Stability, clinical efficacy and antioxidant properties of honeybush extracts in semi-solid formulations

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ABSTRACT

Background: Honeybush extracts (*Cyclopia* spp.) can be incorporated into skin care products to treat conditions such as skin dryness and can function as an anti-oxidant. **Objective:** To formulate Honeybush formulations and test it for antioxidant activity, skin penetration and skin hydrating effects. **Materials and Methods:** Semi-solid formulations containing either *Cyclopia maculata* (2%) or *Cyclopia genistoides* (2%) underwent accelerated stability studies. Membrane release studies, Franz cell skin diffusion and tape stripping studies were performed. Antioxidant potential was determined with the TBA-assay and clinical efficacy studies were performed to determine the formulations' effect on skin hydration, scaliness and smoothness after two weeks of treatment on the volar forearm. **Results:** The formulations were unstable over three months. Membrane release, skin diffusion studies and tape stripping showed that both formulations had inconclusive results due to extremely low concentrations mangiferin and hesperidin present in the Franz cell receptor compartments, stratum corneum-epidermis and epidermis-dermis layers of the skin. Honeybush extracts showed antioxidant activity with concentrations above 0.6250 mg/ml when compared to the toxin; whereas mangiferin and hesperidin did not show any antioxidant activity on their own. The semisolid formulations showed the potential to emit their own antioxidant activity. Both formulations improved skin smoothness, although they did not improve skin hydration compared to the placebos. *Cyclopia maculata* reduced the skin scaliness to a larger extent than the placebos and *Cyclopia genistoides*. **Conclusion:** Honeybush formulations did not penetrate the skin but did however show antioxidant activity and the potential to be used to improve skin scaliness and smoothness.

KEYWORDS: Antioxidant, *Cyclopia genistoides, Cyclopia maculata*, Honeybush, Skin aging, Transdermal delivery

INTRODUCTION

Skin aging is the end result of a constant deterioration process that impairs cellular deoxyribonucleic acid (DNA) and proteins.[1] Cutaneous aging can be divided into two very diverse types, i.e. chronological skin aging (intrinsic) and photo-aging (extrinsic).[1, 2] Intrinsic skin aging is a common and anticipated process characterized by physiological modifications in the skin function.[1] Extrinsic aging is primarily caused by overexposure to UV (ultraviolet) radiation from sunlight. Other exogenous factors include exposure to tobacco smoke, infrared radiation, ozone, airborne particulate matter and malnutrition.[3]

A number of synthetic skincare products (comprised of active ingredients such as diethanolamine, monoethanolamine, triethanolamine and sodium laureth sulfate) are available in the anti-aging market, but can however have certain side-effects including irritant and allergic contact dermatitis, phototoxicity and photo-allergic reactions. In contrast, natural skin care products are generally hypo-allergenic and are quickly absorbed by the superficial layers of the skin. Owing to their noteworthy influence on skin aging, herbal cosmetic products have grown increasingly popular in recent times.[4]

These herbal extracts for topical application deserves to be considered as a cosmeceutical because of their use of treating skin conditions and a wide variety of dermatological disorders for centuries.[5] It can be designed to protect the skin against exogenous and endogenous agents, balancing dermal homeostasis lipids altered by dermatosis and aging. Plants with a high level of flavonoids such as Honeybush, have the potential to reduce skin inflammation and to scavenge free radicals[5], penetrating the dermal and epidermal layers while counteracting the aging of the human skin.

The term Honeybush applies to several different species of *Cyclopia*. These plants are all woody, fynbos shrubs with golden yellow stems and hairless, stalkless leaves.[6] With the focus

nowadays on “food as medicine”; the use of Honeybush has shifted from a non-medicinal beverage and folklore medicinal drink, back to an organic herbal tea with several medicinal properties.[7] Honeybush tea does not contain caffeine and is rich in phenolic compounds which are believed to have important antioxidant activity.[6] These phenolic compounds are known to be mangiferin, hesperidin, hesperetin and isosakuranetin.[8]

As traditional medicine Honeybush was utilized to treat digestive problems, promote lactation and cure skin rashes.[9] Additionally it functioned as restorative and an expectorant in chronic catarrh and pulmonary tuberculosis but was later on also known for its anti-inflammatory, antioxidant, antimutagenic, phytooestrogenic and antimicrobial effects with a relative low toxicity.[7]

The number of available toiletries en cosmetic products containing Honeybush lags behind other herbal products such as rooibos. At this time no clinical trial information or results of human studies are available to prove the health promoting effects of Honeybush and its active ingredients on the human skin.[9]

Subsequently; the aim of this study was to: (1) develop some cream formulations containing either *Cyclopia maculata* or *Cyclopia genistoides*; (2) determine the stability of these formulations; (3) conduct membrane release studies to determine whether the actives (i.e. the bioactive flavonoids, mangiferin and hesperidin) were released from the formulations; (4) investigate the topical delivery of these creams by determining whether mangiferin and hesperidin diffused through the skin (i.e. with Franz cell skin diffusion studies) or into the skin (i.e. with tape stripping); (5) determine the antioxidant properties (of the extracts alone, in formulation and of the actives i.e. mangiferin and hesperidin) and (6) test the clinical efficacy (i.e. skin hydration and topography) of the Honeybush formulations on human subjects.

MATERIALS AND METHODS

Materials

The active ingredients, mangiferin and hesperidin were obtained from Sigma-Aldrich Corporation (Johannesburg, South Africa). The *C. maculata* hot water extracts was sponsored by ARC Infrutec-Nietvoorbij (Stellenbosch, South Africa). The *C. genistoides* (Novel-T® Organic Honeybush PE) powder was obtained from Afriplex (Pty) Ltd (Johannesburg, South Africa). The other ingredients used in the formulation of the semi-solid products were obtained as follow: liquid paraffin, methylparaben, propylparaben, propylene glycol and cetyl stearyl alcohol were obtained from Merck Laboratory Supplies (Johannesburg, South Africa). Cremophor® A6 and Cremophor® A25 was obtained from BASF Chemicals (Johannesburg, South Africa). Potassium dihydrogen orthophosphate and sodium hydroxide used for the preparation of phosphate buffered solution (PBS) were supplied by Merck Laboratory Supplies (Johannesburg, South Africa). Phosphoric acid, ethanol and HPLC (high performance liquid chromatography) analytical grade methanol was obtained from Merck Laboratory Supplies (Johannesburg, South Africa). Ascorbic acid, dimethyl sulfoxide (DMSO) and iron(III)chloride was obtained from Merck Chemicals (Johannesburg, South Africa). The 1,1,3,3-Tetramethoxypropane (TEP), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma-Aldrich Corporation (Steinheim, Germany). Hydrogen peroxide was purchased from a local pharmacy. Deionized HPLC grade water prepared with a Milli-Q® water purification system (Millipore, Milford, USA) was used throughout the entire study.

Methods

Formulation of a cosmeceutical cream with Honeybush extracts as the active ingredient

Two semi-solid Honeybush extract (incorporating either 2% *C. maculata* or 2% *C. genistoides*) creams containing mangiferin and hesperidin (bioactive flavonoids present in Honeybush

extracts) as the active ingredients were formulated. The formula for the Honeybush creams is given in Table 1. Part A (oil phase) and part C (water) was heated separately to approximately 80 °C. Part C was then added to part A with rigorous stirring by utilizing a homogenizer at a speed of 13 500 rpm. Mixture B was heated until the active ingredient dissolved and was subsequently mixed with the combined part A and C. The formulation was stirred while cooling to room temperature. The placebo was prepared similarly, but without the addition of the Honeybush extracts.

HPLC method for the concentration assay and in vitro skin diffusion samples

A HPLC method was developed and validated in terms of linearity, accuracy, precision (intraday and interday), ruggedness and repeatability in the Analytical Technology Laboratory (ATL) of the North-West University (NWU), Potchefstroom Campus, South Africa. An Agilent® 1200 Series HPLC equipped with an Agilent® 1200 pump, autosampler injection mechanism and UV-detector was used (Agilent Technologies, Palo Alto, CA). The apparatus was interfaced with Chemstation Rev. A.06.02 data acquisition and analysis software. High performance silica based, reversed phase Agela® Venusil XBP C_{18} (2) column, (150 mm × 4.6 mm) with a 5 µm particle size was used (Agela® Technologies, Newark, DE). Two mobile phases were used of which the first mobile phase consisted of a mixture of 1 ml phosphoric acid in 1000 ml of HPLC water and the second mobile phase of acetonitrile. The gradient elution was employed starting at 85% phosphoric acid solution and 15% acetonitrile for the first minute, followed by a linear increase to 95% acetonitrile after 10 min. The composition was kept at 95% acetonitrile until 15 min elapsed; thereafter the system was re-equilibrated at starting conditions for 5 min.

The operating flow rate was set to 1.0 ml/min and the injection volume to 20 µl. The UV-detector was set at 210 nm with a total runtime of 20 min. The retention time of mangiferin, hesperidin, methylparaben, propylparaben and BHT was 6.2, 7.2, 8.7, 9.6 and 10.4 min, respectively (Figure 1).

Standard preparation

The concentration mangiferin and hesperidin present in both of the extracts (i.e. *C. genistoides* and *C. maculata*) were determined by utilizing the pure mangiferin and hesperidin as standards. The standards injected into the HPLC for the concentration assay (stability testing) were prepared by dissolving in a 100 ml volumetric flask: Honeybush extracts (50.0 mg); methylparaben (2.0 mg); propylparaben (0.4 mg) and BHT (2.0 mg). It was then made up to volume with methanol/HPLC water (50:50) to obtain a standard solution containing 500 µg/ml, 20 µg/ml, 4 µg/ml and 20 µg/ml of Honeybush extract, methylparaben, propylparaben and BHT, respectively. Subsequently it was injected into the HPLC in duplicate.

For the membrane release studies, calibration curves were constructed for *C. genistoides* cream ranging in mangiferin concentration of 0.58 – 3.14 µg/ml and hesperidin concentration of 0.29 – 1.75 µg/ml. The mangiferin concentration range was 1.44 – 7.98 µg/ml and the hesperidin concentration range was 0.42 – 1.90 µg/ml for *C. maculata* cream during the membrane study. The calibration curves constructed for the *C. genistoides* cream during the diffusion study ranged in a mangiferin concentration of 0.61 – 3.62 µg/ml and a hesperidin concentration of 0.63 – 2.30 µg/ml. The solvent used to prepare the standards of the membrane release and skin diffusion studies were a mixture of methanol and HPLC water (50:50).

Stability testing

A stability program was followed according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Tripartite Guideline[10] and according to the Medicines Control Council (MCC) of South Africa.[11] The two different semi-solid formulations were stored at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH. Various stability tests, such as concentration assay, pH, zeta-potential, particle size, visual appearance and mass loss were investigated on month 0, 1, 2 and 3.

Concentration assay

Each Honeybush extract formulation at each storage condition was weighed off (2 g) in 100 ml volumetric flasks in duplicate. The cream samples were made up to volume with methanol. The solutions were filtered and injected into the HPLC in duplicate for concentration assays. The concentrations of the following ingredients in the different formulations were determined with HPLC analysis: mangiferin, hesperidin, methylparaben, propylparaben and BHT. The validation of the HPLC analysis, as well as the chromatographic conditions was discussed above.

pH

The pH of all the formulations (at all storage conditions) were measured in triplicate with a Mettler Toledo Seven Multi pH meter (Mettler Toledo AG, Giesen, Germany) equipped with a glass Mettler Toledo InLab® 410 (Switzerland) electrode.

Zeta-potential

Samples were prepared by weighing off 1.0 g of each formulation under each storage condition in a 100 ml volumetric flask. The cream samples were made up to volume with methanol and the zeta-potential of each sample was determined by injecting the prepared samples into a Malvern Zetasizer 2000 (Worcestershire, United Kingdom). The zeta-potential of each formulation at each storage condition was measured in triplicate.

Particle size

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

Visual appearance

Photos of each formulation under each storage condition were taken with a camera (Pentax® Optio E40) and compared to each other.

Mass loss

A Mettler Toledo (Mettler Toledo AG, Giesen, Germany) balance was used to determine (in triplicate) the mass loss after each time interval of all the formulations.

Diffusion experiments

Skin preparation

Abdominal skin of Caucasian female patients was obtained after cosmetic abdominoplastic surgery. Ethical approval for obtaining and preparing the skin was provided by the Research Ethics Committee of the North-West University under the title "*In vitro* transdermal delivery of drugs through human skin" (NWU-00114-11-A5). The skin was prepared as previously described by Otto *et al.*[12] by utilizing a Zimmer electric dermatome model 8821 (Zimmer, Dover, OH, USA) to cut the skin to a thickness of approximately 400 µm and a width of 2.5 cm. The skin was subsequently cut into circular pieces with a diameter of approximately 15 mm, placed on filter paper and stored in aluminum foil at -20 °C until used.

Membrane release and skin diffusion studies

Vertical Franz diffusion cells with a donor (top) capacity of approximately 1 ml and receptor (bottom) capacity of approximately 2 ml and a diffusional area of 1.075 cm^2 were used in the membrane and skin permeation studies. For the membrane release and skin permeation studies, the cellulose acetate membranes and skin circles (SC facing upwards) were mounted between the receptor and donor compartments, respectively. The receptor compartments were filled with PBS (pH 7.4) (prepared according to the British Pharmacopoeia[13]) while care was taken to

avoid the entrapment of air bubbles under the surface. The donor compartments were filled with approximately 1 ml of the semi-solid formulation to keep the skin saturated. It was then covered with Parafilm® to avoid evaporation. The diffusion cells were placed in a tray on a Variomag® stirrer plate in a 37 °C Grant® water bath in order to accomplish a skin temperature of 32 °C.

Membrane release studies (minimum of n = 6 per study) were performed with the two formulations (i.e. *C. genistoides* and *C. maculata* creams) prior to the skin diffusion studies in order to determine whether the mangiferin and hesperidin were released from the formulations. The entire content of the receptor phases was withdrawn and replaced with fresh PBS (pH 7.4) on an hourly basis (hours 1, 2, 3, 4, 5 and 6). Due to extremely low, unquantifiable concentrations depicted during HPLC analysis (with method described previously), a second membrane diffusion study was executed where only a single withdrawal of the entire content of the receptor phases for the formulations were performed after 6 h.

During the skin diffusion studies a total of twelve Franz cells were used for each study. Ten Franz cells contained the active formulation; whereas two Franz cells contained a placebo formulation of the cream used during the experiment. Another skin diffusion study was performed consisting of twelve Franz cells that contained a 0.003% *C. genistoides* formulation commercially available on South African markets.

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

Tape stripping

Tape stripping was performed after the completion of the 12 h diffusion studies to determine the penetration of mangiferin and hesperidin into the uppermost layers of the skin. The following method, previously described by Pellet *et al.*[14], was followed: diffusion cells were carefully taken apart at the end of the diffusion study and the pieces of skin were pinned onto a piece of Parafilm® to a solid surface. The pieces of skin were dabbed dry with tissue. Pieces of 3M Scotch® Magic™ Tape was cut into appropriate sizes to cover the diffused area. The first tape strip was discarded, as it was seen as part of the cleaning procedure. The next 15 strips (SC-epidermis) were placed in a vial filled with enough PBS (pH 7.4) to cover the strips. An indication of the complete removal of the SC is when the viable epidermal layer glistens. The vials were kept overnight at 4 °C. The remaining skin (epidermis-dermis) was cut into pieces to enlarge the surface area. It was placed in vials filled with enough PBS (pH 7.4) to cover the skin pieces and were kept overnight at 4 °C. The tape samples were filtered and analyzed by HPLC. The skin samples were homogenized and filtered in turn to be analyzed by HPLC.

In vitro TBA (thiobarbituric acid) assay

The TBA-assay used during this study was modified from the method used by Ottino and Duncan.[15] This assay is a common technique used to determine the extent of lipid peroxidation in biological samples and can therefore be used to determine whether the Honeybush extracts have antioxidant effects against lipid peroxidation.[16] Malondialdehyde (MDA) is a key degradation product of lipid peroxidation and functions as an indicator for measuring the degree of lipid peroxidation.[16] The TBA assay is based on the reaction of MDA to two molecules of TBA through an acid-catalyzed nucleophilic-addition reaction to produce a pinkish-red chromagen, which can be removed with butanol and measured spectrophotometrically.[15, 17] However; this assay is based on *in vitro* chemical reactions and bears no resemblance to biological systems[18] and care needs to be taken not to base the bioactivity of the Honeybush extracts exclusively on this assay. Additionally, this technique has been condemned for its

inclination to overrate the MDA content and its lack in specificity. Nevertheless; this assay has shown to be capable to detect minute TBA alterations in plant and animal tissue[17, 19] and can be distinguished as a trustworthy lipid peroxidation estimator.[20]

Preparation of Honeybush extracts

Four different concentrations of both *C. maculata* and *C. genistoides* extracts were prepared. Extracts with each of the following concentrations of 0.3125 mg/ml, 0.6250 mg/ml, 1.2500 mg/ml and 2.5000 mg/ml were dissolved in a water/methanol mixture of equal parts. These low concentrations were used for testing as the amount of active that diffuses through the skin is usually very low. The actives in the Honeybush extracts (i.e. mangiferin and hesperidin) were made up to represent the approximate concentrations present in the abovementioned concentrations. For mangiferin, four concentrations of approximately 0.0169 mg/ml, 0.0338 mg/ml, 0.0676 mg/ml and 0.1352 mg/ml were dissolved in a water/methanol mixture of equal parts. Hesperidin concentrations of approximately 0.0046 mg/ml, 0.0092 mg/ml, 0.0184 mg/ml and 0.0368 mg/ml were dissolved in a water/methanol mixture. Lastly, 5 ml of each of the two different semi-solid formulations were dissolved in 5 ml water/methanol mixture.

Animals and preparation of rat brain homogenate

Whole rat brain homogenates from adult male Sprague-Dawley albino rats (between 200 to 250 g in weight) are customarily used as a rich source of membrane lipids to measure general lipid peroxidation (in order to ascertain whether a compound will potentiate or attenuate lipid peroxidation). The animals were housed in a windowless well-ventilated constant environment (CER) room with light cycles of 12 h. The temperature was maintained at 21 ± 1 °C and a humidity of 55 ± 5%. The animals received standard laboratory chow and water *ad libitum*. The North-West University (Potchefstroom Campus) Animal Ethics Committee approved the experimental assay performed under ethical code 05D05 and conformed to the University's

Regulations Act concerning animal experiments. The rats were decapitated and the whole brain of each rat was rapidly excised. The whole rat brain was homogenized in 0.1 M PBS (pH 7.4) to give an ultimate concentration of approximately 10% (w/v). PBS (pH 7.4) was chosen as it does not scavenge free radicals.[21]

Preparation of the standard

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

Method

The method involved placing 160 µl rat brain homogenate with 20 µl of the toxin combination in a series of tubes. The toxin combination consisted of 10 µl hydrogen peroxide (5 mM H_2O_2) to generate $OH^{\bullet}$ and induce lipid peroxidation in the rat brain homogenates[22] as well as 5 µl ascorbic acid (1.4 mM) and 5 µl ferric (III) chloride (4.88 mM $FeCl_3$) which increases the generation of $OH^{\bullet}$.

Thereafter 20 µl of either the Trolox (vitamin E, positive control, with its own antioxidant activity) or one of the different concentrations of Honeybush extracts, actives or semi-solid formulations were added. The control solution consisted of 160 µl rat brain homogenate, 20 µl PBS (pH 7.4) and 20 µl of water and methanol mixture of equal parts; whereas the toxin (negative control) consisted of 160 µl rat brain homogenates, 20 µl of the toxin combination (as mentioned above) and 20 µl of water and methanol mixture of equal parts. To induce lipid peroxidation the tubes were incubated in an oscillating water bath at 37 °C for 60 min.

Thereafter the tubes were centrifuged for 20 min at 2000 x g to remove all the insoluble proteins. The supernatant of each tube was removed and 100 µl methanolic BHT (0.5 mg/ml), 200 µl TCA (10%) and 100 µl TBA (0.33%) were added to this fraction. The chain-breaking antioxidant, BHT, was added to prevent the amplification of the lipid peroxidation during the assay, TCA to start the acid-heating hydrolysis reaction (acid-catalyzed nucleophilic addition reaction) and to precipitate proteins and TBA to form a pink chromagen when it binds to the formed MDA.[16]

In order to release the protein-bounded MDA (through hydrolysis), the tubes were sealed and the mixtures heated to 60 °C for 60 min in an oscillating water bath. The samples were subsequently cooled on crushed ice until it reached room temperature. Butanol (400 µl) was then added in order to extract the TBA-MDA complexes and the tubes were then centrifuged at 2000 x g for 10 min. The supernatant (200 µl) was transferred into a 96-well multiplate and the absorbance was read at 532 nm spectrophotometrically (butanol was used as a blank sample). Absorbance values lower than that of Trolox indicates almost definite antioxidant activity through the reduction in MDA formation in peroxidizing lipid systems. Results were compared to that of the toxin, a mixture known to have very low levels of antioxidant activity.

Clinical efficacy of semisolid formulations containing Honeybush extracts

Non-invasive skin measurements

Skin hydration

The Corneometer® CM 825 (Courage-Khazaka, Cologne, Germany) measurement is based on capacitance measurement of a dielectric medium, in this case the skin. Changes in water content of the SC are converted to arbitrary units (AU). The depth of the measurement is very small (i.e. 10 – 20 µm of the SC) in order to avoid the influence of water present in the deeper skin layers.[23, 24]

Skin topography

The Visioscan® VC 98 (Courage-Khazaka, Cologne, Germany) provides the possibility to analyze skin topography. An image of skin area (6 mm x 8 mm) is taken with a built-in CCD camera. The topography of the captured skin image can be analyzed by utilizing the surface evaluation of living skin (SELS) software that generates parameters such as skin scaliness and smoothness.[25]

Human subjects

The study has been carried out according to Helsinki declaration (Ethical principles of medical research involving human subjects), under the project title "(*In vivo*) Cosmetic efficacy studies" (NWU-00097-10-A5). All participants complied with both the inclusion and exclusion criteria. A group of sixteen healthy female subjects between 40 and 65 years of age participated in a two-week treatment phase. Exclusion criteria included: history of any kind of allergic reaction 30 days prior to test, history of eczema, pregnant/lactating women, recent treatment with Honeybush products, uncontrolled systemic disease, having undergone cosmetic surgery within the previous year, having any dermatological illnesses that may interfere with treatment or interpretation of results, having a recent history of intolerance to drugs and/or cosmetic products, treatment with topical or systemic drugs that could influence the test results, conditions that may interfere with neuromuscular function, psoriasis within six months prior to the study, involvement in a clinical investigation 30 days prior to this study and possible exposure to intensive doses of UV radiation during the study. All subjects signed an informed consent form and participants could discontinue their participation at any time during the study.[23]

Treatment protocol

The treatment protocol was conducted according to a comparatively similar study performed by Li *et al*.[23] The treatment sites were on both (left and right) volar forearms. Subjects were

instructed to follow normal skin cleansing routines and to refrain from any use of lotions or soaps other than Dove® soap on the volar forearms for seven days before entering the study (washout period). This cleansing routine was also followed for the remainder of the study. On day 8 (T_0) the participants visited the laboratory schedule. Three sites of 3 x 2 cm on each arm were marked with a Codman® surgical marker, of which one site on each arm functioned as an untreated control site. The baseline measurements (T_0) were taken with the three instruments mentioned earlier on the six different areas before product application.

During the next two weeks (14 days) each site was treated with the assigned cream, i.e. the placebo (applied on both left and right volar forearm of each volunteer), *C. maculata* cream (applied on either the left or right volar forearm of each volunteer) or *C. genistoides* cream (applied on either the left or right volar forearm of each volunteer). Therefore; each Honeybush formulation has its own corresponding placebo values which coincide with the same arm (either left or right) the specific Honeybush formulation was applied on, i.e. *C. genistoides* vs. $placebo_{CG}$ and *C. maculata* vs. $placebo_{CM}$. The treatments were applied on the correct marked skin areas twice daily (between 6:00 and 08:00 in the morning and between 18:00 and 20:00 in the evenings), according to the randomized position double blind placebo controlled study guidelines. The amount of product placed on the marked areas was 1 – 3 µl/cm^2 (1 – 3 mg/cm^2). For measurement days, subjects refrained from applying the treatment in the morning, although the treatments were applied directly after the measurements were completed. The volunteers did however apply the treatments the evening prior to the measurements. The final measurements were made after the two week period passed (T_1).

Environmental conditions

All measurements were conducted in the Cosmetics Efficacy Laboratory (CEL) of the North-West University (Potchefstroom Campus), South Africa under controlled temperature and humidity conditions (22 ± 2 °C and 50 ± 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.

Data analysis

Data analysis for release and skin diffusion studies

The average percentage diffused (%) as well as the average concentration (μg/cm^2; amount per area) of the actives that was released after 6 h or penetrated the skin after 12 h was calculated for the membrane release and skin diffusion studies, respectively.

Data analysis for antioxidant experiments

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

Data analysis for clinical efficacy experiments

In terms of all the parameters measured in this study, the effects of the different treatments (i.e. placebos, *C. maculata* cream and *C. genistoides* cream) after two weeks (T_1) of treatment are presented as percentage change relative to the initial conditions (T_0) as determined by Equation 1.

$$\% \text{ Change} = \left[\frac{T_1 - T_0}{T_0} \times 100\right] \qquad \text{Equation 1}$$

Statistical analysis

Statistical analysis for antioxidant experiments

GraphPad InStat 3 software was used for the statistical analysis of data. Results are given as the mean ± 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 were considered to be significant when $p < 0.05$ when compared to the toxin (#). When $p < 0.001$ it is considered extremely significant (***). When $p < 0.01$ it is considered fairly significant (**), while $p < 0.05$ is considered as significant (*). A $p > 0.05$ is considered to be not significant (ns).

Statistical analysis for clinical efficacy experiments

Statistical analyses for the clinical efficacy study were carried out by using Microsoft Excel 2010. In order to test the statistical significant differences between the different treatments and the different times, the student t-test was performed. A p-value < 0.05 indicate statistical significant differences.

RESULTS AND DISCUSSION

Stability testing

Concentration assay

The assay concentration of mangiferin in the *C. maculata* cream showed that the mangiferin concentration was within the acceptable limits (5% change of initial value) at storage conditions 25 °C/60% RH over the period of 3 months as well as after 1 month at 30 °C/60% RH and 40 °C/75% RH. The concentration of mangiferin in the *C. maculata* cream did however show significant change at 30 °C/60% RH and 40 °C/75% RH after 2 and 3 months of storage. The concentration hesperidin in the *C. maculata* cream did not remain within the acceptable limits and showed significant change. However, the hesperidin concentration stayed within acceptable limits in the *C. maculata* cream after 1 and 2 months of storage conditions at 25 °C/60% RH. It was also within acceptable limits after 1 month storage at 30 °C/60% RH.

In the *C. genistoides* cream, the concentrations of mangiferin and hesperidin did not fall within the acceptable limits and showed significant change. Although, the mangiferin concentration stayed within acceptable limits after 1 month of storage at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH. Additionally, the hesperidin concentration did not show a significant change at 30 °C/60% RH after 1 month.

This could indicate the active bioflavonoids present in Honeybush extracts lack sufficient stability in semi-solid formulations or that interaction with the different ingredients in the formulation could have occurred. The degradation of the actives can also be due to insufficient protection by the preservatives. It is suggested that future formulations containing Honeybush extracts should be stored at room temperature in a cool and dry environment for optimal preservation.

Overall the methylparaben, propylparaben and BHT in the *C. maculata* cream showed little change in concentration. However at 25 °C/60% RH after 2 months of storage, BHT was more than 5% higher and methylparaben more than 5% lower compared to the initial concentrations. After 3 months of storage propylparaben was not within the acceptable limits of the ICH at 30 °C/60% RH and 40 °C/75% RH in the *C. maculata* cream.

In the *C. genistoides* cream the methylparaben concentration was generally not within the acceptable ICH[10] limits, except after 1 month of storage at all three storage conditions. Propylparaben concentration was outside the acceptable limits after 1 month at 30 °C/60% RH, after 2 months at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH and after 3 months at 40 °C/75% RH. BHT showed little change in concentration from its initial concentration, except at 25 °C/60% RH over the 3 month period where its concentration increased with more than 5%. The small change in BHT concentration in both formulations can be ascribed to the fact that Honeybush extracts are known to have their own antioxidant activity; hence a saturated supply of antioxidants was present in the formulation.

pH

The pH of both the creams did not remain stable over the 3 month-period. The pH of the formulation could influence the stability of the actives. The *C. maculata* formulation proved to be the more stable formulation with a maximum pH change of 20% stored at 40 °C/75% RH, while the *C. genistoides* formulation with a maximum pH change of 31% stored at 40 °C/75% RH. This could indicate that the vast variations in pH are a clear indication of the instability of mangiferin and hesperidin.

Zeta-potential

The zeta-potential can be directly affected by several influencing factors, one being the abovementioned pH. The general distinction between stable and unstable suspensions is usually 25 to 30 mV (positive of negative) as stated by Malvern[26] as well as Kirby & Hasselbrink[27]. Particles with zeta-potentials more positive than + 25 mV or more negative than - 25 mV are usually considered stable due to the repelling forces between the particles, forcing the formulation into suspension. Very low zeta-potentials were present during stability testing, indicating incipient instability. Particles within the formulations may adhere to one another and form aggregates of successively increasing size, which may settle out under the influence of gravity. This may possibly lead to coagulation or flocculation of particles and later lead to total phase separation of the formulation. The drop in pH in both formulations gave the zeta-potentials a more negative charge due to higher concentrations of hydronium ions released over time, but these values still remained too low, ensuring incipient instability when storage times are increased. However, in relative terms, the *C. genistoides* formulation proved to be the more "stable" formulation, with the highest increase in zeta-potential although incipient instability was present at both formulations.

Particle size

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

Visual appearance

The visual appearance and color of both creams showed a radical change over the 3 month-period. The *C. maculata* formulation started with a glossy, light caramel colored cream and ended as matt, dark clayed paste-like textured cream. The *C. genistoides* formulation changed from a glossy, light caramel colored formulation to a glossier, burned dark toffee colored formulation with a tea-like fluid present on the top surface of the cream. The *C. maculata* cream proved to be the more stable formulation as it did not undergo phase separation such as was evident in the *C. genistoides* cream.

Mass loss

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

visual appearance after 3 months. The *C. maculata* cream became a paste-like formulation after 3 months.

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

Diffusion experiments

Membrane release studies

In both formulations, extremely low concentrations were detected in the receptor phases of the Franz cells after penetration through the cellulose acetate membranes. This could be an indication that the actives have insufficient physiochemical properties for satisfactory release of the actives and that the active concentrations within the formulations are too low for accurate detection during HPLC analysis.

In the *C. maculata* formulation, only three out of the ten diffusion cells showed release and therefore did not justify a full skin diffusion study. The HPLC method was barely capable of detecting these minuscule concentrations. In the *C. genistoides* cream however, a maximum of five out of the ten diffusion cells showed release and therefore a full skin diffusion study was performed. Both mangiferin and hesperidin in the *C. genistoides* cream had better release than that of the *C. maculata* formulation. When examining the average concentration ($\mu g/cm^2$) after 6 h, *C. genistoides* cream released mangiferin and hesperidin approximately fourteen and seven times better than the *C. maculata* cream, respectively. The *C. genistoides* cream also had extremely low concentrations and the HPLC detection was difficult since the peaks were so

small that they had to be integrated by hand. Therefore, it is difficult to draw any accurate conclusions on these observations.

Diffusion studies

The aim of the diffusion studies was to determine whether the mangiferin and hesperidin was delivered topically to its site of action (as determined with tape stripping) and not transdermally. However, it was important to establish whether the actives would also diffuse into systemic circulation (transdermally). Results indicated that only 0.250 μg/cm^2 mangiferin diffused through the skin after 12 h. With concentrations too low for accurate quantification, the results were considered inconclusive. No mangiferin or hesperidin was detected in the receptor phases when the 0.003% *C. genistoides* cream (currently available on South African market) was tested. Possible reasons for this poor performance by these formulations can be attributed to several factors. Firstly, the human skin has exceptional properties of which functioning as a physiochemical obstruction is one of its key properties.[28] The extent of skin diffusion is mainly reliant on physiological factors of the skin as well as the physiochemical characteristics of the active.[29] Mangiferin, with a molecular weight of 422.34 Dalton (Da)[30] can cross the skin to some extent, but hesperidin with a molecular weight of 610.56 Da[31] will show great effort in crossing the skin according to the "500 Dalton rule", i.e. molecules being larger than 500 Da (Da) cannot penetrate the corneal layer of the skin, while smaller molecules are able to pass through the corneal layer, surpassing transcutaneously.[28] As seen from the results shown above this statement seem to be true. Although mangiferin did pass the corneal layer, it was almost in unquantifiable amounts.

Secondly, poor penetration can also be due to the physiochemical considerations for passive transdermal delivery of a formulation such as drug lipophilicity. A drug molecule must first be released from the formulation and partition into the uppermost SC layer, before diffusion through the entire thickness of the skin can occur.[32] Ideally, a drug must possess a reasonable

solubility in both water and oils for proper skin penetration. It also needs to have an aqueous solubility of more than 1 mg/ml and a log P (octanol-water partition coefficient) in the range of 1 to 2.[33] The actives, mangiferin and hesperidin, have very poor solubility in water[34], i.e. mangiferin has an aqueous solubility of approximately 0.111 mg/ml and a log P value of 2.73[30], while hesperidin with an aqueous solubility of approximately 4.95 mg/ml and a log P value of 1.78.[35] Hesperidin has an ideal log P value for the diffusion through the SC, but its big particle size might be hindering.

According to Barry[36] particles between 3 µm and 10 µm can concentrate in the hair follicles, while particles less than 3 µm penetrate follicles and the SC alike. The *C. maculata* formulation had an average particle size of 22.25 µm with the *C. genistoides* formulation with an average of 14.09 µm. Thus, it can be agreed upon that particles larger than 10 µm stay on the skin's surface or have extreme difficulty to penetrate the skin due to its bigger particle size.

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

Tape stripping

During the skin diffusion studies it was found that mangiferin (2% *C. genistoides* cream) was the only active to cross the skin in extremely low concentrations after application. Tape stripping results showed in addition that there were very low concentrations mangiferin detected in the SC-epidermis as well as the epidermis-dermis, indicating that very poor skin penetration took place due to the very low concentrations present in the semisolid formulation. Hesperidin was not able to cross the SC, epidermis or dermis.

No detectable concentrations of mangiferin and hesperidin were present in the SC-epidermis and epidermis-dermis layers of the skin with the commercial Honeybush product currently available on the South African market.

These skin diffusion studies can be compared to a similar study done by Huang *et al*.[18] that investigated the transport of aspalathin, a unique flavonoid constituent of rooibos tea, across the human skin. These vertical Franz cell diffusion studies were conducted for both pure aspalathin solutions and extracts from unfermented (green) rooibos (*Aspalathus linearis*) aerial plant material across human abdominal skin. The results obtained showed that only a portion of 0.07% of the initial aspalathin dose penetrated the different layers of the skin for the green rooibos extract solution and 0.08% for the pure aspalathin solution.[18]

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

Antioxidant activity

The results of the TBA-assay used to determine the antioxidant properties of the *C. maculata* and *C. genistoides* extracts and formulations are given in Figure 2.

Antioxidant properties of C. maculata extracts

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

However; after the comparison of *C. maculata* extracts with Trolox (3.716 ± 0.320 nmole/mg) it was clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations were below Trolox.

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

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

Antioxidant properties of C. genistoides extracts

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

After the comparison of *C. genistoides* extracts with Trolox (3.716 ± 0.320 nmole/mg) it was clear that concentration 2.500 mg/ml of the extract concentrations emitted its own antioxidant activity, as this concentration had a MDA value below that of the Trolox.

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

Statistical analysis of the results obtained with the *C. genistoides* extracts, showed that the 1.250 mg/ml concentration were statistically extremely significant from the toxin with a p-value of < 0.001. Concentration 2.500 mg/ml was considered statistically fairly significant from the toxin with a p-value of < 0.01, while concentrations 0.3125 and 0.625 mg/ml were considered to be not significantly different from the toxin with a p-value of > 0.05.

Antioxidant properties of Cyclopia semisolid formulations

Both the *C. genistoides* as well as the *C. maculata* semisolid formulation showed potential antioxidant activity when compared with the toxin. This was indicated by a decrease in the MDA formation, which in turn leads to a lower degree of lipid peroxidation and higher $OH^{\bullet}$ radical scavenging abilities of the formulations.

After comparison of the *Cyclopia* semisolid formulations with Trolox (8.497 ± 0.254 nmole/mg) it was observed that both these formulations had a MDA value below that of the Trolox, indicating that both the semisolid formulations emitted their own antioxidant activity.

When comparing both the semisolid formulations to both toxin and Trolox, it was observed that both the *C. genistoides* and *C. maculata* formulations fell below the negative- (toxin) and positive (Trolox) control. Hence, the *C. genistoides* semisolid formulation had the best potential for antioxidant activity due to the highest reduction in MDA formation in the peroxidizing lipid system. Focusing on the statistical analysis of the *Cyclopia* semisolid formulations it was observed that both formulations were statistically extremely significant with a p-value of < 0.001.

Antioxidant properties of mangiferin standard

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

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

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

Statistical analysis of the results obtained with the mangiferin standard concentrations, indicated that concentration 0.0338 mg/ml was considered statistically fairly significant from the toxin with a p-value of < 0.01, while the standard concentrations 0.0169, 0.067 and 0.1352 mg/ml was considered statistically not significant from the toxin with a p-value of $p > 0.05$.

Antioxidant properties of hesperidin standard

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

When comparing the mangiferin standard concentrations with Trolox (2.771 ± 0.305 nmole/mg) it was clear that none of the extract concentrations emitted their own antioxidant activity, as none of the concentrations were below Trolox.

After comparison of the standard concentrations to both toxin and Trolox, it was noticed that all four concentrations fell above the negative- (toxin) and positive (Trolox) control. This clearly indicates a very low potential for possible antioxidant activity as well as a low level of lipid peroxidation inhibition and $OH^{\bullet}$ radical scavenging abilities of all the standards.

Statistical analysis of the hesperidin standard concentrations showed that concentration 0.0046 and 0.0184 mg/ml was considered statistically extremely significant from the toxin with a p-value of < 0.001, while the standard concentrations 0.0092 and 0.0368 mg/ml was considered statistically fairly significant from the toxin with a p-value of $p < 0.01$.

Clinical efficacy

Skin hydration

By using the Corneometer® CM 825, it was possible to detect if any change in skin hydration took place during the two week trial period. With the focus on skin aging, one of the most preventative actions one can take is by the proper hydration of the skin. It is important to note that the subjects in this particular study all had moderately dry skin before testing started.

Corneometer® results in Figure 3a show that the *C. maculata* cream hydrated the skin more than its $placebo_{CM}$; whereas the *C. genistoides* cream had a similar skin hydrating effect than its $placebo_{CG}$ (after two weeks of treatment, T_1). Statistical analysis showed that there was no significant statistical difference between the Honeybush formulations and their respective placebos or between the Honeybush formulations themselves when their skin hydration effects were compared to each other. There was however significant statistical differences present when creams were compared to their own performance from when the study started (T_0) until the study was completed (T_1), with *C. maculata* cream ($p = 0.009$), *C. genistoides* cream ($p = 0.036$) and the $placebo_{CG}$ ($p = 0.036$). This indicated that these last named treatments had a skin hydrating

effect over time. However, none of the Honeybush formulations statistically significantly enhanced skin hydration compared to their respective placebos.

Skin scaliness

Skin scaliness (Figure 3b), as measured with the Visioscan®, decreased after treatment with all the formulations. The *C. maculata* formulation decreased skin scaliness the most, followed by placebo$_{CM}$, placebo$_{CG}$ and then the *C. genistoides* formulation.

There was a statistical significant difference between the measured skin scaliness from T_0 to T_1 when treated with placebo$_{CM}$ ($p = 0.018$), placebo$_{CG}$ ($p = 0.044$) and *C. maculata* cream ($p = 0.0005$), indicating that the effect of these treatments (to decrease skin scaliness) were time dependent. A statistical significant difference was also observed after 2 weeks of treatment (T_1) between the placebo$_{CM}$ and *C. maculata* cream ($p = 0.028$), as well as between the *C. maculata* and *C. genistoides* creams ($p = 0.004$).

Skin smoothness

A graphical representation of the results obtained for skin smoothness, as measured with the Visioscan®, is given in Figure 3c. From the results it is evident that both Honeybush formulations were superior in increasing skin smoothness when compared to their respective placebos. Both *C. maculata* and *C. genistoides* creams increased smoothness statistically significantly over time, with p-values of 0.001 and 0.020, respectively. A statistical significant difference was also observed after two weeks of treatment (T_1) between the placebo$_{CM}$ and *C. maculata* ($p = 0.00007$) as well as between placebo$_{CG}$ and *C. genistoides* ($p = 0.040$) indicating that both Honeybush formulations increased skin smoothness statistically significantly better than their respective placebos.

Conclusion

Stability testing showed that none of the formulations completely met the ICH and MCC's criteria for stability,[10, 11] as both formulations had undergone a change in pH, zeta-potential, particle size, color, and concentration of the active ingredients over the 3 month-period.

There were no profound concentrations of mangiferin (0.003 µg/ml) and hesperidin (0.000 µg/ml) present in the target sites, i.e. the SC-epidermis or the epidermis-dermis. When comparing the results of the 2% *C. genistoides* results to that of the commercial product (0.000 µg/ml), it can be seen that the *C. genistoides* cream performed 677 times better, even though the concentrations of mangiferin detected were extremely low. This is however, a clear indication that Honeybush extract concentrations above 2% are needed for better and more conclusive skin diffusion results in the future. The only problem with higher concentrations of Honeybush extracts is that the aesthetics of the cosmeceutical cream will be diminished due to the change from an already inconsistent caramel colored cream to a brown toffee colored cream. This will make the commercial buyer skeptical due to potential of discoloring the skin.

Results of the TBA-assay showed that both semisolid formulations had the potential to emit their own antioxidant activity when compared to the Trolox (antioxidant). The *C. genistoides* formulation had the lowest value in *in vitro* MDA formation in rat brain tissue and attenuated lipid peroxidation better than that of the *C. maculata* formulation, pointing towards a better hydroxyl radical scavenging ability.

Both *C. maculata* and *C. genistoides* extracts showed some potential antioxidant activity as the concentrations increased above 0.6250 mg/ml (when compared to the Toxin). However, the *C. genistoides* extract, at a concentration of 2.500 mg/ml, showed to have the most potential antioxidant effect as its MDA level was lower than the MDA levels of both the toxin and Trolox.

In contrast, mangiferin and hesperidin as single entities did not show any promising *in vitro* antioxidant activity during the TBA-assay. The different standards showed an increase or equal

concentrations in MDA formation in rat brain tissue *in vitro* when compared to the Toxin. This increase in 2TBA-MDA complex formation indicates higher levels of lipid peroxidation and the $OH^{\bullet}$ radical scavenging inability of the standards. These results show the various bioactive flavonoids present in the Honeybush extracts have a synergistic antioxidant effect on each other. Therefore, it can be concluded that the combination of various actives within Honeybush extracts in various concentrations are of absolute importance for potential antioxidant activity.

The clinical efficacy results showed that *C. maculata* and *C. genistoides* appeared to have similar effects on the skin (except for skin scaliness). The *C. maculata* formulation enhanced the hydration of the skin (as measured with the Corneometer®) more than its $placebo_{CM}$, although this effect was not statistically significant. The *C. maculata* cream, *C. genistoides* cream and the $placebo_{GM}$ showed to have a skin hydrating effect over time. Investigation of the skin's appearance of scaliness indicated that all the treatments reduced this parameter; however the *C. maculata* cream proved to be far more superior compared to the other treatments. The *C. genistoides* cream on the other hand were less effective than its $placebo_{CG}$. Results showed that both the Honeybush extracts was statistically significantly better than their respective placebos in increasing the smoothness of the skin.

Although the Honeybush extract formulation showed some antioxidant activity and the potential to be used to improve certain skin parameters, future studies should be done to improve the stability of the Honeybush extract formulations, as well as to see whether the delivery of the actives (i.e. mangiferin and hesperidin) into the skin can be improved.

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DISCLAIMER

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

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Table 1: Formula of cream

Ingredient	%	Function
Part A		
Cetyl stearyl alcohol	7.0%	Thickening agent
Liquid paraffin	12.0%	Oil phase of emulsion
Cremophor® A6	1.5%	Emulsifier
Cremophor® A25	1.5%	Emulsifier
Methylparaben	0.2%	Preservative
Propylparaben	0.04%	Preservative
BHT	0.2%	Antioxidant
Part B		
Honeybush extract*	2.0%	Active ingredient
Propylene glycol	8.0%	Solvent
Part C		
Water	To 100%	Solvent

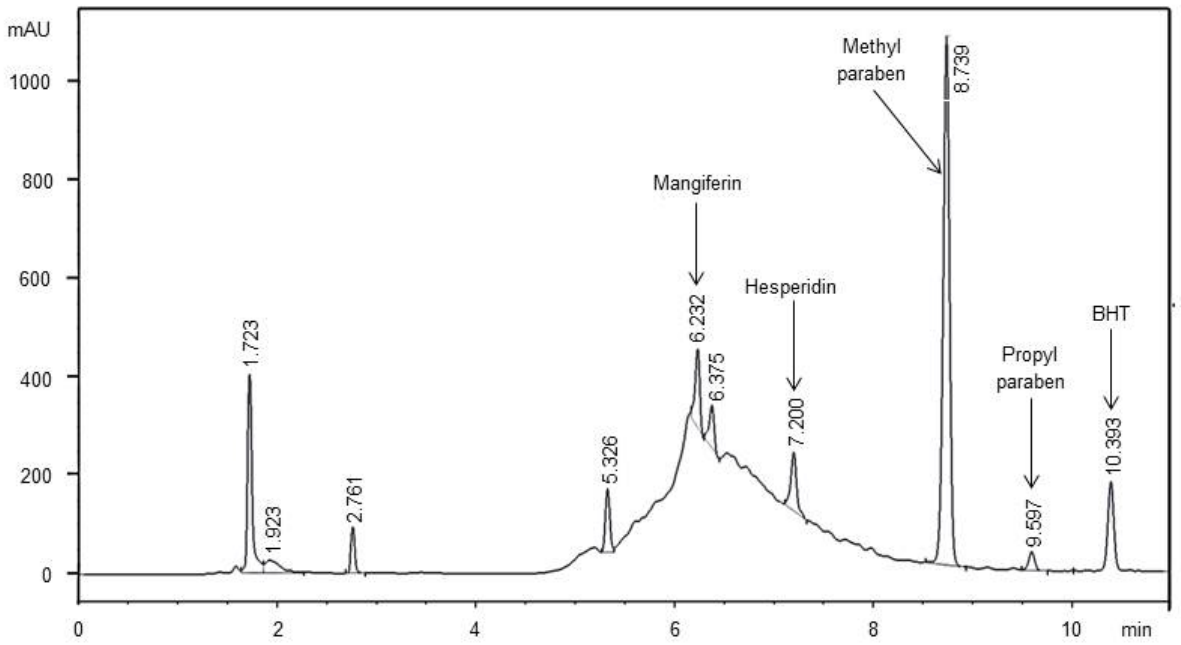
* Either *C. maculata* or *C. genistoides*

Figure legends

Figure 1: HPLC chromatogram illustrating the retention time of mangiferin, hesperidin, methylparaben, propylparaben and BHT.

Figure 2: The attenuation of lipid peroxidation by different concentrations of *C. maculata*- and *C. genistoides* extracts, *C. maculata* cream (CM Crm) and *C. genistoides* cream (CG Crm), as well as different concentrations of mangiferin and hesperidin in whole rat brain homogenates *in vitro*. Each bar represents the mean ± S.E.M. (n = 5). **p < 0.01; ***p < 0.001 vs. toxin (#), ns = not significant

Figure 3: Percentage change in skin hydration (a), skin scaliness (b) and skin smoothness (c) after 2 weeks of treatment (T_1), relative to the initial conditions (T_0).



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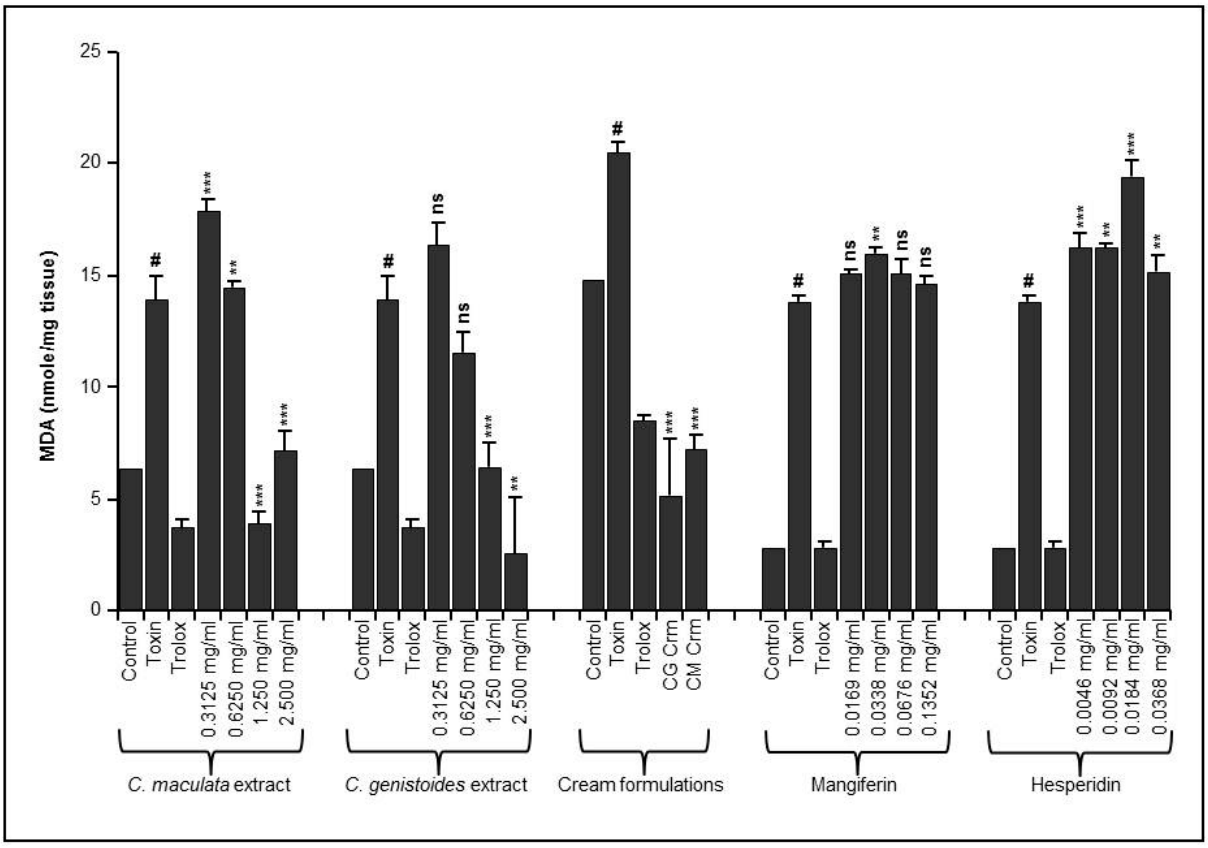

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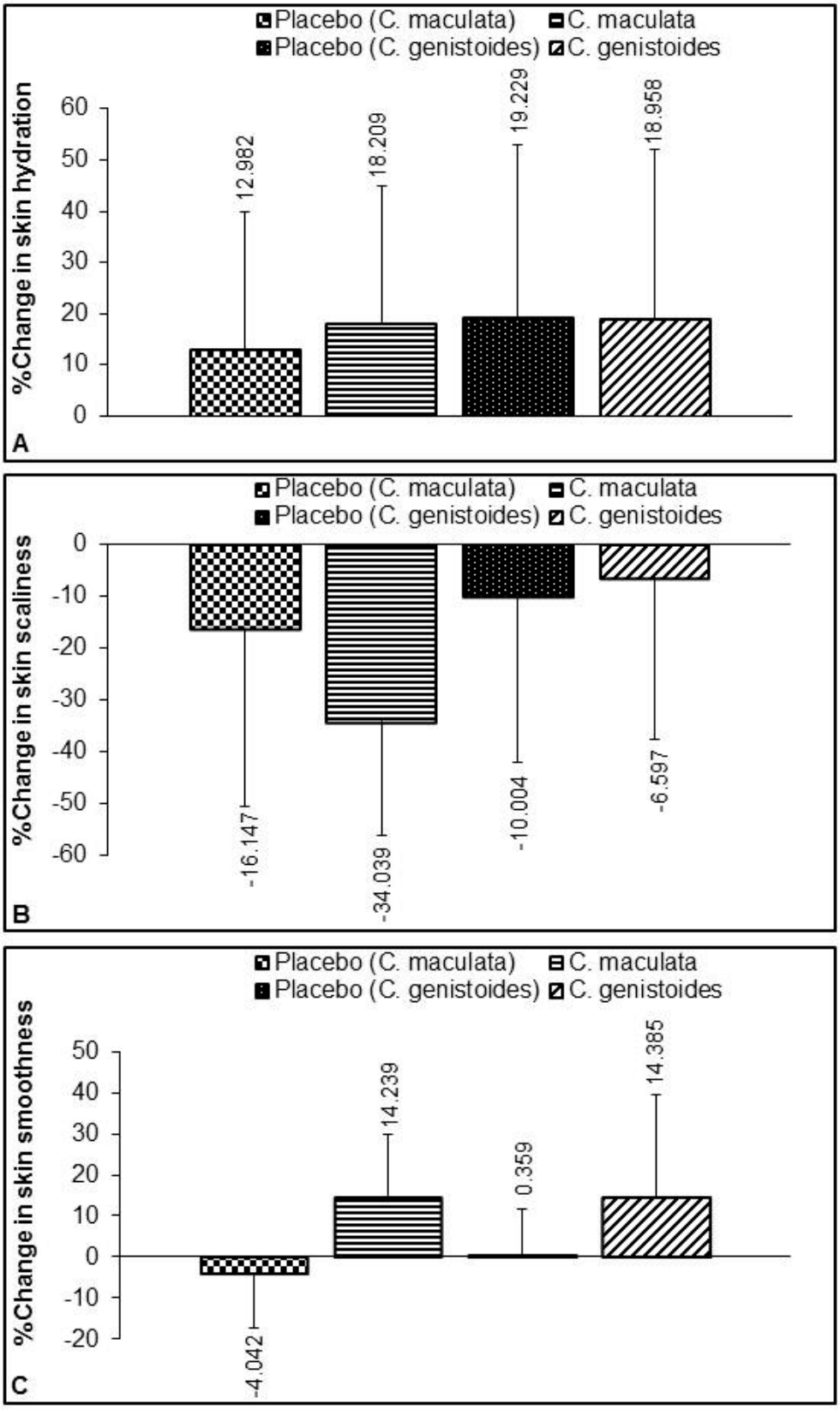

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