Ethanolic extracts of South African plants, *Buddleja saligna* Willd. and *Helichrysum odoratissimum* (L.) Sweet, as multifunctional ingredients in sunscreen formulations

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Highlights

•The use of South African plants in sunscreen formulations as multifunctional ingredients.

•Stabilizing and photostabilizing effect of South African plant extracts.

•Characterisation of plant extracts through GC-MS analysis.

•Cosmetically safe extracts as observed through *in vivo* irritancy and mutagenic testing.

•Significant antioxidant activity.

Abstract

Exposure to solar ultraviolet (UV) radiation is a major contributing factor to the increasing number of skin cancer cases. Interest has grown to use plant extracts as natural ingredients in cosmetic formulations due to their photoprotective effect, antioxidant and anti-inflammatory activity, as well as other biological activities. The aim of this study was to evaluate the biological activity of two South African plant extracts, *Helichrysum odoratissimum* (L.) Sweet. and *Buddleja saligna* Willd., and to successfully incorporate these extracts into sunscreen formulations (o/w emulsions) due to their reported biological activity. Ethanolic extracts were prepared from the leaves and stems of *H. odoratissimum* and *B. saligna* and evaluated for their antioxidant activity, mutagenic potential and antiproliferative activity against human dermal fibroblasts (MRHF). The extracts were further characterized using gas chromatography-mass spectrometry (GC-MS). Thereafter, the extracts were incorporated into separate sunscreen formulations to evaluate the *in vivo*

dermal irritancy potential, *in vivo* sun protection factor, *in vitro* UVA protection, photostability and long term stability of the formulation, to confirm that by incorporating the extracts, the stability or photoprotective effect of the sunscreen formulation was not reduced and that these formulation were considered safe for topical application. Three separate sunscreen formulations were prepared; the base sunscreen formulation (formulation A), the base sunscreen formulation containing *B. saligna* (formulation B) and *H. odoratissimum* (formulation C) respectively. Both extracts showed significant radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with a fifty percent inhibitory concentration (IC_{50}) of 5.13 ± 0.07 and 8.16 ± 0.34 µg/mL for *H. odoratissimum* and *B. saligna* respectively. No mutagenic activity was observed when the extracts were tested in the Ames assay using *Salmonella typhimurium* (TA98 and TA100). The PrestoBlue® cell viability assay was used to determine the antiproliferative activity of the extracts against MRHF cells, both extracts showed an IC_{50} value >90 µg/mL. Photoprotective activity was measured using *in vivo* sun protection factor (SPF) test method according to South African (SANS 1557) and International (ISO 24444) standards as well as the *in vitro* UVA SPF testing procedure (ISO 24443). The SPF results showed that the formulations had broad-spectrum UV protection with SPF values of 15.8±0.41, 16.1±0.66 and 16.0±0.49 and UVAPF values of 6.47±0.06, 6.45±0.06 and 6.47±0.07 for formulation A, B and C respectively. Furthermore, the formulations remained stable under normal and extreme conditions and the plant extracts did not affect the photoprotective effect of the sunscreen formulations and contributed towards the formulations stability. Additionally, each of the formulations were photostable, whereas the formulations with the addition of the extracts showed an incremental increase in photostability when compared to the base formulation. Both these extracts have been previously reported to display antiproliferative activity against skin cancer cell lines (previously published data), with an IC_{50} value of

31.80 ± 0.35 µg/mL (human malignant melanoma, UCT-MEL-1) for *B. saligna* and IC_{50} values of 15.50 ± 0.20 (human epidermoid carcinoma, A431) and 55.50 ± 6.60 µg/mL (human malignant melanoma, A375) for *H. odoratissimum*, contributing towards the medicinal benefit of using these extracts as ingredients into sunscreen formulations. Therefore, *Helichrysum odoratissimum* and *Buddleja saligna* could be considered as useful and viable additives to sunscreen formulations due to their reported biological activity.

Keywords: South African medicinal plants; *Helichrysum odoratissimum* L. (Sweet); *Buddleja saligna* (Willd.); Photoprotective activity; Sun protection factor; Antioxidant; Antiproliferative activity; Dermal irritancy; Mutagenicity; Stability; GC-MS analysis

Abbreviation: 4-NQO: 4-Nitroquinoline 1-oxide; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); DMEM: Dulbecco's modified Eagle's medium ; DMSO: Dimethyl sulfoxide; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FDA: US Food and Drug Administration; GC-MS: Gas chromatography-mass spectrometry ; GRASE: Generally recognized as safe and effective; IC_{50}: Fifty percent inhibitory concentration; MEDp: Minimal erythema dose (protected skin); MEDu: Minimal erythema dose (unprotected skin); MRHF: Human dermal fibroblasts; NER: Nucleotide excision repair; PABA: Aminobenzoic acid; ROS: Reactive oxygen species; SPF: Sun protection factor; UVR: Ultraviolet radiation

1. Introduction

Solar ultraviolet radiation (UVR) is one of the main contributors to the increasing number of skin cancer cases worldwide. Acute and chronic exposure to UVR can lead to changes in the skin, such as erythema, thickening of the skin, UV induced pigmentation, skin aging and damage to cells, fibrous tissue and blood vessels in the skin (World Health Organization, 2017). UV rays are divided into three main types; UVA (320-400nm), UVB (280-320nm) and UVC (200-280nm) rays, however only UVA and UVB rays are able to reach the Earth's surface, whereas UVC is mostly absorbed by the ozone layer (The Skin Cancer Foundation, 2019). In the United States, solar UV radiation accounts for approximately 90% of non-melanoma and 86% of melanoma cases (Epstein and Wang, 2017; Koh et al., 1996; Parkin et al., 2011).

UVA is the most prevalent type of solar radiation, accounting for up to 95% of UVR. The intensity of these UV rays remains the same throughout the day and year, and are able to pass through clouds and glass. UVA is able to penetrate the skin and reach the deeper dermis layer where it causes photo-aging and wrinkling. Furthermore, UVA damages the keratinocytes in the epidermis layer of the skin, which can lead to the development of skin cancer. UVA damage is caused mainly due to the production of reactive oxygen species (ROS) which indirectly damages DNA through a series of reactions (D'Orazio et al., 2013).

Although UVB rays are less prevalent than UVA, these are far more intense, causing skin reddening and burning. These rays differ from UVA in that they are not able to pass through glass. They only penetrate the epidermis layer of the skin and differ in intensity throughout the day based on altitude, cloud coverage, location and the time of day (The Skin Cancer Foundation, 2019). UVB causes direct DNA damage, which can lead to the development of skin cancer. Pyrimidine base pairs, such as thymine and cytosine, in DNA

absorbs UVB. This can lead to two types of DNA lesions when two pyrimidine base pairs are situated next to each other; 1) two covalent bonds can form between these base pairs forming dimers known as cyclobutane pyrimidine dimers (CPDs) and 2) a single bond can form between the carbon atoms on the ring structures forming a 6-4 photoproduct (6-4 PPs). These mutations form kinks in the DNA strand causing disruption during transcription and replication, however these can be repaired by nucleotide excision repair (NER), which excises damaged base pairs from the DNA. If these base pair mutations are not corrected and transcription takes place, there is the possibility of base pairs matching incorrectly, for instance instead of the thymine dimers pairing with adenine base pairs, the thymine dimers pair with cytosine dimers, which can cause permanent mutations (Clancy, 2008; Goodsell, 2001).

On the 21st February 2019, the US Food and Drug Administration (FDA) proposed a new rule for the use of active sunscreen ingredients, both physical and chemical sunscreen ingredients. The FDA proposed that the use of the physical filters; zinc oxide and titanium dioxide are generally recognized as safe and effective (GRASE), whereas the chemical filters; torlamine salicylate and aminobenzoic acid (PABA) should not be used in sunscreen products as these are not recognized as GRASE. For the remaining 12 active sunscreen ingredients, more research is required to determine whether these are recognized as GRASE (FDA, 2019).

The efficacy of a sunscreen or sunscreen ingredient to protect against UVB radiation is measured by the sun protection factor (SPF). UVB is mostly associated with the appearance of erythema, therefore to calculate the SPF of a sunscreen, the minimal dose of UVB radiation required to induce erythema on skin protected by a sample/ sunscreen (MEDp) and that of unprotected skin (MEDu) is determined. The effectiveness of the sunscreen

product is directly proportional to how high or low the SPF is. The Skin Cancer Foundation has defined an SPF 15 sunscreen to filter out about 93% of UVB radiation, whereas SPF 30 and SPF 50 filter out approximately 97 and 98% respectively (The Skin Cancer Foundation, 2012).

Over the past few years there has been a growing trend to use natural resources, such as plants, as ingredients into cosmetic products. Plant extracts are sought after due to their wide range of medicinal properties such as; antiseptic and antibacterial activity, anti-inflammatory, antioxidant and photoprotective properties (Cefali et al., 2016; Mouffouk et al., 2020; Sahu et al., 2019). In a study by Baldisserotto et al (2018), *Moringa oleifera* Lam. leaf extracts were incorporated into sunscreen formulations due to their significant antioxidant and antiproliferative activity against human melanoma (Colo38) cells. The aim of the study was to characterize and evaluate *M. oleifera* extracts as herbal sun care photo-complexes (Baldisserotto et al., 2018). Additionally, Mouffouk et al (2020) evaluated the anti-inflammatory, antioxidant and hemostatic activities of *Linaria scariosa* Desf. methanolic extract as a skincare agent for cosmetic and pharmaceutical applications (Mouffouk et al., 2020). Studies have been conducted on the use of natural products from plants; such as quercetin, resveratrol, curcumin, silymarin and caretonoids; as well as whole plant extracts for examples; *Camellia sinensis* (L.) Kuntze, *Aloe vera* (L.) Burm.f. and *Krameria triandra* Ruiz & Pav., and their potential photoprotective activity (Korać and Khambholja, 2011). In a study by Magcwebeba et al (2016), two well-known South African plants, *Aspalathus linearis* (Burm.f.) R. Dahlgren (Rooibos) and *Cyclopia genistoides* (L.) Vent. (Honeybush), have been shown to prevent UVB-induced reduction of cell growth and aid in the removal of UVB damaged keratinocytes and therefore may be considered useful in the prevention of photo-induced inflammation (Magcwebeba et al., 2016).

The efficacy of a sunscreen formulation is not only associated with the protective effect against UVA/ UVB and the boosting activity but is also regulated by various other biological activities such as; the stability and safety, as well as antioxidant, antimutagenic and antiproliferative properties (Radice et al., 2016). Buso et al (2018), therefore describes that extracts or compounds with multifunctional biological activities, relating to preventing skin problems associated with UV radiation, be incorporated in sunscreen formulations which would enhance the effectivity of the formulation (Buso et al., 2019).

Buddleja saligna (Willd.) is an indigenous South African evergreen plant which is traditionally used for the treatment of coughs, colds and urinary problems as well as the treatment of sores and thrush (Chukwujekwu et al., 2016; Hutchings et al., 1996). *Helichrysum odoratissimum* (L.) Sweet, a strongly aromatic shrub, distributed widely throughout Southern Africa, is extensively used as a traditional medicine for numerous ailments such as coughs, abdominal pains and fever, as well as the treatment and alleviation of various skin disorders. An extract prepared from the leaves is used for eczema, whereas the ground leaves or leaf pulp is used as a dressing for wounds and burns (Lourens et al., 2008). A paste from the flowers has also been used for the treatment of acne and pimples (Cleversley, 2016).

Although the two plants have been broadly used in traditional medicine, both *B. saligna* and *H. odoratissimum* have not been extensively explored for their biological activity. These two South African plants were selected for this study based on previous published results by the authors (Twilley et al., 2017a, Twilley et al., 2020) in which ethanolic extracts from both *B. saligna* and *H. odoratissimum* were found to have significant antiproliferative activity against various skin cancer cell lines as well as their use in traditional medicine for several skin related ailments. The aim of this study was therefore,

to develop two stable o/w emulsion sunscreen formulations containing the selected South African plant extracts, which have significant antiproliferative activity and other multifunctional biological properties, in order to complement the therapeutic potential of a sunscreen formulation, without reducing the photoprotective effect and stability of the formulation. Additionally, the plant extracts were characterized in order to identify potential compounds which have been reported for their complementary therapeutic effect in photoprotection.

2. Materials and Methods

2.1 Chemicals and reagents

Human dermal fibroblasts (MRHF) (Cellonex, CFIB-FL) and Trypsin/ EDTA were purchased from Separation Scientific SA (Pty) Ltd. Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), amphotericin B, penicillin and streptomycin were obtained from ThermoFisher Scientific (Pty) Ltd (Johannesburg, South Africa). Cell culture plates and flasks were acquired from Lasec SA (Pty) Ltd (Midrand, South Africa). The 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, actinomycin D, dimethyl sulfoxide (DMSO), absolute ethanol, 4-nitroquinoline 1-oxide, tetrasodium EDTA, sodium hydrogen phosphate, disodium hydrogen phosphate, Oxoid nutrient broth No.2, biotin, histidine and D-(+)-glucose were purchased from Sigma Aldrich (Johannesburg, South Africa). Xanthan gum was obtained from Protea Chemicals (Germiston, South Africa), whereas the glycerin and Germaben II were purchased from Fourchem (Johannesburg, South Africa). Crodex M, Crodamol STS, Crodamol SFX and Solaveil^{TM} XT-100 was supplied by Croda (Boksburg, South Africa). *Salmonella typhimurium* TA98 and TA100 strains were sourced from Moltox (North Carolina, USA). Solidifying nutrient agar was purchased from Becton Dickinson (New Jersey, USA). The

P2 Standard (ISO 24444) was purchased from Solar Light Company, Inc (Philadeliphia, USA). The 8mm Finn Chambers on Scanpore was supplied by SmartPractice (Phoenix AZ, USA). The sodium lauryl sulfate was purchased from Saar Chem (Johannesburg, South Africa).

2.2 Plant collection

Leaves and stems of *Buddleja saligna* (Willd.) (PRU 122167) were collected during summer (2015) from the Manie van der Schijff Botanical Gardens, University of Pretoria, South Africa, whereas the leaves and stems of *Helichrysum odoratissimum* L. (Sweet) (PRU 96677) were collected in Venda, Limpopo, South Africa during summer (2010). The plant material was identified by Ms Magda Nel and Mr Jason Sampson from the University of Pretoria, and voucher specimens were deposited in the HGWJ Schweickerdt Herbarium, Pretoria, South Africa. The plant material was shade dried at room temperature and powdered using an IKA MF 10 universal grinder. Plant species names were validated using http://mpns.kew.org/mpns-portal and www.theplantlist.org.

2.3 Plant extraction

The powdered plant material (5kg) of both *B. saligna* and *H. odoratissimum* was extracted using absolute ethanol (7L) and left on a shaker for 72h. The extracts were filtered through a Büchner funnel using Whatman No. 1 filter paper. Extraction using ethanol was repeated twice. Thereafter, the filtrate of each plant was evaporated to a dry extract under reduced pressure using a Büchi Rotavapor R-200 at 45°C to obtain a yield of 14.5 and 6.20% for *B. saligna* and *H. odoratissimum* respectively. The extracts were kept at 4ºC until further use.

2.4. Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis of the extracts was performed using a LECO Pegasus 4D GC-TOFMS (LECO Africa (Pty) Ltd., Kempton Park, South Africa) including an apolar Rxi-5SiMS (30 m × 0.25 mm ID × 0.2 µm film thickness) (Restek, Bellefonte, PA, USA) capillary column. Ultra-high purity grade helium (99.999 %) (Afrox, Gauteng, South Africa) was used as a carrier gas at a constant flow rate of 1 mL/min. The injector temperature was maintained at 250ºC and the inlet was operated in a splitless mode (splitless time 30s). The GC oven temperature programme was 40ºC (3 min) at 10ºC/min to 300ºC (5 min). The MS solvent delay was 5 min, and the total GC-MS running time was 36 min. The MS transfer line temperature was set at 280ºC and the ion source temperature was set at 230ºC. The electron energy was 70 eV in the electron impact ionization mode (EI +), the data acquisition rate was 10 spectra/s, the mass acquisition was 40-550 Daltons, and the detector voltage was set at 1750 V.

2.5. In vitro antioxidant assay

The DPPH assay was performed according to a method described by Berrington and Lall, (2012). Stock concentration of the extracts (2 mg/mL) and ascorbic acid (10mg/mL) were prepared. Distilled water (200µl) was added to the first row wells of a 96-well plate and 110µl to the remaining wells. Samples (20µl) were added to the top wells, in triplicate, followed by serial dilutions with final concentrations ranging from 3.9-500 and 0.78-100 µg/mL for the extracts and ascorbic acid respectively. Ethanol was added as the negative control. Ethanolic DPPH (90µl) was added to each of the wells, whereas only ethanol was added to a second set of colour control plates. The plates were incubated for 30 min, covered with aluminium foil. Absorbance values were measured at 515 nm using a BIO-TEK Power-Wave XS multi-plate reader (A.D.P, Weltevreden Park, South Africa).

GraphPad Prism 4 software was used to calculate the IC_{50} values from the percentage DPPH scavenging activity, which was calculated according to the below equation.

$$\%\ \boldsymbol{DPPH\ inhibition} = \frac{\boldsymbol{Absorbance\ control - Absorbance\ sample}}{\boldsymbol{Absorbance\ control}} \times \mathbf{100}$$

2.6. Mutagenicity

The extracts were tested for potential mutagenicity/ genotoxicity using the Ames test (Maron and Ames, 1983) according to the method described by Makhafola et al (2014). *Salmonella typhimurium* strains TA98 and TA100 were used, which detect frame-shift mutations and base-pair substitutions respectively. The bacterial stock (100 µl) was incubated in Oxoid Nutrient broth No.2 (10mL) for 16 h at 37ºC with constant shaking. After incubation, 100µL of bacteria were added to test tubes containing 500µL of 0.2M sodium phosphate buffer (0.2M sodium hydrogen phosphate and disodium hydrogen phosphate, pH=7.4) and 100µL of sample. This was followed by addition of 2mL of top agar containing 0.5mM biotin-histidine. Extracts were tested at concentrations of 5, 0.5 and 0.05 mg/mL. Controls included 10% DMSO and sterile distilled water as negative controls, and the positive control, 4-nitroquinoline 1-oxide (4-NQO) at 2 µg/mL. The prepared mixture was vortexed and gently poured over a petri dish containing minimal agar and incubated for 48 h at 37ºC. Thereafter, the number of revertant colonies were counted. Samples were tested in triplicate.

2.7. Antiproliferative activity

Human dermal fibroblasts (MRHF) were used to determine the *in vitro* toxic potential of the extracts according to the method described by Lall et al (2013). Cells were cultured in flasks using Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal

bovine serum (FBS), 1% antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin) and 1% amphotericin B (250 µg/ml), at 5% CO_2 and 37ºC. Sub-culturing was done under sterile conditions using Trypsin-EDTA (0.25%). Detached cells were seeded in 96-well plates at 1×10^5 cells/mL and incubated overnight at 5% CO_2 and 37ºC to allow for adherence. Thereafter, cells were exposed to the extracts and controls for 72 h, in triplicate. Samples included the positive control, actinomycin D at a concentration ranging from 3.9×10^{-4} – 0.05 µg/mL, a vehicle control (DMSO at 2%), untreated cells, a PrestoBlue® control (with no cells) and cells treated with the extracts at concentrations ranging from 3.13 - 400 µg/mL. After the cells were exposed to the samples for 72 h, 20µL of the cell viability reagent, PrestoBlue® was added, after which the cells were incubated for a further 2 h. The fluorescence was measured at an excitation of 560 nm and an emission of 590 nm using a VICTOR® Nivo™ microplate reader (Perkin Elmer Inc, Massachusetts, USA). The percentage cell viability was calculated using the below equation, where after an IC_{50} value was calculated for each sample.

$$(\%\ \boldsymbol{cell\ viability}) = \frac{\boldsymbol{Fluor.sample - fluor.of\ prestoblue\ control}}{\boldsymbol{Fluor.DMSO\ vehicle\ control - fluor.prestoblue\ control}} \times \mathbf{100}$$

2.8. Preparation of emulsion and stability testing

The emulsions were prepared using the ingredients listed in Table 1. A broad-spectrum physical UV filter, titanium dioxide (Solaveil™ XT-100), was selected to obtain an SPF of 15. The formulations underwent high shear mixing to assist in the dispersion of the UV filter. To assist with the incorporation of the UV filter, a thickening agent, xanthan gum, was added. The O/W emulsions comprised of two main phases, phase A (aqueous phase) and phase B (oil phase). The ingredients of phases A and B were weighed into separate beakers, mixed and heated to 65°C. Thereafter, phase A was poured into phase B with continuous stirring using a Silverson® high shear mixer (Silverson, USA) at 5000 rpm for 15 min, followed by cooling down to 40°C with constant stirring. Thereafter, the 10% w/v of the extracts (stock concentration of 6.0 mg/mL) and preservative were added, mixing after the addition of each until homogenous. Three sunscreen formulations were prepared; the base sunscreen formulation (formulation A) and formulations with the additional of the *B. saligna* (formulation B) extract and *H. odoratissimum* (formulation C) extract respectively.

Physical stability of the three formulations was conducted using cycle testing (pH analysis, droplet size analysis and phase separation). Cycle testing was conducted by alternating the storage of the formulation at 40°C for 24 hrs and at 4°C for 24 hrs for six cycles. Thereafter, formulations were alternated between 40°C for 24 hrs and -18°C for 24 hrs for an additional five cycles. The pH (EZDP PP-203, Gondo Electronic Co, Ltd, Taiwan) and median droplet size (d_{50}) (Mastersizer 3000™, Malvern Panalytical Ltd, United Kingdom) of the formulations was measured after each 48 h cycle test. Additionally centrifugation testing was conducted after the 48 h cycle tests at 3000 rpm for 30 minutes (Eppendorf™ 5810R, Sigma Aldrich, St. Louis, Missouri, USA) to identify any signs of phase separation.

Table 1

Composition of the base sunscreen formulation (formulation A) and the sunscreen formulations containing *Buddleja saligna* (formulation B) and *Helichrysum odoratissimum* (formulation C)

Ingredients	Application	INCI/Chemical Name	Formulation (% ingredients)		
			A	B	C
Phase A (aqueous)					
Water	Diluent/solvent	Aqua	74.7	67.2	6.72
EDTA	Chelating agent	Tetrasodium EDTA	0.10	0.10	0.10
Glycerin	Humectant	Glycerin	1.80	1.60	1.60
Xanthan Gum	Thickener	Xanthan Gum	0.30	0.30	0.30
Phase B (oil)					
Crodex M	Emulsifier	Cetostearyl Alcohol (and) Potassium Cetyl Phosphate	6.20	5.60	5.60
Crodamol STS	Emollient	PPG-3 Benzyl Ether Myristate	2.00	1.80	1.80
Crodamol SFX	Emollient	PPG-3 Benzyl Ether Ethylhexanoate	2.00	1.80	1.80
Solaveil™ XT-100	UV filter	Titanium Dioxide, C12-15 Alkyl Benzoate, Polyhydroxystearic Acid, Stearic Acid & Alumina	11.8	10.6	10.6
Phase C					
Plant extract	Active ingredient	Reconstituted ethanolic plant extract (6.0 mg/mL)	-	10.0	10.0
Germaben II	Preservative	Propylene Glycol (and) Diazolidinyl Urea (and) Methylparaben (and) Propylparaben	1.00	1.00	1.00

2.9. In vivo skin irritation

Irritancy potential of the extracts was conducted according to the Declaration of Helsinki and the Guidelines for "Good Laboratory Practice in the Conduct of Clinical Trials in Human Participation in South Africa". Permission to conduct the study was approved by the Research and Ethics Committee of the Sefako Makgatho Health Sciences University (MREC/H/48/2014: CR; as renewed) with written informed consent from all the subjects. Twenty adult (aged 18-65 years old) female volunteers were recruited for this study, of which five had sensitive skin. Each of the volunteers complied with the exclusion and inclusion criteria and consent forms were signed by each volunteer before the study commenced. Samples were applied to the inner forearm of each volunteer, in a randomized pattern, at 0 h and repeated after 24 hrs at the same position. Samples included the extracts at 6.0 mg/mL, the positive control sodium lauryl sulphate (SLS) at 1% and the negative control, de-ionised water. Occluded application of the samples to the skin using 8mm aluminium Finn Chambers on Scanpore® was performed. Test sites were monitored at 0, 24, 48, 72 and 96 hrs after application. Test sites were covered with the chambers for the first 2 x 23 hrs, thereafter the chambers were removed. Colour assessments were performed by visual scores and an instrumental score using the Minolta Cr400 Chromameter using the a* values, which measures colour on the red/green axis. To quantify the erythema response of the skin at each test site when compared to the baseline, the Delta a* values were calculated at each time point as follows:

$$\mathbf{Delta\ a} * (\mathbf{at\ time\ t}) = (\mathbf{sample\ a} * \mathbf{at\ time\ t} - \mathbf{sample\ a} * \mathbf{at\ time\ 0}) - (\mathbf{Baseline\ a} * \mathbf{at\ time\ t} - \mathbf{Baseline\ a} * \mathbf{at\ time\ 0})$$

2.10. In vivo sun protection factor (SPF) evaluation

Permission to conduct the study was approved by the Research and Ethics Committee of the Sefako Makgatho Health Sciences University (MREC/H/158 /2014: CR). The *in vivo* SPF assessment of *B. saligna* and *H. odoratissimum*, at a concentration of 6.0 mg/mL, in a sunscreen formulation, was performed according to the South African Bureau of Standard (SANS 1557) and the International Standard, ISO 24444. All volunteers signed informed consent before the study commenced. Ten healthy human volunteers were recruited for the study, all with skin phototypes II. A multiport Solar Simulator (xenon lamp) was used to induce UV at four different sites on the skin; unprotected skin (MEDu), skin protected with an SPF 15 reference standard (P2) (MEDp) and skin protected with the sunscreen formulation (Formulation A, B and C respectively) (MEDp), where MED represents the lowest dose of UV needed to induce erythema after 16-24 hrs. The samples were applied to the skin at a concentration of 2 mg/cm^2. Results were calculated by the original values (n = 10) and expressed as mean. The SPF was calculated using the following equation:

$$\mathbf{SPF} = \frac{\mathbf{MED\ of\ protected\ skin\ (MEDp)}}{\mathbf{MED\ of\ unprotected\ skin\ (MEDu)}}$$

2.11. In vitro UVA protection factor and photostability

The *in vitro* UVA assessment and phototstability of the sunscreen formulations was performed according to the International Standard, ISO 24443. The formulations (1.3 mg/cm^2) were applied to a polymethylmethacrylate (PMMA) plate and spread evenly over the roughened surface. The plate was stored in the dark at room temperature for 30 min before use. A blank plate, which was treated with enough glycerine to coat the entire surface, was included. Thereafter, the plates were placed in the light-path of a UV-2000S ultraviolet transmittance analyser (Labsphere, USA). The absorbance of UV

radiation through the samples was measured from 290-400 nm at 1 nm intervals on 4 different locations. Thereafter, photostability was measured where the plates were UV-irradiated and new absorbance measurements were obtained. A total of four test plates were prepared to establish the UVA protection and photostability of the sample by calculating the final UVA protection factor (UVAPF), the SPF *in vivo*/ UVAPF ratio and the critical wavelength.

2.12. Statistical analysis

Results were reported as mean ± SD (n=3; mutagenicity, cell viability and antioxidant; n=19 for irritancy; n=10 for SPF and n=4 for UVAPF). The IC_{50} values were calculated using nonlinear regression analysis of the sigmoidal dose response curves using GraphPad Prism 4. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey's (irritancy test) and Dunnett's (photoprotection and mutagenicity) multiple comparison test using the GraphPad Prism 4 statistical software. Significant difference was indicated as footnotes in the results section for each of the experiments.

3. Results

3.1. GC-MS analysis

GC-MS analysis is used to separate volatile compounds within a complex sample and provides a tentative identification of compounds present in a sample (Rispail et al., 2005). GC-MS chromatogram analysis of the extracts showed multiple peaks indicating the presence of numerous phytochemical compounds. The mass spectra of the constituents were compared to the NIST08 Mass Spectral Library to characterize and identify the compounds depending on their similarity to the library database. The identified compounds,

molecular formula, molecular weights, concentration (peak area %) and similarity to the NIST08 library are presented in Tables 2 and 3.

In the *B. saligna* extract, four different chemical compounds were identified, of which oleanolic acid was the prevailing compound (55.05 %) (Table 2). Numerous compounds were identified within the *H. odoratissimum* extract, of which 6-hydroxy-4-methoxy-2,3-dimethyl-benzaldehyde was the most predominant compound present (20.23%) followed by tau-cadinol (6.53%), tetradecane (5.97%) and hexadecane (5.08%) (Table 3).

Table 2

Chemical composition of the ethanolic extract of *Buddleja saligna*

Peak #	Compounds'	Molecular weight	Formula	Similarity[a]	Area %[b]
1	Hexadecanoic acid, ethyl ester	284	$C_{18}H_{36}O_2$	808	12.982
2	Decanesioic acid, dibutyl ester	314	$C_{18}H_{34}O_4$	815	18.148
3	Heptacosane	380	$C_{27}H_{56}$	886	13.822
4	Oleanolic acid	456	$C_{30}H_{48}O_3$	810	55.047
	Total				**100**

[a]Mass spectral similarity to NIST08 library; [b]Relative peak area

Table 3

Chemical composition of the ethanolic extract of *Helichrysum odoratissimum*

Peak #	Compounds'	Molecular weight	Formula	Similarity	Area %
1	Camphene	136	$C_{10}H_{16}$	845	0.37935
2	2(5H)-Furanone, 5,5-dimethyl-	112	$C_6H_8O_2$	846	0.52592
3	2(3H)-Furanone, dihydro-5-methyl-	100	$C_5H_8O_2$	878	0.52592
4	N,N,O-Triacetylhydroxylamine	159	$C_6H_9NO_4$	893	0.16362
5	Decane	142	$C_{10}H_{22}$	907	0.26491
6	Eucalyptol	154	$C_{10}H_{18}O$	919	2.0637
7	Pentanoic acid, 2-methyl-3-oxo-, ethyl ester	158	$C_8H_{14}O_3$	805	2.6968
8	Undecane	156	$C_{11}H_{24}$	884	1.045
9	2-Hexanone, 6-bromo-	178	$C_6H_{11}BrO$	844	0.29935
10	3-(5-Methylfuryl)-N-furamidopropionamide	262	$C_{13}H_{14}N_2O_4$	759	0.085077
11	Benzoic acid, ethyl ester	150	$C_9H_{10}O_2$	943	0.11488
12	Terpineol	154	$C_{10}H_{18}O$	856	0.502
13	Dodecane	170	$C_{12}H_{26}$	910	2.0203
14	3,5-Diamino-1,2,4-triazole	99	$C_2H_5N_5$	866	0.22534
15	Benzothiazole	135	C_7H_5NS	894	0.76639
16	Acetate, 4-hydroxy-3-methyl-2-butenyl-	144	$C_7H_{12}O_3$	745	1.1397
17	Cyclohexanol, 2,4-dimethyl-	128	$C_8H_{16}O$	784	1.3549
18	Bicyclo[3.1.1]heptan-3-ol, 2,6,6-trimethyl-, [1R-(1à,2á,3à,5à)]-	154	$C_{10}H_{18}O$	730	0.65906

19	Tetradecane	198	$C_{14}H_{30}$	934	5.9697
20	Pseudosolasodine diacetate	499	$C_{31}H_{49}NO_4$	719	1.6777
21	Hexanoic acid, anhydride	214	$C_{12}H_{22}O_3$	831	0.5751
22	2,5-Dimethyl-2-(2-tetrahydrofuryl)tetrahydrofuran	170	$C_{10}H_{18}O_2$	819	0.99226
23	à-Muurolene	204	$C_{15}H_{24}$	865	0.70557
24	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aá,8aà)-	204	$C_{15}H_{24}$	893	3.848
25	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	204	$C_{15}H_{24}$	833	2.7374
26	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1à,4aá,8aà)]-	204	$C_{15}H_{24}$	756	1.3376
27	à-Calacorene	200	$C_{15}H_{20}$	719	1.3376
28	p-Nitrophenyl hexanoate	237	$C_{12}H_{15}NO_4$	882	0.77898
29	Hexadecane	226	$C_{16}H_{34}$	932	5.0805
30	á-copaene	204	$C_{15}H_{24}$	793	0.87134
31	tau-Cadinol	222	$C_{15}H_{26}O$	876	6.5325
32	2-Naphthalenemethanol, decahydro-à,à,4a-trimethyl-8-methylene-, [2R-(2à,4aà,8aá)]-	222	$C_{15}H_{26}O$	866	4.9037
33	à-Cadinol	222	$C_{15}H_{26}O$	820	1.3497
34	2-Propen-1-ol, 2-bromo-, acetate	178	$C_5H_7BrO_2$	619	0.13795
35	Myo-Inositol, 2-C-methyl-	194	$C_7H_{14}O_6$	783	3.2175
36	Nonadecane	268	$C_{19}H_{40}$	929	3.2453
37	2-Fluoro-6-trifluoromethylbenzoic acid, 4-cyanophenyl ester	309	$C_{15}H_7F_4NO_2$	743	0.026951

38	Pyrimidine-2,4,6(1H,3H,5H)-trione, 1-benzyl-5-[1-(2-diethylaminoethylamino)propylidene]-	372	$C_{20}H_{28}N_4O_3$	740	0.17284
39	1,2-Benzenedicarboxylic acid, dihexyl ester	334	$C_{20}H_{30}O_4$	877	0.22972
40	Dodecanoic acid	200	$C_{12}H_{24}O_2$	813	0.33526
41	3-(tert-Butyl)-4-methoxyphenyl 2,2,2-trifluoroacetate	276	$C_{13}H_{15}F_3O_3$	661	0.49714
42	Kaur-16-ene	272	$C_{20}H_{32}$	825	2.8161
43	Undecanoic acid, ethyl ester	214	$C_{13}H_{26}O_2$	844	2.8161
44	Heneicosane	296	$C_{21}H_{44}$	925	1.3852
45	Unknown 1	226	$C_8H_6N_2O_6$	495	0.26318
46	Carbonic acid, monoamide, N-(2,4-dimethoxyphenyl)-, propargyl ester	235	$C_{12}H_{13}NO_4$	615	0.10322
47	7-Amino-3-phenylcoumarin	237	$C_{15}H_{11}NO_2$	605	0.33609
48	4-tert-pentylphenol, trifluoroacetate ester	260	$C_{13}H_{15}F_3O_2$	582	0.044778
49	Benzaldehyde, 6-hydroxy-4-methoxy-2,3-dimethyl-	180	$C_{10}H_{12}O_3$	660	19.591
50	Octadecanoic acid, 17-methyl-, methyl ester	312	$C_{20}H_{40}O_2$	520	0.0377
51	1-Iodo-2-methylundecane	296	$C_{12}H_{25}I$	874	1.313
52	Benzaldehyde, 6-hydroxy-4-methoxy-2,3-dimethyl-	180	$C_{10}H_{12}O_3$	520	0.63795
53	4H,5H-Pyrano(4,3-b)pyran-4,5-dione, 2,3-dihydro-3-à-hydroxy-2-á-methyl-7-propenyl-	236	$C_{12}H_{12}O_5$	646	3.3373
54	9,10-Anthracenedibutanol, 9,10-dihydro-	324	$C_{22}H_{28}O_2$	591	0.16009

55	Acetic acid, [2-[(2-propenylamino)carbonyl]phenoxy]-	235	$C_{12}H_{13}NO_4$	771	0.065837
56	1-Butanone, 1,1'-(2,4,6-trihydroxy-m-phenylene)di-	266	$C_{14}H_{18}O_5$	629	0.35475
57	(Z)-8-(but-3-yn-1-yl)-5-(pent-2-en-4-yn-1-yl)octahydroindolizine	241	$C_{17}H_{23}N$	638	0.10241
58	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	709	0.28155
59	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	711	0.42599
60	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	746	0.301
61	Lupulon	414	$C_{26}H_{38}O_4$	654	1.3595
62	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	612	1.3595
63	Unknown 2	347	$C_{19}H_{30}BNO_4$	473	0.40922
64	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	713	0.18329
65	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	716	0.21977
66	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	641	0.38166
67	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	722	0.12957
68	Hexasiloxane, tetradecamethyl-	458	$C_{14}H_{42}O_5Si_6$	727	0.19224
	Total				**100**

[a]Mass spectral similarity to NIST08 library; [b]Relative peak area

3.2. Antioxidant activity

The antioxidant activity of the extracts was evaluated using the DPPH assay, which is based on the ability of an antioxidant to donate a hydrogen atom to the DPPH free radical, which has a single electron on the nitrogen atom (Kedare and Singh, 2011). Results showed that the extracts had high antioxidant activity with IC_{50} values of 5.13 ± 0.07 and 8.16 ± 0.34 µg/ml for *H. odoratissimum* and *B. saligna* respectively, which were compared to that

of the positive control, ascorbic acid (IC_{50} = 1.98 ± 0.006 µg/ml), which is a well-known antioxidant. The results for *H. odoratissimum* were previously published by the authors (Twilley et al., 2017a).

3.3. Mutagenicity

The results obtained for the mutagenic potential of the extracts are depicted in Table 4. In this assay, *Salmonella typhimurium* bacterial strains are used which have mutations that do not allow the bacteria to synthesis histidine and therefore are unable to grow on histidine poor growth medium. Thus, in the presence of an added mutagen, the preexisting mutants' gene function can be restored, thereby allowing the bacteria to synthesize histidine and permitting the growth of the bacteria on the histidine poor medium. Therefore, bacterial cells which revert to histidine independence (His^+) are able to form colonies. The trace amounts of histidine that were added allow for some of the bacterial cells to grow on the medium, which in many cases is required for mutagenesis to occur (Mortelmans and Zeiger, 2000). The Ames test identifies a genotoxic/ mutagenic sample if the number of revertant colonies formed on the plate containing the test sample is twice the number of colonies formed on the DMSO/sterile water only control plate (Verschaeve and Van Staden, 2008). It was evident that both extracts showed statistically different (P<0.01) results from the positive control, at each of the tested concentrations. Furthermore, the plates containing each of the extracts did not form more than twice the number of revertant colonies as in the DMSO control plate, therefore the extracts had no mutagenic effect on the *Salmonella* strains.

Table 4

Mutagenic effects of the *Buddleja saligna* and *Helichrysum odoratissimum* ethanolic extracts using *Salmonella typhimurium* strains TA98 and TA100.

Concentration	*Buddleja saligna* extract	*Helichrysum odoratissimum* extract
	***Salmonella typhimurium* TA98 revertants ± SD[c]**	
5 mg/mL	19.33 ± 0.58*	36.67 ± 4.04*
0.5 mg/mL	25.33 ± 1.15*	50.00 ± 6.08*
0.05 mg/mL	15.33 ± 0.58*	61.00 ± 9.89*
10% DMSO[a] control	20.33 ± 0.58*	46.25 ± 10.53*
2 µg/mL 4-NQO[b]	210.00 ± 4.36γ	249.50 ± 13.44γ
	***Salmonella typhimurium* TA100 revertants ± SD**	
5 mg/mL	137.00 ± 1.73*	119.33 ± 10.02*
0.5 mg/mL	133.67 ± 0.58*	129.33 ± 8.39*
0.05 mg/mL	115.33 ± 1.54*	127.67 ± 20.50*
10% DMSO control	113.33 ± 0.58*	142.00 ± 12.51*
2 µg/mL 4-NQO	1064.67 ± 1.53γ	1064.67 ± 1.53γ

[a]Dimethyl sulfoxide control; [b]4-nitroquinoline 1-oxide, positive control for mutagenicity; [c]Standard deviation. Values are expressed as mean number of revertants per plate ± SD (n=3). Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparison test, where $P<0.01$ (*) was statistically different to the positive control (γ).

3.4.Cell viability

The antiproliferative effects of the extracts on human dermal fibroblast cells were evaluated using the PrestoBlue® cell viability reagent, which is based on the ability of viable cells to

convert resazurin to resorufin (Lall et al., 2013). Actinomycin D was used as the positive control to induce toxicity in the cells, which showed an IC_{50} value of 0.022 ± 0.002 µg/ml. Similar results were obtained for both the extracts with IC_{50} values of 91.19 ± 0.69 and 90.62 ± 0.21 µg/ml for *B. saligna* and *H. odoratissimum* respectively.

3.5.Stability testing of formulation

The change in pH over each cycle test for the three sunscreen formulations is depicted in Figure 1a. A minor increase in pH between cycle 0 and cycle 1 was noted where after, the pH remained constant. The average pH values obtained over all the cycle testing for each of the formulations were determined as; 6.95 ± 0.01, 6.87 ± 0.17 and 6.81 ± 0.17 respectively for the base formulation and the formulations containing *B. saligna* and *H. odoratissimum*. This showed that there was no significant variation in the pH values over the complete cycle test period.

The results of the median droplet size, d_{50}, at the end of each 48 hr cycle test for each of the sunscreen formulations is depicted in Figure 1b. There was an evident decrease in the median droplet size over the six fridge-oven cycles. This was followed by an increase in droplet size over the first three freezer-oven cycles. The samples containing plant extracts showed a smaller variation in droplet size over the fridge-oven cycles than the base sunscreen formulation suggesting a possible stabilising effect. Each of the sunscreen formulations further underwent centrifugation at the end of each cycle. No separation, where the solid phase separated from the liquid phase or where the two liquid phases separated from each other, was observed after the cycle tests for each of the sunscreen formulations.

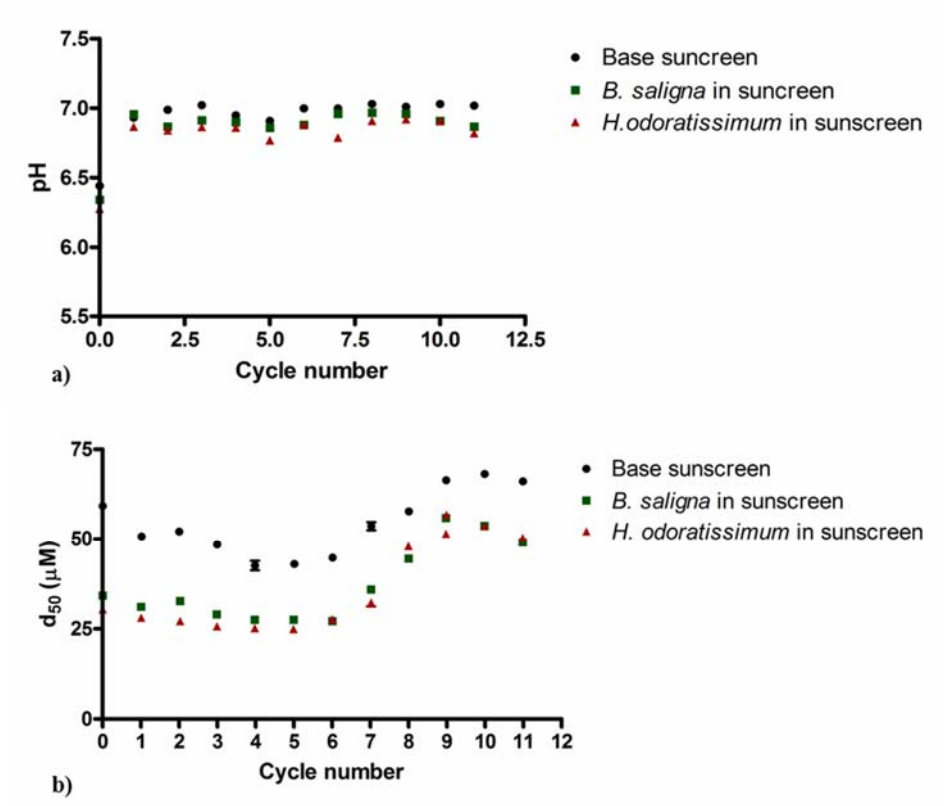

Figure 1. a) pH measurements and b) median droplet size after each cycle testing for the base sunscreen formulation, the sunscreen formulation containing the *Buddleja saligna* extract and the sunscreen formulation containing the *Helichrysum odoratissimum* extract.

3.6.Irritancy testing

Twenty volunteers were recruited, however only 19 completed the study, as the 20th volunteer could not complete the study due to circumstances unrelated to the study. No adverse events occurred during the study. After application of the extracts, the erythema was measured after 0, 24, 48, 72 and 96 hrs, both visually and with a chromameter (Figure 2a & b). The irritancy potential of the positive control was significantly higher to that of de-ionised water control and both the extracts, whereas there was no statistical different between the extracts and the de-ionised water control (Figure 2c).

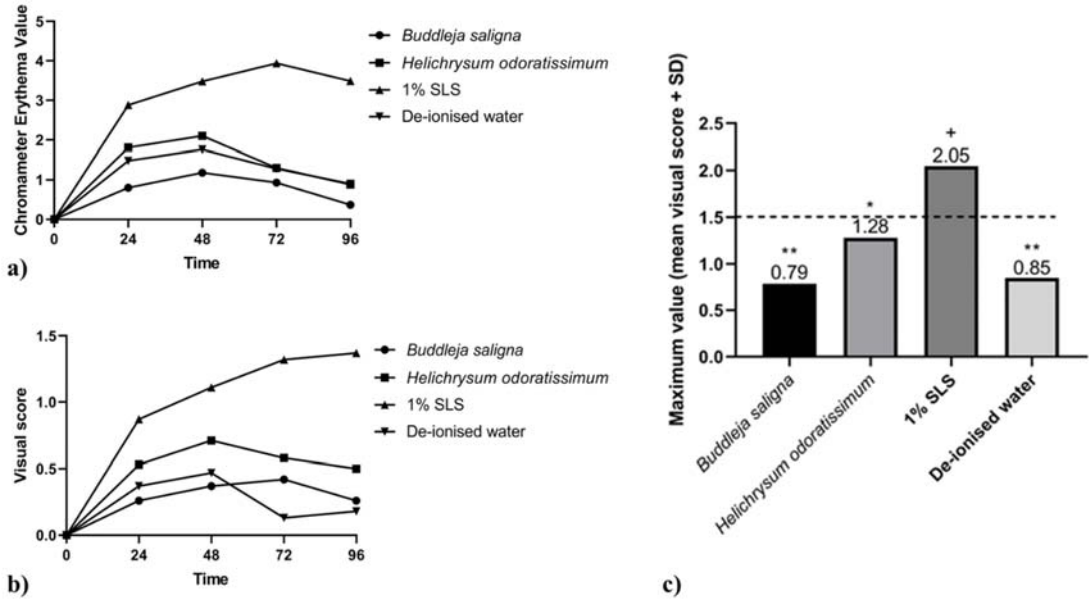

Figure 2. Erythema values measured after 0, 24, 48, 72 and 96 hours after application of the extracts. The erythema values were measured **a**) using a chromamater and **b**) using visual assessment. Graphs represent mean ± SEM (n=19). The visual scores were used to determine **c**) the maximum erythema value (mean + SD) of both the extracts compared to the positive (1% sodium lauryl sulfate) and negative (de-ionised water) controls. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison test, where $P<0.01$ (*) and $P<0.001$ (**) was statistically different to the positive control (+) (n=19)

Visual mean scores (+ SD) showed that the positive control was a high irritant after 48 h of application; 1.35 (24 h), 1.75 (48 h), 2.03 (72 h) and 2.05 (96 h), whereas de-ionised water showed no irritancy at each of the time intervals; 0.69 (24 h), 0.85 (48 h), 0.45 (72 h) and 0.52 (96 h). *Buddleja saligna* showed similar results to that of the negative control; 0.70 (24 h), 0.79 (48 h), 0.71 (72 h) and 0.60 (96 h), whereas *H. odoratissimum* showed slightly higher erythema scores; 1.10 (24 h), 1.28 (48 h), 1.02 (72 h) and 0.96 (96 h).

The chromameter results were used to verify the visual scores of erythema. The results shown below are for the delta a*, i.e. the increase in a* between baseline and the given time point. The positive control was confirmed to be a highly irritant at each of the time

intervals; 2.88 (24 h), 3.48 (48 h), 3.94 (72 h) and 3.49 (96 h), whereas de-ionised water was much lower; 1.48 (24 h), 1.76 (48 h), 1.28 (72 h) and 0.90 (96 h). The readings for *B. saligna* were even lower than that of de-ionised water; 0.80 (24 h), 1.18 (48 h), 0.93 (72 h) and 0.37 (96 h), whereas the reading of *H. odoratissimum* was higher than de-ionised water but still lower than that of the positive control; 1.81 (24 h), 2.10 (48 h), 1.30 (72 h) and 0.88 (96 h).

3.7.In vivo SPF and in vitro UVA evaluation

In Table 5, the SPF and the UVAPF results for the three sunscreens are depicted. It was evident that the results for each of the parameters (SPF, UVAPF, critical wavelength, UVA/ UVB ratio and UVA balance) did not differ significantly (P>0.05), indicating that the extracts did not reduce or interfere with the photoprotective activity of the SPF filter.

Table 5

Photoprotection results of the base sunscreen formulation (A) and the sunscreen with the addition of the *Buddleja saligna* (formulation B) and *Helichrysum odoratissimum* (formulation C) extracts

Sample	SPF[a]	UVAPF[b]	Critical wavelength	SPF *in vivo*/ UVAPF[c]	UVA balance
Base sunscreen (formulation A)	15.8 ± 0.41	6.47 ± 0.06	379.19	2.32	39%
Formulation B	16.1 ± 0.66	6.45 ± 0.06	379.50	2.33	39%
Formulation C	16.0 ± 0.49	6.47 ± 0.07	379.00	2.32	39%

[a]Sun protection factor; [b]UVA protection factor; [c]Claimed SPF (15) divided by UVAPF. Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparison test. Results were not statistically different (P>0.05) and therefore no significance was indicated. For SPF (n=10), and for UVAPF (n=4).

Table 6

In vitro UVA results, pre-and post-irradiation, of the base sunscreen formulation (A) and the sunscreens with the addition of the *Buddleja saligna* (formulation B) and *Helichrysum odoratissimum* (formulation C) extracts

Sample	*In vitro* SPF[a]	T[b] (UVA) (%)	T (UVB) (%)	Critical wavelength	UVA/UVB[c] ratio
Pre-irradiation					
Base sunscreen (formulation A)	12.32 ± 0.11	20.47 ± 0.12	8.00 ± 0.08	379.25	0.681
Formulation B	11.45 ± 0.17	21.32 ± 0.17	8.33 ± 0.12	379.56	0.673
Formulation C	12.38 ± 0.09	20.27 ± 0.08	7.69 ± 0.06	379.00	0.677
Post-irradiation					
Base sunscreen (formulation A)	11.94 ± 0.14	20.87 ± 0.31	8.26 ± 0.16	379.19	0.681
Formulation B	11.41 ± 0.07	21.38 ± 0.09	8.36 ± 0.07	379.50	0.673
Formulation C	12.18 ± 0.14	20.44 ± 0.13	7.81 ± 0.09	379.00	0.678

[a]Sun protection factor; [b]Spectral transmission; [c]Absorption of a 1.3 mg/square cm film was measured between 290 nm and 400 nm. The ratio of areas under the curve between 290-320 nm (UVB region) is compared with the area under the curve between 320-400 nm (UVA region). Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparison test. Results were not statistically different (P>0.05) and therefore no significance was indicated (n=4).

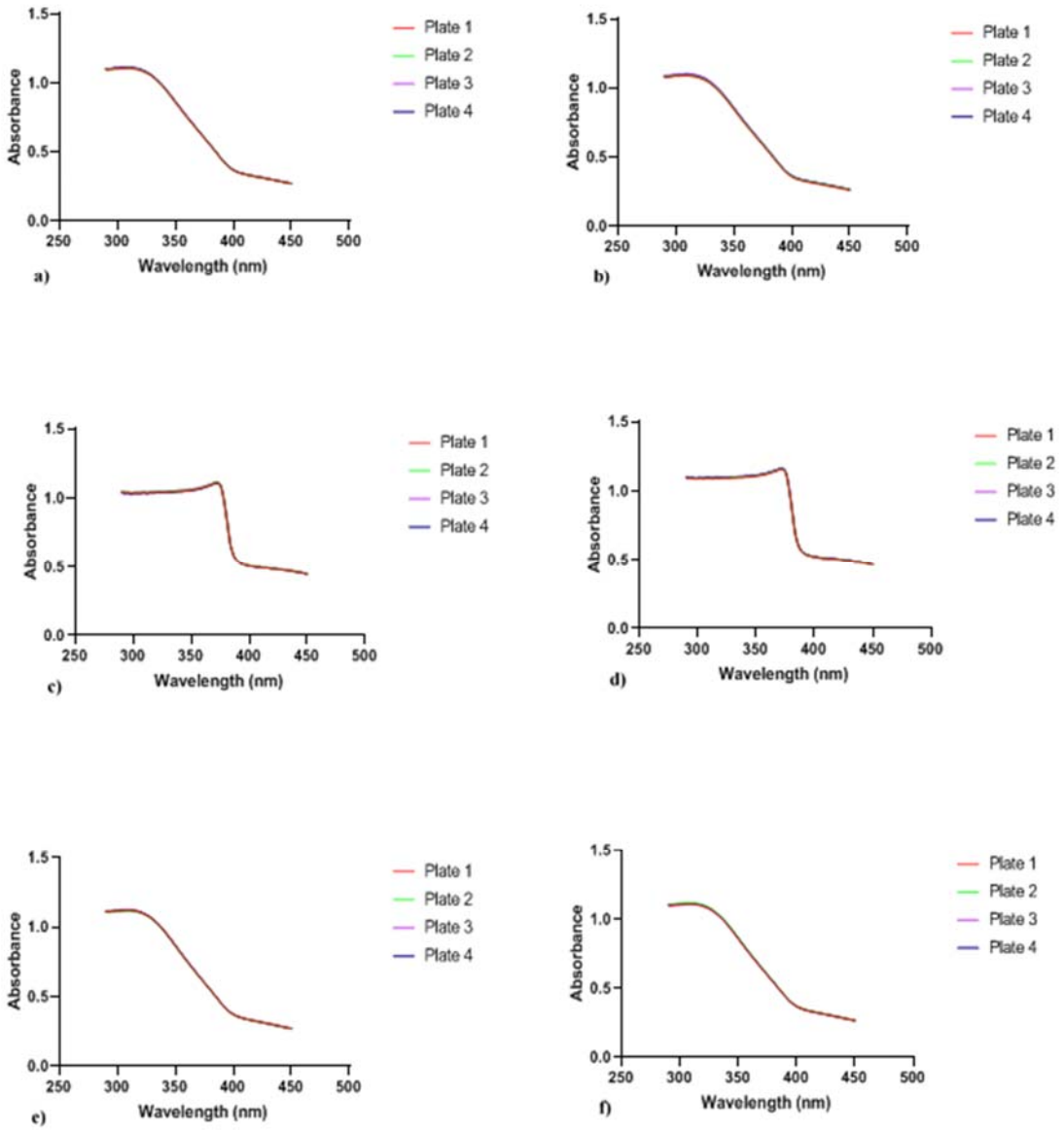

Figure 3: Mean absorbance spectra (n=4) for the UVA protection factor (UVAPF) determination: **a**) pre- and **b**) post irradiation for the base sunscreen formulation; **c**) pre- and **d**) post-irradiation for the sunscreen containing the *Buddleja saligna* extract and **e**) pre-and **f**) post-irradiation for the sunscreen containing the *Helichrysum odoratissimum* extract

3.8.Photostability

The *in vitro* UVA SPF for each of the sunscreen formulations was further measured pre-irradiation and post-irradiation in order to determine the photostability of the sunscreens (Table 6, Figure 3). There was no significant difference in each of the tested parameters post-irradiation, indicating the photostability of the sunscreen formulations. Both extracts showed a lower decrease in *in vitro* SPF compared to the base sunscreen formulation post-irradiation. The sunscreen with the addition of the extracts showed a reduction of 0.04 and 0.2 in SPF for *B. saligna* and *H. odoratissimum* respectively, whereas the base sunscreen showed a decrease of 0.38 post-irradiation, indicating that the extracts contributed to an incremental photoprotective effect.

4. Discussion

The skin is one of the body's primary defenses against external environmental factors and toxins. Equipped with antioxidant defense mechanisms, the skin helps to maintain the balance between free radicals and antioxidants (Thiele et al., 2005). Ultraviolet radiation can overcome these defense mechanisms, causing an excess accumulation of free radicals in the skin, which are able to damage DNA by binding to lipids and proteins (Narendhirakannan and Hannah, 2013). Exposure to ultraviolet radiation, both UVA and UVB, can therefore lead to the development of skin cancer, therefore protecting the skin against UV radiation is important (Clancy, 2008; D'Orazio et al., 2013; Goodsell, 2001). Emphasis is being placed on the use of plants, and their isolated compounds, for their antioxidant activity which could potentially contribute towards the photoprotectant properties of sunscreens (Cefali et al., 2016).

In the present study both *B. saligna* and *H. odoratissimum* showed high antioxidant activity, which was comparable to that of the positive control. A report by Phongpaichit et al (2007) stated that a plant with high antioxidant activity has an IC_{50} value between 10-50 µg/mL. Two methanolic extracts from the leaves and stems of *Buddleja saligna* respectively have been previously reported for their antioxidant activity, ranging from 93.8-94.9 % inhibition of DPPH (at a concentration of 0.1mg/mL), 98.8-100% inhibition of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical and a total antioxidant activity ranging from 490.98-1546.98 µmol Fe (II)/ g extract. The extracts were also examined for their total polyphenolic content (15.65-25.31 mg tannic acid/ g of dry material), total flavonoids (0.83-1.61 mg quercetin/ g dry material) and total flavonol content (0.10-0.76 mg quercetin/ g dry material), which was significant (Adedapo et al., 2009). In a study by Acevedo et al (2014) a methanolic extract was prepared from the leaves of *Buddleja cordata*. Antioxidant tests revealed an IC_{50} of 64.19 ± 2.09 µg/ml in the DPPH assay, showing significantly lower activity than what was obtained for *B. saligna* and *H. odoratissimum* in the present study. *Buddleja cordata* furthermore, showed an IC_{50} of 133.60 ± 35.20 and 1.85 ± 0.10 µg/mL against the superoxide and hydroxyl radical respectively. The major antioxidant compound identified within *B. cordata*, which constituted 10% of the extract weight, was verbascoside, which in a previous study showed an IC_{50} of 7.18 ± 0.08 µg/mL, which is similar to what we reported for *B. saligna* and *H. odoratissimum* (Acevedo et al., 2014; Frum et al., 2007). In the same study by Acevedo et al (2014), the methanolic leaf extract of *B. cordata* was tested to determine whether it had the potential to decrease UVB induced erythema in SKH-1 mice. Results revealed that the extract showed less redness in mice when compared to mice that had unprotected skin and were irradiated with UVB (Acevedo et al., 2014). In a further study by the same researchers, verbascoside, which was identified in *B. cordata*, was tested for its

photoprotective effect against UVB. It was found that the topical application of verbascoside was able to protect SKH-1 mice from inflammation caused by UVB. Both the extract and verbascoside were able to inhibit skin tumour development (Espinosa-González et al., 2016). The same group of authors (Avila Acevedo et al., 2005), prepared a methanolic extract from the aerial parts of *Buddleja scordioides*, and found that the extract had an SPF of 3 ± 0.09 on the skin of guinea pigs. Verbascoside was also identified in *B. scordiodies*, and was found to have an SPF of 24 ± 0.7.

With regards to *H. odoratissimum,* the antioxidant activity of the organic extract prepared from the leaves has only been documented by the authors of this study in another publication (Twilley et al., 2017a). However, there are studies that have been conducted on the essential oil extractions of *H. odoratissimum* (Asekun et al., 2007), which included the DPPH inhibitory activity of an essential oil extraction prepared from the leaves of *H. odoratissimum* showing an IC_{50} >100ppm (Asekun et al., 2007; Frum and Viljoen, 2006). A species in the same genus, *Helichrysum kraussii*, showed similar activity to that of the *H. odoratissimum* in the present study, with an IC_{50} of 4.66 ± 0.05 µg/mL (Twilley et al., 2017b). In a study by Jarzycka et al (2013) a sunscreen formulation, containing a polyphenolic fraction, prepared from *Helichrysum arenarium*, was tested for photoprotective activity. *Helichrysum arenarium* (10% wt.), which was solubilized in an emulsion, showed *in vitro* SPF results of 6.80 ± 0.26 (post-irradiation: 5.60 ± 0.17), an UVAPF of 6.96 ± 0.21 (post-irradiation: 6.35 ± 0.06), a UVA/UVB ratio of 1.06 (post-irradiation: 0.82) and a critical wavelength of 387.0 (post-irradiation: 382.0). Additionally, when *H. arenarium* (10% wt.) was combined with *Crataegus monogyna* (10% wt.), the photoprotective activity significantly increased to an SPF of 19.51 ± 4.19 (post-irradiation: 17.51 ± 4.09), an UVAPF of 16.58 ± 1.67 (post-irradiation: 16.00 ± 0.56), a UVA/UVB ratio of 0.95 (post-irradiation: 0.97) and a critical wavelength of 385.6 (post-irradiation:

386.0). On its own, *C. monogyna* showed similar results to when *H. arenarium* was tested alone, further emphasizing the increased SPF when tested in combination (Jarzycka et al., 2013).

Determining the mutagenic potential of a plant is important in order to assess whether a sample has the potential to induce mutations in DNA, resulting in possible carcinogenesis. In this study both *H. odoratissimum* and *B. saligna* were found to be non-mutagens against both the tested strains of *Salmonella*, as they did not show a significant increase in the number of revertant colonies when compared to the negative DMSO control. Furthermore, both the extracts did not increase the colony numbers above the 2-fold increase criterion for mutagenicity, therefore the extracts did not cause base-pair substitutions or frame-shift mutations. In a study by Verschaeve and Van Staden (2008), three species of *Helichrysum* were tested, and two showed significant mutagenic potential against *Salmonella typhimurium* TA98, namely *H. simillimum* and *H. herbacea*, with a 9.4 and 2.9-fold increase respectively in the number of revertant colonies when compared to the negative control. The third species, *H. regulosum,* showed a 1.7-fold increase, which is below the threshold considered mutagenic. *Helichrysum simillimum* was also considered mutagenic when using the TA100 strain, showing a 4.9-fold increase in colony numbers. Each of the extracts were prepared from the whole plant and extracted using 90% methanol and chloroform respectively and in each case the chloroform extracts did not show mutagenic activity. In the same study, the chloroform and 90% methanolic extracts prepared from the leaves of *B. saligna* did not show mutagenic activity. The chloroform extract, on the contrary showed antimutagenic potential in both the TA98 and TA100 strains of *Salmonella typhimurium*, suggesting that this extract should be further tested to determine whether it has any additional therapeutic potential (Verschaeve and Van Staden, 2008). No reports on the mutagenic or antimutagenic potential of *H. odoratissimum* were found.

Furthermore, results obtained from the cell viability assay, showed that both the extracts inhibited MRHF cells in a concentration-dependent manner, however IC_{50} values were noted at concentrations >90 µg/mL, indicating that high concentrations of the extracts were needed to have a significant antiproliferative effect. In previously published data (Twilley et al., 2017a) an ethanolic leaf and stem extract of *H. odoratissimum* showed significant antiproliferative activity on skin cancer cell lines with IC_{50} values of 15.50 ± 0.20 and 55.50 ± 6.60 µg/mL on human epidermoid carcinoma (A431) and human malignant melanoma (A375) respectively, resulting in a selectivity index (SI) of 5.85 and 1.63 when compared to the antiproliferative activity obtained on non-cancerous MRHF cells. Previously published results on the ethanolic leaf and stem extract of *B. saligna* (Twilley et al., 2020), showed significant antirproliferative activity on human malignant melanoma (UCT-MEL-1) cells with an IC_{50} value of 31.80 ± 0.35 µg/mL and an IC_{50} of 58.65 ± 5.42 µg/mL against human keratinocytes, resulting in a SI of 1.84. Compared to the MRHF cells, an SI value of 1.55 was obtained for *B. saligna* which is comparable to that obtained on the HaCat cells. The SI value is an indication of whether the extract is more targeted towards the cancerous cells (SI > 1) or the non-cancerous cells (SI < 1) (Badisa et al., 2009) and therefore it can be noted that both the extracts show a high toxicity towards cancerous cells than the non-cancerous cells. Eucalyptol (1,8-cineole), which was identified in the GC-MS analysis (Table 3) as a main constituent present within the ethanolic extract of *H. odoratissimum*, has been reported to inhibit UVB-induced skin carcinogenesis by inhibiting UVB-induced cyclooxygenase-2 (COX-2) protein and mRNA expression and the inhibition of prostaglandin E_2 (PGE_2) expression in HaCat cells (Lee et al., 2017). Constituents present within *B. saligna*, such as oleanolic acid and ursolic acid have been reported to inhibit melanoma (SK-MEL-28) proliferation and angiogenesis in a chorioallantoic membrane (CAM) melanoma model respectively (Caunii et al., 2017).

Considering the parameters evaluated to determine the stability of the sunscreen formulations, each were considered stable formulations, with the extracts displaying a stabilizing effect when the median droplet size was measured after the cycle testing. The pH of the samples showed small variances with the average pH remaining close to the pH of skin, making it suitable for skincare application. None of the samples showed phase separation under centrifugation indicating stability and good resistance to high stress conditions.

The irritancy potential of *B. saligna* and *H. odoratissimum* was determined as natural products, such as plant extracts, have been reported to cause allergic reactions of the skin such as contact dermatitis and phytophoto-dermatitis (Rios et al., 2005). The maximum irritancy potential of the two plant extracts as well as the positive and negative controls, were calculated from the visual score where the SD value was added to the mean value of each of the tested samples (Figure 2c). The irritancy values were classified according to the following: mean + SD >1.5 = sample is an irritant; mean + SD ≤ 1.5 and > negative control (de-ionised water) = sample has low irritancy potential and; mean + SD ≤ negative control = sample is a non-irritant (Komane et al., 2017). According to the classification, *B. saligna* was classified as a non-irritant whereas *H. odoratissimum* was a low irritant. Although, *H. odoratissimum* was classified as a low irritant, it should be noted that the irritancy testing was conducted using the neat reconstituted plant extracts (6.0mg/mL) and not using the sunscreen formulations with the incorporated extracts. The neat testing was performed in order to evaluate the highest potential of irritancy that could be displayed when using the extracts.

When considering the photoprotective activity of a sample or a sunscreen, the UVA protection factor (UVAPF) should be at least 1/3 of the sun protection factor (SPF)

(Gonçalves et al., 2015). In 2011, the US Food and Drug Administration (FDA), issued a rule stating that a sunscreen can be labelled as broad-spectrum (breadth of UVA and UVB protection) if the measured critical wavelength is at least 370 nm. Higher critical wavelength values equate to a better UVA protection (Duev et al., 2013). In the current study, *B. saligna* and *H. odoatissimum* were tested in a sunscreen formulation with an SPF 15 respectively. Results revealed that there was no significant difference in SPF of the base sunscreen formulation (SPF: 15.8 ± 0.41; UVAPF: 6.47 ± 0.06; critical wavelength: 379.19; UVA/UVB ratio: 2.32 and UVA balance: 39%) when compared to the formulations containing *B. saligna* (SPF: 16.1 ± 0.66; UVAPF: 6.45 ± 0.06; critical wavelength: 379.50; UVA/UVB ratio: 2.33 and UVA balance: 39%) and *H. odoratissimum* (SPF: 16.0 ± 0.49; UVAPF: 6.47 ± 0.07; critical wavelength: 379.00; UVA/UVB ratio: 2.32 and UVA balance: 39%), indicating that the addition of the extracts did not interfere with the photoprotectant properties of the UV filter (Table 5). However, the sunscreen formulations had both broad-spectrum and UVA protection. Pre-and post-irradiation measurements furthermore, revealed that the sunscreen formulations remained photostable and that irradiation did not have a significant effect on the photostability of the sunscreens, however the formulations containing the extracts showed increased photostability when compared to the base sunscreen formulation (Table 6, Figure 3).

5. Conclusion

Considering the characteristics required to develop an effective sunscreen formulation as defined by (Buso et al., 2019), *H. odoratissimum* and *B. saligna* could be considered as valuable additions into sunscreen formulations, as they displayed an increased stabilizing and photoprotective effect, showed significant antioxidant activity, are non-mutagenic and could be considered safe for cosmetic use as evaluated by the irritancy test. Additionally

each of the extracts have been reported to have antiproliferative activity against skin cancer cell lines, as previously reported by the authors, therefore, both extracts can be considered for use as multifunctional ingredients in sunscreen formulation. Although the DPPH antioxidant activity of the two ethanolic extracts was significant, future studies would include verifying the antioxidant activity by performing cell-based antioxidant studies, such as the cellular antioxidant assay (CAA), which is described as a more biological applicable assay to predict *in vivo* antioxidant activity (Li et al., 2015).

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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