Revista Brasileira de Farmacognosia 26 (2016) 521–529

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Physical stability and clinical efficacy of *Crocodylus niloticus* oil lotion

Telanie Venter[a], Lizelle Triféna Fox[a], Minja Gerber[a], Jan L. du Preez[a], Sterna van Zyl[b], Banie Boneschans[b], Jeanetta du Plessis[a,*]

[a] *Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa*
[b] *Centre for Pharmaceutical and Biomedical Services, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa*

ARTICLE INFO

Article history:
Received 4 December 2015
Accepted 25 March 2016
Available online 23 May 2016

Keywords:
Formulation
Stability
Crocodylus niloticus oil
Lotion
Anti-ageing
Skin hydration

ABSTRACT

The stability and the anti-ageing, skin hydrating and anti-erythema effects of a commercialized *Crocodylus niloticus* Laurenti, 1768, Crocodylidae, oil lotion was determined. The lotion was stored at controlled conditions over six months during which several stability tests were performed. For the clinical efficacy studies lotion was applied on volar forearm skin (female volunteers) and compared to a liquid paraffin-containing reference product. Skin hydrating and anti-ageing effects were determined with a Corneometer®, Cutometer® and Visioscan®, following single (3 h) and multiple applications (12 weeks). The Vapometer® and Mexameter® were utilized to determine this lotion's anti-erythema effects on sodium lauryl sulfate irritated skin. The lotion demonstrated good stability over 6 months. The reference product increased skin hydration and decreased skin wrinkles to a larger extent than the *C. niloticus* lotion after a single application, whereas the *C. niloticus* lotion decreased skin scaliness better than the reference product. During the long-term study, the reference product overall increased skin hydration more than the *C. niloticus* lotion, whereas *C. niloticus* lotion increased skin elasticity to a larger extent than the reference product. *C. niloticus* lotion increased skin wrinkles and decreased skin scaliness over 12 weeks. Compared to non-treated, irritated skin, *C. niloticus* lotion demonstrated some potential anti-inflammatory characteristics.

Introduction

Skin ageing has forever fascinated and inspired researchers in search of solutions that would help prevent wrinkle formation. Additionally, the skin represents an excellent and accessible model organ which allows both intrinsic and extrinsic ageing factors to be studied; of which both promote to the multifaceted phenomenon of ageing. Chronological (intrinsic) ageing affects the skin in a manner similar to other organs, whilst extrinsic ageing is related to environmental damage (mainly ultraviolet-induced) of the dermal connective tissue of the skin. There is evidence that the process of intrinsic and extrinsic ageing have at least in part overlapping biological-, biochemical-, and molecular mechanisms (Ma et al., 2001).

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. An ageing dermis results in increasingly tight, inelastic tissue that is less capable of undergoing modifications in response to stress (Diridollou et al., 2001).

Natural oils are widely used in cosmetics and can be used to treat a growing number of conditions (Nielsen, 2006). Natural oils contain a range of fatty acids, which contribute towards various favorable properties in personal care and cosmetic products. The use of animal oils in cosmetics has increased over the past few years.

Oil is harvested from the fat of *Crocodylus niloticus* Laurenti, 1768, Crocodylidae, which is native to Africa and can reach up to 7 m in length and up to 730 kg in weight. By-products from *C. niloticus*, such as the oil, are widely accepted as folk remedies by Chinese communities around the world, although its beneficial effects have not yet been proven. *C. niloticus* oil can be used as cosmetic or lubricant and therefore rivals with other natural oils (*i.e.* emu- and ostrich oil) that possess similar benefits (Shim-Prydon and Camacho-Barreto, 2007).

Numerous claims of positive results are reported with regards to topically applied, *C. niloticus* oil-containing product. These include the fading of freckles, uneven dark tones, sun spots, acne, pimple marks, dark lines, wrinkles and laugh lines. Additionally, it aids in

* Corresponding author.
E-mail: Jeanetta.DuPlessis@nwu.ac.za (J. du Plessis).

http://dx.doi.org/10.1016/j.bjp.2016.03.011

preventing skin discoloration and has demonstrated the ability to control rashes and dryness to make the skin softer, brighter and more attractive (Lötter, 2009).

A study by Buthelezi et al. (2012) identified sixteen fatty acids from the oil of *C. niloticus*, with the major components being oleic-, palmitic- and linoleic acid. These researchers found that *C. niloticus* oil had shown antimicrobial activity against *Klebsiella pneumoniae* and *Staphylococcus aureus*. In addition, the oil had also demonstrated anti-fungal specificity against *Candida albicans*. Anti-inflammatory assays indicated that a fairly short acting anti-inflammatory response had occurred after oral administration of the *C. niloticus* oil; whereas an acute, somewhat long acting anti-inflammatory response had been observed after topical application of the oil (Buthelezi et al., 2012). Researchers during another study found that oil from *Crocodylus siamensis* had enhanced cutaneous burn wound healing and improved skin regeneration and collagen deposition (Li et al., 2012).

These studies identified the potential use of the oil from the *Crocodylus* species in wound care management, but also as an anti-inflammatory and antimicrobial agent. Little research on its anti-ageing and skin hydrating effects on the human skin is, however, reported in the available literature. The first aim of this study therefore was to determine the stability of a commercially available *C. niloticus* oil lotion product. Secondly, non-invasive electrical instruments were used to determine the skin hydration and anti-ageing effects in human volunteers, after single and multiple applications of the *C. niloticus* oil lotion, compared to a reference product, containing liquid paraffin. Thirdly, the *C. niloticus* oil lotion was tested to determine its anti-erythema effects on sodium lauryl sulfate (SLS) irritated skin.

Materials and methods

Materials

Commercial *Crocodylus niloticus* Laurenti, 1768, Crocodylidae, oil (20%) lotion was obtained from a crocodile and reptile park in South Africa. The reference product to which the *C. niloticus* oil lotion was compared was of the comparable base composition, but instead contained liquid paraffin (20%). Due to commercial-in-confidence, the authors are unable to disclose the exact composition of this formulation. SLS was purchased from Merck Laboratory Supplies (Midrand, South Africa). Deionized high performance liquid chromatography (HPLC) grade water, prepared with a Milli-Q® water purification system (Millipore, Milford, MA, USA), was used throughout this study.

Methods

Stability testing

The purpose of stability testing during this study was to provide evidence on how the quality of a drug substance would vary over time under the influence of a range of environmental factors of temperature and humidity (International Conference on Harmonization, 2003). For the purpose of this study, the *C. niloticus* oil lotion was stored in its original packaging (50 ml plastic container) at accelerated stability conditions of 25 °C/60% relative humidity (RH), 30 °C/60% RH and 40 °C/75% RH over a period of 6 months, according to the guidelines provided by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Tripartite Guideline (ICH, 2003). The stability tests being performed at months 0, 1, 2, 3 and 6 included pH, viscosity, visual appearance, zeta-potential, droplet size and mass loss.

pH

The pH of the lotion at each storage condition was measured in triplicate with a Mettler Toledo pH meter (Mettler Toledo AG, Giessen, Germany), equipped with a glass Mettler Toledo InLab® 410 (Schwerzenbach, Switzerland) electrode.

Viscosity

A Brookfield Viscometer (Model DV II+, Middleboro, MA, USA), mounted on a Helipath D20733 stand and fitted with a T-bar spindle was used to determine the viscosity of the lotion at each storage condition. The lotion was placed in a pre-heated water bath to reach a temperature of 25 °C. The viscosity reading was measured every 10 s for the duration of 5 min. Approximately thirty-two readings were generated and the average viscosity determined.

Visual appearance

The visual appearance of the formulation at each stability test condition was assessed by comparing the color of the lotion to the initial color and appearance of the *C. niloticus* oil lotion. Photos were taken of each formulation, placed at the same location in the laboratory each time that appearance was evaluated. A digital camera was placed approximately 15 cm away from the formulations.

Zeta-potential

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

Droplet size

Initially, approximately 0.5 g of the formulation from each stability storage condition was mixed with approximately 3 ml of HPLC water to moisten the formulation into a uniform, liquid dispersion. Each lotion sample was prepared in triplicate. Thereafter, these mixtures were further diluted with approximately 4.5 ml of HPLC water, mixed well and injected into a Malvern Mastersizer 2000 (Worcestershire, United Kingdom). In addition, the wet cell Hydro 2000 SM was also used to serve as the interface between the sample dispersion accessory and the optical unit. Samples were analyzed in triplicate at a rotating speed of 1500 rpm.

Finally, the statistics of the distribution were calculated from the results, using the derived diameters $D[m,n]$, an internationally recognized method of defining the mean and other moments of particle size.

Mass loss

The mass of the formulation was measured prior to placing it on stability using a Shimadzu scale (Kyoto, Japan). Thereafter, the mass of the formulation at each stability condition (over 6 months) was determined in triplicate and deducted from the initial mass measurements.

Clinical efficacy testing

Clinical study protocol

Guidelines for Good Clinical Practice (GCP) was followed and this study was carried out in accordance with the Helsinki declaration (Ethical principles of medical research involving human subjects) and had been pre-approved by the Ethics Committee of the North-West University (Potchefstroom campus, South Africa) under the project title of "(*In vivo*) Cosmetic efficacy studies" (NWU-00097-10-A5).

All subjects signed an informed consent form and were informed that they were free to discontinue their participation at any time during the study, without any consequences. A group of healthy female subjects, between the ages of 40 and 65 years, participated in the study. Important variables, such as sex and race, can influence biophysical measurements (Berardesca, 2011). Consequently, only female volunteers were included in the study so as to attain a uniform population in which these factors are regulated. Testing only commenced after a 7 day washout period during which participants followed their normal skin cleansing routines, but they were only allowed to use the supplied Dove® soap bars.

All participants complied with both the inclusion (above) and exclusion criteria. The exclusion criteria were:

1. Any previous treatment with *C. niloticus* oil lotion.
2. Any history of eczema.
3. Any episode of psoriasis within 6 months prior to the study.
4. The occurrence of any allergic skin reaction 30 days prior to the study.
5. Any uncontrolled systemic disease.
6. Any dermatological illnesses that could interfere with treatment, or the interpretation of results.
7. Any taking of a topical or systemic drug that could influence the test outcomes.
8. Any recent history of intolerance to cosmetic products.
9. Having any condition that could interfere with neuromuscular function.
10. Pregnant or lactating women, or women who may possibly have been exposed to high doses of UV radiation recently.

The chosen anatomical test area for this study was the volar forearm, as it is hairless, contains a small amount of sebaceous glands and offers a relatively large available skin surface area (Bazin and Fanchon, 2006).

C. niloticus oil lotion was compared to a reference product (lotion) with similar base composition, but containing liquid paraffin, instead of *C. niloticus* oil. Since the *C. niloticus* oil lotion is a commercial product, its composition cannot be disclosed. The liquid paraffin, present in the reference product, is a well-known, commonly used occlusive (Draelos, 2000). An occlusive increases the water content of the stratum corneum (SC), by reducing transepidermal water loss (TEWL) by forming a hydrophobic barrier over the skin (Wiechers and Barlow, 1999; Kraft and Lynde, 2005). It is believed that occlusives diffuse into the intercellular lipid domains which add to their efficiency (Kraft and Lynde, 2005).

On the mornings of measurement, any use of alcohol, caffeine and vasoactive medications were prohibited, to prevent the possible alteration of the microcirculation of the skin and hence any indirect impact on the skin hydration profile. All measurements were conducted under controlled temperature and humidity conditions (20–25 °C and 50 ± 10% RH). The subjects were allowed to acclimate to the room conditions for at least 30 min before any measurements were taken (Darlenski and Fluhr, 2011).

Single application (short-term) and multiple applications (long-term) hydration and anti-ageing studies

These studies were performed to investigate the skin hydration and anti-ageing effects of the *C. niloticus* oil lotion after single (short-term) and multiple (long-term) applications, compared to a reference product. A summary of these studies is given in Table 1.

The short-term study served as a pilot study for predicting product efficacy (Berardesca, 1997).

A group of nine subjects participated in the short-term study. Two treatment areas (3.5 cm × 1.5 cm) were marked on the dominant forearm with a Codman® surgical marker. A baseline reading (*T*0) was taken, after which the *C. niloticus* oil lotion and the reference lotion were applied on the marked squares. Thereafter, measurements were taken at 1 h (*T*1), 2 h (*T*2) and 3 h (*T*3) after application of the lotions.

Table 1
Summary of the short- and long-term studies.

	Short-term study	Long-term study
Amount of volunteers	9	18–22[a]
Duration	3 h	12 weeks
Measurement times	*T*0: baseline *T*1: 1 h *T*2: 2 h *T*3: 3 h	*T*0: baseline *T*1: 2 weeks *T*2: 4 weeks *T*3: 8 weeks *T*4: 12 weeks
Instruments and parameters used	Corneometer®: skin hydration Visioscan®: SEw, SEsc	Corneometer®: skin hydration Visioscan®: SEw, SEsc Cutometer®: *R*2, *R*5, *R*7

SEw, wrinkling; SEsc, scaliness.
[a] Amount of volunteers depended on outliers in data set that were removed.

During the long-term study the two treatment areas (3.5 cm × 1.5 cm) were located on the non-dominant forearm. A baseline reading (*T*0) was taken, followed by measurements after 2 (*T*1), 4 (*T*2), 8 (*T*3) and 12 (*T*4) weeks of treatment. The reference product and *C. niloticus* oil lotion were applied on the marked squares twice daily, between 6 to 8 am in the mornings and between 6 and 8 pm in the evenings. The amount of product spread onto the marked areas with a gloved-covered finger was 1–3 μl/cm^2 (1–3 mg/cm^2) (measured with a syringe). The subjects also received a timetable on which to document the times at which they applied the lotion. On measurement days, the subjects were not allowed to apply the treatment in the mornings, but only in the evening prior to taking the measurements.

Skin ageing is a complicated process and is portrayed by a decline in elasticity, and an increase in wrinkling, dryness and roughness (Gilchrest, 1989). The non-invasive Corneometer® CM 825, Visioscan® VC 98 and Cutometer® dual MPA 580 from Courage-Khazaka (Cologne, Germany) are well-suited to determine the changes in these skin parameters caused by ageing.

These instruments were used during both the short-and long-term studies to determine the skin hydration and anti-ageing effects of the *C. niloticus* oil- and reference lotions. In addition, the Cutometer® dual MPA 580 was utilized for the long-term study.

The Corneometer® measures the water content of the SC (skin hydration) by means of the capacitance method. Since water has the highest di-electrical constant in the skin, increased capacitance values would therefore be indicative thereof that the water content of the skin (skin hydration) has increased. The mean change in water content of the SC (after three measurements) was converted into arbitrary units (AU) (Berardesca, 1997; Darlenski and Fluhr, 2011).

The Visioscan® was used to analyze the changes in skin topography and it comprises of a high resolution camera and a halogenide light source. Ultraviolet light uniformly illuminates the skin surface, and a built-in camera takes images of the skin area (6 mm × 8 mm); where after they are displayed as high resolution black and white images to clearly show the wrinkles and skin surface properties. Computer software make use of the Surface Analysis of Living Skin (SELS) method to analyze the image data and calculate several SELS parameters which are used to evaluate the skin surface; both quantitatively and qualitatively (Udompataikul et al., 2009). The parameters used during this study were wrinkling (SEw) and scaliness (SEsc).

SEsc is indicative of the dryness level of the skin. Consequently, stratum corneum dehydration can be evaluated by this parameter. A smaller value of SEsc indicates a higher level of skin hydration with less scaliness (Kim et al., 2004). Therefore, a decrease in SEsc

is an indication of the capability of the applied formulations to increase skin hydration.

The width and number of skin wrinkles are assessed by Sew, which is evaluated by the proportion of vertical and horizontal wrinkles (Choi et al., 2013). A higher SEw value indicates that more and wider wrinkles are present (Udompataikul et al., 2009) and therefore, it is predicted to decrease when the formulation possess anti-ageing properties.

The Cutometer® MPA 580 measures the mechanical properties of the skin (*i.e.* skin elasticity) (Darlenski et al., 2011). The decrease in skin elasticity, associated with an increase in age, is not as evident as the other signs of ageing (*i.e.* wrinkles) and therefore it is important to also investigate the elasticity of the skin (Ahn et al., 2007). The Cutometer® MPA 580 mechanically deforms the upper skin layer by way of applying a negative pressure that pulls the upper layer of the skin into the probe opening. Thus, the skin resistance (firmness), when pulled into the probe opening, and the skin's capability to recuperate from such deformation (elasticity), are measured. These values are calculated and represented by a deformation curve from which elasticity (*R*) parameters were calculated.

The overall elasticity (*R*2), net elasticity (*R*5) and the ratio of elastic recovery to the total deformation (*R*7) parameters are all related to skin elasticity (Choi et al., 2013) and were found to be the most suitable indicators of the influence of ageing on the skin's mechanical properties (Ahn et al., 2007) and were therefore investigated during this study. These parameters are associated with the functioning of the skin's elastic fibers and are indicative of any changes, due to genetic, physiological, or acquired pathological skin conditions (Dobrev, 2002).

A negative correlation exists between the *R*2, *R*5 and *R*7 (especially) parameters and age. A decrease in the biological elasticity of the skin is observed in both actinic and biological ageing, which is reflected by a decrease in the *R*5 and *R*7 values. It was determined that especially the *R*7 parameter, correlated with age. The net elasticity, *R*5, may be solely influenced by the skin's elastic fibers, whereas *R*7 could be influenced by changes in both skin viscosity and elasticity. As a result, *R*7 correlated with ageing more than *R*5. Due to the *R*7 parameter having a higher correlation with age than the *R*2 parameter, it suggests that the elastic fibers degrade quicker with ageing than the other skin components (Ryu et al., 2008).

Anti-inflammatory effects of C. niloticus *oil (erythema study)*

Erythema is a result of skin inflammation and irritation and can be characterized by an increase in skin redness instigated by hyperaemia of superficial skin tissue due to capillary dalation. This phenomenon can be used to assess the anti-erythema (anti-inflammatory) potential of *C. niloticus* oil lotion by way of measuring the erythema value of irritated skin before and after treatment with *C. niloticus* oil lotion. The level of skin redness is directly related to the concentration of haemoglobin in the skin which intensifies with hyperaemia of the superficial skin tissue.

The anti-erythema effects of the formulations were determined by conducting a study in accordance with the guidelines on SLS exposure tests from the Standardization Group of the European Society of Contact Dermatitis (Tupker et al., 1997). Four areas of 2.5 cm × 1.0 cm on the dominant vertical forearm of twelve subjects were marked with a Codman® surgical marker. Two non-invasive instruments, namely the Mexameter® MX 18 (Courage-Khazaka, Cologne, Germany) and Vapometer® (Delfin Technologies Ltd., Kuopio, Finland) were used. Baseline readings (*T*0) were taken with these two instruments, prior to applying a 1% w/v SLS solution on four areas under occlusion, using Finn Chambers® (internal diameter of 8 mm containing filter papers) on Scanpor® (SmartPractice®, Mednom, Cape Town, South Africa). The Finn Chambers® containing the 1% w/v SLS solution were applied for 24 h to induce erythema. In order to prevent false readings resulting from the hyperhydration effect of the SLS and the occlusive effect of the Finn Chambers® (Darlenski et al., 2011), the first measurement (*T*1) was taken 24 h after removal of the Finn Chambers®. Thereafter, the reference lotion, *C. niloticus* oil lotion and hydrocortisone cream (1% w/v, positive control) was each applied to three of the four areas where SLS had caused irritation to the skin. The fourth irritated area was left untreated, to serve as a negative control. The second measurement (*T*2) was taken 24 h after application.

The Mexameter® was employed to assess the haemoglobin (erythema) content in the upper layer of the skin. Blood is coloured red as haemoglobin reflects red (λ = 660 nm) light and absorbs green (λ = 568) light. The dissimilarities in light absorption by haemoglobin result in a certain intensity of reflected light which can be measured. The Mexameter® probe emits the specific light wavelengths and a receiver measures the light reflected by the skin. As the quantity of emitted light is defined, the quantity of light absorbed by the skin can be calculated. The redness (erythema) value is expressed as arbitrary units ranging from 0 to 999.

It was expected that the haemoglobin content values would be higher after irritation (*T*1) than the baseline readings (*T*0). A decrease in these (*T*1) values (at *T*2) was indicative of the effectiveness of the formulation as an anti-erythema agent.

The VapoMeter® (Delfin, Finland) is a closed chamber instrument which measures skin surface TEWL. It is regarded as the most suitable non-invasive method to examine the impaired barrier function of skin irritated with SLS (Fluhr et al., 2001). TEWL parameters indicate the degree of efficacy by intact skin to prevent unrestrained water loss through evaporation from the SC. TEWL reflects permeability and efficient skin barrier function (Darlenski et al., 2011; Darlenski and Fluhr, 2011). TEWL values increase after application of SLS (*T*1) and a decrease in these values (at *T*2) would be indicative of the formulations' abilities to help repair the barrier function of the skin.

Data analysis

Data analysis for clinical efficacy experiments

All of the parameters being measured during the short- and long-term studies, at all of the treatment areas, were presented as a percentage of change, relative to the initial condition (*T*0), by utilizing Eq. (1).

$$\%\,\text{Change} = \left[\frac{Tn - T0}{T0} * 100\right] \quad (1)$$

where *Tn* represents the value for *n* = 1, 2 and 3 h in the short-term study, and for *n* = 2, 4, 8 and 12 weeks in the long-term study. The percentage change during the erythema study after treatment (*T*2), relative to the conditions after irritation (*T*1), was calculated by using Eq. (2).

$$\%\,\text{Change} = \left[\frac{T2 - T1}{T1} * 100\right] \quad (2)$$

Statistical analysis

Statistical analysis was carried out using Prism. As measurements were repeated over time and every volunteer was exposed to each of the different treatments, a two-way RM (repeated measures) ANOVA (analysis of variance) was used to analyze the data (short- and long-term studies) for statistical significant differences.

This was followed by pairwise comparisons with a Bonferroni adjustment between the levels of time and between the different treatments to establish which means are statistically significantly different (Anderson et al., 2011). Data obtained from the anti-erythema study was analyzed by two-way RM ANOVA

Table 2
The percentage change in physical properties at different stability test conditions of *C. niloticus* oil lotion after a 6 month period.

	25 °C/60% RH (%)	30 °C/60% RH (%)	40 °C/75% RH (%)
pH	−1.40	−1.76	−3.28
Viscosity	−10.90	−32.10	−46.00
Zeta-potential	−2.50	−5.00	+6.50
Mass loss	−0.56	−0.63	−0.73
Droplet size	47.45	40.18	50.73

to determine the statistical significant difference between the different treatments and the irritated, untreated skin values. Statistical significance was tested at a 5% (0.05) level of significance; therefore a p-value □0.05 indicated statistically significant differences between the compared values. Outliers were eliminated from the data set by utilizing standardized values (z-scores). Data was treated as an outlier when the z-score was less than −3, or higher than +3 (Anderson et al., 2011).

Results and discussion

Stability testing

Table 2 summarizes the percentage change in the physical properties and approximate increase in droplet size (μm) of the *C. niloticus* oil lotion, stored at different stability test conditions after a 6 month period.

As reflected by the small percentage change, the pH of the lotion remained stable over 6 months of testing. Even though it is common knowledge that an increase in temperature decreases viscosity; it is also apparent that the RH did not influence viscosity as much as the temperature did, *i.e.* when comparing the change (21.2%) in viscosity when RH stayed the same (25 °C/60% RH with 30 °C/60% RH) with the change (13.9%) in viscosity when RH increased (30 °C/60% RH with 40 °C/75% RH). Nonetheless, although a large decrease in viscosity was observed over the different storage conditions, which may have been a warning of potential instability, the lotion (physically) had a suitable texture and could be applied satisfactorily. The zeta-potential of the lotion at all three controlled stability conditions remained within the same range and could be regarded as stable over the 6 months test period. The small weight decrease was indicative thereof that the lotion remained stable over the 6 months and that the containers had sealed sufficiently well to avoid evaporation during the stability period. Over the 6 months of stability testing, the change in particle size increased more than 40% over the different storage conditions, which may be attributed to the agglomeration of the particles. None of the lotion's stored at all three stability conditions showed any significant changes in color over the 6 months of evaluation.

Clinical efficacy

Short-term study

Fig. 1 illustrates that the reference product caused an increase in skin hydration to a slightly higher extent than the *C. niloticus* oil lotion, after 1, 2 and 3 h following application. Statistical analysis with a two-way RM ANOVA showed that no statistically significant difference (p-value = 0.664) was, however, observed between the reference product and the *C. niloticus* oil lotion at any one of the time points. Statistical analysis (two-way RM ANOVA) showed that the Corneometer® values changed statistically significantly over time ($p < 0.0001$).

The Bonferroni multiple comparisons test between the levels of time revealed that both the *C. niloticus* oil lotion and the reference product had statistically significantly increased skin hydration over time ($p < 0.05$) when compared to the initial conditions (*T*0), *i.e.* *T*1, *T*2 and *T*3 were statistically significantly different from *T*0. This was indicative thereof that the skin hydration effects of these two formulations were somewhat time dependent. For the *C. niloticus* oil lotion a statistical significant difference was also observed between *T*1 and *T*3.

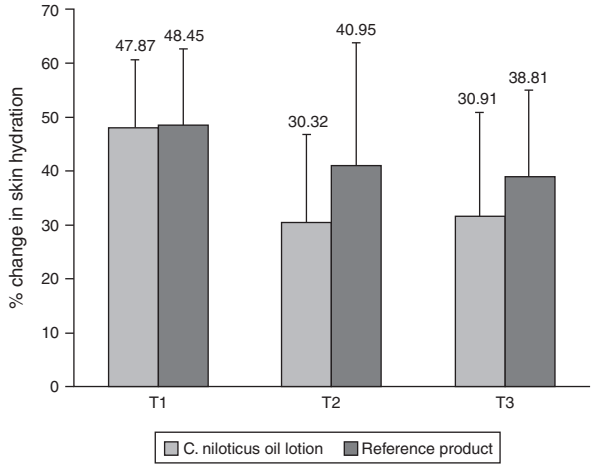

Fig. 1. Percentage change in skin hydration as measured by the Corneometer® during the short-term study ($n = 9$).

The SE_W parameter (Fig. 2A) showed that both formulations had decreased the extent of wrinkles after 1, 2 and 3 h following application, compared to initial conditions. The reference product had, however, decreased SE_W more than the *C. niloticus* oil lotion after 1 and 2 h of application, whereas the *C. niloticus* oil lotion decreased SE_W more than the reference product after 3 h following application. As determined with two-way RM ANOVA, none of these differences was statistically significant, *i.e.* there was no statistical significant difference (p-value = 0.394) observed between the *C. niloticus* oil lotion and the reference product at any of the times measured. There was also no statistical significant difference seen over time (p-value = 0.075; two-way RM ANOVA).

The *C. niloticus* oil lotion had decreased the SE_{SC} (Fig. 2B) more than the reference product after 1, 2 and 3 h following application, although not statistically significantly ($p = 0.941$; two-way RM ANOVA). However, a p-value of 0.041 (two-way RM ANOVA) revealed a statistical significant difference between the different levels of time. The Bonferroni post test revealed that the *C. niloticus* oil lotion had decreased the SE_{SC} statistically significantly over time, from baseline (*T*0) to *T*1, *T*2 as well as *T*3.

From the results of the short-term study, it was decided to conduct a long-term study to determine how these formulations would perform over a longer period of time, especially since both formulations' skin hydration capabilities were found to be somewhat time dependent.

Long-term study

According to Fig. 3A, both formulations had caused an increase in skin hydration, although the reference product had increased the hydration state of the skin to a larger extent than the *C. niloticus* oil lotion after 2, 8 and 12 weeks of application. The *C. niloticus* oil lotion had increased skin hydration more than the reference product after 4 weeks of application. Statistical analysis (two-way RM ANOVA) showed that the treatment type had a statistically significant effect ($p < 0.001$) on the values obtained with the Corneometer®. Pairwise comparisons with a Bonferroni adjustment between the different treatments showed a statistical significant difference between *C. niloticus* oil lotion and the reference product at *T*1, *T*2 and *T*3. Consequently, it can be said that the reference product

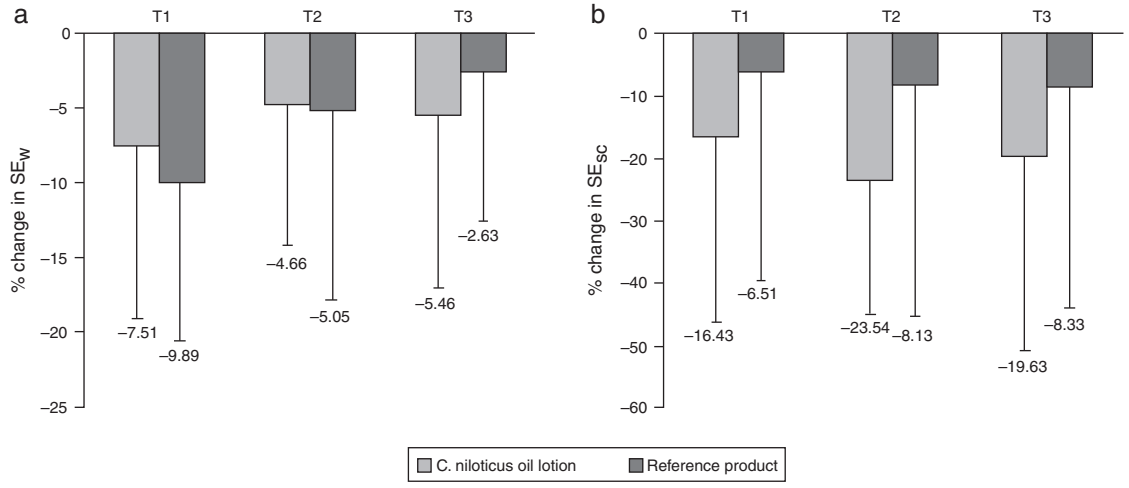

Fig. 2. Percentage change in (a) SE_W ($n = 9$) and (b) SE_{SC} ($n = 9$) as measured by the Visioscan® during the short-term study.

enhanced skin hydration statistically significantly more than the *C. niloticus* oil lotion after 2 and 8 weeks of treatment.

It was furthermore found that skin hydration changed statistically significantly over time ($p < 0.001$; two-way RM ANOVA). Statistical significant differences were observed (with Bonferroni method) for the *C. niloticus oil* lotion between *T*0 vs. *T*1, *T*2, *T*3 as well as *T*4; between *T*1 vs. *T*4 and *T*2 vs. *T*4. For the reference product, statistical significant differences were observed between initial conditions (*T*0) and subsequent measurements (*T*1, *T*2, *T*3 and *T*4) as well as between *T*1 vs. *T*2. This showed that the skin hydration effects of the *C. niloticus* oil lotion as well as the reference product were time dependent.

These results correlated with the results found during the short-term study. The superior skin hydration effect of the reference product could have been ascribed to the occlusive, *i.e.* the liquid paraffin, present in the formulation. As was stated above, this occlusive is a well-known and commonly used moisturizer (Draelos, 2000).

An evaluation of the results obtained with the Cutometer® indicated that the *C. niloticus* oil lotion had generally improved skin

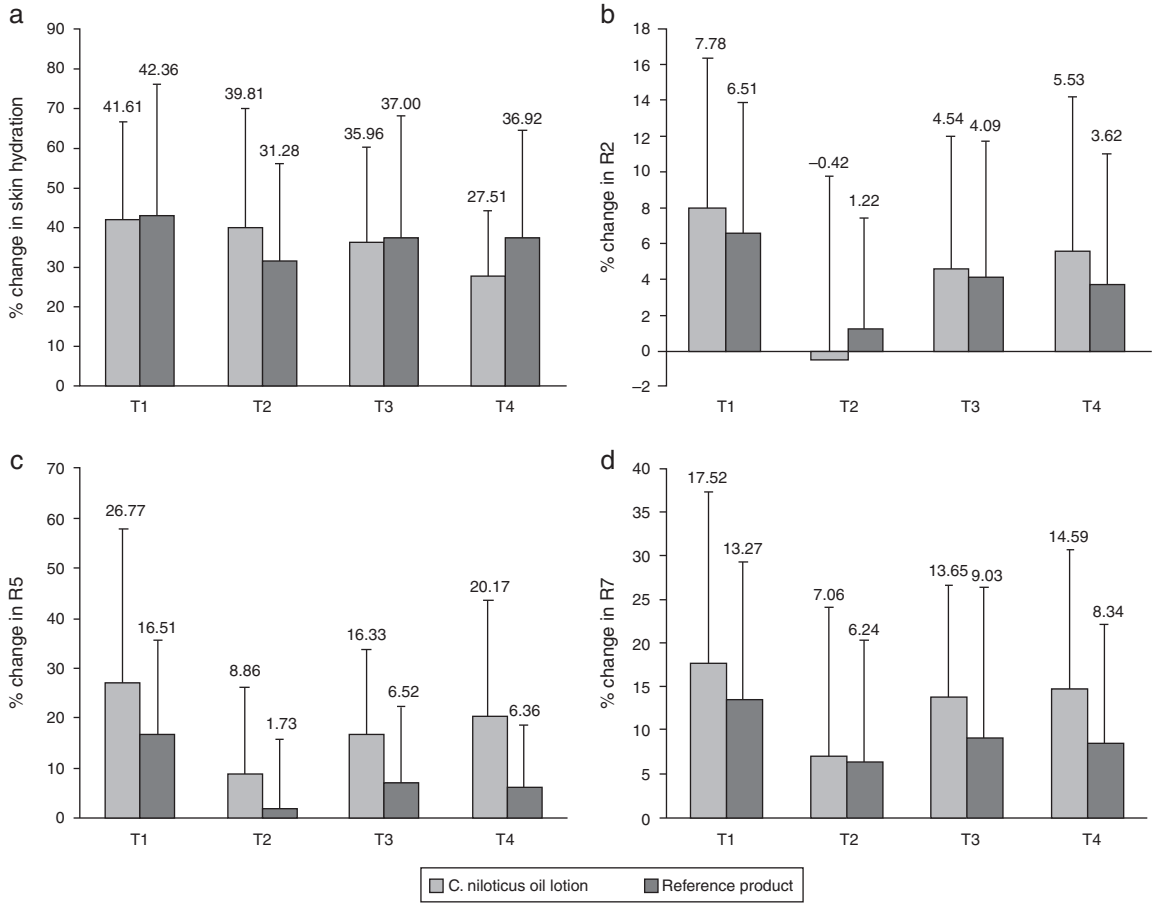

Fig. 3. Percentage change in (a) skin hydration ($n = 22$), (b) *R*2 parameter ($n = 22$), (c) *R*5 parameter ($n = 22$) and (d) *R*7 parameter ($n = 22$) during the long-term study.

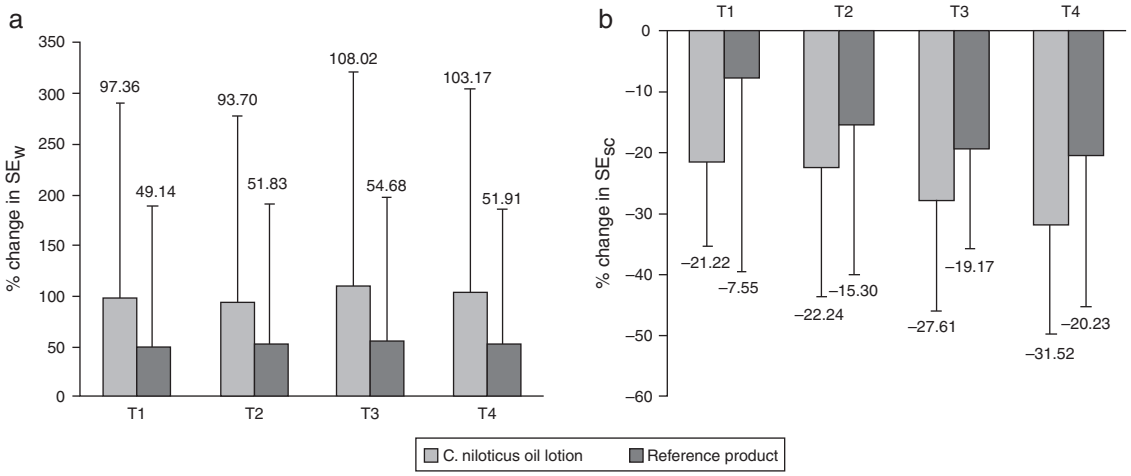

Fig. 4. Percentage change in (a) SE_W ($n = 22$) and (b) SE_{SC} ($n = 18$) as measured by the Visioscan® during the long-term study.

elasticity more than the reference product. The *C. niloticus* oil lotion had increased the overall elasticity (*R*2) (Fig. 3B) of the skin more than the reference product during the long-term study, except after 4 weeks of treatment. Analysis with two-way RM ANOVA indicated that time as well as treatment type showed a statistical significant effect ($p < 0.0001$). The Bonferroni multiple comparisons test indicated the following statistical significant differences between the levels of time for the *C. niloticus* oil lotion: *T*0 vs. *T*1, *T*0 vs. *T*4, *T*1 vs. *T*2, *T*2 vs. *T*3 and *T*2 vs. *T*4. Statistical significant differences were observed for the reference product between *T*0 vs. *T*1 and *T*1 vs. *T*2. Between the different treatments, statistical significant differences were only observed after 4 weeks (*T*2) of treatment.

A two-way RM ANOVA indicated that the treatment type had a statistical significant effect ($p = 0.005$) on the *R*5 values. Both the reference product as well as the *C. niloticus* oil lotion had increased the *R*5 parameter (Fig. 3C), although the latter formulation increased it the most throughout the long-term study. A statistically significant difference ($p < 0.0001$; two-way RM ANOVA) was observed over time for the R5 parameter. For the *C. niloticus* oil lotion, statistical significant differences (Bonferroni) were observed between *T*0 vs. *T*1, *T*3 as well *T*4, *T*1 vs. *T*2 and *T*2 vs. *T*4. Statistical significant differences were shown between *T*0 vs. *T*1 and *T*1 vs. *T*2, *T*3 as well as *T*4 when volunteers were treated with the reference product.

Fig. 3D illustrates that the *C. niloticus* oil lotion had increased the ratio of elastic recovery to total deformation (*R*7 parameter) more than the reference product over the 12 weeks of treatment. A two-way RM ANOVA revealed that both time as well as treatment type had a statistical significant effect on the R7 values. *C. niloticus* oil lotion showed to have statistical significant differences between certain time points (as determined by Bonferroni adjustment), *i.e.* *T*0 vs. *T*1, *T*3 as well as *T*4 and between *T*1 vs. *T*2. For the reference product statistical significant changes were observed between times *T*0 vs. *T*1 as well as *T*3. Statistical analysis indicated that the *C. niloticus* oil lotion enhanced the *R*7 parameter statistically significantly more than the reference product at *T*1, *T*2 and *T*3.

In a study by Choi et al. (2013), a positive relationship was found between skin hydration and the *R*2 and *R*5 parameters. This correlation was also observed in the long-term study, as both formulations had increased skin hydration and the *R*2, *R*5 and *R*7 parameters. Even though the reference product had increased skin hydration more than the *C. niloticus* oil lotion, the *C. niloticus* oil lotion had increased the elasticity parameters to a larger extent than the reference product. It would therefore be incorrect to assume that one product would enhance skin elasticity more than the other, if purely based upon their abilities to enhance skin hydration.

Generally, it is assumed that skin elasticity would increase with an increase in skin hydration. Contrary to the expectation, however, Wiechers and Barlow (1999) found that enhanced skin moisturization would not automatically result in improved skin elasticity. Except for water, it is unlikely that a molecule would possess both elasticity enhancing and moisturization capabilities (Wiechers and Barlow, 1999). Following the long-term application of a moisturizer, Jemec and Na (2002) suggested that the skin hydration level would be a poor predictor of skin mechanics. They found that although the hydration state of the skin had improved (increased skin capacitance), no change had been observed with regards to any of the skin mechanical parameters (*i.e.* distensibility, hysteresis and elasticity), when compared to the control.

Results obtained with the Visioscan® during the long-term study with regards to the SE_W parameter, contrasted with the results obtained during the short-term study. As illustrated by Fig. 4A, the *C. niloticus* oil lotion had increased this parameter to a larger extent than the reference product. Results showed that the *C. niloticus* oil lotion had not reduced skin wrinkles (SE_W) as had been expected in light of its demonstrated improved skin hydration and skin elasticity effects. A study by Choi et al. (2013) had found a negative correlation between SE_W and the hydration level of the skin, suggesting that wrinkles were reduced by an increase in skin hydration (Choi et al., 2013). This correlation was, however, not replicated during this current study. It was determined by way of two-way RM ANOVA that time also had a statistical significant effect on skin wrinkles ($p = 0.004$). A statistically significant difference (after pairwise comparisons with a Bonferroni adjustment) was observed over time (*i.e.* between *T*0 vs. *T*1, *T*2, *T*3 as well as *T*4), with regards to the *C. niloticus* oil lotion treatment. The increase in the SE_W parameter was inconsistent with the SE_{SC} parameter values.

The SE_{SC} parameter results obtained during the long-term study correlated well with the results obtained during the short-term study, as it was found that the *C. niloticus* oil lotion had decreased scaliness to a larger extent than the reference product (Fig. 4B). Only time was shown (by means of two-way RM ANOVA) to statistically significantly affect skin scaliness ($p < 0.0001$). The Bonferroni multiple comparisons test revealed statistical significant differences between *T*0 vs. *T*1, *T*2, *T*3 as well as *T*4 and between *T*1 vs. *T*4. For the reference product statistical significant changes in skin scaliness were observed between *T*0 vs. *T*1, *T*2, *T*3 as well as *T*4. These results indicate that the effect of both *C. niloticus* oil lotion as well as

Table 3
Percentage change in skin erythema (haemoglobin and TEWL) from untreated irritation (*T*1) to treated skin (*T*2).

Treatment	Haemoglobin	TEWL
Irritated skin without treatment	2.90 ± 11.10	77.24 ± 136.90
Crocodylus niloticus oil lotion	0.36 ± 9.76	76.27 ± 135.50
Reference product	−0.82 ± 15.93	62.58 ± 116.94
Hydrocortisone (1% w/v)	−0.66 ± 20.86	84.35 ± 144.70

the reference product was time dependent. Pairwise comparisons with a Bonferroni adjustment between the different treatments showed no statistical significant difference at any of the measurement times.

Erythema study

The percentages changes in skin erythema, as expressed by the haemoglobin content (Mexameter®, $n = 12$) and TEWL (Vapometer®, $n = 12$), from the untreated skin irritation (*T*1) condition to after treatment (*T*2) with the different formulations, are summarized in Table 3.

None of the treatments had a significant effect on the haemoglobin content of irritated skin after one application. The reference product had decreased the skin haemoglobin content the most, followed by the positive control, *i.e.* the hydrocortisone cream. The *C. niloticus* oil lotion slightly increased the haemoglobin content of the skin, although to a lesser extent than the irritated, untreated skin. No statistically significant differences ($p > 0.05$) were observed between any of the treated skin or the untreated, irritated skin (determined with two-way RM ANOVA).

The Vapometer® results showed that none of the treatments had lowered the TEWL values (no negative percentage change had been observed), but had in fact increased these values. The reference product did, however, show a lower percentage increase from *T*1 after one application (*T*2), compared to the other treated skin and irritated, untreated skin; followed by an even lesser impact by the *C. niloticus* oil lotion. Held et al. (2001) tested several moisturizers to determine their abilities of promoting the recovery of SLS irritated human skin. It was found that the moisturizers had accelerated barrier regeneration, compared to irritated, untreated skin. It was, however, suggested that this may not have been the case for all moisturizers. Moisturizers with higher lipid content had been found to have improved the barrier recovery more that those moisturizers with a lower lipid content (Held et al., 2001).

In this study, the reference product may have accelerated the recovery of the irritated skin, due to its protective and occlusive effect, which, as previously suggested, may have restricted the evaporation of water from the skin to allow the skin to recover undisturbed (Held et al., 2001).

Interestingly enough, the hydrocortisone cream had demonstrated a higher percentage change in TEWL from *T*1, when compared to irritated, untreated skin. However, as shown by two-way RM ANOVA none of the treatments had statistically significantly differed from each other, or from the irritated, untreated skin ($p > 0.05$).

Results obtained during the erythema study showed that *C. niloticus* oil lotion may have promising anti-inflammatory effects, when compared to irritated, untreated skin. During this study, the anti-erythema efficacy of the formulations were only determined after a single application. It could therefore be postulated that the treatments would neither have adequate time, nor a sufficient number of applications to demonstrate whether, or not, it would significantly decrease haemoglobin levels and TEWL. Additionally, the small number of volunteers used during this study may also not have been an adequately representative sample size from which to draw scientific conclusions and it is recommended that the *C. niloticus* oil lotion be tested on a larger population size to confirm its possible anti-inflammatory capabilities.

In conclusion, *C. niloticus* oil lotion increased skin hydration, but to a lesser extent than the reference product. The *C. niloticus* oil lotion also increased skin elasticity over a longer treatment period and may have potential anti-inflammatory effects. In future, the effect of pure *C. niloticus* oil (*i.e.* not in formulation) should also be tested and the formulation should be optimized to effectively deliver the *C. niloticus* oil into the skin layers.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work centre on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Authors' contributions

TV (PhD student) contributed in planning and execution of the laboratory work (stability and *in vivo* study), analysis of the data and drafting of the paper. LTF contributed to analysis of the data and drafting the paper. MG contributed to planning the project, assisting with data analysis and critical reviewing of the manuscript. JLDP contributed to the stability studies and critical reviewing of the manuscript. SVZ contributed to planning and execution of the *in vivo* clinical cosmetic efficacy study, analysis of the data and critical reviewing of the manuscript. BB contributed to and supervised the execution of the *in vivo* clinical cosmetic efficacy study and its data analysis. JDP designed the study and contributed to critical reviewing of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This study was carried out with the financial support of the National Research Foundation (NRF) of South Africa (Grants no. IFRR81178 and CPRR13091742482) and the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) at the North-West University, Potchefstroom Campus, South Africa. Prof Antoon Lötter is also acknowledged for his valuable input during this study. The authors also thank the crocodile and reptile park in South Africa for the generous supply of *C. niloticus* oil and *C. niloticus* oil lotion. Special thanks and acknowledgement to Mrs. Marike Cockeran (MSc Statistics), the Subject Specialist from MUSA (Medicine usage in South Africa), North-West University, Potchefstroom Campus who assisted with the statistical data analysis.

Disclaimer

Any opinion, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

References

Ahn, S., Kim, S., Lee, H., Moon, S., Chang, I., 2007. Correlation between a Cutometer® and quantitative evaluation using Moire topography in age-related skin elasticity. Skin Res. Technol. 13, 280–284.

Anderson, D.R., Sweeney, D.J., Williams, T.A., 2011. Statistics for Business and Economics, 11th ed. South-Western Cengage Learning, Mason, OH.

Bazin, R., Fanchon, C., 2006. Equivalence of face and volar forearm for the testing of moisturizing and firming effect of cosmetics in hydration and biomechanical studies. Int. J. Cosmet. Sci. 28, 453–460.

Berardesca, E., 1997. EEMCO guidance for the assessment of stratum corneum hydration: electrical methods. Skin Res. Technol. 3, 126–132.

Berardesca, E., 2011. Factors influencing measurements. In: Fluhr, J.W. (Ed.), Practical Aspects of Cosmetic Testing. Springer-Verlag, Berlin Heidelberg, pp. 89–99.

Buthelezi, S., Southway, C., Govinden, U., Bodenstein, J., Du Toit, K., 2012. An investigation of the antimicrobial and anti-inflammatory activities of crocodile oil. J. Ethnopharmacol. 143, 325–330.

Choi, J.W., Kwon, S.H., Huh, C.H., Park, K.C., Youn, S.W., 2013. The influences of skin visco-elasticity, hydration level and aging on the formation of wrinkles: a comprehensive and objective approach. Skin Res. Technol. 19, e349–e355.

Darlenski, R., Callaghan, T., Fluhr, J.W., 2011. Antiageing and antiwrinkle products. In: Fluhr, J.W. (Ed.), Practical Aspects of Cosmetic Testing. Springer-Verlag, Berlin Heidelberg, pp. 143–153.

Darlenski, R., Fluhr, J.W., 2011. Moisturizers and emollients. In: Fluhr, J.W. (Ed.), Practical Aspects of Cosmetic Testing. Springer-Verlag, Berlin Heidelberg, pp. 123–141.

Diridollou, S., Vabrey, V., Bersony, M., Vaillantz, L., Black, D., Lagarde, J.M., Gergoirey, J.M., Gall, Y., Patay, F., 2001. Skin ageing changes of physical properties of human skin in vivo. Int. J. Cosmet. Sci. 23, 353–362.

Dobrev, H.P., 2002. A study of human skin mechanical properties by means of Cutometer®. Folia Med. (Plovdiv.) 44, 5–10.

Draelos, Z., 2000. Atlas of Cosmetic Dermatology. Churchill Livingstone, New York.

Fluhr, J.W., Kao, J., Jain, M., Ahn, S.K., Feingold, K.R., Elias, P.M., 2001. Generation of free fatty acids from phospholipids regulates stratum corneum acidification and integrity. J. Invest. Dermatol. 117, 44–51.

Gilchrest, B.A., 1989. Skin aging and photoaging: an overview. J. Am. Acad. Dermatol. 21, 610–613.

Held, E., Lund, H., Agner, T., 2001. Effect of different moisturizers on SLS-irritated human skin. Contact Dermat. 44, 229–234.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q1A (R2), http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf (accessed 05.15) 2003. Stability Testing of New Drug Substances and Products.

Jemec, G.B.E., Na, R., 2002. Hydration and plasticity following long-term use of a moisturizer: a single-blind study. Acta Derm. Venereol. 82, 322–324.

Kim, S.Y., Kim, S.J., Lee, J.Y., Kim, W.G., Park, W.S., Sim, Y.C., Lee, S.J., 2004. Protective effects of dietary soy isoflavones against UV-induced skin-aging in hairless mouse model. J. Am. Coll. Nutr. 23, 157–162.

Kraft, J.N., Lynde, C.W., 2005. Moisturizers: what they are and a practical approach to product selection. Skin Ther. Lett. 10, 1–8.

Li, H.L., Chen, L.P., Hu, Y.H., Qin, Y., Liang, G., Xiong, Y.X., Chen, Q.X., 2012. Crocodile oil enhances cutaneous burn wound healing and reduces scar formation. Acad. Emerg. Med. 19, 265–273.

Lötter, A.P., 2009. Uses for Crocodile Oil and Crocodile Oil Containing Products (unpublished)., pp. 1–3.

Ma, W., Wlaschek, M., Tantchava-Pooa, I., Schneider, L.A., Naderi, L., Razi-Wolf, Z., Schueller, J., Scharffetter-Kockanek, K., 2001. Chronological ageing and photoageing of the fibroblasts and the dermal connective tissue. Clin. Dermatol. 26, 592–599.

Nielsen, J.B., 2006. Natural oils affect the human skin integrity and the percutaneous penetration of benzoic acid dose-dependently. Basic Clin. Pharmacol. Toxicol. 98, 575–581.

Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Ryu, H.S., Joo, Y.H., Kim, S.O., Park, K.C., Youn, S.W., 2008. Influence of age and regional differences on skin elasticity as measured by the Cutometer®. Skin Res. Technol. 14, 354–358.

Shim-Prydon, G., Camacho-Barreto, H., RIRDC publication no. 06/117, RIRDC Project no. DAQ 320A 2007. New animal products: new uses and markets for by-products and co-products of crocodile, emu, goat, kangaroo and rabbit: a report for the Rural Industries Research and Development Corporation. Australian Government: Rural Industries Research and Development Corporation, https://rirdc.infoservices.com.au/downloads/06-117 (accessed 02.15).

Tupker, R.A., Willis, C., Berardesca, E., Lee, C.H., Fartasch, M., Agner, T., Serup, J., 1997. Guidelines on sodium lauryl sulfate (SLS) exposure test: a report from the Standardization Group of the European Society of Contact Dermatitis. Contact Dermat. 37, 53–69.

Udompataikul, M., Sripiroj, P., Palungwachira, P., 2009. An oral nutraceutical containing antioxidants, minerals and glycosaminoglycans improves skin roughness and fine wrinkles. Int. J. Cosmet. Sci. 31, 427–435.

Wiechers, J.W., Barlow, T., 1999. Skin moisturization and elasticity originate from at least two different mechanisms. Int. J. Cosmet. Sci. 21, 425–435.