</b>	1.0 ml/min
<b>Injection volume:</b>	20 µl
<b>Retention time:</b>	Mangiferin eluted first after 4.8 min, followed by hesperidin (5.9 min), methyl paraben (7.5 min), propyl paraben (9.2 min) and BHT (14.4 min).
<b>Run time:</b>	15 min with a post running time of 5 min.

### A.3 Preparation of standard and samples

#### A.3.1 Standard preparation

The following ingredients were weighed to obtain a standard solution of 100% and were dissolved in a 50 ml volumetric flask. It was then made up to volume with methanol/HPLC water:

- Honeybush extracts: 50.0 mg
- Methyl paraben: 2.0 mg
- Propyl paraben: 0.4 mg
- BHT: 2.0 mg

#### A.3.2 Placebo preparation

To prepare a 100% placebo sample the following ingredients were weighed off and heated together:

- Cetyl alcohol: 7.14 g
- Cremophor<sup>®</sup> A 6: 1.59 g
- Cremophor<sup>®</sup> A 25: 1.55 g
- Liquid paraffin: 12.02 g
- Propylene glycol: 8.13 g

### A.3.3 Sample preparation

1 g cream was weighed in a 50 ml volumetric flask and made up to volume with methanol to obtain a 100% sample.

## A.4 Validation parameters

### A.4.1 Linearity

The linearity of an analytical method is described as its capability, within a given range, to obtain test results that are directly proportional to the concentration of analyte in the sample (ICH, 2005:5).

Linearity of the analytes was determined by injecting ten different injection volumes into the HPLC. A 100% standard solution was prepared and the different injection volumes were injected in duplicate into the HPLC.

#### A.4.1.1 Linear regression analysis

The linearity of the active ingredients was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration ( $\mu\text{g/ml}$ ).

The data are described by a linear equation:

$$y = mx + c$$

**Equation A.1**

Where:

- y = peak area ratios of the different active ingredients
- m = slope
- x = concentration of the different active ingredients in  $\mu\text{g/ml}$
- c = y-intercept

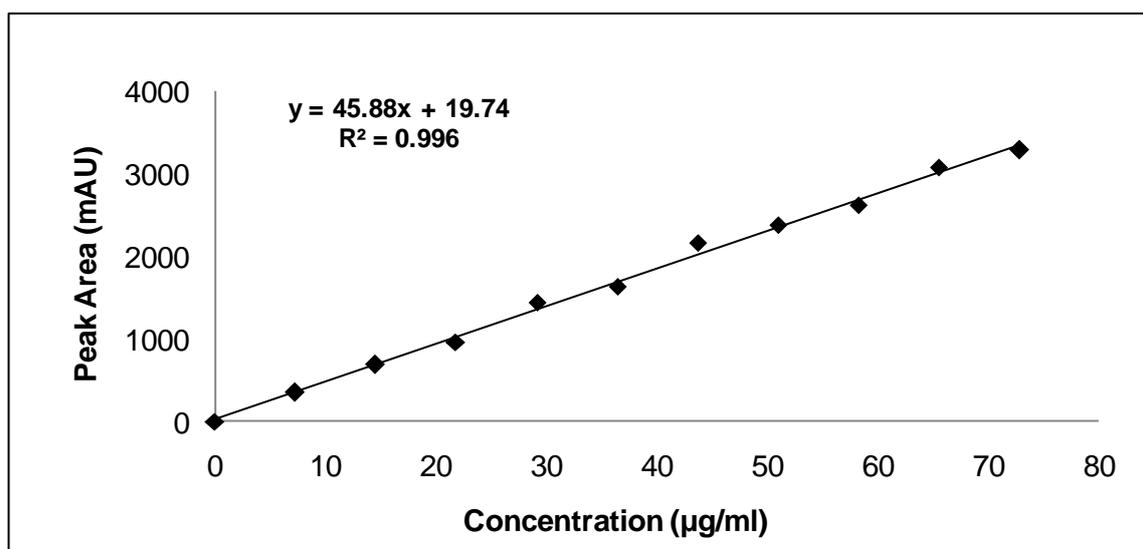
The acceptance criteria for linear regression analysis should yield a regression coefficient ( $r^2$ ) of  $\geq 0.99$ .

##### A.4.1.1.1 Mangiferin

The method was linear for mangiferin over the concentration range 7.0 – 75.0  $\mu\text{g/ml}$ . The regression value ( $r^2 = 0.996$ ) obtained, was within the acceptance criteria and indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

**Table A.1:** Peak area ratio values of mangiferin standards

Standard ( $\mu\text{g/ml}$ )	Peak area ratio
7.29	347.40
14.59	681.71
21.88	958.33
29.17	1443.83
36.47	1644.88
43.76	2163.00
51.05	2379.97
58.35	2627.74
65.64	3086.33
72.94	3291.90
<b>Slope</b>	6.20
<b>y-intercept</b>	- 9.15
<b><math>r^2</math></b>	0.99



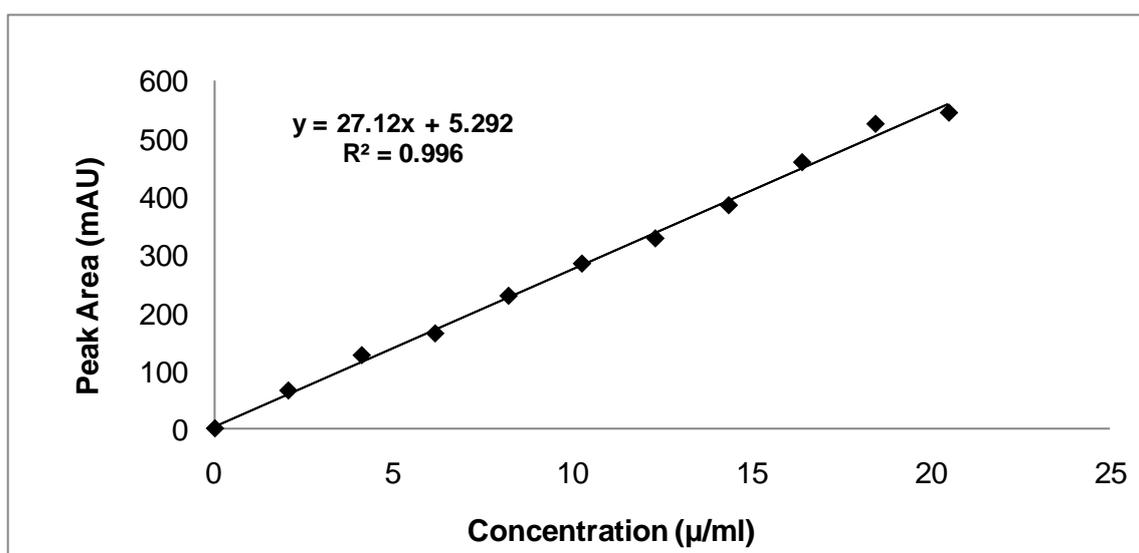
**Figure A.1:** Linear regression curve of mangiferin standards

#### A.4.1.1.2 Hesperidin

The method was linear for hesperidin over the concentration range 2.0 – 25.0  $\mu\text{g/ml}$ . The regression value ( $r^2 = 0.996$ ) obtained, was within the acceptance criteria and indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

**Table A.2:** Peak area ratio values of hesperidin standards

Standard ( $\mu\text{g/ml}$ )	Peak area ratio
2.05	65.38
4.10	126.34
6.15	163.97
8.19	228.94
10.24	284.59
12.29	328.14
14.34	385.37
16.39	459.70
18.44	526.02
20.48	545.28
<b>Slope</b>	6.20
<b>y-intercept</b>	- 9.15
<b>r<sup>2</sup></b>	0.99



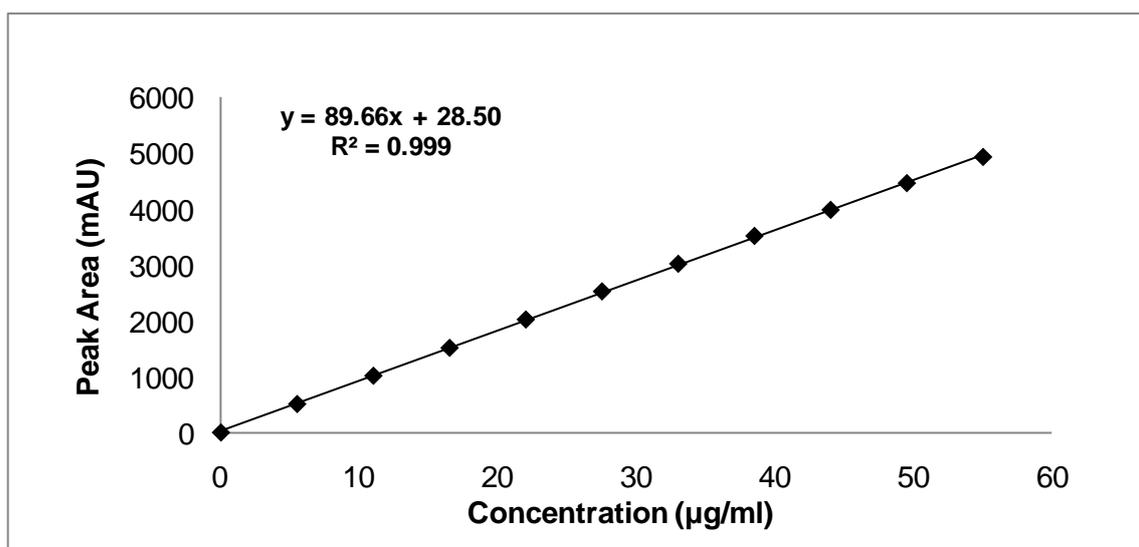
**Figure A.2:** Linear regression curve of hesperidin standards

#### A.4.1.1.3 Methyl paraben

The method was linear for methyl paraben over the concentration range 5.0 – 60.0  $\mu\text{g/ml}$ . The regression value ( $r^2 = 0.999$ ) obtained, was within the acceptance criteria and indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

**Table A.3:** Peak area ratio values of methyl paraben standards

Standard ( $\mu\text{g/ml}$ )	Peak area ratio
5.51	508.78
11.01	1014.07
16.52	1512.66
22.02	2019.46
27.53	2521.70
33.03	3014.95
38.54	3512.83
44.04	3978.36
49.55	4456.27
55.05	4924.10
<b>Slope</b>	6.20
<b>y-intercept</b>	- 9.15
<b>r<sup>2</sup></b>	0.99



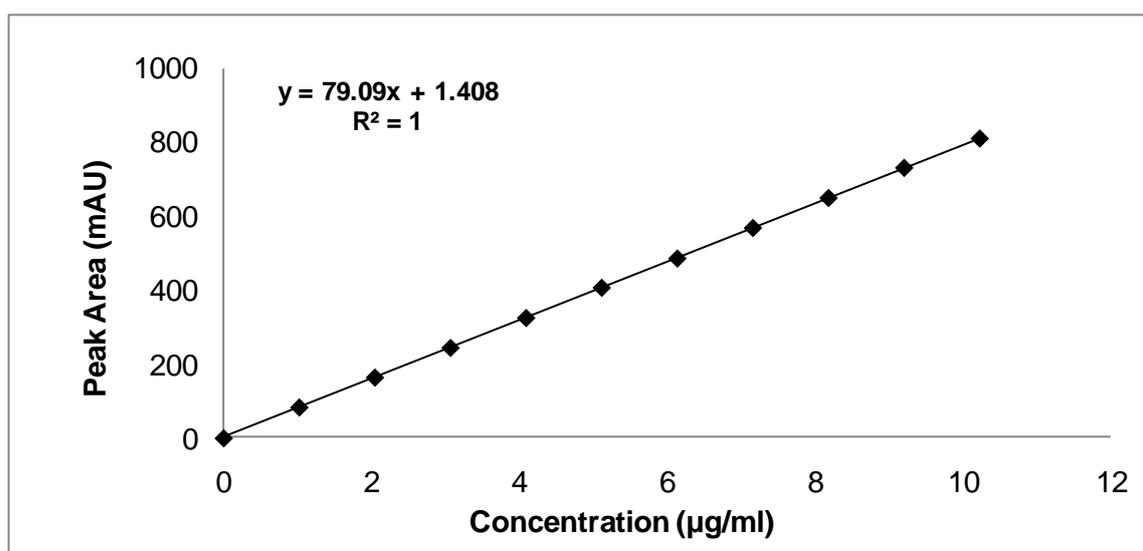
**Figure A.3:** Linear regression curve of methyl paraben standards

#### A.4.1.1.4 Propyl paraben

The method was linear for propyl paraben over the concentration range 1.0 – 15.0  $\mu\text{g/ml}$ . The regression value ( $r^2 = 1$ ) obtained, was within the acceptance criteria and indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

**Table A.4:** Peak area ratio values of propyl paraben standards

Standard ( $\mu\text{g/ml}$ )	Peak area ratio
1.03	83.47
2.05	164.30
3.08	244.67
4.10	325.49
5.13	407.29
6.15	486.53
7.18	569.07
8.20	650.30
9.23	731.82
10.25	811.23
<b>Slope</b>	6.20
<b>y-intercept</b>	- 9.15
<b>r<sup>2</sup></b>	0.99



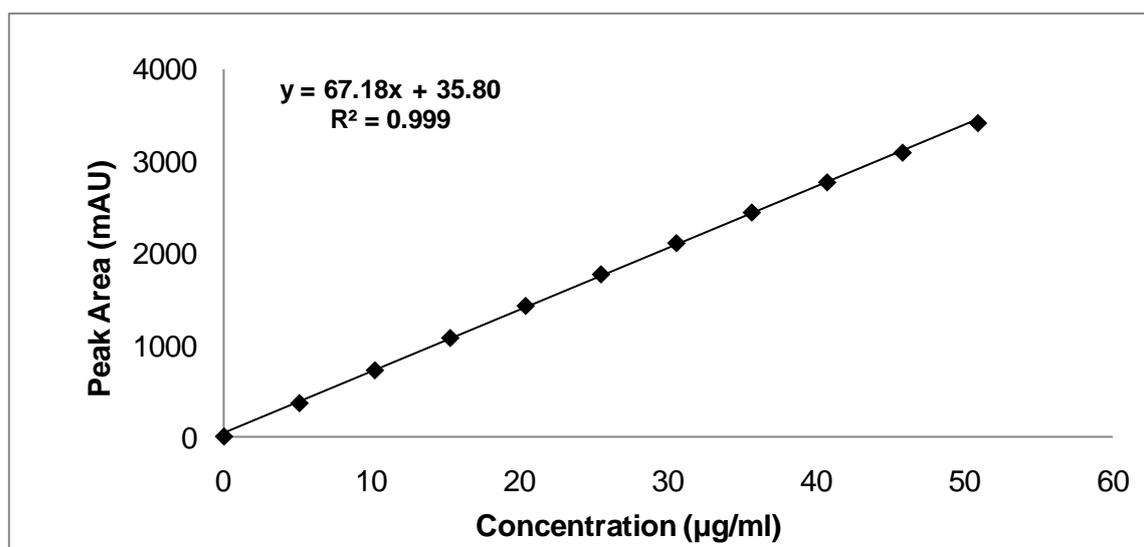
**Figure A.4:** Linear regression curve of propyl paraben standards

#### A.4.1.1.5 BHT

The method was linear for BHT over the concentration range 5.0 – 55.0  $\mu\text{g/ml}$ . The regression value ( $r^2 = 0.999$ ) obtained, was within the acceptance criteria and indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

**Table A.5:** Peak area ratio values of BHT standards

Standard ( $\mu\text{g/ml}$ )	Peak area ratio
5.09	363.48
10.18	722.22
15.27	1075.06
20.36	1425.13
25.45	1769.39
30.54	2110.26
35.63	2444.56
40.72	2773.56
45.81	3097.39
50.90	3420.18
<b>Slope</b>	6.20
<b>y-intercept</b>	- 9.15
<b>r<sup>2</sup></b>	0.99



**Figure A.5:** Linear regression curve of BHT standards

#### A.4.2 Accuracy

The accuracy of an analytical procedure represents the proximity of the concentration value found in the sample to the true concentration value. The percentage recovery is an indication of the accuracy of the system. According to acceptance criteria, the percentage recovery should be between 98 – 102% (ICH, 2005:9). The standard deviation (SD) and the percentage relative standard deviation (%RSD) are reflected in the data tables below.

A 100% standard solution was prepared as described in Section A.3.1.

A placebo solution was prepared (as described in Section A.3.2), and separated into nine 50 ml volumetric flasks. The first three flasks contained 0.8 g (80%) placebo solution, the next three flasks contained 1.0 g (100%) placebo solution and the last three flasks contained 1.2 g (120%) placebo solution.

The different placebo solutions were then spiked with the standard. The following procedure was then followed:

- 8 ml of standard was added to the 80% placebo solutions;
- 10 ml of standard was added to the 100% placebo solutions; and
- 12 ml of standard was added to the 120% placebo solutions.

The placebo solutions with the spiked standard were made up to volume with methanol. The different samples were injected in duplicate into the HPLC.

#### A.4.2.1 Accuracy analysis

##### A.4.2.1.1 Mangiferin

**Table A.6:** Accuracy parameters of mangiferin

Concentration spiked (µg/ml)	Peak area 1	Peak area 2	Mean	Recovery (µg/ml)	%
7.02	210.04	218.61	214.32	7.21	102.74
7.02	207.66	214.36	211.01	7.11	101.22
7.02	208.15	199.84	203.99	6.88	97.99
8.37	259.90	251.69	255.80	8.55	102.22
8.37	259.40	251.81	255.61	8.55	102.15
8.37	258.63	256.48	257.55	8.61	102.90
10.53	320.39	329.27	324.83	10.78	102.37
10.53	326.69	323.95	325.32	10.80	102.52
10.53	316.86	323.90	320.38	10.64	101.01
				<b>Mean</b>	101.68
				<b>SD</b>	1.44
				<b>%RSD</b>	<b>1.41</b>

Over the range of 80 – 120% of the sample concentration, the method yielded an average mangiferin recovery of 101.68%; which was within the accepted criteria.

#### A.4.2.1.2 Hesperidin

**Table A.7:** Accuracy parameters of hesperidin

Concentration spiked (µg/ml)	Peak area 1	Peak area 2	Mean	Recovery (µg/ml)	%
1.88	50.35	50.07	50.21	1.92	101.93
1.88	50.55	51.16	50.86	1.94	103.04
1.88	50.48	50.36	50.42	1.92	102.29
2.35	63.15	62.25	62.70	2.32	98.70
2.35	63.91	63.12	63.52	2.35	99.82
2.35	62.68	63.56	63.12	2.33	99.28
2.82	76.76	76.16	76.46	2.76	98.00
2.82	77.17	76.09	76.63	2.77	98.20
2.82	76.00	76.20	76.10	2.76	97.59
				<b>Mean</b>	99.87
				<b>SD</b>	1.92
				<b>%RSD</b>	<b>1.93</b>

Over the range of 80 – 120% of the sample concentration, the method yielded an average hesperidin recovery of 99.87%; which was within the accepted criteria.

#### A.4.2.1.3 Methyl paraben

**Table A.8:** Accuracy parameters of methyl paraben

Concentration spiked (µg/ml)	Peak area 1	Peak area 2	Mean	Recovery (µg/ml)	%
8.28	795.98	701.25	798.61	8.38	101.12
8.28	799.86	793.54	796.70	8.39	101.32
8.28	793.33	701.49	797.41	8.23	99.44
10.35	934.08	923.53	928.81	10.56	102.06
10.35	925.27	926.64	925.95	10.37	100.21
10.35	921.25	923.52	922.39	10.69	103.25
12.42	1128.15	1129.28	1128.72	12.44	100.14
12.42	1121.37	1127.36	1124.37	12.53	100.88
12.42	1126.24	1126.20	1126.22	13.14	105.83
				<b>Mean</b>	101.58
				<b>SD</b>	1.84
				<b>%RSD</b>	<b>1.81</b>

Over the range of 80 – 120% of the sample concentration, the method yielded an average methyl paraben recovery of 101.58%; which was within the accepted criteria.

#### A.4.2.1.4 Propyl paraben

**Table A.9:** Accuracy parameters of propyl paraben

Concentration spiked (µg/ml)	Peak area 1	Peak area 2	Mean	Recovery (µg/ml)	%
2.47	143.56	143.67	144.62	2.47	100.04
2.47	145.95	149.49	148.72	2.53	102.72
2.47	144.96	147.39	146.18	5.51	101.71
3.08	173.12	178.18	176.65	3.03	98.38
3.08	170.89	175.64	173.27	2.94	95.56
3.08	170.28	178.64	174.46	2.96	96.19
3.70	221.47	204.07	213.77	3.74	101.25
3.70	214.57	210.18	212.38	3.74	101.07
3.70	212.19	211.83	212.01	3.65	98.73
				<b>Mean</b>	99.52
				<b>SD</b>	2.34
				<b>%RSD</b>	<b>2.35</b>

Over the range of 80 – 120% of the sample concentration, the method yielded an average propyl paraben recovery of 99.52%; which was within the accepted criteria.

#### A.4.2.1.5 BHT

**Table A.10:** Accuracy parameters of BHT

Concentration spiked (µg/ml)	Peak area 1	Peak area 2	Mean	Recovery (µg/ml)	%
12.02	623.24	622.26	627.75	12.23	101.76
12.02	626.06	627.77	626.92	13.01	108.22
12.02	623.46	627.32	625.39	12.21	101.60
15.03	776.17	771.26	773.72	15.14	100.77
15.03	780.70	766.25	773.49	15.09	100.46
15.03	777.84	770.56	774.20	15.04	100.08
18.03	946.53	927.33	936.93	18.37	101.87
18.03	985.66	972.26	978.96	18.30	101.48
18.03	995.76	970.41	983.08	18.33	101.65
				<b>Mean</b>	101.99
				<b>SD</b>	2.28
				<b>%RSD</b>	<b>2.24</b>

Over the range of 80 – 120% of the sample concentration, the method yielded an average BHT recovery of 101.99%; which was within the accepted criteria.

### A.4.3 Precision

The precision of an analytical procedure expresses the proximity of agreement among a sequence of measurements obtained from numerous sampling of the similar homogenous material under set conditions. Precision was investigated in terms of intraday (repeatability) variation and interday (reproducibility) variation, under normal operating conditions (ICH, 2005:10).

#### A.4.3.1 Intraday precision (Repeatability)

The intraday precision was determined by performing HPLC analysis on three different samples of three different concentrations (80%, 100% and 120%) during the same day. The acceptance criteria according to the United States Pharmacopoeia (USP) for interday precision must have a repeatability of more than 2%. The different samples were injected in duplicate into the HPLC.

##### A.4.3.1.1 Mangiferin

**Table A.11:** Intraday precision parameters of mangiferin

Mass (g)	Peak area 1	Peak area 2	Mean	Concentration spiked (µg/ml)	Concentration injected (µg/ml)	%
0.81	320.36	327.57	323.96	7.19	51.60	103.19
0.81	313.14	328.88	321.01	7.13	51.70	103.39
0.83	324.04	321.87	322.96	7.17	50.20	100.39
1.11	422.96	427.48	425.22	9.44	49.53	99.06
1.10	424.96	427.33	426.14	9.46	50.43	100.87
1.01	403.58	410.39	406.98	9.04	52.34	104.68
1.29	506.23	508.96	507.60	11.27	50.91	101.82
1.34	495.92	509.16	502.54	11.16	48.69	97.39
1.25	509.76	492.73	501.24	11.13	51.94	103.87
					<b>Mean</b>	101.63
					<b>SD</b>	2.28
					<b>%RSD</b>	<b>2.24</b>

The %RSD of 2.24. This value is acceptable.

#### A.4.3.1.2 Hesperidin

**Table A.12:** Intraday precision parameters of hesperidin

Mass (g)	Peak area 1	Peak area 2	Mean	Concentration spiked ( $\mu\text{g/ml}$ )	Concentration injected ( $\mu\text{g/ml}$ )	%
0.81	184.83	201.77	193.30	6.89	49.42	98.84
0.80	193.32	198.86	196.09	6.99	50.69	101.39
0.83	199.68	205.30	202.49	7.22	50.52	101.04
1.11	255.85	257.85	256.85	9.16	48.02	96.05
1.01	256.61	256.75	256.68	9.15	48.76	97.53
1.00	258.39	257.38	257.89	9.19	53.24	106.48
1.29	305.11	307.09	306.10	10.91	49.28	98.57
1.33	309.44	306.90	308.17	10.98	47.93	95.87
1.25	306.26	304.96	305.61	10.89	50.83	101.66
					<b>Mean</b>	99.71
					<b>SD</b>	3.15
					<b>%RSD</b>	<b>3.16</b>

The %RSD of 3.16. This value is acceptable.

#### A.4.3.1.3 Methyl paraben

**Table A.13:** Intraday precision parameters of methyl paraben

Mass (g)	Peak area 1	Peak area 2	Mean	Concentration spiked ( $\mu\text{g/ml}$ )	Concentration injected ( $\mu\text{g/ml}$ )	%
0.81	631.78	632.44	632.11	7.32	52.48	104.96
0.81	625.61	620.00	622.81	7.22	52.39	104.78
0.83	649.84	636.34	643.09	7.43	51.98	103.97
1.11	855.85	857.85	856.85	9.56	50.16	100.32
1.10	856.61	856.75	856.68	9.56	50.96	101.92
1.01	865.39	853.38	859.39	9.59	55.53	111.06
1.29	1005.11	1007.09	1006.10	11.05	49.93	99.86
1.33	1009.44	1006.90	1008.17	11.07	48.33	96.65
1.25	1006.26	1004.96	1005.61	11.05	51.56	103.12
					<b>Mean</b>	104.23
					<b>SD</b>	2.86
					<b>%RSD</b>	<b>2.74</b>

The %RSD of 2.74. This value is acceptable.

#### A.4.3.1.4 Propyl paraben

**Table A.14:** Intraday precision parameters of propyl paraben

Mass (g)	Peak area 1	Peak area 2	Mean	Concentration spiked (µg/ml)	Concentration injected (µg/ml)	%
0.81	626.87	625.05	625.96	7.18	51.51	103.01
0.80	625.35	623.40	624.38	7.04	51.07	102.14
0.83	623.94	624.57	624.26	7.28	50.96	101.93
1.11	776.39	773.99	775.19	9.40	49.31	98.62
1.10	779.56	779.18	779.37	9.47	50.47	100.94
1.00	772.69	776.63	774.66	9.33	54.06	108.12
1.29	929.08	926.29	927.68	11.54	52.12	104.24
1.34	928.00	927.18	927.59	11.24	49.03	98.07
1.25	920.84	920.05	920.44	11.21	52.31	104.63
					<b>Mean</b>	102.41
					<b>SD</b>	2.92
					<b>%RSD</b>	<b>2.85</b>

The %RSD of 2.85. This value is acceptable.

#### A.4.3.1.5 BHT

**Table A.15:** Intraday precision parameters of BHT

Mass (g)	Peak area 1	Peak area 2	Mean	Concentration spiked (µg/ml)	Concentration injected (µg/ml)	%
0.81	345.53	344.32	344.93	6.89	49.65	99.30
0.81	346.86	345.44	346.15	6.91	50.13	100.26
0.83	339.07	344.72	341.90	7.54	52.81	105.61
1.11	431.42	439.99	435.71	9.58	50.23	100.46
1.10	435.01	435.07	435.04	9.56	50.96	101.93
1.01	433.01	436.24	434.63	9.04	52.35	104.71
1.29	567.43	569.00	568.22	11.10	50.13	100.25
1.34	569.05	571.52	570.29	11.52	50.27	100.54
1.25	564.18	574.99	569.59	11.09	51.77	103.54
					<b>Mean</b>	101.84
					<b>SD</b>	2.12
					<b>%RSD</b>	<b>2.08</b>

The %RSD of 2.08. This value is acceptable.

#### A.4.3.2 Interday precision (Reproducibility)

The interday precision was determined by performing HPLC analysis on three homogenous samples of the same known concentration on three consecutive days. According to USP requirements interday precision must be better than 5%. Due to the physiological nature of the samples and variability, interday precision ranges can vary from as low as 3% and to as high as 6% (FDA, 2001:8). Samples were injected in duplicate.

##### A.4.3.2.1 Mangiferin

**Table A.16:** Interday precision parameters of mangiferin

<b>Mass (g)</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Between days</b>
1.03	99.06	113.87	113.86	
1.03	100.87	114.24	113.07	
1.03	104.68	110.80	115.82	
<b>Mean</b>	101.54	112.97	114.25	109.59
<b>SD</b>	2.34	1.55	1.15	5.72
<b>%RSD</b>	2.31	1.37	1.01	<b>5.22</b>

The interday precision was acceptable at 5.22%. Repeatability was within acceptable limits and the assay should perform well.

##### A.4.3.2.2 Hesperidin

**Table A.17:** Interday precision parameters of hesperidin

<b>Mass (g)</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Between days</b>
1.03	96.05	108.11	109.72	
1.03	97.53	103.98	112.24	
1.03	106.48	107.78	109.98	
<b>Mean</b>	100.02	106.62	110.64	105.76
<b>SD</b>	4.61	1.87	1.13	4.38
<b>%RSD</b>	4.61	1.76	1.02	<b>4.14</b>

The interday precision was acceptable at 4.14%. Repeatability was within acceptable limits and the assay should perform well.

#### A.4.3.2.3 Methyl paraben

**Table A.18:** Interday precision parameters of methyl paraben

<b>Mass (g)</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Between days</b>
1.03	100.32	115.37	113.84	
1.03	101.92	110.34	111.34	
1.03	111.06	114.92	112.70	
<b>Mean</b>	104.43	113.54	112.63	110.20
<b>SD</b>	4.73	2.27	1.02	4.10
<b>%RSD</b>	4.53	2.00	0.91	<b>3.72</b>

The interday precision was acceptable at 3.72%. Repeatability was within acceptable limits and the assay should perform well.

#### A.4.3.2.4 Propyl paraben

**Table A.19:** Interday precision parameters of propyl paraben

<b>Mass (g)</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Between days</b>
1.03	98.62	111.86	106.44	
1.03	100.94	107.13	110.45	
1.03	108.12	111.73	111.88	
<b>Mean</b>	102.56	110.24	109.59	107.47
<b>SD</b>	4.04	2.20	2.30	3.48
<b>%RSD</b>	3.94	2.00	2.10	<b>3.24</b>

The interday precision was acceptable at 3.24%. Repeatability was within acceptable limits and the assay should perform well.

#### A.4.3.2.5 BHT

**Table A.20:** Interday precision parameters of BHT

<b>Mass (g)</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Between days</b>
1.03	100.46	97.85	105.65	
1.03	101.93	96.51	104.14	
1.03	104.71	99.49	106.76	
<b>Mean</b>	102.36	97.95	105.52	101.94
<b>SD</b>	1.76	1.22	1.07	3.10
<b>%RSD</b>	1.72	1.24	1.02	<b>3.04</b>

The interday precision was acceptable at 3.04%. Repeatability was within acceptable limits and the assay should perform well.

#### **A.4.4 Ruggedness**

##### **A.4.4.1 Sample stability**

A 100% sample was left on the autosampler tray and analysed at hourly intervals for a period of 12 h to determine the sample stability. Sample solutions should not be used for a period longer than it takes to degrade by 2%. Should degradation occur, special precautions must be followed to compensate for the loss in analyte concentration.

All the active ingredients (mangiferin, hesperidin, methyl paraben, propyl paraben and BHT) are stable after a period of 12 h, as seen in Table A.21.

**Table A.21:** Sample stability parameters for mangiferin, hesperidin, methyl paraben, propyl paraben and BHT

Time	Mangiferin		Hesperidin		Methyl paraben		Propyl paraben		BHT	
	Peak area	%	Peak area	%	Peak area	%	Peak area	%	Peak area	%
0	2547.96	100.00	486.37	100.00	4369.32	100.00	1026.39	100.00	2165.01	100.00
1	2548.49	100.02	485.35	99.79	4375.62	100.14	1024.91	99.86	2169.17	100.19
2	2545.53	99.90	487.54	100.24	4368.33	99.98	1025.54	99.92	2176.01	100.51
3	2568.74	100.82	485.99	99.92	4369.51	100.00	1017.63	99.15	2169.40	100.20
4	2544.52	99.86	484.73	99.66	4374.38	100.12	1026.22	99.98	2164.62	99.98
5	2550.62	100.10	487.85	100.30	4373.20	100.09	1024.48	99.81	2164.25	99.96
6	2547.40	99.98	486.37	100.00	4367.56	99.96	1026.06	99.97	2163.72	99.94
7	2547.75	99.99	485.44	99.81	4372.09	100.06	1026.95	100.05	2167.24	100.10
8	2551.62	100.14	485.50	99.82	4371.60	100.05	1027.53	100.11	2169.17	100.19
9	2547.57	99.98	487.35	100.20	4376.21	100.16	1026.53	100.01	2165.01	100.00
10	2543.55	99.83	487.21	100.17	4375.58	100.14	1034.82	100.82	2164.62	99.98
11	2546.98	99.96	489.28	100.60	4373.89	100.10	1032.99	100.64	2169.23	100.19
12	2548.83	100.03	482.58	99.22	4382.58	100.30	1025.88	99.95	2169.88	100.22
<b>Mean</b>	2549.20	100.05	486.27	99.98	4373.07	100.09	1026.61	100.02	2167.49	100.11
<b>SD</b>	6.03	0.24	1.60	0.33	3.90	0.09	3.92	0.38	3.33	0.15
<b>%RSD</b>	0.24	0.24	0.33	0.33	0.09	0.09	0.38	0.38	0.15	0.15

#### A.4.4.2 System repeatability

In an attempt to estimate the repeatability of the peak areas and retention times of the different actives, a 100% sample was injected 6 times. It was done on the same day and under the same conditions. The peak area and retention times should have a RSD of 2% or less (Du Preez, 2008:6).

##### A.4.4.2.1 Mangiferin

**Table A.22:** Variations in response (%RSD) of the detection system regarding peak area and retention time of mangiferin

Injection	Peak area (mAU*s)	Retention time (min)
1	3015.30	4.88
2	3007.78	4.87
3	3019.51	4.87
4	3019.99	4.87
5	3013.69	4.87
6	3010.96	4.87
<b>Mean</b>	3014.54	4.87
<b>SD</b>	4.36	0.01
<b>%RSD</b>	0.14	0.11

The variation in response (%RSD) proved to be excellent with a value of 0.14 for peak area and 0.11 for retention time and was within the accepted range.

##### A.4.4.2.2 Hesperidin

**Table A.23:** Variations in response (% RSD) of the detection system regarding peak area and retention time of hesperidin

Injection	Peak area (mAU*s)	Retention time (min)
1	592.11	5.95
2	588.18	5.94
3	597.27	5.95
4	593.19	5.94
5	595.53	5.94
6	594.62	5.95
<b>Mean</b>	593.48	5.95
<b>SD</b>	2.88	0.00
<b>%RSD</b>	0.49	0.05

The variation in response (%RSD) proved to be excellent with a value of 0.49 for peak area and 0.05 for retention time and was within the accepted range.

#### A.4.4.2.3 Methyl paraben

**Table A.24:** Variations in response (% RSD) of the detection system regarding peak area and retention time of methyl paraben

Injection	Peak area (mAU*s)	Retention time (min)
1	5029.77	7.54
2	5035.17	7.54
3	5041.20	7.54
4	5032.00	7.53
5	5023.70	7.54
6	5025.73	7.53
<b>Mean</b>	5031.26	7.54
<b>SD</b>	5.84	0.00
<b>%RSD</b>	0.12	0.06

The variation in response (%RSD) proved to be excellent with a value of 0.12 for peak area and 0.06 for retention time and was within the accepted range.

#### A.4.4.2.4 Propyl paraben

**Table A.25:** Variations in response (% RSD) of the detection system regarding peak area and retention time of propyl paraben

Injection	Peak area (mAU*s)	Retention time (min)
1	787.97	9.23
2	789.46	9.23
3	783.09	9.23
4	794.03	9.23
5	789.21	9.23
6	782.46	9.23
<b>Mean</b>	787.70	9.23
<b>SD</b>	3.96	0.00
<b>%RSD</b>	0.50	0.00

The variation in response (%RSD) proved to be excellent with a value of 0.50 for peak area and 0.00 for retention time and was within the accepted range.

#### A.4.4.2.5 BHT

**Table A.26:** Variations in response (% RSD) of the detection system regarding peak area and retention time of BHT

Injection	Peak area (mAU*s)	Retention time (min)
1	2361.64	14.40
2	2369.56	14.40
3	2384.66	14.40
4	2375.46	14.40
5	2357.96	14.40
6	2359.17	14.40
<b>Mean</b>	2368.08	14.40
<b>SD</b>	9.62	0.00
<b>%RSD</b>	0.41	0.00

The variation in response (%RSD) proved to be excellent with a value of 0.41 for peak area and 0.00 for retention time and was within the accepted range.

### A.5 Conclusion

The HPLC method was found to be reliable and sensitive enough for the determination of the concentration of mangiferin, hesperidin, methyl paraben, propyl paraben and BHT in semi-solid formulations.

The method performed well and should be appropriate to analyse all the analytes in the formulations during stability testing, quality control and batch release purposes.

## References

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ICH **see** International Conference of Harmonisation

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## APPENDIX B

# FORMULATION OF A COSMECEUTICAL CREAM WITH HONEYBUSH EXTRACTS AS THE ACTIVE INGREDIENT

### B.1 Introduction

In our everyday life, the significance of cosmetics is playing an even greater function than a few years back. The increasing quantity of consumers and quantities being consumed are impressive. Topical products can be separated into two groups i.e. cosmetics and pharmaceuticals (Kligman, 1993:37). The classification of cosmetics under the law varies to some extent among countries, although in universal terms “cosmetics” points towards any article intended to be used by means of rubbing, sprinkling or by related application to the human body for cleaning, beautifying, promoting attractiveness, changing the appearance of the human body and to preserve the healthiness of skin and hair (Mitsui, 1997:3). According to the Food, Drug and Cosmetic Act (FDC) a cosmetic is not allowed to have any activity (i.e., without affecting structure or function) (Vermeer, 2000:9).

The expression “cosmeceutical” defined by Dr. Albert Kligman approximately 20 years ago as a hybrid class of products lying on the spectrum between drugs and cosmetics, is currently used to explain a cosmetic product that exerts a pharmaceutical therapeutic benefit, but not fundamentally a biologic therapeutic benefit (Choi & Berson, 2006:163). During this study cosmeceuticals were formulated.

### B.2 Development program for the formulation of cosmeceutical products

The development procedure of a product consists of a preformulation, early formulation and final formulation segment. These three segments are discussed in the subsequent section.

#### B.2.1 Pre-formulation

Pre-formulation comprises those studies that ought to be carried out prior to commencement of formulation development. The main objective of the pre-formulation process is to allow the logical development of stable, harmless, efficacious dosage forms and it is primarily concerned with the classification of the physicochemical properties of the drug substance. In the pre-formulation stage the final route of drug administration should be decided (Walters & Brain, 2002:321).

## **B.2.2 Early formulation**

A “trial-and-error” approach was used during the early stages of formulation. Previously tested formulations were used and altered accordingly to specific needs.

## **B.2.3 Final formulation**

The final formulation was chosen to best suite the active ingredient and the creams were formulated in bulk for storage and stability testing purposes. A stability programme according to the International Conference on Harmonisation (ICH, 2003:5) was followed at three different storage temperatures (25, 30 and 40 °C) and humidities (60 and 75%) for three months.

## **B.3 Preservation of semisolid formulations**

Preservatives are supplementary to cosmetics to restrain the increase of bacteria which have contaminated them, by this means averting deterioration of the product. Restraining the increase of microorganisms is called microbiostasis and preservatives make use of these activities to prevent product deterioration. Preservatives do not have such a powerful effect by themselves; in general the ones used blend in well with the ingredients of the cosmetic and progressively kill off the contaminating microorganisms eventually. Typical examples of these preservatives are paroxybenzoates which are universally known as parabens. Parabens are also widely used in food products (Mitsui, 1997:201).

According to Mitsui (1997:202) the most important characteristics of preservatives used in cosmetics include the following:

- Efficacy against several species of microorganisms
- Water solubility or easy dissolution in frequently used cosmetic ingredients
- High safety and no irritation
- Neutral with no effect on product pH
- No decline of the effectiveness of product constituents
- No unfavourable consequences on product appearance
- Stability over wide temperature and pH range

## **B.4 Formulation of a cream**

Creams are skin care cosmetics which have been in all-purpose use ever since way back in times gone by. Many diverse forms of creams have been created right through history and the

extensive range of creams that is being produced these days is the outcome of progress in surface chemistry, higher levels of cosmetic production methods and the development of new beauty treatments (Mitsui, 1997:341).

#### **B.4.1 Purpose and function of cream**

Creams are semisolid emulsions for external application in which two liquids that are immiscible are made into an even dispersion. This can be done by dispersing the one liquid as droplets, the dispersed phase, into the other liquid, the continuous phase or dispersion medium. Therefore, the most important purposes of a cream are to preserve the moisture balance and to keep skin hydrated and flexible through the provision of water, humectants and oils (Mitsui, 1997:341-342).

In addition to creams which merely moisturise the skin, there are other products which have added functions such as stimulating the circulation, cleansing the skin and treating various skin conditions. By altering the quantity of water, humectants and oil in the formulation, a cream can be used for different skin types, skin conditions, cosmetic routines and preferences (Mitsui, 1997:342).

#### **B.4.2 Main ingredients of a cream**

Mitsui (1997:342) stated clearly that a number of key ingredients of a cream contain oily ingredients, aqueous ingredients, surfactants, preservatives, chelating agents, perfumes and pharmaceutical agents.

Creams can be separated into two groups of emulsions, specifically an oil-in-water (O/W) or water-in-oil (W/O) emulsion. In the case of O/W emulsions, generally hydrophilic surfactants are used. The oily ingredients used can also differ from those with zero polarity, to those with high polarity. In W/O emulsions, lipophilic surfactants are generally used. A cream with an oily nature desires a W/O emulsion. In case of a cream with a light feeling nature, the choice would be an O/W emulsion (Mitsui, 1997:343). An O/W cream was formulated during this study.

### **B.5 Formulation of a cream containing different honeybush extracts**

The purpose of this study was to formulate a cream containing two different honeybush extracts namely *Cyclopia maculata* or *Cyclopia genistoides*. Several different formulations were prepared and the best formulation was selected on grounds of appearance, homogeneity and overnight stability.

### B.5.1 Ingredients used in the manufacturing of different honeybush extracts creams

The ingredients used in the formulations which were selected as candidates to continue with this study, are given in Table B.1. The supplier, as well as the batch number, is given.

**Table B.1:** Ingredients used in the selected formulations

Ingredient	Supplier	Batch number
Liquid paraffin	Merck Chemicals	1035428
Cremophor® A 6	BASF Chemicals	65753075LO
Cremophor® A 25	BASF Chemicals	12903975LO
Methyl paraben	Merck Chemicals	K42067957
Propyl paraben	Merck Chemicals	K41917527
BHT	Merck Chemicals	K42115274
Novel-T® Organic Honeybush PE	Afriplex (Pty) Ltd	60787
Cyclopia Maculata hot water extract	ARC Infrutec-Nietvoorbij	ARC169
Cetylstearyl alcohol	Merck Chemicals	1119714
Propylene Glycol	Merck Chemicals	S5433688 018

### B.5.2 Formula of creams containing honeybush extracts

The formula of the cream is given in Table B.2. During this study two different honeybush extracts were used consisting of either *Cyclopia maculata* extracts or *Cyclopia genistoides* extracts.

**Table B.2:** Formula of cream

Ingredient	%	Function
<b>A:</b> Cetylstearyl alcohol	7.0%	Thickening agent
Liquid paraffin	12.0%	Oil phase of emulsion
Cremophor® A 6	1.5%	Emulsifier
Cremophor® A 25	1.5%	Emulsifier
Methyl paraben	0.2%	Preservative
Propyl paraben	0.04%	Preservative
BHT	0.2%	Anti-oxidant
<b>B:</b> Honeybush extract	2.0%	Active ingredient
Propylene glycol	8.0%	Solvent
<b>C:</b> dH <sub>2</sub> O	to 100%	Solvent

### **B.5.3 Procedure to prepare a cream containing honeybush extracts**

- Heat the mixture A (oil phase) and mixture C (water) separately to approximately 80 °C.
- Add mixture C (water) to the obtained solution mixture A (oil phase) with rigorous stirring using a homogeniser at speed 13 500 rpm.
- Heat mixture B until the active ingredient is dissolved.
- Mix mixture B with mixture A/C and continue to stir while cooling to room temperature.

### **B.5.4 Outcomes of cream containing honeybush extracts**

The cream applied easily and was not too oily. It had a homogeneous caramel texture and a sweet odour.

## **B.6 Conclusion**

Two different honeybush extracts were formulated into the following semi-solid formulations:

- A 2% *Cyclopia maculata* cream; and
- A 2% *Cyclopia genistoides* cream

The final formulations were prepared in adequate amounts for stability testing. All the formulations were inspected for appearance and texture. Both formulations were found suitable prior to storage at the three different temperatures used throughout stability testing.

Appendix C will reflect the stability test evaluations done on these formulations as well as the results, examination and discussion of the results..

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## APPENDIX C

### STABILITY TESTING OF SEMI-SOLID FORMULATIONS

#### C.1 Introduction

The principle of stability testing is to present data on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature and humidity (ICH, 2003:5).

The first phase of stability testing is to examine whether or not there are any transformations in the physico-chemical properties of the semi-solid formulations. The following characteristics should be considered:

- Chemical changes: colour change, colour fading, fragrance change, staining and crystallisation.
- Physical changes: separation, sedimentation, aggregation, blooming, sweating, gelling, unevenness, evaporation, solidification, softening and cracking (Mitsui, 1997:191).

An intermediate stability study as described by the ICH was conducted with a minimum testing frequency of four time points, including the initial and final time of testing (ICH, 2003:12). This was done on two semi-solid formulations containing two different extracts that were formulated (as discussed in Appendix B). These formulations were stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH. The following parameters were determined on month 0, 1, 2, and 3:

- Concentration assay
- pH
- Viscosity
- Mass loss
- Zeta potential
- Particle size
- Visual appearance assessment

According to the Medicine Control Council of South Africa (MCC) (2006:13) a significant change for a drug product is defined as:

- a 5% potency change in the active ingredient assay from its initial value;
- any degradation products exceeding its acceptance criterion;
- failure to meet the acceptance criteria for appearance (e.g., colour, phase separation, caking, hardness); and
- failure to meet the acceptance criterion for pH.

For this study batches of approximately 2000 ml were manufactured for each formulation. Small plastic containers were used to divide the different formulations into smaller batches for stability testing at different temperatures and humidities. The assay method used for the stability testing of the products was fully validated as discussed in Appendix A.

## C.2 Methods

### C.2.1 Concentration assay

In order to ensure the accuracy of the test results, all tests were done under Good Laboratory Practice (GLP) conditions. The concentrations of the following ingredients in the different formulations were determined by HPLC analysis: mangiferin, hesperidin, methyl paraben, propyl paraben and BHT. The validation of the HPLC analysis, as well as the chromatographic conditions is discussed in Appendix A.



**Figure C.1:** Agilent® 1200 Series HPLC

### C.2.1.1 Standard preparation

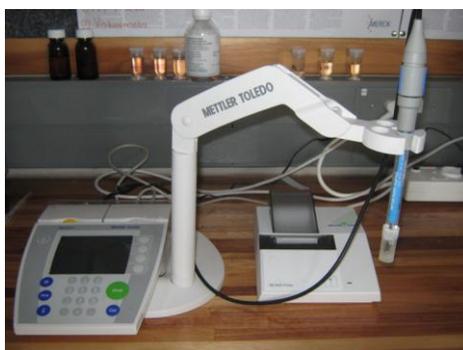
The following ingredients were weighed and dissolved in a 100 ml volumetric flask: honeybush extracts (50.0 mg), methyl paraben (2.0 mg), propyl paraben (0.4 mg) and BHT (2.0 mg). It was then made up to volume with methanol/HPLC water. The standard solution was injected into the HPLC in duplicate.

### C.2.1.2 Sample preparation

Each formulation (1 g) at each condition was weighed in 50 ml volumetric flasks in triplicate. The cream samples were made up to volume with methanol. The solutions were filtered and injected into the HPLC in duplicate.

### C.2.2 pH

The apparatus used to measure the pH of the formulations was a Mettler Toledo pH meter (Schwerzenbach, Switzerland) together with a glass Mettler Toledo Inlab<sup>®</sup> 410 electrode. The apparatus was calibrated with Mettler Toledo buffer solutions at pH 4.01, 7.00 and 10.01 with a slope at 25 °C, prior to pH measuring of the samples. The pH of each formulation at each condition was measured in triplicate.



**Figure C.2:** Mettler Toledo pH meter

### C.2.3 Viscosity

Viscosity is the resistance to flow, caused by inner friction (Marriott, 2002:41; Brookfield, 1998:2). Rheology may be defined as the science of the flow of matter, which is a means of classification of fluids and semisolids in the pharmacy sector (Marriott, 2002:41).

Viscosity of the different formulations was measured by means of a Brookfield Viscometer (Stoughton, Massachusetts, USA). A viscometer is an instrument used to measure the viscosity of a fluid, semi-solid or solid suspension. It measures the viscosity by determining the resistance to a rotating spindle immersed in the sample medium.



**Figure C.3:** Brookfield Viscometer

The direct sample temperature was controlled by a Brookfield temperature controller, circulating water at 25 °C in a water bath. Appropriate Helipath spindles (Stoughton, Massachusetts, USA) were placed in the formulations and the rate was specified. A Helipath D20733 was used to move the viscometer up and down in the different creams. The spindle turns at a specific rate, measured in rounds per minute (rpm) and the viscosity reading was measured every 10 sec for 5 min. Approximately 32 readings were obtained and the average viscosity was determined.

#### **C.2.4 Mass loss**



**Figure C.4:** Mettler Toledo balance

Filled plastic containers were weighed with a calibrated Mettler Toledo balance (Schwarzenbach, Switzerland) to determine if a loss in mass occurred. The mass of each formulation at each condition was determined in triplicate. After the indicated time intervals the

mass of each formulation was determined and was subtracted from the original mass to determine the total mass loss.

### **C.2.5 Zeta potential**

The stability of particle suspensions, colloidal dispersions, emulsions and other related systems is strongly influenced by the electrical charges that exist at the particle-liquid interface. The parameter we need to measure, to determine these electrical charges, is the zeta potential (Kirby & Hasselbrink, 2004:187; Malvern 2011:1; Malvern, 2000:1.1).



**Figure C.5:** Malvern Zetasizer 2000

Samples for the measurement of zeta potential are by definition particles dispersed in a liquid. Each formulation (1 g) at each condition was weighed off in 100 ml volumetric flasks. The cream samples were made up to volume with methanol. The zeta potential of each sample was measured by means of injecting the prepared samples into a Malvern Zetasizer 2000 (Worcestershire, United Kingdom). The zeta potential of each formulation at each condition was measured in triplicate.

### **C.2.6 Particle size**

The stability, chemical reactivity, opacity and material strength of many materials are affected by the size and characteristics of the particles within them.

Approximately 0.5 g of each formulation was mixed with approximately 3 ml HPLC water to wet the formulation to a uniform wet dispersion. These mixtures were made up with approximately 4.5 ml HPLC water, mixed well and injected into a Malvern Mastersizer 2000 (Worcestershire, United Kingdom). In addition the wet cell Hydro 2000 SM was used, which serves as the interface between the sample dispersion accessory and the optical unit. Samples were analysed in duplicate at speed 1500 rpm.

Finally, the statistics of the distribution were calculated from the results using the derived diameters  $D [m,n]$  – an internationally agreed method of defining the mean and other moments of particle size. Reflecting in the results as seen in Section C.3.6,  $D (0.5)$  refers to the size in microns ( $\mu\text{m}$ ) at which 50% of the sample is smaller and 50% larger. This value is also known as the Mass Median Diameter (MMD) (Taylor, 2002:474; Malvern, 2000:6.3).



**Figure C.6:** Malvern Mastersizer 2000 with wet cell, Hydro 2000SM

### **C.2.7 Colour and visual appearance assessment**

The visual appearance of each stored trial batch was visually assessed. Appearances were compared with the initial colour and appearance of the particular formulation. Photos were taken with a digital camera (Pentax<sup>®</sup> Optio E40).

## **C.3 Results and discussion**

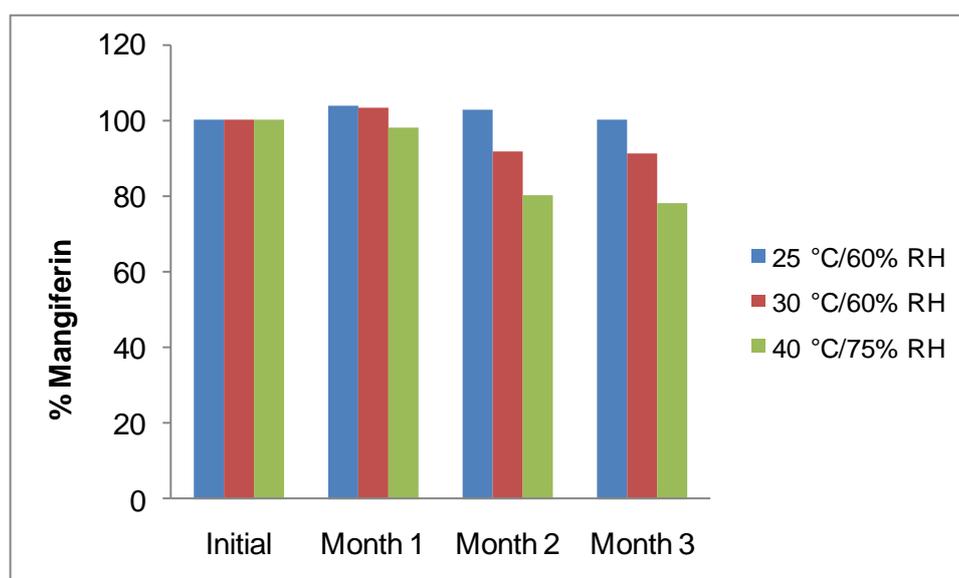
### **C.3.1 Concentration assay**

The percentage of the active ingredients was measured in the initial cream. It was also measured at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH on months 1, 2 and 3 as described in Section C.2.1.

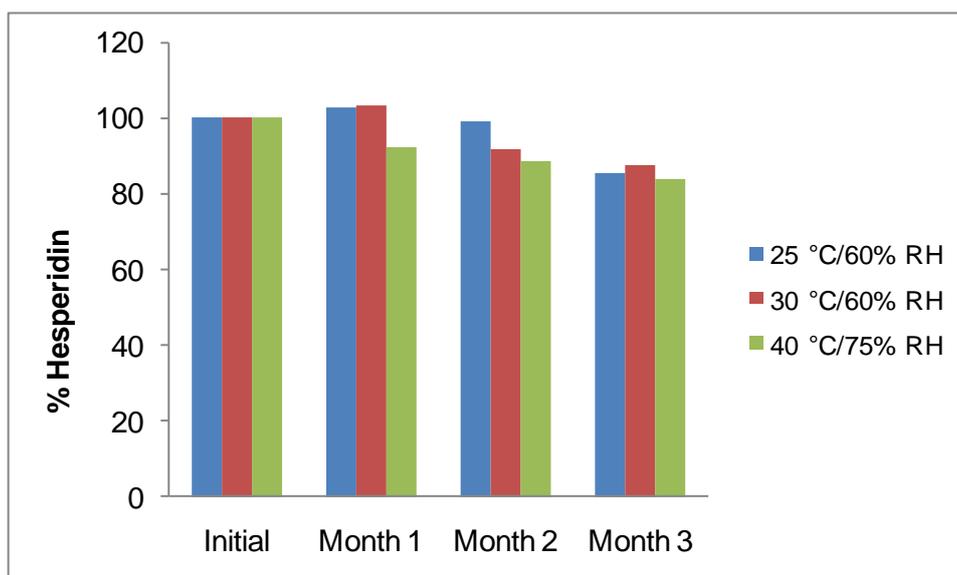
### C.3.1.1 *Cyclopia maculata* cream

**Table C.1:** Percentage of each active ingredient in *Cyclopia maculata* cream at the different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>Mangiferin</b>	100.00	103.77	102.65	99.86
<b>Hesperidin</b>	100.00	102.62	98.78	85.22
<b>Methyl paraben</b>	100.00	106.32	93.11	103.13
<b>Propyl paraben</b>	100.00	99.14	100.11	99.64
<b>BHT</b>	100.00	105.54	106.02	105.85
<b>30 °C/60% RH</b>				
<b>Mangiferin</b>	100.00	103.40	91.50	91.39
<b>Hesperidin</b>	100.00	103.24	91.84	87.27
<b>Methyl paraben</b>	100.00	102.37	96.45	99.92
<b>Propyl paraben</b>	100.00	98.59	96.81	90.52
<b>BHT</b>	100.00	105.52	101.42	101.12
<b>40 °C/75% RH</b>				
<b>Mangiferin</b>	100.00	98.15	80.02	77.91
<b>Hesperidin</b>	100.00	92.13	88.61	83.82
<b>Methyl paraben</b>	100.00	102.16	96.46	97.72
<b>Propyl paraben</b>	100.00	99.58	96.99	92.79
<b>BHT</b>	100.00	100.24	97.04	98.24



**Figure C.7:** Percentage of mangiferin (active ingredient) in *C. maculata* cream at the different conditions after each time interval



**Figure C.8:** Percentage of hesperidin (active ingredient) in *C. maculata* cream at the different conditions after each time interval

The concentrations of mangiferin in the *C. maculata* cream showed significant change at 30 °C/60% RH and 40 °C/75% RH after 2 and 3 months. Hence, it did not remain within the acceptable limits (5% change in initial value). However, mangiferin did stay within acceptable limits in the *C. maculata* cream at storing conditions 25 °C/60% RH over the period of 3 months, as well as after 1 month at 30 °C/60% RH and 40 °C/75% RH.

The concentrations of hesperidin in the *C. maculata* cream showed significant change and did not remain within the acceptable limits (5% change in initial value). However, hesperidin did stay within acceptable limits in the *C. maculata* cream at storing conditions 25 °C/60% RH and 30 °C/60% RH after 1 month.

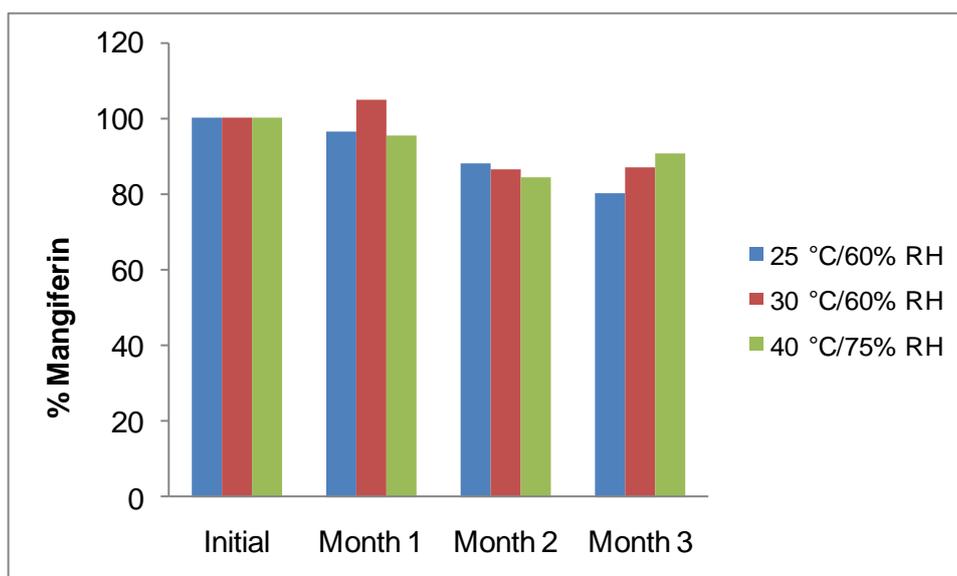
The concentrations of methyl paraben, propyl paraben and BHT in the *C. maculata* cream showed little change and remained within the acceptable limits of the ICH. Though at 25 °C/60% RH (month 2), methyl paraben was more than 5% lower and BHT more than 5% higher than the initial concentration. Propyl paraben did not remain within the acceptable limits of the ICH at 30 °C/60% RH and 40 °C/75% RH after month 3.

The degradation of all the active ingredients may be due to the fact of insufficient protection by the anti-oxidants and preservatives.

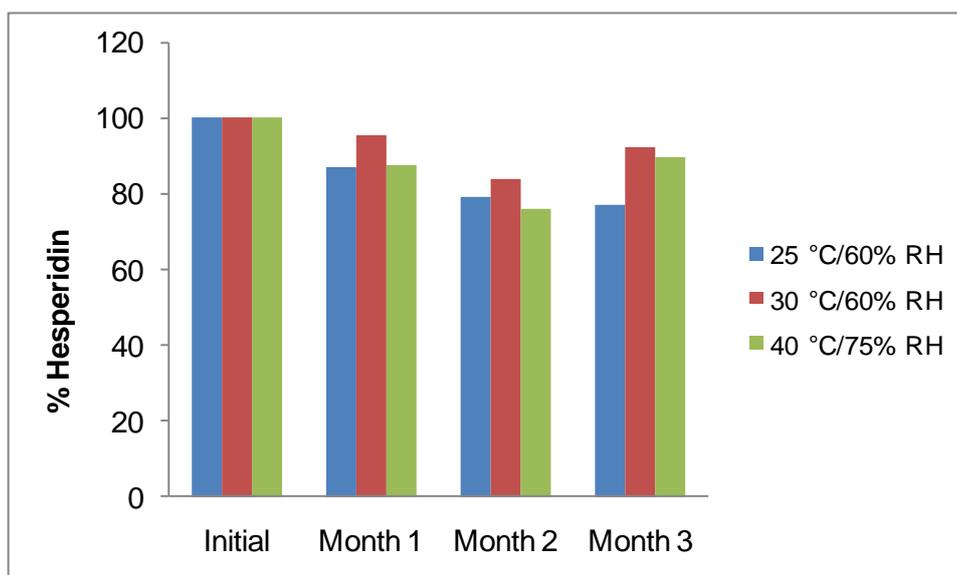
### C.3.1.2 *Cyclopia genistoides* cream

**Table C.2:** Percentage of each active ingredient in *Cyclopia genistoides* cream at the different condition after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>Mangiferin</b>	100.00	96.17	88.28	80.32
<b>Hesperidin</b>	100.00	87.20	79.33	76.80
<b>Methyl paraben</b>	100.00	103.93	94.34	94.77
<b>Propyl paraben</b>	100.00	104.92	92.42	98.64
<b>BHT</b>	100.00	114.46	107.83	105.54
<b>30 °C/60% RH</b>				
<b>Mangiferin</b>	100.00	104.66	86.27	86.94
<b>Hesperidin</b>	100.00	95.22	84.09	92.24
<b>Methyl paraben</b>	100.00	96.07	92.36	93.29
<b>Propyl paraben</b>	100.00	107.52	93.10	97.76
<b>BHT</b>	100.00	104.96	104.7	101.69
<b>40 °C/75% RH</b>				
<b>Mangiferin</b>	100.00	95.23	84.26	90.73
<b>Hesperidin</b>	100.00	87.76	75.81	89.76
<b>Methyl paraben</b>	100.00	100.75	92.56	91.69
<b>Propyl paraben</b>	100.00	104.17	91.79	94.09
<b>BHT</b>	100.00	103.04	103.17	95.74



**Figure C.9:** Percentage of mangiferin (active ingredient) in *C. genistoides* cream at the different conditions after each time interval



**Figure C.10:** Percentage of hesperidin (active ingredient) in *C. genistoides* cream at the different conditions after each time interval

The concentrations of mangiferin and hesperidin in the *C. genistoides* cream showed significant change and did not remain within the acceptable limits (5% change in initial value). However, mangiferin did stay within acceptable limits in the *C. genistoides* cream at storing conditions 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH after 1 month. In addition, hesperidin did stay within acceptable limits in the *C. genistoides* cream at 30 °C/60% RH after 1 month.

Methyl paraben in the *C. genistoides* cream did not remain within the acceptable limits of the ICH, except after 1 month at storage conditions 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH.

The concentrations of propyl paraben did not remain within the acceptable limits of the ICH after month 1 at 30 °C/60% RH, month 2 at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH or after month 3 at 40 °C/75% RH.

BHT in the *C. genistoides* cream showed little change and remained within the acceptable limits of the ICH, except at 25 °C/60% RH over the 3-month period.

The degradation of all the active ingredients can be due to the fact of insufficient protection by the anti-oxidants and preservatives.

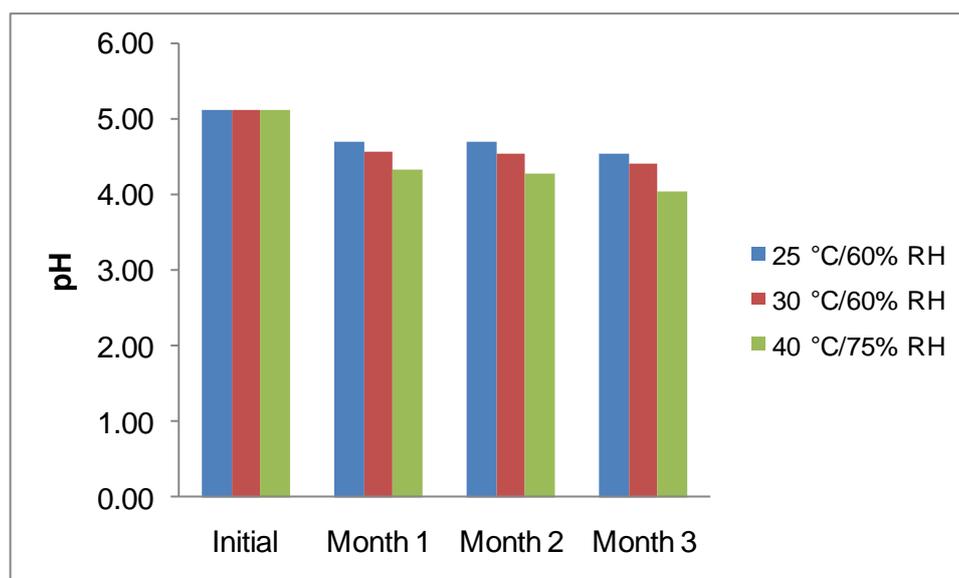
### C.3.2 pH

The pH was measured of the initial cream. It was also measured at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH on months 1, 2 and 3 as described in Section C.2.2.

### C.3.2.1 *Cyclopi* *maculata* cream

**Table C.3:** pH of *Cyclopi* *maculata* cream at the different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>1</b>	5.13	4.68	4.71	4.51
<b>2</b>	5.08	4.69	4.68	4.52
<b>3</b>	5.08	4.69	4.68	4.53
<b>Average</b>	5.10	4.69	4.69	4.52
<b>SD</b>	0.02	0.00	0.01	0.01
<b>%RSD</b>	0.46	0.10	0.30	0.18
<b>30 °C/60% RH</b>				
<b>1</b>	5.13	4.56	4.58	4.40
<b>2</b>	5.08	4.55	4.52	4.41
<b>3</b>	5.08	4.55	4.52	4.40
<b>Average</b>	5.10	4.55	4.54	4.40
<b>SD</b>	0.02	0.00	0.03	0.00
<b>%RSD</b>	0.46	0.10	0.62	0.11
<b>40 °C/75% RH</b>				
<b>1</b>	5.13	4.33	4.29	4.04
<b>2</b>	5.08	4.33	4.27	4.04
<b>3</b>	5.08	4.33	4.27	4.04
<b>Average</b>	5.10	4.33	4.28	4.04
<b>SD</b>	0.02	0.00	0.01	0.00
<b>%RSD</b>	0.46	0.00	0.22	0.00



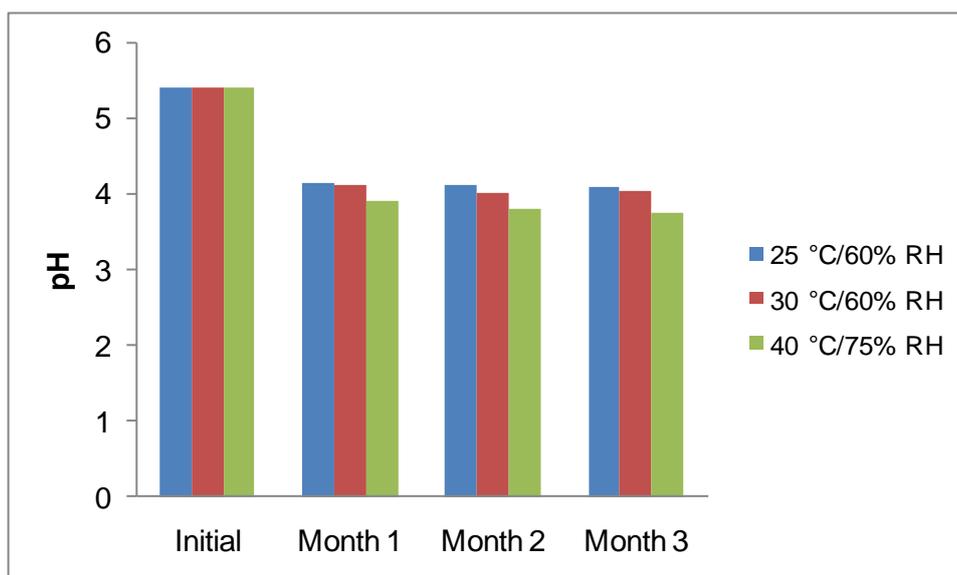
**Figure C.11:** pH of *C. maculata* cream at the different conditions after each time interval

The pH of the *C. maculata* cream did not remain stable over the 3-month period with a significant change in pH. The biggest decrease in pH over the 3-month period was the cream stored at 40 °C/75% RH which had a decrease of approximately 21%. The formulation stored at 30 °C/60% RH had a decrease of approximately 14% after 3 months while the cream stored at 25 °C/60% RH showed a decrease of approximately 11% after 3 months. Therefore, the prediction could be made that the formulation will not remain stable after a 3-month storage period.

### C.3.2.2 *Cyclopia genistoides* cream

**Table C.4:** pH of *Cyclopia genistoides* cream at different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>1</b>	5.39	4.13	4.11	4.09
<b>2</b>	5.39	4.14	4.12	4.11
<b>3</b>	5.39	4.14	4.12	4.11
<b>Average</b>	5.39	4.14	4.12	4.10
<b>SD</b>	0.00	0.00	0.00	0.01
<b>%RSD</b>	0.00	0.11	0.11	0.23
<b>30 °C/60% RH</b>				
<b>1</b>	5.39	4.10	4.00	4.02
<b>2</b>	5.39	4.11	4.02	4.03
<b>3</b>	5.39	4.11	4.02	4.03
<b>Average</b>	5.39	4.11	4.01	4.03
<b>SD</b>	0.00	0.00	0.01	0.00
<b>%RSD</b>	0.00	0.11	0.23	0.12
<b>40 °C/75% RH</b>				
<b>1</b>	5.39	3.90	3.81	3.74
<b>2</b>	5.39	3.90	3.81	3.74
<b>3</b>	5.39	3.90	3.82	3.74
<b>Average</b>	5.39	3.90	3.81	3.74
<b>SD</b>	0.00	0.00	0.00	0.00
<b>%RSD</b>	0.00	0.00	0.12	0.00



**Figure C.12:** pH of *C. genistoides* cream at the different conditions after each time interval

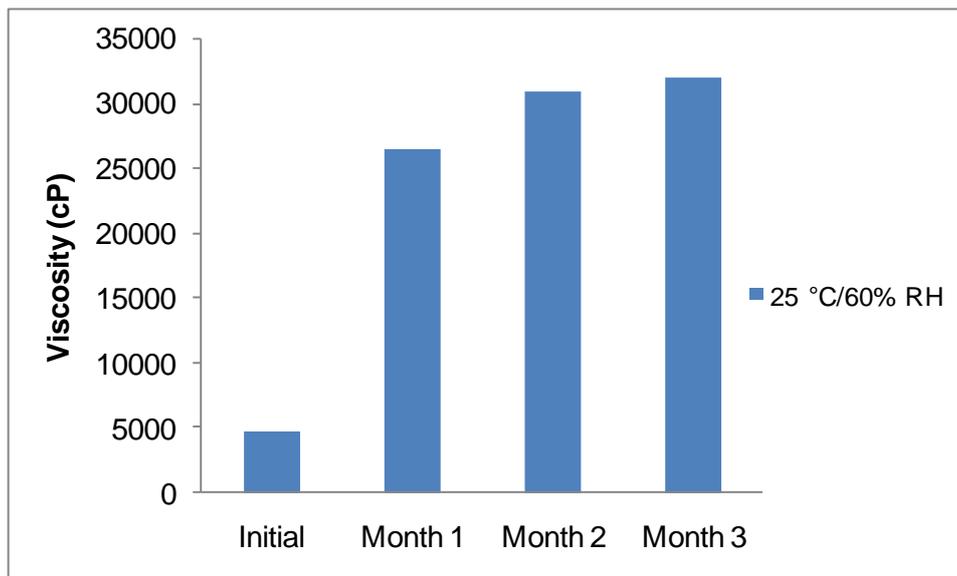
The pH of the *C. genistoides* cream did not remain stable over the 3-month period with a significant change in pH. The largest decrease in pH after 3 months was observed for the cream stored at 40 °C/75% RH with a decrease of approximately 31%. The formulation stored at 30 °C/60% RH had a decrease of approximately 25% after 3 months while the cream stored at 25 °C/60% RH showed a decrease of approximately 24% after 3 months. Therefore, the prediction could be made that the formulation will not remain stable after a 3-month storage period.

### C.3.3 Viscosity

The viscosity was measured of the initial creams. It was also measured at 25 °C/60% RH on month 1, 2 and 3 as described in Section C.2.3.

**Table C.5:** Viscosity (cP) of *Cyclopia* creams at 25 °C/60% RH after each time interval

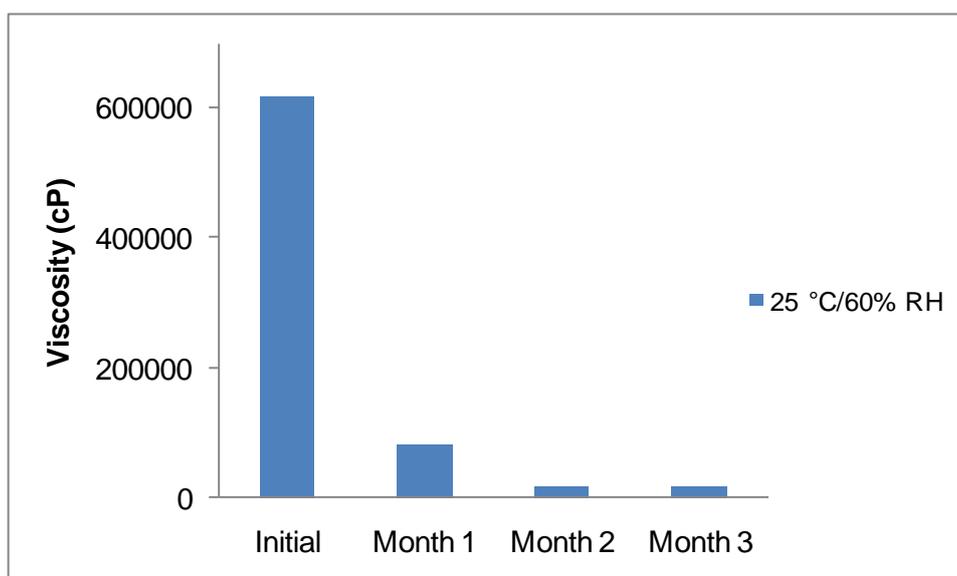
	Initial	Month 1	Month 2	Month 3
<b><i>C. maculata</i> cream</b>				
<b>Average</b>	4698.21	26448.90	30803.97	31868.20
<b>SD</b>	1170.16	40232.01	5792.15	2297.88
<b>%RSD</b>	24.91	53.57	33.57	17.30
<b><i>C. genistoides</i> cream</b>				
<b>Average</b>	678468.51	83463.20	16998.51	18844.33
<b>SD</b>	83899.62	17172.3	1865.38	33.35
<b>%RSD</b>	12.36	7.95	4.02	1.81



**Figure C.13:** The change in viscosity (cP) for *C. maculata* cream over a 3-month period at 25 °C/60% RH

The viscosity of the *C. maculata* cream changed radically over the 3-month period. The *C. maculata* cream showed an increase in viscosity from 4698.21 cP (month 0) to 31868.20 cP (month 3).

An increase in viscosity can be due to the settling process of the cream before the formulation went into the final resting stage as well as loss of moisture during stability testing over the period of 3 months, giving the cream a higher viscosity value.



**Figure C.14:** The change in viscosity (cP) for *C. genistoides* cream over a 3-month period at 25 °C/60% RH

The viscosity of the *C. genistoides* cream had dramatic changes in its viscosity over the 3-month period. The *C. genistoides* cream showed a decrease in viscosity from 678468.51 cP (month 0) to 18844.33 cP (month 3).

The immense decrease in viscosity could be contributed to the changes in the formulation, the reactions between the different ingredients, such as the breakdown of the active ingredients as seen in the assay and different incompatibilities between the honeybush extracts and the constituents.

It may also be due to the fact that the cream was still settling before it went into the final resting stage before stability testing started. Broken Van der Waals forces between the molecules could also have occurred during the previous viscosity measurements on the same cream, also leading to a decrease in viscosity. This large decrease in viscosity is a clear indication of possible instability and phase separation.

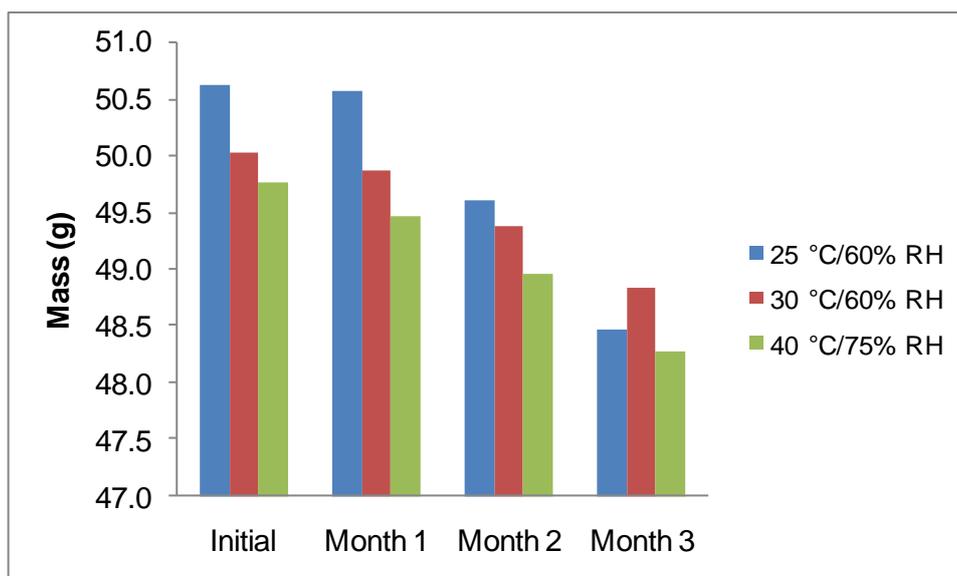
### C.3.4 Mass loss

The mass loss was measured for the initial creams. It was also measured at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH on month 1, 2 and 3 as described in Section C.2.4.

#### C.3.4.1 *Cyclopia maculata* cream

**Table C.6:** Mass (g) of *Cyclopia maculata* cream at different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>1</b>	50.61	50.56	49.60	48.47
<b>2</b>	50.61	50.56	49.60	48.47
<b>3</b>	50.61	50.56	49.60	48.47
<b>Average</b>	50.61	50.56	49.60	48.47
<b>30 °C/60% RH</b>				
<b>1</b>	50.02	49.86	49.37	48.83
<b>2</b>	50.02	49.86	49.37	48.83
<b>3</b>	50.02	49.86	49.37	48.83
<b>Average</b>	50.02	49.86	49.37	48.83
<b>40 °C/75% RH</b>				
<b>1</b>	49.76	49.46	48.97	48.27
<b>2</b>	49.76	49.46	48.97	48.27
<b>3</b>	49.76	49.46	48.96	48.27
<b>Average</b>	49.76	49.46	48.96	48.27



**Figure C.15:** The change in mass (g) for *C. maculata* cream at the different conditions after each time interval

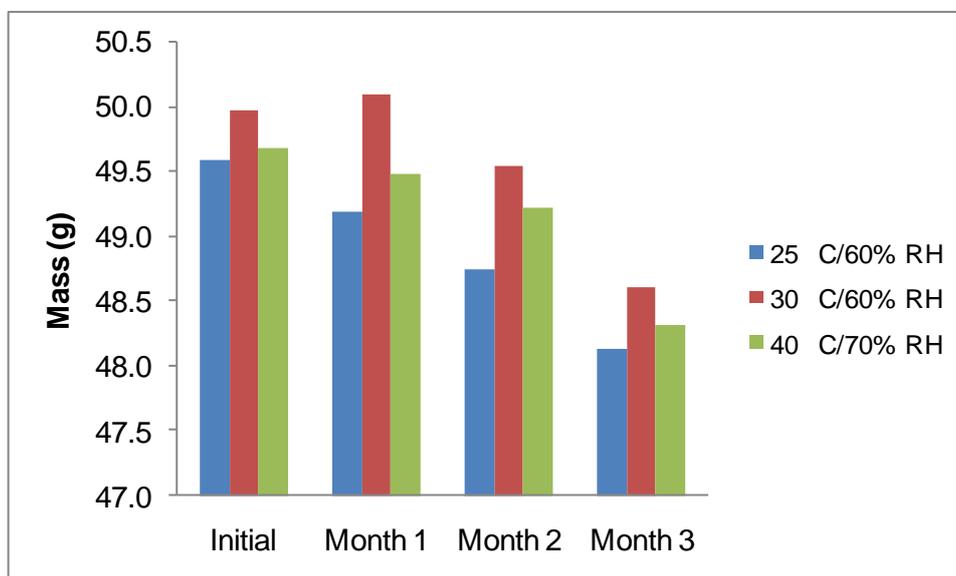
The loss in mass of the *C. maculata* cream did remain relatively stable over the 3-month period with no significant change in mass. The biggest decrease in mass was the cream stored at 25 °C/60% RH with a decrease of approximately 4%. The formulation stored at 30 °C/60% RH confirmed a decrease of approximately 2% while the cream stored at 40 °C/75% RH also showed a decrease of approximately 3%. These decreases in mass indicate that the storage containers did not seal sufficiently enough, allowing evaporation during storage periods.

#### C.3.4.2 *Cyclopia genistoides* cream

**Table C.7:** Mass (g) of *Cyclopia genistoides* cream at different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>1</b>	49.59	49.18	48.74	48.13
<b>2</b>	49.59	49.18	48.74	48.13
<b>3</b>	49.59	49.18	48.74	48.13
<b>Average</b>	49.59	49.18	48.74	48.13
<b>30 °C/60% RH</b>				
<b>1</b>	49.97	50.08	49.54	48.60
<b>2</b>	49.97	50.08	49.54	48.60
<b>3</b>	49.97	50.08	49.54	48.60
<b>Average</b>	49.97	50.08	49.54	48.60

40 °C/75% RH				
1	49.68	49.48	49.22	48.32
2	49.68	49.48	49.22	48.32
3	49.68	49.48	49.22	48.32
<b>Average</b>	49.68	49.48	49.22	48.32



**Figure C.16:** The change in mass (g) for *C. genistoides* cream at the different conditions after each time interval

The loss in mass of the *C. genistoides* cream did remain relatively stable over the 3-month period with no significant change in mass. However, the biggest decrease in mass was the cream stored at 25 °C/60% RH with a decrease of approximately 3%. This could indicate that the storage containers did not seal sufficiently enough, permitting evaporation during storage periods. The containers used for storage at lower temperatures did not seal well enough for limiting evaporation and preventing moisture loss.

The formulation stored at 30 °C/60% RH confirmed a decrease of approximately 2% while the cream stored at 40 °C/75% RH also showed a decrease of approximately 2%. This could be due to the higher humidity and possible porosity of the storage containers, allowing moisture through.

### C.3.5 Zeta potential

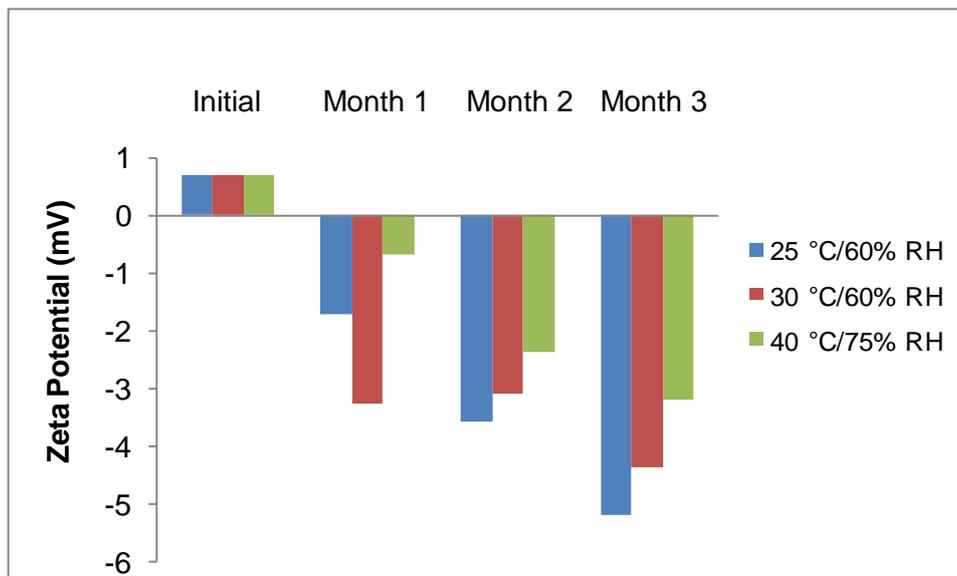
The zeta potential was determined of the initial creams immediately after it was made (0h), as well as at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH on months 1, 2 and 3 as described in Section C.2.5. Figures C.17 and C.18 illustrate an overview of the changes between months 0 and 3 for the different formulations.

A value of 25 mV (positive or negative) can be seen as the arbitrary value that separates low-charged surfaces from highly-charged surfaces. A high zeta potential (positive or negative) is electrically stabilised, while low zeta potentials tend to coagulate or flocculate.

### C.3.5.1 *Cyclopia maculata* cream

**Table C.8:** Zeta potential (mV) of *Cyclopia maculata* cream at the different conditions after each time interval

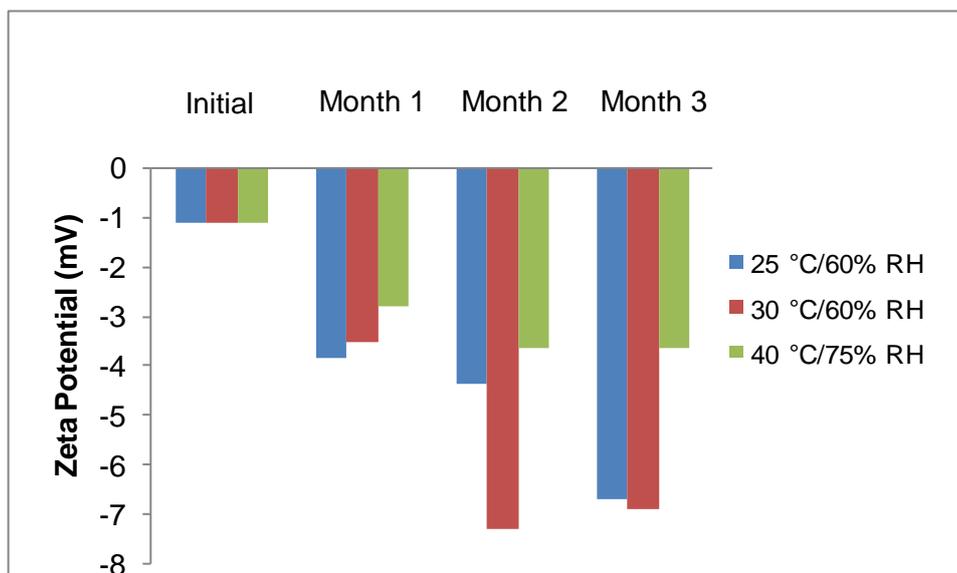
	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>1</b>	0.60	-1.90	-3.80	-5.40
<b>2</b>	0.70	-1.70	-3.50	-5.10
<b>3</b>	0.80	-1.60	-3.40	-5.10
<b>Average</b>	0.70	-1.73	-3.57	-5.20
<b>SD</b>	0.08	0.12	0.17	0.14
<b>%RSD</b>	11.66	-7.20	-4.77	-2.72
<b>30 °C/60% RH</b>				
<b>1</b>	0.60	-3.40	-3.20	-4.70
<b>2</b>	0.70	-3.20	-3.10	-4.30
<b>3</b>	0.80	-3.20	-3.00	-4.10
<b>Average</b>	0.70	-3.27	-3.10	-4.37
<b>SD</b>	0.08	0.09	0.08	0.25
<b>%RSD</b>	11.66	-2.89	-2.63	-5.71
<b>40 °C/75% RH</b>				
<b>1</b>	0.60	-0.80	-2.50	-3.60
<b>2</b>	0.70	-0.70	-2.40	-3.40
<b>3</b>	0.80	-0.60	-2.20	-2.60
<b>Average</b>	0.70	-0.70	-2.37	-3.20
<b>SD</b>	0.08	0.08	0.12	0.43
<b>%RSD</b>	11.66	-11.66	-5.27	-13.50



**Figure C.17:** The change in zeta potential (mV) for *C. maculata* cream at the different conditions after each time interval

Zeta-potential of the *C. maculata* cream at all three stability controlled conditions did not stay within the range of less than -25 mV or more than +25 mV from months 0 to 3. Although all three stability testing conditions changed from a positive to a negative charged formulation, the zeta potential values were low. The afore-mentioned indicate that the formulation stored at the different stability conditions are instable and may tend to coagulate or flocculate.

### C.3.5.2 *Cyclopia genistoides* cream



**Figure C.18:** The change in zeta potential (mV) for *C. genistoides* cream at the different conditions after each time interval

**Table C.9:** Zeta potential (mV) of *Cyclopia genistoides* cream at the different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>1</b>	-1.20	-3.90	-4.40	-7.00
<b>2</b>	-1.20	-3.90	-4.40	-6.60
<b>3</b>	-1.00	-3.80	-4.30	-6.50
<b>Average</b>	-1.13	-3.87	-4.37	-6.70
<b>SD</b>	0.09	0.05	0.05	0.22
<b>%RSD</b>	-8.32	-1.22	-1.08	-3.22
<b>30 °C/60% RH</b>				
<b>1</b>	-1.20	-3.60	-7.40	-7.20
<b>2</b>	-1.20	-3.50	-7.30	-6.80
<b>3</b>	-1.00	-3.50	-7.30	-6.70
<b>Average</b>	-1.13	-3.53	-7.33	-6.90
<b>SD</b>	0.09	0.05	0.05	0.22
<b>%RSD</b>	-8.32	-1.33	-0.64	-3.13
<b>40 °C/75% RH</b>				
<b>1</b>	-1.20	-2.90	-3.80	-3.80
<b>2</b>	-1.20	-2.80	-3.60	-3.70
<b>3</b>	-1.00	-2.70	-3.60	-3.50
<b>Average</b>	-1.13	-2.80	-3.67	-3.67
<b>SD</b>	0.09	0.08	0.09	0.12
<b>%RSD</b>	-8.32	-2.92	-2.57	-3.40

Zeta potential of the *C. genistoides* cream at all three stability controlled conditions did not stay within the range of less than -25 mV or more than +25 mV from months 0 to 3. Although all three stability testing conditions changed from a positive to a negative charged formulation, the zeta potential values were low. The afore-mentioned indicate that the formulation stored at the different stability conditions are instable and may tend to coagulate or flocculate.

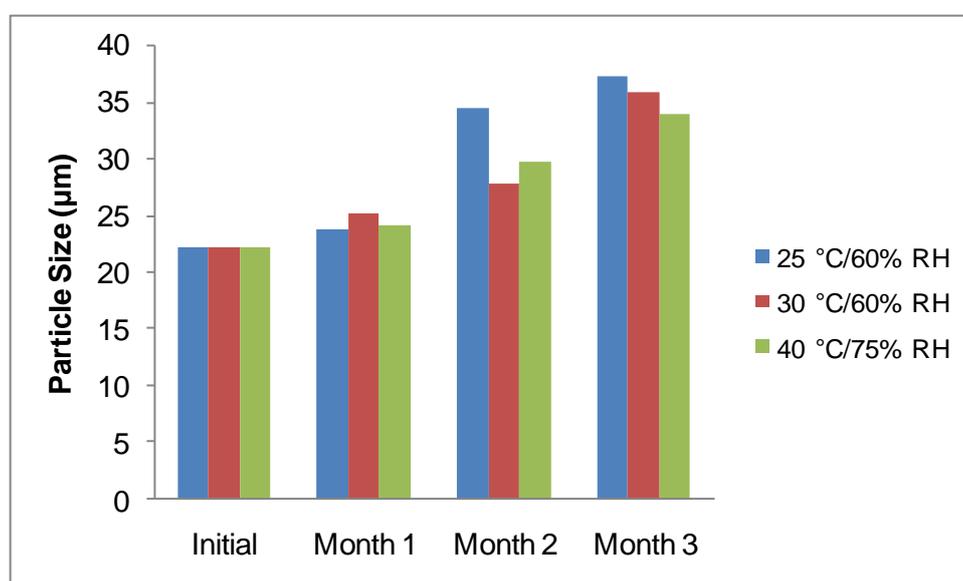
### C.3.6 Particle size

The particle size was determined on the initial creams, as well as at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH on months 1, 2 and 3 as described in Section C.2.6. Figures C.19 and C.20 illustrate an overview of the changes between months 0 and 3 of the different formulations. Reflecting in the results, D (0.5) refers to the size in microns (µm) at which 50% of the sample is smaller and 50% larger. This value is also known as the Mass Median Diameter (MMD) (Taylor, 2002:474; Malvern, 2000:6.3).

### C.3.6.1 *Cyclopia maculata* cream

**Table C.10:** Average particle size ( $\mu\text{m}$ ) of *Cyclopia maculata* cream at the different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>D (0.5)</b>	22.25	23.78	34.44	37.25
<b>30 °C/60% RH</b>				
<b>D (0.5)</b>	22.25	25.09	27.75	35.88
<b>40 °C/75% RH</b>				
<b>D (0.5)</b>	22.25	24.17	29.64	33.85



**Figure C.19:** The change in average particle size ( $\mu\text{m}$ ) for *C. maculata* cream at the different conditions after each time interval

In the *C. maculata* cream the average particle size increased after the 3-month period in all three stability controlled conditions. The cream stored at 25 °C/60% RH increased from an initial average particle size of 22.25  $\mu\text{m}$  to a final average particle size of 37.25  $\mu\text{m}$  after 3 months. This is an increase of approximately 15  $\mu\text{m}$ . The cream stored at 30 °C/60% RH demonstrated an increase of approximately 14  $\mu\text{m}$  after 3 months of stability testing, while the formulation stored at 40 °C/75% RH confirmed a final increase in average particle size of approximately 12  $\mu\text{m}$ .

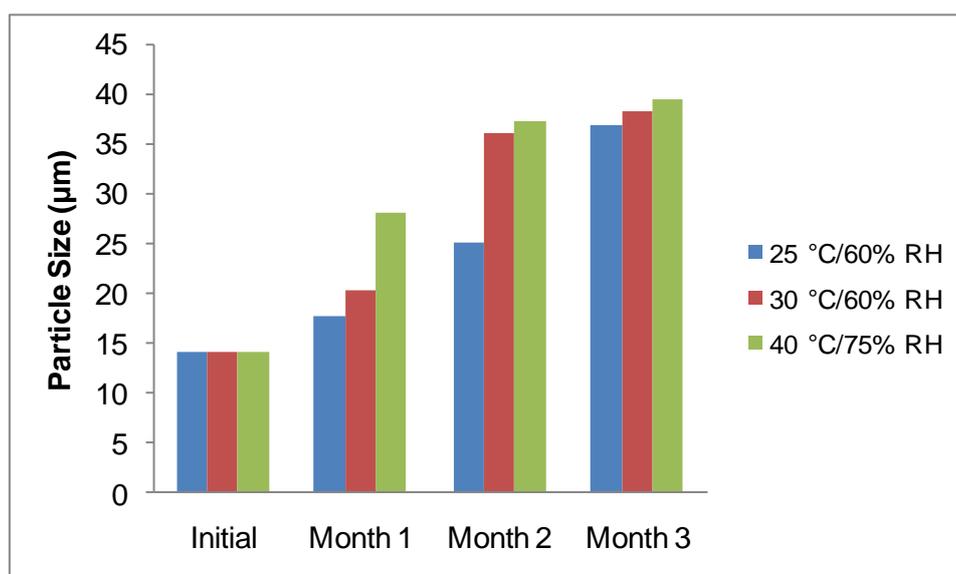
Evident during these different conditions, the particles in dispersion demonstrated that they adhered to one another to form aggregates of successively increasing size, which will possibly

settle out under the influence of gravity. This is called flocculation. In this cream the average particles increased over the 3-month period, showing that instability was present. This can be confirmed by the low zeta potentials, also indicating that flocculation was imminent.

### C.3.6.2 *Cyclopia genistoides* cream

**Table C.11:** Average particle size ( $\mu\text{m}$ ) of *Cyclopia genistoides* cream at the different conditions after each time interval

	Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>				
<b>D (0.5)</b>	14.09	17.67	25.10	36.77
<b>30 °C/60% RH</b>				
<b>D (0.5)</b>	14.09	20.24	36.00	38.20
<b>40 °C/75% RH</b>				
<b>D (0.5)</b>	14.09	28.09	37.25	39.51



**Figure C.20:** The change in average particle size ( $\mu\text{m}$ ) for *C. genistoides* cream at the different conditions after each time interval

In the *C. genistoides* cream the average particle size increased after the 3-month period in all three stability controlled conditions. The cream stored at 25 °C/60% RH increased from an initial average particle size of 14.09  $\mu\text{m}$  to a final average particle size of 36.77  $\mu\text{m}$  after 3 months. This is an increase of approximately 23  $\mu\text{m}$ . The cream stored at 30 °C/60% RH demonstrated an increase of approximately 24  $\mu\text{m}$  after 3 months of stability testing, while the formulation stored at 40 °C/75% RH confirmed a final increase in average particle size of approximately 25  $\mu\text{m}$  after 3 months.

Evident during these storage conditions, the particles in dispersion demonstrated that they adhered to one another to form aggregates of successively increased size, which possibly will settle out under the influence of gravity. This is called flocculation. In this cream the average particles increased over the 3-month period, showing that instability was present. This can be confirmed by the low zeta potentials, also indicating that flocculation was imminent.

The *C. maculata* cream proved to be the more stable formulation during the particle size analysis attributable to the lowest increase in the average particle size.

### **C.3.7 Visual appearance assessment**

Visual appearance assessment was done on the initial cream, as well as at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH on months 1, 2 and 3 as described in Section C.2.7.

In Table C.12 and Table C.13 the change in colour during the 3-month period of each formulation is given, respectively.

### C.3.7.1 *Cyclopia maculata* cream

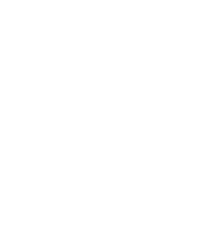
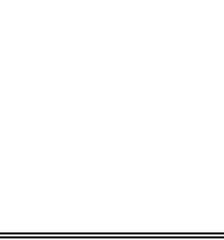
**Table C.12:** Change in colour of *Cyclopia maculata* cream at the different conditions after each time interval

Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>			
			
<b>30 °C/60% RH</b>			
			
<b>40 °C/75% RH</b>			
			

The overall colour of the *C. maculata* formulation changed from a light caramel colour to a dark toffee colour over the 3-month period. The most intensive colour changes appeared at the 40 °C/75% RH stage, where the cream appeared to become almost a dark clay colour. This clearly shows that moisture content has decreased during the 3-month period, undoubtedly evident by the decrease in surface shine in the pictures. Moderate colour changes were evident at both the formulations stored at 25 °C/60% RH and 30 °C/60% RH. Reasonable moisture loss was also evident in the decrease in surface shine in the picture giving the creams a clay-like texture after the 3-month period expired. All three formulations show a definite change in colour and texture appearance with the cream becoming almost a paste-like formulation.

### C.3.7.2 *Cyclopia genistoides* cream

**Table C.13:** Change in colour of *Cyclopia genistoides* cream at the different conditions after each time interval

Initial	Month 1	Month 2	Month 3
<b>25 °C/60% RH</b>			
			
<b>30 °C/60% RH</b>			
			
<b>40 °C/75% RH</b>			
			

The overall colour of the *C. genistoides* formulation changed from a light caramel colour to a dark, burned toffee colour over the 3-month period. The most intensive colour changes appeared during the 40 °C/75% RH stage, where the cream appeared to become almost a dark, burned clay colour. However, it appears to have gained a glossier look starting from the initial testing to the final testing stages. This could indicate that the cream either gained moisture or phase separation could be imminent by the water substance clearly visible on top of the cream. Intense colour changes was also evident in both the formulations stored at 25 °C/60% RH and 30 °C/60% RH. Reasonable moisture increase was also noticed in the increase in glossiness in the picture and the brownish, tea-like water substance floating on top of the cream. This gave all the formulations a fluid-like, diluted texture after the 3-month period expired making this specific formulation unsuitable for future use due to possible imminent phase separation.

## C.4 Conclusion

The assay concentrations of mangiferin and hesperidin in both the formulations showed significant change and did not remain within the acceptable limits (5% change of initial value). This could indicate that the active bioflavonoids present in honeybush extracts lack sufficient stability in semi-solid formulations and that interaction with the different ingredients in the formulation could have occurred. The only active ingredient (mangiferin) that did stay within acceptable limits is the *C. maculata* cream stored 25 °C/60% RH. This is a clear indication that future formulations containing honeybush extracts should be stored at room temperatures in a cool and dry environment for optimal preservation of the active ingredients within these extracts. The preservatives in both formulations also did not remain within the acceptable limits. Propyl paraben in the *C. maculata* cream and methyl paraben in the *C. genistoides* cream did not remain within the limits of 5% according to the ICH. Possible reasons for this degradation of the active ingredient can be due to the fact that insufficient protection by the preservatives occurred. The antioxidant concentration of BHT showed little change in both formulations. This can be contributed to the fact that honeybush extracts are known to emit their own anti-oxidant activity, therefore a saturated supply of antioxidants were present in the formulation.

The following three factors are co-dependent of each other and will be discussed together. These three factors are the pH, zeta potential and particle size of the formulations. Looking at the pH of both the creams, neither remained stable over the 3-month period. The pH of the formulation may influence the stability of the actives in the formulation. Human skin is very sensitive to extreme pH ranges, while the stratum corneum can be extremely resistant to changes in pH, tolerating a pH range of 3 to 9 (Barry, 2002:511). The *C. maculata* formulation proved to be the more stable formulation with a maximum pH change of 20% stored at 40 °C/75% RH, while the *C. genistoides* formulation had a maximum pH change of 31% stored at 40 °C/75% RH. This could indicate that the vast variations in pH are a clear indication of in the instability of mangiferin and hesperidin.

The zeta potential can be directly affected by several influencing factors, one being the above mentioned pH. The general distinction between stable and unstable suspensions is usually 25 to 30 mV (positive or negative) as stated by Malvern (2011:3) as well as Kirby & Hasselbrink (2004:187). Particles with zeta potentials more positive than + 25 mV or more negative than - 25 mV are usually considered stable due to the repelling forces between the particles, forcing the formulation into suspension. In this stability study very low zeta potentials were present, indicating incipient instability. Particles within the formulations may adhere to one another and form aggregates of successively increasing size, which may settle out under the influence of gravity. This may possibly lead to coagulation or flocculation of particles and later lead to total

phase separation of the formulation. Once again the magnitude of the zeta potential gives a clear indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential (above 25 mV), they will tend to repel each other and there will be no tendency for the particles to come together, indicating the stability of the colloidal system (Kirby & Hasselbrink, 2004:178). The drop in pH in both formulations gave the zeta potential a more negative charge due to higher concentrations of hydronium ions released over time, but these values still remained too low, ensuring incipient instability when storage times are increased. However, in relative terms, the *C. genistoides* formulation proved to be the more “stable” formulation, with the highest increase in zeta potential although incipient instability was present at both formulations.

Another factor to consider is the average size of the particles in dispersion. Both formulations had an average particle size increase over the three months. Their low zeta potential values, predicted that there will be a very low repellent force between the particles in dispersion to prevent flocculation and sedimentation as time passed. In relative terms, it could be concluded that the *C. maculata* formulation had the lowest increase in average particle size, also considering that both formulations had very low zeta potentials. Therefore, both creams showed imminent flocculation, sedimentation and possible total phase separation as time will progress. It is also important to remember that average particle size also plays an integral role in skin penetration. Particles smaller than 3 µm have the ability to penetrate both the skin’s follicles and the stratum corneum. Particles between 3 and 10 µm can enter the skin follicles, while particles larger than 10 µm stay on the skin’s surface (Barry, 2002:508).

The viscosity of both creams changed radically over the 3-month period. The *C. genistoides* formulation showed an immense decrease in overall viscosity. Possible explanations for this tendency can be attributed to the low zeta potentials detected during this study, indicating imminent flocculation, sedimentation and possible phase separation. Interactions between the different ingredients within the formulations, evident the breakdown of the actives could also have an effect on the decrease in viscosity. The formulation could also have been settling before it went into the final resting stage before stability testing started. Broken Van der Waals forces between the molecules could also have occurred during the previous viscosity measurements on the same cream, also leading to a decrease in viscosity (Attwood, 2002:80). However, this vast decrease in viscosity is a clear indication of possible instability and phase separation, clearly evident in the visual appearance demonstrated in Table C.13.

An increase in viscosity as seen in the *C. maculata* cream may be due to loss of moisture during stability testing over the period of 3 months, giving the cream a higher viscosity value. Low zeta potentials were detected for the *C. maculata* formulations and predicted that imminent instability

was present. However, the increase in viscosity is clearly evident in the paste-like appearance of the formulations during visual appearance analysis shown in Table C.12. The *C. maculata* formulation appeared to be the more stable formulation, due to the imminent phase separation present in the *C. genistoides* cream. Moisture loss was the most attributable factor responsible for the increase in viscosity in the *C. maculata* cream.

The visual appearance and colour of both creams showed a radical change over the 3-month period. The *C. maculata* formulation started with a glossy, light caramel coloured cream and ended as matt, dark clayed paste-like textured cream, supporting the data that a decrease in viscosity was present. The *C. genistoides* formulation changed from a glossy, light caramel coloured formulation to a glossier, burned dark toffee coloured formulation with a tea-like fluid present on the top surface of the cream. The massive decrease in viscosity supports this appearance, with imminent phase separation clearly evident. In this instance, the *C. maculata* cream proved to be the more stable formulation due to the lack in phase separation evident in the *C. genistoides* cream.

The mass of the both creams did not remain stable over the 3-month period. The *C. maculata* formulation showed the highest degree of moisture loss between the two formulations with the highest decrease in mass loss in the formulations stored at 25 °C/60% RH. This could be due to the loss of moisture due to the conveying of moisture from high moisture content within the cream to an atmosphere lower in moisture content. Containers not sealing as desired, could also increase the loss of mass due to the escape of moisture to the surrounding atmosphere due to the possible porosity of the storage containers. These results are also supported by the data retrieved during the estimation of change in the viscosity as well as in the visual appearance after 3 months. The *C. maculata* cream showed a massive decrease in viscosity and becoming a paste-like formulation after 3 months.

The lowest decrease in mass was present in the formulations stored at 30 °C/60% RH in both extracts. This could be due to the quicker reach of equilibrium between the moisture content within the cream and the surrounding atmosphere due to the favourable temperature relative to the humidity. The *C. genistoides* formulation proved to be the more stable formulation, with the lowest average decrease in mass loss during the 3-month storage period. In the future, sealable amber glass containers will be more effective to use.

Unfortunately the change in pH, zeta potential, particle size, colour, viscosity and concentration of the active ingredients in the formulations showed incipient instability of the products over the 3-month period. None of the formulations completely met the ICH and MCC's criteria for stability.

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## APPENDIX D

### FRANZ CELL DIFFUSION STUDIES

#### D.1 Introduction

Skin penetration studies have a central function in the optimisation of drug and formulation design in dermal and transdermal delivery. *In vitro* diffusion studies indicate the transdermal delivery of an active ingredient, while tape stripping techniques determine the topical delivery of an active ingredient (Leveque *et al.*, 2004:323).

All the diffusion studies were done by using cellulose acetate membranes to determine drug release from the formulations, or dermatomed human skin to determine the transdermal diffusion. In this study the vertical Franz cell diffusion and tape stripping methods were used to determine the release, transdermal and topical delivery of mangiferin and hesperidin in honeybush extracts. The target site for this specific study was the stratum corneum and the dermal layer of the skin.

#### D.2 Methods

##### D.2.1 Skin preparation

Abdominal skin of Caucasian female patients was obtained after cosmetic abdominoplastic surgery. Ethical approval under the title "*In vitro* transdermal delivery of drugs through human skin" (NWU-00114-11-A5) was permitted by the Ethics Committee of the North-West University (Potchefstroom, South Africa) and skin was obtained with informed consent from the donors.

The fresh skin was sponged down with deionised water and dried with a paper tissue. The exterior of the skin was once wiped clean with an ethanol-moistened cotton swab to eliminate possible fat residual from the subcutaneous fat layer and surface sebaceous lipids.

A skin layer of a thickness of approximately 400 µm including the stratum corneum, viable epidermis and upper dermis with a width of 2.5 cm was prepared using an electrical dermatome (Zimmer Inc., Warsaw, IN, USA). The skin was positioned dermal side down on filter paper and stored in aluminium foil at -20 °C until used. The time of storage did not surpass 6 months. One hour preceding the diffusion experiments, the skin was thawed at room temperature and cut into circular pieces (approximately 15 mm in diameter).

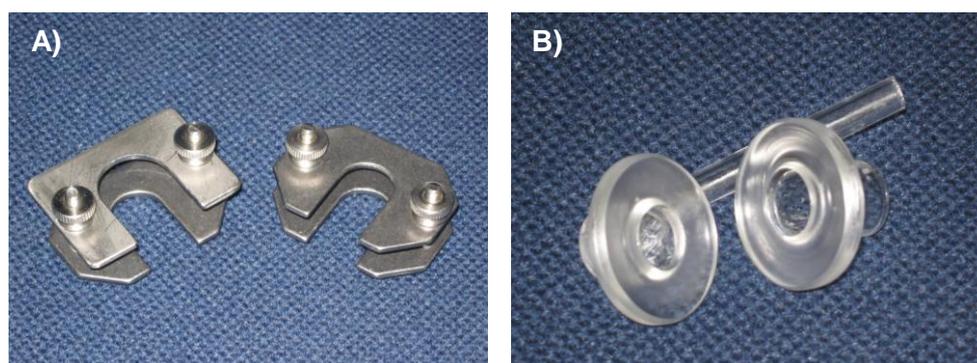
## D.2.2 Preparation of phosphate buffer solution (pH 7.4)

Phosphate buffer solution (PBS), acting as the receptor phase, was freshly prepared before each diffusion study as follows:

- Potassium dihydrogen orthophosphate (13.620 g) was weighed and dissolved in 500.0 ml HPLC water.
- Sodium hydroxide (3.147 g) was weighed and dissolved in 786.8 ml HPLC water.
- The two solutions were mixed and the pH was set to 7.4 with 10% orthophosphoric acid.
- The solution was filtered and degassed.

## D.2.3 Diffusion studies

Vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and a diffusion area of 1.075 cm<sup>2</sup> were used in this study. In each experiment twelve Franz cells were used. Franz cells 1 to 10 contained the active formulation, namely 2% *C. maculata* or 2% *C. genistoides* creams. Franz cells 11 and 12 contained a placebo formulation of the cream used during the experiment. Another diffusion study was performed consisting of twelve Franz cells that contained a 0.003% *C. genistoides* formulation commercially available on South African markets. All these Franz cells consisted of a donor (top) and receptor (bottom) compartment. The donor phase, containing the above-mentioned semi-solid formulations with their active ingredients (2% *C. maculata*-, 2% *C. genistoides*- or 0.003% commercial creams), was placed in the donor compartment and the receptor phase (PBS, pH 7.4) was placed in the receptor compartment.

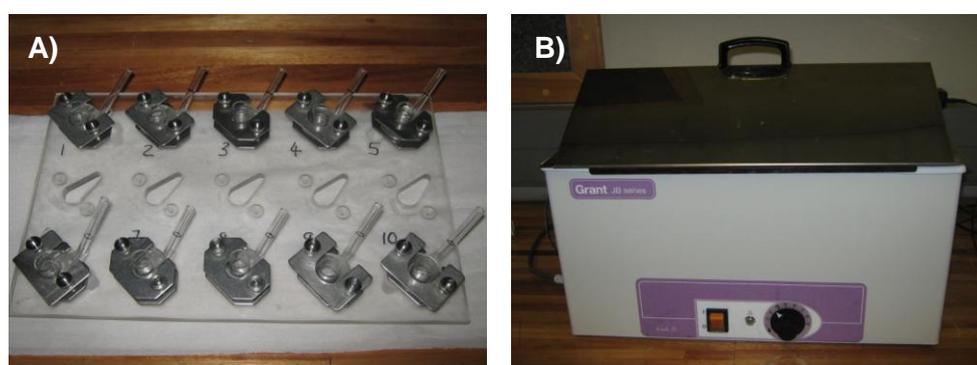


**Figure D.1:** A) Horseshoe clamps and B) vertical Franz diffusion cell with donor and receptor compartments

Miniature magnetic stirring bars were placed in the receptor compartments of each Franz cell to continue stirring throughout the experiment. The diffusion cells were placed in a tray on a

Variomag<sup>®</sup> stirrer plate. The skin circles or cellulose acetate membranes were mounted among the receptor and donor compartment with the stratum corneum facing upwards (towards the donor compartment). Dow Corning<sup>®</sup> high vacuum grease was used to seal the cells to prevent any leakages. The donor and receptor compartments were secured with a metal horseshoe clamp. Thereafter the receptor compartments were filled with PBS (pH 7.4) while care was taken to avoid the entrapment of air bubbles under the surface.

The donor compartments were filled with approximately 1 ml of the semi-solid formulation to keep the skin saturated. It was then covered with Parafilm<sup>®</sup> to avoid evaporation. The Franz cells were placed in a 37 °C Grant<sup>®</sup> water bath in order to accomplish a skin temperature of 32 °C.



**Figure D.2:** A) Assembled Franz diffusion cells and B) Grant<sup>®</sup> water bath

The complete content of the receptor phases was withdrawn at exact time intervals and substituted with fresh PBS (pH 7.4) that was pre-heated to 37 °C. HPLC vials were then filled with the withdrawn PBS (receptor phase). The concentration of mangiferin and hesperidin was immediately analysed by HPLC.

#### D.2.4 Membrane diffusion

The aim of the membrane studies was to determine whether mangiferin and hesperidin are released from the formulations. The method as discussed in Section D.2.3 was used for the membrane diffusion studies. The entire content of the receptor phases was withdrawn and replaced with fresh PBS (pH 7.4) on an hourly basis (hours 1, 2, 3, 4, 5 and 6). Due to extremely low, unquantifiable concentrations depicted during HPLC analysis, a second membrane diffusion study was executed where only a single withdrawal of the entire content of the receptor phases for the formulations was performed after 6 h.

### **D.2.5 Skin diffusion**

The method as discussed in Section D.2.3 was used for the skin diffusion studies. The complete content of the receptor phases was withdrawn and substituted with fresh PBS (pH 7.4) after 20, 40, 60, 80, and 100 min, as well as 2, 4, 6, 8, 10 and 12 h. Due to extremely low, unquantifiable concentrations noticed during HPLC analysis, a second diffusion study was executed where only a single withdrawal of the entire content of the receptor phases for the formulations was performed after 12 h. Tape stripping was performed after the diffusion studies of 12 h were completed (see Section D.2.6).

### **D.2.6 Tape stripping**

According to Lademann *et al.* (2009:317) tape stripping is a straightforward and competent method for the evaluation of quality and effectiveness of cosmetic and dermatological formulations. After topical application and penetration of formulations, the cell layers of the stratum corneum are sequentially separated from the same skin region by means of adhesive films. The tape strips enclose the quantity of penetrated formulation, which can be analysed by chemical methods (Lademann *et al.*, 2009:317).

Towards the end of the diffusion study the diffusion cells were carefully separated and the segments of skin were attached onto a piece of Parafilm<sup>®</sup>, stapled to a fixed surface. The diffused area could undoubtedly be seen. The segments of skin, tapped dry with tissue. Pieces of 3M Scotch<sup>®</sup> Magic<sup>™</sup> Tape were cut into sizes large enough to envelop the diffused area, and small enough not to extend beyond the areas outside the diffused area. The first tape strip was discarded, as it is seen as part of the cleaning procedure. The next 15 strips (stratum corneum-epidermis) were placed in a vial filled with 5 ml PBS (pH 7.4) to cover the strips. A clear indication of the complete removal of the stratum corneum is when the viable epidermal layer appears to glisten. The vials were set aside overnight at 4 °C.

The remaining skin was cut into pieces to enlarge the surface area. It was placed in vials filled with 2 ml PBS (pH 7.4) to cover the skin pieces and were kept overnight at 4 °C. The tape samples were filtered and examined by HPLC. The skin samples were homogenised and filtered in turn to be analysed by HPLC.

### **D.2.7 HPLC analysis of mangiferin and hesperidin**

A HPLC method has already been developed and validated in conjunction with Prof. Jan du Preez from the Analytical Technology Laboratory at the North-West University, Potchefstroom Campus, South Africa.

An Agilent® 1200 Series HPLC equipped with an Agilent® 1200 pump, autosampler injection mechanism and UV-detector was used (Agilent Technologies, Palo Alto, CA). The apparatus was interfaced with Chemstation Rev. A.06.02 data acquisition and analysis software. High performance silica based, reversed phase Agela® Venusil XBP C<sub>18</sub> (2) column, (150 mm × 4.6 mm) with a 5 µm particle size was used (Agela® Technologies, Newark, DE).

The two mobile phases consisted of a mixture of 1 ml phosphoric acid in 1000 ml of HPLC water and acetonitrile. The operating flow rate was 1.0 ml/min and the injection volume was 20 µl. The UV-detector was set at 210 nm for the detection of mangiferin and hesperidin. The retention time of mangiferin was 4.8 min and that of hesperidin was 5.9 min. The total running time was 20 min. Analysis was performed in a controlled laboratory environment at 25 °C. The solvent used was PBS.

### D.2.8 Data analysis

For the diffusion studies the average percentage diffused (%) as well as the average concentration (µg/cm<sup>2</sup>) of the active ingredients that penetrated the skin after 12 h was calculated.

For the membrane studies the average percentage released (%) was determined as well as the average concentration (µg/cm<sup>2</sup>) of active ingredient that penetrated the skin was calculated after 6 h.

## D.3 Results and discussion

### D.3.1 Membrane release studies

**Table D.1:** Data obtained from membrane release studies after 6 h for 2% *C. maculata* cream

<b><i>C. maculata</i></b>	<b>Average %released after 6 h (%)</b>	<b>Average concentration after 6 h (µg/cm<sup>2</sup>)</b>
<b>Mangiferin</b>	0.001	0.418
<b>Hesperidin</b>	0.003	0.602

**Table D.2:** Data obtained from membrane release studies after 6 h for 2% *C. genistoides* cream

<i>C. genistoides</i>	Average %released after 6 h (%)	Average concentration after 6 h ( $\mu\text{g}/\text{cm}^2$ )
Mangiferin	0.016	5.950
Hesperidin	0.012	4.416

In both formulations, as seen in Tables D.1 and D.2, extremely low concentrations were detected in the receptor phase of the Franz cell after penetration through the cellulose acetate membranes. This could be an indication that the active ingredients have insufficient physico-chemical properties for satisfactory release of the active ingredients and that the active ingredient concentrations within the formulations are too low for accurate detection during HPLC analysis.

In the *C. maculata* formulation, only three of the ten diffusion cells showed release and therefore did not justify a full skin diffusion study. The HPLC method was barely capable of detecting these minuscule concentrations of *C. maculata*. In the *C. genistoides* cream however, a maximum of six the ten diffusion cells showed release and therefore a full skin diffusion study was performed. Both mangiferin and hesperidin in the *C. genistoides* cream had better release than that of the *C. maculata* formulation. When examining the average concentration ( $\mu\text{g}/\text{cm}^2$ ) after 6 h, *C. genistoides* cream released mangiferin and hesperidin approximately fourteen and seven times better than the *C. maculata* cream, respectively. The *C. genistoides* cream also had extremely low concentrations and the HPLC detection was difficult since the peaks were so small that they had to be integrated by hand. Therefore, it is difficult to draw any accurate conclusions on these observations.

### D.3.2 Diffusion studies

**Table D.3:** Data obtained from skin diffusion studies after 12 h with 2% *C. genistoides* cream

	Average % diffused after 12 h (%)	Average concentration ( $\mu\text{g}/\text{cm}^2$ ) after 12 h
<b>2% <i>C. genistoides</i> cream</b>		
Mangiferin	0.001	0.250
Hesperidin	0.000	0.000
<b>0.003% <i>C. genistoides</i> commercial cream</b>		
Mangiferin	0.000	0.000
Hesperidin	0.000	0.000

Two diffusion studies were conducted on the 2% *C. genistoides* formulation. The first diffusion study consisted of extractions every 20 min up to 2 h and thereafter 2 hourly up to 12 h. Due to unmeasurable concentrations that diffused in the above mentioned study, a second diffusion study was initiated with only one extraction after 12 h. Results from the second diffusion study were analysed and only 0.250 µg/cm<sup>2</sup> mangiferin diffused through the skin. With concentrations too low for accurate quantification, the results were considered inconclusive. Two diffusion studies were also conducted on the honeybush formulation (0.003% *C. genistoides* cream) currently available on South African markets. One diffusion study was extracted every 20 min up to 2 h and thereafter 2 hourly up to 12 h and the other after 12 h. In both studies no mangiferin or hesperidin was detected.

Possible reasons for this poor performance by these formulations can be attributed to several factors. First the human skin has exceptional properties of which functioning as a physico-chemical obstruction is one of its key properties (Bos & Meinardi, 2000:165). Skin is a complex organ that serves to protect the underlying tissues against external physical, chemical, immunological and pathogenic intrusion and free radical attacks, while retaining moisture and providing thermal regulation (Ho, 2004:49). The extent of skin diffusion is mainly reliant on physiological factors of the skin as well as the physico-chemical characteristics (Idson, 1971:615). To some extent, poor penetration can be attributed to active molecules being larger than 500 Dalton (Da) which cannot penetrate the corneal layer of the skin, while smaller molecules are able to pass through the corneal layer, surpassing transcutaneously (Bos & Meinardi, 2000:165). Mangiferin, with a molecular weight of 422.34 Da (Wauthoz *et al.*, 2007:114) can cross the skin to some extent, but hesperidin with a molecular weight of 610.56 Da (Garg *et al.*, 2001:656) will show great effort in crossing the skin according to the “500 Dalton rule” (Bos & Meinardi, 2000:165). As seen from the results shown above this statement seems to be true. Although mangiferin did pass the corneal layer, it was almost in unquantifiable amounts.

Secondly, poor penetration can also be due to the physico-chemical considerations for passive transdermal delivery of a formulation such as drug lipophilicity. The stratum corneum is lipophilic (Naik *et al.*, 2000:319). A drug molecule must first be released from the formulation and partition into the uppermost stratum corneum layer, before diffusion through the entire thickness of the skin can occur (Naik *et al.*, 2000:319). Ideally, a drug must possess a reasonable solubility in both water and oils for proper skin penetration. It also has to have an aqueous solubility of more than 1 mg/ml and a log P (octanol-water partition coefficient) in the range of 1 to 2 (Hadgraft, 1996:165). The active ingredients, mangiferin and hesperidin, have very poor solubility in water (Majumbar & Srirangam, 2009:1221), i.e. mangiferin has an aqueous solubility of approximately 0.111 mg/ml and a log P value of 2.73 (Wauthoz *et al.*,

2007:115), while hesperidin with an aqueous solubility of approximately 4.95 mg/ml and a log P value of 1.78 (Srirangam & Majumbar, 2010:60). Hesperidin has an ideal log P value for the diffusion through the stratum corneum, but its big particle size might be hindering successful.

According to Barry (2002:508) particles between 3 µm and 10 µm can concentrate in the hair follicles, while particles less than 3 µm penetrate follicles and the stratum corneum alike. As seen in Appendix C, the *C. maculata* formulation had an average particle size of 22.25 µm with the *C. genistoides* formulation with an average of 14.09 µm. Thus, it can be agreed that particles larger than 10 µm stay on the skin's surface or have extreme difficulty to penetrate the skin due to its bigger particle size.

However, the real reason for this poor performance by both the formulations could be attributed to the fact that extremely low concentrations of mangiferin and hesperidin are present in both the 2% and 0.003% *Cyclopia* formulations. Extremely low mangiferin (20.302 mg) and hesperidin (29.544 mg) concentrations within the 2% *Cyclopia genistoides* formulation were released from both the formulations, resulting in inaccurate HPLC quantifications.

### D.3.3 Tape stripping

**Table D.4: Data obtained from tape stripping studies**

	Average concentration in stratum corneum-epidermis (µg/ml)	Average concentration in epidermis-dermis (µg/ml)
<b>2% <i>C. genistoides</i> cream</b>		
<b>Mangiferin</b>	0.003	0.005
<b>Hesperidin</b>	0.000	0.000
<b>0.003% <i>C. genistoides</i> commercial cream</b>		
<b>Mangiferin</b>	0.000	0.000
<b>Hesperidin</b>	0.000	0.000

During HPLC analysis, mangiferin (2% *C. genistoides* cream) was the only active ingredient after application to cross the skin in extremely low concentrations. There were also very low concentrations detected in the stratum corneum-epidermis as well as in the epidermis dermis, indicating that very poor skin penetration took place due to the very low concentrations present in the semisolid formulation. Hesperidin was not able to cross the stratum corneum, dermis or epidermis.

No detectable concentrations of mangiferin and hesperidin were present in the stratum corneum-epidermis and epidermis-dermis in the honeybush product currently available on South African markets.

These skin diffusion studies can be compared to a similar study done by Huang *et al.*, (2008:699) that investigated the transport of aspalathin, a unique flavonoid constituent of rooibos tea, across the human skin. These vertical Franz cell diffusion studies were conducted for both pure aspalathin solutions and extracts from unfermented (green) rooibos (*Aspalathus linearis*) aerial plant material across the human abdominal skin. The results obtained showed that only a portion of 0.07% of the initial aspalathin dose penetrated the different layers of the skin for the green rooibos extract solution and 0.08% for the pure aspalathin solution (Huang *et al.*, 2008:699).

The incorporation of rooibos extracts as well as honeybush extracts in topical cosmetic formulations, has become a trend in cosmeceuticals to directly target the skin as the target site of action to fight against ultraviolet radiation damage and photo-ageing through the antioxidant properties of both extracts. The *in vitro* release of the bioactive flavonoids such as mangiferin and hesperidin can be compared to that of aspalathin. Permeation across the skin with the highly resistant stratum corneum from both extracts was relatively low and should be taken into consideration in the future preparation and formulation of cosmeceutical products containing honeybush extracts that aim to provide anti-ageing and protective effects in the skin.

#### **D.4 Conclusion**

It can be concluded that the fraction of a drug that penetrates the skin through any particular route depends on the physico-chemical characteristics of the drug, in particular the particle size, the solubility and its partition coefficient. The site and the condition of the skin as well as the vehicle components can temporarily change the properties of the stratum corneum and inhibit proper transdermal diffusion. The release of the active ingredient from the vehicle also plays an integral part in skin diffusion as well as concentration present within the formulation (Barry, 2002:508).

Although freezing of skin for purposes of storage is scientifically acceptable as it does not affect its integrity, it must be kept in mind during the interpretation of the results that the metabolic systems found in viable skin may no longer be functioning and permeation of aqueous soluble substances across skin that was frozen for a period of time may be greater than that of fresh skin (Brain *et al.*, 2005:681).

There were no profound concentrations of mangiferin (0.003 µg/ml) and hesperidin (0.000 µg/ml) present in the stratum corneum-epidermis or the epidermis-dermis. When comparing the results of the 2% *C. genistoides* results to that of the commercial product (0.000 µg/ml), it can be seen that the *C. genistoides* cream performed 677 times better, even though the concentrations of mangiferin detected were extremely low. This is however, a clear

indication that honeybush extract concentrations above 2% are needed for better and more conclusive skin diffusion results in the future. The only problem with higher concentrations of honeybush extracts is that the aesthetics of the cosmeceutical cream will be diminished due to the change from an already inconsistent caramel coloured cream to a brown toffee coloured cream. This will make the commercial buyer sceptical due to the potential of discolouring the skin.

Further attention needs to be given to the formulation of mangiferin and hesperidin into topical preparations in order to improve drug release and skin permeability while further investigations are, however, necessary to determine the exact mechanisms involved and the factors that may play a role in the transdermal transport of mangiferin and hesperidin across the human skin.

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## APPENDIX E

### ANTIOXIDANT PROPERTIES OF HONEYBUSH EXTRACTS

#### E.1 Introduction

Natural products have provided vital resources of drugs since ancient times and an important component of today's drugs is in some way imitative from natural sources. In recent years, a new awareness in acquiring biologically active compounds from natural sources has been noticed. Consequently, a wide range of bioactive assays, isolation methods and spectroscopic methods has been developed (Pérez-Bonilla *et al.*, 2006:311). Diverse assays can be used to examine biological activity, *in vitro* and *in vivo* (Choi *et al.*, 2002:1161). According to Masoko & Eloff (2007:231), the most commonly used techniques for screening antioxidant activity, are those that engage the production of free radical species which are then neutralised by antioxidant compounds. On the other hand one must take into account that antioxidant capacity is not accurately rated. The assay is rigorously based on chemical reactions and bears no resemblance to biological systems. The legitimacy of the data is restricted to a stringent chemical sense with context analysis. Any claims concerning bioactivity of a sample based exclusively on this assay would be overstated, unscientific and in isolation (Huang *et al.*, 2005:1842).

To establish the antioxidant activity of the two different *Cyclopia* species, it is essential to perform an assay illustrating this mechanism of action. In this study the thiobarbituric acid (TBA)-assay demonstrates the ability of extracts/compounds to scavenge OH• (hydroxyl radical). Malondialdehyde (MDA), a major degradation product of lipid hydroperoxides, will be the marker for assessing the extent of lipid peroxidation. This compound is of particular concern, since it has been shown to be mutagenic, carcinogenic and implicated in other pathological processes such as the formation of fluorescent pigments typical of cellular ageing (Botsoglou *et al.*, 1994:1931). The most common method for measuring MDA in biological samples seems to be the thiobarbituric acid test, which is based on spectrophotometric quantification of the pink complex formed after reaction of MDA with two molecules of TBA.

#### E.2 TBA-Assay

The TBA-assay is one of the most commonly used techniques for the determination of lipid peroxidation in biological samples and involves the reaction between TBA and malondaldehyde (MDA) equivalents (Ottino & Duncan, 1997:1147). MDA, a key degradation product of lipid peroxidation, serves as an indicator for measuring the degree of lipid peroxidation. MDA acts in

response to two molecules of TBA through an acid-catalysed nucleophilic-addition reaction yielding a pinkish-red chromagen, which can be removed with butanol and measured by spectrophotometric quantisation due to an absorbance maximum at 532 nm (Hodges *et al.*, 1999:604). Even though this technique has been condemned for its lack of specificity and its inclination to overrate the MDA content, it has been shown to be responsive to minute TBA alterations in animal and plant tissue and is presently accepted as a consistent estimator of lipid peroxidation (Hodges *et al.*, 1999:604).

### **E.2.1 Preparation of honeybush extracts**

Four different concentrations of both *Cyclopia maculata* and *Cyclopia genistoides* extracts were prepared. Extracts with each of the following concentrations of 0.3125 mg/ml, 0.6250 mg/ml, 1.250 mg/ml and 2.500 mg/ml were dissolved in a water/methanol mixture of equal parts. The active ingredients in the honeybush extracts were made up to represent the approximate concentrations present in the abovementioned concentrations. For mangiferin, approximately four concentrations of 0.0169 mg/ml, 0.0338 mg/ml, 0.0676 mg/ml and 0.1352 mg/ml were dissolved in a water/methanol mixture of equal parts. Hesperidin concentrations of approximately 0.0046 mg/ml, 0.0092 mg/ml, 0.0184 mg/ml and 0.0368 mg/ml were dissolved in a water/methanol mixture. Lastly 5 ml of each of the two different semi-solid formulations were dissolved in 5 ml water/methanol mixture.

### **E.2.2 Test animals**

*In vitro* experiments were performed on whole rat brain homogenates from adult male Sprague-Dawley albino rats weighing between 200 to 250 g. The animals were housed in a windowless, well-ventilated constant environment (CER) room under a diurnal lighting cycle: 12 h light; 12 h darkness. Ambient temperature of the animal room was maintained at  $21 \pm 1$  °C, with a humidity of  $55 \pm 5\%$ . The animals received standard laboratory chow and water *ad libitum* and the induction of stress was minimised at all times. The North West University (Potchefstroom Campus) Animal Ethics Committee approved the experimental assay performed under ethical code 05D05 and conforms to the University's Regulations Act concerning animal experiments.

### **E.2.3 Chemicals and reagents**

Ascorbic acid (vitamin C), dimethyl sulphoxide (DMSO) and iron(III)chloride ( $\text{Fe}_3\text{Cl}$ ) were purchased from Merck Chemicals (Wadeville, Gauteng, South Africa). 1,1,3,3-Tetramethoxypropane (98%) (TEP), 2-thiobarbituric acid (98%) (TBA), BHT, trichloroacetic acid (TCA) and trolox (vitamin E) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen

peroxide was purchased at a local pharmacy. All other chemicals and reagents used were of the highest chemical purity.

Phosphate buffer solution (PBS) consisted of 137 mM NaCl (sodium chloride), 2.7 mM KCl (potassium chloride), 10 mM Na<sub>2</sub>HPO<sub>4</sub> (di-sodium hydrogen orthophosphate anhydrous) and 2 mM KH<sub>2</sub>PO<sub>4</sub> (potassium dihydrogen orthophosphate) in 1000 ml Milli-Q water. The pH of the solution was ascertained to be 7.4 and the solution was stored in the refrigerator.

BHT (0.5 g/L) was dissolved in methanol; TCA (10%) and TBA (0.33%) were prepared in Milli-Q water. TBA is light sensitive and was always prepared fresh and protected from light by covering the container with aluminium foil.

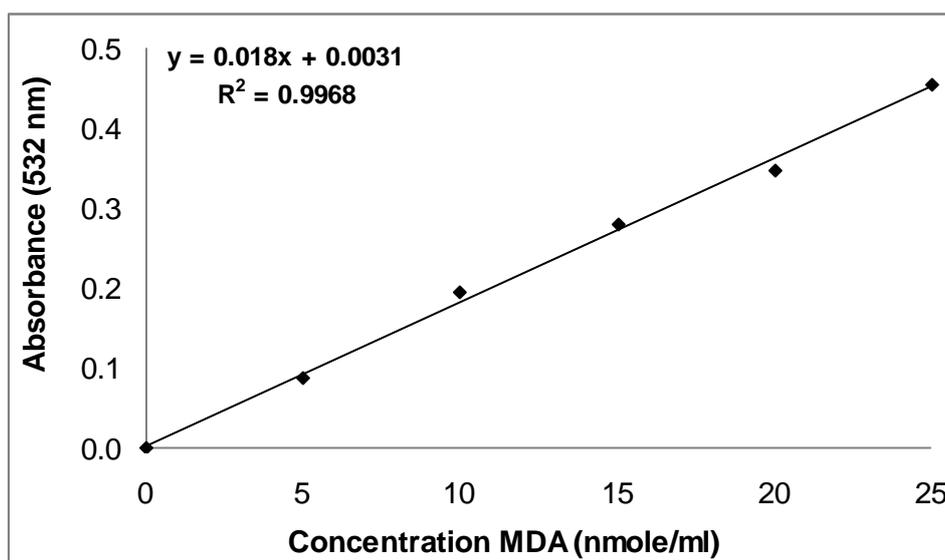
Hydrogen peroxide (5 mM H<sub>2</sub>O<sub>2</sub>) was used, as the toxin, to generate OH<sup>•</sup> and initiate lipid peroxidation in the rat brain homogenates (Garcia *et al.*, 2000:1). Ascorbic acid (1.4 mM) and FeCl<sub>3</sub> (4.88 mM) were added to increase the generation of OH<sup>•</sup> according the following reactions (Cui *et al.*, 2004:774):



Trolox was used throughout the experiments as the positive control, by emitting its own anti-oxidant activity. Values lower than that of trolox indicate almost definite antioxidant activity, by the reduction in MDA formation in peroxidising lipid systems. The control solution consisted of 160 µl rat brain homogenates, 20 µl PBS and 20 µl of water and methanol mixture of equal parts. Results were compared to that of the toxin, a mixture known to have very low levels of antioxidant activity.

#### **E.2.4 Preparation of standard**

TEP/MDA was used as a standard. A series of reaction tubes, each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 1 ml. A calibration curve was generated by measuring the absorbance at 10 nmole/ml intervals, in the range of 0 – 50 nmole/ml at a detection wavelength of 532 nm using an ultraviolet (UV)-visible spectrophotometer. The absorbance of the TBA/MDA-complex was plotted against the known concentration of MDA.



**Figure E.1:** MDA standard curve generated from TEP

### E.2.5 Tissue preparation

Rat brain homogenate is a valuable model for determining the efficiency of agents to attenuate or potentiate lipid peroxidation and is commonly used as a rich source of membrane lipids to measure general lipid peroxidation. Rats were sacrificed by decapitation and the whole brain of each rat was rapidly excised. The whole rat brain was homogenised in 0.1 M PBS (pH 7.4) to give an ultimate concentration of roughly 10% (w/v). PBS buffer was used as it has been shown not to scavenge free radicals (Auddy *et al.*, 2003:134).

### E.2.6 Method

To establish the potential antioxidant activity of extracts, 1 ml rat brain homogenate containing toxin combination and varying concentrations of extracts, actives and semi-solids were incubated in an oscillating water bath for 60 min at 37 °C, in order to induce lipid peroxidation. After incubation, the content was centrifuged at 2000 x g for 20 min, removing all insoluble proteins. The supernatant was removed from each tube and the termination of the incubation period was followed by the addition of 0.5 ml methanolic BHT (0.5 mg/ml), 1 ml TCA (10%) and 0.5 ml TBA to this fraction. Amplification of lipid peroxidation during the assay was prevented by adding the chain-breaking antioxidant BHT to the sample, TCA to start the acid-heating hydrolysis reaction (acid-catalysed nucleophilic addition reaction) and to precipitate proteins and TBA to bind to the formed MDA and form the pink chromogen (Halliwell & Chirico, 1993:719S). The tubes were sealed (marbles) and the mixtures heated to 60 °C in an oscillating water bath for 60 min, to release the protein-bound MDA through hydrolysis. Following the incubation, the samples were cooled on crushed ice until it reached room

temperature and the TBA-MDA complexes were extracted with 2 ml butanol and centrifuged at 2000 x g for 10 min. The absorbance was read at 532 nm.

### E.2.7 Data collection

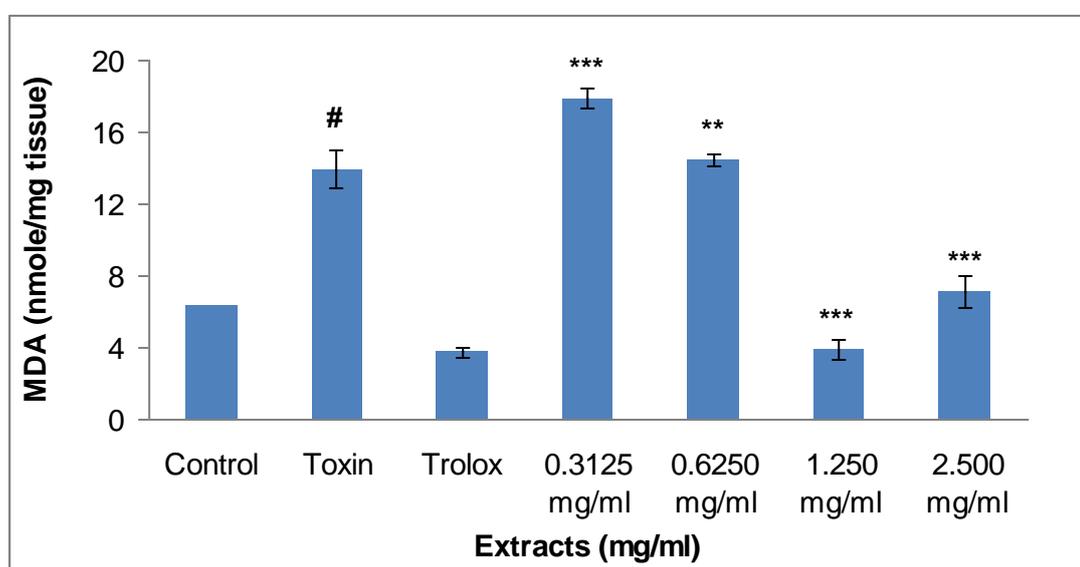
The absorbance values obtained were converted to MDA levels (nmole MDA) from the calibration curve generated with TEP. Results and the extent to which lipid peroxidation occurred were expressed as nmole MDA/mg tissue.

## E.3 Results and discussion

### E.3.1 Statistical analysis

GraphPad InStat 3 software was used for the statistical analysis of data. Results are given as the mean  $\pm$  S.E.M (standard error of the mean) of 5 repeats (n = 5). Data were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman Keuls test for multiple comparisons. Difference between groups was considered to be significant when  $p < 0.05$  when compared to the toxin (#). When  $p < 0.001$  it is considered extremely significant (\*\*\*). When  $p < 0.01$  it is considered fairly significant (\*\*), while  $p < 0.05$  is considered as significant (\*). A  $p > 0.05$  is considered to be not significant (ns).

### E.3.2 Antioxidant properties of *Cyclopia maculata* extracts



**Figure E.2** The attenuation of lipid peroxidation by different concentrations of *C. maculata* extracts in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. toxin (#)

When comparing the *Cyclopia maculata* extracts with the toxin it is evident that extract concentrations 0.3125 mg/ml and 0.6250 mg/ml did not demonstrate sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.920 \pm 1.065$  nmole/mg), in turn showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. On the contrary, when comparing the *Cyclopia maculata* extracts with the toxin it is apparent that extracts concentrations 1.250 mg/ml and 2.500 mg/ml showed potential antioxidant activity by means of decreasing the MDA formation, which in turn leads to lower degree of lipid peroxidation and higher OH<sup>•</sup> radical scavenging abilities of the extracts.

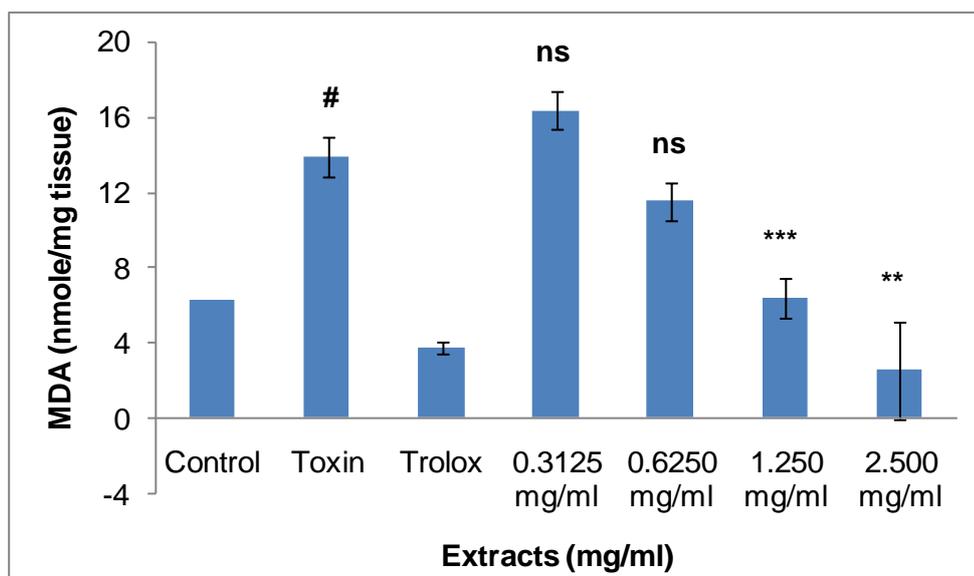
After the comparison of *Cyclopia maculata* extracts with trolox ( $3.716 \pm 0.320$  nmole/mg) it is clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations were below trolox.

When comparing the extract concentrations to both toxin and trolox, it is observed that concentrations 1.250 and 2.500 mg/ml were the only two concentrations that fell between the negative (toxin) and positive (trolox) control. Therefore, optimum synergistic activity could be predicted between the concentration range of 1.250 mg/ml and 2.500 mg/ml. Hence, the 1.250 mg/ml concentration had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidising lipid system.

Focusing on the statistical analysis of the *Cyclopia maculata* extracts it is observed that concentrations 0.3125, 1.250 and 2.500 mg/ml were statistically extremely significant with a p-value of  $< 0.001$ . Concentration 0.625 mg/ml was considered statistically fairly significant with a p-value of  $< 0.01$ .

### **E.3.3 Antioxidant properties of *Cyclopia genistoides* extracts**

When comparing the *Cyclopia genistoides* extracts with the toxin it is evident that extract concentration 0.3125 mg/ml did not demonstrate sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.920 \pm 1.065$  nmole/mg), sequentially showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. On the contrary, when comparing the *Cyclopia genistoides* extracts with the toxin it is apparent that extracts concentrations 0.625, 1.250 and 2.500 mg/ml showed potential antioxidant activity by means of decreasing the MDA formation, which in turn leads to a lower degree of lipid peroxidation and higher OH<sup>•</sup> radical scavenging abilities of the extracts.



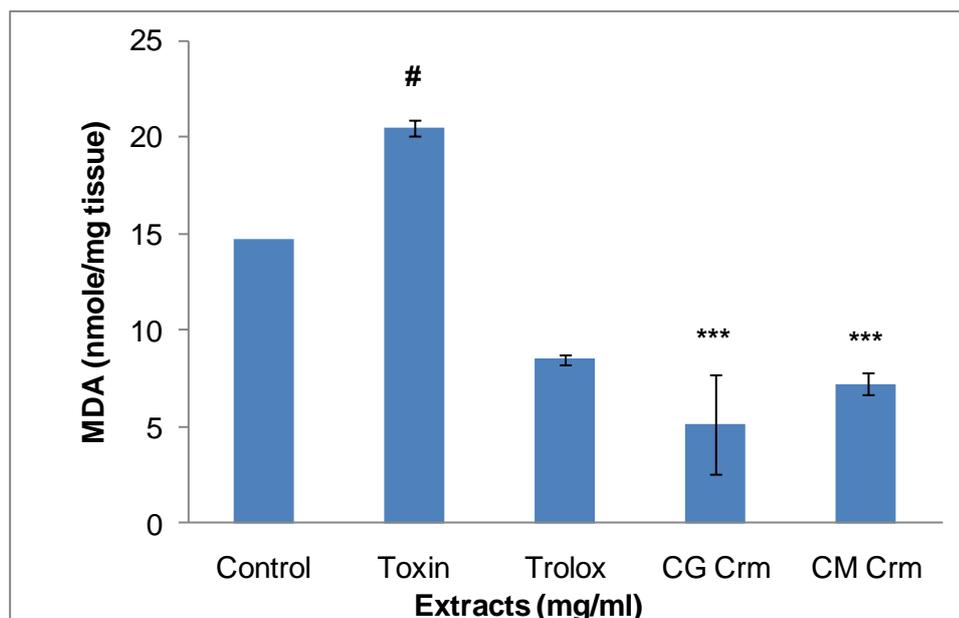
**Figure E.3** The attenuation of lipid peroxidation by different concentrations of *C. genistoides* extracts in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). \*\*p < 0.01; \*\*\*p < 0.001 vs. toxin (#); ns = not significant

After the comparison of *Cyclopia genistoides* extracts with trolox ( $3.716 \pm 0.320$  nmole/mg) it is clear that concentration 2.500 mg/ml of the extract concentrations emitted its own antioxidant activity, as this concentration had a MDA value below that of the trolox.

When comparing the extract concentrations to both toxin and trolox, it is observed that concentrations 0.6250 and 1.250 mg/ml were the only two concentrations that fell between the negative (toxin) and positive (trolox) control, while 2.500 mg/ml was below both negative and positive controls. Therefore, optimum synergistic activity could be predicted between the concentration range of 0.625 mg/ml and 2.500 mg/ml. Hence, the 2.500 mg/ml concentration had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidising lipid system.

Focusing on the statistical analysis of the *Cyclopia genistoides* extracts it is observed that concentration 1.250 mg/ml was statistically extremely significant with a p-value of < 0.001. Concentration 2.500 mg/ml was considered statistically fairly significant with a p-value of < 0.01, while concentrations 0.3125 and 0.625 mg/ml were considered to be not significant with a p-value of > 0.05.

### E.3.4 Antioxidant properties of *Cyclopia* semisolid formulations



**Figure E.4** The attenuation of lipid peroxidation by *C. maculata* cream (CM Crm) and *C. genistoides* cream (CG Crm) in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). \*\*\*p < 0.001 vs. toxin (#)

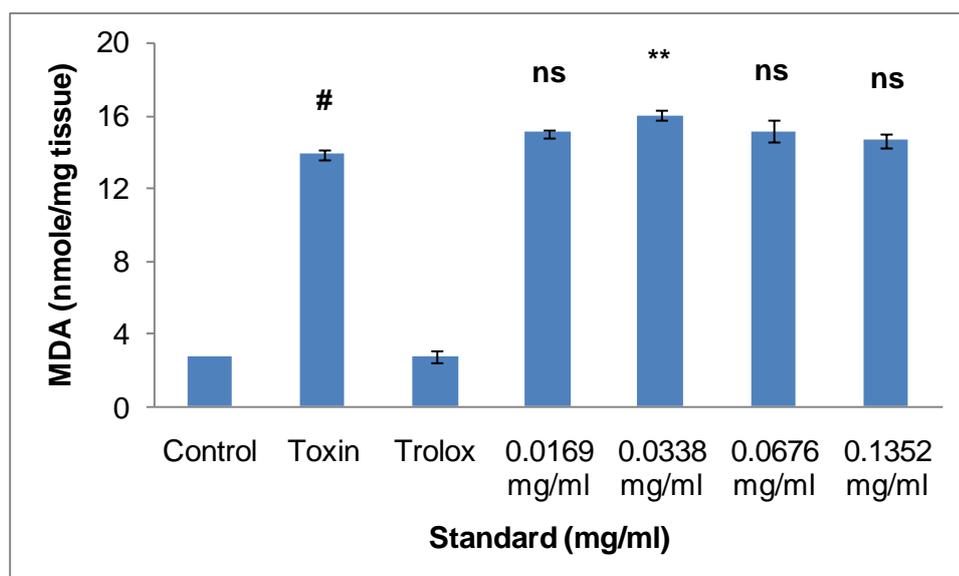
When comparing the *Cyclopia* semisolid formulations with the toxin it is evident that both the *Cyclopia genistoides* and the *Cyclopia maculata* semisolid formulation showed potential antioxidant activity by means of decreasing the MDA formation, which in turn leads to a lower degree of lipid peroxidation and higher OH<sup>•</sup> radical scavenging abilities of the formulations.

After the comparison of the *Cyclopia* semisolid formulations with trolox (8.497  $\pm$  0.254 nmole/mg) it is clear that both the semisolid formulations emitted their own antioxidant activity, as both these formulations had a MDA value below that of the trolox.

When comparing both the semisolid formulations to both toxin and trolox, it is observed that both the *Cyclopia genistoides* and *Cyclopia maculata* formulations fell below the negative- (toxin) and positive (trolox) control. Hence, the *Cyclopia genistoides* semisolid formulation had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidising lipid system.

Focusing on the statistical analysis of the *Cyclopia* semisolid formulations it is observed that both formulations were statistically extremely significant with a p-value of < 0.001.

### E.3.5 Antioxidant properties of mangiferin standard



**Figure E.5** The attenuation of lipid peroxidation by different concentrations of mangiferin in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). \*\*p < 0.01 vs. toxin (#); ns = not significant

When comparing the mangiferin standard concentrations with the toxin it is evident that extract concentrations 0.0169, 0.0338, 0.0676 and 0.1352 mg/ml did not demonstrate sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.840 \pm 0.239$  nmole/mg), in turn showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower OH<sup>•</sup> radical scavenging abilities of the extracts. When comparing the mangiferin standard concentrations with the toxin it is apparent that none of the standard concentrations showed any potential antioxidant activity by means of decreasing the MDA formation

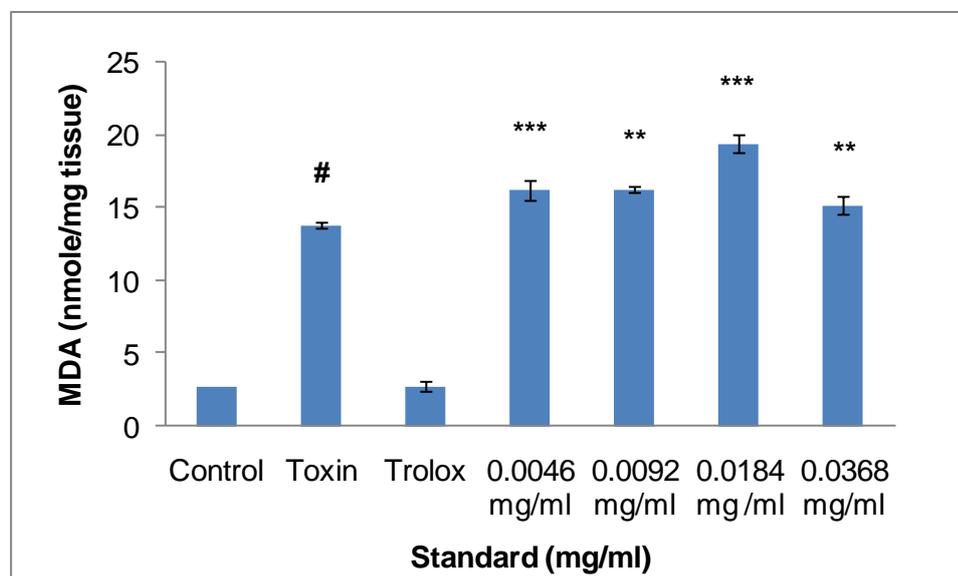
After the comparison of mangiferin standard concentrations with trolox ( $2.771 \pm 0.305$  nmole/mg) it is clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations were below trolox.

When comparing the standard concentrations to both toxin and trolox, it is observed that all four concentrations fell above the negative (toxin) and positive (trolox) control. This clearly indicates a very low potential for possible antioxidant activity as well as a low level of lipid peroxidation inhibition and OH<sup>•</sup> radical scavenging abilities of all the standards.

Focusing on the statistical analysis of the mangiferin standard concentrations, it is observed that concentration 0.0338 mg/ml was considered statistically fairly significant with a p-value of

< 0.01, while the standard concentrations 0.0169, 0.067 and 0.1352 mg/ml were considered statistically not significant with a p-value of  $p > 0.05$ .

### E.3.6 Antioxidant properties of hesperidin standard



**Figure E.6:** The attenuation of lipid peroxidation by different concentrations of hesperidin in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. ( $n = 5$ ). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. toxin (#)

When comparing the hesperidin standard concentrations with the toxin it is evident that extract concentrations 0.0046, 0.0092, 0.0184 and 0.0368 mg/ml did not exhibit sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $13.840 \pm 0.239$  nmole/mg), in turn showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicates a higher degree of lipid peroxidation and lower  $\text{OH}^\bullet$  radical scavenging abilities of the extracts. When comparing the hesperidin standard concentrations with the toxin it is apparent that none of the standard concentrations showed any potential antioxidant activity by means of decreasing the MDA formation

After the comparison of mangiferin standard concentrations with trolox ( $2.771 \pm 0.305$  nmole/mg) it is clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations was below trolox.

When comparing the standard concentrations to both toxin and trolox, it is observed that all four concentrations fell above the negative- (toxin) and positive (trolox) control. This clearly indicate a very low potential for possible antioxidant activity as well as a low level of lipid peroxidation inhibition and  $\text{OH}^\bullet$  radical scavenging abilities of the all the standards.

Focusing on the statistical analysis of the hesperidin standard concentrations, it is observed that concentrations 0.0046 and 0.0184 mg/ml were considered statistically extremely significant with a p-value of  $< 0.001$ , while the standard concentrations 0.0092 and 0.0368 mg/ml were considered statistically fairly significant with a p-value of  $p < 0.01$ .

#### **E.4 Conclusion**

Both *Cyclopia maculata* and *Cyclopia genistoides* extracts showed potential antioxidant activity as the concentrations increased above 0.6250 mg/ml. Extraction concentrations of 0.3125 mg/ml and 0.6250 mg/ml did not show sufficient *in vitro* antioxidant activity when compared to the toxin, showing an increase in MDA formation in rat brain tissue *in vitro*. This increase in 2TBA-MDA complex formation demonstrates a higher level of lipid peroxidation and the inability to effectively scavenge  $\text{OH}^\bullet$  radicals.

Lipid peroxidation, the oxidative deterioration of the polyunsaturated lipids in various substances, leads through formation of hydroperoxides to short-chain aldehydes, ketones and other oxygenated compounds, considered to be responsible for the development of ageing in living species (Botsoglou *et al*, 1994:1931). The honeybush extract concentrations between 1.250 mg/ml and 2.500 mg/ml showed the most promise for potential antioxidant activity.

Both semisolid formulations showed promising results when compared to the toxin, an oxidant. Both formulations showed potential in emitting their own antioxidant activity when compared to trolox, an antioxidant. The *Cyclopia genistoides* formulation had the lowest value in *in vitro* MDA formation in rat brain tissue and attenuated lipid peroxidation better than that of the *Cyclopia maculata* formulation, pointing towards the better in hydroxyl scavenging ability.

The standard concentrations of mangiferin and hesperidin did not show any promising *in vitro* antioxidant activity during the TBA-assay. The different standards showed an increase or equal concentrations in MDA formation in rat brain tissue *in vitro* when compared to the toxin, the oxidant. This increase in 2TBA-MDA complex formation indicates higher levels of lipid peroxidation and the  $\text{OH}^\bullet$  radical scavenging inability of the standards. None of the standards could be compared to the results obtained for trolox, an antioxidant with strong free radical scavenging abilities. Honeybush extracts contain various bioactive flavonoids that have a synergistic antioxidant effect on each other. Mangiferin and hesperidin as single entities could not emit antioxidant activity on their own. Combinations of various active ingredients within honeybush extracts in various concentrations are of absolute importance for potential antioxidant activity.

However, it is of utmost importance to keep in mind that antioxidant capacity is not accurately rated during lipid peroxidation studies. This assay is strictly based on chemical reactions and bears no resemblance to biological systems. The legitimacy of the data is restricted to a stringent chemical sense with context analysis. Any claims concerning bioactivity of a sample based exclusively on this assay would be overstated, unscientific and in isolation (Huang *et al.*, 2005:1842).

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## APPENDIX F

# CLINICAL EFFICACY OF SEMISOLID FORMULATIONS CONTAINING HONEYBUSH EXTRACTS

### F.1 Introduction

Moisturisers are the most universally used topically applied manufactured product for the management of dehydrated skin conditions (Crowther *et al.*, 2008:567). Water is crucial to skin function and the level of hydration is directly related to the vital plasticity and barrier function of the skin (Martinsen & Grimnes, 2008:87). The use of natural products in cosmetic formulations is increasing. This is due to their low mammalian toxicity and their various active ingredients used to care for the skin. Honeybush extracts could also possibly be used in skin care, such as dryness, eczema, acne, free-radical scavenging, anti-inflammatory, anti-ageing and skin protection against UV damage (Aburjai & Natsheh, 2003:987). The flavonoids present in honeybush extracts are phenolic derivatives which display a remarkable spectrum of biological activities (Chuarienthong *et al.*, 2010:100).

In this Appendix, the focus will be on the moisturising effects of two different honeybush species on the human skin. The use of non-invasive electrical instruments will determine the skin hydration, skin surface smoothness and skin erythema as a result of the application of these two formulations in human volunteers. The two semisolid formulations containing honeybush extracts are a 2% *Cyclopia maculata* cream and a 2% *Cyclopia genistoides* cream.

### F.2 Materials and Methods

#### F.2.1 Non-invasive skin measurements

##### F.2.1.1 Skin hydration

The Corneometer<sup>®</sup> CM 825 (Courage-Khazaka Electronic, Cologne, Germany) measurement is based on capacitance measurement of a dielectric medium, in this case the skin. It uses fringing field capacitance sensors to measure the dielectric constant of the skin. The dielectric constant of the skin will change with water content. This allows for any changes in skin hydration to be measured by the precision measuring capacitor. These changes in water content of the stratum corneum are converted to arbitrary units (AU). On the probe head there is a fine piece of glass to ensure that only the capacitance changes due to water content are identified. Even small changes in water can be detected. The measurement time is short at

only 1 sec minimising occlusion effects. The depth of the measurement is 10 – 20 µm, analysing the stratum corneum and ensuring that deeper skin layers do not influence the measurements (Li *et al.*, 2001:24).

#### **F.2.1.2 Skin topography**

The Visioscan® VC 98 (Courage-Khazaka Electronic, Cologne, Germany) provides the possibility to analyse skin topography. Two special halogenide lights, arranged on opposite sides, illuminate the skin uniformly. The spectrum of the light, the intensity and arrangement are chosen to monitor the stratum corneum without reflections from deeper layers in the skin. This specialised light excludes almost all undesired light reflections on the skin, showing a very sharp, non glossy image of the skin and hair. An image of skin area (6 mm x 8 mm) is taken with a built-in CCD camera. The connection of the Visioscan® VC 98 to the personal computer is possible by an image digitalisation unit which configures the image in 256 gray level pixel by pixel, where 0 resembles the colour black and 255 resembles the colour white. The topography of the captured skin image can be analysed by utilising the surface evaluation of living sels (SELS) software that generates parameters such as skin entropy, scaliness and roughness.

#### **F.2.1.3 Melanin and haemoglobin content of skin**

The Mexameter® MX 18 (Courage-Khazaka Electronic, Cologne, Germany) measures the content of melanin and haemoglobin (erythema) in the skin. These two components are largely responsible for skin pigmentation. The measurement is based on the absorption principle. The special probe of the Mexameter® MX 18 emits light of three defined wavelengths. A receiver measures the light reflected by the skin. The positions of the emitter and receiver guarantee that only diffused and scattered light is measured. As the quantity of the emitted light is defined, the quantity of light absorbed by the skin can be calculated. The melanin is measured at two wavelengths. These two wavelengths have been chosen in order to achieve different absorption rates by the melanin pigments. For the erythema measurement, two different wavelengths are used to measure the absorption capacity of the skin. One of these wavelengths corresponds to the spectral absorption peak of haemoglobin. The other wavelengths have been chosen to avoid other colour influences. The achieved results are shown on as indices on the screen on a scale from 0 – 999.

### **F.2.2 Formulations**

Three semisolid formulations were used in the study. It included a 2% *Cyclopia maculata* formulation, a 2% *Cyclopia genistoides* formulation and a placebo formulation containing no active honeybush extracts. The components of the oil phase were mineral oil (12.0%), cetyl

stearyl alcohol (7.0%), Cremophor<sup>®</sup> A 6 (1.5%), Cremophor<sup>®</sup> A 25 (1.5%) and preservatives. The water phase contained deionised water (67.56%). The third phase contained the active honeybush extracts (2.0%) dissolved in propylene glycol (8.0%).

### F.2.3 Subjects

The study has been carried out according to Helsinki declaration (Ethical principles of medical research involving human subjects), under the project title “(In vivo) Cosmetic efficacy studies” (NWU-00097-10-A5). A group of eighteen healthy female subjects between 40 and 65 years of age participated in a two-week treatment phase. A seven day washout period took place before testing started, where participants followed their normal skin cleansing routines but were only allowed to use Dove<sup>®</sup> soap that were supplied. All participants complied with both the inclusion and exclusion criteria. All subjects signed an informed consent form and participants could discontinue their participation at any time during the study (Li *et al.*, 2001:24).

### F.2.4 Treatment protocol

The treatment protocol was conducted according to a comparatively similar study performed by Li *et al.* (2002:25). The treatment sites were on both (left and right) volar forearms. Subjects were instructed to follow their normal skin cleansing routines and to refrain from any use of lotions or soaps other than Dove<sup>®</sup> soap on the volar forearms for seven days before entering the study.

On day 8 (T<sub>0</sub>) the participants visited the laboratory according to a pre-organised time schedule. Three sites of 3 x 2 cm on each arm were marked with a Codman<sup>®</sup> surgical marker. The three measurements (T<sub>0</sub>) were taken with the three instruments on the six different areas before product application and were the baseline values for each area.

During the next two weeks (14 days) each site was treated with the assigned cream. The placebo, *C. maculata* cream or *C. genistoides* cream were applied on the correct marked square twice daily, according to the randomised double blind placebo controlled study guidelines. Each subject received a treatment programme and must use the placebo, as well as the two active formulations in the morning and evening. The amount of product put on the marked areas is 1 – 3 µl/cm<sup>2</sup> (1 – 3 mg/cm<sup>2</sup>). The subjects also received a timetable to document the time they applied the cream. Creams have to be applied between 06:00 to 08:00 in the mornings and between 18:00 to 20:00 in the evenings. For measurement days, subjects refrained from applying the treatment in the morning. They did however apply treatment the evening prior to the measurements. The final measurements were made after the two week period passed (T<sub>1</sub>).

## F.2.5 Environmental conditions

All measurements were conducted under controlled temperature and humidity conditions ( $22 \pm 2$  °C and  $50 \pm 10\%$  RH) according to the guidelines for standardised hydration measurement. The subjects acclimated to the room conditions for at least 30 min before any measurements were made.

## F.2.6 Statistical analysis

All the parameters were calculated as follow:

- The difference in the various skin measurements at ( $T_1$ ) relative to the initial conditions ( $T_0$ ) and was taken as a percentage change between the measurements calculated for the placebo, *C. maculata* and *C. genistoides* treatments, respectively.

$$\%Change = \frac{T_1 - T_0}{T_0} \times 100 \quad \text{Equation F.1}$$

- The parameters were further subjected to appropriate statistical analysis to determine any significant differences between the normalised parameters for the various treatments.

## F.3 Results and Discussion

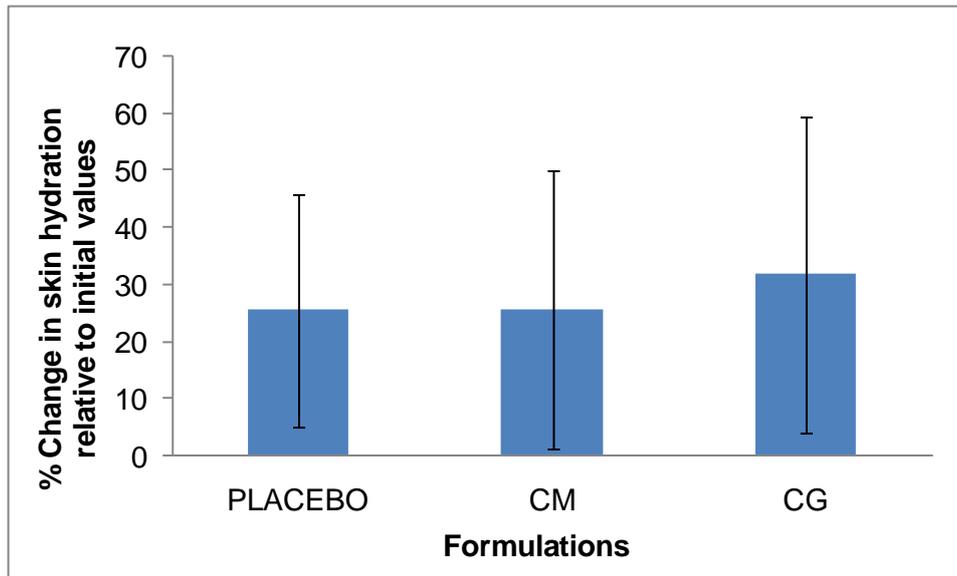
### F.3.1 Statistical analysis

Results are given as the mean  $\pm$  S.E.M. of 5 repeats ( $n = 5$ ). Data were analysed by one-way analysis of variance (ANOVA) followed by the T-test (paired two sample for means). Differences between groups were considered to be significant when  $p < 0.05$ .

### F.3.2 Skin hydration

By using the Corneometer<sup>®</sup> CM 825, it was possible to detect whether any change in skin hydration took place during the two week trial period. With the focus on skin ageing, one of the most preventative actions one can take is by the proper hydration of the skin. It is important to note that the subjects in this particular study all had moderately dry skin before testing started. Focussing on all that data, there was no significant statistical change in the skin after two weeks when formulations were compared to each other. There were, however, significant statistical differences present when creams were compared to their own performance from when the study started ( $T_0$ ) until the study was completed ( $T_1$ ), with *C. maculata* ( $p = 0.001$ ), *C. genistoides* ( $p = 0.0004$ ) and the placebo ( $p = 0.0006$ ).

The *C. genistoides* ( $31.8 \pm 27.7\%$ ) cream performed better than the placebo ( $25.5 \pm 20.4\%$ ) and the *C. maculata* cream ( $25.0 \pm 24.4\%$ ). The *C. maculata* cream as well as the placebo had a similar degree of skin hydration after the two weeks of skin treatment. The *C. genistoides* cream compared to the placebo had a slightly higher value, but had the highest variation in accuracy. The *C. genistoides* will be the cream to consider between the three formulations to have the best skin hydration effects.

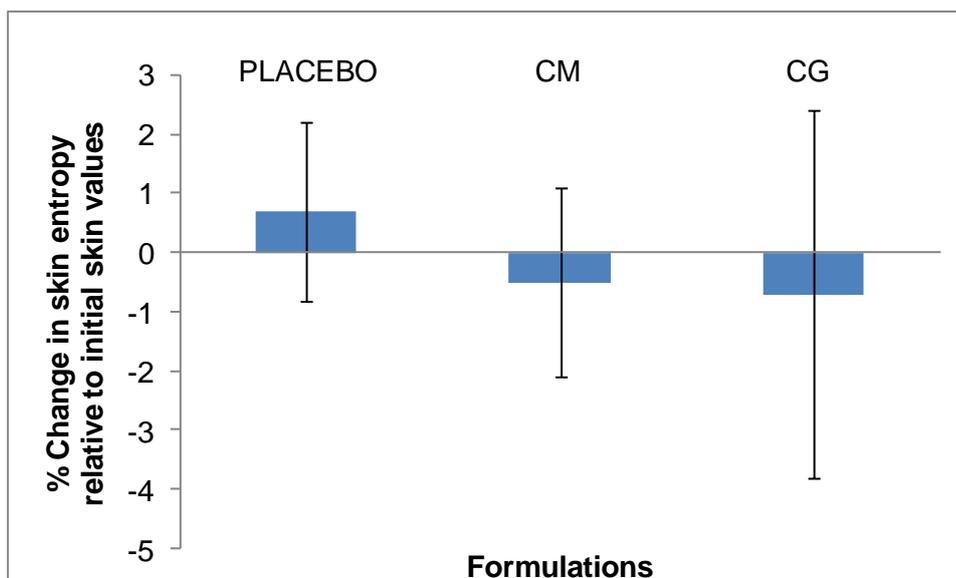


**Figure F.1:** Percentage change in skin hydration of placebo, *Cyclopia maculata* (CM) and *Cyclopia genistoides* (CG) formulations after 14 days ( $T_1$ )

### F.3.3 Skin entropy

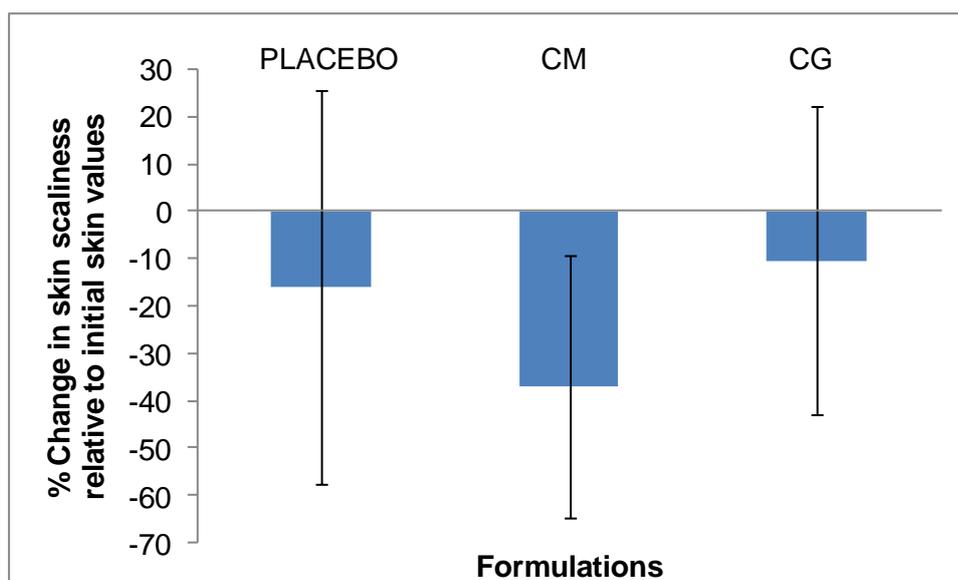
By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin entropy took place in the two week trial period. Focussing on all that data presented during the trial, there were no significant statistical differences in the skin entropy after two weeks when formulations were compared to each other as *Cyclopia genistoides* had a p-value of 0.459, *Cyclopia maculata* had a p-value of 0.251 and the placebo had a p-value of 0.134.

The results also indicated that the placebo showed an increase in skin entropy with a percentage of  $0.7 \pm 1.5\%$ . This gives the skin a higher level of order, indicating the increase in the appearance of skin smoothness. Both *Cyclopia genistoides* ( $-0.7 \pm 3.1\%$ ) and *Cyclopia maculata* ( $-0.5 \pm 1.6\%$ ) showed a decrease in skin entropy, which in turn indicates that honeybush does not have the ability to improve the appearance of a smooth skin complexion.



**Figure F.2:** Percentage change in skin entropy of placebo, *Cyclopia maculata* (CM) and *Cyclopia genistoides* (CG) formulations after 14 days ( $T_1$ )

#### F.3.4 Skin scaliness



**Figure F.3:** Percentage change in skin scaliness of placebo, *Cyclopia maculata* (CM) and *Cyclopia genistoides* (CG) formulations after 14 days ( $T_1$ )

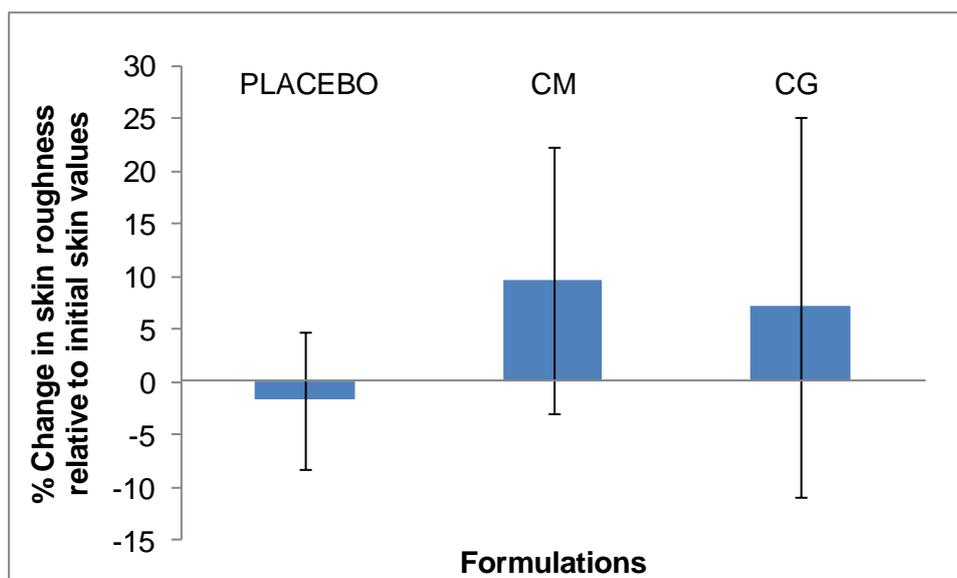
By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin scaliness took place in the two week trial period. There was a significant statistical difference in skin scaliness in subjects using the *Cyclopia maculata* formulation with a p-value of 0.002, but no significant statistical differences in skin scaliness when *Cyclopia genistoides* ( $p = 0.279$ ) and the placebo ( $p = 0.075$ ) formulations were applied.

All three formulations showed an improvement in skin scaliness after two weeks of treatment by reducing the skin's appearance of dryness. *Cyclopia maculata* ( $-36.8 \pm 27.7\%$ ) achieved the best results in improving skin scaliness, followed by placebo ( $-16.0 \pm 41.5\%$ ) and lastly *Cyclopia genistoides* ( $-10.3 \pm 32.52\%$ ). It is very important to note that variations between the subjects were very inconsistent, which may interfere with truthful conclusions.

### F.3.5 Skin roughness

By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin roughness took place in the two week trial period. Skin roughness indicates the degree of unevenness visible during analysis. There was a significant statistical difference in skin roughness in subjects using the *Cyclopia maculata* ( $p = 0.03$ ), but no significant statistical change in skin roughness when *Cyclopia genistoides* ( $p = 0.247$ ) and the placebo ( $p = 0.350$ ) was used.

Both *Cyclopia maculata* ( $9.6 \pm 12.7\%$ ) and *Cyclopia genistoides* ( $7.1 \pm 18.0\%$ ) increased roughness in the skin. The placebo ( $-1.8 \pm 6.5\%$ ) did however perform the best in reducing the skin roughness and giving the skin a more even texture.



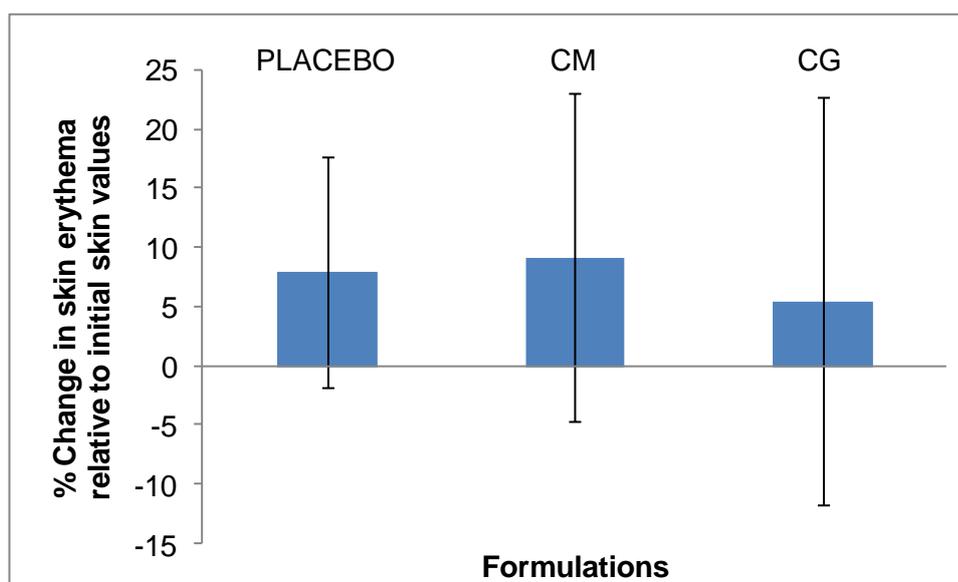
**Figure F.4:** Percentage change in skin roughness of placebo, *Cyclopia maculata* (CM) and *Cyclopia genistoides* (CG) formulations after 14 days ( $T_1$ )

### F.3.6 Skin erythema

By using the Mexameter<sup>®</sup> MX 18, it was possible to detect if any change in skin erythema took place in the two week trial period and to establish whether any skin irritations had occurred during the study period by measuring the degree of skin erythema within the skin. Focussing on all the data presented during the trial, there were no significant statistical differences in the skin

erythema after two weeks when formulations (*Cyclopia genistoides* ( $p = 0.464$ ), *Cyclopia maculata* ( $p = 0.557$ ) and the placebo ( $p = 0.785$ )) were compared to each other.

*Cyclopia genistoides* ( $5.6 \pm 17.2\%$ ), *Cyclopia maculata* ( $9.2 \pm 13.9\%$ ) and the placebo ( $8.0 \pm 9.7\%$ ), did increase the degree of erythema in the skin slightly. It is very important to note that variations between the subjects were very inconsistent, which may interfere with truthful conclusions.



**Figure F.5:** Percentage change in skin erythema of placebo, *Cyclopia maculata* (CM) and *Cyclopia genistoides* (CG) formulations after 14 days ( $T_1$ )

#### F.4 Conclusion

Numerous reasons can be attributed to these indecisive results. Three categories or sources of variation including instrumental, environmental and individual (person-linked factors) variables may interfere with measurements (Berardesca, 2011:90).

The calibration of the instruments being used to evaluate the skin must be checked repeatedly. This is to facilitate successful and consistent inter-laboratory assessment of results surmounting the outcome of the inter-instrumental variability; an added calibration method integrating a calibration for a genuine gold reference standard can be implemented. Variation may also emerge throughout the “ageing process” of the apparatus, and for that reason, the same piece of equipment can give diverse results over the years (Berardesca, 2011:90). In this study the newly purchased investigative instruments were calibrated before every testing day. In the future, more regular calibrations between skin analyses could improve variable results.

Another instrument-related variable can be the surface area and contact time during measurements (Berardesca, 2011:91). It is always desirable to evaluate the skin on a straight or horizontal plane to evade skin curving. If there is direct contact among the apparatus and the skin, the pressure of the probe on the skin exterior must always be held constant. Measuring areas should be well defined and in case of devices with small probes, uninterrupted adjacent measurements in the same skin area are recommended to decrease the standard deviation. When focusing on the contact time of the probe applied to the skin, the contact time must be as short as possible to evade occlusive effects which may possibly modify the skin surface (Berardesca, 2011:91). In this study, meticulous efforts went into taking accurate measurements. However, there is always room for improvement when it comes using exactly the same techniques on every subject without applying different pressures, holding the probe at different angles during different measurements and reading different measurements on different skin areas as to all the previous measurements. One of the difficulties experienced during this study was that the marked skin areas were inconsistent due to the fading of the marked areas. Subjects also applied the cream irregularly, covering the whole marked area, but also going outside the marked area. This can lead to lower concentrations of cream applied to the specific areas, resulting in inconsistent and variable results. Another difficulty was that readings weren't taken at the exact same skin area between the initial measurements before the study started and the final measurement at the end of the study. This can also influence the variability between readings and can result in inconclusive findings.

Environment-related variables can be a result of air convections, the ambient air temperature, the ambient air humidity, different light sources and skin cleansing (Berardesca, 2011:92). Air convections could be the most important source of interruption resulting in swift fluctuations of the measurements (Berardesca, 2011:91). Disturbances such as people moving around in the room, the opening and closing of doors, breathing across the measurement zone and air conditioning are all contributing factors to variable and inconclusive results. These disturbances are very difficult to avoid, but by using a covering box over the marked skin area, covered with cotton cloth and an open top could be the answer to more accurate readings and avoiding unnecessary air turbulence. This is particularly indicated for capacitance and other skin hydration measurements, since air flow on the skin surface can change the moisture content and the skin temperature. It is therefore very important that the temperature and relative humidity should be recorded in this particular box (Berardesca, 2011:91). The vast variability between readings in the study can definitely be an outcome of air convections present during measurements.

Ambient air temperature and relative humidity manipulate the skin temperature both directly (by convection) and indirectly (by central thermoregulatory effects) (Berardesca, 2011:92).

Variations in the temperature and relative humidity in the measuring area can influence the stratum corneum hydration. Seasonal differences must be avoided. Tests were conducted in a controlled temperature and relative humidity environment during the spring season, when climate changes are at a minimum. In elderly individuals, it has been revealed that stratum corneum lipid levels of diverse body locations were in short supply in the winter in contrast with spring and summer. Sweating in the summer and a cold feeling in the winter appear to be noticeable problems. Consequently it is unmistakable that geographical variations may also have an effect on measurements. A clear distinction must be made between the temperature of the measuring room and the climate where the volunteers live. An adjustment time of more or less 30 min is absolutely compulsory (Berardesca, 2011:92). In this study volunteers were aged between 40 and 65 years of age. Variations in age can have major variable results due to aged skin and different levels of skin hydration and elasticity.

Light sources in close proximity to the investigation site disturbing ambient air temperature, the probe temperature and the temperature of the skin's exterior of the volunteer should be avoided (Berardesca, 2011:92). Different skin cleansing routines by the volunteers could also alter the micro-environment and manipulating the skin barrier function. Contact with water or water-containing products might end in elevated water loss from the surface and stratum corneum hydration which may obstruct numerous biophysical parameters ranging from micro-texture, cell cohesion, stratum corneum mechanical properties and friction coefficient. It is also imperative to take into account that agents in cleansers may deposit on the skin surface and alter its chemical composition leading to mistakes in reading capacitance measurements (Berardesca, 2011:93). In this study no restrictions were placed on how many times per day the volunteer may be exposed to water by ways of showering and bathing. If a volunteer had a bath or shower right before measurements were taken, it can lead to immense skin variations between a volunteer who had a shower or bath the night before measurements. The use of different skin cleaning devices was not limited. Devices such as skin buffs or a wash cloth can disrupt the stratum corneum as well as the level of hydration.

Individual-related variables include the age, sex, race, anatomical sites, sweating, skin surface temperature, skin damage or diseases and lastly, intra-and inter-individual variation (Berardesca, 2011:95). Age, sex and race are very important variables that can affect the skin function and biophysical measurements. Ageing skin is characterised by the alteration in water content, corneocyte size and the loss in mechanical function. As a result, all these variables must be controlled or standardised when planning a product efficiency study. Studies must be planned within the similar racial groups, age range and gender, except when the rationale of the trial itself is to emphasize these dissimilarities (Berardesca, 2011:93). In this study female, Caucasian subjects between the ages of 40 and 65 years were used to minimise variation.

Variations in anatomical sites differ widely. Skin thickness and connective tissue vary tremendously according to the different sites of the body. Therefore, the structural organisation of the dermis of the face, scalp, back, forearm, legs, palms and soles differs greatly from site to site. Dermal thickness decreases with age and is susceptible to endocrine influences. Measurements taken on the volar forearms can show a significant higher transepidermal water loss (TEWL) on the dominant forearm than the one on the non-dominant forearm (Rodrigues & Pereira, 1998:135), but is not confirmed by all investigators. The volar forearm is representative of the face for measuring skin hydration and biomechanical properties, and relevant for the assessment of the efficacy of cosmetic products destined for facial use (Bazin & Fanchon, 2006:453).

Physical, thermal and emotional sweating are essential variables which need to be controlled (Shahidullah *et al.*, 1969:722). A pre-measurement of the skin temperature after 30 min in the temperature-controlled room should be taken into account. This is of particular significance for instruments measuring blood flow, skin colour (erythema) and thermography. Physical activity must be kept to a minimum to avoid excessive body heat. It must be mentioned that it is not possible to manage insensible perspiration (Berardesca, 2011:94-95). In this study, no pre-measurements of skin temperature were made. This can also be one of the explanations for the variations in the results.

Lastly, intra- and inter-individual variations occur and are also dependent on the measuring device (Oestmann *et al.*, 1993:130). Certain skin sites, including some parts of the forehead, the palm of the hand and wrist should be avoided because of their very high inter-individual variability (Berardesca, 2011:95). In this study, it was clearly seen in the results that there was great inter- and intra-individual variability between the left and right volar forearms, but also in the same forearm in different skin sites where creams were applied in specified areas. The areas nearest to the wrist showed very inconsistent results. This proves that more care should be taken in selecting skin sites in the future to prevent these variabilities.

Once again, the immense variation between the participants had a definite influence on the results. Not only did the results have great variability between different participants, but also great variation between the left and right arm of each individual, making accurate predictions very difficult.

In future greater care needs to go into applying the cream only in clearly marked areas, avoiding the wrist area completely. Great variations between the different skin areas as well as in the dominant and non-dominant arms must be reduced to a minimum. Taking better care in measurement techniques and environment changes will also ensure more accurate results.

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## APPENDIX G

# SKIN PHARMACOLOGY AND PHYSIOLOGY: GUIDE FOR AUTHORS

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4. Short Communications
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Authors are required to disclose any sponsorship or funding arrangements relating to their research and all authors should disclose any possible conflicts of interest. Conflict of interest statements will be published at the end of the article.

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**Full address:** The exact postal address of the corresponding author complete with postal code must be given at the bottom of the title page. Please also supply phone and fax numbers, as well as e-mail address.

**Key words:** Please supply 3–10 key words in English that reflect the content of the paper.

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**Footnotes:** Avoid footnotes.

**Tables and illustrations:** Tables and illustrations (both numbered in Arabic numerals) should be sent in separate files. Tables require a heading and figures a legend, also in a separate file. Due to technical reasons, figures with a screen background should not be submitted. When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm).

Black and white half-tone and color illustrations must have a final resolution of 300 dpi after scaling, line drawings one of 800–1,200 dpi.

## G.6 Color Illustrations

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In the text, identify references by Arabic numerals [in square brackets]. Material submitted for publication but not yet accepted should be noted as ‘unpublished data’ and not be included in the reference list. The list of references should include only those publications which are cited in the text. Do not alphabetize; number references in the order in which they are first mentioned in the text. The surnames of the authors followed by initials should be given. There should be no punctuation other than a comma to separate the authors. Preferably, please cite all authors. Abbreviate journal names according to the Index Medicus system. Also see International Committee of Medical Journal Editors: Uniform requirements for manuscripts submitted to biomedical journals ([www.icmje.org](http://www.icmje.org)).

### *Examples*

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Chatel J-M, Bernard H, Orson FM: Isolation and characterization of two complete Ara h 2 isoforms cDNA. *Int Arch Allergy Immunol* 2003;131:14–18.

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Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. *Int Arch Allergy Immunol* DOI: 10.1159/000063858.

(c) *Monographs: Matthews DE, Farewell VT:*

Using and Understanding Medical Statistics, ed 3, revised. Basel, Karger, 1996.

(d) *Edited books: Parren PWHI, Burton DR:*

DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, Dubois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124–137.

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