

**Characterisation, toxicology and clinical effects of crocodile oil in skin products**

by

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## ABSTRACT

Natural oils are regularly used in cosmetics and as treatment for numeral skin conditions (Nielsen, 2006:575). The natural products industry is a multibillion dollar industry and has grown tremendously over the past few years. Natural oils used in cosmetics contain a range of fatty acids which contribute to several valuable properties in cosmetic\_ and personal care products. Fatty acids are divided into saturated acids and unsaturated acids (Vermaak *et al.*, 2011:920,922).

Because of the popularity and wide diversity of skin care products, it is necessary to create products that will distinguish themselves from the rest of the commercial products. To include natural oils in skin care products is a new way to prevent skin ageing, as well as other dermatological conditions. In this study, a natural oil, namely crocodile oil was used.

Crocodile oil is obtained from the fat of the Nile crocodile (*Crocodylus niloticus*). Crocodile oil has the same composition as human skin oil. It only differs with regard to the percentages of the ingredients present. Crocodile oil contains saturated and unsaturated fatty acids. Because of the similar composition as human skin oil, crocodile oil will rarely be allergenic when applied to human skin and therefore will be a very accepted and harmless product to use (Croc city, 2012).

There are many claims of positive results when crocodile oil containing products have been used. It includes fading of freckles, treatment of acne and pimple marks, dark lines, wrinkles and laugh lines. It also includes vanishing of dark shadows, sun spots and other discolorations. It helps prevent discolorations from forming and makes the skin softer, brighter and more attractive. It also controls rashness and dryness (Croc city, 2012).

Because of crocodile oil's anti-ageing, anti-fungal and anti-bacterial effects claimed by crocodile oil suppliers, and due to the fact that little scientific data is available on crocodile oil, it was decided to investigate the claims.

In this study, the aims and objectives were to use natural oil, namely crocodile oil, and investigate the fatty acid profile, anti-microbial and anti-fungal activity, anti-oxidant activity, toxicity studies, stability determination of crocodile oil lotion and clinical efficacy testing of the anti-ageing effects.

To determine the fatty acid profile of crocodile oil, fatty acid methyl ester (FAME) analysis with gas chromatography were used. Identification of FAME peaks in the samples was made by comparing the relative retention times of FAME peaks from samples to those of reference

standards. The composition of fatty acids in crocodile oil compared well to fatty acids found in human skin oil.

Anti-microbial and anti-fungal tests were done by Envirocare Laboratories, North-West University, Potchefstroom. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Brasiliensis*, *Propionibacterium acnes* and *Trichophyton rubrum* cultures were used to determine the anti-microbial and anti-fungal activity of crocodile oil. Unfortunately no activity was observed.

The anti-oxidant properties of crocodile oil and crocodile oil lotion were determined by using the most commonly used method for measuring Malondialdehyde (MDA) in biological samples, namely the thiobarbituric acid (TBA) test. This method is based on spectrophotometric quantification of the pink complex formed after reaction of MDA with two molecules of TBA. No anti-oxidant activity was observed in the oil or the lotion.

Toxicity studies were performed by Dr. D. Goosen (BVSc Hons. Pret.) from Tswane University of Technology (Pretoria, South Africa). The studies showed that the lotion had no toxicity in the skin sensitisation, acute dermal toxicity and acute dermal irritation studies.

To determine the stability of the crocodile oil lotion, the formulated products were stored at 25 °C / 60% RH (relative humidity), 30 °C / 60% RH and 40 °C / 75% RH for 6 months in the original packaging as well as a glass container. The stability tests included pH, viscosity, visual appearance assessment, zeta-potential, droplet size and mass loss. The crocodile cream lotion was stable over the 6 months period in both containers.

Clinical efficacy testing was performed at the CEL (Clinical Efficacy Laboratory) of the North-West University, Potchefstroom, South Africa. A short-term study over a period of 3 h was performed to investigate the hydrating effects of crocodile oil lotion. A long-term study over a period of 12 weeks was performed to examine the anti-ageing effects of crocodile oil lotion. An erythema study was also conducted to test the anti-erythema properties of crocodile oil lotion. Although the crocodile oil lotion as well as the placebo lotion showed an increase in skin hydration, there was no significant difference between the two treatments. Crocodile oil lotion also showed no anti-erythema properties.

Keywords: natural oils, crocodile oil, stability testing, anti-oxidant, clinical efficacy, toxicity testing

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## UITTREKSEL

Natuurlike olies word gereeld gebruik in kosmetiese produkte en as behandeling van verskeie veltoestande (Nielsen, 2006:575). Die natuurlike produkte bedryf is 'n multi-biljoen dollar industrie en het die laaste paar jaar geweldig gegroei. Natuurlike olies wat in kosmetiese produkte gebruik word bevat 'n verskeidenheid vetsure wat 'n positiewe bydrae maak tot die voordelige eienskappe in kosmetiese en persoonlike sorg produkte. Vetsure word onderverdeel in versadigde en onversadigde vetsure (Vermaak *et al.*, 2011:920,922).

As gevolg van die gewildheid en wye verskeidenheid van velsorgprodukte, is dit nodig om produkte te ontwikkel wat hulle sal onderskei van die ander produkte op die mark. Om natuurlike olies in velsorgprodukte in te sluit is 'n nuwe manier om veroudering en ander dermatologiese toestande te voorkom. In hierdie studie is 'n natuurlike olie, naamlik krokodil olie, gebruik.

Krokodil olie word verkry van die vet van die Nyl krokodil (*Crocodylus niloticus*). Krokodil olie het dieselfde samestelling as menslike vel olie, behalwe dat die persentasie van die bestanddele verskil. Krokodil olie bevat versadigde en onversadigde vetsure. Omdat die samestelling baie ooreenstem met dié van menslike vel olie, behoort krokodil olie selde allergenies te wees wanneer dit aan die vel blootgestel word. Daarom is krokodil olie 'n baie aanvaarbare en veilige produk om te gebruik (Croc city, 2012).

Daar is talle aannames van positiewe resultate wanneer krokodil olie-bevattende produkte gebruik word. Dit sluit in die verligting van sproete, behandeling van aknee, donker lyne, plooië en laglyntjies. Dit sluit ook die verdwyning van donker kolle, sonskade kolle en ander verkleurings in. Krokodil olie help om die vel sagter, helderder en meer aantreklik te maak. Dit beheer ook veluitslae en droogheid (Croc city, 2012).

As gevolg van krokodil olie se anti-veroudering – , anti-fungale – en anti-bakteriële werking wat deur krokodil olie verskaffers beweer word, en as gevolg van die feit dat min data oor krokodil olie beskikbaar is, is daar besluit om die aannames te ondersoek.

Die doel van hierdie studie was om 'n natuurlike olie, naamlik krokodil olie, se vetsuurprofiel, anti-fungale werking en anti-bakteriële werking, anti-oksidadant aktiwiteit, toksisiteit studies, stabiliteitsbepaling en kliniese effektiwiteit te bepaal.

Om die vetsuurprofiel van krokodil olie te bepaal, is vetsuur metiel ester analise met gas chromatografie gebruik. Die identifikasie van die pieke in die monsters is bepaal deur dit te vergelyk met die relatiewe retensietye van die pieke van monsters met verwysings standaarde.

Die inhoud van die vetsure van krokodil olie het goed ooreengestem met die vetsure wat in menslike vel olie aangetref word.

Anti-bakteriese en anti-fungale aktiwiteit van krokodil olie en krokodil olie room is bepaal by Envirocare Laboratoriums, Noord-Wes Universiteit, Potchefstroom. *Staphylococcus aureus*, *Esterichia coli*, *Pseudomanas aeruginosa*, *Candida albicans*, *Brasiliensis*, *Propionibacterium acnes* en *Trichophyton rubrum* kulture is gebruik om die aktiwiteit te bepaal. Ongelukkig is geen aktiwiteit waargeneem nie.

Die anti-oksidadant eienskappe van krokodil olie en krokodil olie room is bepaal deur die algemeenste metode, naamlik die tio-barbituraatsuur toets te gebruik om Malondialdehyd (MDA) in biologiese monsters te toets. Hierdie metode berus op die spektrofotometriese bepaling van die pienk kompleks wat gevorm word na die reaksie van MDA met twee molekule van TBA. Geen anti-oksidadant aktiwiteit is by die krokodil olie of krokodil olie room waargeneem nie.

Toksisiteit studies is gedoen by die Tswane Universiteit van Tegnologie (Pretoria, Suid-Afrika) deur Dr. D. Goosen (BVSc Hons. Pret.). Die studies het getoon dat krokodil olie room geen toksiese effekte het wanneer dit aan die vel blootgestel word nie.

Om die stabiliteit van krokodil olie room te bepaal, is die geformuleerde produk vir 6 maande in die oorspronklike verpakking en 'n glas verpakking by 25 °C / 60% RH (relatiewe humiditeit), 30 °C / 60% RH en 40 °C / 75% RH gestoor. Die stabiliteitstoetse het pH, viskositeit, visuele voorkoms bepaling, zeta potensiaal, deeltjie grootte en massa verlies ingesluit. Die krokodil olie room in albei verpakings was stabiel oor 6 maande.

Kliniese effektiwiteit bepaling is uitgevoer by die CEL (Kliniese effektiwiteit Laboratorium) van die Noord-Wes Universiteit, Potchefstroom, Suid- Afrika. 'n Korttermynstudie is oor 'n periode van 3 ure uitgevoer om die hidrerende effekte van krokodil olie room te bepaal. 'n Langtermynstudie is oor 'n periode van 12 weke uitgevoer om die anti-verouderings effekte te ondersoek. 'n Eriteemstudie is ook uitgevoer om die krokodil olie room se anti-eriteem eienskappe te bepaal. Alhoewel die krokodil olie room sowel as die plasebo room die vel se hidrase verhoog het, was daar geen statistiese verskil tussen die twee behandelings nie. Krokodil olie room het ook geen anti-eriteem eienskappe getoon nie.

Sleutelwoorde: natuurlike olies, krokodil olie, stabiliteitstoetse, anti-oksidadant, kliniese effektiwiteit, toksisiteit bepaling

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## FOREWORD

In this study we aimed at investigating the characterisation, toxicology and clinical effects of crocodile oil in skincare products. Crocodile oil is obtained from the fat of the Nile Crocodile and has the same composition as human skin oil - it only differs with regard to the percentages of the ingredients present. Because of the similar composition as human skin oil, crocodile oil will rarely be allergenic when applied to human skin and therefore will be a very accepted and safe product to use.

This thesis is presented in the so-called article format, which includes introductory chapters and full length articles for publication in a pharmaceutical journal. The data procured during the studies are attached in the appendices. The articles in this thesis are to be submitted for publication in *Molecules*, *Skin Pharmacology and Physiology* and *Journal of Natural Medicines* of which the complete guides for authors are included in Appendices G-I.

My PhD study was a great and unforgettable journey. I've gained countless experience in my field of study as well as other aspects of life. I am looking forward to the future and upcoming new chapters.

## CHAPTER 1

### INTRODUCTION AND STATEMENT OF THE PROBLEM

The treatment of ageing skin has become very popular over the last decade. Ageing skin is characterised by wrinkles, sagging skin and decreased laxicity (Jenkins, 2002:801). The skin is a continuous external sheet that covers the body. Due to its outside visibility and aesthetic value, people tend to give a lot of attention to skin (Boissieux *et al.*, 2000:15). Because of the wide awareness of both women and men to prevent skin ageing, the use of anti-ageing products has become very well-established.

Over the last few decades, the popularity and variety of anti-ageing products has grown immensely. Due to the growth in recognition and variety, it is necessary to create products that are unique in every possible way.

Natural oils are extensively used in cosmetics and as treatment for a growing number of conditions (Nielsen, 2006:575). According to Vermaak *et al.* (2011:920,922) the natural products industry is a multibillion dollar industry and has grown enormously in the past few years. Natural oils used in cosmetics, contain a range of fatty acids which contribute to several beneficial properties in cosmetic and personal care products. Natural oils mainly contain fatty acids. The unsaturated fatty acids namely omega-3, -6, -7 and -9 are responsible for the positive effects on human skin (Croc city, 2012).

Fatty acids are divided into saturated and unsaturated acids. Fatty acids are usually insoluble in water and are sometimes referred to as fixed oils or fats (Vermaak *et al.*, 2011:922). Fatty acids are very important as formulation agents and vehicles in pharmacy and as components of cosmetics and soaps. The most common fatty acids include omega-3 and omega-6 fatty acids. Common oils used in oral and topical formulations include cocoa, olive, almond and coconut oils (Heinrich *et al.*, 2004:65). The use of animal oils also increased over the past few years, and some of the oils from animal origin are described in Chapter 2. It includes crocodile -, emu - and fish oil.

Crocodile oil has the same composition as human skin oil. It only differs with regard to the percentages of the ingredients present. Because of the similar composition as human skin oil, crocodile oil will rarely be allergenic when applied to human skin and therefore will be a very accepted and safe product to use (Croc city, 2012).

Crocodile oil and crocodile oil containing products currently on the market, are used for the following (Croc city, 2012):

- Treatment of dermatitis
- Treatment of scrapes, acne, razor bumps, bed sores, haemorrhoids and anal fissures
- Alleviation of pain and inflammation of arthritic conditions
- Treatment of discolorations and pigmentation of skin-like brown spots, freckles and menopausal darkening
- Treatment of dry, flaky, itchy and flocking skin (like in ageing), nappy rash, athlete's feet, jock-itch and irritation of head skin

There are many claims of positive results when crocodile oil containing products have been used. It includes fading of freckles, acne, pimple marks, dark lines, wrinkles and laugh lines. It also includes vanishing of uneven dark tones, dark shadows, sun spots and other discolorations. It helps prevent discolorations from forming and makes the skin softer, brighter and more attractive. It also controls rashness and dryness (Croc city, 2012).

Because of the very positive claims of crocodile oil, as described above, and because of the fact that no confirmation could be found in literature that any thorough scientific study has been done before, it was decided to investigate the characteristics, toxicology and clinical effects of crocodile oil and crocodile oil lotion by looking at the following:

- fatty acid profile of crocodile oil;
- anti-microbial and anti-fungal activity of crocodile oil and crocodile oil lotion;
- anti-oxidant activity of crocodile oil and crocodile oil lotion;
- toxicity studies of crocodile oil;
- stability determination of crocodile oil lotion and
- clinical efficacy testing in human volunteers of anti-ageing effects.

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## CHAPTER 2

# NATURAL OILS FOR THE TREATMENT OF SKIN CONDITIONS

### 2.1 Introduction

Natural oils are extensively used in cosmetics and as treatment for a growing number of conditions (Nielsen, 2006:575). According to Vermaak *et al.* (2011:920) the natural products industry is a multibillion dollar industry and has grown enormously over the past few years.

Oils extracted from plant sources have a rich history of use by local people as a source of food, energy, medicine and for cosmetic applications. It has been used in the production of lubricants, soaps and personal care products, as well as in the topical treatment of various conditions such as hair dandruff, muscle spasms, varicose veins and wounds. In recent years, the demand for seed oils as ingredients in cosmetics has greatly increased as the industry has been seeking for natural alternatives (Vermaak *et al.*, 2011:920).

Natural oils used in cosmetics contain a range of fatty acids which contribute to several beneficial properties in cosmetic and personal care products. Fatty acids are divided into saturated acids and unsaturated acids (Vermaak *et al.*, 2011:922). Fatty acids are usually insoluble in water and are sometimes referred to as fixed oils or fats. Fatty acids are very important as formulation agents and vehicles in pharmacy and as components of cosmetics and soaps (Heinrich *et al.*, 2004:65). The most common fatty acids include omega-3 and omega-6 fatty acids.

The omega-3 and -6 fatty acids are naturally occurring lipids, appearing in high concentrations in certain fish, particularly in coldwater and oily species, and plants such as flax seed oil (Stoll *et al.*, 1999:332). Other natural oils discussed in this chapter include fish oil, crocodile oil, emu oil and essential oils like tea tree oil, olive oil, avocado oil, marula oil, grapeseed oil and coconut oil.

Essential oils may be acceptable natural alternatives to synthetic skin penetration enhancers. Essential oils may also be considered as potential natural antioxidants and could perhaps be formulated as a part of daily supplements or additives to prevent oxidative stress that contributes to many degenerative diseases, including ageing (Edris, 2007:314,315). According to Edris (2007:309), essential oils can also be used in the treatment of cancer, cardiovascular diseases including atherosclerosis, thrombosis and diabetes. It is also an antiviral and

antibacterial agent and can be used in aromatherapy and massage therapy. Essential oils are known for their medicinal properties and are used in embalment, preservation of foods and as analgesic, sedative, anti-inflammatory, spasmolytic and local anaesthetic remedies (Bakkali *et al.*, 2007:447).

In this chapter the treatment of skin conditions including ageing, acne, psoriasis, eczema and bacterial and fungal infections with natural oils are discussed.

## **2.2 The structure and functions of human skin**

According to Menon (2002:3) and DeBenedictis *et al.* (2001:573), the skin is the largest organ in the human body and covers approximately 1.5 to 2.0 m<sup>2</sup> of the average human's body surface. It is the heaviest and most versatile organ of the body by representing almost 16% of a human's total body weight (Sanders *et al.*, 1999:168). The skin is a protective barrier with immunological and sensory functions (Foldvari, 2000:417), that provides a multifunctional interface between us and our surroundings (Naik *et al.*, 2000:318). It plays a very important role in thermo-regulation and performs endocrine functions like vitamin D synthesis and peripheral conversion of prohormones (Menon, 2002:4).

The skin consists of two layers, namely the epidermis and the dermis. The epidermis contains numerous nerve endings but is without blood or lymphatic vessels. It is approximately 0.1 mm thick, except on the palm and sole, where its thickness can exceed 1 mm (Fornage, 1995: 174). The epidermis is a self-renewing, stratified epithelium that functions as the interface between the human body and outer environment. The epidermis protects against mechanical, chemical and microbial attacks and functions as a permeability barrier by preventing water loss from the dermis. The epidermis also has immunological functions and provides some protection to the skin from ultraviolet (UV) light via the pigment system (Wickett & Visscher, 2006:98). The stratified epidermis is divided into four distinct layers namely, the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Venus *et al.*, 2011:471).

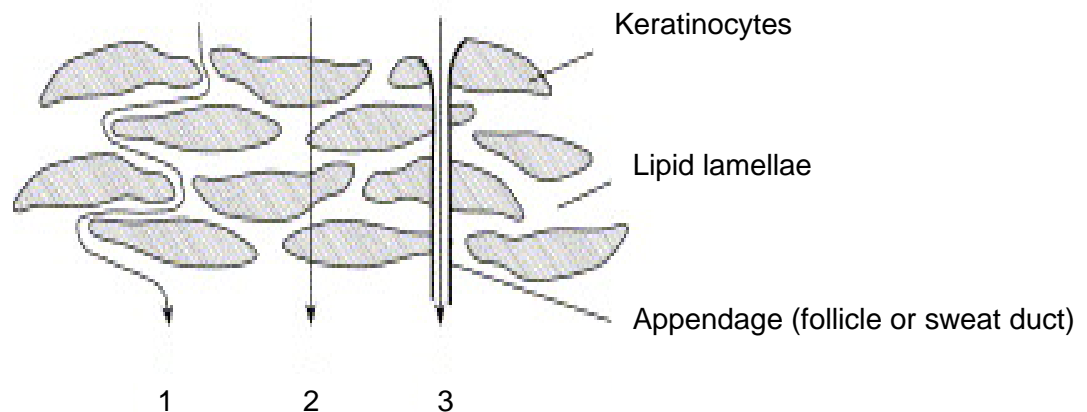
The stratum corneum is the outermost portion of the epidermis and provides a protective barrier that limits the penetration of topical contaminants and prevents dehydration of the underlying tissue (Kuempel *et al.*, 1998:135). It is the definitive boundary or frontier structure that sharply separates the body's vulnerable organs and tissues from the variable and sometimes hazardous outside world (Bernard *et al.*, 2007:1317). Physically, the stratum corneum consists of an array of flat, multilateral, keratin-filled cells embedded in a matrix of lamellar lipids (Kuempel *et al.*, 1998:135). In this two-compartment system the only continuous phase is the intercellular domain which seems to be the major rate-determining pathway by which most

drugs cross the stratum corneum (Moghimi *et al.*, 1996:103). Lipids present in the stratum corneum originate from a mixture of polar and neutral lipids, typical of other tissues, which are replaced by more nonpolar mixtures, including ceramides, free sterol and free fatty acids, as well as variable amounts of triglycerides, sterols, esters and other nonpolar compounds depending on race, age and gender (Bernard *et al.*, 2007:1317).

The dermis is directly adjacent to the epidermis and provides the mechanical support for the skin (Bouwstra *et al.*, 2003:2). It is a tough, resilient layer that protects the body against mechanical injury and contains specialised structures (Venus *et al.*, 2011:471). The dermis is largely acellular, but is rich in blood vessels, lymphatic vessels and nerve endings. Hair follicles, sebaceous glands and sweat glands are found in the dermis and might serve as additional but limited pathways for drug absorption (Foldvari, 2000:418).

### 2.3 Routes of transport through human skin

Based on the physiology of the skin (as seen in Figure 2.1), three possible pathways exist for passive transport of chemicals through the skin to the vascular network.



**Figure 2.1:** 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 (Ho, 2003:50).

According to Hadgraft (2004:292), there has been much debate over the past decades regarding the route of penetration of drugs through the skin, but experimental evidence suggests that under normal circumstances, the predominant route is through the intercellular spaces. Percutaneous absorption of pharmaceuticals for either systemic or local delivery is a desirable process and can be attained by the combination of appropriate solute properties for skin transport with appropriate dosage form designs. Compounds have been applied to the skin

for many centuries, and drugs in the form of plant or animal extracts have been applied for the relief of a variety of local disorders (Roberts *et al.*, 2002:89). Benefits of transdermal drug delivery systems have emerged over the past years as technologies have evolved. These include the potential for sustained release which is useful for drugs with short biological half-lives, requiring frequent oral or parenteral administration and controlled input kinetics, which are particularly indispensable for drugs with narrow therapeutic indices (Naik *et al.*, 2000:319).

However, transdermal drug delivery has several limitations. Because of the highly organised structure of the stratum corneum, it is the major permeability barrier to external materials, and is regarded as the rate-limiting factor in the penetration of therapeutic agents through the skin (Foldvari, 2000:418). Because of the challenge to produce a systemic effect through transdermal drug delivery, penetration enhancers are needed.

Penetration enhancers promote drug diffusion by disturbing the structure of the stratum corneum and/or deeper layers. The specific mechanism can fall into one of three categories: (1) disruption of the highly ordered structure of intercellular lipid channels, (2) interaction with corneocyte intracellular protein components, and (3) enhanced partitioning of the drug in the presence or absence of the enhancer compound (Foldvari, 2000:419). According to Barry (1983:160) clinical investigators and chemical warfare experts suggested that there are substances which could temporarily diminish the impermeability of the skin. Such materials, if they are safe and non-toxic, could be used in dermatology to enhance the penetration rate of drugs and even to treat patients systemically by means of the dermal route.

According to Naik *et al.* (2000:321) the most extensively investigated enhancement strategy, involves the use of chemicals that can reversibly compromise the skin's barrier function and consequently allow the entry of otherwise poorly penetrating molecules into the membrane and through to the systemic circulation. Most chemical enhancers affect the intercellular lipid bilayers in the stratum corneum. This creates various types of "openings" in the bilayers. The nature of these "openings" can vary. It can be triggering of a thermodynamic imbalance within the lipid domains leading to increased lipid fluidity or creation of actual microscopically visual pores (Dayan, 2007:37).

## **2.4 Fatty acids**

Crocodile oil and other natural oils mainly contain saturated and unsaturated fatty acids. The unsaturated fatty acids namely omega-3, -6, -7 and -9 are responsible for the positive effects on human skin (Croccity, 2012).

Fatty acids have an even number of carbon atoms, in the range of 16-26. Fatty acids with only single bonds between adjacent carbon atoms are referred to as saturated, whereas those with at least one C=C double bond are called unsaturated. The polyunsaturated fatty acids have two or more double bonds and are named according to the position of these bonds and the total chain length. For example, docosahexaenoic acid (DHA; 22:6) is an omega-3 (n-3) fatty acid with 22 carbon atoms and 6 double bonds. The term 'n-3' indicates that, counting from the methyl (CH<sub>3</sub>) end of the molecule, the first double bonds are located between the third and fourth carbons. As the degree of unsaturation in fatty acids increases, the melting point decreases which confers the attribute of fluidity on n-3 polyunsaturated fatty acids (Ruxton *et al.*, 2004:450).

**Table 2.1:** Common fatty acids used in cosmetic products

Common name	Carbon atoms	Double bonds	Scientific name	Sources
Butyric acid	4	0	butanoic acid	butterfat
Caproic acid	6	0	hexanoic acid	butterfat
Caprylic acid	8	0	octanoic acid	coconut oil
Capric acid	10	0	decanoic acid	coconut oil
Lauric acid	12	0	dodecanoic acid	coconut oil
Myristic acid	14	0	tetradecanoic acid	palm kernel oil
Palmitic acid	16	0	hexadecanoic acid	palm oil
Palmitoleic acid	16	1	9-hexadecenoic acid	animal fats
Stearic acid	18	0	octadecanoic acid	animal fats
Oleic acid	18	1	9-octadecenoic acid	olive oil
Ricinoleic acid	18	1	12-hydroxy-9-octadecenoic acid	castor oil
Vaccenic acid	18	1	11-octadecenoic acid	butterfat
Linoleic acid	18	2	9,12-octadecadienoic acid	grape seed oil
Alpha-linolenic acid (ALA)	18	3	9,12,15-octadecatrienoic acid	flaxseed (linseed) oil
Gamma-linolenic acid (GLA)	18	3	6,9,12-octadecatrienoic acid	borage oil
Arachidic acid	20	0	eicosanoic acid	peanut oil, fish oil
Gadoleic acid	20	1	9-eicosenoic acid	fish oil
Arachidonic acid (AA)	20	4	5,8,11,14-eicosatetraenoic acid	liver fats
Eicosapentanoic acid (EPA)	20	5	5,8,11,14,17-eicosapentaenoic acid	fish oil
Behenic acid	22	0	docosanoic acid	grapeseed oil

Erucic acid	22	1	13-docosenoic acid	grapeseed oil
DHA	22	6	4,7,10,13,16,19-docosahexaenoic acid	fish oil
Lignoceric acid	24	0	tetracosanoic acid	small amounts in most fats

Fatty acids are usually insoluble in water and are sometimes referred to as fixed oils or fats (Heinrich *et al.*, 2004:65). Fatty acids consist of the elements carbon (C), hydrogen (H) and oxygen (O) arranged as a carbon chain skeleton with a carboxyl group (-COOH) at one end. Saturated fatty acids have all the hydrogen that the carbon atoms can hold, and therefore, have no double bonds between the carbons (Zamora, 2005). The three most common saturated fatty acids (myristic, palmitic and stearic acids) differ in two methylene groups. The unsaturated fatty acids contain a varying number of double bonds. This, together with the length of the carbon chain, is indicated after the name of the fatty acid. The polyunsaturated fatty acids contain three or more double bonds and are particularly beneficial in the diet as antioxidants (Heinrich *et al.*, 2004:65). Table 2.1 gives the chemical name and descriptions of some common fatty acids (Zamora, 2005).

Triglycerides are the main constituents of vegetable oils and animal fats. A triglyceride (also called triacylglycerol) is a chemical compound formed from one molecule of glycerol and three fatty acids (Zamora, 2005).

Omega 3 and 6 fatty acids are two very common unsaturated essential fatty acids and are discussed below.

#### **2.4.1 Omega-3 fatty acids**

The omega-3 fatty acids (also known as 'n-3' fatty acids) are a group of naturally occurring lipids, appearing in high concentrations in certain fish, particularly in coldwater and oily species, and plants such as flax seed oil, perilla oil and others (Stoll *et al.*, 1999:332). Omega-3 fatty acids are long-chain, polyunsaturated fatty acids (PUFAs). Unlike saturated fats, which have been shown to have negative health consequences, omega-3 fatty acids are PUFAs that have been associated with many health benefits (Freeman, 2000:159). There are three predominant naturally occurring omega-3 fatty acids: DHA, eicosapentanoic acid (EPA) and  $\alpha$ -linolenic acid (Stoll *et al.*, 1999:332). Linolenic acid is an omega-3 fatty acid found in plants (Freeman, 2000:159).

Omega-3 fatty acids are polyunsaturated; with their first double bond exactly 3 carbons from the lipophilic end of the molecule. A series of double bonds recur every third carbon atom. The

presence of multiple double bonds in the carbon chain produces a more highly folded molecule than more saturated fatty acids. In addition, the melting point of the omega-3 fatty acids is much lower than for most saturated fatty acids, which explains why membranes containing a high content of omega-3 fatty acids may be more fluid at a given body temperature, when compared to membranes comprised of more saturated fatty acids. The major difference among the different omega-3 fatty acids is the length of the carbon chain and the number of double bonds (Stoll *et al.*, 1999:333).

According to Freeman (2000:159) omega-3 fatty acids have been found to be helpful in treating hypertension, Crohn's disease, rheumatoid arthritis and asthma. It has also been reported to decrease the risk of primary cardiac arrest and coronary artery disease and decrease serum triglycerides.

#### **2.4.2 Omega-6 fatty acids**

Omega-6 PUFAs are characterised by the presence of at least 2 carbon-carbon double bonds, with the first bond at the sixth carbon from the methyl terminus. Linoleic acid, an 18-carbon fatty acid with 2 double bonds, is the primary dietary omega-6 PUFA (Harris *et al.*, 2009:902).

Conjugated linoleic acid is unique because unlike most naturally occurring fatty acids, it is present in food from animal sources (MacDonald, 2000:116S). According to MacDonald (2000:113S) linoleic acid can be used in treating cancer and atherosclerosis.

### **2.5 Natural oils**

Natural oils that are high in fatty acids and glycerides are used as components of oral formulations and vehicles for injections of pharmaceuticals. Common oils used in oral and topical formulations include cocoa-, olive-, almond- and coconut oils (Heinrich *et al.*, 2004:65).

#### **2.5.1. Crocodile oil**

Crocodile oil is obtained from the fat of the Nile crocodile (*Crocodylus niloticus*). According to Magnino *et al.* (2009:164) the Nile crocodile is native to Africa and can reach up to 7 m in length. It is Africa's largest crocodylian and can weigh up to 730 kg. The meat fat composition of crocodiles is known to contain high levels of palmitic (16:0), palmitoleic (16:1c9), stearic (18:0), oleic (18:1c9) and linoleic (18:2n6) acids. Crocodiles are monogastric animals and therefore their diet strongly influences the fatty acid composition of the fat. Fish based diets result in greater amounts of longer fatty acids compared to chicken and beef diets (Osthoft *et al.*, 2009:64).

Crocodile oil has the same composition as human skin oil. It only differs with regard to the percentages of the ingredients present. Crocodile oil contains saturated and unsaturated fatty acids. Because of the similar composition as human skin oil, crocodile oil will rarely be allergenic when applied to human skin and therefore will be a very well-accepted and safe product to use (Croc city, 2012).

There are many claims of positive results when crocodile oil-containing products are used. It includes fading of freckles, acne, pimple marks, dark lines, wrinkles and laugh lines. It also includes vanishing of uneven dark tones, dark shadows, sun spots and other discolorations. It helps prevent the forming of discoloration and makes the skin softer, brighter and more attractive. It also controls rashness and dryness. Because of the similar composition as human skin oil, crocodile is therefore a very popular and safe product to use (Croc city, 2012). In Table 2.2 crocodile oil ingredients are compared to human skin oil (Croc city, 2012).

**Table 2.2:** Crocodile oil compared to human skin oil

	<b>Crocodile oil %</b>	<b>Human skin oil %</b>
<b>Palmitoleic acid (omega-7)</b>	6.00	3.80
<b>Palmitic acid</b>	23.00	20.20
<b>Myristic acid</b>	0.94	2.10
<b>Stearic acid</b>	6.00	11.20
<b>Oleic acid (omega-9)</b>	39.00	30.80
<b>Linoleic acid (omega-6)</b>	20.00	15.10
<b>Alpha linoleic acid (omega-3)</b>	1.37	0.30

### 2.5.2. Emu oil

Emu oil is obtained from the fat of the Emu (*Dromaius novaehollandiae*). According to Suman *et al.* (2010:623), the Emu is the second largest bird in the world and is the largest avian species native to Australia. In the United States more than one million Emus are raised as a specialty livestock for meat, oil and leather. Emu oil has received attention for its possible therapeutic, notably anti-inflammatory and cosmetic benefits (Pegg *et al.*, 2006:194).

### 2.5.3 Fish oil

According to Choi *et al.* (2010:1694) much work has been conducted on the diverse health advantages related to the consumption of fish oil. These health advantages appear to be due to its high n-3 PUFA content. Health advantages include the prevention of atherosclerosis, anti-inflammatory and immunosuppressive effects (Silva *et al.*, 1996:75). According to Thomas *et al.*

(2007:207) topically applied fish oil was found to put forth an anti-inflammatory activity against erythema.

## **2.6 Essential oils**

According to Edris (2007:308) aromatic plants have been used since ancient times for their preservative and medicinal properties, and to provide aroma and flavour to food. The pharmaceutical properties of aromatic plants are partially attributed to essential oils.

Essential oils are volatile, natural, complex compounds characterised by a strong odour and are formed by aromatic plants as secondary metabolites (Bakkali *et al.*, 2007:447). An essential oil is produced by steam distillation of vegetable plant matter. Plant matter can be any part of a botanical species including stems, branches, fruits, flowers, seeds, roots, bark, needles and leaves. During the distillation process, the vapours are condensed, collected and separated from the condensation water (Stewart, 2005:51).

Essential oils are very complex natural mixtures which can contain about 20-60 components at quite different concentrations. The components include two groups of distinct biosynthetic origin. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterised by low molecular weight (Bakkali *et al.*, 2007:447).

Essential oils may be acceptable natural alternatives to synthetic skin penetration enhancers. They are characterised by their relatively low price and promising penetration enhancing activities. Due to the popularity of these essential oils, their toxicities are well documented and found to be relatively low compared with most synthetic penetration enhancers (Edris, 2007:315).

Essential oils may also be considered as potential natural antioxidants and could perhaps be formulated as a part of daily supplements or additives to prevent oxidative stress that contributes to many degenerative diseases, including ageing (Edris, 2007:314). According to Edris (2007:309), essential oils can also be used in the treatment of cancer, cardiovascular diseases including atherosclerosis, thrombosis and diabetes. It is also an antiviral and antibacterial agent and can be used in aromatherapy and massage therapy. Essential oils and fatty acids are compared in Table 2.3 (Adapted from Stewart, 2005:55).

**Table 2.3:** Essential oils compared to fatty acids

<b>Essential oils</b>	<b>Fatty acids</b>
Distilled from plant parts	Pressed from seeds
Tiny molecules	Large molecules
Molecules built from rings and short chains	Molecules built from long chains
Aromatic and volatile	Nonaromatic and nonvolatile

Essential oils are known for their medicinal properties and are used in embalment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and local anaesthetic remedies (Bakkali *et al.*, 2007:447).

Although clinical applications of essential oils and their components have been limited, some components have been incorporated into creams, lotions, drops or liposomal formulations that are applied externally for treatment of skin diseases or for cosmetic use, whilst other components have been used in inhalation solutions for respiratory infections. Recent studies have shown the activity of essential oils as penetration enhancers for antiseptics and as restorers of antimicrobial activity against resistant species. However, oral delivery is seldom included in these assays due to the potential toxicity associated with essential oils administered through this route (Solorzano-Santos & Miranda-Novales, 2011:1).

### **2.6.1 Tea tree oil**

Tea tree oil has been used as a botanical medicine in various forms over the centuries, and over 70 years for medicinal use as an essential oil (Halcon & Milkus, 2004:402). Tea tree oil is probably the most popular essential oil in the world with innumerable uses. The oil is derived by steam distillation of leaves of the Australian native tea tree, *Melaleuca alternifolia*. Tea tree oil is a complex mixture of about 100 different compounds mainly monoterpenes and their corresponding alcohols. The main constituent is terpinen-4-ol (Reichling *et al.*, 2006:222). It is a clear, mobile liquid with no visible trace of water and has a distinct odour (Halcon & Milkus, 2004:403).

Tea tree oil is reputed to have several medicinal properties including antibacterial, antifungal, antiviral and anti-inflammatory and analgesic properties. In recent years, it has especially gained popularity as a topical antimicrobial agent. In skincare products it is marketed for cleaning, healing, and relieving itching, hotspots, abrasions and other minor rashes and irritations (Reichling *et al.*, 2006:222). It is highly regarded as an antiseptic essential oil. Common uses for tea tree oil include treatment of fungal infections like candida and ringworms and skin conditions like acne and sores (Essential Science Publishing, 2006:84). Tea tree oil also has a

long history of clinical use in the treatment of foot problems such as tinea pedis and toenail onychomycosis. Other dermatologic studies have been conducted with tea tree oil in the treatment of dandruff, head lice, and recurrent herpes labialis (Halcon & Milkus, 2004:402).

### **2.6.2 Olive oil**

Although olive oil has only recently been included in modern cosmetics, this oil has been used on the skin for thousands of years (Badiu *et al.*, 2010:1128). It contains several active ingredients, including polyphenols, squalene, fatty acids, triglycerides, tocopherols, carotenoids and sterols (Baumann & Weisberg, 2010:1117).

There are several cutaneous indications for olive oil including atopic dermatitis, burns, contact dermatitis, eczema, pruritus, psoriasis, rosacea, seborrhoea and various inflammations (Baumann & Weisberg, 2010:1117). In contemporary times, olive oil is not generally known as a first-line treatment for cutaneous disorders, but it has come to be considered an effective therapeutic option for several conditions (Baumann & Weisberg, 2010:1121). Olive oil is often used in combination treatments for treatment of psoriasis, fungal and bacterial infections and anal fissures and hemorrhoids (Baumann & Weisberg, 2010:1121).

Olive oil is found in most skincare products, including bar and liquid soaps, bath oils, soaks for nails, lip balms, massage oils, shampoos and moisturisers (Baumann & Weisberg, 2010:1122). It acts as a moisturising agent in many organic cosmetics that will not clog pores (Essential Science Publishing, 2006:169).

Olive oil appears to be an effective therapeutic option for several conditions and shows promise for future inclusion in photoprotective products. The fact that several constituents in olive oil are known to exhibit significant anti-oxidant activity, along with the results of recent studies yielding evidence of anti-inflammatory and anticarcinogenic effects conferred by olive oil, provide reasons for future research and optimism regarding the expansion of medical and dermatologic applications (Baumann & Weisberg, 2010:1123).

### **2.6.3 Marula oil**

Marula oil is clear and has a pleasant nutty aroma. The oil is classified as medium rich and is silky to the touch, making it ideal as massage oil. Like many other fixed oils, marula oil is rich in mono-unsaturated fatty acids which make the oil very stable. The oil is particularly rich in oleic acid and can be considered an excellent source of natural oleic acid. Marula oil is similar to olive oil in terms of the high content of oleic acid. Therefore it can be used as starting material

for the production of cocoa butter equivalents that can be used in the food and cosmeceutical industries (Vermaak *et al.*, 2011:928).

Marula oil improves skin hydration and smoothness and also reduces skin redness. Oleic acid is known to exert good anti-oxidant activity and as marula oil is reported to contain a high content of this acid, it could be expected to also exhibit antioxidative properties (Vermaak *et al.*, 2011:929).

#### **2.6.4 Avocado oil**

The avocado (*Persea Americana*) is a species widely cultivated around the world for its edible fruits, which are rich in volatile oil. Avocado oil has medicinal uses for wound healing and to stimulate hair growth as well as treating dysentery and diarrhea (Ding *et al.*, 2007:388).

#### **2.6.5 Grapeseed oil**

Grapeseed (*Vitis vinefera*) is a global well-known oilseed crop containing 8 – 15% oil. This oil is becoming increasingly popular for cooking, pharmaceutical, cosmetic and medical purposes. This is primarily due to its high levels of unsaturated fatty acids namely oleic and linoleic acid (Passos *et al.*, 2009:48).

#### **2.6.6 Coconut oil**

All parts of the coconut (*Cocos nucifera*) are useful and especially the oil from the nuts is valued as an emollient and used as an ingredient in remedies for skin infections. Coconut oil is extracted from mature coconuts that have fallen to the ground. Modified coconut oil containing PUFAs in the form of mono-, di- and triglycerides, is useful as a constituent of a barrier lipid mixture in cosmetic and pharmaceutical formulations to protect and prevent drying of the skin. Coconut oil was formerly the main ingredient in marine soaps because coconut oil soap was, unlike other soaps, not readily precipitated by salt solutions (Aburjai & Natsheh, 2003:988).

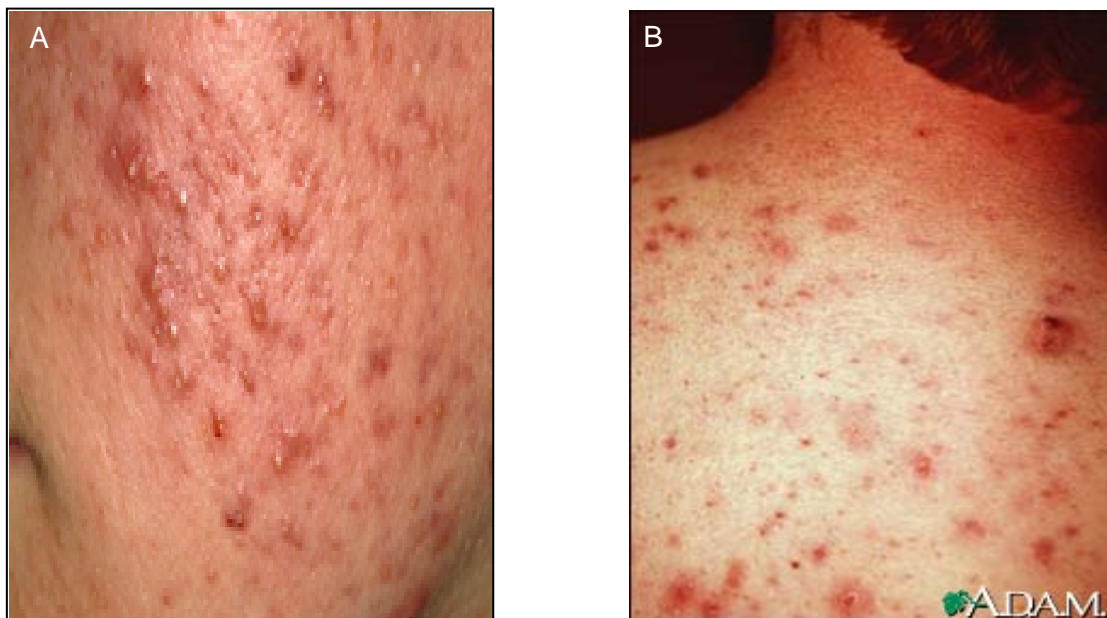
### **2.7 Skin conditions**

Natural oils as described above are extensively used in cosmetics and as treatment for a growing number of skin ailments (Nielsen, 2006:575). Some of these conditions including acne, ageing, psoriasis, eczema and bacterial and fungal infections are described in the coming paragraphs.

#### **2.7.1 Acne**

Acne vulgaris, more commonly known as acne, is the most common skin disease treated by physicians (Krowchuk, 2000:8414). According to Gollnick and Cunliffe (2003:1) it affects almost

80% of adolescents and young adults aged 11 to 30. Some reports estimate that as many as 90% of all teenagers are affected by acne, with virtually 100% of teenagers experiencing some degree of comedone formation (Olutunmbi *et al.*, 2008:171). Acne is a multifactorial disease affecting the pilosebaceous units of the skin and the etiological factors include abnormal keratinisation, increased sebum production, bacterial infection with *Propionibacterium acnes* and production of inflammation (Huber & Walch, 2005:23; Cordain, 2005:84). Barry (1983:38) defines acne as a chronic inflammatory disease, characterised by comedones, papules, pustules and sometimes cysts, involving the sebaceous glands and follicles. Several variants of acne are recognised, including infantile acne, which occurs on the face during the first few months and usually settles spontaneously, and occupational acne, resulting from exposure to oil, coal tar, chlorinated hydrocarbons or insecticides. *Acne vulgaris* commonly affects the face, chest and upper back, and usually presents during puberty (Long, 2002:48). Figure 2.2 shows different forms of acne.



**Figure 2.2:** Different forms of acne: A) comedones and pustules on the face (Dermnet, 2011) and B) scarring on the back (Medline Plus, 2011).

The assessment of acne severity divides acne into four main groups. Mild acne requires topical therapy, moderate acne requires topical and oral antibiotic therapy, severe acne requires topical and oral therapy with very regular review and very severe acne requires urgent referral to a hospital specialist for large doses of antibiotics, steroids or oral isotretinoin (Brajac *et al.*, 2004:21). Grading the severity of acne as mild, moderate or severe is a useful initial assessment. Mild disease comprises open and closed comedones with sparse inflammatory lesions. In moderate acne, papules and pustules are more numerous and in severe acne

extensive lesions occur and may include nodules and scarring (Layton, 2005:44). According to Long (2002:49), the aims for treatment of acne are to reduce the bacterial population of the hair follicles, to encourage the shedding of comedones, to reduce the rate of sebum production and to reduce the degree of inflammation. Topical treatments aim at preventing the formation of new comedones and can take several months to deliver maximal benefit (Shaw & Kennedy, 2007:386). Topical treatments include benzoyl peroxide, topical antibiotics and topical retinoids (Layton, 2005:44). Oral treatments include antibiotics, isotretinoin and hormonal therapy (Gollnick & Cunliffe, 2003:12). Treating acne should centre around 1) opening the pore and 2) killing *P. acnes* (White, 1999:307).

According to Redmond (1998:29) acne is associated with significant negative psychosocial effects that can influence sufferers' overall well-being. Acne adversely affects a person's body image and self-esteem and is associated with increased anxiety and depression. It is repeatedly cited as a cause of low self-esteem.

One of the main problems with the treatment of acne is the relatively slow response, regardless of which topical or systemic preparation is used. Patients should be advised that it may be 6 months before significant improvement can be seen. This slow efficacy leads to poor compliance, which leads to bacterial resistance and less effective response to treatment (Layton, 2005:46).

Because of the potential negative effects caused by acne, as well as adverse effects from acne treatment, it is extremely important to find treatments with faster response and without the possible adverse reactions.

### **2.7.2 Ageing**

Ageing causes a functional deficit in the skin through structural and molecular degradation. This degradation results in clinical changes, including wrinkling, colour changes, laxity and non-elasticity. Ageing dermis results in an increasingly rigid, inelastic tissue, less capable of undergoing modification in response to stress (Diridollou *et al.*, 2001:354). Ageing of the skin is a complex biological process involving both genetically determined and environmental factors and can be divided into intrinsic and extrinsic ageing (Ferreira *et al.*, 2010:444). Ageing is a process in which both intrinsic and extrinsic determinants lead progressively to a loss of structural integrity and physiological function (Farage *et al.*, 2008:87).

Intrinsic ageing, which is largely genetically determined, affects the skin in a manner similar to most internal organs (Ferreira *et al.*, 2010:444). The intrinsic rate of skin ageing in any individual can be dramatically influenced by personal and environmental factors (Farage *et al.*,

2007:87). Intrinsic skin ageing factors include ethnicity, anatomical variation and hormonal changes in cutaneous tissues (Farage *et al.*, 2007:88).

Extrinsic ageing, more commonly termed photo-ageing, is caused by environmental exposure (Ferreira *et al.*, 2010:444). Extrinsic skin ageing factors include lifestyle influences like smoking, and exposure to UV light (Farage *et al.*, 2007:90). Suggested mechanisms include DNA damage to both nuclear and mitochondrial DNA and skin collagen degradation caused by an increase in matrix metalloproteinases induced by UV radiation (Leung & Harvey, 2002:1187). According to Ma *et al.* (2001:592) there is evidence that intrinsic and extrinsic ageing have at least in part overlapping biological, biochemical and molecular mechanisms.

Photo-ageing of the skin is a complex biological process affecting various layers of the skin with the major damage seen in the connective tissue of the dermis. At the histological level, photo-aged skin is mainly characterised by a loss of mature dermal collagen (Ma *et al.*, 2001:593). According to Sommerfeld (2007:711) a major consequence of photo-ageing of the skin is wrinkles. More changes associated with sun damage include pigment changes like hypopigmentation, hyperpigmentation, seborrhoeic warts and dry skin.



**Figure 2.3:** Clinical appearance of photoaged skin in sun-exposed areas of the A) face and B) neck, revealing leathery, coarsely wrinkled, yellowish skin and reduced resilience (Ma *et al.*, 2001:593).

The appearance and mechanical function of human skin undergo profound changes with increasing chronological age and crucially with cumulative exposure to external factors such as UV radiation and smoking (Naylor *et al.*, 2011:250). Clinically, chronological ageing causes a

thinner and less elastic skin compared to the skin in childhood (Sommerfeld, 2007:711). Figure 2.3 shows the clinical appearance of ageing skin in sun-exposed areas.

As human skin ages, each layer undergoes several biological changes. One of the most visible results of these changes is an increase in the frequency of skin wrinkles as we get older. In the stratum corneum, the ability to bind water within the layer decreases with age. It is the water that breaks down the hydrogen bonds in the keratin and thus plasticises the stratum corneum. Reducing the moisture content of this layer, results in a stiffer stratum corneum. This leads to a progression of skin wrinkling with age (Flynn & McCormack, 2010:442). Wrinkles in facial skin are one of the most characteristic morphological changes of ageing (Ferreira *et al.*, 2010:444).

### 2.7.3 Psoriasis

Psoriasis vulgaris is an inflammatory skin disease that is generally chronic and multifactorial (Puig *et al.*, 2009:386; Carretero *et al.*, 2010:600). It is caused by genetic and environmental factors and is characterised by well-defined plaques and silvery scales. It affects 2-3% of the population (Bae *et al.*, 2011:1). Psoriasis is mainly seen in adults, with onset most frequently at around the age of 20. It is regarded as an auto-immune disease in which genetic and environmental factors play a significant role (Berth-Jones, 2009:235). Trauma and streptococcal infections are the main triggering factors of psoriasis. Other factors include endocrine factors, drugs, sunlight, alcohol, smoking and stress (Kawada *et al.*, 2003:63).



**Figure 2.4:** Different forms of *Psoriasis vulgaris*: A) chronic plaque psoriasis (Berth-Jones, 2009:236) and B) guttate psoriasis (Berth-Jones, 2009:239).

There are several types of psoriasis including plaque, guttate, inverse, erythrodermic, and pustular that affects the skin, nails and less frequently the joints (Bae *et al.*, 2011:1). The most frequent manifestation (up to 80%) is chronic plaque psoriasis (Kawade *et al.*, 2003:63). Scaly, erythematous plaques develop in a fairly symmetrical distribution and most commonly affect the scalp, elbows, knees, umbilicus, genitalia, sacrum and shins. Plaques may range in size from a few millimeters to a large part of the trunk or limb. Occasionally, instead of scaling, the surface of plaques is covered by hard, thickened, firmly adherent keratin. Disease activity fluctuates spontaneously over a variable timescale of months or years (Berth-Jones, 2009:235). Figure 2.4 shows two forms of Psoriasis vulgaris.

A wide range of treatments are currently available, although patients reflect a strong degree of dissatisfaction with the treatments (Puig *et al.*, 2009:386). Long-term therapy is often required to control the symptoms (Carretero *et al.*, 2010:600). As a general rule, topical treatments are safest. Second-line treatments, including phototherapy and systemic agents, should only be used when topical treatments are inadequate (Berth-Jones, 2009:236).

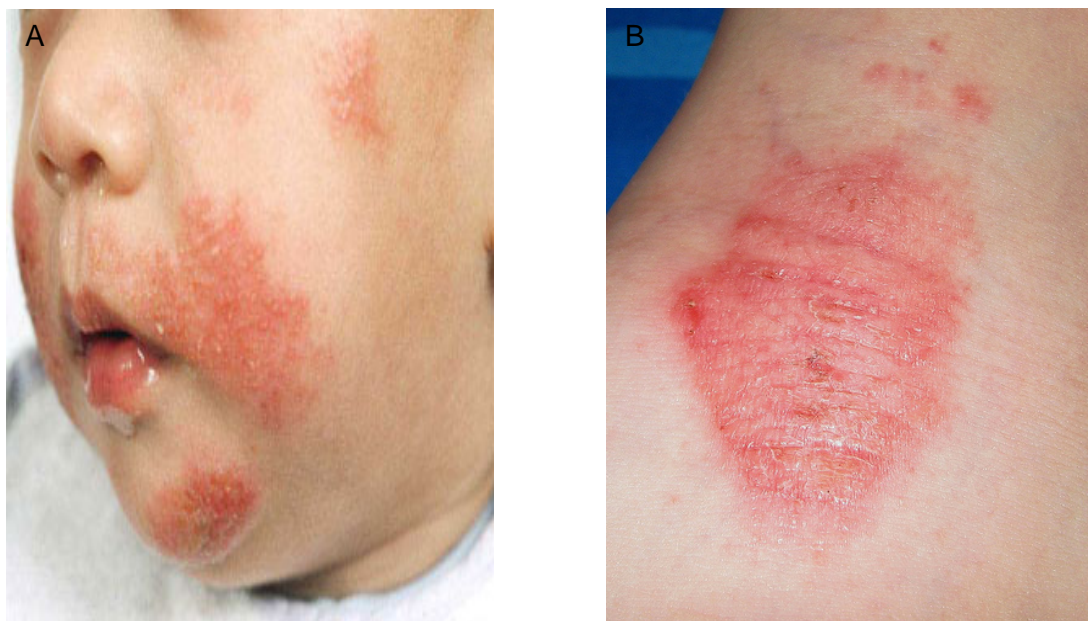
Most psoriasis sufferers to some degree are embarrassed by their disease. In many cases a significant change of lifestyle with avoidance of activities such as sports, can be seen. The psychological impact and significant comorbidities of psoriasis are often underestimated and can have a great impact on quality of life (Berth-Jones, 2009:236; Grozdev *et al.*, 2011:538).

#### **2.7.4 Eczema**

Eczema, also known as atopic dermatitis is a chronic-relapsing inflammatory skin condition that can be distressing and, when severe, can be functionally and socially disabling (Foster *et al.*, 2010:708). It is a cutaneous immune defect that disrupts the skin barrier, thereby increasing an individual's susceptibility to infection with bacteria, fungi and viruses. Diagnostic criteria commonly include pruritus, a history of asthma or hayfever, dry skin, erythema, rash onset in children less than two years of age, visible flexural dermatitis and a history of flexural involvement. Eczema can occur across the lifespan but is particularly prevalent in infancy (Camfferman *et al.*, 2010:359). It affects up to 15-20% of children in developed countries, representing a twofold to threefold increase during the past decades (Foster *et al.*, 2010:708; Kelly & Hourihane, 2011:406).

The central event of the condition is chronic itching frequently resulting in damage to the skin, bleeding, secondary infections and sleep loss. Atopic eczema skin lesions are poorly defined and several types of skin lesions may occur simultaneously or consecutively in the same person. Frequent rubbing and scratching can lead to a reaction known as *lichenification*. This

is a term used to describe thickening of the skin. This reaction takes place mainly in the epidermis and it imparts a brownish appearance to the skin with an exaggeration of skin markings. The distribution of eczema on the body varies with age, affecting primarily the cheeks, forehead and scalp when it first manifests itself in infancy. At this stage it appears as a red, sometimes scaly rash. As the infant grows older the distribution on the affected regions of the body may change. In particular there is greater involvement on all surfaces of the limbs and eventually on the flexural and extensor surfaces, most notably in the elbow creases, behind the knees and around the neck. The persistence of atopic eczema into later life is most common when the infantile experience of the disease is most severe. Atopic eczema in adulthood is often located on the hands (McNally *et al.*, 1998:729). Figure 2.5 shows different forms of atopic eczema.



**Figure 2.5:** Different forms of atopic eczema: A) infantile eczema (Morris, 2009) and B) eczema on the hand (Langley, 2009).

Patients with severe atopic eczema require prolonged treatment with large amounts of highly potent topical corticosteroids and systemic treatment or both. Frequently used options for systemic treatment of atopic eczema include cyclosporine and systemic corticosteroids. Although proved effective, a proportion of patients have a contra-indication for cyclosporine treatment because of ineffectiveness or side-effects. Moreover, long-term use of cyclosporine raises concerns over toxicity. Systemic corticosteroids are used frequently to suppress exacerbations, although high-level evidence is lacking (Schram *et al.*, 2011:353). As health care costs are increasing, it is necessary to look for cheaper alternatives with less side-effects.

## 2.7.5 Bacterial and fungal infections

The types of organisms that cause primary skin and soft tissue infections are diverse, and include bacterial, viral and fungal pathogens as well as parasites (Laube, 2004: 69). The choice of bacterial populations that were used in this study relied on the assumptions made by the manufacturers of the crocodile oil-containing products.

***Staphylococcus aureus***: Cellulitis is a bacterial infection of the lower dermis and subcutaneous soft tissue. It is a common condition in the elderly and frequently affects the legs (Laube, 2004: 70). The major causative organism is *Staphylococcus aureus* (Cox *et al.*, 1998: 634). Furunculosis and carbunculosis are other infections that can occur because of *Staphylococcus aureus* bacteria (Laube, 2004: 75).

***Pseudomonas aeruginosa***: Necrotising fasciitis is an infection causing rapidly advancing deep tissue necrosis. It is caused by a number of organisms including gram-negative bacteria and anaerobes such as *P. aeruginosa* (Laube, 2004: 73).

***Escherichia coli***: Folliculitis is a superficial infection of the pilosebaceous follicles, usually in areas with short, coarse hair such as the scalp, neck and beard area. It is caused by *Enterobacter* species such as *E. coli*. This occurs usually in patients on prolonged courses of antibiotics.

***Trichophyton rubrum***: *T. rubrum* are frequently the pathogens found in nail infections.

The above named bacteria will be used in this study to determine whether crocodile oil and crocodile oil lotion can be used to treat infections caused by them.

## 2.8 Summary

The skin is the biggest and most versatile organ in the body and represents almost 16% of a human's total body weight (Menon, 2002:3; DeBenedictis *et al.*, 2001:573; Sanders *et al.*, 1999:168). It assumes several important physiological functions and represents also a social interface between an individual and other members of society. This is the main reason its age-dependent modifications are in the forefront of dermatological research and of the anti-ageing cosmetic industry (Robert *et al.*, 2009:336).

Because of the popularity and wide variety of skincare products, it is necessary to create products that distinct them from the rest on the market. The use of natural oils in skincare products is a new way to prevent skin ageing as well as other dermatological conditions.

Natural ingredients are continually gaining popularity and the use of plant extracts in cosmetic formulation is on the rise. A cosmetic formulation including active principles of natural origin can protect the skin against exogenous or endogenous harmful agents, and help to remedy many skin conditions. In addition, natural products can be used in hair care. Aromatic plants and oils have been used for thousands of years in cosmetics, and for their medicinal and culinary applications.

Essential oils are known for their medicinal properties and are used in embalment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and local anaesthetic remedies (Bakkali *et al.*, 2007:447). It also imparts many other benefits such as a pleasant aroma, especially in perfumes and to impart shine or conditioning in a hair care product and for emolliency or improving the elasticity of the skin (Aburjai & Natsheh, 2003:997).

Although clinical applications of essential oils and their components have been limited, some components have been incorporated into creams, lotions, drops or liposomal formulations that are applied externally for treatment of skin diseases or for cosmetic use (Solorzano-Santos & Miranda-Novales, 2011:1).

In this study, the aims and objectives were to use natural oil, namely crocodile oil, and to investigate the fatty acid profile, anti-microbial and anti-fungal activity, anti-oxidant activity, toxicity studies, stability and shelf-life determination of crocodile oil lotion, sensitivity testing of crocodile oil and clinical efficacy testing of anti-ageing effects.

## 2.9 References

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## CHAPTER 3

### ARTICLE FOR PUBLICATION IN THE JOURNAL *MOLECULES*

Chapter 3 is written in an article format for the purpose of publication in the journal *Molecules*. 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.

## NATURAL OILS FOR THE TREATMENT OF SKIN CONDITIONS

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## **Abstract**

Natural oils are broadly used in skincare products and as treatment for a growing number of conditions. Natural oils used in skincare products contain a range of fatty acids which contribute to several beneficial properties in cosmetics and personal care products. Fatty acids are divided into saturated- and unsaturated acids. Triglycerides are the main constituents of vegetable oils and animal fats. A triglyceride is a chemical compound formed from one molecule of glycerol and three fatty acids. The most common fatty acids include omega-3 and omega-6. The omega-3 and -6 fatty acids are naturally occurring lipids, appearing in high concentrations in certain fish and plants such as flax seed oil. The natural oils discussed in this article include fish -, crocodile -, emu - and essential oils like tea tree -, olive -, avocado -, marula -, grapeseed - and coconut oil. The aim of this article is to give a comprehensive summary of natural oils used to treat several skin conditions including acne, psoriasis, eczema, ageing and bacterial and fungal infections. The discussions on these natural oils have been organized into the following classes: fatty acids including omega-3 and omega-6 fatty acids, essential oils and natural oils.

**Keywords:** natural oils; essential oils; skin conditions, skin care products

## 1 Introduction

Natural oils are extensively used in skincare products and as treatment for a growing number of conditions [1]. The natural products industry is a multibillion dollar industry and has grown enormously in the past few years [2].

Oils extracted from plant sources have a rich history of use by people as a source of food, energy, medicine and for cosmetic applications. It has been used in the production of lubricants, soaps and personal care products, as well as in the topical treatment of various conditions such as hair dandruff, muscle spasms, varicose veins and wounds. In recent years, demand for seed oils as ingredients in cosmetics has greatly increased as the industry seeks natural alternatives [2].

Natural oils used in skin care products contain a range of fatty acids which contribute to several beneficial properties in cosmetic and personal care products. Fatty acids are divided into saturated acids and unsaturated acids [2]. Fatty acids are usually insoluble in water and are sometimes referred to as fixed oils or fats. Fatty acids are very important as formulation agents and vehicles in pharmacy and as components of skin products and soaps [3]. The most common fatty acids include omega-3 and omega-6 fatty acids.

The omega-3 and -6 fatty acids are naturally occurring lipids, appearing in high concentration in certain fish, particularly in coldwater and oily species, and plants such as flax seed oil [4]. Other natural oils discussed in this article include fish -, crocodile -, emu - and essential - like tea tree -, olive -, avocado -, marula -, grapeseed - and coconut oil. These are just a few of the many natural oils that are available.

Essential oils may be acceptable natural alternatives to synthetic skin penetration enhancers. Essential oils may also be considered as potential natural antioxidants and could perhaps be formulated as a part of daily supplements if taken orally to prevent oxidative stress that contributes to many degenerative diseases, including ageing [5]. Essential oils can also be used in the treatment of cancer, cardiovascular diseases including atherosclerosis, thrombosis and diabetes. It is also an antiviral and antibacterial agent and can be used in aromatherapy and massage therapy [5]. Essential oils are known for their medicinal properties and are used in embalment, preservation of foods and as analgesic, sedative, anti-inflammatory, spasmolytic and local anesthetic remedies [6].

In this article the treatment of skin conditions including ageing, acne, psoriasis, eczema and bacterial and fungal infections with natural oils are discussed.

## 2 Fatty acids

Natural oils mainly contain saturated and unsaturated fatty acids. The unsaturated fatty acids namely omega-3, -6, -7 and -9 are responsible for the positive effects on human skin [7].

Fatty acids have an even number of carbon atoms, in the range of 16-26. Fatty acids with only single bonds between adjacent carbon atoms are referred to as saturated, whereas those with at least one C=C double bond are called unsaturated. The polyunsaturated fatty acids have two or more double bonds and are named according to the position of these bonds and the total chain length. For example, docosahexaenoic acid (DHA; 22:6) is an omega-3 (n-3) fatty acid with 22 carbon atoms and 6 double bonds. The term 'n-3' indicates that, counting from the methyl (CH<sub>3</sub>) end of the molecule, the first double bonds are located between the third and fourth carbons. As the degree of unsaturation in fatty acids increases, the melting point decreases which confers the attribute of fluidity on n-3 polyunsaturated fatty acids [8].

**Table 1:** Common fatty acids used in cosmetic products

Common name	Carbon atoms	Double bonds	Scientific name	Sources
Butyric acid	4	0	butanoic acid	butterfat
Caproic acid	6	0	hexanoic acid	butterfat
Caprylic acid	8	0	octanoic acid	coconut oil
Capric acid	10	0	decanoic acid	coconut oil
Lauric acid	12	0	dodecanoic acid	coconut oil

Myristic acid	14	0	tetradecanoic acid	palm kernel oil
Palmitic acid	16	0	hexadecanoic acid	palm oil
Palmitoleic acid	16	1	9-hexadecenoic acid	animal fats
Stearic acid	18	0	octadecanoic acid	animal fats
Oleic acid	18	1	9-octadecenoic acid	olive oil
Ricinoleic acid	18	1	12-hydroxy-9-octadecenoic acid	castor oil
Vaccenic acid	18	1	11-octadecenoic acid	butterfat
Linoleic acid	18	2	9,12-octadecadienoic acid	grape seed oil
Alpha-linolenic acid (ALA)	18	3	9,12,15-octadecatrienoic acid	flaxseed (linseed) oil
Gamma-linolenic acid (GLA)	18	3	6,9,12-octadecatrienoic acid	borage oil
Arachidic acid	20	0	eicosanoic acid	peanut oil, fish oil
Gadoleic acid	20	1	9-eicosenoic acid	fish oil
Arachidonic acid (AA)	20	4	5,8,11,14-eicosatetraenoic acid	liver fats
Eicosapentanoic acid (EPA)	20	5	5,8,11,14,17-eicosapentaenoic acid	fish oil

Behenic acid	22	0	docosanoic acid	grapeseed oil
Erucic acid	22	1	13-docosenoic acid	grapeseed oil
DHA	22	6	4,7,10,13,16,19- docosahexaenoic acid	fish oil
Lignoceric acid	24	0	tetracosanoic acid	small amounts in most fats

Fatty acids are usually insoluble in water and are sometimes referred to as fixed oils or fats [3]. Fatty acids consist of the elements carbon (C), hydrogen (H) and oxygen (O) arranged as a carbon chain skeleton with a carboxyl group (-COOH) at one end. Saturated fatty acids have all the hydrogen that the carbon atoms can hold, and therefore, have no double bonds between the carbons [26]. The three most common saturated fatty acids (myristic, palmitic and stearic acids) differ in two methylene groups. The unsaturated fatty acids contain a varying number of double bonds. This, together with the length of the carbon chain, is indicated after the name of the fatty acid. The polyunsaturated fatty acids contain three or more double bonds and are particularly beneficial in the diet as antioxidants [3]. Table 1 gives the chemical name and descriptions of some common fatty acids [9].

Triglycerides are the main constituents of vegetable oils and animal fats. A triglyceride (also called triacylglycerol) is a chemical compound formed from one molecule of glycerol and three fatty acids [9].

Omega-3 and -6 fatty acids are two very common unsaturated essential fatty acids and are discussed below.

## 2.1 Omega-3 (linolenic) fatty acids

The omega-3 fatty acids (also known as 'n-3' fatty acids) are a group of naturally occurring lipids, appearing in high concentration in certain fish, particularly in coldwater and oily species,

and plants such as flax seed oil, perilla oil and others [4]. Omega-3 fatty acids are long-chain, polyunsaturated fatty acids (PUFAs). Unlike saturated fats, which have shown to have negative health consequences, omega-3 fatty acids are PUFAs that have been associated with many health benefits [10]. There are three predominant naturally occurring omega-3 fatty acids: DHA, eicosapentanoic acid (EPA) and  $\alpha$ -linolenic acid [4]. Linolenic acid is an omega-3 fatty acid found in plants [10].

Omega-3 fatty acids are polyunsaturated; with their first double bond exactly 3 carbons from the lipophilic end of the molecule. A series of double bonds recur every third carbon atom. The presence of multiple double bonds in the carbon chain produces a more highly folded molecule than more saturated fatty acids. In addition, the melting point of the omega-3 fatty acids is much lower than for most saturated fatty acids, which explains why membranes containing a high content of omega-3 fatty acids may be more fluid at a given body temperature, when compared with membranes comprised of more saturated fatty acids. The major difference among the different omega-3 fatty acids is the length of the carbon chain and the number of double bonds [4].

Omega-3 fatty acids have been found to be helpful in treating hypertension, Crohn's disease, rheumatoid arthritis and asthma. It has also been reported to decrease the risk of primary cardiac arrest and coronary artery disease and to decrease serum triglycerides [10].

## **2.2 Omega-6 (linoleic) fatty acids**

Omega-6 PUFAs are characterized by the presence of at least 2 carbon-carbon double bonds, with the first bond at the sixth carbon from the methyl terminus. Linoleic acid, an 18-carbon fatty acid with 2 double bonds, is the primary dietary omega-6 PUFA [11].

Conjugated linoleic acid is unique because unlike most naturally occurring fatty acids, it is present in food from animal sources. Linoleic acid can be used in treating cancer and atherosclerosis [12].

### **3 Natural oils**

Natural oils that are high in fatty acids and glycerides are used as components of oral formulations and vehicles for injections of pharmaceuticals. Common oils used in oral and topical formulations include cocoa -, olive -, almond - and coconut oils [3]. The use of animal oils has also increased over the past few years, and some of the oils from animal origin are described in the following paragraphs. It includes crocodile oil, emu oil and fish oil.

#### **3.1 Oils from animal origin**

##### **3.1.1 Crocodile oil**

Crocodile oil is obtained from the fat of the Nile crocodile (*Crocodylus niloticus*). The Nile crocodile is native to Africa and can reach up to 7 m in length. It is Africa's largest crocodilian and can weigh up to 730 kg [13]. The meat fat composition of crocodiles is known to contain high levels of palmitic (16:0), palmitoleic (16:1c9), stearic (18:0), oleic (18:1c9) and linoleic (18:2n6) acids. Crocodiles are monogastric animals and therefore their diet strongly influences the fatty acid composition of the fat. Fish based diets result in greater amounts of longer fatty acids compared to chicken and beef diets [14].

Crocodile oil has the same composition as human skin oil. It only differs with regard to the percentages of the ingredients present. Crocodile oil contains saturated and unsaturated fatty acids. Because of the similar composition as human skin oil, crocodile oil will rarely be allergenic when applied to human skin and therefore will be a very accepted and safe product to use [7].

There are many claims of positive results when crocodile oil containing products were used. It includes fading of freckles, acne, pimple marks, dark lines, wrinkles and laugh lines. It also includes vanishing of uneven dark tones, dark shadows, sun spots and other discolorations. It helps prevent discolorations from forming and makes the skin softer, brighter and more attractive. It also controls rashness and dryness. Because of the similar composition as human skin oil, crocodile oil will be a very popular and safe product to use [7]. In Table 2 crocodile oil ingredients are compared to human skin oil [7].

**Table 2:** Crocodile oil compared to human skin oil

	<b>Crocodile oil %</b>	<b>Human skin oil %</b>
<b>Palmitoleic acid (omega-7)</b>	6.00	3.80
<b>Palmitic acid</b>	23.00	20.20
<b>Myristic acid</b>	0.94	2.10
<b>Stearic acid</b>	6.00	11.20
<b>Oleic acid (omega-9)</b>	39.00	30.80
<b>Linoleic acid (omega-6)</b>	20.00	15.10
<b>Alpha linoleic acid (omega-3)</b>	1.37	0.30

### **3.1.2 Emu oil**

Emu oil is obtained from the fat of the Emu (*Dromaius novaehollandiae*). The Emu is the second largest bird in the world and is the largest avian species native to Australia [15]. In the United States more than one million Emus are raised as a specialty livestock for meat, oil and leather. Emu oil has received attention for its possible therapeutic, notably anti-inflammatory and cosmetic benefits [16]. In Table 3 emu oil ingredients are compared to human skin oil [17].

**Table 3:** Emu oil compared to human skin oil

	<b>Emu oil %</b>	<b>Human skin oil %</b>
<b>Palmitoleic acid (omega-7)</b>	3.20	3.80
<b>Palmitic acid</b>	20.30	20.20
<b>Myristic acid</b>	0.30	2.10
<b>Stearic acid</b>	10.10	11.20
<b>Oleic acid (omega-9)</b>	51.60	30.80
<b>Linolenic acid</b>	0.50	0.30

### **3.1.3 Fish oil**

Much work has been conducted on the diverse health advantages related to the consumption of fish oil. These health advantages appear to be due to its high n-3 PUFA content [18]. Health advantages include the prevention of atherosclerosis, anti-inflammatory and immunosuppressive effects [19]. Topically applied fish oil was found to put forth an anti-inflammatory activity against erythema [20].

### **3.2 Essential oils**

Aromatic plants have been used since ancient times for their preservative and medicinal properties, and to provide aroma and flavor to food. The pharmaceutical properties of aromatic plants are partially attributed to essential oils [5].

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites [6]. An essential oil is produced by steam distillation of vegetable plant matter. Plant matter can be any part of a botanical species

including stems, branches, fruits, flowers, seeds, roots, bark, needles and leaves. During the distillation process, the vapors are condensed, collected and separated from the condensation water [21].

Essential oils are very complex natural mixtures which can contain about 20-60 components at quite different concentrations. The components include two groups of distinct biosynthesical origin. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight [6].

Essential oils may be acceptable natural alternatives to synthetic skin penetration enhancers. They are characterized by their relatively low price and promising penetration enhancing activities. Due to the popularity of these essential oils, their toxicities are well documented and found to be relatively low compared to most synthetic penetration enhancers [5].

Essential oils may also be considered as potential natural antioxidants and could perhaps be formulated as a part of daily supplements or additives to prevent oxidative stress that contributes to many degenerative diseases, including ageing [5]. Essential oils can also be used in the treatment of cancer, cardiovascular diseases including atherosclerosis, thrombosis and diabetes. It is also an antiviral and antibacterial agent and can be used in aromatherapy and massage therapy [5]. Essential oils and fatty acids are compared in Table 4 [21].

**Table 4:** Essential oils compared to fatty acids

<b>Essential oils</b>	<b>Fatty acids</b>
Distilled from plant parts	Pressed from seeds
Tiny molecules	Large molecules
Molecules built from rings and short chains	Molecules built from long chains
Aromatic and volatile	Nonaromatic and nonvolatile

Essential oils are known for their medicinal properties and are used in embalment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and local anaesthetic remedies [6].

Although clinical applications of essential oils and their components have been limited, some components have been incorporated into creams, lotions, drops or liposomal formulations that are applied externally for treatment of skin diseases or for cosmetic use, whilst other components have been used in inhalation solutions for respiratory infections. Recent studies have shown the activity of essential oils as penetration enhancers for antiseptics and as restorers of antimicrobial activity against resistant species. However, oral delivery is seldom included in these assays due to the potential toxicity associated with essential oils administered through this route [22].

Currently almost 3000 essential oils are known to man, of which 300 are commercially important for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. In this article only a few of the most common essential oils are described [6].

### **3.2.1 Tea tree oil**

Tea tree oil has been used as a botanical medicine in various forms over the centuries, and over 70 years for medicinal use as an essential oil [23]. Tea tree oil is probably the most popular

essential oil in the world with innumerable uses. The oil is derived by steam distillation of leaves of the Australian native tea tree, *Melaleuca alternifolia*. Tea tree oil is a complex mixture of about 100 different compounds mainly monoterpenes and their corresponding alcohols. The main constituent is terpinen-4-ol [40]. It is a clear, mobile liquid with no visible trace of water and has a distinct odor [23].

Tea tree oil is reputed to have several medicinal properties including antibacterial, antifungal, antiviral, anti-inflammatory and analgesic properties. In recent years, it has especially gained popularity as a topical anti-microbial agent. In skincare products it is marketed for cleaning, healing, and relieving itching, hotspots, abrasions and other minor rashes and irritations [24]. It is highly regarded as an antiseptic essential oil. Common uses for tea tree oil include treatment of fungal infections like candida and ringworms and skin conditions like acne and sores [25]. Tea tree oil also has a long history of clinical use in the treatment of foot problems such as tinea pedis and toenail onychomycosis. Other dermatological studies have been conducted with tea tree oil in the treatment of dandruff, head lice, and recurrent herpes labialis [23].

### **3.2.2 Olive oil**

Although olive oil has only recently been included in modern cosmetics, this oil has been used on the skin for thousands of years [26]. It contains several active ingredients, including polyphenols, squalene, fatty acids, triglycerides, tocopherols, carotenoids and sterols [27].

There are several cutaneous indications for olive oil including atopic dermatitis, burns, contact dermatitis, eczema, pruritus, psoriasis, rosacea, seborrhoea and various inflammations [27]. In contemporary times, olive oil is not generally known as a first-line treatment for cutaneous disorders, but it has come to be considered an effective therapeutic option for several conditions. Olive oil is often used in combination treatments for treatment of psoriasis, fungal and bacterial infections and anal fissures and hemorrhoids [27].

Olive oil is found in most skincare products, including bar and liquid soaps, bath oils, soaks for nails, lip balms, massage oils, shampoos and moisturizers [27]. It acts as a moisturizing agent in many organic cosmetics that will not clog pores [25].

Olive oil appears to be an effective therapeutic option for several conditions and shows promise for future inclusion in photoprotective products. The fact that several constituents in olive oil are known to exhibit significant antioxidant activity, along with the results of recent studies yielding evidence of anti-inflammatory and anticarcinogenic effects conferred by olive oil, provide reasons for future research and optimism regarding the expansion of medical and dermatologic applications [27].

### **3.2.3 Marula oil**

Marula oil is clear and has a pleasant nutty aroma. The oil is classified as medium rich and is silky to the touch, making it ideal as a massage oil. Like many other fixed oils, marula oil is rich in mono-unsaturated fatty acids which make the oil very stable. The oil is particularly rich in oleic acid and can be considered an excellent source of natural oleic acid. Marula oil is similar to olive oil in terms of the high content of oleic acid. Therefore it can be used as starting material for the production of cocoa butter equivalents that can be used in the food and cosmeceutical industries [2].

Marula oil improves skin hydration and smoothness as well as reduces skin redness. Oleic acid is known to exert good anti-oxidant activity and as marula oil is reported to contain a high content of this acid, it could be expected to also exhibit anti-oxidative properties [2].

### **3.2.4 Avocado oil**

The avocado (*Persea Americana*) is a species widely cultivated around the world for its edible fruits, which are rich in volatile oil. Avocado oil has medicinal uses for wound healing and to stimulate hair growth as well as treating dysentery and diarrhea [28].

### **3.2.5 Grapeseed oil**

Grapeseed (*Vitis vinefera*) is a global well-known oilseed crop containing 8-15% oil. This oil is becoming increasingly popular for cooking, pharmaceutical, cosmetic and medical purposes. This is primarily due to its high levels of unsaturated fatty acids namely oleic and linoleic acid [29].

### **3.2.6 Coconut oil**

All parts of the coconut (*Cocos nucifera*) are useful and especially the oil from the nuts is valued as an emollient and used as an ingredient in remedies for skin infections. Coconut oil is extracted from mature coconuts that have fallen to the ground. Modified coconut oil containing PUFAs in the form of mono-, di- and triglycerides, is useful as a constituent of a barrier lipid mixture in cosmetic and pharmaceutical formulations to protect and prevent drying of the skin. Coconut oil was formerly the main ingredient in marine soaps because coconut oil soap was, unlike other soaps, not readily precipitated by salt solutions [30].

## **4 The structure and functions of human skin**

When talking about the use of natural oils for the treatment of skin conditions, it is important to look at the structure and functions of human skin as well as the different skin layers where treatment is most needed.

The skin is the largest organ in the human body and covers approximately 1.5 to 2.0 m<sup>2</sup> of the average human's body surface [31, 32]. It is the heaviest and most versatile organ of the body by representing almost 16% of a human's total body weight [33]. The skin is a protective barrier with immunological and sensory functions [34] that provides a multifunctional interface between us and our surroundings [35]. It plays a very important role in thermo-regulation and performs endocrine functions like vitamin D synthesis and peripheral conversion of prohormones [31].

The skin consists of two layers, namely the epidermis and the dermis. The epidermis contains numerous nerve endings but is without blood or lymphatic vessels. It is approximately 0.1 mm thick, except at the palm and sole, where its thickness can exceed 1 mm [36]. The epidermis is a self-renewing, stratified epithelium that functions as the interface between the human body and outer environment. The epidermis protects against mechanical, chemical and microbial attacks and functions as a permeability barrier by preventing water loss from the dermis. The epidermis also has immunological functions and provides some protection to the skin from ultraviolet (UV) light via the pigment system [37]. The stratified epidermis is divided into four distinct layers namely, the stratum basale, stratum spinosum, stratum granulosum and stratum corneum [38].

The stratum corneum is the outermost portion of the epidermis and provides a protective barrier that limits the penetration of topical contaminants and prevents dehydration of the underlying tissue [39]. It is the definitive boundary or frontier structure that sharply separates the body's vulnerable organs and tissues from the variable and sometimes hazardous outside world [40]. Physically, the stratum corneum consists of an array of flat, multilateral, keratin-filled cells embedded in a matrix of lamellar lipids [39]. In this two-compartment system the only continuous phase is the intercellular domain which seems to be the major rate-determining pathway by which most drugs cross the stratum corneum [41]. Lipids present in the stratum corneum originate from a mixture of polar and neutral lipids, typical of other tissues, which are replaced by more nonpolar mixture, including ceramides, free sterol and free fatty acids, as well as variable amounts of triglycerides, sterols, esters and other nonpolar compounds depending on race, age and gender [40].

The dermis is directly adjacent to the epidermis and provides the mechanical support for the skin [42]. It is a tough, resilient layer that protects the body against mechanical injury and contains specialized structures [38]. The dermis is largely acellular, but is rich in blood vessels, lymphatic vessels and nerve endings. Hair follicles, sebaceous glands and sweat glands are found in the dermis and might serve as additional but limited pathways for drug absorption [34]. For the treatment of skin conditions with natural oils, absorption into the dermis and epidermis, and not necessary systemic absorption, are important.

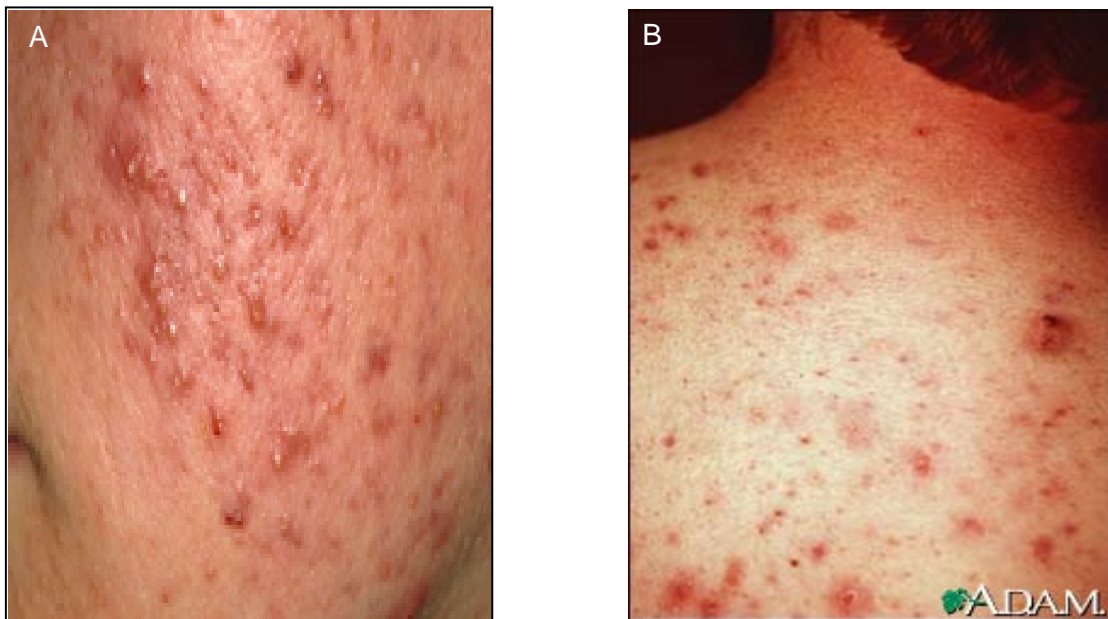
## **5 Skin conditions that are regularly treated with natural oils**

Natural oils as described above are extensively used in cosmetics and as treatment for a growing number of skin ailments [1]. Some of these conditions including acne, ageing, psoriasis, eczema and bacterial and fungal infections are described in the coming paragraphs.

### **5.1 Acne**

Acne vulgaris, more commonly known as acne, is the most common skin disease treated by physicians [43]. Acne affects almost 80% of adolescents and young adults aged 11 to 30 [44]. Some reports estimate that as many as 90% of all teenagers are affected by acne, with virtually 100% of teenagers experiencing some degree of comedone formation [45]. Acne is a multifactorial disease affecting the pilosebaceous units of the skin and the etiological factors

include abnormal keratinisation, increased sebum production, bacterial infection with *Propionibacterium acnes* and production of inflammation [46, 47]. Acne can be defined as a chronic inflammatory disease, characterized by comedones, papules, pustules and sometimes cysts, involving the sebaceous glands and follicles [48]. Several variants of acne are recognized, including infantile acne, which occurs on the face during the first few months and usually settles spontaneously, and occupational acne, resulting from exposure to oil, coal tar, chlorinated hydrocarbons or insecticides. *Acne vulgaris* commonly affects the face, chest and upper back, and usually presents during puberty [49]. Figure 1 shows different forms of acne.



**Figure 1:** Different forms of acne: A) comedones and pustules on the face [50] and B) scarring on the back [51].

The assessment of acne severity divides acne into four main groups. Mild acne requires topical therapy, moderate acne requires topical and oral antibiotic therapy, severe acne requires topical and oral therapy with very regular review and very severe acne requires urgent referral to a hospital specialist for large doses of antibiotics, steroids or oral isotretinoin [52]. Grading the severity of acne as mild, moderate or severe is a useful initial assessment. Mild disease comprises open and closed comedones with sparse inflammatory lesions. In moderate acne, papules and pustules are more numerous and in severe acne extensive lesions occur and may include nodules and scarring [53]. The aims for treatment of acne are to reduce the bacterial

population of the hair follicles, to encourage the shedding of comedones, to reduce the rate of sebum production and to reduce the degree of inflammation [49]. Topical treatments aim at preventing the formation of new comedones and can take several months to deliver maximal benefit [54]. Topical treatments include benzoyl peroxide, topical antibiotics and topical retinoids [53]. Oral treatments include antibiotics, isotretinoin and hormonal therapy [44]. Treating acne should center around 1) opening the pore and 2) killing *P. acnes* [55].

Acne is associated with significant negative psychosocial effects that can influence sufferers' overall well-being. Acne adversely affects a person's body image and self-esteem and is associated with increased anxiety and depression. It is repeatedly cited as a cause of low self-esteem [56].

One of the main problems with the treatment of acne is the relatively slow response, regardless of which topical or systemic preparation is used. Patients should be advised that it may be 6 months before significant improvement can be seen. This slow efficacy leads to poor compliance, which leads to bacterial resistance and less effective response to treatment [53].

Because of the potential negative effects caused by acne, as well as adverse effects from acne treatment, it is extremely important to find treatments with faster response and without the possible adverse reactions.

Tea tree oil has broad-spectrum anti-microbial and anti-inflammatory activity *in vitro*. The use of 5% tea tree oil for the treatment of acne showed the efficacy of tea tree oil gel against *Propionibacterium acnes* [57].

## **5.2 Ageing**

Ageing causes a functional deficit in the skin through structural and molecular degradation. This degradation results in clinical changes, including wrinkling, color changes, laxity and non-elasticity. Ageing dermis results in an increasingly rigid, inelastic tissue, less capable of undergoing modification in response to stress [58]. Ageing of the skin is a complex biological process involving both genetically determined and environmental factors and can be divided into intrinsic and extrinsic ageing [59]. Ageing is a process in which both intrinsic and extrinsic determinants lead progressively to a loss of structural integrity and physiological function [60].

Intrinsic ageing, which is largely genetically determined, affects the skin in a manner similar to most internal organs [59]. The intrinsic rate of skin ageing in any individual can be dramatically influenced by personal and environmental factors [60]. Intrinsic skin ageing factors include ethnicity, anatomical variation and hormonal changes in cutaneous tissues [60].

Extrinsic ageing, more commonly termed photo-ageing, is caused by environmental exposure [59]. Extrinsic skin ageing factors include lifestyle influences like smoking, and exposure to UV light [60]. Suggested mechanisms include DNA damage to both nuclear and mitochondrial DNA and skin collagen degradation caused by an increase in matrix metalloproteinases induced by UV radiation [61]. There is evidence that intrinsic and extrinsic ageing have at least in part, overlapping biological, biochemical and molecular mechanisms [62].

Photo-ageing of the skin is a complex biological process affecting various layers of the skin with the major damage seen in the connective tissue of the dermis. At the histological level, photo-aged skin is mainly characterized by a loss of mature dermal collagen [62]. A major consequence of photo-ageing of the skin is wrinkles. More changes associated with sun damage include pigment changes like hypopigmentation, hyperpigmentation, seborrhoeic warts and dry skin [63].



**Figure 2:** Clinical appearance of photoaged skin in sun-exposed areas of the A) face and B) neck, revealing leathery, coarsely wrinkled, yellowish skin and reduced resilience [62].

The appearance and mechanical function of human skin undergo profound changes with both increasing chronological age and crucially with cumulative exposure to external factors such as UV radiation and smoking [64]. Clinically, chronological ageing causes a thinner and less elastic skin compared to the skin in childhood [63]. Figure 2 shows the clinical appearance of ageing skin in sun-exposed areas.

As human skin ages, each layer undergoes several biological changes. One of the most visible results of these changes is an increase in the frequency of skin wrinkles as we get older. In the stratum corneum, the ability to bind water within the layer decreases with age. It is the water that breaks down the hydrogen bonds in the keratin and thus plasticizes the stratum corneum. Reducing the moisture content of this layer, results in a stiffer stratum corneum. This leads to a progression of skin wrinkling with age [65]. Wrinkles in facial skin are one of the most characteristic morphological changes of ageing [59].

Grapeseed oil can prevent ageing because of the radical scavenging action and inhibition of lipid peroxidation that contributes to the anti-oxidant properties of grapeseed oil [66].

### **5.3 Psoriasis**

Psoriasis vulgaris is an inflammatory skin disease that is generally chronic and multifactorial [67,68]. It is caused by genetic and environmental factors and is characterized by well-defined plaques and silvery scales. It affects 2-3% of the population [69]. Psoriasis is mainly seen in adults, with onset most frequently at around the age of 20. It is regarded as an auto-immune disease in which genetic and environmental factors play a significant role [70]. Trauma and streptococcal infections are the main triggering factors of psoriasis. Other factors include endocrine factors, drugs, sunlight, alcohol, smoking and stress [71].



**Figure 3:** Different forms of *Psoriasis vulgaris*: A) chronic plaque psoriasis and B) guttate psoriasis [70].

There are several types of psoriasis including plaque, guttate, inverse, erythrodermic, and pustular that affects the skin, nails and less frequently the joints [59]. The most frequent manifestation (up to 80%) is chronic plaque psoriasis [71]. Scaly, erythematous plaques develop in a fairly symmetrical distribution and most commonly affect the scalp, elbows, knees, umbilicus, genitalia, sacrum and shins. Plaques may range in size from a few millimeters to a large part of the trunk or limb. Occasionally, instead of scaling, the surface of plaques is covered by hard, thickened, firmly adherent keratin. Disease activity fluctuates spontaneously over a variable timescale of months or years [70]. Figure 3 shows two forms of *Psoriasis vulgaris*.

A wide range of treatments are currently available, although patients reflect a strong degree of dissatisfaction with the treatments [67]. Long-term therapy is often required to control the symptoms [68]. As a general rule, topical treatments are safest. Second-line treatments, including phototherapy and systemic agents, should only be used when topical treatments are inadequate [70].

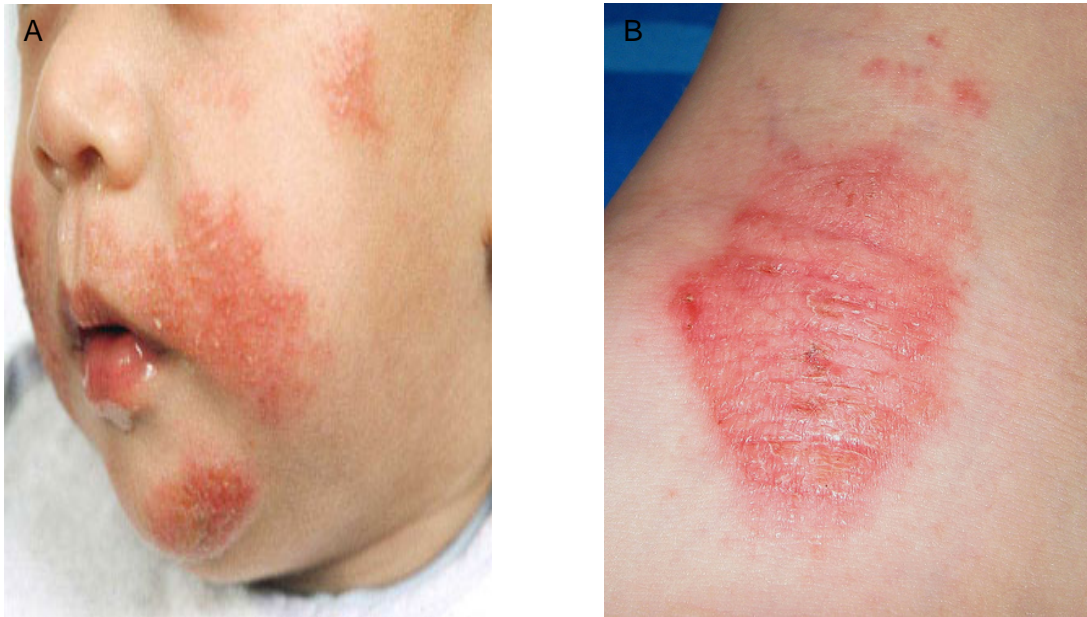
Most psoriasis sufferers are to some degree embarrassed by their disease. In many cases a significant change in lifestyle, with avoidance of activities such as sports, can be seen. The psychological impact and significant comorbidities of psoriasis are often underestimated and can have a great impact on quality of life [70, 72].

Topical and oral fish oil can be useful in the treatment of psoriasis. Olive oil is also a natural oil that contains flavonoids, anti-oxidants and anti-bacterial ingredients that are important in the treatment of psoriasis [73].

#### 5.4 Eczema

Eczema, also known as atopic dermatitis is a chronic-relapsing inflammatory skin condition that can be distressing and, when severe, can be functionally and socially disabling [74]. It is a cutaneous immune defect that disrupts the skin barrier, thereby increasing an individual's susceptibility to infection with bacteria, fungi and viruses. Diagnostic criteria commonly include pruritus, a history of asthma or hay fever, dry skin, erythema, rash onset in children less than two years of age, visible flexural dermatitis and a history of flexural involvement. Eczema can occur across the lifespan but is particularly prevalent in infancy [75]. It affects up to 15-20% of children in developed countries, representing a twofold to threefold increase during the past decades [74, 76].

The central event of the condition is chronic itching frequently resulting in damage to the skin, bleeding, secondary infections and sleep loss. Atopic eczema skin lesions are poorly defined and several types of skin lesions may occur simultaneously or consecutively in the same person. Frequent rubbing and scratching can lead to a reaction known as *lichenification*. This is a term used to describe thickening of the skin. This reaction takes place mainly in the epidermis and it imparts a brownish appearance to the skin with an exaggeration of skin markings. The distribution of eczema on the body varies with age, affecting primarily the cheeks, forehead and scalp when it first manifests itself in infancy. At this stage it appears as a red, sometimes scaly rash. As the infant grows older the distribution on the affected regions of the body may change. In particular there is greater involvement on all surfaces of the limbs and eventually on the flexural and extensor surfaces, most notably in the elbow creases, behind the knees and around the neck. The persistence of atopic eczema into later life is most common when the infantile experience of the disease is most severe. Atopic eczema in adulthood is often located on the hands [77]. Figure 4 shows different forms of atopic eczema.



**Figure 4:** Different forms of atopic eczema: A) infantile eczema [78] and B) eczema on the hand [79].

Patients with severe atopic eczema require prolonged treatment with large amounts of highly potent topical corticosteroids, systemic treatment or both. Frequently used options for systemic treatment of atopic eczema include cyclosporine and systemic corticosteroids. Although proven effective, a proportion of patients have a contra-indication for cyclosporine treatment because of ineffectiveness or side-effects. Moreover, long-term use of cyclosporine raises concerns over toxicity. Systemic corticosteroids are used frequently to suppress exacerbations, although high-level evidence is lacking [80]. As health care costs are increasing, it is necessary to look for cheaper alternatives with less side-effects.

Oils from certain vegetables and fish can be used in the treatment of eczema because of the essential fatty acids present that influence skin physiology via their effects on the skin barrier function [81].

## **5.5 Bacterial and fungal infections**

The types of organisms that cause primary skin and soft tissue infections are diverse, and include bacterial, viral and fungal pathogens as well as parasites [82].

***Staphylococcus aureus:*** Cellulitis is a bacterial infection of the lower dermis and subcutaneous soft tissue. It is a common condition in the elderly and frequently affects the legs [82]. The

major causative organism is *Staphylococcus aureus* [83]. Furunculosis and carbunculosis are more infections that can occur because of *Staphylococcus aureus* bacteria [82].

***Pseudomonas aeruginosa:*** Necrotising fasciitis is an infection causing rapidly advancing deep tissue necrosis. It is caused by a number of organisms including gram-negative bacteria and anaerobes such as *P. aeruginosa* [82].

***Escherichia coli:*** Folliculitis is a superficial infection of the pilosebaceous follicles, usually in areas with short, coarse hair such as the scalp, neck and beard area. It is caused by *Enterobacter* species such as *E. coli*. This occurs usually in patients on prolonged courses of antibiotics.

***Trichophyton rubrum:*** *T. rubrum* are frequently the pathogens found in nail infections.

Data clearly shows the anti-bacterial and anti-fungal activity of tea tree oil [84].

## **6 Conclusion**

The skin is the biggest and most versatile organ in the body and represents almost 16% of a human's total body weight [31, 32, 33]. It assumes several important physiological functions and represents also a social interface between an individual and other members of society. This is the main reason its age-dependent modifications are in the forefront of dermatological research and of the anti-ageing cosmetic industry [85].

Because of the popularity and wide variety of skin care products, it is necessary to create products that will distinguish themselves from the rest on the market. The use of natural oils in skin care products is a new way to prevent skin ageing as well as other dermatological conditions.

Natural ingredients are continually gaining popularity and the use of plant extracts in cosmetic formulation is on the rise. A cosmetic formulation including active principles of natural origin can protect the skin against exogenous or endogenous harmful agents, and help to remedy many skin conditions. In addition, natural products can be used in hair care. Aromatic plants and oils have been used for thousands of years in cosmetics, and for their medicinal and culinary applications.

Essential oils are known for their medicinal properties and are used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and local anesthetic remedies [6]. It also imparts many other benefits such as a pleasant aroma, especially in perfumes and to impart shine or conditioning in a hair care product and for emolliency or improving the elasticity of the skin [30].

Although clinical applications of essential oils and their components have been limited, some components have been incorporated into creams, lotions, drops or liposomal formulations that are applied externally for treatment of skin diseases or for cosmetic use [22].

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## **CHAPTER 4**

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

Chapter 4 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 H. 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.

## **Physical stability and clinical efficacy against ageing of crocodile oil lotion**

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**Keywords:** crocodile oil, stability testing, clinical efficacy testing

## 1 Abstract

**Background:** The successful formulation of cosmeceutical products requires that it should be evaluated to determine its efficacy and stability over a certain time period. The positive claims of crocodile oil lotion used on ageing skin, was the motivation to determine the clinical efficacy against ageing of crocodile oil lotion. **Purpose of the study:** The aim of this study was to determine the stability of a commercial crocodile oil lotion over a period of 6 months and the anti-ageing efficacy over a period of 12 weeks. **Procedures:** Crocodile oil lotion was stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH for 6 months. The following stability tests were done: pH, viscosity, visual appearance assessment, zeta-potential, droplet size and mass loss. For the clinical efficacy testing, non-invasive electrical instruments were used to determine the skin hydration, skin surface smoothness and skin erythema as a result of the application of the formulation in human volunteers. **Results and Conclusion:** The crocodile oil lotion showed good stability over the 6 months period. The clinical efficacy study showed no positive effect of crocodile oil lotion over the placebo lotion.

## 2 Introduction

The successful formulation of cosmeceutical products requires that the products should be put under certain conditions and tested to determine their stability over a certain period of time. Like other products, the stability of cosmeceuticals must be matched to the expected period of usage as well as to the user's requirements. It is important to guarantee product quality by paying sufficient attention to the time required to distribute the product from the manufacturer to the consumer and the actual period the product will be used. It is not sufficient to simply guarantee the feeling on use and performance of a cosmeceutical; it's also important to consider the safety and stability during usage [8].

The first stage of stability testing is to establish baseline values against which changes over time can be measured by observing whether or not there are any changes in the physicochemical properties of the semi-solid formulations. Clinical changes like color change and crystallization, as well as physical changes must be investigated [8].

The purpose of stability testing is to provide evidence on how the quality of a drug substance varies with time under the influence of a variety of environmental factors such as temperature and humidity [4].

Ageing of the skin has fascinated researchers for many years. Not only to ultimately prevent wrinkle formation, but also because skin represents an excellent and accessible model organ, allowing the study of intrinsic and extrinsic factors. Chronological (intrinsic) ageing affects the skin in a manner similar to other organs. Extrinsic ageing is related to environmental, mainly UV-induced, damage of the dermal connective tissue of the skin. There is evidence that these processes, intrinsic and extrinsic ageing, have at least in part overlapping biological-, biochemical-, and molecular mechanisms [7].

Ageing causes a functional deficit in the skin through structural and molecular degradation. This degradation results in clinical changes including wrinkling, color changes, laxity and non-elasticity. Ageing dermis results in increasing stiff, inelastic tissue and is less capable of undergoing modifications in response to stress [1].

The skin of elderly persons is structurally and functionally different from that of other age groups. The epidermis is thinner and has a slower cell turnover rate, resulting in less resistance to external injury and prolonged wound healing. Changes of dermal collagen and elastin result in wrinkles. Histologically there is dermal thinning, fragmented collagen fibers, reduced elastin and fewer fibroblast and mast cells. Blood flow and sweating are reduced. Hair becomes gray and sparse and nail growth is slower. The majority of skin changes are thought to be a result of cumulative exposure to environmental factors, especially chronic UV exposure [5].

As the proportion of the ageing population in industrialized countries continues to amplify, the dermatological concerns of the elderly grow in medical importance. The rate of ageing is significantly different among different populations, as well as among different anatomical sites even within a single individual. The intrinsic rate of skin ageing in any person can also be dramatically influenced by personal and environmental factors, particularly the amount of exposure to ultraviolet light. Photo damage, which considerably accelerates the visible ageing of skin, also seriously increases the risk of cutaneous neoplasms [3].

In this study, crocodile oil cream was stored in the original packaging (50 ml HDPE plastic container) at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH for 6 months. The following stability tests were done on months 0, 1, 2, 3 and 6: pH, viscosity, visual appearance assessment, zeta-potential, droplet size and mass loss.

For the clinical efficacy testing, the focus was on the anti-ageing effects of crocodile oil lotion on the human skin. Non-invasive electrical instruments were used to determine the skin hydration, skin surface smoothness and skin erythema as a result of the application of the formulation in

human volunteers. Crocodile oil lotion and a placebo lotion (containing liquid paraffin instead of crocodile oil) were applied to the skin of the volunteers and measurements were taken. An increase in skin hydration measurements showed an increase in skin hydration. To determine skin scaliness, the RKU was determined. For this parameter to show a decrease in skin scaliness, the value had to be nearer to 3. SEW was measured to determine skin roughness and wrinkles. A decrease in this parameter showed decrease in skin roughness and wrinkles. For the determination of skin elasticity, an increase in this parameter showed an increase in skin elasticity.

### **3 Materials and methods**

#### **3.1 Materials**

Crocodile oil and 20% Crocodile oil lotion were obtained from Croc City Crocodile and Reptile Park (Johannesburg, South Africa). The Corneometer<sup>®</sup> CM 825, Visioscan<sup>®</sup> VC 98, Mexameter<sup>®</sup> MX 18, Cutometer<sup>®</sup> dual MPA 580 and Skin-pH-Meter<sup>®</sup> PH 905 were purchased from Courage-Khazaka Electronics (Cologne, Germany). Sodium Lauryl Sulphate (SLS) was purchased from Merck Laboratory Supplies (Midrand, South Africa). Deionized HPLC grade water prepared with a Milli-Q<sup>®</sup> water purification system (Millipore, Milford, USA) was used throughout the entire study. A Mettler Toledo pH meter (made in Schwerzenbach, Switzerland) and Mettler Toledo Inlab<sup>®</sup> 410 electrode was used. A Brookfield Viscometer (model DV II, Stoughton, Massachusetts, USA) with helipath (Stoughton, Massachusetts, USA) was used with a T-bar spindle (Stoughton, Massachusetts, USA). A digital camera (Blackberry<sup>®</sup> Bold 9300), Malvern Zetasizer 2000 (Worcestershire, United Kingdom) and Shimadzu (Kyoto, Japan) scale were used.

#### **3.2 Methods**

##### **3.2.1 Stability testing**

For the purpose of this article, crocodile oil cream was stored in the original packaging (50 ml plastic container) at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH for 6 months.

###### **3.2.1.1 pH**

The pH of each formulation at each condition was measured in triplicate.

### **3.2.1.2 Viscosity**

The formulation was placed in the water bath to reach a temperature of 25 °C. The T-bar spindle 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.

### **3.2.1.3 Visual appearance**

The visual appearance of each formulation was assessed by comparing the color of the cream to the initial color and appearance of the particular formulation. Photos were taken and each formulation was placed in the same position and site each time. The camera was placed approximately 15 cm away from the formulations.

### **3.2.1.4 Zeta potential**

The formulation (1 g) at each condition was weighed off in triplicate in 100 ml volumetric flasks. The cream samples were made up to volume with 0.1 M potassium chloride solution. The zeta-potential of each sample was measured by means of injecting the prepared samples into a Malvern Zetasizer 2000. The zeta-potential of the formulation at each condition was measured in triplicate.

### **3.2.1.5 Droplet size**

Approximately 0.5 g of the formulation was mixed with approximately 3 ml HPLC water to wet the formulation to a uniform wet dispersion. This was done in triplicate. These mixtures were made up with approximately 4.5 ml HPLC water, mixed well and injected into a Malvern Mastersizer 2000. 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 analyzed in triplicate 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.

### **3.2.1.6 Mass loss**

The mass of the formulation at each condition was determined in triplicate.

## **3.2.2 Clinical efficacy testing**

### **3.2.2.1 Non-invasive skin measurements**

#### **3.2.2.1.1 Skin hydration**

The Corneometer<sup>®</sup> CM 825 probe was placed vertically onto the area to be measured and wasn't moved during the measurement. A spring inside the probe head provided constant pressure on the skin. The Corneometer-probe started the measurement when in contact with the skin. A beep signaled that the measurement had been carried out successfully. The display showed the measuring value.

Repeated measurements on the same skin area led to a moisture increase due to occlusion, as water was accumulated under the probe head and could not evaporate. This water influenced the measurement and the measuring values became higher even though the water content in the stratum corneum had not changed. Therefore the measurements were repeated about 5 seconds between each measurement. The probe head was cleaned thoroughly.

#### **3.2.2.1.2 Skin topography**

The skin to be measured was marked with a stencil and pen to find the exact spot again for the next measurement. A double sided sticking ring was placed on the measuring rectangle. The camera's measuring head was removed and placed on the ring. Thereafter the camera was put back onto the measuring head and carefully positioned into the right position. Photos were taken by pushing the small button on the Visioscan<sup>®</sup>.

#### **3.2.2.1.3 Skin elasticity**

The probe was applied to the skin at a right angle and was not pressed too tightly onto the skin. Pressing too tightly could lead to disturbed blood circulation, thus influencing the measurement. The probe was held very steady during the measurement. A spring in the probe head ensured constant pressure on the skin. The probe was placed onto the marked skin area. During the first phase of the measurement the skin was sucked into the probe opening by negative pressure. Then the negative pressure was cut off and the skin relaxed in the second phase of the measurement. The probe was held still throughout both phases of the measurement.

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

The Mexameter<sup>®</sup>-probe head was placed straight onto the skin and held still on the area to be measured. The measurement was triggered by the skin contact and after one second the results for melanin and erythema were displayed accompanied by an acoustical signal.

#### **3.2.2.1.5 Skin pH**

Before starting the measurement, the pH meter's probe was washed with distilled water. Excessive water was shaken off to have a moist probe. Too much water on the membrane might influence the measuring result or delay a stable value on the screen. The probe-head was placed closely onto the skin surface. The measurement was started by pressing the button on the side of the probe. The measuring value appeared immediately.

#### **3.2.2.1.6 Vapor loss**

The Vapometer<sup>®</sup> was placed straight onto the skin and held still on the area to be measured. The measurement was triggered by the skin contact and after about 10 sec the results were displayed, accompanied by an acoustical signal.

#### **3.2.2.2 Human subjects**

This 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 healthy female subjects between 40 and 65 years of age participated in the study. 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 bars that were supplied. All participants complied to 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 [6].

#### **3.2.2.3 Treatment protocol**

##### **3.2.2.3.1 Short term study**

The treatment sites were on the dominant forearm. A group of 11 subjects participated in the study. 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 forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory according to a pre-organized time schedule. Three sites of 3.5 x 1.5 cm on the dominant arm were marked with a Codman<sup>®</sup> surgical marker. Three instruments, namely Corneometer<sup>®</sup> CM 825, Visioscan<sup>®</sup> VC 98 and Cutometer<sup>®</sup> dual MPA 580 were used. Three measurements on the three different areas were taken with the three instruments before product application and were the baseline values for each area.

After  $T_0$  measurements the cream and placebo cream were applied on the marked squares. On the third square no cream was applied. Measurements were taken after 1 hour ( $T_1$ ), 2 hours ( $T_2$ ) and 3 hours ( $T_3$ ).

### **3.2.2.3.2 Erythema study**

The treatment sites were on the dominant forearm. A group of 12 subjects participated in the study. 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 forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory according to a pre-organized time schedule. Five sites of 2.5 x 1 cm on the dominant arm were marked with a Codman<sup>®</sup> surgical marker. Three instruments, namely Mexameter<sup>®</sup> MX 18, Skin-pH-Meter<sup>®</sup> PH 905 and Vapometer<sup>®</sup> were used. Three measurements on the five different areas were taken with the three instruments before a 1% sodium lauryl sulphate (SLS) solution was applied on 4 sites using Finn Chambers. The Finn Chambers were removed after 24 hours, and 24 hours after the removal the subjects returned to the laboratory.  $T_1$  measurements were taken with the three instruments. Thereafter the placebo cream, cream and cortisone cream were applied to 3 of the 4 sites where SLS irritated the skin. 24 hours after application the subjects returned to the laboratory for  $T_2$  measurements.

### **3.2.2.3.3 Long term study**

The treatment protocol was conducted according to a comparatively similar study performed by Li *et al.* [6]. The treatment sites were on the non-dominant forearm. 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 forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory according to a pre-organized time schedule. Three sites of 3.5 x 1.5 cm on the non-dominant arm were marked with a Codman<sup>®</sup> surgical

marker. Three measurements were taken with the three instruments on the three different areas before product application and were the baseline values for each area.

During the next 12 weeks each site was treated with the assigned cream. The placebo and crocodile oil cream were applied on the marked squares twice daily, according to the double blind placebo controlled study guidelines. On the third square no cream was applied. Each subject received a treatment programme and used the placebo as well as the active formulation in the morning and evening. The amount of product put onto the marked areas was  $1 - 3 \mu\text{l}/\text{cm}^2$  ( $1 - 3 \text{ mg}/\text{cm}^2$ ). The subjects also received a timetable to document the time they applied the cream. Creams had 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. Measurements were taken after two weeks ( $T_1$ ), four weeks ( $T_2$ ), eight weeks ( $T_3$ ) and twelve weeks ( $T_4$ ).

#### **3.2.2.4 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.

### **3.3 Data analysis**

#### **3.3.1 Data analysis for clinical efficacy experiments**

All the parameters were calculated as follows:

The difference in the various skin measurements at  $T_1$ ,  $T_2$ ,  $T_3$  and  $T_4$  relative to the initial conditions ( $T_0$ ) and was taken as a percentage change between the measurements calculated for the placebo and active treatments, respectively. Thereafter, the untreated skin measurements were subtracted from the values obtained, as seen in the following formula.

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

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

### **3.4 Statistical analysis**

#### **3.4.1 Short term study**

For this study crocodile oil lotion was used as Treatment A, the placebo cream as Treatment B and UNTR refers to Untreated skin.

For skin hydration determination, a mixed model was fitted to the data since there were missing values and repeated measures were taken over both time and treatment. An AR(1) covariance structure was specified. For the determination of skin scaliness a mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed no significant difference between treatments or exposure times or interactions for time and treatment.

For skin roughness determination a mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed no significant difference between treatments or exposure times or interactions for time and treatment.

For the stipulation of skin elasticity, ANOVA analysis was performed on all Cutometer<sup>®</sup> variables to determine if difference in means between treatment groups were significant. More robust tests, the Welch and Brown-Forsythe tests, were performed in addition to the ANOVA test since the assumption of equal variances was questioned as Levene's test was insignificant at a 5% level but not a 10% level. The non-parametric Kruskal-Wallis test was also performed since some deviation from normality was observed using QQ-plots. All four tests concluded insignificant differences. Cohen's d-value was calculated for pairwise comparisons to assess the practical significance of observed differences. Practically visible differences [2] were observed between treatment B and the untreated skin for mean values of Cutometer<sup>®</sup> parameter R2 ( $d = 0.51$ ) and Cutometer<sup>®</sup> parameter R8 ( $d = 0.49$ ). However, since all participants were exposed to all treatments a mixed model was also fitted to account for repeated measures over treatment. An AR(1) covariance structure was selected, as an unstructured covariance structure showed a worse fit. Again, no significant treatment effect was found for any of the Cutometer<sup>®</sup> variables.

#### **3.4.2 Erythema study**

For this study crocodile oil lotion was used as Treatment B, the placebo cream as Treatment C, a 1% cortisone cream as Treatment D and UNTR refers to skin without irritation or treatment. Treatment A refers to irritated skin without any treatment.

For the skin erythema, skin pH and vapor loss determination, a mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis.

### **3.4.3 Long term study**

For this study crocodile oil lotion was used as Treatment A, the placebo cream as Treatment B and UNTR refers to Untreated skin.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis of skin hydration, skin roughness and skin elasticity. For stipulation of skin scaliness, a mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed no significant effects.

## **4 Results and Discussion**

### **4.1 Stability testing**

As seen in Table 1, the pH of the cream remained stable over the six months-period. Although the large decrease in viscosity may be a warning of instability, the change in the cream's viscosity was not that significant. To put it in perspective and compare the viscosity of the cream to other substances, the change was not that considerable. Water's viscosity is 1 cP whereas ethylene glycol is 24 cP, honey is 2000 cP, molasses are 5000 cP and pork fat is 100000 cP. The cream still had a good texture and could be applied without any problem. The zeta potential of the cream at all three stability controlled conditions stayed within the same range and can be seen as stable over the 6 months-period. The small decrease in mass indicated that the cream was stable over the 6 months-period and that the containers sealed sufficiently to avoid evaporation during storage periods. The cream's color stored at all 3 conditions didn't show any significant change over the 6 months-period. During the 6 months at different storage conditions, the particles in dispersion demonstrated that they adhered to one another to form aggregates and increasing in size. Although the average particles increased over the 6 months period and could show that instability was present, the increase was very small.

## **4.2 Clinical efficacy**

### **4.2.1 Short term study**

Type III fixed effects revealed significant factors for time ( $p < 0.0001$ ), treatment ( $p < 0.0001$ ) as well as interaction between time and treatment ( $p < 0.0001$ ). Multiple pairwise comparisons with Bonferroni adjustment revealed significant differences between treatment A and Untreated ( $p = 0.001$ ), treatment B and Untreated ( $p < 0.0001$ ), 30 min and 60 min ( $p < 0.0001$ ) and 30 min and 120 min ( $p < 0.0001$ ).

From Figure 1(A) it can be seen that both treatment A and B had an immediate effect on skin hydration. It can be assumed that external factors may also have contributed to the observed skin hydration levels as can be seen from the rise in skin hydration levels for the untreated areas after and hour's exposure. The effect of both treatment A and B seemed to diminish over time, however, it was difficult to explain the upward trend of treatment A after 120 min. Although both treatment A and B showed an increase in skin hydration, there was not a significant difference between the two treatments.

Figure 1(B) reveals an initial decrease in skin scaliness for both treatment A and B. It is again notable that the environment may have played a role in the observed scaliness, since untreated area also showed a decrease after 120 min. Treatment A seemed to reduce scaliness soon after application, however the effects seemed to reverse itself over time. After 180 min the untreated skin seemed to appear almost similar to the skin treated with Treatment A.

Figure 1(C) shows that after 60 min a difference was observed between the treated skin and the untreated skin (however not statistically significant). However no treatment showed a decrease and therefore it can be said that no improvement in skin roughness was observed over the 3h period.

No improvement in skin elasticity was observed over the 3h period for both treatments.

### **4.2.2 Erythema study**

For skin erythema, type III fixed effects only revealed significant differences between treatments ( $p < 0.0001$ ). Multiple pairwise comparisons with Bonferroni adjustment revealed significant differences between the four treatments and the untreated skin (all  $p$ -values  $< 0.0001$ ). However, no significant differences were found between the treatments.

For skin pH, type III fixed effects only revealed somewhat significant differences between treatments ( $p=0.063$ ). Pairwise comparisons with Bonferroni adjustment revealed a somewhat significant difference between treatment C and the untreated skin ( $p= 0.056$ ).

For skin vapor loss, type III fixed effects only revealed significant differences between treatments ( $p=0.003$ ). Pairwise comparisons with Bonferroni adjustment revealed significant differences between Untreated and treatment B ( $p=0.006$ ), Untreated and treatment C ( $p=0.025$ ) and Untreated and treatment D ( $p=0.012$ ). From Figure 2(A) it can be seen that no treatment (B, C or D) showed significant difference towards the irritated skin with any treatment (A). From Figure 2(B) it can be seen that none of the treatments (B, C or D) showed significant differences towards the irritated skin that had no treatment (A). From Figure 2(C) it can be seen that no treatment (B, C or D) showed significant difference towards the irritated skin with no treatment (A).

Therefore it can be concluded that the crocodile oil lotion didn't show any effect towards irritated skin.

#### **4.2.3 Long term study**

For skin hydration, type III fixed effects only revealed significant differences between treatments ( $p<0.0001$ ). The interaction effect between time and treatment was also significant ( $p=0.025$ ), however the F-value for treatment ( $F=23.157$ ) was higher than that for time ( $F = 1.046$ ) indicating that the interaction effect might be induced by treatment effect ( $F =2.462$ ). However from figure 4(A) the interaction did seem important. Multiple pairwise comparisons with Bonferroni adjustment revealed significant differences between Untreated and Treatment A ( $p<0.0001$ ) and Untreated and Treatment B ( $p<0.0001$ ).

For determination of skin roughness, type III fixed effects revealed a significant treatment effect ( $p=0.053$ ). Multiple pairwise comparisons with Bonferroni adjustment revealed significant differences between Treatment A and Treatment B ( $p=0.049$ ).

Table 2 provides an overview of the statistical findings of skin elasticity. For parameter R2 multiple comparisons with Bonferroni adjustment revealed significant differences between Untreated and Treatment A ( $p=0.002$ ) and Untreated and B ( $p<0.0001$ ). From Figure 3(C) it is also clear that the interaction effect could not be ignored. Multiple comparisons for parameter R6 with Bonferroni adjustment revealed significant differences as seen in Table 3. Multiple comparisons for R7 with Bonferroni adjustment revealed significant differences between

Treatment B and the untreated skin ( $p=0.007$ ). In Table 4 pairwise comparisons revealed significant differences between week 2 and the remaining weeks. Figure 4.3(D) confirms the importance of the interaction effect observed

From Figure 3(A) it can be seen that both treatment A and B had an immediate effect on skin hydration. It can be assumed that external factors may also have contributed to the observed skin hydration levels as can be seen from the rise in skin hydration levels for the untreated areas after 8 weeks exposure. The effect of both treatment A and B seemed to diminish over time, however. Although both treatment A and B showed an increase in skin hydration, there was not a significant difference between the two treatments.

Treatment B showed a significant decrease in SEW value and stayed almost the same throughout the 12 week period as seen in Figure 3(B). This showed that skin smoothness improved over the 12 week period. Treatment A showed an increase in SEW value after 2 weeks and although it changed throughout the period, the change between week 2 and week 12 was not significant. The increase in SEW value showed that skin smoothness did not improve over the 12 week period.

In Figure 3(D) a statistical difference can be seen on week 2 between Untreated skin and Treatment A and Untreated skin and Treatment B. After 4 weeks there was no significant difference between the Untreated skin and Treatment A and B. After 8 and 12 weeks, the Untreated skin showed better elasticity than Treatment A and B. It can be assumed that external factors may have contributed to the rise in skin hydration levels as seen from the hydration levels for the untreated areas after the 12 weeks exposure.

For the long term study both the crocodile oil lotion and placebo lotion showed an increase in skin hydration. After 2 weeks the skin hydration was at its highest point, and again after 12 weeks. Although both treatments showed an increase in skin hydration, there was not a significant difference between the two treatments. No treatment showed any effects toward skin scaliness. Although the crocodile oil lotion didn't show a positive effect on skin roughness, the placebo lotion showed a positive effect on skin roughness over the 12 weeks period. When looking at parameter R8 for skin elasticity, both treatments showed an increase in elasticity on week 2. However, after 4 weeks, the effects started to decrease and after 12 weeks the untreated skin showed more elasticity than the treated skin.

## **5 Conclusion**

The pH, particle size, color, zeta potential and mass loss remained stable over the 6 months-period in both containers. This shows that the original packaging of the cream was sufficient to assure stability.

The results of the short term clinical study indicated that the crocodile oil lotion, as well as the placebo lotion, increased the skin hydration, although no statistical significant difference was seen between the two treatments over 3 h. The results also indicated that both the treatments showed a decrease in skin scaliness after 60 min, but after 3 h no effect could be seen. Although both treatments showed a decrease in skin scaliness, no statistical significant difference was seen between the two treatments. Neither treatment showed any change in skin roughness and skin elasticity over the 3 h period. The results obtained for the erythema clinical study clearly showed that none of the treatments showed any significant difference towards the non-treated, irritated skin when looking at the skin redness, skin pH and vapor loss of the skin. From the results of the long term clinical study, followed that the crocodile oil lotion, as well as the placebo lotion, increased the skin hydration, although no statistical significant difference was seen between the two treatments over 12 weeks. The biggest increase in skin hydration could be seen after the first two weeks of treatment. No improvement could be seen in skin scaliness and skin elasticity after 12 weeks with both treatments. Crocodile oil lotion also didn't improve skin roughness over the 12 weeks-period.

It would thus be concluded that the Crocodile oil lotion is stable but did not have any statistically significant benefits above the use of the placebo lotion in the treatment of ageing skin.

## **6 Acknowledgements**

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**Tables:**

**Table 1:** The change in physical properties at different conditions of Crocodile oil lotion over a 6 months-period

	<b>25 °C/60% RH</b>	<b>30 °C/60% RH</b>	<b>40 °C/75% RH</b>
<b>pH</b>	-1.4%	-1.76%	-3.28%
<b>Viscosity</b>	-10.9%	-32.1%	-46.0%
<b>Zeta potential</b>	-2.5%	-5.0%	+6.5%
<b>Mass loss</b>	-0.56%	-0.63%	-0.73%
<b>Droplet size</b>	2.6 µm	2.2 µm	2.8 µm

**Table 2:** Cutometer parameters and their respective p-values

<b>Measure</b>	<b>Comparison</b>	<b>p-value</b>
<b>R2</b>	Time Effect (Type III)	0.124
	Treatment Effect (Type III)	0.00001*
	Interaction Effect (Type III)	0.065*
<b>R6</b>	Time Effect (Type III)	0.498
	Treatment Effect (Type III)	0.00001*
	Interaction Effect (Type III)	0.730
<b>R7</b>	Time Effect (Type III)	0.362
	Treatment Effect (Type III)	0.008*
	Interaction Effect (Type III)	0.235
<b>R8</b>	Time Effect (Type III)	0.00001*
	Treatment Effect (Type III)	0.208
	Interaction Effect (Type III)	0.00001*

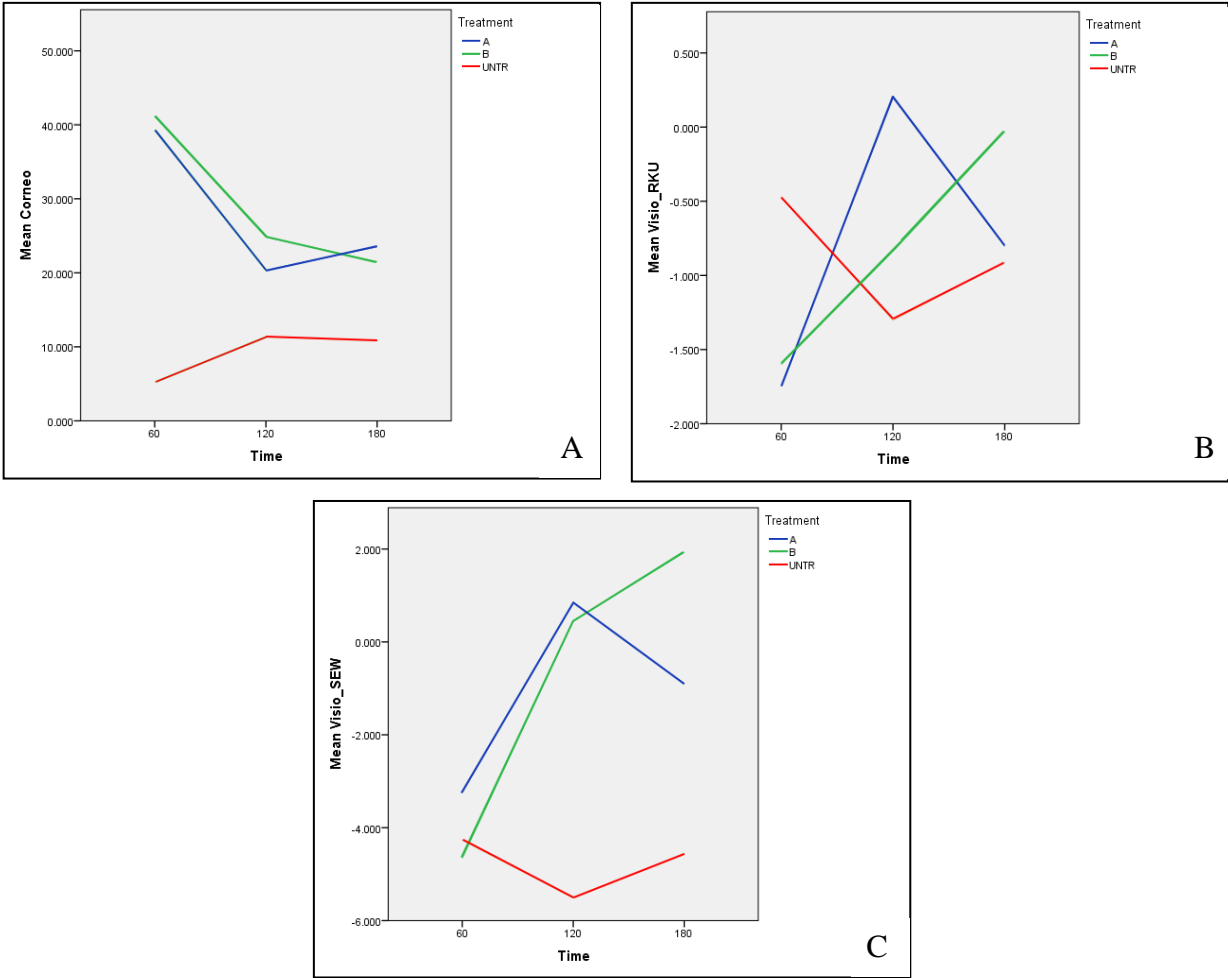
**Table 3:** Cutometer parameter R6 and their respective p-values

<b>Comparison</b>	<b>p-value</b>
A vs B	0.014
A vs UNTR	0.061
B vs UNTR	0.0001

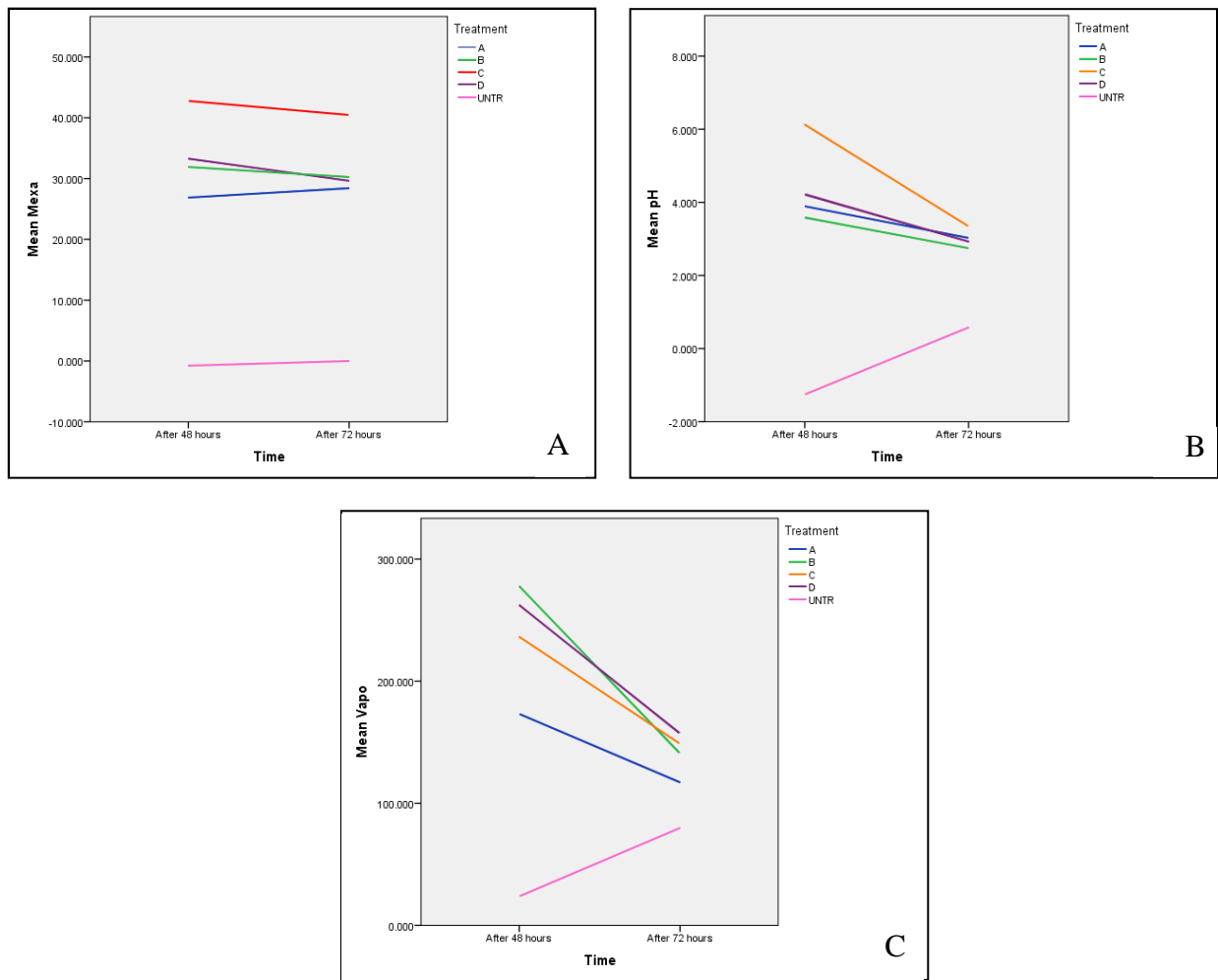
**Table 4:** Pairwise comparisons between treatment weeks

<b>Week</b>	<b>Sig</b>
2 vs 4	0.00001
2 vs 8	0.001
2 vs 12	0.009

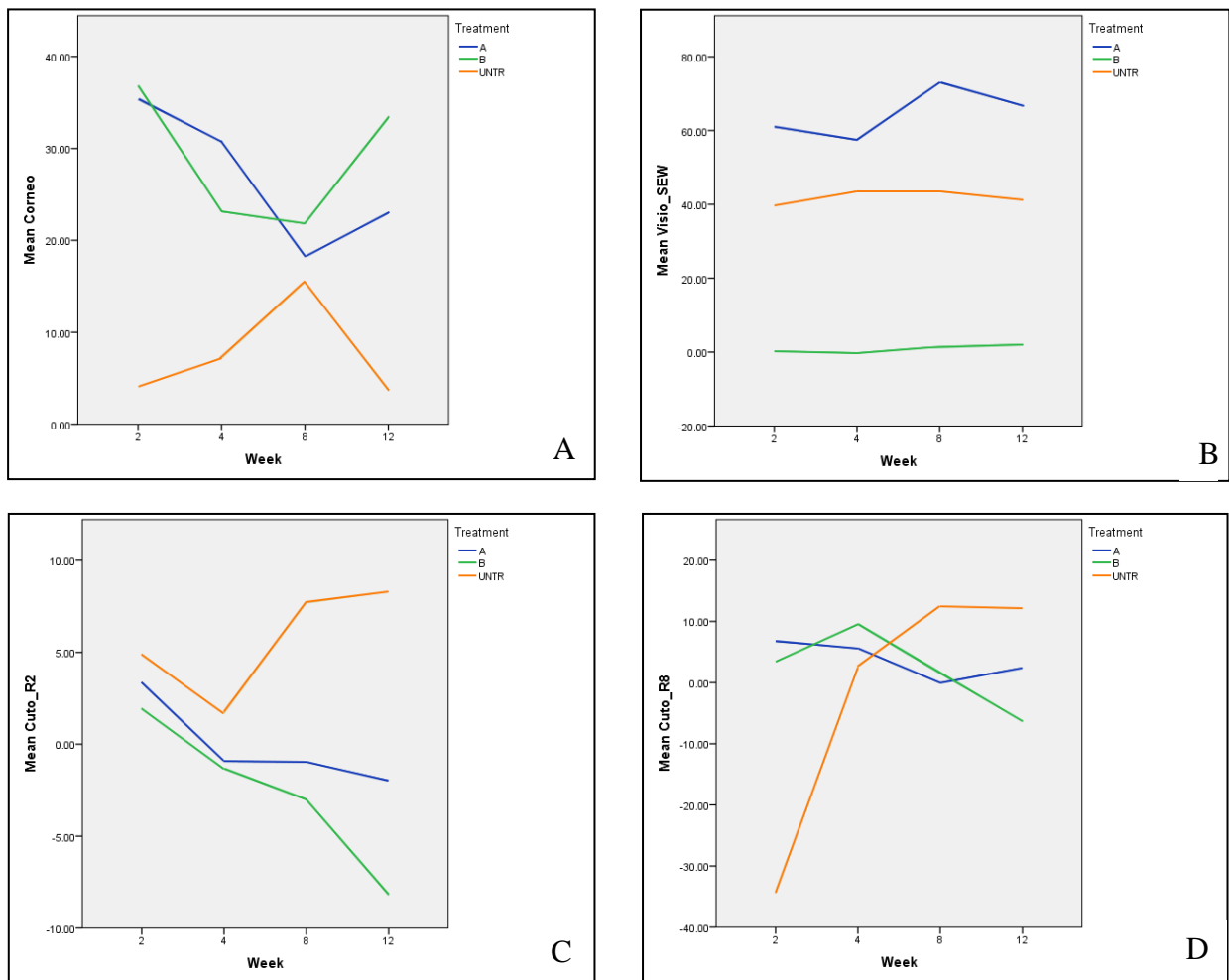
**Figures:**



**Figure 1:** % Change in skin hydration (A), skin scaliness (B) and skin roughness (C) over 180 min for short term study



**Figure 2:** % Change in skin redness (A), skin pH (B), and skin Vapometer<sup>®</sup> readings (C) over 72 h for erythema study



**Figure 3:** % Change in skin hydration (A), skin roughness (B), Cutometer<sup>®</sup> readings for parameter R2 (C) and R8 (D) over 12 weeks for long term study

## **CHAPTER 5**

### **ARTICLE FOR PUBLICATION IN THE *JOURNAL OF NATURAL MEDICINE***

Chapter 5 is written in an article format for the purpose of publication in the *Journal of Natural Medicine*. The complete guide for authors of this journal is given in Appendix H. For publishing purposes Chapter 5 contains no formatting, except for double-line spacing and font specification written in Times New Roman 12 as described by the instruction 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.

## **Crocodile oil: Investigation into chemical composition and biological effects**

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## **Abstract**

**Context:** There is a growing interest in the use of natural products. Recently, crocodile oil and crocodile oil containing products emerged and claims have been made that it can be used for treatment of ageing skin and various other skin diseases. **Objective:** This study focused on the determination of the fatty acid profile, anti-bacterial, anti-fungal and anti-oxidant properties and dermal toxicity effects of crocodile oil. **Materials/Methods:** Gas chromatography (GC) was employed for the quantitative analysis of the fatty acid methyl esters of palmitic acid, stearic acid, palmitoleic acid, oleic acid, linoleic acid and linolenic acid. Anti-bacterial and anti-fungal tests were performed to investigate the anti-fungal and anti-bacterial properties of crocodile oil and -lotion. Thiobarbituric acid (TBA) assay was used to investigate the anti-oxidant properties. Skin sensitization, acute dermal toxicity and dermal irritation potential studies were done to inspect the toxicity of crocodile oil lotion. **Results and discussion:** The fatty acid analysis showed that crocodile oil compared well to human skin oil. Crocodile oil and crocodile oil lotion presented no anti-fungal, anti-bacterial or anti-oxidant properties. Toxicity studies showed that crocodile oil lotion had no skin sensitization, dermal toxicity and dermal irritation effects. **Conclusion:** Results obtained for the fatty acid composition of crocodile oil in this study compared well to values found in literature. Crocodile oil and crocodile oil lotion presented no anti-fungal, anti-bacterial or anti-oxidant properties. Skin sensitization, dermal toxicity and dermal irritation studies on crocodile oil lotion showed that it is a safe product to use.

**Keywords:** crocodile oil, crocodile oil lotion, anti-oxidant, toxicity studies, fatty acid profile

## 1 Introduction

There are claims that crocodile oil and crocodile oil containing products can be used for treatment of dry, flaky, itchy and flocking skin and pigmentation of skin-like brown spots, freckles and menopausal darkening like in ageing [1].

The ageing process is a very complex biological process. Factors contributing to the ageing process include external influences such as nutrition, smoking, alcohol, environmental conditions as well as individual genetic factors. Particular attention in this respect has been paid to the biological action of free radicals, especially to oxygen species like  $\text{OH}^\bullet$  (hydroxyl radicals), which are causing oxidative stress. It appears that these radicals, in addition to the nitrogen oxides, are one of the major factors for a forced ageing, DNA-damage and for a number of other diseases [2]. Therefore the anti-oxidant activity of crocodile oil and crocodile oil lotion was determined to identify whether the positive anti-ageing results of the products are because of the anti-oxidant activity or maybe because of a different working mechanism.

An anti-oxidant is defined as “any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” [3].

Ageing is a complicated process where recent evidence suggests that several of its most important mechanisms are linked by means of cellular damage caused by reactive oxygen species (ROS). Oxidative damage may be a major factor in the loss of physiological functions that occur in ageing. This is because in aerobic organisms the mitochondrial electron transport chain plays an important role in energy production and is a significant source of ROS that damage DNA, RNA, and proteins in cells [4].

Although aerobic lifestyles are advantageous in many ways, the utilization of oxygen by cells for many of their biochemical reactions, results in the formation of highly reactive free radical products. These free radicals and products of free radical reactions can cause reversible and

irreversible damage to macromolecular targets such as DNA, proteins, and cellular membranes. A particularly important consequence of free radical damage in many cells is the peroxidation of poly-unsaturated fatty acids (PUFAs), which results in the formation of lipid peroxides and aldehydes. These products can cause extensive damage to membrane structure and integrity, and several lines of evidence have implicated lipid peroxidation with chemical and physical changes within membranes [5].

Several methods have been developed to measure the free radical scavenging capacity (RSC), regardless of the individual compounds which contribute towards the total capacity of a natural product in scavenging free radicals. The methods are typically based on the inhibition of the accumulation of oxidized products, since the generation of free radical species is inhibited by the addition of anti-oxidants and this gives rise to a reduction of the end point by scavenging free radicals. The reliable method to determine RSC, involves the measurement of the disappearance of free radicals or other colored radicals with a spectrophotometer [6]. Free radicals produced by radiation, chemical reactions and several redox reactions of various compounds may contribute to protein oxidation, DNA damage, lipid peroxidation in living tissues and cells [7].

To determine the anti-oxidant properties of crocodile oil, it is necessary to perform an assay illustrating this mechanism of action. In this study, the thiobarbituric acid (TBA)-assay demonstrates how the compound scavenges  $\text{OH}^\bullet$ . Malondialdehyde (MDA) which is a major degradation product of lipid hydroperoxides was the marker for assessing the extent of lipid peroxidation. MDA is of great concern because it has been shown to be part of pathological processes in the formation of pigments which is typical in ageing [8].

In this study, the most commonly used method for measuring MDA in biological samples was used, namely the TBA test. This method is based on spectrophotometric quantification of the pink complex formed after reaction of MDA with two molecules of TBA.

For the toxicity studies the Series 870 Health Effects test guidelines have been harmonized between the Office of Pesticide Programs (OPP) and the Office of Prevention, Pesticides, and Toxic Substances (OPPTS) and, where possible, with the Organization for Economic Co-operation and Development (OECD) [9], [10], [11] and South African test guidelines. The Ethics Committee of the Tshwane University of Technology verified that the animal facility operated within the standards and rules of the National Laboratory Animal Ethical Code of Conduct and that the animals were kept according to recognized international standards in animal husbandry practice. This study was conducted according to the Environmental Protection Agency (EPA) Guidelines and complied with registration requirements in South Africa, USA and Europe.

Because of crocodile oil's anti-ageing, anti-fungal and anti-bacterial effects claimed by crocodile oil suppliers and due to the fact that no scientific data is available for crocodile oil, it was decided to investigate the claims.

Anti-oxidant studies were done to investigate the anti-ageing effects. Anti-microbial and anti-fungal tests were done to examine claims of acne treatment and fungal infections treatment. The fatty acid profile of crocodile oil was determined to compare it to the fatty acid profile of human skin oil and dermal toxicity studies were performed to identify whether crocodile oil lotion had no toxic effects like claimed.

The purpose of the study was to:

- identify the fatty acid profile of crocodile oil,
- determine the anti-oxidant, anti-bacterial and anti-fungal properties of crocodile oil and crocodile oil lotion as well as
- the potential of crocodile oil to induce skin sensitization, acute dermal toxicity and dermal irritation.

## **2 Materials and Methods**

### **2.1 Materials**

Ascorbic acid (vitamin C), dimethyl sulphoxide (DMSO) and iron(III)chloride ( $\text{FeCl}_3$ ) were purchased from Merck Chemicals (Wadeville, Gauteng, South Africa). 1,1,3,3-Tetramethoxypropane (98%) (TEP), 2-thiobarbituric acid (98%) (TBA), butyl hydroxy toluene (BHT), trichloroacetic acid (TCA) and trolox (vitamin E) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide was purchased at a local pharmacy. Deionized HPLC grade water was used throughout the study (Elix 5 and Milli-Q academic purification systems, Millipore, Johannesburg). All other chemicals and reagents used were of the highest chemical purity.

### **2.2 Fatty acid methyl ester analysis with gas chromatography**

#### **2.2.1 Preparation of sample**

The crocodile oil was transesterified using trimethylsulphonium hydroxide (TMSH) [12] and analyzed by GC as described below. The oil sample was dissolved in 200  $\mu\text{L}$  chloroform to which 200  $\mu\text{L}$  TMSH was added. The sample was then made up to volume (2.5 ml) with methanol. The sample was placed on a vortex to ensure complete mixture of the sample before being injected onto the GC. A 0.5  $\mu\text{l}$  sample was injected in duplicate onto the GC, with a split ratio of 1:100 and an inlet port temperature of 230  $^\circ\text{C}$ .

#### **2.2.2 Gas chromatography conditions**

Fatty acid methyl esters (FAME) were analyzed with a Shimadzu 2010 gas chromatograph in a controlled laboratory environment at 25  $^\circ\text{C}$ . The instrument was controlled and results processed using Shimadzu GC solution software. A SGE BP x 70 glass capillary column with inner diameter of 0.32 mm, film thickness of 0.25  $\mu\text{m}$  and length of 60 m was used. Column oven temperature was programmed from 180  $^\circ\text{C}$  held for 1 min, increased to 225  $^\circ\text{C}$  at a rate of 10  $^\circ\text{C}/\text{min}$  held for 4 min and finally increased at a rate of 10  $^\circ\text{C}/\text{min}$  to 256  $^\circ\text{C}$  held for 1 min.

Nitrogen was employed as carrier gas at a flow rate of 1.07 ml/min. Peaks were detected with a flame ionization detector at a temperature of 310 °C.

### **2.3 Anti-bacterial and anti-fungal properties of crocodile oil and crocodile oil lotion**

Anti-microbial and anti-fungal tests were done by Envirocare Laboratories, North-West University, Potchefstroom, South Africa. *Staphylococcus aureus*, *Esterichia coli*, *Pseudomanas aeruginosa*, *Candida albicans*, *Brasiliensis*, *Propionibacterium acnes* and *Trichophyton rubrum* cultures were used to determine the anti-microbial and anti-fungal activity of crocodile oil. The choice of bacterial populations that were used in this study relied on the assumptions made by the manufacturers of the crocodile oil-containing products.

### **2.4 Anti-oxidant properties of crocodile oil and crocodile oil lotion**

#### **2.4.1 Preparation of materials used in anti-oxidant determination**

BHT (0.5 g/L) was dissolved in methanol; TCA (10.00%) and TBA (0.33%) were prepared in water. Due to the fact that TBA is light sensitive, it was always freshly prepared and protected from light by covering the container with aluminum foil.

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 5 mM) was used as the toxin to generate OH<sup>°</sup> and initiate lipid peroxidation in the rat brain homogenates [13]. Ascorbic acid (1.4 mM) and FeCl<sub>3</sub> (4.88 mM) were added to increase the generation of OH<sup>°</sup>.

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

#### **2.4.2 Preparation of crocodile oil samples**

Four different concentrations of crocodile oil were prepared by dissolving it in methanol to give 10, 30, 50 and 90% (v/v) concentrations. The crocodile oil lotion was prepared by dissolving the lotion in methanol to make a 50% concentration.

#### **2.4.3 Preparation of PBS (pH 7.4) buffer for anti-oxidant activity determination**

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.

#### **2.4.4 Test animals for anti-oxidant determination**

*In vitro* experiments were performed on whole rat brain homogenates from adult male Sprague-Dawley albino rats weighing between 200 and 250 g. The animals were housed in a windowless, well-ventilated constant environment room (CER) 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 minimized 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.

#### **2.4.5 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 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 UV-visible spectrophotometer. The absorbance of the TBA/MDA-complex was plotted against the known concentration of MDA.

#### **2.4.6 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 homogenized by a manual glass Teflon Homogenizer in 0.1 M PBS (pH 7.4) to give an ultimate concentration of roughly 10% (w/v).

#### **2.4.7 TBA-assay for anti-oxidant determination of crocodile oil and crocodile oil lotion**

To establish the potential antioxidant activity of extracts, 1 ml rat brain homogenate containing toxin combination and varying concentrations of the oil and lotion were incubated in an oscillation water bath (GFL 1083, Burgwedel, Germany) 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), 1.0 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 [14]. 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. Thereafter the absorbance was determined at 532 nm.

#### **2.5 Determination of the dermal toxicity potential of crocodile oil lotion**

In order to determine the possible dermal toxicity of crocodile oil, the skin sensitization, acute dermal toxicity and dermal irritation potential were investigated. The experiments were

conducted according to the Environmental Protection Agency (EPA) Guidelines and complied with registration requirements in South Africa, USA and Europe.

The principles of the following guidelines were also incorporated in this study:

- Guidelines for the Registration of Botanical Pesticides, Quality Assurance and Toxicology: South African Departments of Agriculture and Health; 2003 [15].
- Guidelines for Industry, Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies: March 1, 1995 (60 FR 11264) CDER, Rockville, MD, USA [16].
- Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals. Guideline 406: Skin Sensitization (July 1992) [17].
- The Assessment of Systemic Exposure in Toxicity Studies: CDER, Rockville, MD, USA [18].
- Guidelines for the Registration of Disinfectants, Quality Assurance and Toxicology: South African Departments of Agriculture and Health.

The following experiments were performed:

#### 1. Skin sensitization

The purpose of the study was to identify whether the test substance has skin sensitization potential. Determination of the potential to cause or elicit skin sensitization reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity. Information derived from skin sensitization tests serves to identify possible hazards to a population exposed repeatedly to a test substance.

#### 2. Acute dermal toxicity

The purpose of the study was to identify whether the test substance has dermal toxicity potential. Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a substance or multiple doses given within a 24 h period. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity

is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from short-term exposure by the dermal route. Data from an acute study may serve as a basis for classification and labeling. It is an initial step in establishing a dosage regimen in sub chronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route. An evaluation of acute toxicity data will include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

### 3. Acute dermal irritation

The purpose of the study was to identify whether the test substance has dermal irritation potential. Determination of the irritant and/or corrosive effects on the skin of mammals is useful in the assessment and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance. This test is designed to serve as a quality control test for newly produced products.

#### **2.5.1 Ethics approval**

The Ethics Committee of the Tshwane University of Technology verified that the animal facility operated within the standards and rules of the National Laboratory Animal Ethical Code of Conduct and that the animals were kept according to recognized international standards in animal husbandry practice. The protocol was submitted to the Animal Ethics Committee of Tshwane University of Technology for ethical committee approval, but as this is a study required by regulatory authorities and was done according to EPA/OECD protocols, the committee took notice thereof. No official approval was needed.

## **2.5.2 Housing conditions of animals used in toxicity determination**

The rats were acclimatized in the animal unit of La-Bio Research, Pretoria, South Africa, for a period of 5 days before the start of the study at a room temperature of between 19-23 °C and a humidity of 45-75%. A 12 h day/night light cycle was a constant in the animal unit. The light intensity was kept between 70-100 Lux. The animals were kept in a micro-barrier unit consisting of individually ventilated cages. The rats were housed in cages where they could conduct their species-specific behavior. The physical size of these cages was in accordance with European standards. Water, food and bedding were autoclaved before being used in the cages. Rat/mice feed procured from Epol<sup>®</sup> (Randburg, South Africa) were fed to the animals. Each animal was assigned a unique identification number. A system to randomly assign animals to test groups and control groups was used.

## **2.5.3 Experimental design**

The experimental design is described in Tables 1 and 2.

## **2.6 Data analysis**

### **2.6.1 Data analysis for anti-oxidant 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.

## **2.7 Statistical analysis**

### **2.7.1 Statistical analysis for anti-oxidant 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. Difference between groups was considered to be significant when  $p < 0.05$  when compared to the toxin (#). When  $p < 0.001$  it was considered extremely significant. When  $p <$

0.01 it was considered fairly significant, while  $p < 0.05$  was considered as significant. A  $p > 0.05$  was considered to be not significant (ns).

### **3 Results and Discussion**

#### **3.1 Fatty acid methyl ester analysis with gas chromatography**

Identification of FAME peaks in the samples was made by comparing the relative retention times of FAME peaks from samples to those of standards obtained from SIGMA. Results of the percentage fatty acid composition from the GC for crocodile oil are shown in Table 3.

From the data collected, it could be seen that crocodile oil presented high levels of oleic acid (41.4%), medium levels of linoleic- (22.9%) and palmitic acid (22.5%) and low levels of stearic- (5.0%), palmitoleic- (7.2) and linolenic acid (0.45%). In Table 4 the percentage fatty acid composition in crocodile oil to human skin oil are compared [1].

The composition of fatty acids in crocodile oil compared well to fatty acids found in human skin oil [1]. Palmitoleic acid, oleic acid and linoleic acid presented with much higher concentrations of each fatty acid in crocodile oil than in human skin oil. Palmitic acid found in crocodile oil showed a slightly higher percentage than found in human skin oil and stearic acid in crocodile oil was much lower than found in human skin oil.

#### **3.2 Anti-bacterial and anti-fungal properties of crocodile oil and crocodile oil lotion**

No anti-bacterial and anti-fungal activity was observed.

#### **3.3 Anti-oxidant properties of crocodile oil and crocodile oil lotion**

When comparing the different concentrations of the crocodile oil with the toxin in Figure 1, it was evident that the oil concentrations 10, 30, 50 and 90% did not exhibit sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $1.01 \pm 0.12$  nmole/mg), consecutively showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicated a higher degree of lipid peroxidation and lower  $\text{OH}^\circ$  radical scavenging abilities of the extracts. When comparing the different concentrations of the

crocodile oil with the toxin it was clear that none of the concentrations showed any potential antioxidant activity by means of decreasing the MDA formation.

When the different concentrations of crocodile oil were compared with trolox ( $0.32 \pm 0.06$  nmole/mg) it was clear that none of the concentrations showed their own antioxidant activity, as none of the concentrations was below trolox.

### **3.4 Dermal toxicity of crocodile oil lotion**

#### **3.4.1 Skin sensitization of crocodile oil lotion**

Two animals were euthanized because of skin mutilation (No 9 on day 20 & no.24 on day 21). Animal weights indicated that the animals in both the test and control group maintained or gained weight over the study period. This showed that the test substance had no general adverse affect on the animals' health.

Skin sensitivity assessment showed that the test substance caused no skin sensitization in the animals tested.

#### **3.4.2 Acute dermal toxicity of crocodile oil lotion**

No signs of clinical abnormalities were observed during the study. All animals gained weight according to normal standards for Sprague Dawley rats in the laboratory. No macro pathological abnormalities were observed.

#### **3.4.3 Acute dermal irritation of crocodile oil lotion**

All 3 animals showed no signs of erythema or edema after patch removal and at any time of observation thereafter (Table 4). The control regions on the animals (where no test substance was added) showed no symptoms of either erythema or edema. The study was terminated after 72 h as it was unnecessary to continue because of the absence of any irritation. It is therefore concluded that the Crocodile lotion causes no dermal irritation.

#### 4 Conclusion

Identification of the relative retention times of FAME peaks from the samples were compared to those of standards obtained from SIGMA, to ensure authenticity of the oils. This was a good indication of the reliability of the method used and the suitability for the intended purpose.

Crocodile oil and crocodile oil lotion showed no anti-bacterial and anti-fungal activity.

Crocodile oil concentrations of 10, 30, 50 and 90% as well as the 50% lotion did not show *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 demonstrated a higher level of lipid peroxidation and the inability to effectively scavenge OH<sup>o</sup> radicals. Although there is no literature available about the anti-oxidant properties of crocodile oil, there are data showing that other animal oils, like fish oil, showed anti-oxidant activity. This anti-oxidant activity showed effects on lipid metabolism and significant interest has arisen regarding the potential of fish oils as therapeutic agents in the management of patients with vascular diseases. Fish oils may be concerned in the development of atherosclerosis because of their potential impact upon lipid peroxidation [19].

However, it is very important to realize that the assay described in this article is strictly based on chemical reactions and bear no similarity to biological systems. The validity of the data is limited to a strict chemical sense with no context interpretation [20]. Due to the complexity of the composition of biological samples, it is very difficult separating each antioxidant and studying it individually [20].

The skin sensitization, dermal toxicity and dermal irritation studies on crocodile oil lotion showed no toxicity and therefore is a safe and harmless product to use.

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**Declaration of interest**

The authors report no declaration of interest. The authors would like to express their appreciation towards the National Research Foundation (NRF) of South Africa and the Unit for Drug Research and Development of the North-West University, Potchefstroom Campus, South Africa for the financial support.

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**Tables:****Table 1:** Animals used in dermal toxicity testing

<b>Animals</b>	<b>Skin sensitization</b>	<b>Acute dermal toxicity</b>	<b>Acute dermal irritation</b>
Species	Duncan Hartley Guinea Pig	Sprague Dawley Rats	New Zealand White Albino Rabbit,
Source	South African Vaccine Producers (Pty) Ltd, Johannesburg, South Africa	South African Vaccine Producers (Pty) Ltd, Johannesburg, South Africa	Durheim Rabbit Centre, Delmas
Weight	450-820 g	150-200 g	Approximately 3.5 kg
Age	9 weeks	Between 8 and 12 weeks	16 to 20 weeks
Sex	Males	Females. Females were nulliparous and not pregnant	Males
Number	30	15	3

**Table 2:** Experimental design of dermal toxicity testing

Procedures	Skin sensitization	Acute dermal toxicity	Acute dermal irritation
Test groups	Two groups: <ul style="list-style-type: none"> <li>• Test group: 20 male animals</li> <li>• Negative control: 10 male animals.</li> </ul>	Three test groups with 5 animals in each group.  Dosage groups: 5000, 2000 & 1000 mg/kg.	Three animals in one test group.
Dosage	0.5 ml administered via a closed patch to the test group on day 0, 8 and 14.  Negative control group: bare patch with 0.5 ml distilled water.  Day 28 (test and control groups): test substance (0.5 ml) administered to an area adjacent to the induction area via a closed patch for 6 h.	Animals weighed immediately before dosing.  Dosages calculated at 5000, 2000 & 1000 mg/kg.	0.5 ml of test substance applied, covered with gauze and non-irritation, non-porous plaster.  Test site: small area (approximately 6 cm <sup>2</sup> ) of skin and covered with a gauze patch.
Exposure	Fur clipped from the dorsal area of the trunk of the animals 24 h before dosing. Topical	Fur clipped from the dorsal area of the trunk of the animals 24 h before dosing. Test substance	Fur clipped from the dorsal area of the trunk of the animals 24 h before dosing. Test

	administration via a closed patch on days 0, 8, and 14 for induction. Topical challenge of the untreated flank for 6 h on day 28.	applied uniformly (approximately 10% of the body surface area). Observation after 24 h.	substance applied to three animals. Exposure period of 4 h.
Observation	Animals observed daily for normal vitality and weighed weekly.	Animals observed every hour for the first 4 h and thereafter daily for 14 days and were weighed weekly.	Observations for skin irritation were done at 1, 24, 48 and 72 h.
Clinical examination	Animals examined directly after removal of the induction patch and 24 and 48 h after removal of the challenge patch and the responses scored and recorded according to the grades in the Table 3.	Observations included the following evaluation criteria: <ul style="list-style-type: none"> <li>• skin and fur;</li> <li>• eyes;</li> <li>• mucus membranes;</li> <li>• respiratory and circulatory effects and</li> <li>• autonomic effects.</li> </ul>	Animals were examined for dermal irritation and was scored according to the grades in Table 3 within 30-60 min, and at 24, 48, 72 h after removal of the patch.

**Table 3:** GC results of the fatty acid composition in percentage ( $\% \pm SD$ ,  $n = 4$ ) of crocodile oil compared to human skin oil values as obtained from literature [1].

	Saturated fatty acids		Mono-unsaturated fatty acids		Poly-unsaturated fatty acids	
	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	Linolenic
	16:0	18:0	16:1	18:1	18:2	18:3
<b>Crocodile oil</b>	22.5 $\pm$ 1.2	5.3 $\pm$ 0.2	7.2 $\pm$ 0.3	41.4 $\pm$ 0.2	22.9 $\pm$ 0.7	0.5 $\pm$ 0.3
<b>Human skin oil</b>	20.2	11.2	3.8	30.8	15.1	0.3
<b>Canola oil (standard)</b>	4.3	2.0	0.0	55.4	29.0	9.7

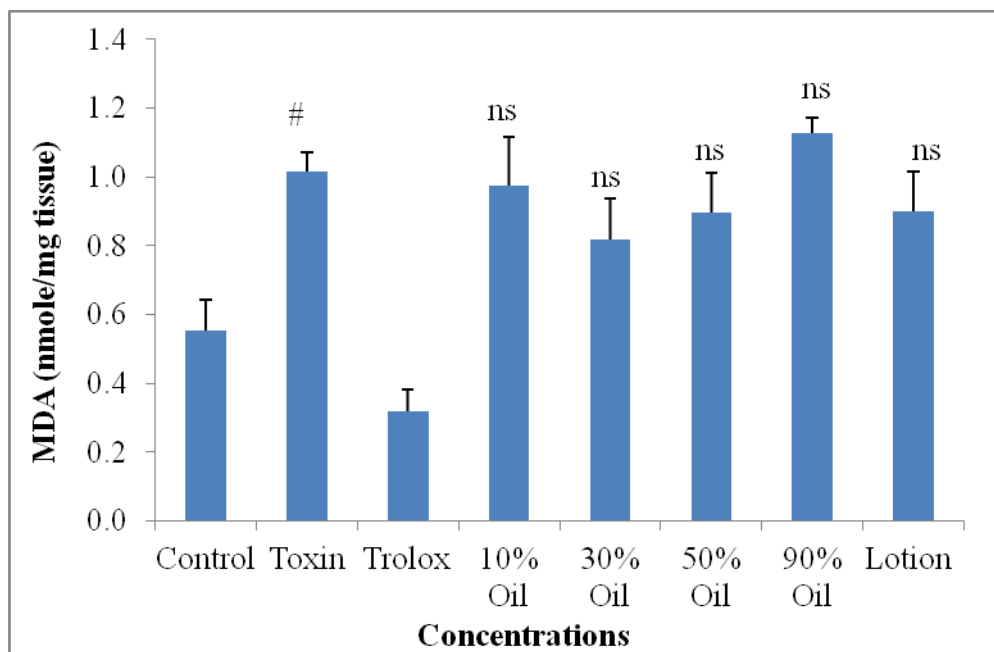
**Table 4:** Scale for evaluation of skin reaction for skin sensitization and dermal irritation

	Value
<b>Erythema and Eschar or mation:</b>	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible	5
<b>Edema formation:</b>	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Maximum possible	5

**Figure legends:**

**Figure 1:** The attenuation of lipid peroxidation by different concentration of crocodile oil in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). ns (p > 0.05) vs. toxin (#).

**Figures:**



**Figure 1:** The attenuation of lipid peroxidation by different concentration of crocodile oil in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. (n = 5). ns (p > 0.05) vs toxin (#).

## CHAPTER 6

### FINAL CONCLUSIONS AND FUTURE PROSPECTS

There are many claims of positive results when crocodile oil containing products have been used. It includes fading of freckles, acne, pimple marks, dark lines, wrinkles and laugh lines. Because of the similar composition as human skin oil, crocodile oil will thus be a very popular and safe product to use (Croc city, 2012).

Crocodile oil is obtained from the fat of the Nile crocodile (*Crocodylus niloticus*). According to Magnino *et al.* (2009:164) the Nile crocodile is native to Africa and can reach up to 7 m in length. It is Africa's largest crocodilian and can weigh up to 730 kg.

Crocodile oil has the same composition as human skin oil. It only differs with regard to the percentages of the ingredients present. Crocodile oil contains saturated and unsaturated fatty acids. Because of the similar composition as human skin oil, crocodile oil will rarely be allergenic when applied to human skin and therefore will be a very accepted and safe product to use (Croc city, 2012).

Because of crocodile oil's anti-ageing, anti-fungal and anti-bacterial effects claimed by crocodile oil suppliers and due to the fact that few scientific data is available for crocodile oil, it was decided to investigate the claims.

In this study the aims and objectives were to use natural oil, namely crocodile oil, and investigate the following:

- fatty acid profile of crocodile oil;
- anti-microbial and anti-fungal activity of crocodile oil and crocodile oil lotion;
- anti-oxidant activity of crocodile oil and crocodile oil lotion;
- toxicity studies of crocodile oil lotion;
- stability determination of crocodile oil lotion and
- clinical efficacy testing of anti-ageing effects.

The fatty acid profile of crocodile oil was determined by FAME analysis with GC. Identification of FAME peaks in the samples was made by comparing the relative retention times of FAME peaks from samples to those of standards obtained from SIGMA. The composition of fatty acids in crocodile oil compared well to fatty acids found in human skin oil.

Anti-microbial and anti-fungal tests were done by Envirocare Laboratories, North-West University, Potchefstroom. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Brasiliensis*, *Propionibacterium acnes* and *Trichophyton rubrum* cultures were used to determine the anti-microbial and anti-fungal activity of crocodile oil. Unfortunately no activity was observed.

The anti-oxidant properties of crocodile oil and crocodile oil lotion were determined by using the most commonly used method for measuring MDA in biological samples namely the TBA test. This method is based on spectrophotometric quantification of the pink complex formed after reaction of MDA with two molecules of TBA. No anti-oxidant activity was seen in the oil as well as the lotion.

Toxicity studies showed that the lotion showed no toxicity in the skin sensitisation, acute dermal toxicity and acute dermal irritation studies.

Stability tests were done of the crocodile oil lotion over a 6 months-period. The formulated products were stored under different conditions namely, 25 °C / 60% RH, 30 °C / 60% RH and 40 °C / 75% RH in the original packaging as well as a glass container. The pH, viscosity, visual appearance, zeta-potential, droplet size and mass loss were determined on months 0, 1, 2, 3 and 6. The crocodile cream lotion was stable over the six months period.

Clinical efficacy testing of the anti-ageing effects of crocodile oil demonstrated that both the crocodile oil lotion and placebo lotion showed an increase in skin hydration in the short term study as well as the long term study. There was however no significant difference between the two treatments.

Due to the lack of scientific data available for crocodile oil, this study can be considered as a great contribution to the cosmetic and pharmaceutical field of study. Although the claims of positive results couldn't be proofed in this study, it can still be considered as positive input into the study of crocodile oil.

Future prospects for further investigation include the following:

- chemical stability testing of crocodile oil lotion;
- anti-oxidant studies on incubated skin cells;
- anti-inflammatory activity of crocodile oil;
- clinical efficacy testing where skin temperature before each measurement are taken and
- clinical studies on patients with skin disorders.

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# APPENDIX A

## FATTY ACID PROFILE OF CROCODILE OIL

### A.1 Introduction

Fatty acids are usually insoluble in water and are sometimes referred to as fixed oils or fats. Fatty acids are very important as formulation agents and vehicles in pharmacy and as components of cosmetics and soaps (Heinrich *et al.*, 2004:65). To determine the fatty acid composition of crocodile oil in this study, gas chromatography (GC) was employed for the total quantitative analysis of the fatty acid methyl esters of:

- palmitic acid (C16:0);
- stearic acid (C18:0);
- palmitoleic acid (C16:1);
- oleic acid (C18:1);
- linoleic acid (C18:2) and
- linolenic acid (C18:3).

Different analytical techniques have been used for studying the profile of oils namely thin layer chromatography, reserved-phase high-performance liquid chromatography and high-temperature gas-liquid chromatography (Cunha & Oliveira, 2006:518). In this study GC was used.

### A.2 Method

#### A.2.1 Fatty acid methyl ester analysis with gas chromatography

The method for fatty acid analysis was developed by Professor J.L.F. Kock from the Department of Microbial, Biochemical and Food Biotechnology, Faculty of Natural and Agricultural Sciences at the University of the Free State, Bloemfontein, South Africa.

##### A.2.1.1 Preparation of sample

The crocodile oil was transesterified using trimethylsulphonium hydroxide (TMSH) (Butte, 1983:142) and analysed by GC as described below. The oil sample was dissolved in 200  $\mu$ L chloroform to which 200  $\mu$ L TMSH was added. The sample was then made up to volume (2.5 ml) with methanol. The sample was placed on a vortex to ensure complete mixture of the sample before being injected onto the GC. A 0.5  $\mu$ l sample was injected in duplicate onto the GC, with a split ratio of 1:100 and an inlet port temperature of 230 °C.

### A.2.1.2 Gas chromatography conditions

**Analytical instrument:** Fatty acid methyl esters (FAME) were analysed with a Shimadzu 2010 gas chromatograph in a controlled laboratory environment at 25 °C. The instrument was controlled and results processed using Shimadzu GC solution software.

**Column:** A SGE BP x 70 glass capillary column with inner diameter of 0.32 mm, film thickness of 0.25 µm and length of 60 m was used. Column oven temperature was programmed from 180 °C held for 1 min, increased to 225 °C at a rate of 10 °C/min held for 4 min and finally increased at a rate of 10 °C/min to 256 °C held for 1 min.

**Gas phase:** Nitrogen was employed as carrier gas at a flow rate of 1.07 ml/min.

**Detection:** Peaks were detected with a flame ionisation detector at a temperature of 310 °C.

## A.3 Results and discussion

### A.3.1 Fatty acid methyl ester analysis

Identification of FAME peaks in the samples was made by comparing the relative retention times of FAME peaks from samples with those of standards obtained from SIGMA. Results of the percentage fatty acid composition from the GC for crocodile oil are shown in Table A.1.

**Table A.1:** GC results of the fatty acid composition in percentage (%) of crocodile oil

	Saturated fatty acids		Mono-unsaturated fatty acids		Poly-unsaturated fatty acids	
	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	Linolenic
	16:0	18:0	16:1	18:1	18:2	18:3
<b>Sample 1</b>	24.5	5.3	6.8	41.7	21.7	0.0
<b>Sample 2</b>	21.6	4.9	7.7	41.1	23.2	0.6
<b>Sample 3</b>	22.2	5.0	7.0	41.4	23.3	0.6
<b>Sample 4</b>	21.6	4.7	7.3	41.5	23.4	0.6
<b>Canola oil (standard)</b>	4.3	2.0	0.0	55.4	29.0	9.7

### A.3.2 Discussion

From the data collected, it could be seen that crocodile oil presented high levels of oleic acid (41.4%), medium levels of linoleic- (22.9%) and palmitic acid (22.5%) and low levels of stearic- (5.0%), palmitoleic- (7.2) and linolenic acid (0.45%). In Table A.2 the percentage fatty acid composition in crocodile oil to human skin oil are compared.

**Table A.2:** Fatty acid composition in percentage (%) of crocodile oil compared to human skin oil (Croc city, 2012).

	<b>Crocodile oil %</b>	<b>Human skin oil %</b>
<b>Palmitoleic acid (omega-7)</b>	7.2	3.80
<b>Palmitic acid</b>	22.5	20.20
<b>Stearic acid</b>	5.0	11.20
<b>Oleic acid (omega-9)</b>	41.4	30.80
<b>Linoleic acid (omega-6)</b>	22.9	15.10

The composition of fatty acids in crocodile oil compared well to fatty acids found in human skin oil. Palmitoleic acid, oleic acid and linoleic acid showed much higher concentrations of each fatty acid in crocodile oil than in human skin oil. Palmitic acid found in crocodile oil showed a slightly higher percentage than found in human skin oil and stearic acid in crocodile oil was much lower than found in human skin oil.

### A.4 Summary

Results obtained for the fatty acid composition of crocodile oil in this study compared well to the values found in literature (Croc city, 2012). Identification of the relative retention times of FAME peaks from the samples were compared to those of standards obtained from SIGMA, to ensure authenticity of the oils. This was a good indication of the reliability of the method used and the suitability for the intended purpose.

## A.5 References

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## APPENDIX B

# ANTI-OXIDANT ACTIVITY OF CROCODILE OIL AND CROCODILE OIL LOTION

### B.1 Introduction

Crocodile oil and crocodile oil containing products are used for treatment of dry, flaky, itchy and flaking skin and pigmentation of skin-like brown spots, freckles and menopausal darkening like in ageing.

The ageing process is a very complex biological process. Factors contributing to the ageing process include external influences such as nutrition, smoking, alcohol, environmental conditions as well as individual genetic factors. Particular attention in this respect has been paid to the biological action of free radicals, especially to oxygen species like  $\text{OH}^\bullet$  (hydroxyl radicals), which are causing oxidative stress. It appears that these radicals in addition to the nitrogen oxides, are one of the major factors for a forced ageing, DNA-damage and for a number of other diseases (Getoff, 2007:1577). Therefore the anti-oxidant activity of crocodile oil and crocodile oil lotion was determined to identify whether the positive anti-ageing results of the products are because of the anti-oxidant activity or maybe because of a different working mechanism.

There is a growing interest in the use of natural products, as consumer awareness of their possible beneficial health effects increases. An imbalance between formation of reactive oxygen species and anti-oxidants *in vivo* is suggested to play a major role in multiple diseases, including ageing. An anti-oxidant is defined as “any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate” (Tang *et al.*, 2004:1575).

Ageing is a complicated process where recent evidence suggests that several of its most important mechanisms are linked by means of cellular damage caused by reactive oxygen species (ROS). Oxidative damage may be a major factor in the loss of physiological functions that occur in ageing. This is because in aerobic organisms the mitochondrial electron transport chain plays an important role in energy production and is a significant source of ROS that damage DNA, RNA, and proteins in cells (Huang & Manton, 2004:1100).

Although aerobic lifestyles are advantageous in many ways, the utilisation of oxygen by cells for many of their biochemical reactions, results in the formation of highly reactive free radical

products. These free radicals and products of free radical reactions can cause reversible and irreversible damage to macromolecular targets such as DNA, proteins, and cellular membranes. A particularly important consequence of free radical damage in many cells is the peroxidation of PUFAs, which results in the formation of lipid peroxides and aldehydes. These products can cause extensive damage to membrane structure and integrity, and several lines of evidence have implicated lipid peroxidation with chemical and physical changes within membranes (Ottino & Duncan, 1997:1145).

Several methods have been developed to measure the free radical scavenging capacity (RSC), regardless of the individual compounds which contribute towards the total capacity of a natural product in scavenging free radicals. The methods are typically based on the inhibition of the accumulation of oxidised products, since the generation of free radical species is inhibited by the addition of anti-oxidants and this gives rise to a reduction of the end point by scavenging free radicals. The reliable method to determine RSC, involves the measurement of the disappearance of free radicals or other coloured radicals with a spectrophotometer (Choi *et al.*, 2002:1161). Free radicals produced by radiation, chemical reactions and several redox reactions of various compounds may contribute to protein oxidation, DNA damage, lipid peroxidation in living tissues and cells (Choi *et al.*, 2006:130).

To determine the anti-oxidant properties of crocodile oil, it is necessary to perform an assay illustrating this mechanism of action. In this study the thiobarbituric acid (TBA)-assay demonstrates how the compound scavenges  $\text{OH}^\bullet$  (hydroxyl radical). Malondialdehyde (MDA) which is a major degradation product of lipid hydroperoxides will be the marker for assessing the extent of lipid peroxidation. MDA is of great concern because it has shown to be part of pathological processes in the formation of pigments which is typical in ageing (Botsoglou *et al.*, 1994:1931).

In this study the most commonly used method for measuring MDA in biological samples was used, namely the thiobarbituric acid test. This method is based on spectrophotometric quantification of the pink complex formed after reaction of MDA with two molecules of TBA.

## **B.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 MDA equivalents. It is a simple and rapid technique used for determining the amount of lipid peroxidation occurring in cells (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 quantification due to an absorbance maximum at 532 nm. Even though this technique has been condemned for its lack of specificity and its inclination to overrate the MDA content, it has 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).

### **B.2.1 Preparation of crocodile oil samples**

Four different concentrations of crocodile oil were prepared by dissolving it in methanol to give 10% (v/v), 30% (v/v), 50% (v/v) and 90% (v/v) concentrations. The crocodile oil lotion was prepared by dissolving the lotion in methanol to make a 50% concentration.

### **B.2.2 Test animals**

*In vitro* experiments were performed on whole rat brain homogenates from adult male Sprague-Dawley albino rats weighing between 200 and 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.

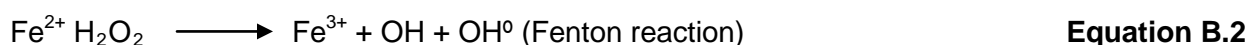
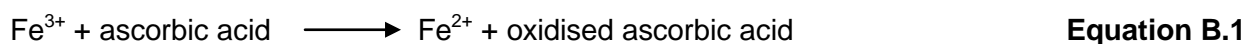
### **B.2.3 Chemicals and reagents**

Ascorbic acid (vitamin C), dimethyl sulphoxide (DMSO) and iron(III)chloride ( $\text{FeCl}_3$ ) 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  $\text{Na}_2\text{HPO}_4$  (di-sodium hydrogen orthophosphate anhydrous) and 2 mM  $\text{KH}_2\text{PO}_4$  (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. Due to the fact that TBA is light sensitive, it was always freshly prepared and protected from light by covering the container with aluminium foil.

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 5 mM) was used, as the toxin, to generate  $\text{OH}^\bullet$  and initiate lipid peroxidation in the rat brain homogenates (Garcia *et al.*, 2000:1). Ascorbic acid (1.4 mM) and  $\text{FeCl}_3$  (4.88 mM) were added to increase the generation of  $\text{OH}^\bullet$  according to the following reactions (Cui *et al.*, 2004:774):

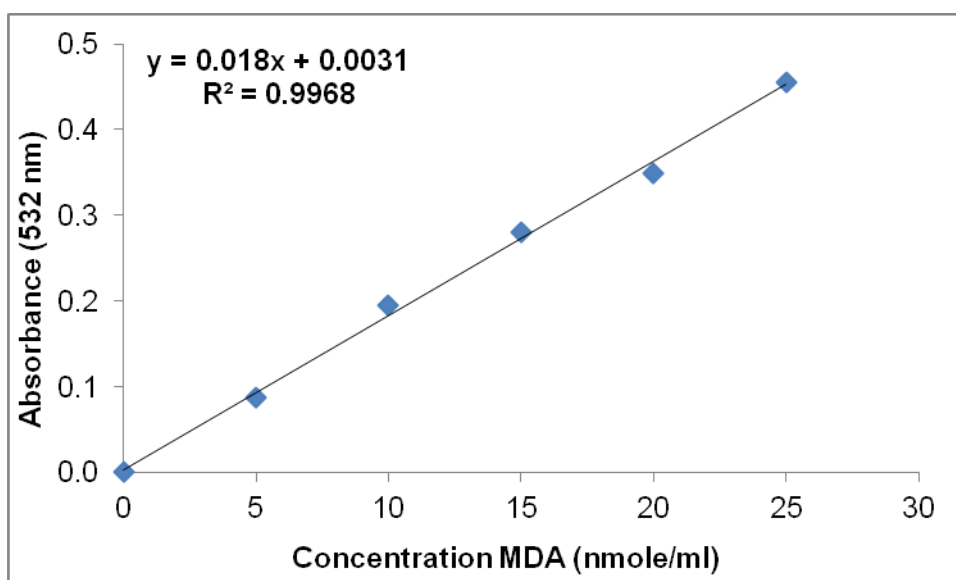


Hydroxyl radicals generated via Fenton reaction from hydrogen peroxide, which is produced *in vivo* in the brain, may initiate and propagate the degenerative reaction in the cell membranes known as lipid peroxidation (Garcia *et al.*, 2000:1).

Trolox was used throughout the experiments as the positive control, by emitting its own antioxidant activity. Values lower than that of trolox indicated almost definite antioxidant activity, by the reduction in MDA formation in peroxidising lipid systems. The control solution consisted of 160  $\mu\text{l}$  rat brain homogenates, 20  $\mu\text{l}$  PBS and 20  $\mu\text{l}$  of methanol. Results were compared to that of the toxin, a mixture known to have very low levels of antioxidant activity.

#### **B.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 a UV-visible spectrophotometer. The absorbance of the TBA/MDA-complex was plotted against the known concentration of MDA.



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

### B.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 by a manual glass Teflon Homogeniser in 0.1 M PBS (pH 7.4) to give an ultimate concentration of roughly 10% (w/v).

### B.2.6 Method of TBA-Assay

To establish the potential antioxidant activity of extracts, 1 ml rat brain homogenate containing toxin combination and varying concentrations of the oil and lotion were incubated in an GFL 1083 oscillation water bath (Burgwedel, Germany) 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), 1.0 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. Thereafter the absorbance was determined at 532 nm.

### **B.2.7 Data collection**

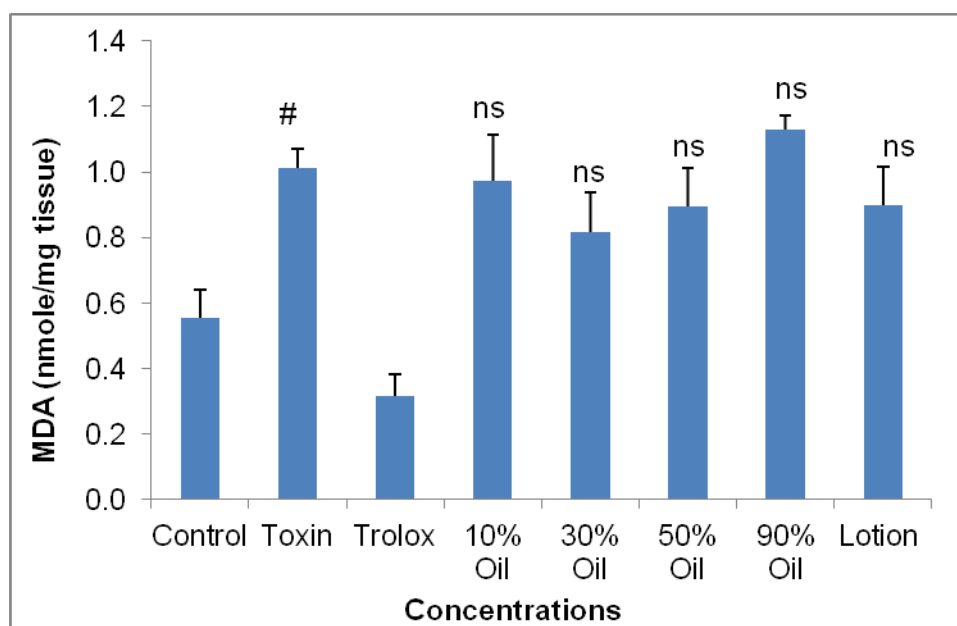
The absorbance values obtained were converted to MDA levels (nmole MDA) from the calibration curve generated with TEP. Results and the extent to which lipid peroxidation occurred were expressed as nmole MDA/mg tissue.

## **B.3 Results and discussion**

### **B.3.1 Statistical analysis**

GraphPad InStat 3 software was used for the statistical analysis of data. Results were 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 was considered extremely significant. When  $p < 0.01$  it was considered fairly significant, while  $p < 0.05$  was considered as significant. A  $p > 0.05$  was considered to be not significant (ns).

### B.3.2 Anti-oxidant properties of crocodile oil



**Figure B.2:** The attenuation of lipid peroxidation by different concentrations of crocodile oil in whole rat brain homogenates *in vitro*. Each bar represents the mean  $\pm$  S.E.M. ( $n = 5$ ). ns ( $p > 0.05$ ) vs toxin (#).

When comparing the different concentrations of the crocodile oil with the toxin it is evident that the oil concentrations 10, 30, 50 and 90% did not exhibit sufficient *in vitro* antioxidant activity when compared to that of the toxin ( $1.01 \pm 0.12$  nmole/mg), consecutively showing significant increase in MDA formation in rat brain tissue *in vitro*. This increase in the 2TBA/MDA-complex formation indicated a higher degree of lipid peroxidation and lower  $\text{OH}^\bullet$  radical scavenging abilities of the extracts. When comparing the different concentrations of the crocodile oil with the toxin it was clear that none of the concentrations showed any potential antioxidant activity by means of decreasing the MDA formation.

When the different concentrations of crocodile oil were compared with trolox ( $0.32 \pm 0.06$  nmole/mg) it was clear that none of the concentrations showed their own antioxidant activity, as none of the concentrations was below trolox.

### B.4 Conclusion

Crocodile oil concentrations of 10, 30, 50 and 90% as well as the 50% lotion did not show *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.

Although there is no literature available about the anti-oxidant properties of crocodile oil, there are data showing that other animal oils, like fish oil, showed anti-oxidant activity. This anti-oxidant activity showed effects on lipid metabolism and significant interest has arisen regarding the potential of fish oils as therapeutic agents in the management of patients with vascular diseases. Fish oils may be concerned in the development of atherosclerosis because of their potential impact upon lipid peroxidation (McGrath *et al.*, 1996:280). Cole *et al.* (2005:135) says that omega-3 fatty acids have a long history of use and proven health benefits for cardiovascular disease because of the anti-oxidant properties.

However, it is very important to realise that the assay described in this Appendix is strictly based on chemical reactions and bear no similarity to biological systems. The validity of the data is limited to a strict chemical sense with no context interpretation (Huang *et al.*, 2005:1853). Due to the complexity of the composition of biological samples, it is very difficult separating each antioxidant and studying it individually (Huang *et al.*, 2005:1841).

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## APPENDIX C

### TOXICITY STUDIES OF CROCODILE OIL LOTION

#### C.1 Introduction

Toxicity studies were performed by Dr. D. Goosen (BVSc Hons. Pret.) from Tswane University of Technology (Pretoria, South Africa). The Series 870 Health Effects test guidelines have been harmonised between the Office of Pesticide Programs (OPP) and the Office of Prevention, Pesticides, and Toxic Substances (OPPTS) and, where possible, with the Organisation for Economic Co-operation and Development (OECD) and South African test guidelines. The Ethics Committee of the Tshwane University of Technology verified that the animal facility operated within the standards and rules of the National Laboratory Animal Ethical Code of Conduct and that the animals were kept according to recognised international standards in animal husbandry practice. This study was conducted according to the Environmental Protection Agency (EPA) Guidelines and complied with registration requirements in South Africa, USA and Europe.

The purpose of the study was to identify whether crocodile oil lotion had skin sensitisation, acute dermal toxicity and dermal irritation potential.

#### C.2 Safety precautions

Laboratory coats, face masks and gloves were worn at all times and all work was performed in a well-ventilated area. If any spills occurred, the test item as well as diluents was dried using paper towels which were discarded into biohazard waste.

#### C.3 Animal husbandry

The rats were acclimatised in the animal unit of La-Bio Research for a period of 5 days before the start of the study at a room temperature of between 19-23 °C and a humidity of 45-75%. A 12 h day/night light cycle was a constant in the animal unit. The light intensity was kept between 70-100 Lux. The animals were kept in a micro-barrier unit consisting of individually ventilated cages. The rats were housed in cages where they could conduct their species-specific behaviour. The physical size of these cages was in accordance to European standards. Water, food and bedding were autoclaved before being used in the cages. Rat/mice feed procured from Epol<sup>®</sup> were fed to the animals. Each animal was assigned a unique identification number. A system to randomly assign animals to test groups and control groups was used.

## **C.4 Ethics approval**

The Ethics Committee of the Tshwane University of Technology verified that the animal facility operated within the standards and rules of the National Laboratory Animal Ethical Code of Conduct and that the animals were kept according to recognised international standards in animal husbandry practice. The protocol was submitted to the Animal Ethics Committee of Tshwane University of Technology for ethical committee approval, but as this is a study required by regulatory authorities and was done according to EPA/OECD protocols, the committee took notice thereof. No official approval was needed.

## **C.5 Skin sensitisation**

### **C.5.1 Introduction**

The purpose of the study was to identify whether the test substance has skin sensitisation potential. Determination of the potential to cause or elicit skin sensitisation reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity. Information derived from skin sensitisation tests serves to identify possible hazards to a population exposed repeatedly to a test substance.

The Series 870 Health Effects test guidelines have been harmonised between OPP and OPPTS and, where possible, with OECD and South African test guidelines. This study was conducted according to EPA Guideline 870.100 and 870.2600 Skin Sensitisation (Buehler Method) and complied with registration requirements in South Africa, USA and Europe. The principles of the following guidelines were also incorporated in this study plan:

- Guidelines for the Registration of Botanical Pesticides, Quality Assurance and Toxicology: South African Departments of Agriculture and Health; 2003
- Guidelines for Industry, Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies: March 1, 1995 (60 FR 11264) CDER, Rockville, MD, USA
- Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals. Guideline 406: Skin Sensitization (July 1992)

### **C.5.2 Animals**

Species: Duncan Hartley Guinea Pig  
Source: South African Vaccine Producers (Pty) Ltd, Johannesburg, South Africa  
Acclimatisation: 6 days  
Weight: 450-820 g

Age: 9 weeks  
Sex: Male  
Number: 30

### **C.5.3 Experimental design**

Following initial exposure to a test substance, the animals were subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state had been induced. Sensitisation was determined by examining the reaction to the challenge exposure and comparing this reaction to that of the initial induction exposure.

The test animals were initially exposed to the test substance by topical application (induction exposure). Following a rest period of 10 to 14 days (the induction period), during which an immune response could develop, the animals were exposed to a topical challenge dose. The extent and degree of skin reaction to the challenge exposure was compared to that demonstrated by control animals that underwent sham treatment during induction and then received the challenge exposure.

All of the surviving animals were sacrificed at the end of the study. The experimental technique in native animals was assessed by the use of a positive control substance known to have mild-to-moderate skin-sensitising properties.

### **C.5.4 Experimental procedure**

#### **C.5.4.1 Test Groups**

Only animals with healthy intact skin were used and were naïve animals. The main Study had two groups. One group (test group) consisted of 20 male animals exposed to the test substance and the other group of 10 male animals that acted as the negative control group. Animal no 9 in the test group was mutilated by its sibling no 10 in the same cage to such an extent that it had to be removed from the study.

#### **C.5.4.2 Dosage**

##### **C.5.4.2.1 Induction**

The undiluted test substance (0.5 ml) was administered via a closed patch on days 0, 8 and 14 to the test group. An undiluted test substance was used as it is known to be a non-irritant. The negative control group received only a bare patch with 0.5 ml distilled water on it at the same time.

### C.5.4.2.2 Challenge

On day 28, the undiluted test substance (0.5 ml) was administered to an area adjacent to the induction area via a closed patch for 6 h for both the test and control groups.

### C.5.4.3 Exposure and exposure duration

Fur was clipped from the dorsal area of the trunk of the animals 24 h before dosing. Care was taken to avoid abrading the skin. Topical administration of the test substance was via a closed patch on days 0, 8, and 14 for induction, with topical challenge of the untreated flank for 6 h on day 28.

### C.5.4.4 Observation period

Animals were observed daily for normal vitality and were weighed weekly. Readings for irritations were made after removing the induction patches.

### C.5.4.5 Observation of animals

Animals were weighed the day before induction started, and the day before every application of the test substance and at termination of the study. Animals were examined directly after removal of the induction patch and 24 and 48 h after removal of the challenge patch for signs of erythema and oedema and the responses scored and recorded according to the grades in the Table C.1.

**Table C.1:** Evaluation of skin reaction for skin sensitisation and dermal irritation

	Value
<b>Erythema and Eschar Formation:</b>	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible	5
<b>Oedema Formation:</b>	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4
Maximum possible	5

## **C.5.5 Pathology**

The animals were euthanised on day 29 with CO<sub>2</sub>.

## **C.5.6 Results**

Two animals were euthanised because of skin mutilation (no 9 on day 20 & no 24 on day 21). Animal weights indicated that the animals in both the test and control group maintained or gained weight over the study period. This showed that the test substance had no general adverse affect on the animal's health.

Skin sensitivity assessment showed that the test substance caused no skin sensitisation in the animals tested.

## **C.6 Acute dermal toxicity**

### **C.6.1 Introduction**

The purpose of the study was to identify whether the test substance has skin dermal toxicity potential.

Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a substance or multiple doses given within a 24 h period. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from short-term exposure by the dermal route.

Data from an acute study may serve as a basis for classification and labelling. It is an initial step in establishing a dosage regimen in sub-chronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route. An evaluation of acute toxicity data will include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

The Series 870 Health Effects test guidelines have been harmonised between OPP and OPPTS and, where possible, with OECD and South African test guidelines. This study was conducted according to EPA Guideline 870.100 and 870.1200 Acute Dermal Toxicity and should comply with registration requirements in South Africa, USA and Europe. The principles of the following guidelines are incorporated in this study plan:

- Guidelines for the Registration of Botanical Pesticides, Quality Assurance and Toxicology: South African Departments of Agriculture and Health; 2003
- The Assessment of Systemic Exposure in Toxicity Studies: CDER, Rockville, MD, USA
- Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals. Guideline 402: Acute Dermal Toxicity

### **C.6.2 Animals**

Species:	Sprague Dawley Rats
Source:	South African Vaccine Producers (Pty) Ltd, Johannesburg, South Africa
Acclimatisation:	5 days
Weight:	150-200 g
Age:	Between 8 and 12 weeks
Sex:	Females. Females were Nulliparous and non pregnant
Number:	15 rats

### **C.6.3 Experimental design**

Because of the similar composition to human skin oil, crocodile oil is considered to be of low toxicity when applied to human skin and therefore will be a very accepted and safe product to use. Based on this 3 dosage levels were used, namely: 1000, 2000 and 5000 mg/kg.

Five females were used for each dosage level as they are more prone to toxicity. The test substance was administered over a period of 24 h. All the animals were observed for at least 14 days. No animals were moribund or suffered any discomfort or pain during the study performed on them. All the animals were euthanised at the end of the study by CO<sub>2</sub>. As there were no macro pathological lesions, no organs were taken for pathology.

### **C.6.4 Experimental procedure**

#### **C.6.4.1 Test Groups**

There were 3 test groups with 5 animals in each group. Dosage groups were 5,000, 2000 & 1000 mg/kg.

#### **C.6.4.2 Dosage**

Animals were weighed immediately before dosing. Dosages were calculated at 5000, 2000 & 1000 mg/kg for each rat according to their body weight.

#### **C.6.4.3 Exposure and exposure duration**

Fur was clipped from the dorsal area of the trunk of the test animals 24 h before dosing. Care was taken to avoid abrading the skin, which would have altered its permeability. The test substance was applied uniformly over a shaved area which was approximately 10% of the body surface area. The area started at the scapulae (shoulders) to the wing of the ileum (hip bone) and half way down the flank on each side of the animal.

The test substance was held in contact with the skin with a porous gauze dressing (< 8-ply) and non-irritating tape for 24 h after application. It also ensured that the animals could not ingest the test substance. At the end of the exposure period residual test substance was washed off using water.

#### **C.6.4.4 Observation period**

Animals were observed clinically every hour for the first 4 h for any sign of ill health and adverse effects during exposure to the test substance and thereafter daily for 14 days. All the animals were euthanised at the end of the study.

#### **C.6.4.5 Observation of animals**

##### **C.6.4.5.1 Clinical examination**

No animals died during the observation period. Observations were recorded in detail using explicitly defined scales according to LBR standard operating procedure. Observations included the following evaluation criteria:

- the skin and fur;
- the eyes;
- mucus membranes;
- respiratory and circulatory effects and
- autonomic effects.

##### **C.6.4.5.2 Animal weights**

Individual weights of the animals were determined 24 hours before the test substance was administered, weekly thereafter and at termination of the study.

## C.6.5 Pathology

At the end of the study, all the animals were weighed and euthanised with CO<sub>2</sub>. Necropsies were performed on all animals in the study. Necropsies were performed immediately after euthanasia. None of the animals had any macro-pathological lesions.

## C.6.6 Results

### C.6.6.1 Clinical results

No signs of clinical abnormalities were observed during the study.

### C.6.6.2 Animal weights

All animals gained weight according to normal standards for Sprague Dawley rats in the laboratory.

### C.6.6.3 Post mortem

No macro pathological abnormalities were observed.

### C.6.6.4 Evaluation of results

As no mortalities or toxic symptoms have been observed in this study at a dosage of 5000, 2000 or 1000 mg/kg, it is concluded that according to Toxic Categories (Table C.2) as per OPPTS 870.1000 the acute Dermal Toxicity of crocodile lotion falls in Category IV.

**Table C.2:** Toxicity categories

Study	Category I	Category II	Category III	Category IV
Acute Dermal	Up to and including 200 mg/kg	> 200 through 2000 mg/kg	> 2000 through 5000 mg/kg	> 5000 mg/kg

## C.7 Acute dermal irritation

### C.7.1 Introduction

Determination of the irritant and/or corrosive effects on the skin of mammals is useful in the assessment and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance. This test is designed to serve as a quality control test for newly produced products. The aim of the study was to determine the acute dermal irritation of crocodile oil lotion.

The Series 870 Health Effects test guidelines have been harmonised between OPP and OPPTS and, where possible, with OECD and South African test guidelines. This study was conducted according to EPA Guideline 870.100 and 870.2500 Acute Dermal Irritation and should comply with registration requirements in South Africa, USA and Europe. The principles of the following guidelines were incorporated in this study:

- Guidelines for the Registration of Disinfectants, Quality Assurance and Toxicology: South African Departments of Agriculture and Health
- The Assessment of Systemic Exposure in Toxicity Studies: CDER, Rockville, MD, USA
- Organisation for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals. Guideline 404: Acute Dermal Irritation

### **C.7.2 Animals**

Species:	New Zealand White Albino Rabbit Durheim Rabbit Centre, Delmas, South Africa
Weight:	Approximately 3.5 kg
Age:	16 to 20 weeks
Sex:	Males
Number:	3

Albino rabbits were procured from the Durheim Rabbit Centre. Only young healthy animals with no skin lesions were admitted to the study. A veterinarian confirmed the health status of all animals.

### **C.7.3 Experimental design**

The substance tested was applied in a single dose to the skin of the test animals, each animal serving as its own control. As no irritation was expected, the three animals were tested simultaneously.

The degree of irritation was read and scored at specified intervals and was further described to provide a complete evaluation of the effects. The duration of the study was sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed. No animals were moribund or suffered severe pain or distress during the study. Separate animals were not used for an untreated control group. Adjacent areas of untreated skin of each animal served as a control for the test.

## **C.7.4 Experimental procedure**

### **C.7.4.1 Dosage**

A dose of 0.5 ml undiluted cream was applied to the test site and covered with 8-ply gauze and non-irritation non-porous plaster.

### **C.7.4.2 Exposure and exposure duration**

Fur was clipped from the dorsal area of the trunk of the test animals 24 h before dosing. Care was taken to avoid abrading the skin, which would alter its permeability. As no irritation was expected, the test substance was applied to three animals, each receiving one patch for an exposure period of 4 h.

### **C.7.4.3 Application of the test substance**

Undiluted (100%) test substance was applied to a small area (approximately 6 cm<sup>2</sup>) of skin and covered with a gauze patch, which was held in place with non-irritating tape. Access by the animal to the patch and resultant ingestion/inhalation of the test substance was prevented by proper coverage of the test patch with non-irritating 3M-Nexcare cloth tape. Residual test substance was washed off with distilled water immediately after patch removal.

### **C.7.4.4 Observation period**

Observations for skin irritation were done at 1, 24, 48 and 72 h after application of the test substance. The study was terminated after 72 h as no irritation was observed in any of the three animals at any stage after the application of the substance.

### **C.7.4.5 Observation of animals**

After removal of the patch, animals were examined for signs of erythematic and oedema and the responses scored within 30-60 min, and at 24, 48, 72 h after removal of the patch. Dermal irritation was scored and recorded according to the grades in Table C.1

## **C.7.6 Results**

All 3 animals showed no signs of erythema or oedema after patch removal and at any time of observation thereafter (Table C.1). The control regions on the animals (where no test substance was added) showed no symptoms of either erythema or oedema. The study was terminated after 72 h as it was unnecessary to continue because of the absence of any irritation.

It is therefore concluded that the crocodile lotion causes no dermal irritation.

## **C.8 Conclusion**

The skin sensitisation, dermal toxicity and dermal irritation studies on crocodile oil lotion showed no toxicity and therefore are a safe and harmless product to use.

## C.9 References

Guideline for Industry, Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies. March 1, 1995 (60 FR 11264).

Guidelines for the Registration of Botanical Pesticides, Quality Assurance and Toxicology: South African Departments of Agriculture and Health, 2003

OECD (1998) A Harmonized Integrated Hazard Classification System for Human Health and Environmental effects of Chemicals. ENV/MC/HCL (98) 14/REV2.

OECD (2000) Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation. Environmental Health and Safety Monograph series on Testing and assessment No. 19, September 2000. [<http://www.oecd.org/test/monos/htm>]

OECD (2000). Classification Revised Step 2 Proposal for Harmonized classification criteria for Mixtures. ENV/JM/HCL (2000)9/REV8. The conduct of acute toxicity studies, September 1997, E.P.A document #737-R-97-002.

Organization for Economic Cooperation and Development. OECD Guidelines for the Testing of Chemicals. OECD Guideline 423: Acute Oral Toxicity: Acute Toxic Class Method. Adopted: 22 March 1996.

The Assessment of Systemic Exposure in Toxicity Studies: CDER, Rockville, MD, USA.

## **APPENDIX D**

### **STABILITY TESTING OF CROCODILE OIL LOTION**

#### **D.1 Introduction**

The successful formulation of cosmeceutical products requires that the products should be placed under certain conditions and tested to determine their stability over a certain period of time. Like other products, the stability of cosmeceuticals must be matched to the expected period of usage as well as to the user's requirements. It is important to guarantee product quality by paying sufficient attention to the time required to distribute the product from the manufacturer to the consumer and the actual period the product will be used. It is not sufficient to simply guarantee the feeling on use and performance of a cosmeceutical, it's also important to consider the safety and stability during usage (Mitsui, 1997:191).

The first stage of stability testing, is to establish baseline values against which changes over time can be measured by observing whether or not there are any changes in the physicochemical properties of the semi-solid formulations. The following changes must be investigated:

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

If no changes in the physicochemical properties of the formulations are observed, the formulations are stored at different temperatures and humidities for 6 months and stability tests are done at fixed time intervals.

The purpose of stability testing is to provide evidence 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). In this study, crocodile oil cream was stored in the original packaging (50 ml plastic container) as well as in a glass container (50 ml) at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH for 6 months. Both types of containers were sealed properly with a lid. The following stability tests were done on months 0, 1, 2, 3 and 6:

- pH
- Viscosity
- Visual appearance assessment
- Zeta-potential
- Droplet size
- Mass loss

In this study the chemical stability of the different triglycerides was not tested. Because of the extensive methods that are necessary in testing oils and their triglycerides, it was decided that it will be appropriate to study it in the future.

According to the ICH (2003:13) a significant change for a drug product is defined as:

- A 5% change in 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).
- Failure to meet the acceptance criterion for pH.

## **D.2 Methods**

### **D.2.1 pH**

The apparatus that was used to measure the pH of the formulations was a Mettler Toledo pH meter (made in Schwerzenbach, Switzerland). A Mettler Toledo Inlab<sup>®</sup> 410 electrode was used and the apparatus was calibrated each time before use. The pH of each formulation at each condition was measured in triplicate.



**Figure D.1:** Mettler Toledo pH meter

## **D.2.2 Viscosity**

Rheology is the science of the flow of a product and its study begins with gathering data on the product's viscosity. Viscosity is the resistance to flow caused by internal friction (Brookfield, 1008:2). According to Marriott (2007:42) rheology may be defined as the study of the flow and deformation properties of matter.

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. The spindle turns at a specific rate, measured in rpm. A Brookfield Viscometer (model DV II, Stoughton, Massachusetts, USA) with helipath (Stoughton, Massachusetts, USA) was used. The formulations were placed in the water bath to reach a temperature of 25 °C. A T-bar spindle (Stoughton, Massachusetts, USA) 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.



**Figure D.2:** Brookfield Viscometer

### D.2.3 Visual appearance assessment

The visual appearance of each formulation was assessed by comparing the colour of the cream with the initial colour and appearance of the particular formulation. Photos were taken with a digital camera (Blackberry® Bold 9300) and each formulation was placed in the same position and site each time. The camera was placed approximately 15 cm away from the formulations.

### D.2.4 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 needed to measure to determine these electrical charges was the zeta-potential (Malvern 2011:1; Malvern, 2000:1.1).

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 triplicate in 100 ml volumetric flasks. The cream samples were made up to volume with 0.1 M potassium chloride solution. 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.



**Figure D.3:** Malvern Zetasizer 2000

### D.2.5 Droplet size

Approximately 0.5 g of each formulation was mixed with approximately 3 ml HPLC water to wet the formulation to a uniform wet dispersion. This was done in triplicate. These mixtures were made up with approximately 4.5 ml HPLC water, well-mixed 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 triplicate 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 E.3.5,  $D (0.5)$  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) (Malvern, 2000:6.3)



**Figure D.4:** Malvern Mastersizer 2000 with wet cell Hydro 2000 SM

### D.2.6 Mass loss

The apparatus that was used to determine the mass loss of each formulation was a Shimadzu (Kyoto, Japan) scale. The mass of each formulation at each condition was determined in triplicate.



**Figure D.5:** Shimadzu scale

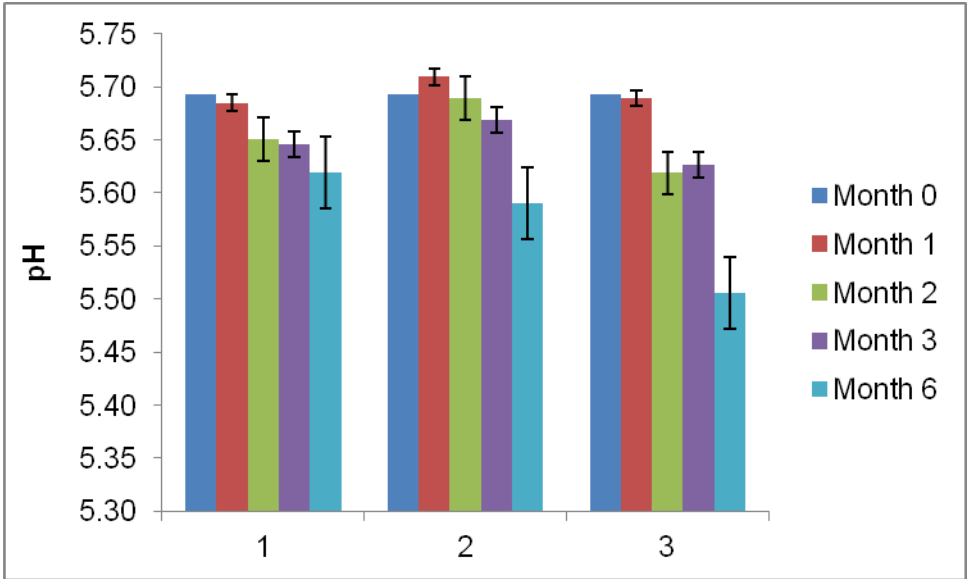
## D.3 Results and discussion

### D.3.1 pH

The pH was measured on 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, 3 and 6 as described in Section D.2.1.

Figures D.6 and D.7 illustrate an overview of the changes between months 0 and 6 of the cream in different containers.

D.3.1.1 CREAM IN ORIGINAL PACKAGING



**Figure D.6:** The change in pH between month 0 and 6 for cream in original packaging at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 3.

**Table D.1:** pH of cream in original packaging at different conditions after each time interval

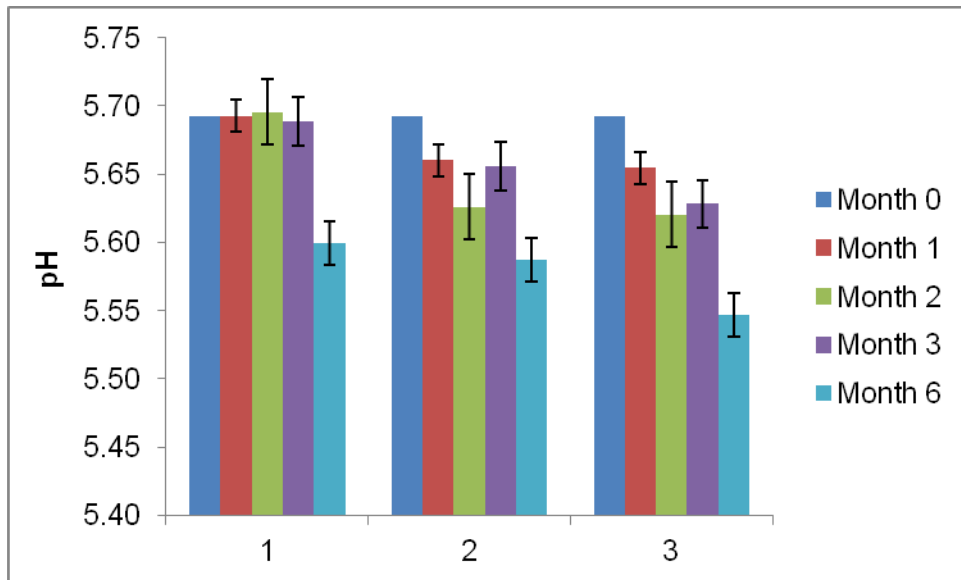
	<b>Initial</b>	<b>Month 1</b>	<b>Month 2</b>	<b>Month 3</b>	<b>Month 6</b>
<b>25 °C/60% RH</b>					
<b>1</b>	5.70	5.69	5.65	5.65	5.62
<b>2</b>	5.69	5.69	5.65	5.65	5.62
<b>3</b>	5.69	5.68	5.65	5.65	5.62
<b>Average</b>	5.69	5.69	5.65	5.65	5.62
<b>SD</b>	0.0030	0.0004	0.0010	0.0010	0.0030
<b>%RSD</b>	0.040	0.010	0.010	0.010	0.050
<b>30 °C/60% RH</b>					
<b>1</b>	5.70	5.71	5.69	5.67	5.59
<b>2</b>	5.69	5.71	5.69	5.67	5.59
<b>3</b>	5.69	5.71	5.69	5.67	5.59
<b>Average</b>	5.69	5.71	5.69	5.67	5.59
<b>SD</b>	0.0020	0.0020	0.0020	0.0020	0.0000
<b>%RSD</b>	0.040	0.040	0.040	0.040	0.000
<b>40 °C/75% RH</b>					
<b>1</b>	5.70	5.69	5.62	5.63	5.51
<b>2</b>	5.69	5.69	5.62	5.63	5.51
<b>3</b>	5.69	5.69	5.62	5.62	5.51
<b>Average</b>	5.69	5.70	5.62	5.63	5.51
<b>SD</b>	0.0020	0.0008	0.0005	0.0020	0.0008
<b>%RSD</b>	0.040	0.010	0.008	0.040	0.020

The pH of the cream stored in the original packaging remained stable over the six months period. The biggest decrease (3.28%) in pH was observed from the cream stored at 40 °C/75% RH namely 0.187 pH units which is within acceptable limits.

D.3.1.2 CREAM IN GLASS CONTAINER

**Table D.2:** pH of cream in glass container at different conditions after each time interval

	<b>Initial</b>	<b>Month 1</b>	<b>Month 2</b>	<b>Month 3</b>	<b>Month 6</b>
<b>25 °C/60% RH</b>					
<b>1</b>	5.70	5.70	5.70	5.69	5.60
<b>2</b>	5.69	5.69	5.670	5.69	5.60
<b>3</b>	5.69	5.69	5.69	5.69	5.60
<b>Average</b>	5.69	5.69	5.70	5.69	5.60
<b>SD</b>	0.002	0.003	0.003	0.003	0
<b>%RSD</b>	0.04	0.58	0.05	0.06	0
<b>30 °C/60% RH</b>					
<b>1</b>	5.70	5.66	5.62	5.66	5.59
<b>2</b>	5.69	5.66	5.63	5.66	5.59
<b>3</b>	5.69	5.66	5.63	5.65	5.59
<b>Average</b>	5.69	5.66	5.63	5.66	5.59
<b>SD</b>	0.002	0.002	0.001	0.002	0.001
<b>%RSD</b>	0.04	0.03	0.03	0.04	0.01
<b>40 °C/75% RH</b>					
<b>1</b>	5.70	5.65	5.62	5.63	5.55
<b>2</b>	5.69	5.66	5.62	5.63	5.55
<b>3</b>	5.69	5.66	5.62	5.63	5.55
<b>Average</b>	5.69	5.65	5.62	5.63	5.55
<b>SD</b>	0.002	0.001	0.001	0.001	0.001
<b>%RSD</b>	0.04	0.02	0.02	0.03	0.01



**Figure D.7:** The change in pH between month 0 and 6 for cream in glass container at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 3.

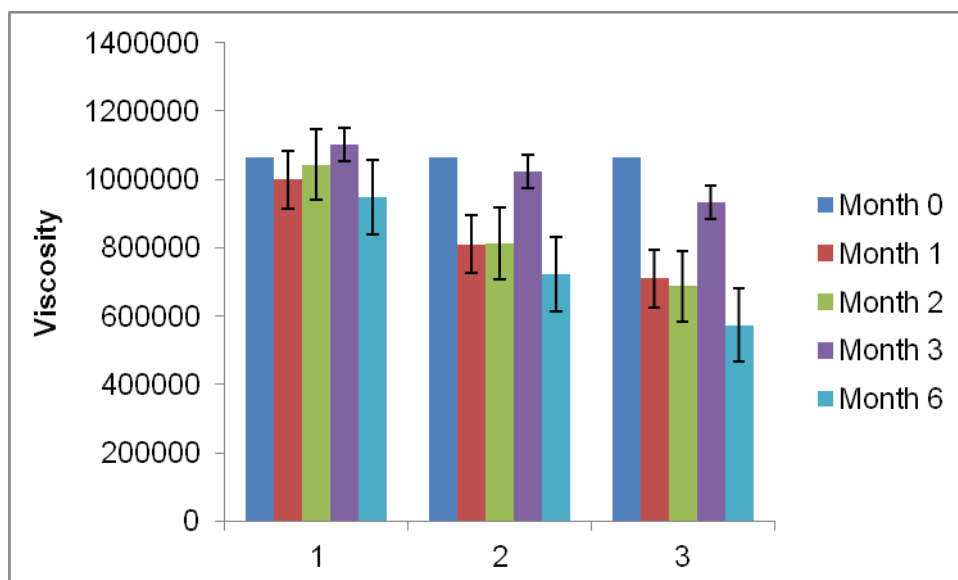
The pH of the cream stored in a glass container remained stable over the six months period. The biggest decrease (2.56%) in pH was the cream stored at 40 °C/75% RH namely 0.052 pH units which it within acceptable limits.

### D.3.2 Viscosity

The viscosity was measured on 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, 3 and 6 as described in Section D.2.2. Figures D.8 and D.9 illustrate an overview of the changes between months 0 and 6 of the cream in different containers.

**Table D.3:** Viscosity of cream (Pa.s) at different conditions after each time interval

	Initial	Month 1	Month 2	Month 3	Month 6
<b>25 °C/60% RH</b>					
<b>Average</b>	1063.30	998.68	1042.62	1101.08	946.46
<b>SD</b>	195.33	90.70	83.95	103.12	211.30
<b>%RSD</b>	18.37	9.08	8.05	9.37	22.33
<b>30 °C/60% RH</b>					
<b>Average</b>	1063.30	809.30	811.91	1021.56	722.33
<b>SD</b>	195.33	64.95	180.35	88.26	160.99
<b>%RSD</b>	18.37	8.03	22.21	8.64	22.29
<b>40 °C/75% RH</b>					
<b>Average</b>	1063.30	710.23	686.85	932.36	573.87
<b>SD</b>	195.33	72.96	79.43	69.60	112.71
<b>%RSD</b>	18.37	10.27	11.56	7.46	19.64



**Figure D.8:** The change in viscosity between month 0 and 6 for cream at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 30.

The viscosity of the cream stored at all the condition changed over the six months period. The cream stored at 25 °C/60% RH showed a decrease in viscosity of 10.9%, where the cream stored at 30 °C/60% RH and 40 °C/75% RH showed a decrease of 32.1% and 46.0%, respectively. Although the large decrease in viscosity may be a warning of instability, the change in the cream's viscosity was not that significant. To put it in perspective and compare the viscosity of the cream to other substances the change was not that considerable. Water's

viscosity is 1 cP whereas ethylene glycol is 24 cP, honey 2000 cP, molasses 5000 cP and pork fat 100000 cP. The cream still had a good texture and could be applied without any problem.

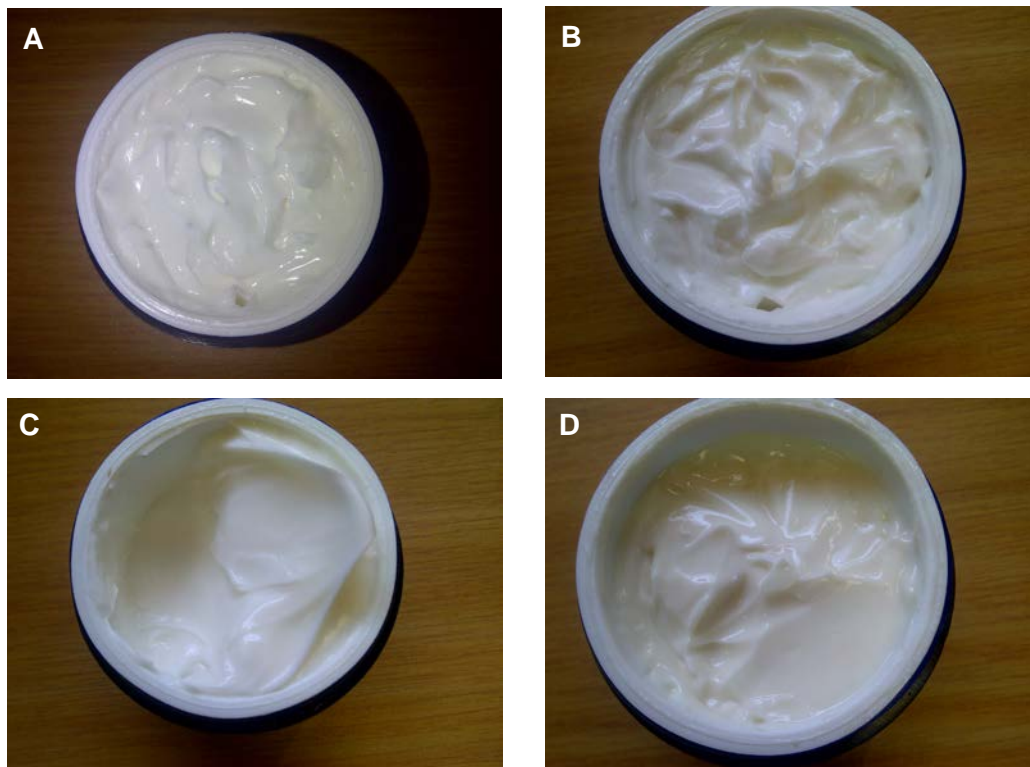
### D.3.3 Visual appearance assessment

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, 3 and 6 as described in Section D.2.3.

In Figures D.9 and D.10 the change in colour during the six months period of the cream in each container is given.

#### D.3.3.1 CREAM IN ORIGINAL PACKAGING

The cream's colour stored at all 3 conditions didn't show any significant change over the six months period



**Figure D.9:** The change in colour of cream stored in original packaging from A) month 0 to month 6 at B) 25 °C/60% RH, C) 30 °C/60% RH and D) 40 °C/75% RH

### D.3.3.2 CREAM IN GLASS CONTAINER

The cream's colour stored at all 3 conditions didn't show any significant change over the six months period.



**Figure D.10:** The change in colour of cream stored in glass container from A) month 0 to month 6 at B) 25 °C/60% RH, C) 30 °C/60% RH and D) 40 °C/75% RH

### D.3.4 Zeta-potential

The zeta-potential was determined on the initial creams as well as the formulations stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH after months 1, 2, 3 and 6 as described in Section D.2.4. Figures D.11 and D.12 illustrate an overview of the changes between months 0 and 6 of the cream in different containers.

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-potential tend to coagulate or flocculate (Jelvehgari *et al.*, 2010:1240).

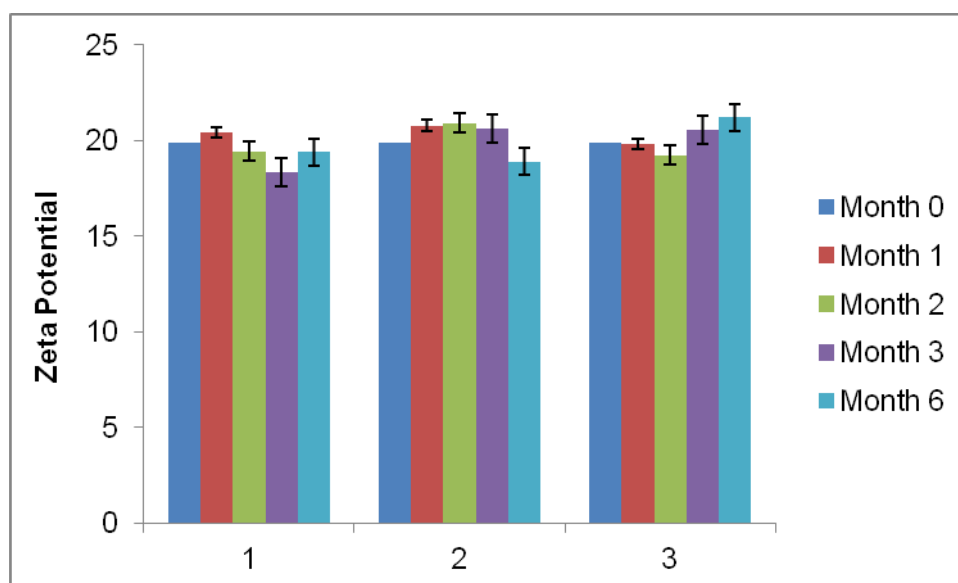
#### D.3.4.1 CREAM IN ORIGINAL PACKAGING

The zeta potential 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 6. Although all three stability testing conditions did not stay within this range, the values stayed within the same range and can be seen as stable over the 6 months period. The cream stored at 25 °C/60% RH and 30 °C/60% RH showed a decrease in zeta-potential of 2.5% and 5.0%, respectively, whereas the cream stored at 40 °C/75% RH showed an increase of 6.5%.

**Table D.4:** Zeta potential (mV) of cream in original packaging at different conditions after each time interval

	<b>Initial</b>	<b>Month 1</b>	<b>Month 2</b>	<b>Month 3</b>	<b>Month 6</b>
<b>25 °C/60% RH</b>					
<b>1</b>	17.2	20.8	18.9	19.9	13.4
<b>2</b>	21.4	19.4	16.4	21.3	19.7
<b>3</b>	19.3	19.4	20.5	21.4	20.0
<b>4</b>	18.3	19.9	13.9	22.5	19.8
<b>5</b>	21.2	22.3	21.3	17.5	22.0
<b>6</b>	20.6	18.8	18.6	20.7	19.6
<b>7</b>	20.9	21.0	21.9	20.4	23.0
<b>8</b>	20.1	21.6	21.1	15.9	15.0
<b>9</b>	20.8	20.6	22.3	5.4	22.0
<b>Average</b>	19.9	20.4	19.4	18.3	19.4
<b>SD</b>	1.4	1.1	2.6	5.0	3.0
<b>%RSD</b>	6.8	5.3	13.6	27.1	15.6
<b>30 °C/60% RH</b>					
<b>1</b>	17.2	20.3	21.7	20.6	22.3
<b>2</b>	21.4	17.4	23.2	21.7	18.4
<b>3</b>	19.3	23.5	23.3	19.7	19.7
<b>4</b>	18.3	22.3	22.4	22.2	10.3
<b>5</b>	21.2	20.9	22.8	21.3	22.9
<b>6</b>	20.6	21.5	22.1	19.7	19.9
<b>7</b>	20.9	21.0	17.4	19.8	14.6
<b>8</b>	20.1	17.7	17.5	18.7	22.7
<b>9</b>	20.8	22.4	17.8	21.8	19.1
<b>Average</b>	19.9	20.8	20.9	20.6	18.9
<b>SD</b>	1.4	1.9	2.4	1.1	3.9
<b>%RSD</b>	6.8	9.4	11.5	5.5	20.6
<b>40 °C/75% RH</b>					
<b>1</b>	17.2	19.5	20.3	22.0	23.6
<b>2</b>	21.4	17.5	17.7	23.0	21.3
<b>3</b>	19.3	20.8	18.7	11.5	20.4
<b>4</b>	18.3	21.3	23.0	22.4	22.0
<b>5</b>	21.2	21.1	11.2	19.4	20.5
<b>6</b>	20.6	19.3	21.1	23.6	21.7
<b>7</b>	20.9	16.3	18.1	20.9	18.5
<b>8</b>	20.1	20.8	22.8	22.0	21.3
<b>9</b>	20.8	21.9	20.2	20.0	21.6

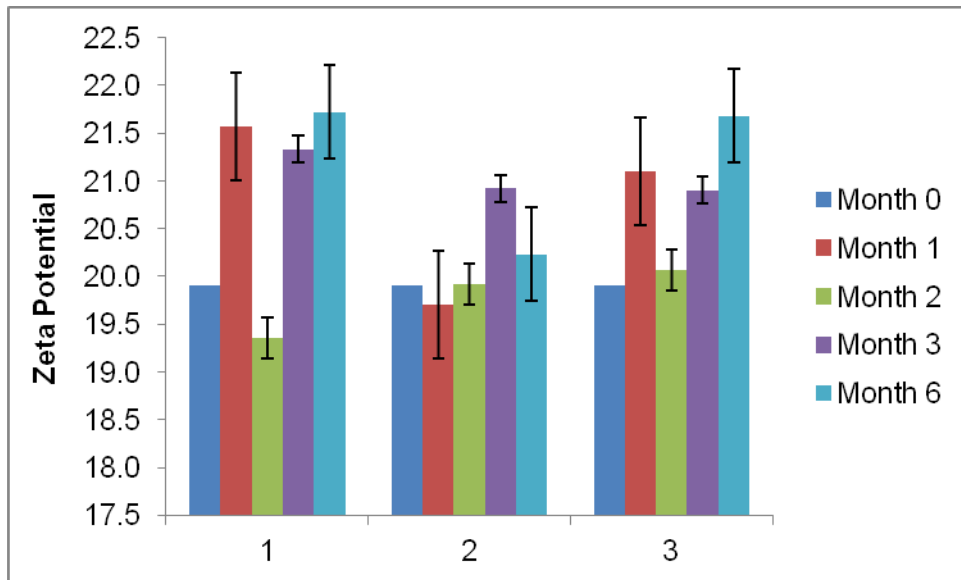
<b>Average</b>	19.9	19.8	19.2	20.5	21.2
<b>SD</b>	1.4	1.8	3.3	3.4	1.3
<b>%RSD</b>	6.8	8.9	17.4	16.8	6.1



**Figure D.11:** The change in zeta-potential between month 0 and 6 for cream in original packaging at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 9.

#### D.3.4.2 CREAM IN GLASS CONTAINER

The zeta-potential 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 6. Although all three stability testing condition did not stay within this range, the values are still high and can be seen as stable over the 6 months period. The cream stored at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH showed an increase in zeta-potential of 9.0%, 1.5% and 9.0%, respectively.



**Figure D.12:** The change in pH between month 0 and 6 for cream in glass container at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 9.

**Table D.5:** Zeta-potential (mV) of cream in glass container at different conditions after each time interval

	<b>Initial</b>	<b>Month 1</b>	<b>Month 2</b>	<b>Month 3</b>	<b>Month 6</b>
<b>25 °C/60% RH</b>					
<b>1</b>	17.2	21.2	14.3	22.1	22.8
<b>2</b>	21.4	18.1	18.5	15.2	22.3
<b>3</b>	19.3	20.8	25.2	23.1	22.8
<b>4</b>	18.3	26.0	22.4	21.9	22.4
<b>5</b>	21.2	20.1	9.4	21.8	21.7
<b>6</b>	20.6	20.3	16.5	22.8	18.9
<b>7</b>	20.9	25.9	22.6	21.3	22.0
<b>8</b>	20.1	22.0	22.4	23.1	20.6
<b>9</b>	20.8	19.7	22.9	20.7	22.0
<b>Average</b>	19.9	21.5	19.4	21.3	21.7
<b>SD</b>	1.4	2.6	4.8	2.3	1.2
<b>%RSD</b>	6.8	11.8	24.9	10.8	5.4
<b>30 °C/60% RH</b>					
<b>1</b>	17.2	20.1	17.7	20.1	22.6
<b>2</b>	21.4	15.7	12.3	20.6	21.1
<b>3</b>	19.3	21.0	22.1	19.9	21.4
<b>4</b>	18.3	17.6	19.6	21.7	15.2
<b>5</b>	21.2	21.2	22.6	20.4	16.8
<b>6</b>	20.6	19.8	24.0	23.4	22.3
<b>7</b>	20.9	19.1	22.2	20.6	20.6
<b>8</b>	20.1	21.7	19.2	21.1	20.5
<b>9</b>	20.8	21.1	19.6	20.5	21.6
<b>Average</b>	19.9	19.7	19.9	20.9	20.2
<b>SD</b>	1.4	1.9	3.3	1.0	2.4
<b>%RSD</b>	6.8	9.4	16.5	4.8	11.8
<b>40 °C/75% RH</b>					
<b>1</b>	17.2	18.2	19.0	21.2	21.4
<b>2</b>	21.4	21.7	23.5	21.5	22.4
<b>3</b>	19.3	22.5	17.6	23.4	20.7
<b>4</b>	18.3	20.6	19.6	20.1	22.2
<b>5</b>	21.2	20.0	21.6	22.3	22.6
<b>6</b>	20.6	21.7	18.5	22.8	21.8
<b>7</b>	20.9	22.3	18.6	22.3	21.5
<b>8</b>	20.1	22.7	19.9	14.7	22.2
<b>9</b>	20.8	20.2	22.3	19.8	20.3

<b>Average</b>	19.9	21.1	20.1	20.9	21.7
<b>SD</b>	1.4	1.4	1.9	2.5	0.7
<b>%RSD</b>	6.8	6.6	9.3	11.8	3.4

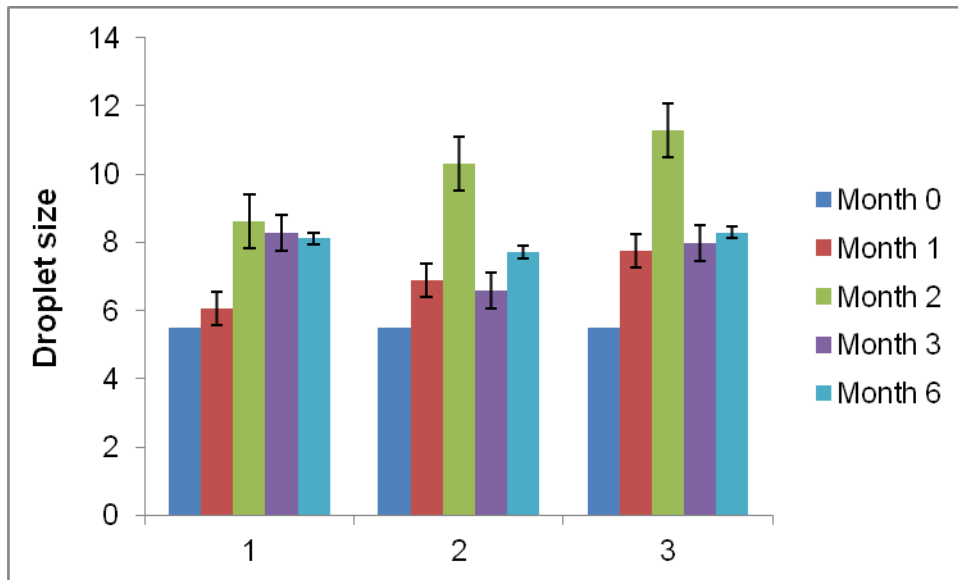
### D.3.5 Droplet size

The droplet size was determined 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, 3 and 6 as described in Section D.2.5. Figures D.13 and D.14 illustrate an overview of the changes in droplet size between months 0 and 6 of the cream in different containers.

#### D.3.5.1 CREAM IN ORIGINAL PACKAGING

**Table D.6:** Average particle size ( $\mu\text{m}$ ) of cream in original packaging at different conditions after each time interval

	<b>Initial</b>	<b>Month 1</b>	<b>Month 2</b>	<b>Month 3</b>	<b>Month 6</b>
<b>25 °C/60% RH</b>					
<b>D (0.5)</b>	5.50	6.05	8.61	8.29	8.11
<b>30 °C/60% RH</b>					
<b>D (0.5)</b>	5.50	6.90	10.31	6.58	7.71
<b>40 °C/75% RH</b>					
<b>D (0.5)</b>	5.50	7.74	11.28	8.00	8.29



**Figure D.13:** The change in droplet size between month 0 and 6 for cream in glass container at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 9.

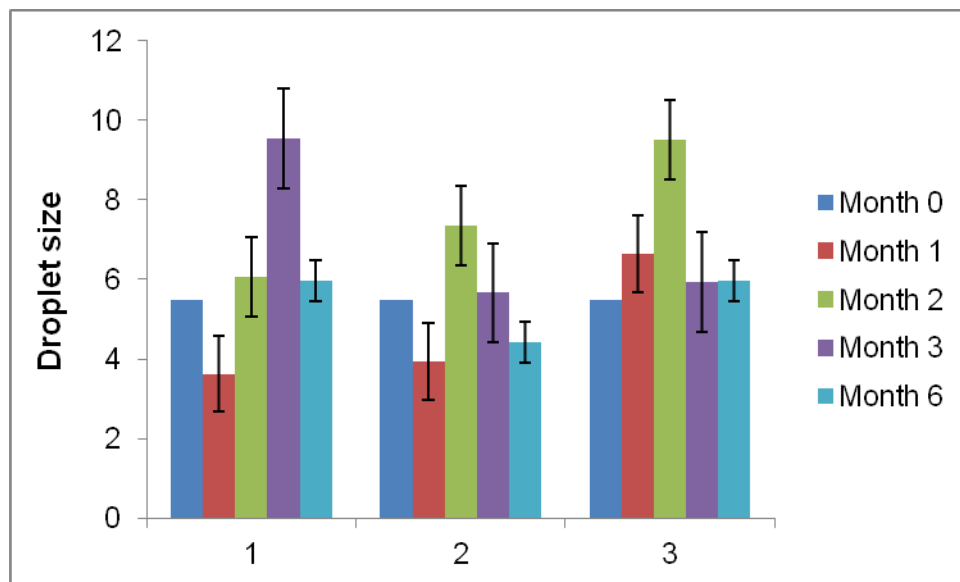
In the crocodile oil cream the average particle size increased after the 6 months period in all three stability controlled conditions. The cream stored at 25 °C/60% RH increased from an initial average particle size of 5.50 µm to a final average particle size of 8.11 µm after 6 months. This is an increase of approximately 2.6 µm. The cream stored at 30 °C/60% RH demonstrated an increase of approximately 2.2 µm after 6 months of stability testing, while the formulation stored at 40 °C/75% RH showed an increase in average particle size of approximately 2.8 µm.

During the 6 months at different storage conditions, the particles in dispersion demonstrated that they adhered to one another to form aggregates and increasing in size. This is called flocculation. Although the average particles increased over the 6 months period and can show that instability was present, the increase was very small.

### D.3.5.2 CREAM IN GLASS CONTAINER

**Table D.7:** Average particle size ( $\mu\text{m}$ ) of cream in glass container at different conditions after each time interval

	Initial	Month 1	Month 2	Month 3	Month 6
<b>25 °C/60% RH</b>					
<b>D (0.5)</b>	5.50	3.63	6.08	9.54	5.97
<b>30 °C/60% RH</b>					
<b>D (0.5)</b>	5.50	3.93	7.35	5.66	4.43
<b>40 °C/75% RH</b>					
<b>D (0.5)</b>	5.50	6.64	9.50	5.93	5.98



**Figure D.14:** The change in droplet size between month 0 and 6 for cream in glass container at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 9.

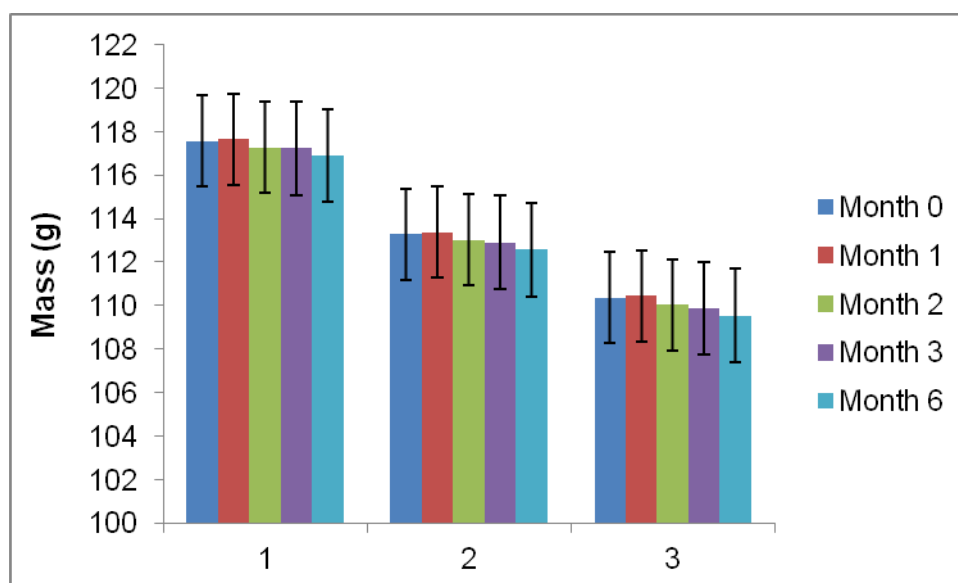
In the crocodile oil cream the average particle size increased after the 6 months period in all three stability controlled conditions. The cream stored at 25 °C/60% RH increased from an initial average particle size of 5.50 µm to a final average particle size of 5.97 µm after 6 months. This is an increase of approximately 0.50 µm. The cream stored at 30 °C/60% RH demonstrated a decrease of approximately 1.10 µm after 6 months of stability testing, while the formulation stored at 40 °C/75% RH showed an increase in average particle size of approximately 0.5 µm.

In this formulation the change in particle size over the 6 months period was very small and it can be said that the cream was stable over the period.

### D.3.6 Mass loss

The mass loss was measured on 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, 3 and 6 as described in Section D.2.6. Figures D.15 and D.16 illustrate an overview of the changes in mass between months 0 and 6 of the cream in different containers.

#### D.3.6.1 CREAM IN ORIGINAL PACKAGING



**Figure D.15:** The change in mass between months 0 and 6 for cream in glass container at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 3.

**Table D.8:** Mass (g) of cream in original packaging at different conditions after each time interval

	<b>Initial</b>	<b>Month 1</b>	<b>Month 2</b>	<b>Month 3</b>	<b>Month 6</b>
<b>25 °C/60% RH</b>					
<b>1</b>	117.56	117.64	117.26	117.23	116.90
<b>2</b>	117.56	117.64	117.26	117.23	116.90
<b>3</b>	117.56	117.64	117.26	117.23	116.90
<b>Average</b>	117.56	117.64	117.26	117.23	116.90
<b>SD</b>	0	4.0x10 <sup>-4</sup>	0	1.42x10 <sup>-14</sup>	4.0x10 <sup>-4</sup>
<b>%RSD</b>	0	4.0x10 <sup>-4</sup>	0	1.21x10 <sup>-14</sup>	4.0x10 <sup>-4</sup>
<b>30 °C/60% RH</b>					
<b>1</b>	113.28	113.36	113.02	112.91	112.57
<b>2</b>	113.28	113.36	113.02	112.91	112.57
<b>3</b>	113.28	113.36	113.02	112.91	112.57
<b>Average</b>	113.28	113.36	113.02	112.91	112.57
<b>SD</b>	0	1.42x10 <sup>-14</sup>	0	0	9.43x10 <sup>-4</sup>
<b>%RSD</b>	0	1.25x10 <sup>-14</sup>	0	0	8.38x10 <sup>-4</sup>
<b>40 °C/75% RH</b>					
<b>1</b>	110.35	110.44	110.02	109.87	109.54
<b>2</b>	110.35	110.44	110.02	109.87	109.54
<b>3</b>	110.35	110.44	110.02	109.87	109.54
<b>Average</b>	110.35	110.44	110.02	109.87	109.54
<b>SD</b>	0	0	0	0	9.43x10 <sup>-4</sup>
<b>%RSD</b>	0	0	0	0	8.61x10 <sup>-4</sup>

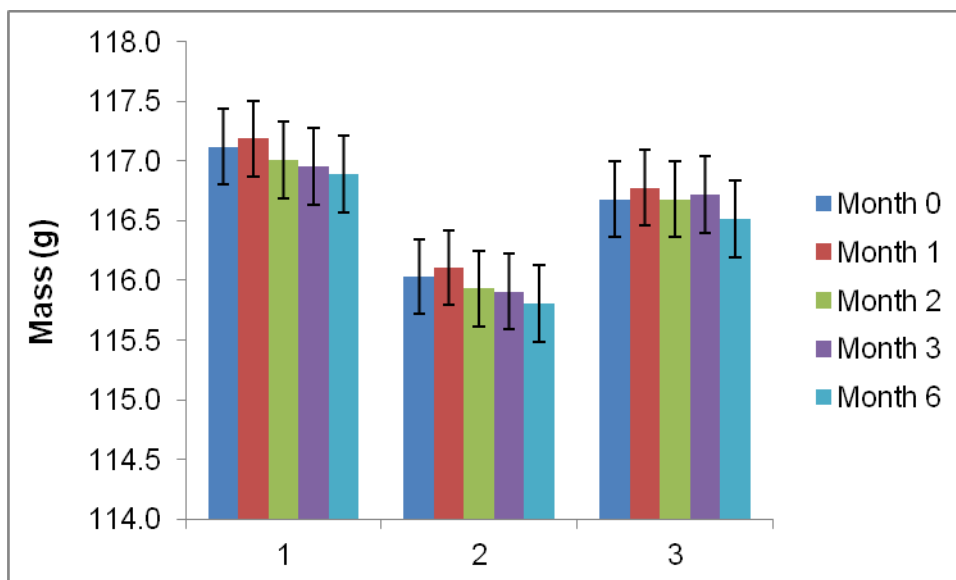
The loss in mass of the crocodile oil cream in the original packaging remained relatively stable over the 6 months period with no significant change in mass. The biggest decrease in mass was the cream stored at 40 °C/75% RH with a decrease of approximately 0.73%. The formulation stored at 30 °C/60% RH showed a decrease of 0.63% while the cream stored at 25 °C/60% RH showed a decrease of 0.56%. The small decrease in mass indicated that the cream was stable over the 6 months period and that the containers sealed sufficiently to avoid evaporation during storage periods.

### D.3.6.2 CREAM IN GLASS CONTAINER

**Table D.9:** Mass (g) of cream in glass container at different conditions after each time interval

	Initial	Month 1	Month 2	Month 3	Month 6
<b>25 °C/60% RH</b>					
<b>1</b>	117.12	117.19	117.01	116.96	116.89
<b>2</b>	117.12	117.19	117.01	116.96	116.89
<b>3</b>	117.12	117.19	117.01	116.96	116.89
<b>Average</b>	117.12	117.19	117.01	116.96	116.89
<b>SD</b>	0	4.71x10 <sup>-4</sup>	0	0	4.71x10 <sup>-4</sup>
<b>%RSD</b>	0	4.02x10 <sup>-4</sup>	0	0	4.03x10 <sup>-4</sup>
<b>30 °C/60% RH</b>					
<b>1</b>	116.03	116.11	115.93	115.90	115.81
<b>2</b>	116.03	116.11	115.93	115.91	115.80
<b>3</b>	116.03	116.11	115.93	115.90	115.81
<b>Average</b>	116.03	116.11	115.93	115.90	115.81
<b>SD</b>	0	4.71x10 <sup>-4</sup>	4.71x10 <sup>-4</sup>	4.71x10 <sup>-4</sup>	4.71x10 <sup>-4</sup>
<b>%RSD</b>	0	4.06x10 <sup>-4</sup>	4.07x10 <sup>-4</sup>	4.07x10 <sup>-4</sup>	4.07x10 <sup>-4</sup>
<b>40 °C/75% RH</b>					
<b>1</b>	116.68	116.77	116.68	116.62	116.51
<b>2</b>	116.68	116.77	116.68	116.62	116.51
<b>3</b>	116.68	116.77	116.68	116.62	116.51
<b>Average</b>	116.68	116.77	116.68	116.62	116.51
<b>SD</b>	1.42x10 <sup>-14</sup>	0	0	0.14	4.71x10 <sup>-4</sup>
<b>%RSD</b>	1.22x10 <sup>-14</sup>	0	0	0.12	4.05x10 <sup>-4</sup>

The mass of the cream stored in the glass container remained stable over the 6 months period. The biggest decrease in mass was the formulation stored at 25 °C/60% RH and 30 °C/60% RH. The cream showed a decrease in mass of 0.19% at both conditions. The formulation stored at 40 °C/75% RH showed a decrease of 0.14%. The small decrease in mass indicated that the cream was stable over the 6 months period and that the containers used for storage of the products sealed well enough for limiting evaporation and preventing moisture absorption.



**Figure D.16:** The change in mass between month 0 and 6 for cream in glass container at 25 °C/60% RH (1), 30 °C/60% RH (2) and 40 °C/75% RH (3). n = 3.

#### D.4 Conclusion

The pH of the products in both containers at all conditions indicated stability over the 6 months period. In all the formulations the pH decreased. Although a change in pH was seen, the stratum corneum was remarkably resistant to alteration in pH, tolerating a range of 3 to 9 (Barry, 2002:511).

The viscosity of the cream stored at 30 °C/60% RH and 40 °C/75% RH changed radically over the 6 months period. The cream stored at 25 °C/60% RH showed a decrease in viscosity of 10.9%, whereas the cream stored at 30 °C/60% RH and 40 °C/75% RH showed a decrease of 32.1% and 46.0%, respectively. The decrease in viscosity can be explained as a reaction among some ingredients. The large decrease in viscosity was a warning of instability.

In the crocodile oil cream stored in the original container, the average particle size increased after the 6 months period in all three stability controlled conditions. This demonstrates that the particles adhered to one another and formed aggregates to increase in size. In the crocodile oil stored in the glass container the only decrease in particle size was seen in the cream stored at 30 °C/60% RH. At the other conditions the particle size showed a small increase. Although the average particle size changed over the 6 months period, and can show that instability was present, the change was very small and it can be said that the cream was stable over the period.

The cream's colour stored at all 3 conditions and in both containers didn't show any significant change over the 6 months period.

The zeta potential at all three stability controlled conditions in both containers did not stay within the range of less than -25 mV or more than +25 mV from months 0 to 6. Although it did not stay within this range the values are still high and can be seen as stable over the 6 months period.

The loss of mass in the crocodile oil cream in both containers remained stable over the 6 month-period with no significant change in mass. The small decrease in mass indicated that the cream was stable over the 6 months period and that the containers sealed well enough for limiting evaporation and preventing moisture absorption.

The pH, particle size, colour, zeta potential and mass loss remained stable over the 6 months period in both containers. This shows that the original packaging of the cream was sufficient to assure stability. Only the change in viscosity showed that there was a warning of instability.

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## APPENDIX E

# CLINICAL EFFICACY TESTING OF CROCODILE OIL LOTION AGAINST AGEING OF THE SKIN IN HUMAN VOLUNTEERS

### E.1 Introduction

Ageing of the skin has fascinated researchers for many years. Not only to ultimately prevent wrinkle formation, but also because skin represents an excellent and accessible model organ allowing the study of intrinsic and extrinsic factors. Chronological (intrinsic) ageing affects the skin in a manner similar to other organs. Extrinsic ageing is related to environmental, mainly UV-induced, damage of the dermal connective tissue of the skin. There is evidence that these processes of intrinsic and extrinsic ageing, have at least in part overlapping, biological, biochemical, and molecular mechanisms (Ma *et al.*, 2001:592).

Ageing causes a functional deficit in the skin through structural and molecular degradation. This degradation results in clinical changes including wrinkling, colour changes, laxity and non-elasticity. Ageing dermis results in increasing stiff, inelastic tissue and is less capable of undergoing modifications in response to stress (Diridollou *et al.*, 2001:354).

The skin of the elderly is structurally and functionally different from that of other age groups. The epidermis is thinner and has a slower cell turnover rate, resulting in less resistance to external injury and prolonged wound healing. Changes of dermal collagen and elastin result in wrinkles. Histologically there is dermal thinning, fragmented collagen fibres, reduced elastin and fewer fibroblast and mast cells. Blood flow and sweating are reduced. Hair becomes gray and sparse and nail growth is slower. The majority of skin changes are thought to be a result of cumulative exposure to environmental factors, especially chronic UV exposure (Laube, 2003:70).

As the proportion of the ageing population in industrialised countries continues to amplify, the dermatological concerns of the elderly grow in medical importance. The rate of ageing is significantly different among different populations, as well as among different anatomical sites even within a single individual. The intrinsic rate of skin ageing in any person can also be dramatically influenced by personal and environmental factors, particularly the amount of exposure to UV light. Photo damage, which considerably accelerates the visible ageing of skin, also seriously increases the risk of cutaneous neoplasms (Farage *et al.*, 2008:87).

In this Appendix, the focus is on the anti-ageing effects of crocodile oil lotion on the human skin. Non-invasive electrical instruments were used to determine the skin hydration, skin surface smoothness and skin erythema as a result of the application of the formulation in human volunteers. Crocodile oil lotion and a placebo lotion were applied to the skin of the volunteers and measurements were taken. To determine the clinical effects of crocodile oil lotion, the following parameters were used:

- Skin hydration measured by the Corneometer<sup>®</sup> CM 825 (Courage-Khazaka Electronic, Cologne, Germany). An increase in this parameter showed an increase in skin hydration.
- RKU measured by the Visioscan<sup>®</sup> VC 98 (Courage-Khazaka Electronic, Cologne, Germany) to determine skin scaliness. For this parameter to show a decrease in skin scaliness, the value had to be nearer to 3.
- SEW measured by the Visioscan<sup>®</sup> VC 98 (Courage-Khazaka Electronic, Cologne, Germany) to determine skin roughness and wrinkles. A decrease in this parameter showed decrease in skin roughness and wrinkles.
- R2, 6, 7 and 8 measured by the Cutometer<sup>®</sup> dual MPA 580 (Courage-Khazaka Electronic, Cologne, Germany) to determine skin elasticity. An increase in this parameter shows an increase in skin elasticity.

## **E.2 Materials and methods**

### **E.2.1 Non-invasive skin measurements**

#### **E.2.1.1 Skin hydration**

The Corneometer<sup>®</sup> CM 825 measurement is based on capacitance measurement of a dielectric medium, in this case the skin. 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 (Courage & Khazaka, 2010:5; Li *et al.*, 2001:24).

During the clinical testing, the probe was placed vertically onto the area to be measured and wasn't moved during the measurement (as seen in Figure E.1). A spring inside the probe head provided constant pressure on the skin. The Corneometer®-probe started the measurement when brought into contact with the skin. A beep signalled that the measurement had been carried out successfully. The display showed the measuring value.

Repeated measurements on the same skin area led to a moisture increase due to occlusion, as water was accumulated under the probe head and could not evaporate. This water influenced the measurement and the measuring values became higher even though the water content in the stratum corneum had not changed. Therefore the measurements were repeated about 5 sec between each measurement. The probe head was cleaned thoroughly.



**Figure E.1:** Measurement with Corneometer® CM 825

#### **E.2.1.2 Skin topography**

The Visioscan® VC 98 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 reflection 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 configured the image in 256 grey 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 skin (SELS) software that generates parameters such as skin entropy, scaliness and roughness. The Visioscan® is used to measure the skin surface.

This includes the measurement of skin smoothness, skin roughness, scaliness and wrinkles (Courage & Khazaka, 2009:10; Ferreira *et al.*, 2010:444).

The skin to be measured was marked with a stencil and pen to find the exact spot again for the next measurement. A double sided sticking ring was placed on the measuring rectangle. The camera's measuring head was removed and placed on the ring. Thereafter the camera was put back onto the measuring head and carefully positioned into the right position. Photos were taken by pushing the small button on the Visioscan®.



**Figure E.2:** Measurement with Visioscan® VC 98

### **E.2.1.3 Skin elasticity**

The Cutometer® dual MPA 580 is designed to measure elasticity of the upper skin layer using negative pressure which deforms the skin mechanically. The measuring principle is based on the suction method. Negative pressure is created in the device and the skin is drawn into the opening of the probe and after a defined time released again. Inside the probe, the penetration depth is determined by a non-contact optical measuring system. This optical measuring system consists of a light source and a light receptor, as well as two prisms facing each other, which project the light from transmitter to receptor. The light intensity varies due to the penetration depth of the skin. The resistance of the skin to the negative pressure (firmness) and its ability to return into its original position (elasticity) are displayed as curves (penetration depth in mm/time) in real time during the measurement. This measurement principle allows getting information about the elastic and mechanical properties of skin surface and enables to objectively quantify skin ageing (Courage & Khazaka, 2012a:7).

During the clinical studies, the probe was applied to the skin at a right angle and was not pressed too tightly onto the skin. Pressing too tightly could lead to disturbed blood circulation,

thus influencing the measurement. The probe was held very steady during the measurement. A spring in the probe head ensured constant pressure on the skin. The probe was placed on the marked skin area. During the first phase of the measurement the skin was sucked into the probe opening by negative pressure. Then the negative pressure was cut off and the skin relaxed in the second phase of the measurement. The probe was held still throughout both phases of the measurement.



**Figure E.3:** Measurement with Cutometer<sup>®</sup> dual MPA 580

#### **E.2.1.4 Melanin and haemoglobin content of skin**

The Mexameter<sup>®</sup> MX 18 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<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 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 as indices on the screen on a scale from 0-999 (Courage & Khazaka, 2012b:1).

During the clinical studies, the Mexameter<sup>®</sup>-probe head was placed straight on the skin and held still on the area to be measured. The measurement was triggered by the skin contact and after

one second the results for melanin and erythema were displayed, accompanied by an acoustical signal.



**Figure E.4:** Measurement with Mexameter<sup>®</sup> MX 18

#### **E.2.1.5 Skin pH**

The Skin-pH-Meter<sup>®</sup> PH 905 (Courage-Khazaka Electronic, Cologne, Germany) is used to measure the pH-value and covers an important characteristic of any aqueous solution: its acidity or alkalinity. The measurement of the pH-value is done with a glass electrode. The glass electrode is filled with an inner buffer. This inner buffer is separated from the measuring solution by a special glass membrane and carries away the potential of the internal side of the glass membrane. A so-called reference electrode carries away the potential of the external side of the glass membrane which contacts the measuring solution. The reference electrode is filled with electrolyte and equipped with a diaphragm which ensures the transportation of the ions between the measuring solution and the inner buffer, but prevents the mixing of both substances. If the electrical leakage of both electrodes is connected with a voltmeter, a potential-difference can be read which depends only on the pH-value of the measuring solution. As the excretions of the skin are almost an aqueous solution, pH measurement can be performed directly on the skin surface. With the Skin-pH-Meter<sup>®</sup> PH 905 a highly precise, easy and quick measurement of the pH-value is possible (Courage & Khazaka, 2012c:1).

During the clinical studies, the probe was washed with distilled water, before starting the measurements. Excessive water was shaken off to have a moist probe. Too much water on the membrane might influence the measuring result or delay a stable value on the screen. The probe-head was placed closely onto the skin surface. The measurement was started by pressing the button on the side of the probe. The measuring value appeared immediately.



**Figure E.5:** Measurement with Skin-pH-Meter<sup>®</sup> PH 905

#### **E.2.1.6 Vapour loss**

The Vapometer<sup>®</sup> (Delfin Technologies, Kuopio, Finland) is a portable and battery-operated device containing a humidity sensor HIH 3605-B and uses the unventilated-chamber method of measurement. A closed chamber is formed after touching the skin and the relative humidity (RH) inside the capsule is measured with an electronic hygrosensor. The cylinder-shaped chamber is equipped with sensors for RH and temperature. Water vapour from the skin surface collects in the chamber and causes the humidity to rise with time, slowly at first but linearly thereafter. The flux density is calculated from the slope of the linearly rising part of the curve. After the measurement is complete, the chamber needs to be lifted from the flux source to allow the accumulated water vapour to escape (Roelandt *et al.*, 2011:257).

During the clinical studies, the Vapometer<sup>®</sup> was placed straight on the skin and held still on the area to be measured. The measurement was triggered by the skin contact and after about 10 sec the results were displayed accompanied by an acoustical signal.

#### **E.2.2 Formulations**

Two semisolid formulations were used in this study. It included a 20% crocodile oil formulation and a placebo formulation containing liquid paraffin instead of crocodile oil.

Because the crocodile oil lotion is a commercial product, it's not possible to supply the composition of the lotion.

### **E.2.3 Subjects**

This 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 healthy female subjects between 40 and 65 years of age participated in the study. 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). The inclusion and exclusion criteria and consent forms given to the subjects and completed by them are attached in Appendix F.

### **E.2.4 Treatment protocol**

#### **E.2.4.1 Short term study**

The treatment sites were on the dominant forearm. A group of 11 subjects participated in the study. 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 forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory according to a pre-organised time schedule. Three sites of 3.5 x 1.5 cm on the dominant arm were marked with a Codman<sup>®</sup> surgical marker. Three instruments namely, Corneometer<sup>®</sup> CM 825, Visioscan<sup>®</sup> VC 98 and Cutometer<sup>®</sup> dual MPA 580 were used. Three measurements on the three different areas were taken with the three instruments before product application and were the baseline values for each area.

After  $T_0$  measurements the cream and placebo cream were applied on the marked squares. On the third square no cream was applied. Measurements were taken after 1 h ( $T_1$ ), 2 h ( $T_2$ ) and 3 h ( $T_3$ ).

#### **E.2.4.2 Erythema study**

The treatment sites were on the dominant forearm. A group of 12 subjects participated in the study. 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 forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory according to a pre-organised time schedule. Five sites of 2.5 x 1.0 cm on the dominant arm were marked with a Codman<sup>®</sup> surgical marker. Three instruments, namely Mexameter<sup>®</sup> MX 18, Skin-pH-Meter<sup>®</sup> PH 905 and Vapometer<sup>®</sup> were used. Three measurements on the five different areas were taken with the three instruments before a 1% sodium lauryl sulphate (SLS) solution was applied on 4 sites using Finn Chambers. The Finn Chambers were removed after 24 h, and 24 h after the removal the subjects returned to the laboratory.  $T_1$  measurements were taken with the three instruments. Thereafter the placebo cream, cream and cortisone cream were applied to 3 of the 4 sites where SLS irritated the skin. 24 h after application the subjects returned to the laboratory for  $T_2$  measurements.

#### **E.2.4.3 Long term study**

The treatment protocol was conducted according to a comparatively similar study performed by Li *et al.* (2000:25). The treatment sites were on the non-dominant forearm. 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 forearms for seven days before entering the study.

On day 8 ( $T_0$ ) the participants visited the laboratory according to a pre-organised time schedule. Three sites of 3.5 x 1.5 cm on the non-dominant arm were marked with a Codman<sup>®</sup> surgical marker. Three measurements were taken with the three instruments on the three different areas before product application and were the baseline values for each area.

During the next 12 weeks each site was treated with the assigned cream. The placebo and crocodile oil cream were applied on the marked squares twice daily, according to the double blind placebo controlled study guidelines. On the third square no cream was applied. Each subject received a treatment programme and used the placebo as well as the active formulation in the morning and evening. The amount of product put on the marked areas was 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 had 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. Measurements were taken after two weeks ( $T_1$ ), four weeks ( $T_2$ ), eight weeks ( $T_3$ ) and twelve weeks ( $T_4$ ).

#### **E.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

measurements. The subjects acclimated to the room conditions for at least 30 min before any measurements were made.

### **E.3 Statistical analysis**

All the parameters were calculated as follows:

- The difference in the various skin measurements at  $T_1$ ,  $T_2$ ,  $T_3$  and  $T_4$  relative to the initial conditions ( $T_0$ ) and was taken as a percentage change between the measurements calculated for the placebo and active treatments, respectively.
- Thereafter, the untreated skin measurements were subtracted from the values obtained, as described above.

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

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

### **E.4 Results**

#### **E.4.1 Short term study**

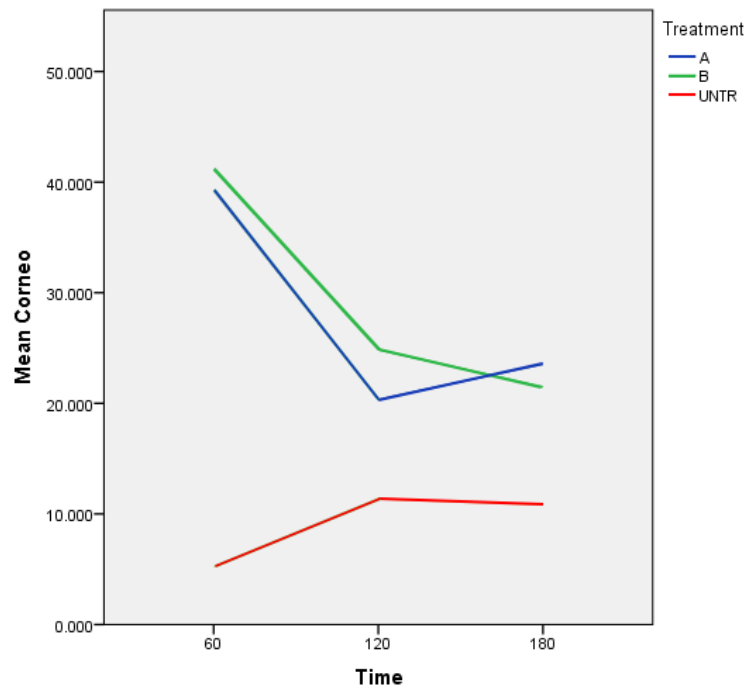
The purpose of this study was to identify whether crocodile oil lotion improved skin hydration over a short period of time, and how long the lotion was giving the hydrating effect. For this study crocodile oil lotion was used as Treatment A, the placebo cream as Treatment B and UNTR refers to untreated skin.

##### **E.4.1.1 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 3 h period. It is important to note that the subjects in this study all had moderately dry skin before testing started.

A mixed model was fitted to the data, since there was missing values and repeated measures were taken over both time and treatment. An AR(1) covariance structure was specified. Type III fixed effects revealed significant factor for time ( $p < 0.0001$ ), treatment ( $p < 0.0001$ ) as well as interaction between time and treatment ( $p < 0.0001$ ).

Multiple pair wise comparisons with Bonferroni adjustment revealed significant differences between Treatment A and untreated ( $p = 0.001$ ), Treatment B and untreated ( $p < 0.0001$ ), as well as between 30 min and 60 min ( $p < 0.0001$ ) and 30 min and 120 min ( $p < 0.0001$ ).



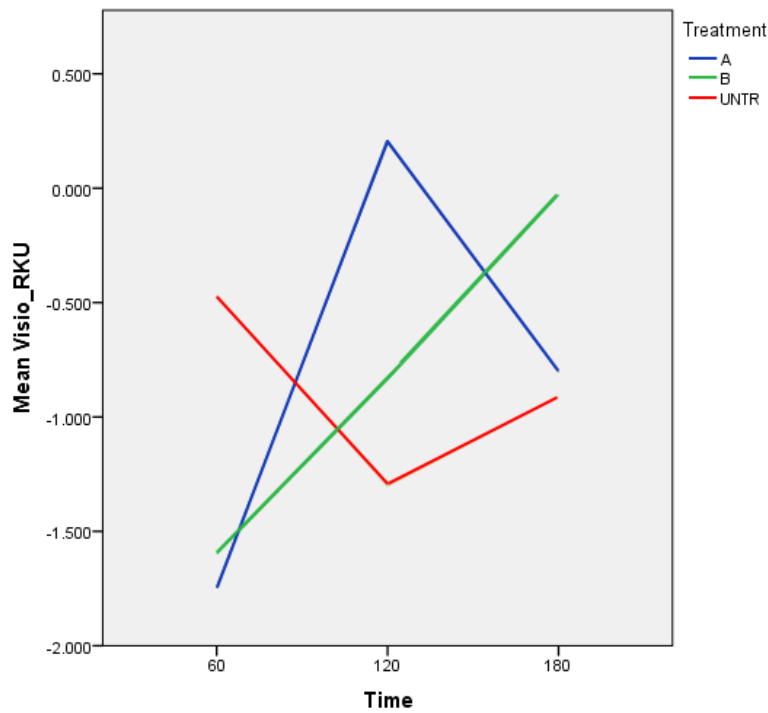
**Figure E.6:** % Change in skin hydration over 180 min for short term study

From Figure E.6 it can be seen that both Treatments A and B had an immediate effect on skin hydration. It can be assumed that external factors may also have contributed to the observed skin hydration levels as can be seen from the rise in skin hydration levels for the untreated areas after hour's exposure. The effect of both Treatments A and B seemed to diminish over time, however, it is difficult to explain the upward trend of Treatment A after 120 min. Although both Treatments A and B showed an increase in skin hydration, there was not a significant difference between the two treatments.

#### E.4.1.2 Skin scaliness (RKU)

By using the Visioscan<sup>®</sup> VC 98, it was possible to detect if any change in skin scaliness took place in the 3 h period by using the RKU parameter. The closer this value is around 3 the smoother the skin.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed no significant difference between treatments or exposure times or interactions for time and treatment.



**Figure E.7:** % Change in skin scaliness over a period of 180 min for short term study

Figure E.7 reveals an initial decrease in skin scaliness for both Treatments A and B. It is again notable that the environment may have played a role in the observed scaliness, since the untreated area also showed a decrease after 120 min. Treatment A seemed to reduce scaliness soon after application, however the effects seemed to reverse itself over time. After 180 min the untreated skin seemed to appear almost similar to the skin treated with Treatment A. The mixed model 95% confidence intervals for the interaction estimated effects confirm this in Table E.1:

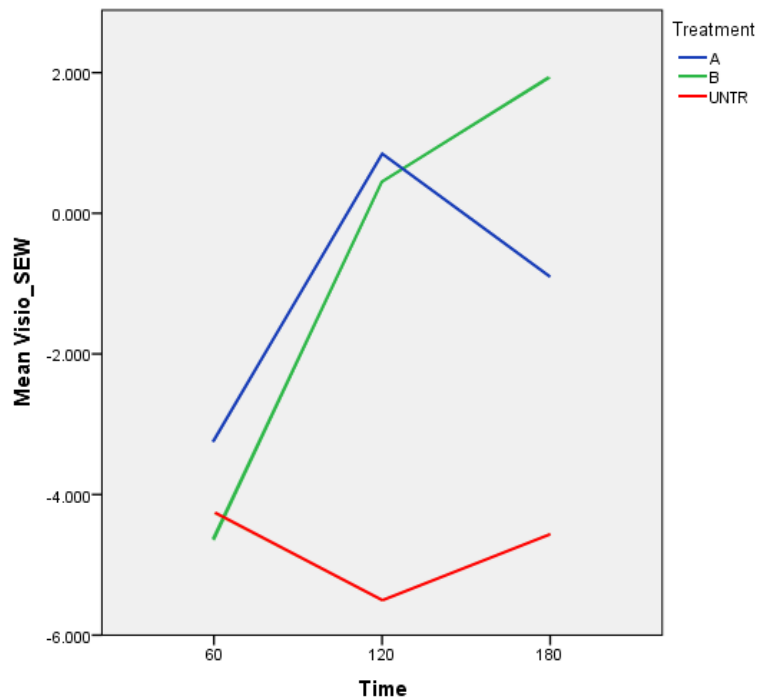
**Table E.1:** Mixed model 95% confidence intervals

Time	Treatment	Mean	Std. Error	df	95% Confidence Interval	
					Lower Bound	Upper Bound
T3 = 180 min	A	-0.803	0.985	56.005	-2.776	1.170
	B	-0.029	0.985	56.005	-2.002	1.944
	UNTR	-0.912	0.985	56.005	-2.885	1.061

#### E.4.1.3 Skin roughness (SEW)

By using the Visioscan® VC 98, it was possible to detect if any change in skin roughness took place in the 3 h period by using the SEW parameter. Skin roughness indicates the degree of

unevenness visible during analysis. If this value decreases, skin roughness decreases and skin smoothness increases.



**Figure E.8:** % Change in skin roughness over 180 min for short term study

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed no significant difference between treatments or exposure times or interactions for time and treatment.

Figure E.8 shows that after 60 min a difference was observed between the treated skin and the untreated skin (however not statistically significant). However, no treatment showed a decrease and therefore it can be said that no improvement in skin roughness was seen over the 3 h period.

#### E.4.1.4 Skin elasticity

By using the Cutometer® dual MPA 580, it was possible to detect if any change in skin elasticity took place. Cutometer readings were measured once after 60 min of exposure by using R2, 6, 7 and 8 parameters.

ANOVA analysis was performed on all Cutometer® variables to determine if difference in means between treatment groups were significant. More robust tests, the Welch and Brown-Forsythe tests were performed in addition to the ANOVA test since the assumption of equal variances

was questioned as Levene's test was insignificant at a 5% level, but not a 10% level. The non-parametric Kruskal-Wallis test was also performed since some deviation from normality was observed using QQ-plots. All four tests concluded insignificant differences. Cohen's d-value was calculated for pair wise comparisons to assess the practical significance of observed differences. Practically visible differences (Ellis & Steyn, 2003:51) were observed between Treatment B and the untreated skin for mean values of Cutometer<sup>®</sup> parameter R2 (d = 0.51) and Cutometer<sup>®</sup> parameter R8 (d = 0.49).

However, since all participants were exposed to all treatments a mixed model was also fitted to account for repeated measures over treatment. An AR(1) covariance structure was selected, as an unstructured covariance structure showed a worse fit. Again, no significant treatment effect was found for any of the Cutometer<sup>®</sup> variables.

#### **E.4.2 Erythema study**

The purpose of this study was to identify whether crocodile oil lotion had anti-erythema effects. The crocodile oil lotion was compared to a negative control (placebo lotion) as well as a positive control (1% cortisone cream).

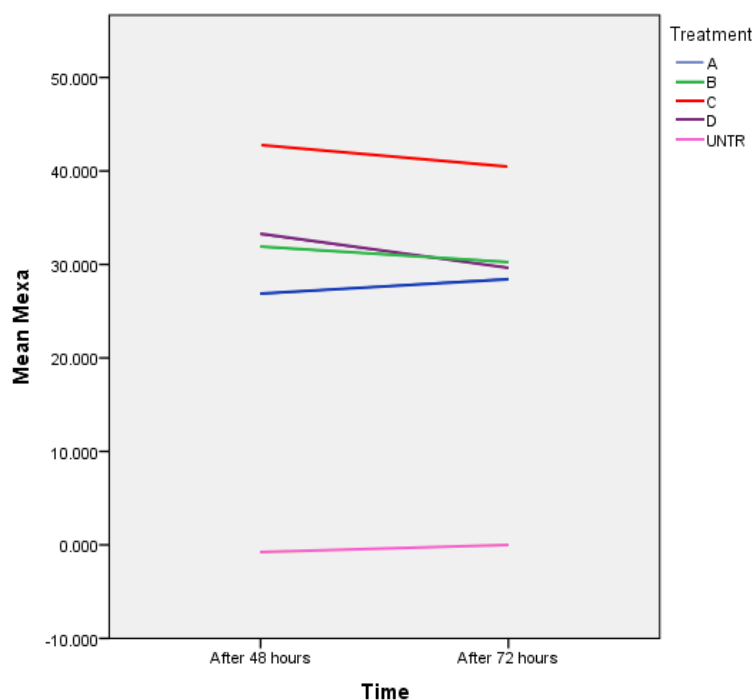
For this study crocodile oil lotion was used as Treatment B, the placebo cream as Treatment C, a 1% cortisone cream as Treatment D and UNTR refers to skin without irritation or treatment. Treatment A refers to irritated skin without any treatment.

##### **E.4.2.1 Skin erythema**

By using the Mexameter<sup>®</sup> MX 18, it was possible to detect whether any change in skin redness took place during the time period. Only two measures were taken – one after 48 h and the second 72 h after exposure.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects only revealed significant differences between treatments ( $p < 0.0001$ ).

Multiple pair wise comparisons with Bonferroni adjustment revealed significant differences between the four treatments and the untreated skin (all p-values  $< 0.0001$ ). However, no significant differences were found between the treatments as seen in Figure E.9.



**Figure E.9:** % Change in skin redness over time for erythema study

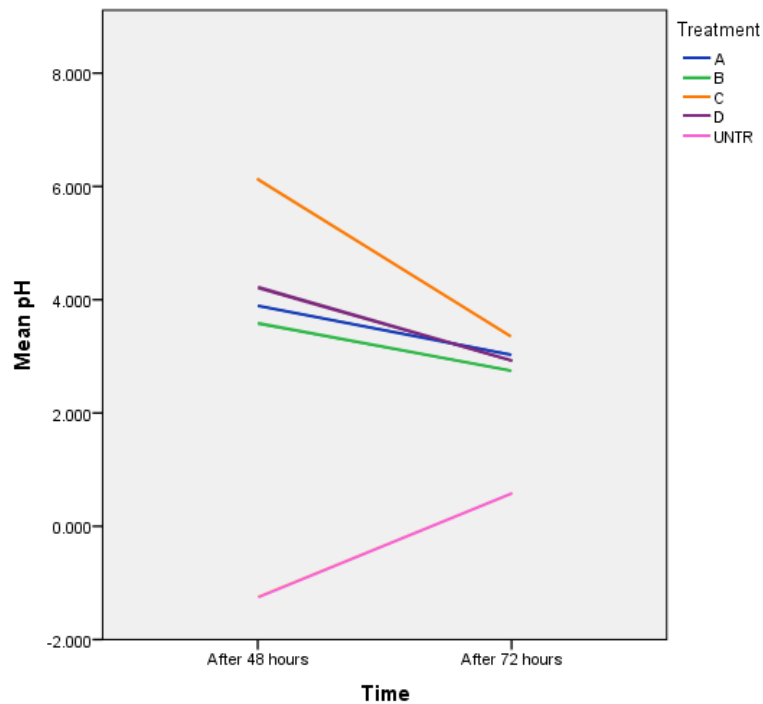
From Figure E.9 it can be seen that none of the Treatments (B, C or D) showed significant difference towards Treatment A (irritated skin with no treatment).

#### E.4.2.2 Skin pH

By using the Skin-pH-Meter® PH 905, it was possible to detect if any change in skin pH took place in the time period.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects only revealed somewhat significant differences between treatments ( $p = 0.063$ ).

Pair wise comparisons with Bonferroni adjustment revealed a somewhat significant difference between Treatment C and the untreated skin ( $p = 0.056$ ).



**Figure E.10:** % Change in skin pH over time for erythema study

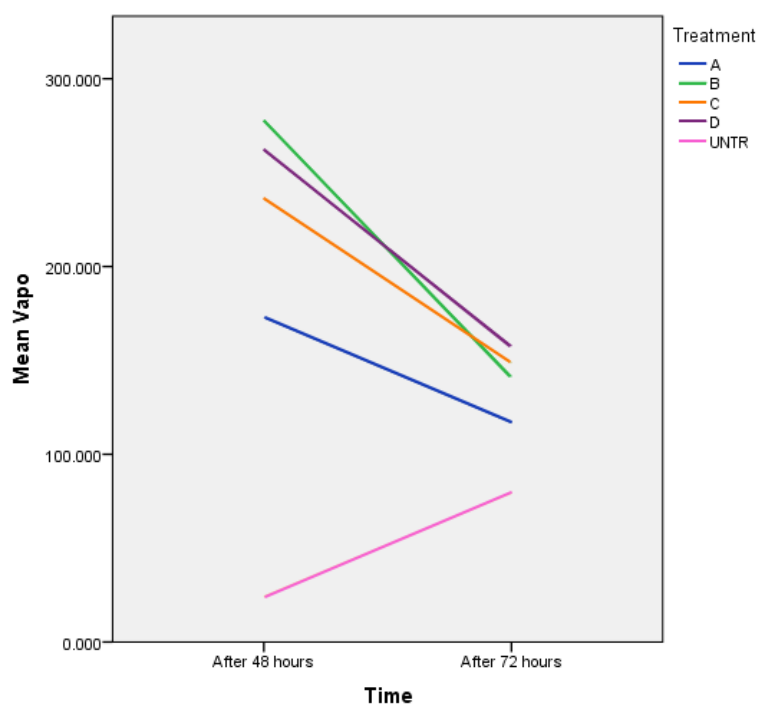
From Figure E.10 it can be seen that none of the Treatments (B, C or D) showed significant difference towards Treatment A (irritated skin with no treatment).

#### E.4.2.3 Vapour loss

By using the Vapometer<sup>®</sup>, it was possible to detect if any change in skin vapour loss took place in the time period.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects only revealed significant differences between treatments ( $p = 0.003$ ).

Pair wise comparisons with Bonferroni adjustment revealed significant, differences between untreated and Treatment B ( $p = 0.006$ ), untreated and Treatment C ( $p = 0.025$ ) and untreated and Treatment D ( $p = 0.012$ ).



**Figure E.11:** % Change in skin Vapometer<sup>®</sup> readings over time for erythema study

From Figure E.11 it can be seen that none of the Treatments (B, C or D) showed significant difference towards Treatment A (irritated skin with no treatment).

### E.4.3 Long term study

The purpose of this study was to identify whether crocodile oil lotion had anti-ageing effects over a 12 week period. For this study crocodile oil lotion were used as Treatment A, the placebo cream as Treatment B and UNTR refers to untreated skin.

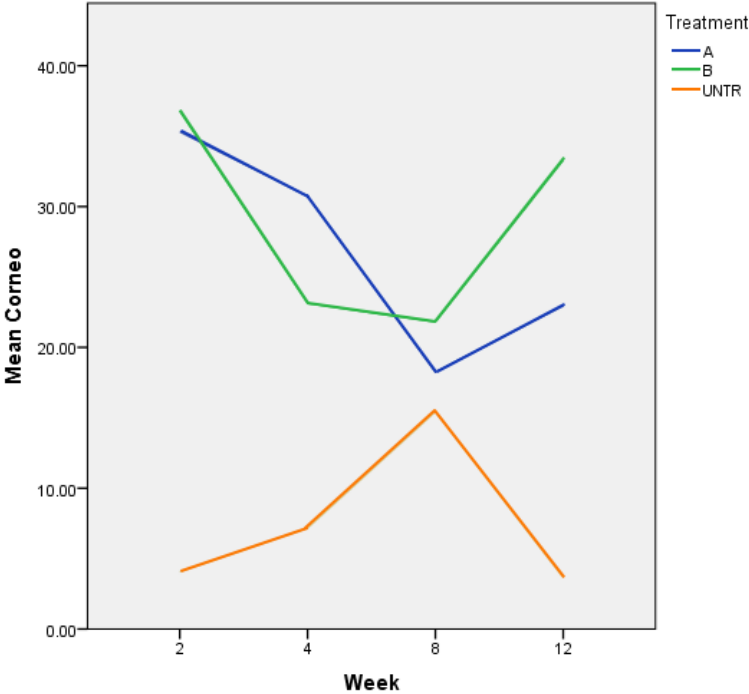
#### E.4.3.1 Skin hydration

By using the Corneometer<sup>®</sup> CM 825, it was possible to detect whether any change in skin hydration took place during the 12 week trial period. With the focus on skin ageing, one of the most preventative actions that can be taken, is by proper hydration of the skin. It is important to note that the subjects in this study all had moderately dry skin before testing started.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects only revealed significant differences between treatments ( $p < 0.0001$ ). The interaction effect between time and treatment was also significant ( $p = 0.025$ ), however the F-value for treatment ( $F = 23.157$ ) was higher than

that for time ( $F = 1.046$ ) indicating that the interaction effect might be induced by treatment effect ( $F = 2.462$ ), however from Figure E.12 the interaction does seem important.

Multiple pair wise comparisons with Bonferroni adjustment revealed significant differences between untreated and Treatment A ( $p < 0.0001$ ) and untreated and Treatment B ( $p < 0.0001$ ).



**Figure E.12:** % Change in skin hydration over 12 weeks for long term study

From Figure E.12 it can be seen that both Treatments A and B had an immediate effect on skin hydration. It can be assumed that external factors may also have contributed to the observed skin hydration levels as can be seen from the rise in skin hydration levels for the untreated areas up to 8 weeks of exposure. The effect of both Treatments A and B seemed to diminish over time, however. Although both Treatments A and B showed an increase in skin hydration after 2 weeks, there was not a significant difference between the two treatments over the 12 week period.

**E.4.3.2 Skin scaliness (RKU)**

By using the Visioscan® VC 98, it was possible to detect if any change in skin scaliness took place during the 12 week period.

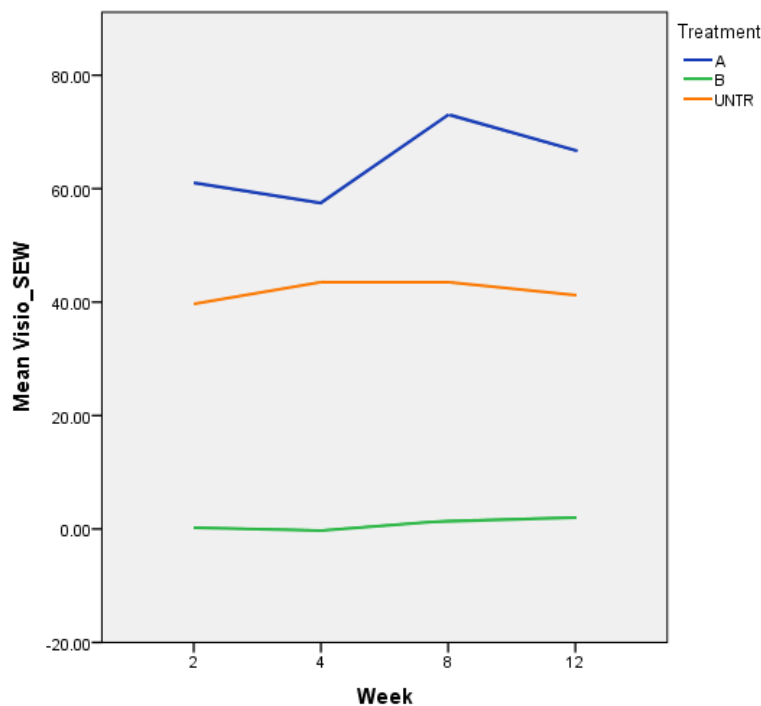
A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed no significant effects.

### E.4.3.3 Skin roughness (SEW)

By using the Visioscan® VC 98, it was possible to detect if any change in skin roughness took place during the 12 week period by using the SEW parameter. Skin roughness indicates the degree of unevenness visible during analysis. If this value decreases, skin roughness decreases and skin smoothness increases.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis. Type III fixed effects revealed a significant treatment effect ( $p = 0.053$ ).

Multiple pair wise comparisons with Bonferroni adjustment revealed significant differences between Treatment A and Treatment B ( $p = 0.049$ ).



**Figure E.13:** % Change in skin roughness over 12 weeks for long term study

Treatment B showed a significant decrease in SEW value and stayed almost the same throughout the 12 week period. This showed that skin smoothness improved over the 12 week period. Treatment A showed an increase in SEW value after 2 weeks and although it changed throughout the period, the change between week 2 and week 12 was not significant. The increase in SEW value showed that skin smoothness did not improve over the 12 week period.

#### E.4.3.4 Skin elasticity

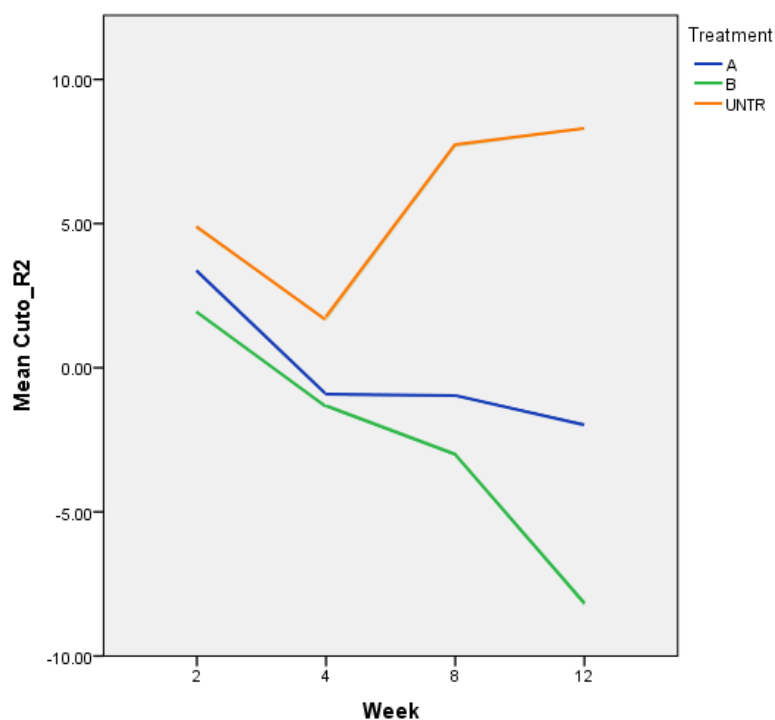
By using the Cutometer<sup>®</sup> dual MPA 580, it was possible to detect if any change in skin elasticity took place during the 12 week period.

A mixed model, with AR(1) covariance structure and fixed repeated measures over time and treatment, was used for statistical analysis of all Cutometer<sup>®</sup> measurements. Table E.2 provides an overview of the findings.

**Table E.2:** Cutometer<sup>®</sup> parameters and their respective p-values

Measure	Comparison	p-value
<b>R2</b>	Time Effect (Type III)	0.124
	Treatment Effect (Type III)	0.00001*
	Interaction Effect (Type III)	0.065*
<b>R6</b>	Time Effect (Type III)	0.498
	Treatment Effect (Type III)	0.00001*
	Interaction Effect (Type III)	0.730
<b>R7</b>	Time Effect (Type III)	0.362
	Treatment Effect (Type III)	0.008*
	Interaction Effect (Type III)	0.235
<b>R8</b>	Time Effect (Type III)	0.00001*
	Treatment Effect (Type III)	0.208
	Interaction Effect (Type III)	0.00001*

\*significant



**Figure E.14:** % Change in Cutometer® readings for parameter R2 over 12 weeks for long term study

For parameter R2 multiple comparisons with Bonferroni adjustment revealed significant differences between untreated and Treatment A ( $p = 0.002$ ) and untreated and Treatment B ( $p < 0.0001$ ). From Figure E.14 it is also clear that the interaction effect could not be ignored.

Multiple comparisons for parameter R6 with Bonferroni adjustment revealed significant differences as seen in Table E.3:

**Table E.3:** Cutometer® parameter R6 and their respective p-values

Comparison	p-value
A vs. B	0.014
A vs. UNTR	0.061
B vs. UNTR	0.0001

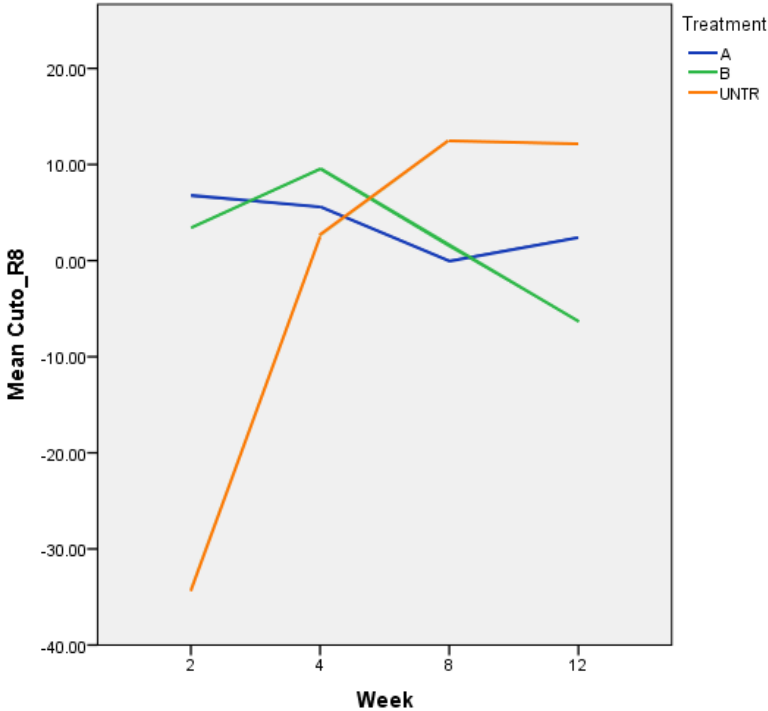
Multiple comparisons for R7 with Bonferroni adjustment revealed significant differences between Treatment B and the untreated skin ( $p = 0.007$ ).

For parameter R8 type III fixed effects revealed significant interactions for week ( $p < 0.0001$ ) as well as interaction between week and treatment ( $p < 0.0001$ ). In Table E.4 pair wise comparisons revealed significant differences between week 2 and the remaining weeks:

**Table E.4:** Pairwise comparisons between treatment weeks

Week	Sig
2 vs. 4	0.00001
2 vs. 8	0.001
2 vs. 12	0.009

Figure E.15 confirms the importance of the interaction effect observed.



**Figure E.15:** % Change in Cutometer® readings for parameter R8 over 12 weeks for long term study

In Figure E.15 a statistical difference can be seen on week 2 between untreated skin and Treatment A and untreated skin and Treatment B. After 4 weeks there was no significant difference between the untreated skin and Treatments A and B. After 8 and 12 weeks, the untreated skin showed better elasticity than Treatments A and B. It can be assumed that external factors may have contributed to the rise in skin hydration levels as seen from the hydration levels for the untreated areas after the 12 week exposure.

**E.5 Discussion**

From the short term study it could be seen that both the placebo- and crocodile oil lotion had an immediate effect on skin hydration after 60 min. The effect seem to diminish over time.

Although both treatments showed an increase in skin hydration, there was not a significant difference between the two treatments. An initial decrease in skin scaliness for both treatments could be seen after 60 min. The crocodile oil lotion seemed to reduce scaliness soon after application, however the effects seemed to reverse itself over time. No improvement in skin smoothness as well as skin elasticity was seen over the 3 h period for both treatments.

For the erythema study the crocodile oil lotion didn't show any effect towards irritated skin.

For the long term study both the crocodile oil lotion and placebo lotion showed an increase in skin hydration. After 2 weeks the skin hydration was at its highest point, and again after 12 weeks. Although both treatments showed an increase in skin hydration, there was not a significant difference between the two treatments. No treatment showed any effects towards skin scaliness. Although the crocodile oil lotion didn't show a positive effect on skin roughness, the placebo lotion showed a positive effect on skin roughness over the 12 week period. When looking at parameter R8 for skin elasticity, both treatments showed an increase in elasticity on week 2. However, after 4 weeks, the effects started to decrease and after 12 weeks the untreated skin showed more elasticity than the treated skin.

A number of non-invasive methods were applied to determine skin properties in an objective way. These types of methods can potentially detect and quantify some subclinical symptoms. However, standardisation between instruments is at present imperfect and measuring the same skin property with different instruments can give different results (Berardesca, 2011:89). Three categories of factors and sources of variation including instrumental – , environmental – and individual variables may interfere with measurements (Berardesca, 2011:90).

The calibration of the instruments should be checked frequently to enable successful and reliable laboratory comparison of results (Berardesca, 2011:90). In this study the instruments were calibrated before every testing day. In the future, more regular calibrations between skin analyses, could improve unpredictable results.

Another instrument-related variable is the surface area and contact time. It is advisable to measure on a horizontal plane to avoid skin curving. If there is a contact between the device and the skin, the pressure of the probe on the skin surface should be held constant. The time the probe is applied to the skin should be as short as possible to avoid occlusive effects which may alter skin surface (Berardesca, 2011:91). In this study, detailed efforts went into taking precise measurements. However, there is always room for improvement when it comes to using exactly the same techniques on every subject. One of the complications experienced during this study was that the marked skin areas were inconsistent due to the fading of the

marked areas. Subjects also applied the lotions irregularly by covering the whole marked area and also going outside the marked area. This can lead to lower concentrations of lotion applied to the specific areas, resulting in conflicting and uneven results.

Environment-related variables include air convections, the ambient air temperature, ambient air humidity, light sources and skin cleansing. Air convections are the main source of disturbance resulting in rapid fluctuations of the measurements. It is commonly produced by disturbances in the room, such as people moving around, opening and closing doors, breathing across the measurement zone and air conditioners. These disturbances are difficult to avoid and the use of a covering box to shield undesirable air turbulence as much as possible, can be of great help (Berardesca, 2011:91). The variability between readings in this study can definitely be an outcome of air convections present during measurements.

The most important effect of the air temperature is that it influences the skin temperature. A distinction must be made between the temperature of the measuring room and the temperature where the subjects live. Therefore an adaptation time of 15-30 min is mandatory. Sweating in summer and feeling cold in winter seem to be obvious problems. As a consequence of this, it is evident that geographical variations also may affect measurements (Berardesca, 2011:92). In this study, 30 min adaptation time was included before measurements were taken. In future this time may be prolonged to assure good adjustment of subjects to the temperature and humidity in the measurement room. Another variable that could influence the study was the season in which the study was performed. The study was performed in winter and continued into early spring. This could definitely have influenced the measurements taken.

Any light source close to the test site, affecting the ambient air temperature, the probe temperature and the temperature of the skin surface of the subjects, should be avoided. Cleansing of the skin with surface active agents and solvents could modify the skin surface due to damage of skin barrier function. Changes in skin hydration could also result from the removal of occlusive substances from the skin surface, like cream and also from the removal of sebum. Exposure of the skin to water-containing products could also result in elevated water loss from the surface of the skin (Berardesca, 2011:92). In this study, no restrictions were placed on how many times per day the subject might be exposed to water. If a subject had a bath or shower right before measurements, it could lead to skin variations between subjects who had a shower or bath the night before measurements. The use of different skin cleaning devices was not limited. Devices such as skin sponges or wash cloths could disrupt the stratum corneum as well as the level of hydration. The type of cleansing agent (Dove<sup>®</sup> soap) was used by all subjects over the period of the study.

Individual related variables like age, sex and race are very important variables influencing skin function, as well as biophysical measurements. Therefore, all these variables should be controlled or standardised when planning a study. In particular, studies should be designed within the same ethnic group, age range and sex. Ageing skin is characterised by alteration in water content and loss of mechanical function (Berardesca, 2011:93). In this study female, Caucasian subjects between the ages of 40 and 65 years were used to minimise variation.

Different anatomical sites differ widely from a physiological point of view, being characterised by different anatomical characteristics. Dermal thickness decreases with age and is greater in men than in women. It varies with the body site and is disposed to endocrine influences. Recently, it has been suggested that for the evaluation of cosmetic formulation, facial skin would be more suitable than the volar forearm which was used in this study (Berardesca, 2011:94).

Physical, thermal and emotional sweating are important variables which need to be controlled. If the ambient air temperature is below 20 °C and the skin temperature is below 30 °C, thermal sweat gland activity is unlikely, provided the skin is not exposed to forced convection and no excessive body heat is produced. A pre-measurement after a 15-30 min rest in a temperature controlled room of 20-22 °C should be taken into account. Physical activity is kept to a minimum but it is impossible to control the insensible perspiration (Berardesca, 2011:94). In this study, no pre-measurements of skin temperature were made. In future this is something that needs to be checked. This can also be one of the explanations for the variations in the results.

Skin damage and diseases are variables that can influence measurements. Cosmetic testing should be avoided in subjects or sites affected by skin disease (Berardesca, 2011:95). In this study subjects had to comply within the inclusion criteria for the study.

Lastly intra- and inter-individual variations can also affect measurements. It should be taken into account that some skin sites, including some parts of the forehead, the palm of the hand and the 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 variability between the different skin sites on the same forearm. The areas nearest to the wrist showed very inconsistent results. This proves that more care should be taken in selecting skin sites in future to prevent these variables.

The immense variation between the participants had a definite influence on the results. In future, greater care needs to go into applying the cream only in clearly marked areas, avoiding the wrist area completely. Taking better care in measurement techniques and environment changes, will also ensure more accurate results.

## **E.6 Conclusion**

The results of the short term study indicated that the crocodile oil lotion as well as the placebo lotion increased the skin hydration, although no statistical significant difference was seen between the two treatments over 3 h. The results also indicated that both the treatments showed a decrease in skin scaliness after 60 min, but after 3 h no effect could be seen. Although both treatments showed a decrease in skin scaliness, no statistical significant difference was seen between the two treatments. Neither treatments showed any change in skin roughness and skin elasticity over the 3 h period.

The results obtained for the erythema study clearly showed that none of the treatments showed any significant difference towards the non-treated, irritated skin when looking at the skin redness, skin pH and vapour loss of the skin.

From the results of the long term study, followed that the crocodile oil lotion, as well as the placebo lotion, increased the skin hydration, although no statistical significant difference was seen between the two treatments over 12 weeks. The biggest increase in skin hydration could be seen after the first two weeks of treatment. No improvement could be seen in skin scaliness and skin elasticity after 12 weeks in both treatments. Crocodile oil lotion also didn't improve skin roughness over the 12 week period.

It would thus be concluded that the use of crocodile oil lotion did not have any statistically significant benefits above the use of the placebo lotion in the treatment of ageing skin.

In future more care needs to go into applying the cream only in clearly marked areas and avoiding the wrist area completely. Better care in measurement techniques and environment changes will also ensure more accurate results. In future it will be more sensible to complete the study in one season, and not between two seasons. This will also ensure more precise results.

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## APPENDIX F

# FORMS USED IN CLINICAL EFFICACY STUDY OF CROCODILE OIL LOTION

### CROCODILE CREAM STUDY PROTOCOL

NR

#### INFORMED CONSENT

<b>STUDY IDENTIFICATION</b>
<b>CHARACTERISATION, TOXICOLOGY AND CLINICAL EFFECTS OF CROCODILE OIL IN SKIN CARE PRODUCTS</b>
<b>INSTITUTION - UNIT FOR DRUG RESEARCH AND DEVELOPMENT NORTH-WEST UNIVERSITY (POTCHEFSTROOM CAMPUS)</b>
<b>INVESTIGATOR -TELANIE VENTER (072 281 3461)</b>

I, the undersigned \_\_\_\_\_ agree to take part in the experimental study that was described to me and which will be supervised by professor Jeanetta du Plessis. I am aware that there are risks such as adverse skin effects, that along with my role in the study, was exhaustively explained (orally and written) to me and that I am free to ask any questions regarding the study.

I am free to retire my participation before the study or to interrupt my participation during the study at anytime. I also accept to inform the investigator of any change in my state of health or pharmacological treatment that might occur during the execution of the study.

I am free to ask for any further information regarding the study or to inform any adverse effects at once, by contacting the investigator at cell phone number: **072 281 3461**.

I have read and signed this consent form, fully aware of the test procedures.

I accept that the data recorded during the study could be subjected to computerised analysis by the investigator, but I am also aware that any information that can identify me will remain confidential throughout the study recordings.

- I agree  
 I do not agree

\_\_\_\_\_  
NAME AND SURNAME

\_\_\_\_\_  
DATE

\_\_\_\_\_  
SIGNATURE

<b>CROCODILE OIL CREAM STUDY PROTOCOL</b>	<b>NR</b>	
<b>PRE-TREATMENT QUESTIONNAIRE</b>		
<b>INCLUSION / EXCLUSION CRITERIA</b>		
<b>Name:</b>		
<b>INCLUSION CRITERIA</b>		
1 Age between 40 and 65 years	<b>YES</b>	<b>NO</b>
2 Good health state with no pathological events for the period immediately before tests	<b>YES</b>	<b>NO</b>
3 Understanding of both English and Afrikaans languages	<b>YES</b>	<b>NO</b>
4 Can be contacted at any time via cell phone or landline	<b>YES</b>	<b>NO</b>
<b>EXCLUSION CRITERIA</b>		
1 Subject out of age bracket for specific test	<b>YES</b>	<b>NO</b>
2 Subject has a history of any kind of allergic reaction 30 days prior to test	<b>YES</b>	<b>NO</b>
3 Subject has a history of eczema	<b>YES</b>	<b>NO</b>
4 Subject has psoriasis within 6 months of enrolment	<b>YES</b>	<b>NO</b>
5 Subject are pregnant or lactating	<b>YES</b>	<b>NO</b>
6 Subject had prior cosmetic procedures or major surgery within the previous 12 months that could affect response evaluation	<b>YES</b>	<b>NO</b>
7 Subject had previous treatment with the products that are tested	<b>YES</b>	<b>NO</b>
8 Subject has uncontrolled systemic disease	<b>YES</b>	<b>NO</b>
9 Subject has any condition that could interfere with neuromuscular function (e.g. Myasthenia gravis/ Eaton-Lambert syndrome/ Amyotrophic lateral sclerosis)	<b>YES</b>	<b>NO</b>
10 Subject has any or dermatological illnesses that could interfere with treatment or interpretation of results	<b>YES</b>	<b>NO</b>
11 Subject is taking topical or systemic drugs that could influence the test results (e.g. topical anti-inflammatory / corticosteroids / etc.)	<b>YES</b>	<b>NO</b>
12 Subject has a <b>recent</b> history of intolerance to drugs and/or cosmetic products	<b>YES</b>	<b>NO</b>
13 Subject is currently involved in another clinical investigation or were involved within a period of 30 days prior to admission to this study	<b>YES</b>	<b>NO</b>
14 Subject is going to expose herself to intensive doses of UV radiation	<b>YES</b>	<b>NO</b>
Dominant hand	<b>LEFT</b>	<b>RIGHT</b>
_____	_____	
<b>SUBJECT SIGNATURE</b>	<b>DATE</b>	

## APPENDIX G

### MOLECULES: GUIDE FOR AUTHORS

#### G.1 Manuscript Preparation

Manuscripts should be prepared in English using a word processor. A template file can be used. MS Word for Macintosh or for Windows .doc or .rtf files is preferred. Manuscripts may be prepared with other software, provided that the full document (with figures, schemes and tables inserted into the text) is exported to a MS Word format for submission. Times or Times New Roman font is preferred. The font size should be 12 pt. and the line spacing "at least" 17 pt. Although our final output is in .pdf format, authors are asked to not send manuscripts in this format as editing them is much more complicated.

Special Notes regarding MS Word files:

Please do not insert any graphics (schemes, figures, etc.) into a movable frame which can superimpose the text and make the layout very difficult.

Most formatting codes will be removed or replaced on processing your article so there is no need to use excessive layout styling. In addition, options such as automatic word breaking, double columns, footnotes, automatic numbering (especially for references) or add-ins like EndNote should not be used. However, bold face, italic, subscripts, superscripts, etc. may be used for emphasis as needed. Authors from countries where right-to-left writing is used should ensure their manuscripts have Western style (left-to-right) formatting.

The standard style of *Molecules* should be followed. Prospective authors should consult any current issue of *Molecules* for examples of this style. A template file may be downloaded from the *Molecules* homepage.

Authors' full mailing addresses, homepage addresses, phone and fax numbers, e-mail addresses and homepages can be included in the title page and these will be published in the manuscripts and the Table of Contents. The corresponding author should be clearly identified. It is the corresponding author's responsibility to ensure that all co-authors are aware of and approve of the contents of a submitted manuscript.

A brief (200 word maximum) Abstract should be provided. The use in the Abstract of numbers to identify compounds should be avoided, unless these compounds are also identified by name.

A list of three to five keywords must be given, and placed after the Abstract. Keywords may be single words or very short sentences.

Although variations in accord with a manuscript's contents are permissible, in general all papers should have the following sections: Introduction, Results and Discussion, Conclusions, Acknowledgments (if applicable), Experimental and References (or References and Notes, if applicable).

Authors are encouraged to prepare figures and schemes in color. Full color graphics will be published free of charge. Conference slides, video sequences, software, etc., can also be included and will be published as supplementary material.

Tables should be inserted into the main text, and numbers and titles supplied for all tables. All table columns should have an explanatory heading. To facilitate layout of large tables, smaller fonts may be used, but in no case should these be less than 10 pt. in size. Authors should use the Table option of MS Word to create tables, rather than tabs, as tab delimited columns are often difficult to format in .pdf for final output.

Figures and schemes should also be placed in numerical order in the appropriate place within the main text. Numbers, titles and legends should be provided for all schemes and figures. These should be prepared as a separate paragraph of the main text and placed in the main text before the figure or scheme. Chemical structures and reaction schemes should be drawn using an appropriate software package designed for this purpose. As a guideline, these should be drawn to a scale such that all the details and text are clearly legible when placed in the manuscript (i.e. text should be no smaller than 8-9 pt.). To facilitate editing we recommend the use of any of the software packages widely available for this purpose: MDL<sup>®</sup> Isis/Draw, ACD/ChemSketch<sup>®</sup>, CS ChemDraw<sup>®</sup>, ChemWindow<sup>®</sup>, etc. Free versions of some of these products are available for personal or academic use from the respective publishers. If another less common structure drawing software is used, authors should ensure the figures are saved in file format compatible with one of these products.

*X-ray crystallographic data.* To avoid publication of extensive compilations of crystallographic data and facilitate refereeing of manuscripts, *Molecules* has entered a data deposition scheme with the Cambridge Crystallographic Data Centre (<http://www.ccdc.cam.ac.uk/>). Authors planning to include such results in their papers should contact the CCDC to deposit the crystallographic data and obtain the corresponding CCDC deposition number(s) before submitting their manuscripts to *Molecules* (for instructions on doing this, see: <http://www.ccdc.cam.ac.uk/conts/depositing.html>). The deposition number(s) (usually provided

by the CCDC within 3 working days) should be included in the manuscript, along with the following text: "CCDC ..... contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via [www.ccdc.cam.ac.uk/conts/retrieving.html](http://www.ccdc.cam.ac.uk/conts/retrieving.html) (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: [deposit@ccdc.cam.ac.uk](mailto:deposit@ccdc.cam.ac.uk)"). This text may be included in the General subsection of the Experimental or as a suitably referenced endnote.

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*References.* For bibliographic citations the reference numbers should be placed in square brackets, i.e., [ ], and placed before the punctuation, for example [4]. or [1-3], and all the references should be listed separately and as the last section at the end of the manuscript. The ACS style for references(<http://pubs.acs.org>) is used for all MDPI journals.

Journal references should cite the title of the paper and its starting and ending pages, thus

8. Bowman, C. M.; Landee, F. A.; Reslock, M. A. Chemically Oriented Storage and Retrieval System. 1. Storage and Verification of Structural Information. *J. Chem. Doc.* 1967, 7, 43-47.

Journal titles should be abbreviated according to standard ISO rules. A useful list of journal title abbreviations is available on the *Molecules* website at <http://www.mdpi.org/molecules/journallist.htm>. Other relevant listings are widely available on the Internet, for example: <http://www.cas.org/sent.html> (CASSI abbreviations).

References to books should cite the author(s), title, publisher, publication date, and page(s).

9. Smith, A. B. *Textbook of Organic Chemistry*; D. C. Jones: New York, 1961; pp 123-126.

In referring to a book written by various contributors, cite author(s) first, as in

10. Winstein, S.; Henderson, R. B. In *Heterocyclic Compounds*; Elderfield, R. C., Ed.; Wiley: New York, 1950; Vol. 1, Chapter 1, p 60.

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## **G.2 Referees**

Authors should suggest at least *five* referees with the appropriate technical expertise, although the Editor will not necessarily approach them. Their addresses, homepage addresses, phone and fax numbers and e-mail addresses should be provided as fully as possible.

## **G.3 English corrections**

This journal is published in English, so it is essential that for proper refereeing and quick publication all manuscripts be submitted in grammatically correct English. For this purpose we ask that non-native English speakers ensure their manuscripts are checked before submitting them for consideration. We suggest that for this purpose your manuscript be revised by an English speaking colleague before submission. Additional fees of 250 CHF will be paid by authors if extensive English corrections must be done by the editors.

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## G.6 Sample Deposit and Exchange

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## G.7 Correct identification of components of *natural products*

The correct identification of the various components of extracts from natural sources is of key importance, and as publishers we are keenly aware of our responsibility to the scientific community in this area. Consequently, for papers on this topic, we have adopted the recommendations of the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), as published in *Flavour Fragr. J.* 2006, 21, 185. These recommendations may be summarized as follows:

Any identification of a natural compound must pass scrutiny by the latest forms of available analytical techniques. This implies that its identity must be confirmed by at least two different methods, for example, comparison of chromatographic and spectroscopic data (including mass, IR and NMR spectra) with those of an authentic sample, either isolated or synthesized. For papers claiming the first discovery of a given compound from a natural source, the authors must provide full data obtained by their own measurements of both the unknown and an authentic sample, whose source must be fully documented. Authors should also consider very carefully

potential sources of artifacts and contaminants resulting from any extraction procedure or sample handling.

## APPENDIX H

# SKIN PHARMACOLOGY AND PHYSIOLOGY: GUIDE FOR AUTHORS

### H.1 Submission

Manuscripts written in English should be submitted to: Online Manuscript Submission or as an e-mail attachment (the preferred word-processing package is MS-Word) to the Editorial Office: spp@karger.ch Professor Dr. med. J. Lademann Editorial Office „Skin Pharmacology and Physiology“ S. Karger AG PO Box CH-4009 Basel (Switzerland) Tel. +41 61 306 1361 Fax +41 61 306 1434

### H.2 Conditions

All manuscripts are subject to editorial review. Manuscripts are received with the explicit understanding that they are not under simultaneous consideration by any other publication. Submission of an article for publication implies transfer of the copyright from the author to the publisher upon acceptance. Accepted papers become the permanent property of „Skin Pharmacology and Physiology“ and may not be reproduced by any means, in whole or in part, without the written consent of the publisher. It is the author's responsibility to obtain permission to reproduce illustrations, tables, etc. from other publications. The journal consists of the following sections: 1. Editorials 2. Reviews 3. Original Papers 4. Short Communications 5. Letters to the Editor.

### H.3 Short Communications

Authors may apply to have their paper treated as a short communication. Condition for this is a brief definite report of important results. Final decision whether a paper will be published as a short communication will be made by the Editors. A publication time of two months after acceptance is intended.

### H.4 Conflicts of Interest

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.

## H.5 Arrangement

**Title page:** The first page of each paper should indicate the title, the authors' names, the institute where the work was conducted, and a short title for use as running head. **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. **Abstract:** Each paper needs an abstract in English of up to 10 lines. The abstract is of utmost importance. It should contain the following information: purpose of the study, procedures, results, conclusions and message of the paper. **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 **Online edition:**

## H.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)).

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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)).

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## **H.9 Supplementary Material**

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### **H.13 Page Charges**

There are no page charges for papers of 5 or fewer printed pages (including tables, illustrations and references). Each additional complete or partial page is charged to the author at CHF 325.00. The allotted size of a paper is equivalent to approximately 16 manuscript pages (including tables, illustrations and references).

### **H.14 Proofs**

Unless indicated otherwise, proofs are sent to the corresponding author and should be returned with the least possible delay. Alterations other than the correction of printer's errors are charged to the author.

### **H.15 Reprints**

Order forms and a price list are sent with the proofs. Orders submitted after the issue is printed are subject to considerably higher prices.

## APPENDIX I

### JOURNAL OF NATURAL MEDICINES: GUIDE FOR AUTHORS

“Journal of Natural Medicines” is pleased to announce the launch of its new online manuscript submission and tracking system. This new system makes it possible for authors to submit manuscripts via the Internet, provides online peer review services, and tracks manuscripts through the review process. Key features include automatic conversion of authors’ submissions into PDF format as well as supporting submissions in various file formats and special characters. Authors are requested to use Editorial Manager for all submissions of new manuscripts.

#### **I.1 Editorial policy**

The Journal of Natural Medicines invites papers that make a significant contribution to the knowledge and understanding of naturally occurring medicines and their related foods and cosmetics, including identification and structure elucidation of natural products, biosynthesis, biotechnology, and pharmacology of herbs, natural products, and Kampo formulas. Papers are also published concerning chemical and botanical identification of herbs or their products where such information contributes to the overall safety of plant-based medicines currently and/or formerly in use. Submission of a paper implies that it has been approved by all the named authors, that it has not been published before, and that it is not under consideration for publication elsewhere.

Acceptance of papers will be decided by the Editorial Board after examination by at least two referees. Manuscripts that require revision and/or correction and/or condensation will be returned to the authors with comments specifying alterations from the referees and/or the Editorial Board.

The following types of papers may be submitted. (i) Original Papers: Original Papers are the usual form of publication of this journal describing original experimental results. (ii) Notes (up to 4 printed pages): Notes are brief articles reporting new facts and/or valuable data. (iii) Rapid Communications (up to 3 printed pages): These are short preliminary reports of novel and significant findings that merit urgent publication. Full details of the Rapid Communication may be published later as a comprehensive original paper, in this or another journal. An explanatory statement is required for urgent publication. (iv) Reviews (up to 12 printed pages): Reviews covering recent discoveries or topics of current interest are generally invited. (v) Mini-Reviews

(up to 6 printed pages): These provide a short overview of a particular subject and are also generally invited. In the case of a non-invited Review or Mini-Review, a short summary describing the outline should be sent to the Chief Editor for approval before submitting a manuscript. (vi) Natural Resource Letters (up to 6 printed pages): Natural Resource Letters are reports on biological screening data, field observations, market surveys, quality evaluation or quantitative methods of crude drugs, etc., which may contribute to the scientific knowledge of the readers.

Documentation of experimental biological materials.

Use the correct scientific name and indicate who identified the biological materials. The herbarium deposit site and voucher number should be recorded. Authors who purchase herbal materials from companies must make provision for their deposit in a herbarium.

Pharmacological investigations of extracts require detailed extract characterization.

This includes botanical characterization of plant material(s), solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). A chromatographic (e.g., HPLC profile recorded at different wavelengths) or chemical characterization (quantities of typical constituents) must be presented. The investigation must clearly indicate a dose/activity dependence in comparison with a reference compound (positive control) together with the appropriate statistics.

Chief Editor: Hiromitsu Takayama

E-mail: [takayama@p.chiba-u.ac.jp](mailto:takayama@p.chiba-u.ac.jp)

## **I.2 Online Submission**

Authors should submit their manuscripts online. Electronic submission substantially reduces the editorial processing and reviewing times and shortens overall publication times. Please log directly onto the link below and upload all your manuscript files following the instructions given on the screen.

<http://www.editorialmanager.com/jonm>

## **I.3 Legal requirements**

The author(s) guarantee(s) that the manuscript will not be published elsewhere in any language without the consent of The Japanese Society of Pharmacognosy and Springer, that the rights of

third parties will not be violated, and that the publisher will not be held legally responsible should there be any claims for compensation.

Authors wishing to include figures or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

#### **I.4 Manuscript preparation**

Manuscripts must be written in English using Times font and typed in double-line spacing with 3-cm margins; pages should be numbered. Authors whose first language is not English are urged to have their manuscript read by a colleague who is a native English speaker and is familiar with their field of work before submitting the paper.

Manuscripts with extensive English language deficiencies will be returned to the author without the full reviewing process.

##### Documentation of experimental biological materials.

Use the correct scientific name and indicate who identified the biological materials. The herbarium deposit site and voucher number should be recorded. Authors who purchase herbal materials from companies must make provision for their deposit in a herbarium.

##### Pharmacological investigations of extracts require detailed extract characterization.

This includes botanical characterization of plant material(s), solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). A chromatographic (e.g., HPLC profile recorded at different wavelengths) or chemical characterization (quantities of typical constituents) must be presented. The investigation must clearly indicate a dose/activity dependence in comparison with a reference compound (positive control) together with the appropriate statistics.

The manuscript should be arranged as follows:

Title page (including an article title, name(s) of author(s), affiliation(s) and address(es) of the author(s), e-mail address, telephone and fax numbers of the corresponding author.)

Abstract (200-250 words)

Four to six keywords

Text of the paper: Introduction; main text (divided into sections, if appropriate); Acknowledgments; References; tables; figure legends

Abbreviations. Abbreviations must be spelled out in full at their initial appearance in the abstract and main text, followed by the abbreviation in parentheses. Thereafter, the abbreviation may be employed.

However, the following need not be defined : AIDS (acquired immunodeficiency syndrome), ATP (adenosine 5'-triphosphate), cAMP (adenosine 3',5'-cyclic monophosphate), CD (circular dichroism), cDNA (complementary DNA), CoA (coenzyme A), COSY (correlated spectroscopy), DNA (deoxyribonucleic acid), ED<sub>50</sub> (50% effective dose), EI-MS (electron ionization mass spectrometry), FAB-MS (fast atom bombardment mass spectrometry), FAD (flavin adenine dinucleotide), GC-MS (gas chromatography-mass spectrometry), HMBC (heteronuclear multiple bond connectivity), HMQC (heteronuclear multiple quantum coherence), HPLC (high-pressure liquid chromatography, high-performance liquid chromatography), HSQC (heteronuclear single quantum coherence), IC<sub>50</sub> (inhibitory concentration, 50%), IR (infrared), LD<sub>50</sub> (50% lethal dose), mRNA (messenger RNA), MS (mass spectrum), NMR (nuclear magnetic resonance, as <sup>13</sup>C-NMR, <sup>1</sup>H-NMR), NOE (nuclear Overhauser effect), NOESY (nuclear Overhauser and exchange spectroscopy), ORD (optical rotatory dispersion), P450 (as in cytochrome P450), RNA (ribonucleic acid), rRNA (ribosomal RNA), TLC (thin-layer chromatography), tRNA (transfer RNA), UV (ultraviolet).

Units. The following units should be used: length (m, cm, mm, μm, nm, Å), mass (kg, g, mg, μg, ng, pg, mol, mmol), volume (l, ml, μl), time (s, min, h, d), temperature (°C, K), radiation (Bq, Ci, dpm, Gy, rad), concentration (M, mM, mol/l, mmol/l, mg/ml, μg/ml, %, %(v/v), %(w/v), ppm, ppb).

Spectral and elemental analysis data. Please report spectral and elemental analysis data in the following format, <sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ:1.25 (3H, d, J=7.0 Hz), 3.55 (1H, q, J=7.0 Hz), 6.70 (1H, m). <sup>13</sup>C-NMR (CDCl<sub>3</sub>) δ:20.9 (q), 71.5 (d), 169.9 (s). IR (KBr) cm<sup>-1</sup>: 1720, 1050, 910. UV λ<sub>max</sub>(H<sub>2</sub>O) nm (log ε):280 (3.25). MS m/z :332 (M<sup>+</sup>), 180, 168. HRFAB-MS m/z:332.1258 (Calcd for C<sub>18</sub>H<sub>20</sub>O<sub>6</sub>: 332.1259). [α]<sub>D</sub><sup>23</sup>—74.5° (c 1.0, MeOH). Anal. Calcd for C<sub>19</sub>H<sub>21</sub>NO<sub>3</sub>:C, 73.29; H, 6.80; N, 4.50. Found: C, 73.30; H, 6.88; N, 4.65.

Nomenclature. The nomenclature of chemical compounds should be in accordance with the nomenclature rules formulated by IUPAC. Alternatively, naming may conform to the nomenclature in the index of Chemical Abstracts or the Ring Index.

X-ray crystal structure analyses. When structure determination by X-ray crystallographic analysis is a central theme of the paper, the data required for registration with the Cambridge Crystallographic Data Base (CCDB) should be attached as supplementary material. Although this does not apply in cases where crystallographic analysis plays only a supplementary role, crystal data (unit cell parameters, space group, Z density) and R-factor should still be noted. Atomic coordinates, bond lengths and angles, thermal parameters, and torsion angles should be presented when they are important for the issues addressed in the paper. When papers have been accepted, the authors are advised to register the data with CCDB.

## **I.5 References**

The list of References should include only works that are cited in the text and that have been published or accepted for publication. Personal communications should be mentioned only in the text. If available, the DOI can be added at the end of the reference in question.

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2. Chen W, Punja Z (2002) Agrobacterium-mediated transformation of American ginseng with a rice chitinase gene. *Plant Cell Rep* 20:1039–1045 DOI 10.1007/s00299-002-0446-z
3. Tsukaya H, Iokawa Y, Kondo M, Ohba H (2005) Large-scale general collection of wild-plant DNA in Mustang, Nepal. *J Plant Res* DOI 10.1007/s10265-005-0196-4
4. Capasso F, Gaginella TS, Grandolini G., Izzo AA (2003) *Phytotherapy*. Springer, Berlin Heidelberg New York
5. Bauer BA (2003) Herbal therapy and the elderly. In: Cherniack P, Cherniack N (eds) *Alternative medicine for the elderly*. Springer, Berlin Heidelberg New York, pp 111–132

## **I.6 Tables and figures**

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Figure legends must be brief, self-sufficient explanations of the illustrations. The legends should be placed at the end of the text.

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Color illustrations should be saved as RGB (8 bits per channel) in TIFF format.

Artwork Guidelines

## **I.7 Conflict-of-interest policy**

Authors should indicate any financial assistance, commercial affiliations, or potential conflicts of interest associated with any work submitted for publication.

## **I.8 Ethical Standards**

Manuscripts that reveal a lack of ethical consideration for human subjects or experimental animals will not be accepted for publication. All clinical research using human subjects or materials should be conducted in accordance with the Recommendations from the Declaration of Helsinki. For animal experiments, authors should follow the experimental animal guidelines of their institution as well as the appropriate government guidelines, such as those published by the Japanese Ministry of Education, Culture, Sports, Science and Technology. Authors must also indicate in the manuscript that the experimental procedures were approved by the appropriate committee (e.g., Experimental Animal Research Committee) at their institute or university.

## **I.9 Electronic Supplementary Material**

Electronic Supplementary Material (ESM) for a paper will be published in the electronic edition of this journal provided the material is:

- submitted in electronic form together with the manuscript
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ESM may consist of:

information that cannot be printed: animations, video clips, sound recordings (use QuickTime, .avi, .mpeg, animated GIFs, or any other common file format); information that is more convenient in electronic form: sequences, spectral data, etc.; large quantities of original data that relate to the paper, e.g., additional tables, large numbers of illustrations (color and black & white), etc.

Legends must be brief, self-sufficient explanations of the ESM. ESM is to be numbered and referred to as S1, S2, etc.

After acceptance for publication, ESM will be published as received from the author in the online version only. Reference will be given in the printed version.

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- Springer Open Choice

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(ii) Charges for excess pages in Notes, Rapid Communications and Natural Resource Letters: JPY 8,000 per printed page in addition to the handling charges.

### **I.12 Date of issue**

The journal will be issued on 10th of January, April, July, and October each year.

(Revised on 7 October 2011)