The stimulating effect of extracts of South African plants on melanin production and their antibacterial activity against *Cutibacterium acnes*

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Highlights

- *Sideroxylon inerme* ethanolic extract showed an MIC of 125 µg/mL against Cutibacterium acnes.
- *Bulbine frutescens* ethanolic extract increased tyrosinase activity by 31.44 ± 1.41% at 200 µg/mL.
- *B. frutescens* ethanolic extract increased melanin production by 8.55 ± 1.66% (at 200 µg/mL).
- *S. inerme* and *B. frutescens* ethanolic extracts showed IC_{50} values >400 µg/mL against human keratinocytes (HaCat).
- *S. inerme* and *B. frutescens* ethanolic extracts show potential use for the treatment of hypopigmentation.

Abstract

Progressive macular hypomelanosis (PMH) is a hypopigmented skin disorder, which is identified by the presence of hypopigmented macules on the trunk and upper extremities. It is caused by the bacteria, *Cutibacterium acnes* which decreases melanogenesis in the affected areas resulting in hypopigmented macules. Current treatments include a combination of ultra-violet radiation and antibiotics, however, these antibiotics cause side effects such as severe rashes, blistering and dryness. Conversely UV radiation leads to increased oxidative stress which can cause premature ageing and susceptibility to skin cancer development. Therefore, there are currently no consistently effective or adequate treatment options for PMH. Furthermore, there are only a few studies which have evaluated the potential of plant extracts or natural products as potential treatments for PMH. In this study, thirty-three extracts, prepared using ethanol, water and dichloromethane from the leaves and twigs of eleven South African medicinal plants, were investigated for their antibacterial activity as well as tyrosinase and melanin stimulatory activity as possible targets for the treatment of PMH.

The *Sideroxylon inerme* L. ethanolic extract showed a noteworthy minimum inhibitory concentration (MIC) of 125 µg/mL against *C. acnes* (ATCC 6919) and had an additive effect when combined with the positive control, tetracycline. The *Bulbine frutescens* (L.) Willd. ethanolic extract (at 200 µg/mL) increased the monophenolase activity of tyrosinase by 31.44 ± 1.41 %. It was furthermore, able to increased melanin production by 8.55 ± 1.66 % (at 200 µg/mL), which was compared to α-melanocyte stimulating hormone (α-MSH), which showed a 13.39 ± 1.44 % increase at 100 µM. Furthermore, *B. frutescens* and *S. inerme* ethanolic extracts showed fifty percent inhibitory concentration (IC_{50}) values > 400 µg/mL against human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cells, indicating low toxicity against keratinocytes and melanocytes.

Consequently, the ethanolic leaf and stem extract of *B. frutescence* and *S. inerme* should be considered for further evaluation as potential treatments of hypopigmentation due to their melanin production and antibacterial activity. Further investigation would include evaluating the irritancy potential of these extracts to determine cosmetic safety as topical treatments and to conduct *in vivo* hypopigmentation trials to determine their efficacy.

Abbreviations

AMPK-SREBP-1, AMP-activated protein kinase/ sterol regulatory element binding protein 1; AP-1, Activator protein; CFU, Colony forming units; EGCG, Epigallocatechin gallate; NF-κβ, Nuclear Factor kappa beta; PMH, Progressive macular hypomelanosis

Key words

Cutibacterium acnes, progressive macular hypomelanosis, antibacterial activity, tyrosinase, melanin production

1. Introduction

There are numerous reports which have focused on the reduction of skin pigmentation with respect to age spots and ultra-violet (UV) radiation damage (Madu et al., 2022; Saleem et al., 2019; Waibel et al., 2019). Age spots and UV induced skin damage is primarily caused by the over-production of melanin. Melanin, present in the melanocytes, can be grouped into two classes, eumelanin, which is a black or brown pigment, and pheomelanin, which is yellow to red. Melanin functions as a broadband UV absorbent and is one of the most important photoprotective factors of the skin (Brenner and Hearing, 2008), where eumelanin scatters and absorbs UV radiation and scavenges reactive oxygen species associated with exposure to UV radiation (Yardman-Frank and Fisher, 2020). Melanosomes, which are the organelles

containing melanin, are transferred by the melanocytes to the keratinocytes where the melanin is localized above the nucleus as a protective cover against UV-induced cellular DNA damage. Upon exposure to UV radiation, an immediate pigment darkening (IPD) of melanin occurs either through chemical changes or potential redistribution of the melanosomes (Maddodi et al., 2012). A delayed tanning (DT) response can also occur after UV exposure which often lasts for several weeks. Consequently, UV radiation plays a major role in the regulation of pigmentation in the skin, which can lead to an overproduction or an underproduction of melanin, leading to several skin related disorders such as age spots, hyperpigmentation, freckling and hypopigmentation (Lee, 2021). Therefore, it is important to investigate both the potential of natural products and/ or medicinal plants to reduce the production of melanin as well as their potential to stimulate melanin production.

There are numerous skin pigmentation disorders such as progressive macular hypomelanosis, post-inflammatory hypopigmentation, vitiligo, chemical leukoderma and *pityriasis alba*. Progressive macular hypomelanosis (PMH) is identified by hypopigmented macules that are symmetrical, ill-defined and found primarily on the trunk and upper extremities, and in rare cases have been known to form facial lesions (McDowell et al., 2021). The melanocytes in the lesions produce significantly less pigment as the melanosomes are undersized and under-developed (Leonard et al., 2020; Westerhof et al., 2004). Westerhof et al (Westerhof et al., 2004) was the first to suggest that *Cutibacterium acnes*, previously known as *Propionibacterium acnes,* is the causative bacteria involved in the pathogenesis of this skin disorder, due to the high-density presence of the bacteria localised in the lesions. Three hypotheses have been suggested for the development of PMH; *C. acnes* produces inhibitory factors that either inhibit melanogenesis, inhibit or alter tyrosinase, or interfere with melanin transfer (McDowell et al., 2021; Westerhof et al., 2004).

With the hypothesis that *C. acnes* is the main cause of PMH, attempts at treating the disease have led to the combinational use of antibiotics and phototherapy. Phototherapy involves the use of light and laser therapy that requires the patient to be exposed to controlled amounts of non-ionising ultraviolet (UV) light, such as UVA, UVB and sunlight (El-Megei et al., 2022). Relyveld et al (Relyveld et al., 2006), reported that antibiotics together with non-ionising UVA radiation treatment, enhanced the re-pigmentation process, by inhibiting *C. acnes* and stimulating melanogenesis. Similarly, antibiotics in combination with narrow band UVB radiation and antibiotics alone, have also been proven to treat PMH (Kim et al., 2012; Thng et al., 2016). The most successful treatment has been the use of antibiotics in combination with narrowband UVB treatment, however this treatment combination did not result in permanent re-pigmentation of the affected areas (Leonard et al., 2020; Thng et al., 2016). The main concern with the current treatments is that they are chronic, lasting up to 18 months, and there is a high likelihood of treatment failure and a relapse of forming hypopigmented skin lesions (El-Megei et al., 2022). Considering the vast reports on the antibacterial and melanin stimulating activity of medicinal plants (Blom van Staden et al., 2017; Pérez-Sánchez et al., 2016; Xu et al., 2017; Zhou et al., 2012), plant extracts should be considered for their potential to treat PMH. There are currently no treatment options for PMH using plant extracts or natural products, and furthermore, the scientific evaluation of plant extracts and natural products for PMH has not been well studied. Two reports by Blom van Staden et al. (2017) and Blom van Staden et al. (2021), which is from the same research group as the current study, reported on plant extracts used for the potential treatment of hypopigmentation and PMH. The study by Blom van Staden et al. (2017) focused on plants traditionally used in Africa for the treatment of various skin diseases. Blom van Staden et al. (2017), reported the antibacterial ant melanin stimulating effect of *Hypericum revolutum* Vahl subs. *revolutum* and *Withania somnifera*, and their potential to be further evaluated for the treatment of PMH. Furthermore, this study

quantified melanin production by determining the concentration of melanin both extracellularly and intracellular using mouse melanoma (B16F10) cells. Blom van staden et al. (2021) focused on the potential of *Aspalathus linearis* (Burm.f.) R. Dahlgren, a plant endemic to the Cape Floristic Region, to stimulate melanogenesis, which was quantified through the amount of tyrosinase production and melanin production using the Fontana-Masson assay. In the present study eleven additional South African plants, from the Cape Floristic Region, were selected, based on the lack of scientific reports regarding their effect on melanin. Consequently, thirty-three extracts (dichloromethane, ethanol and water extracts prepared from eleven plants) were evaluated for their potential to be used as possible alternative and consistent treatment options for PMH by determining their inhibitory activity against the growth of *C. acnes* and to induced melanogenesis, via an increase of tyrosinase monophenolase activity and melanin production within melanocytes.

2. Materials and methods

2.1. Materials

Kojic acid (purity ≥ 98.5%), mushroom tyrosinase, L-tyrosine substrate, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (purity ≥ 99%), tetracycline (purity ≥ 98%), α-melanocyte stimulating hormone, hematoxylin, actinomycin D (purity ≥ 95%) and all analytical grade reagents were supplied by Sigma Aldrich (Johannesburg, South Africa). Dulbecco's Modified Eagle medium, fetal bovine serum, PrestoBlue™ cell viability reagent, phosphate-buffered saline and phenol red trypsin-EDTA (0.25%) was supplied by Thermo Fisher Scientific, (Johannesburg, South Africa). *Cutibacterium acnes* (ATCC 6919), brain heart infusion (BHI) agar and broth was supplied by Anatech Instruments (Pty) Ltd (Randburg, South Africa). The human keratinocyte (HaCat) and the University of Cape Town-Melanoma

1 (UCT-Mel-1) cell lines were donated by Dr. Lester Davids (Department of Human Biology, University of Cape Town).

2.2. Methods

2.2.1. Preparation of plant extracts

The aerial parts (leaves and twigs) of eleven plants were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria. A herbarium specimen of each plant was deposited at the H.G.W.J. Schweickerdt Herbarium (Table 1). Plant species names were validated using The Plant List and Plants of the World Online by Kew Royal Botanical Gardens.

Extracts were prepared using three solvents with a range of polarities, such as dichloromethane (DCM, 3.1 polarity index), ethanol (4.3 polarity index) and water (10.2 polarity index), to extract both polar and non-polar compounds (Abubakar and Haque, 2020). Extract preparation was done according to a method described by Sharma and Lall (2014), with modifications, where plant material was macerated with a blender rather than using a grinder and extracts were freeze dried for complete evaporation of solvents or water. The dichloromethane (DCM) and ethanolic extracts for the non-succulent plants (*Barleria obtusa* Nees, *Cussonia spicata* Thunb., *Hypoestes aristata* (Vahl) Roem. & Schult., *Hypoestes forskaolii* (Vahl) R.Br., *Pelargonium citronellum* J.J.A. Van der Walt, *Pelargonium graveolens* L'Hér and *Sideroxylon inerme* L.), were prepared using air-dried powdered plant material, which was blended using the solvent and shaken for 48 hrs. The menstruum was filtered using a Buchner funnel (Whatman 1.0 filter paper), followed by rotary evaporation (Buchi-R-200) (Heidolph, Hei-Vap value digital HB/G3B, Germany), freeze drying (Alpha 1-2 LDplus) (Christ, Osterode am Harz, Germany) and storage at -20ºC until further use. The succulent plants (*Bulbine frutescens* (L.) Willd., *Carpobrotus dimidiatus* (Haw.) L. Bolus, *Cotyledon orbiculata* L. and

Table 1. Phytochemical groups detected in ethanolic crude extracts

Plant samples[a]	Herbarium number	Phytochemicals					
		Tannins	Alkaloids	Saponins	Terpenes	Flavonoids	Phenolics
Barleria obtusa Nees	PRU 125926	+	+	-	+	+	+
Bulbine frutescens (L.) Willd.	PRU 125216	-	+	+	-	+	+
Carpobrotus dimidiatus (Haw.) L. Bolus	PRU 125927	+	+	+	-	+	+
Cotyledon orbiculata L.	PRU 128848	+	-	+	+	-	-
Cussonia spicata Thunb.	PRU 128851	+	+	+	+	+	+
Hypoestes aristata (Vahl) Roem. & Schult.	PRU 125925	-	+	-	+	+	+
Hypoestes forskaolii (Vahl) R.Br.	PRU 127863	-	+	-	+	+	+
Pelargonium citronellum J.J.A. Van der Walt	PRU 127869	+	+	-	+	+	+
Pelargonium graveolens L'Hér	PRU 128847	+	+	-	+	+	+
Portulacaria afra Jacq.	PRU 128849	+	+	+	-	+	+
Sideroxylon inerme L.	PRU 128850	+	+	-	+	+	+

[a] Leaves and twigs were used to prepare the ethanolic extracts, '+' indicates presence; '-' indicates absence

Portulacaria afra Jacq.) were harvested and immediately macerated in a blender using DCM and ethanol, respectively, shaken for 48 hrs and filtered using a Buchner funnel (Whatman 1.0 filter paper). Thereafter, the extract was rotary evaporated, freeze dried and stored at -20ºC.

The aqueous plant extracts were prepared by making a decoction in hot water, which was then left to cool and placed in the fridge overnight. The aqueous decoction was filtered using a Buchner funnel (Whatman 1.0 filter), frozen in the -20ºC freezer, freeze dried and stored at -20ºC. The aqueous extract of the succulent plants followed a similar procedure, except they were blended in the hot water on the same day of harvesting.

2.2.2. Phytochemical screening

The major phytochemical groups present in the ethanolic extracts were determined as described by Lall et al (Lall et al., 2019), with minor modifications. Ethanol is able to extract both polar and non-polar compounds and, therefore, these were the only extracts tested. Stock concentrations of the extracts were prepared at 15 mg/mL (in dH_2O) to determine the presence of tannins, saponins and terpenes. The presence of tannins was observed, after the addition of 2 mL 5% ferric chloride ($FeCl_3$) (in dH_2O), by the development of a yellow-brown precipitate. The presence of saponins was observed by the formation of froth after being shaken vigorously. Terpenes were observed by the formation of a reddish-brown colour forming on the interface, after the addition of 5 mL chloroform, 2 mL glacial acetic acid and several drops of concentrated H_2SO_4 (85%).

Stock concentrations of the extracts were prepared at 15 mg/mL (in methanol) for the determination of alkaloids, flavonoids and phenolics. Alkaloids were detected by adding 1.5 mL of 1% HCl, heating the solution in a water bath at 90 ºC and then adding a few drops of Dragendroff's reagent to form an orange precipitate. Flavonoids were detected through the immediate appearance of a pink-red colour after 2 drops of hydrochloric acid (HCl) and 0.5 g

magnesium turnings were added to the extracts. Phenolics were detected after 1 mL 1% ferric chloride was added to the extracts and a colour change to blue or green was observed.

2.2.3. Antibacterial activity – minimum inhibitory concentration

The antibacterial activity of the extracts was determined through a broth microdilution method as described by Lall et al. (2013). *Cutibacterium acnes* (ATCC 6919) was cultured on nutrient agar from a Kwik-Stick and incubated for 72 hrs at 37ºC under anaerobic conditions (Anerocult A in an anaerobic jar). Colonies were then inoculated into BHI broth at a concentration of 1.5 × 10^8 colony forming unit (CFU) per mL (CFU/mL) (OD_{600} = 0.132). The extracts and the positive control, tetracycline, were prepared in 10% DMSO at a stock concentration of 2.0 and 0.2 mg/mL, respectively. The samples were serially diluted in a 96-well plate in BHI broth and the bacterial suspension (100 µL) was added to obtain a final concentration range of 3.9 - 500 and 0.3 - 50 µg/mL, respectively. Controls included the DMSO (2.5% v/v) as the vehicle control, a growth media control (0%) and untreated bacterial cells (100%). The plates were incubated in an anaerobic environment for 72 hrs at 37ºC after which, 20 µL of PrestoBlue™ was added to visually determine the minimum inhibitory concentration (MIC) after 2 hrs incubation. The threshold for antibacterial activity of the extracts was determine using the guidelines set by Kuete and Efferth, (2010), where an (MIC < 100 µg/mL) is significant, (100 < MIC ≤ 625 µg/mL) is moderate and (MIC > 625 µg/mL) is weak.

2.2.4. Antibacterial activity – minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined using a method adapted from Afroz et al (Afroz et al., 2020). Prior to the addition of the PrestoBlue™ to the above wells for determination of MIC, half the wells' content (100 µL) was transferred to nutrient broth (100 µL) in a 96-well plate. The 96-well plates were incubated under anaerobic conditions at 37ºC for 72 hrs, after which, 20 µL of PrestoBlue™ was added and the minimum bactericidal concentration (MBC) was visually determined.

2.2.5. Antibacterial activity – combination study

The combined antibacterial activity of the ethanolic *S. inerme* extract, which showed the lowest MIC, and tetracycline was evaluated. The assay was performed as described by Gibango et al. (2020). Stock concentrations of the extract and tetracycline were prepared at 2.0 and 0.2 mg/mL (in 10% DMSO), respectively. The extract and tetracycline were added in nine different ratios from *S. inerme*: tetracycline (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) (Supplementary Table 1). In a sterile 96-well plate, 100 µL of BHI broth was added to each well, followed by 100 µL of the combined ratios, which were serially diluted. Thereafter, 100 µL of bacterial suspension, as described in section 2.2.3, was added to all the wells. Controls include ethanolic *S. inerme* (10:0) (3.91 – 500 µg/mL), tetracycline (0:10) (0.39 – 50 µg/mL), DMSO (2.5% v/v) as the vehicle control, a growth media control (0%) and untreated bacterial cells (100%). The plates were incubated for 72 hrs at 37ºC in anaerobic conditions, where after 20 µL of PrestoBlue™ was added to determine the MICs. The fractional inhibitory concentration (FIC) was calculated using the following formula:

$$\Sigma \text{FIC} = \frac{\text{MIC (Sample A + Sample B)}}{\text{MIC (Sample A)}} + \frac{\text{MIC (Sample A + Sample B)}}{\text{MIC (Sample B)}}$$

Where an FIC < 0.5 = synergistic effect, 0.5 < FIC < 4 = additive effect and FIC > 4 = antagonistic effect (Pillai et al., 2005).

2.2.6. Tyrosinase activity

The extracts were evaluated for modulatory activity of the mushroom tyrosinase enzyme using a method described by Blom van Staden et al. (2021). The extracts and the positive control, kojic acid, were prepared at a stock concentration of 2 mg/mL (in 10% DMSO). The extracts and kojic acid were added to a 96-well plate and serially diluted to obtain final concentration ranges of 3.12-200 and 0.19-12.5 µg/mL, respectively. Controls included the DMSO (2%) as the vehicle control and an untreated enzyme control. To each well, 90 µL of 50 mM potassium

phosphate buffer (pH 6.5) and 20 µL of monophenolase mushroom tyrosinase (333 Units/mL in phosphate buffer) were added and incubated at room temperature for 5 min. Thereafter, 70 µL of L-tyrosine substrate (2 mM) was added. The absorbance was kinetically measured at 492 nm for 30 min with a BIOTEK Power Wave multi-well plate reader (A.D.P., Weltevreden Park, South Africa). The 50% inhibitory concentrations (IC_{50}) were determined using GraphPad Prism 7 software.

2.2.7. Cell culture

The human melanoma (UCT-Mel-1) and human keratinocyte (HaCaT) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% amphotericin B (250 µg/mL), 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were cultured in a humidified incubator set at 5% CO_2 and 37ºC. The cells were grown until a confluent monolayer formed in a T75 cell culture flask and were then sub-cultured using 0.25% trypsin-EDTA.

2.2.8. Antiproliferative activity

Ethanolic extracts of *B. frutescens, C. spicata, P. afra* and *S. inerme* were tested for their antiproliferative activity against human keratinocytes (HaCaT) and human melanoma (UCT-Mel-1) cells as previously described Lall et al. (2013). The extracts which increased tyrosinase activity or showed noteworthy antibacterial activity were evaluated for antiproliferative activity to determine whether the extracts showed potential toxicity against human keratinocytes, or human melanoma cells, which was used for further melanin quantification. The UCT-Mel-1 and HaCaT cells were seeded (100 µL) into a 96-well plate (1×10^5 cells/ well) and incubated in 5% CO_2 at 37ºC for 24 hrs. The extracts and the positive control, actinomycin D were prepared at stock concentrations of 20 and 1 mg/mL (in DMSO), respectively and serially diluted in a 24-well plate in DMEM at concentration ranges of 6.26 – 800 and 7.81×10^{-4} – 0.1 µg/mL. Samples dilutions (100 µL) were transferred, in triplicate, to

the 96-well plates containing the cells to obtain final concentration ranges of 3.13 - 400 and 3.9×10^{-4} - 0.05 µg/mL, respectively. Controls included untreated cells (100%), 1% DMSO treated cells as the vehicle control and a 0% media control. The cells were incubated for 72 hrs, where after, 20 µL of PrestoBlue™ reagent was added to all the wells and incubated for an additional 2 hrs. The fluorescence was measured at an excitation of 530 nm and emission of 625 nm using the Victor Nivo Multimode Microplate plate (PerkinElmer, Midrand, South Africa) reader. Graph Pad Prism 7 software was used to determine the IC_{50} values of the samples. The threshold for antiproliferative activity of the extracts was determined as significant activity (IC_{50} < 100 µg/mL), moderate activity (100 µg/mL < IC_{50} < 300 µg/mL), low activity (300 µg/mL < IC_{50} < 1000 µg/mL) and no activity (IC_{50} > 1000 µg/mL) according to guidelines set by Kuete and Efferth (2015).

2.2.9. Melanin production

The amount of melanin produced by UCT-Mel-1 cells, after treatment with the ethanolic extract of *B. frutescens*, was determined using the Fontana-Masson assay as described by Kloepper et al. (2010), with minor modifications. The ethanolic *B. frutescens* extract showed an increase in tyrosinase activity and an IC_{50} > 400µg/mL, therefore it was further evaluated for its effect on melanin production. The UCT-Mel-1 cells were seeded (1 mL) into 24-well plates at 5×10^4 cells/well and incubated for 24 hrs at 5% CO_2 and 37ºC. The extracts and the positive control, α-melanocyte stimulating hormone (α-MSH), were prepared at stock concentrations of 400 µg/mL and 200 µM (in 1% DMSO), respectively, where after 1 mL was added to the cells to obtain final concentrations of 200 µg/mL and 100 µM, respectively. Controls included untreated cells and 1% DMSO treated cells as the vehicle control. The cells were incubated for another 24 hrs in 5% CO_2 at 37°C

After 24 hrs, the cells were washed with tris buffered saline and fixed using ethanol: acetic acid (2:1). The prepared silver nitrate ($AgNO_3$) working solution (0.15 M), was added to the cells

and incubated for 40 min in the dark at 56ºC. Following incubation, sodium thiosulphate ($Na_2S_2O_3$) in water (0.33 M) was added, enough to cover the cells, for 1 min. The cells were then counter stained with hematoxylin (7 g/L) for 3 min and washed with dH_2O. Thereafter, the cells were dehydrated with 70, 90 and 100% ethanol and visualised with a Zeiss Primo Vert light microscope (Carl Zeiss (Pty) Limited, Randburg, South Africa) and analysed using Image J.

Image J was used to calculate the percentage pigment granule pixels within the square selection and provided a mean, minimum and maximum pigmentation score. The percentage pigmentation activity was then calculated as follows:

$$\%\, Melanin = \left(\frac{Melanin\ content\ of\ sample}{Melanin\ content\ of\ control}\ x\ 100\right)$$

2.2.10. Statistical analysis

Experiments were performed in triplicate with three independent experiments. The results are represented as the mean ± SD (n = 3). GraphPad Prism version 7 was used for statistical analysis to obtain the effective concentrations resulting in 50% of the activity (IC_{50}), which were derived from a sigmoidal dose response curve. A one-way analysis of variance (ANOVA) together with Dunnett's multiple comparison test was used to determine whether the difference between the controls and the treatments were significant, $^{**}p < 0.01$.

3. Results and discussion

3.1. Extraction of plants and phytochemical analysis

Alkaloids, terpenes, flavonoids and phenolics are commonly found in plants that have antibacterial activity. This could provide an explanation to the moderate antibacterial activity observed from the ethanolic *S. inerme* extract (Kim et al., 2010, 2008; Smith et al., 2008). Seven of the selected plants *B. obtusa, C. spicata, H. aristata, H. forskaolii, P. citronellum, P.*

graveolens and *S. inerme* contain the above four mentioned phytochemical groups (Table 1). To date there have been no reports documenting the phytochemical groups found in *B. obtusa*, therefore the results are presented here for the first time. Alkaloids, flavonoids and phenolics were found in each extract except for *C. orbiculata*. Several flavonoids (naringenin, hesperetin, isosakuranetin), terpenes (geniposide, glycyrrhizin, lupenone) and phenolics (rosmarinic acid) have been found to induce melanin synthesis, increase tyrosinase activity, enhance melanin production and gene expression (Niu and Aisa, 2017). The increased tyrosinase activity observed in the present study by *B. frutescens, C. spicata* and *P. afra* could be attributed to the flavonoids and phenolics that were detected in the three ethanolic extracts.

3.2. Antibacterial activity

Cutibacterium acnes has been associated with PMH due to the high density (73.9%) of the bacterium found in PMH lesions on the skin compared to non-lesioned skin (14.2%) (Kim et al., 2008; Petersen et al., 2017). Currently one of the PMH treatments is through the use of antibiotics and narrow band ultraviolet (UV) radiation therapy (Leonard et al., 2020; Relyveld et al., 2006). However, some antibiotics, such as isotretinoin, clindamycin and benzoyl peroxide cause severe side effects (migraines, alteration in eyesight, gastrointestinal bleeding, joint swelling and pain) and have proven to cause skin reactions such as sun sensitivity, severe rashes, peeling, blistering and dryness (Cunha, 2022; Leonard et al., 2020; McDowell et al., 2021; MedlinePlus, 2018; NHS, 2022). Due to the selective pressure applied by antibiotics, resistant strains are becoming increasingly common. Thus, the use of combinational antibiotics are used to reduce the further development of drug resistant *C. acnes* strains (Relyveld et al., 2006).

Of the 33 extracts tested, nine showed antibacterial activity against *C. acnes* (Table 2). *Barleria obtusa* ethanolic, *H. aristata* ethanolic and *H. forskaolii* ethanolic extracts showed MIC values of 250 µg/mL, whereas *H. aristata* DCM, *P. citronellum* ethanolic, *P. graveolens* ethanolic and

S. inerme aqueous and DCM extracts showed MIC values of 500 µg/mL. The *S. inerme* ethanolic extract showed the most noteworthy antibacterial activity with an MIC of 125 µg/mL. *Barleria obtusa* (aqueous and DCM), *B. frutescens* (aqueous, ethanolic and DCM), *C. dimidiatus* (aqueous, ethanolic and DCM), *C. orbiculata* (aqueous, ethanolic and DCM), *C. spicata* (aqueous, ethanolic and DCM), *H. aristata* (aqueous), *H. forskaolii* (aqueous and DCM), *P. citronellum* (aqueous and DCM), *P. graveolens* (aqueous and DCM) and *P. afra* (aqueous, ethanolic and DCM) displayed MIC values > 500 µg/mL.

No reports on the antibacterial activity of *Barleria obtusa* or *Hypoestes aristata* could be found and therefore, is reported here for the first time. *Bulbine frutescens* has been extensively evaluated for its antibacterial activity by Coopoosamy (2011), where three different extractions (aqueous, acetone and ethyl acetate) were prepared from three different plant parts (leaves, roots and rhizomes), resulting in nine extracts. The extracts were evaluated against three gram-positive bacteria strains (*Bacillus subtilis, Micrococcus kritinae* and *Staphylococcus aureus*) and three gram-negative bacteria strains (*Escherichia coli*, *Proteus vulgaris* and *Enterobacter aerogenes*). The highest activity was noted for the acetone root extract which showed an MIC of 1.0mg/mL against *M. kristinae*. *Carpobrotus dimidiatus* aqueous, dichloromethane, ethyl acetate and methanol leaf extracts have previously been evaluated for antibacterial activity against two *Klebsiella pneumoniae* multidrug-resistant strains (carbapenem-resistant (CBR) and extended-spectrum beta-lactamase (ESBL)), where the highest activity was noted for the DCM extracts against CBR (MIC: 0.78 mg/mL), and the lowest activity was observed for the aqueous extract against CBR and the methanol extract against both strains with MIC values of 6.25 mg/mL. These extracts furthermore, showed varying activity against virulence factors associated with bacterial pathogenesis such as biofilm formation, exopolysaccaride production, curli expression and hypermucoviscosity (Adeosun et al., 2023). The antimicrobial activity of *C. orbiculata* has been extensively studied by Aremu et al (2010). Petroluem ether, DCM,

ethanol and aqueous extracts were prepared from the leaves and stems which showed varying activity (MIC values ranging between 1.56 - >12.5 mg/mL) against *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumoniae*. A study by De Villiers et al (2010), reported that the leaf extracts (methanol and aqueous) of *C. spicata* showed activity against *S. aureus*, *E. coli*, *E. faecalis*, *Pseudomonas auruginoa*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae* with varying MIC values, where the methanolic extract overall showed the highest activity and the activity was also dependent on the geographical region in which the plant material was collected. In previous reports, both a methanolic and aqueous leaf extract of *H. forskaolii* showed MIC values > 1000 µg/mL, against *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* (Mothana et al., 2011). An acetone extract prepared from the aerial parts of *P. citronellum* has been reported to have weak activity against *S. aureus* and *B. subtilis* (MIC >1600 µg/mL) (Lalli, 2005). A study by M'hamdi et al (2024), showed the antibacterial activity of *P. graveolens* essential oil (prepared from the stems, leaves and flowers) against *Listeria monocytogenes*, *Salmonella typhimurium*, *S. aureus* and *E. coli*, with MIC values varying between 1.04-2.08 (%), with major components being identified as epi-γ-eudesmol, β-citronellol, geraniol, citronellyl formate and geranyl tiglate. Basson et al (2023), reported on the antibacterial activity of *P. afra* against *E. coli* and *S. aureus*. Eighteen extracts were prepared from the stems, leaves and roots using methanol, cold water, room temperature water, hot water, ethyl acetate and hexane as extraction solvents. Both the cold water and room temperature water extracts did not show a zone of inhibition against both bacterial strains. The hot water extracts and methanol extracts showed zones of inhibition between 12-20 mm, whereas the hexane extracts showed between 13- 18 mm (Basson et al., 2023). In a study conducted by Sharma and Lall (2014), a similar result was obtained as in the present study, where an ethanolic *S. inerme* bark extract displayed an MIC of 250 µg/mL against *C. acnes.* A study by Mzimba et al (2023), reported that a 1:1 DCM: methanol leaf extract of *S. inerme*

showed an MIC of 0.06 mg/mL (60 µg/mL) against *C. acnes*. Against the other tested bacterial strains (*Brevibacterium linens*, *Corynebacterium xerosis*, *S. aureus*, *Staphylococcus capitis*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis*, *Enterobacter cloacae*, *K. pneumoniae* and *P. aeuroginosa*), the *S. inerme* extract showed MIC values ranging between 0.75 – 4.0 mg/mL.

Table 2. Antibacteria activity (MIC) of dichloromethane (DCM), ethanolic and aqueous crude extracts

Plant sample[a]	MIC[b] (µg/mL) on *Cutibacterium acnes*		
	Aqueous	**DCM**	**Ethanolic**
Barleria obtusa	> 500	> 500	250
Bulbine frutescens	> 500	> 500	> 500
Carpobrotus dimidiatus	> 500	> 500	> 500
Cotyledon orbiculata	> 500	> 500	> 500
Cussonia spicata	> 500	> 500	> 500
Hypoestes aristata	> 500	500	250
Hypoestes forskaolii	> 500	> 500	250
Pelargonium citronellum	> 500	> 500	500
Pelargonium graveolens	> 500	> 500	500
Portulacaria afra	> 500	> 500	> 500
Sideroxylon inerme	500	500	125
Tetracycline[c]	0.78		

[a] Leaves and twigs were used to prepare the ethanolic extracts, [b] Minimum inhibitory concentration, [c] Positive control for the antibacterial assay against *Cutibacterium acnes*,

Extracts which showed moderate MIC values were evaluated for their bactericidal activity. *Barleria obtusa* (ethanolic), *H. aristata* (ethanolic and DCM), *H. forskaolii* (ethanolic), *P. citronellum* (ethanolic), *P. graveolens* (ethanolic) and *S. inerme* (aqueous and DCM) extracts showed MBC values >500 µg/mL, whereas the *S. inerme* ethanolic extract showed and MBC

of 500 µg/mL. The positive control, tetracycline, which is used in the treatment of acne vulgaris, specifically follicular *C. acnes*, showed an MIC and MBC of 0.78 and 5.25 µg/mL, respectively (Williams et al., 2012).

A combination of antibiotics is often used for the treatment of bacterial infections as they may show enhanced efficacy, a reduction in the normally required dose of antibiotics and possibly a decrease in bacteria developing resistance (synergism) (Zhang et al., 2004). Therefore, the combined effect of ethanolic *S. inerme* and tetracycline was evaluated (Table 3).

Table 3. Combined effect of ethanolic *Sideroxylon inerme* extract and tetracycline against *Cutibacterium acnes* (ATCC 6919)

Ratio	Ethanolic *Sideroxylon inerme* MIC[a] (µg/mL)	Tetracycline MIC (µg/mL)	FIC value[b]
Sideroxylon inerme ethanolic extract	125^{++}	-	-
9:1	56.25^{**}	0.63^{+}	1.25
8:2	50^{**}	1.25^{**}	2
7:3	43.75^{**}	1.88^{**}	2.75
6:4	37.5^{**}	2.5^{**}	3.5
5:5	15.63^{**}	1.56^{**}	2.13
4:6	12.5^{**}	1.88^{**}	2.5
3:7	9.38^{**}	2.19^{**}	2.88
2:8	6.25^{**}	2.5^{**}	3.25
1:9	3.13^{**}	2.81^{**}	3.63
Tetracycline	-	0.78^{+}	-

[a] Minimum inhibitory concentration; [b] Fractional Inhibitory Index; Note: the MIC reported is the MIC of the serial diluted ratio for ethanolic *S. inerme* and tetracycline. Data was expressed as mean ± SD (n = 3). $^{**}p < 0.01$ indicates significant different when the MIC of the extract combined with the tetracycline was compared to *Sideroxylon inerme* (++) alone and when the MIC of tetracycline combined with *S. inerme* was compare to tetracycline (+) alone.

Only the *S. inerme* ethanolic extract was tested for its combined activity with tetracycline, as it showed the highest antibacterial activity with an MIC value of 125 µg/mL. The interaction between the ethanolic *S. inerme* extract and tetracycline was found to be additive at each of the ratios, the most effective being 9:1 with an FIC value of 1.25. For *S. inerme* and tetracycline, the MIC value decreased from 125 to 56.25 µg/mL and 0.78 to 0.63 µg/mL, respectively. Significance indicates that the extracts MIC (when combined with tetracycline) significantly reduced ($p < 0.01$), whereas the MIC of tetracycline (when combined with *S. inerme*) significantly increased ($p < 0.01$), expect for at a 9:1 combination where the MIC was significantly similar (+).

Previously several compounds have been isolated from *S. inerme.* A study by Momtaz et al. (2008) isolated a polyphenol, epigallocatechin gallate (EGCG)), and a polyphenol flavonoid, procyanidin B1, from the methanolic stem bark extract. Shelembe (2014), reported on the isolation of lutein (carotenoid) and friedelin (triterpenoid) from a hexane/ DCM leaf extract; friedelin, stigmasterol (sterol) and a mixture of α- and β-amyrin (triterpene) from the hexane/DCM bark extract, as well as apocynol B (sesquiterpenoid) from the aqueous methanol stem bark extract. Both phenolics and terpenoids are classes of compound which were found present in the current study. A polyphenol, such as EGCG was shown to reduce inflammation through the inhibition of nuclear factor-kappa beta (NF-κβ) and activator protein 1 (AP-1), induces cytotoxicity and apoptosis in SEB-1 sebocytes and thereby decreases the viability of *P. acnes* (Yoon et al., 2013). A study by Im et al (2012), further confirmed the inhibitory effect of EGCG on cell viability of sebocytes (SZ295) and insulin-like growth factor I (IGF-I) differentiated sebocytes, as well as its inhibitory activity against lipid synthesis in SZ295 and IGF-I sebocytes. Im et al (2012) further determined that EGCG was able to inhibit the protein kinase B/ mammalian target of rapamycin (Akt/mTOR) signalling pathway which may correlate with the anti-lipogenic activity of EGCGin IGF-I differentiated sebocytes. In addition,

inflammatory cytokines (IL-1α and IL-6) know to be upregulated in acne lesions, were inhibited by EGCG in SZ95 sebocytes (Im et al., 2012). Lutein has been shown to supress the up-regulation pyroptotic inflammatory markers in human keratinocytes (HaCat), such as Interleukin (IL)-1β, IL-18, tumor necrosis factor (TNF)-α, matrix metalloproteinase (MMP)-3 and 13, caspase-1, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and 5, toll-like receptor (TLR)-4 and the NOD-like receptor protein (NLPR)-3, thereby reducing the pyrotopic effect caused by *C. acnes* in HaCat cells (Chen et al., 2023). EGCG has previously been shown to reduce acne by reducing sebum through the modulation of the AMP-activated protein kinase/ sterol regulatory element binding protein 1 (AMPK-SREBP-1) signalling pathway in human SEB-1 sebocytes, isolated from patients with acne. Alkaloids, which were shown to be present in the *S. inerme* ethanolic extract, have also been reported to inhibit *C. acnes*. A study by Sun et al (2024), showed that an alkaloid, berberine, inhibited four *C. acne* strains with MIC values ranging between 6.25-12.5 µg/mL and MBC values ranging between 12.5-25 µg/mL.

3.3. Melanin production

Tyrosinase plays a role in the production of eumelanin and pheomelanin (Shuster Ben-Yosef et al., 2010). Seven extracts had an effect on the activity of the tyrosinase enzyme. Five extracts increased tyrosinase activity at a concentration of 200 µg/mL, ethanolic and DCM *B. frutescens* extracts (31.44 ± 1.41 % and 11.07 ± 2.44 % stimulation, respectively), ethanolic and DCM *C. spicata* extracts (11.47 ± 1.10 % and 10.21 ± 1.09 % stimulation, respectively) and ethanolic *P. afra* extract (25.76 ± 1.22 % stimulation), whereas two extracts decreased the activity, namely the ethanolic and DCM extracts of *P. citronellum* (IC_{50} values of 2.33 ± 0.83 and 8.28 ± 7.25 µg/mL, respectively) (Fig. 1).

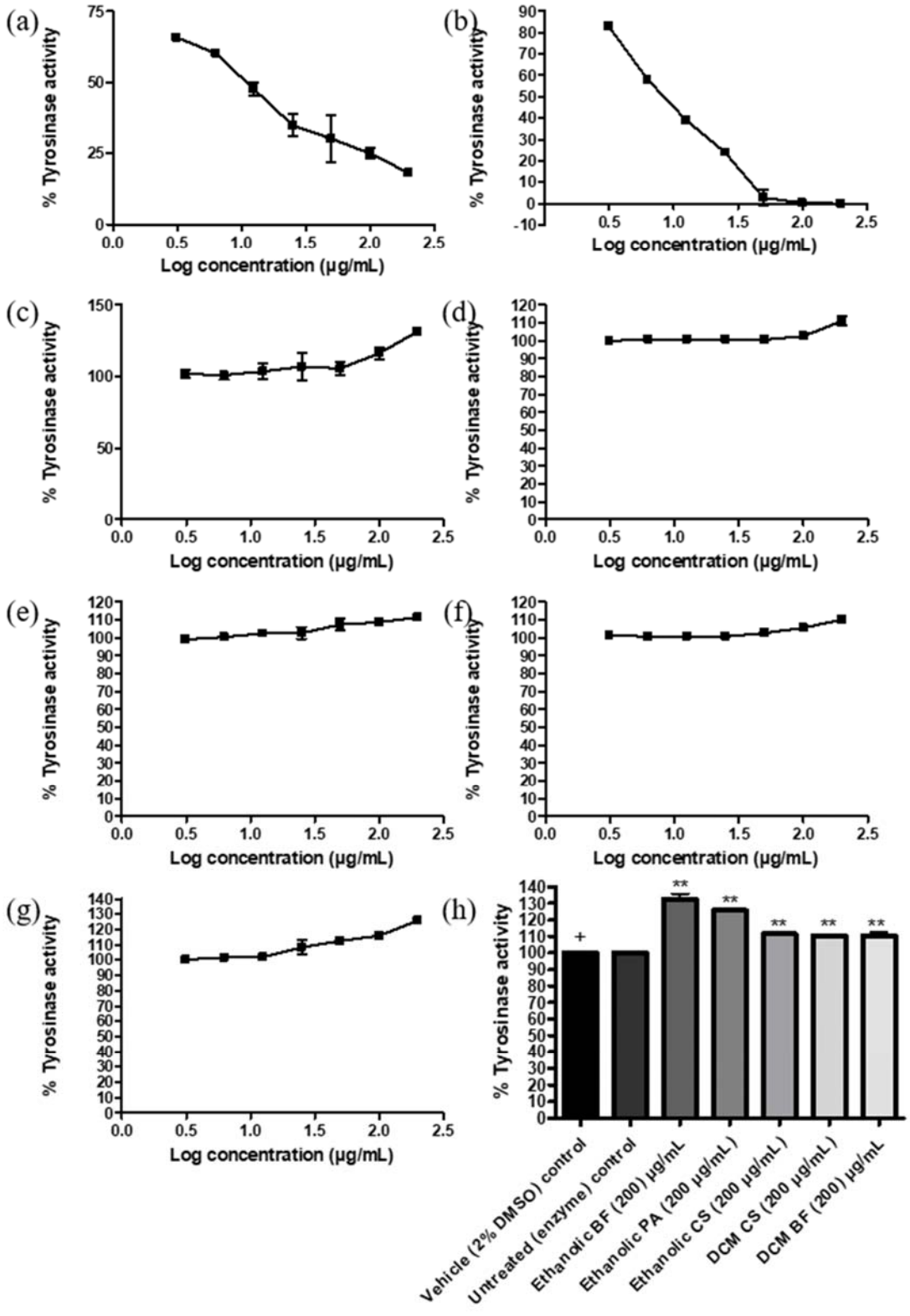

Fig. 1. The tyrosinase modulatory activity of (a) ethanolic *Pelargonium citronellum*, (b) dichloromethane (DCM) *Pelargonium citronellum* (c) ethanolic *Bulbine frutescens*, (d) dichloromethane *Bulbine frutescens* (e)

ethanolic *Cussonia spicata*, (f) dichloromethane *Cussonia spicata* and (g) ethanolic *Portulacaria afra* extracts at concentrations ranging from 3.12 - 200 µg/mL. Data is depicted as the log concentration (µg/mL) versus the tyrosinase activity as mean ± SD (n=3). (h) One-way ANOVA followed by Dunnett's multiple comparison test was used to determine statistical significance ($p < 0.01^{**}$) compared to the vehicle (2% DMSO) control (+).

The ethanolic, aqueous and DCM extracts of *B. obtusa, C. dimidiatus, C. orbiculata, H. aristata, H. forskaolii*, *P. graveolens* and *S. inerme* did not have an effect on the activity of the tyrosinase enzyme. The ethanolic and DCM *P. citronellum* extracts inhibited the tyrosinase enzyme, which was compared to kojic acid (IC_{50}: $7.49 \times 10^{-1} \pm 1.21 \times 10^{-2}$ µg/mL). The ethanolic *P. afra,* ethanolic and DCM *B. frutescens* and the ethanolic and DCM *C. spicata* showed a significant increase ($p < 0.01$) in tyrosinase activity when compared to the vehicle control (Fig. 1).

No previous reports on the effect of *Barleria obtusa*, *C. dimidiatus*, *C. spicata*, *H. aristata*, *H. forskaolii* and *P. citronellum* were found. *Cotyledon orbiculata* was observed to have low inhibitory activity against the tyrosinase enzyme where the 80% methanolic extracts prepared from the early somatic embryos, mature somatic embryos and germinated somatic embryos showed IC_{50} values of 0.76, 0.79 and 0.73 mg/mL, respectively (Zengin et al., 2023). Essential oils prepared from the different phenological stage of *P. graveolens* (vegetative state, begin flowering stage, full flowering stage) showed IC_{50} values of 187.29, 152.39 and 124.49 µg/mL against the tyrosinase enzyme (Al-Mijalli et al., 2022). An 80% methanolic extract prepared from the whole plant of *P. afra* showed >60% inhibition of the tyrosinase enzyme at 1 mg/mL (Tabassum et al., 2023). Momtaz et al (2008), reported that a methanolic and acetone extracts

prepared from the stem bark of *S. inerme* showed noteworthy inhibition of tyrosinase with IC_{50} values of 63 and 82 µg/mL, respectively.

The ethanolic *B. frutescens, C. spicata*, *P. afra* and *S. inerme* extracts were tested for their antiproliferative activity towards UCT-Mel-1 and HaCaT cells to determine their potential toxicity when used as a topical. Each of the above extracts showed IC_{50} values > 400 µg/mL, except for *C. spicata* which showed an IC_{50} value of 172.60 ± 5.73 and 201 ± 4.21 µg/mL against HaCat and UCT-Mel-1, respectively. The ethanolic *B. frutescens* and *P. afra* showed a decrease in UCT-Mel-1 cell viability by 3.48 ± 2.21 and 21.25 ± 10.90%, respectively at 200 µg/mL. These antiproliferative activity was compared to the positive control, actinomycin D, which showed an IC_{50} value of $0.026 \pm 1.2 \times 10^{-3}$ and $0.018 \pm 2.0 \times 10^{-3}$ µg/mL against HaCat and UCT-Mel-1, respectively.

The methanolic bark extract of *S. inerme* has been reported to show toxicity towards mouse melanocytes with an IC_{50} > 100 µg/mL (Momtaz et al., 2008). A methanolic leaf extract of *C. spicata* has been reported to be cytotoxic towards T-cell leukaemia (Jurkat) cancer cells with an IC_{50} value of 27.69 ± 1.09 µg/mL (De Villiers et al., 2010). In a previous report by Pather et al. (2011), the fresh leaf gel of *B. frutescens* showed less than 20% decrease in cell viability of HaCaT cells at 1000 µg/mL (IC_{50} > 1000 µg/mL). Khanyile et al. (2021) reported that a *P. afra* methanolic leaf extract had an IC_{50} value of 4010 µg/mL towards human hepatocellular carcinoma (HepG2) cells.

Only the ethanolic extract of *B. frutescens* was evaluated for its effect on melanin production as it increased tyrosinase activity and showed negligible antiproliferative activity against UCT-Mel-1 and HaCat cells (IC_{50} > 400 µg/mL). The potential of *B. frutescens* ethanolic extract to enhance melanin production was compared to α-MSH, which promotes the production of melanin. The extract (at 200 µg/mL) and α-MSH (at 100 µM) showed an increase in melanin

production by 8.55 ± 1.66 and 13.39 ± 1.44%, respectively (Fig. 2). The silver nitrate oxidises in the presence of melanin in the melanocytes resulting in a brownish-black colour. An increase in melanin macules was observed in the cells treated with the ethanolic *B. frutescens* and α-MSH when compared to the vehicle control cells ($p < 0.01$). This is the first report on the effect of *B. frutescens* on melanogenesis. A study by Abegaz et al. (2002) reported the isolation of four anthraquinones; 4′-*O*-demethylknipholone-4′-*O*-D-glucoside, knipholone, gaboroquinones A and B from the chloroform/methanol root extract of *B. frutescens*. Furthermore, Mukanganyama et al. (2011) isolated isofuranonaphthoquinone from the DCM/ methanol extract of *B. frutescens*. Additionally, Abdissa et al (2014) reported on the isolation of two new compounds from the 1:1 DCM: methanol root extract of *B. frutescens*, which included a xanthone, 8-hydroxy-6-methylxanthone-1-carboxylic acid and a phenylanthraquinone, 6'8-O-dimethylknipholone. However, in a previous study, a xanthone type compound, α-mangostin, showed a decrease in melanin production in B16F10 cells, inhibited the tyrosinase enzyme, and down-regulates the production of the tyrosinase enzyme, the tyrosinase relative protein (TRP)-2 and microphthalmia-associated transcription factor (MITF) (Zhou et al., 2021). Further bioassay guided isolation of *B. frutescens* is required to identify a bioactive compound as well as to determine its mode of action.

In a similar study, an ethanolic *Capparis spinosa* leaf extract has been reported to increase melanin content in B16 murine melanoma cells by 12 and 60% at a concentration of 0.005 and 0.05% (w/v), respectively. It further increased the tyrosinase level by 20-fold after 48 hrs after exposure to the extract at a concentration of 0.03% (w/v) (Matsuyama et al., 2009; Niu and Aisa, 2017).

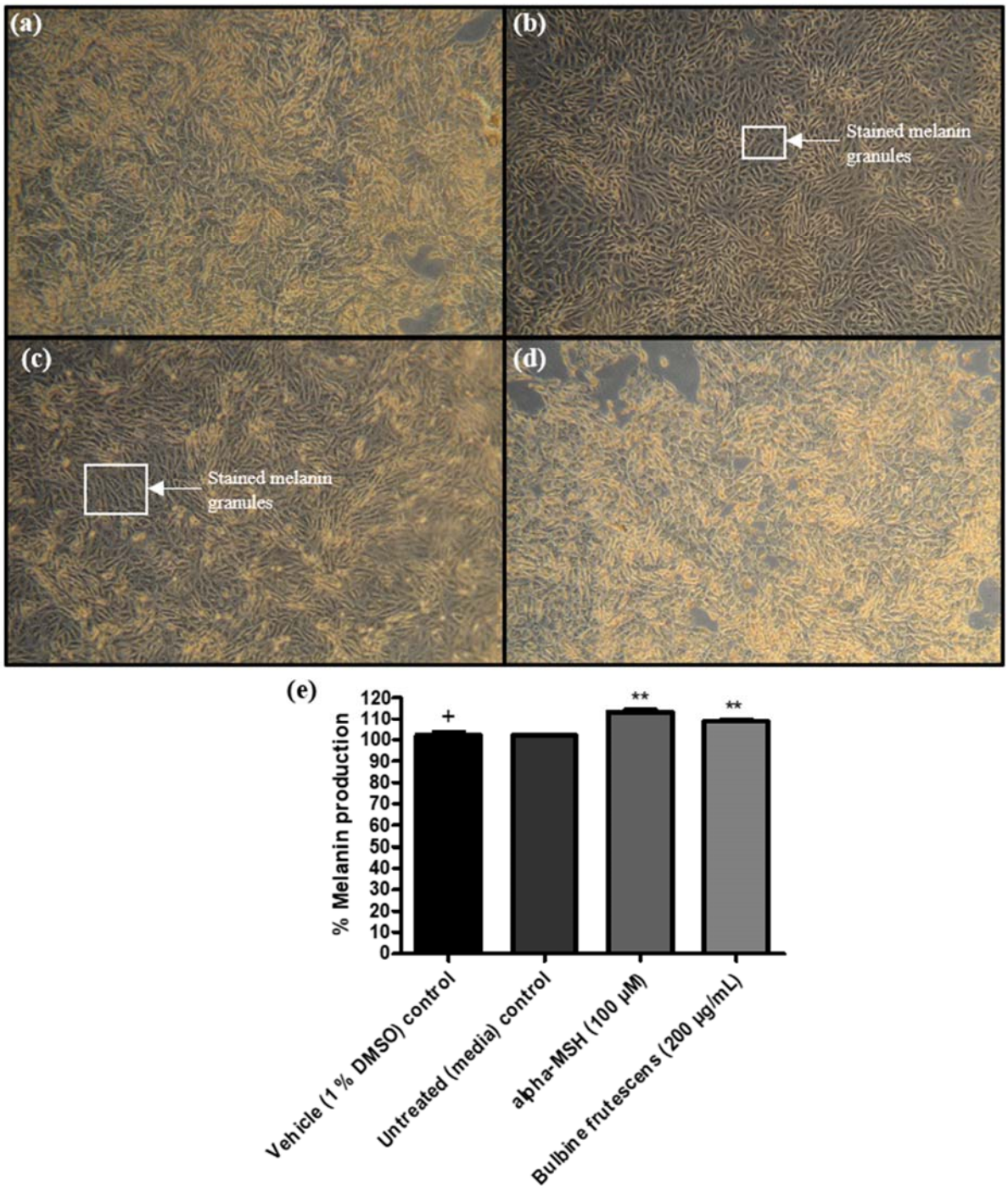

Fig.2. The Fontana-Masson staining of UCT-Mel-1 cells with (a) untreated cells; (b) α-melanocyte stimulation hormone at 100 µM; (c) Bulbine frutescens ethanolic extract at 200 µg/mL; (d) the DMSO (1%) vehicle control after 24 hrs of exposure (e) One-way ANOVA followed by Dunnett's multiple comparison test was used to determine statistical significance ($p < 0.01^{**}$) compared to the vehicle (1% DMSO) control (+).

4. Conclusion

The ethanolic extracts of *S. inerme* and *B. frutescens* have the potential to be used in the treatment for PMH, as an antibacterial and as a melanin increasing topical treatment, respectively. The *S. inerme* ethanolic extract showed noteworthy antibacterial activity against *C. acnes*, whereas the ethanolic *B. frutescens* extract displayed a significant increase in the activity of tyrosinase enzyme and melanin production. Additionally, both ethanolic extracts had low antiproliferative activity towards both UCT-Mel-1 and HaCat cells. To date, no reports on the activity of *B. frutescens* on melanin production have been published.

A further consideration to this study, would have been to conduct an antibacterial assay and quantify melanin production with a combination of *S. inerme* and *B. frutescens*, in order to target both *P. acne* and hypopigmentation and to determine whether a combination of the two extracts would show enhanced activity against PMH. In addition, the Fontana-Masson assay should be followed by an additional quantification assay, such as extracellular and intracellular melanin production in melanocytes, which may provide a more accurate determination of the melanin content. Bioassay guided isolation should also be considered to identify the bioactive compound present in *B. frutescens* and to determine its mode of action. Furthermore, the irritancy potential of the extracts, to determine their cosmetic safety as topical treatments and the *in vivo* hypopigmentation effects, should be evaluated.

Data Availability

The data that supports the findings of this study are available from the corresponding author upon reasonable request

Declaration of Competing Interest

The authors declare that there is no conflict of interest

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References

Abegaz, B.M., Bezabih, M., Msuta, T., Brun, R., Menche, D., Mühlbacher, J., Bringmann, G., 2002. Gaboroquinones A and B and 4'-O-Demethylknipholone-4'-O-β-d-glucopyranoside, phenylanthraquinones from the roots of *Bulbine frutescens*. J. Nat. Prod. 65, 1117–1121. https://doi.org/10.1021/NP0201218

Abdissa, N., Heydenreich, M., Midiwo, J.O., Ndakala, A., Majer, Z., Neumann, B., Stammler, H.G., Sewald, N., Yenesew, A., 2014. A xanthone and a phenylanthraquinone from the roots of *Bulbine frutescens*, and the revision of six seco-anthraquinones into xanthones. Phytochem. Lett., 9, 67-73. https://doi.org/10.1016/j.phytol.2014.04.004

Abubakar, A.R., Haque, M., 2020. Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. J. Pharm. Bioallied Sci. 12, 10. https://doi.org/10.4103/JPBS.JPBS_175_19

Adeosun, I.J., Baloyi, I.T., Cosa, S., 2023. Extracts of selected South African medicinal plants mitigate virulense factors in multifrug-resistant strains of *Klebsiella pneumoniae*. Evid. Based Complement. Alternat. Med. 2023, 3146588. https://doi.org/10.1155/2023/3146588

Afroz, J., Islam, S., Rahman, M., 2020. Determination of antimicrobial activity of tea (*Camellia sinensis*) and coffee (*Coffea arabica*) extracts on common human pathogenic bacteria. Acad. J. Med. Plants 8, 17–22. https://doi.org/10.1080/10942910701675928

Al-Mijalli, S.H., Mrabti, H.N., Assaggaf, H., Attar, A.A., Hamed, M., Baaboua, A.E., Omari, N.E., Menyiy, N.E., Hazzoumi, Z., Sheikh, R.A., Zengin, G., 2022. Chemical profiling and biological activities of *Pelargonium graveolens* essential oils at three different phenological stages. Plants 11(17), 2226. https://doi.org/10.3390/plants11172226

Aremu, A.O., Ndhlala, A.R., Fawole, O.A., Light, M.E., Finnie, J.F., van Staden, J., 2010. *In vitro* pharmacological evaluation and phenolic content of ten South African medicinal plants used as antihelminitics. S. Afr. J. Bot. 76(3), 558-566. https://doi.org/10.1016/j.sajb.2010.04.009

Basson, D.C., Teffo, T.K., Risenga, I.M., 2023. A phytochemical screening, antioxidant and antibacterial activity analysis in the leaves, stems and roots of *Portulacaria afra*. J. Herbmed. Pharmacol. 12(1), 109-117. https:// doi.org/10.34172/jhp.2023.10

Blom van Staden, A., De Canha, M., Nqephe, M., Rademan, S., Kumar, V., Lall, N., 2017. Potential medicinal plants for progressive macular hypomelanosis. S. Afr. J. Bot. 111, 346–357. https://doi.org/10.1016/J.SAJB.2017.04.007

Blom van Staden, A., Oosthuizen, C.B., Lall, N., 2021. The effect of *Aspalathus linearis* (Burm.f.) R.Dahlgren and its compounds on tyrosinase and melanogenesis. Sci. Reports 11, 7020. https://doi.org/10.1038/s41598-021-86410-z

Brenner, M., Hearing, V.J., 2008. The protective role of melanin against UV damage in human skin. Photochem. Photobiol. 84, 549. https://doi.org/10.1111/J.1751-1097.2007.00226.X

Chen, Y., Yi, S., Wang, Q., Xiong, H., Yuan, J., Zhnag, Y., Yang, L., Zhong, G., Li, X., Zhu, T., 2023. Lutein attenuates *Propionibacterium acnes*-induced inflammation by inhibiting

pyroptosis of human keratinocyte cells via TLR4/NLRP3/caspase-1 pathway. Int. Immunopharmacol. 117, 109937. https://doi.org/ 10.1016/j.intimp.2023.109937

Coopoosamy, R.M., 2011. Traditional information and antibacterial activity of four *Bulbine* species (Wolf). Afr. J. Biotechnol.10 (2), 220-224. https://doi.org/10.5897/AJB10.1435

Cunha, J., 2022. Side Effects of brevoxyl gel (benzoyl peroxide gel), warnings, uses [WWW Document]. URL https://www.rxlist.com/brevoxyl-gel-side-effects-drug-center.htm (accessed 10.8.22).

De Villiers, B.J., Van Vuuren, S.F., Van Zyl, R.L., Van Wyk, B.E., 2010. Antimicrobial and antimalarial activity of *Cussonia* species (Araliaceae). J. Ethnopharmacol. 129, 189–196. https://doi.org/10.1016/J.JEP.2010.02.014

El-Megei, H.A., El-Houni, N.O., Ezeddin, S.S., 2022. Therapeutic response of patients with progressive macular hypomelanosis to narrowband ultraviolet B phototherapy at dermatology department, Tripoli Central Hospital, Tripoli, Libya (2008–2018). Libyan J. Med. Sci. 5, 30. https://doi.org/10.4103/LJMS.LJMS_91_20

Gibango, L., Oosthuizen, C.B., Lall, N., 2020. Potential implications of the use of *Rapanea melanophloeos* (L.) Mez against mycobacteria. S. Afr. J. Bot. 132, 388–394. https://doi.org/10.1016/J.SAJB.2020.06.003

Im, M., Kim, S.Y., Sohn, K.C., Choi, D.K., Lee, Y., Seo, Y.J., Kim, C.D., Hwang, Y.L., Zouboulis, C., Lee, J.H., 2012. Epigallocatechin-3-gallate suppresses IGF-I induced lipogenesis and cytokine expression in SZ95 sebocytes. J. Invest. Dermatol. 132(12), 2700-2708. https://doi.org/10.1038/jid.2012.202

Khanyile, A., Sidney Maliehe, T., Siyabonga Shandu, J., Khan, R., 2021. *In vitro* antibacterial, antioxidant, anti-quorum sensing and cytotoxic properties of *Portulacaria afra* leave extract. Biosci. Res. 18, 455–463.

Kim, J.-K., Kim, N., Lim, Y.-H., 2010. Evaluation of the antibacterial activity of rhapontigenin produced from rhapontin by biotransformation against *Propionibacterium acnes*. J. Microbiol. Biotechnol. 20, 82–87.PMID: 20134237

Kim, M.B., Kim, G.W., Cho, H.H., Park, H.J., Kim, H.S., Kim, S.H., Kim, B.S., Ko, H.C., 2012. Narrowband UVB treatment of progressive macular hypomelanosis. J. Am. Acad. Dermatol. 66, 598–605. https://doi.org/10.1016/J.JAAD.2011.04.010

Kim, S.S., Baik, J.S., Oh, T.H., Yoon, W.J., Lee, N.H., Hyun, C.G., 2008. Biological activities of Korean *Citrus obovoides* and *Citrus natsudaidai* essential oils against acne-inducing bacteria. Biosci. Biotechnol. Biochem. 72, 2507–2513. https://doi.org/10.1271/BBB.70388

Kloepper, J.E., Sugawara, K., Al-Nuaimi, Y., Gáspár, E., van Beek, N., Paus, R., 2010. Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. Exp. Dermatol. 19, 305–312. https://doi.org/10.1111/J.1600-0625.2009.00939.X

Kuete, V., Efferth, T., 2015. African flora has the potential to fight multidrug resistance of cancer. Biomed Res. Int. 2015, 1–24. https://doi.org/10.1155/2015/914813

Kuete, V., Efferth, T., 2010. Cameroonian medicinal plants: pharmacology and derived natural products. Front. Pharmacol. 1, 123. https://doi.org/10.3389/FPHAR.2010.00123

Lall, N., Chrysargyris, A., Lambrechts, I., Fibrich, B., Blom, A., Staden, V., Twilley, D., Nuno De Canha, M., Basson Oosthuizen, C., Bodiba, D., Tzortzakis, N., 2019. *Sideritis perfoliata* (Subsp. Perfoliata) nutritive value and its potential medicinal properties. Antioxidants. 8, 521–539. https://doi.org/10.3390/antiox8110521

Lall, N., Henley-Smith, C.J., De Canha, M.N., Oosthuizen, C.B., Berrington, D., 2013. Viability reagent, PrestoBlue, in comparison with other available reagents, utilized in

cytotoxicity and antimicrobial assays. Int. J. Microbiol. 2013. https://doi.org/10.1155/2013/420601

Lalli, J.Y., 2005. *In vitro* pharmacological properties and composition of leaf essential oils and extracts of selected indigenous *Pelargonium* (Geraniaceae) species. Masters Dissertation. University of the Witwatersrand, Johannesburg.

Lee, A-Y., 2021. Skin pigmentation abdormalities and their possible relationship with skin aging. Int. J. Mol. Sci. 22 (7), 3727 - 3746. https://doi: 10.3390/ijms22073727

Leonard, N., Krueger, S., Rashighi, M., 2020. Successful treatment of progressive macular hypomelanosis. Dermatol. Rep. 12, 56–57. https://doi.org/10.4081/DR.2020.8509

Maddodi, N., Jayanthy, A., Setaluri, V., 2012. Shining light on skin pigmentation: The darker and the brighter side of effects of UV radiation. Photochem. Photobiol. 88 (5), 1075-1082. htpps://doi: 10.1111/j.1751-1097.2012.01138.x

Madu, P.N., Syder, N., Elbuluk, N., 2022. Postinflammatory hypopigmentation: a comprehensive review of treatments. J. Dermatolog. Treat. 33(2), 704–708. https://doi.org/10.1080/09546634.2020.1793892

Matsuyama, K., Villareal, M.O., El Omri, A., Han, J., Kchouk, M.E., Isoda, H., 2009. Effect of Tunisian *Capparis spinosa* L. extract on melanogenesis in B16 murine melanoma cells. J. Nat. Med. 63, 468–472. https://doi.org/10.1007/S11418-009-0355-3

McDowell, A., McLaughlin, J., Layton, A.M., 2021. Is *Cutibacterium* (previously *Propionibacterium*) acnes a potential pathogenic factor in the aetiology of the skin disease progressive macular hypomelanosis? J. Eur. Acad. Dermatol. Venereol. 35, 338–344. https://doi.org/10.1111/JDV.16789

MedlinePlus, 2018. Clindamycin: MedlinePlus Drug Information [WWW Document]. URL https://medlineplus.gov/druginfo/meds/a682399.html (accessed 10.8.22).

M'hamdi, Z., Bouymajane, A., Riffi, O., Filali, F.R., Ettarchouch, M., ELhourri, M., Amechrouq, A., 2024. Chemical composition and antibacterial activity of essential oil of *Pelargonium graveolens* and its fractions. Arab. J. Chem. 17 (1), 105375. https://doi.org/10.1016/j.arabjc.2023.105375

Momtaz, S., Mapunya, B.M., Houghton, P.J., Edgerly, C., Hussein, A., Naidoo, S., Lall, N., 2008. Tyrosinase inhibition by extracts and constituents of *Sideroxylon inerme* L. stem bark, used in South Africa for skin lightening. J. Ethnopharmacol. 119, 507–512. https://doi.org/10.1016/J.JEP.2008.06.006

Mothana, R.A.A., Kriegisch, S., Harms, M., Wende, K., Lindequist, U., 2011. Assessment of selected Yemeni medicinal plants for their *in vitro* antimicrobial, anticancer, and antioxidant activities. Pharm. Biol. 49, 200–210. https://doi.org/10.3109/13880209.2010.512295

Mukanganyama, S., Bezabih, M., Robert, M., Ngadjui, B.T., Kapche, G.F.W., Ngandeu, F., Abegaz, B., 2011. The evaluation of novel natural products as inhibitors of human glutathione transferase P1-1. J. Enzyme Inhib. Med. Chem. 26, 460–467. https://doi.org/10.3109/14756366.2010.526769

Mzimba, N.F., Moteetee, A., van Vuuren, S., 2023. Southern African plants used as soap substitutes; phytochemical, antimicrobial, toxicity and formulation potential. S. Afr. J. Bot. 163, 673-683. https://doi.org/10.1016/j.sajb.2023.11.002

NHS, 2022. Side effects of isotretinoin capsules - NHS [WWW Document]. URL https://www.nhs.uk/medicines/isotretinoin-capsules/side-effects-of-isotretinoin-capsules/ (accessed 10.8.22).

Niu, C., Aisa, H.A., 2017. Upregulation of melanogenesis and tyrosinase activity: potential agents for vitiligo. Mol. A J. Synth. Chem. Nat. Prod. Chem. 22.

https://doi.org/10.3390/MOLECULES22081303

Pather, N., Viljoen, A.M., Kramer, B., 2011. A biochemical comparison of the *in vivo* effects of *Bulbine frutescens* and *Bulbine natalensis* on cutaneous wound healing. J. Ethnopharmacol. 133, 364–370. https://doi.org/10.1016/J.JEP.2010.10.007

Pérez-Sánchez, A., Barrajón-Catalán, E., Herranz-López, M., Castillo, J., Micol, V., 2016. Lemon balm extract (*Melissa officinalis*, L.) promotes melanogenesis and prevents UVB-induced oxidative stress and DNA damage in a skin cell model. J. Dermatol. Sci. 84, 169–177. https://doi.org/10.1016/J.JDERMSCI.2016.08.004

Petersen, R.L.W., Scholz, C.F.P., Jensen, A., Brüggemann, H., Lomholt, H.B., 2017. *Propionibacterium acnes* phylogenetic type III is associated with progressive macular hypomelanosis. Eur. J. Microbiol. Immunol. (Bp). 7, 37–45. https://doi.org/10.1556/1886.2016.00040

Pillai, S.., Moellering, R.., Eliopoulos, G.., 2005. Antimicrobial combinations, in: Lorian, V. (Ed.), antibiotics in laboratory medicine. The lippincott Williams & Wilkins Co, Philadelphia, 365–440.

Relyveld, G.N., Kingswijk, M.M., Reitsma, J.B., Menke, H.E., Bos, J.D., Westerhof, W., 2006. Benzoyl peroxide/clindamycin/UVA is more effective than fluticasone/UVA in progressive macular hypomelanosis: a randomized study. J. Am. Acad. Dermatol. 55, 836–843. https://doi.org/10.1016/J.JAAD.2006.03.020

Saleem, M.D., Oussedik, E., Picardo, M., Schoch, J.J., 2019. Acquired disorders with hypopigmentation: A clinical approach to diagnosis and treatment. J. Am. Acad. Dermatol. 80, 1233-1250.e10. https://doi.org/10.1016/J.JAAD.2018.07.070

Sharma, R., Lall, N., 2014. Antibacterial, antioxidant activities and cytotoxicity of plants against *Propionibacterium acnes*. S. Afr. J. Sci. 110, 01–08.

https://doi.org/10.1590/SAJS.2014/20130293

Shelembe, B.., 2014. Phytochemical and elemental studies of two indigenous medicinal plants of South Africa, *Bridelia micrantha* and *Sideroxylon inerme*. Masters Dissertation. University of KwaZulu-Natal, Durban.

Shuster Ben-Yosef, V., Sendovski, M., Fishman, A., 2010. Directed evolution of tyrosinase for enhanced monophenolase/diphenolase activity ratio. Enzyme Microb. Technol. 47, 372–376. https://doi.org/10.1016/J.ENZMICTEC.2010.08.008

Smith, E.C.J., Wareham, N., Zloh, M., Gibbons, S., 2008. 2β-Acetoxyferruginol—A new antibacterial abietane diterpene from the bark of *Prumnopitys andina*. Phytochem. Lett. 1, 49–53. https://doi.org/10.1016/J.PHYTOL.2007.12.006

Sun, L., Yu, Q., Peng, F., Sun, C., Wang, D., Pu, L., Xiong, F., Tian, Y, Peng, C., Zhou, Q. 2024. The antibacterial activity of berberine against *Cutibacterium acnes*: its therapeutic potential in inflammatory acne. Front. Microbiol. 14, 12766383, https:// doi/ 10.3389/fmicb.2023.1276383

Tabassum, S., Ahmad, S., Ali, B., Usman, F., Jabeen, Q., Sajid-ur-Rehman, M., Ahmed, M., Zubair, H.M., Alkazmi, L., Batiha, G.E.S. and Basit, A., 2023. Chemical profiling and evaluation of toxicological, antioxidant, anti-inflammatory, anti-nociceptive and tyrosinase inhibitory potential of *Portulacaria afra* using *in-vitro*, *in-vivo* and *in-silico* studies. Arab. J. Chem. 16(6), 104784. https://doi.org/10.1016/j.arabjc.2023.104784

Thng, S., Long, V., Chuah, S., Tan, V., 2016. Efficacy and relapse rates of different treatment modalities for progressive macular hypomelanosis. Indian J. Dermatol. Venereol. Leprol. 82, 673–676. https://doi.org/10.4103/0378-6323.182797

Waibel, J.., Rudnick, A., Arheart, K.., Nagrani, N., Gonzalez, A., Gianatasio, C., 2019. Re-pigmentation of hypopigmentation: fractional lasers vs laser-assisted delivery of

bimatoprost vs epidermal melanocyte harvesting system. J. Drugs Dermatology. 18, 1090–1096.

Westerhof, W., Relyveld, G.N., Kingswijk, M.M., De Man, P., Menke, H.E., 2004. *Propionibacterium acnes* and the pathogenesis of progressive macular hypomelanosis. Arch. Dermatol. 140, 210–214. https://doi.org/10.1001/ARCHDERM.140.2.210

Williams, H.C., Dellavalle, R.P., Garner, S., 2012. Acne vulgaris. Lancet (London, England) 379, 361–372. https://doi.org/10.1016/S0140-6736(11)60321-8

Xu, P., Su, S., Tan, C., Lai, R.S., Min, Z.S., 2017. Effects of aqueous extracts of *Ecliptae herba*, *Polygoni multiflori radix praeparata* and *Rehmanniae radix praeparata* on melanogenesis and the migration of human melanocytes. J. Ethnopharmacol. 195, 89–95. https://doi.org/10.1016/J.JEP.2016.11.045

Yardman-Frank, J.M., Fisher, D.E., 2020. Skin pigmentation and its control: from ultraviolet radiation to stem cells. Exp. Dermatoly. 30(4), 560-571. https://doi.org/10.1111/exd.14260

Yoon, J.Y., Kwon, H.H., Min, S.U., Thiboutot, D.M., Suh, D.H., 2013. Epigallocatechin-3-gallate improves acne in humans by modulating intracellular molecular targets and inhibiting *P. acnes*. J. Invest. Dermatol. 133, 429–440. https://doi.org/10.1038/JID.2012.292

Zengin, G., Cziáky, Z., Jekõ, J., Kang, K.W., Lorenzo, J.M., Sivanesan, I., 2023. Phytochemical composition and biological activities of extracts from early, mature, and germinated somatic embryos od *Cotyledon orbiculata* L. Plants (Basel). 12(5), 1065. https://doi.org/10.3390/plants12051065

Zhang, L., Duan, C.J., Binkley, C., Li, G., Uhler, M.D., Logsdon, C.D., Simeone, D.M., 2004. A transforming growth factor beta-induced Smad3/Smad4 complex directly activates

protein kinase A. Mol. Cell. Biol. 24, 2169–2180. https://doi.org/10.1128/MCB.24.5.2169-2180.2004

Zhou, J., Shang, J., Ping, F., Zhao, G., 2012. Alcohol extract from *Vernonia anthelmintica* (L.) willd seed enhances melanin synthesis through activation of the p38 MAPK signaling pathway in B16F10 cells and primary melanocytes. J. Ethnopharmacol. 143, 639–647. https://doi.org/10.1016/J.JEP.2012.07.030

Zhou, S., Yotsumoto, H., Tian, Y., Sakamoto, K. 2021. α-Mangostin suppressed melanogenesis in B16F10 murine melanoma cells through GSK3β and ERK signaling pathway. Biochem. Biophys. Rep. 26, 100949. https:// doi.org/10.1016/j.bbrep.2021.100949

Supplementary Table 1. Concentrations (in µg/mL) tested for each combination of *Sideroxylon inerme* (S): tetracycline (T) (9:1-1:9) based on starting concentration of 500µg/mL for *S. inerme* and 50µg/mL for tetracycline, followed by serial dilutions.

	9:1		8:2		7:3		6:4		5:5		4:6		3:7		2:8		1:9	
Dilution #	**S**	**T**	**S**	**T**	**S**	**T**	**S**	**T**	**S**	**T**	**S**	**T**	**S**	**T**	**S**	**T**	**S**	**T**
	Concentration (in µg/mL)																	
1	450.00	5.00	400.00	10.00	350.00	15.00	300.00	20.00	250.00	25.00	200.00	30.00	150.00	35.00	100.00	40.00	50.00	45.00
2	225.00	2.50	200.00	5.00	175.00	7.50	150.00	10.00	125.00	12.50	100.00	15.00	75.00	17.50	50.00	20.00	25.00	22.50
3	112.50	1.25	100.00	2.50	87.50	3.75	75.00	5.00	62.50	6.25	50.00	7.50	37.50	8.75	25.00	10.00	12.50	11.25
4	56.25	0.63	50.00	1.25	43.75	1.88	37.50	2.50	31.25	3.13	25.00	3.75	18.75	4.38	12.50	5.00	6.25	5.63
5	28.13	0.31	25.00	0.63	21.88	0.94	18.75	1.25	15.63	1.56	12.50	1.88	9.38	2.19	6.25	2.50	3.13	2.81
6	14.06	0.16	12.50	0.31	10.94	0.47	9.38	0.63	7.81	0.78	6.25	0.94	4.69	1.09	3.13	1.25	1.56	1.41
7	7.03	0.08	6.25	0.16	5.47	0.23	4.69	0.31	3.91	0.39	3.13	0.47	2.34	0.55	1.56	0.63	0.78	0.70
8	3.52	0.04	3.13	0.08	2.73	0.12	2.34	0.16	1.95	0.20	1.56	0.23	1.17	0.27	0.78	0.31	0.39	0.35