Potential medicinal plants for progressive macular hypomelanosis

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Highlights

- First time plant extracts were investigated for progressive macular hypomelanosis.
- *Hypericum revolutum* and *Withania somnifera* exhibited *P. acnes* inhibitory activity.
- Combinational studies resulted in reduced minimum inhibitory concentrations.
- *Terminalia prunoides* exhibited noteworthy stimulation of melanin production.
- Anti-tyrosinase results were supported by molecular docking analysis.

Abstract

Progressive macular hypomelanosis (PMH) is a hypopigmentation disorder caused by the bacterium identified as *Propionibacterium acnes.* The current treatments for PMH are antibiotics together with ultra violet radiation; however, UV radiation is not a recommended method to increase melanin production. Currently, there are no known plants used traditionally or medicinally for PMH. The objective of this study was to find plants that could stimulate tyrosinase activity induce melanin production and inhibit *Propionibacterium acnes'* growth.

Seventeen ethanol plant extracts, used traditionally in Africa for skin diseases, were screened for their antibacterial activity against *P. acnes*, their effect on monophenolase activity of

tyrosinase and their cytotoxicity and stimulation of melanin production on mouse melanocytes (B16-F10).

Hypericum revolutum Vahl subsp. *revolutum* (Hypericaceae) and *Withania somnifera* L. Dunal (Solanaceae) (twigs and leaves), combined with the known drug tetracycline, exhibited significant antibacterial activity against *P. acnes,* with the minimum inhibitory concentration ranging from 5.47 µg/ml to 14.06 µg/ml. The combination of a known drug with other antibacterial compounds not only decreases the concentration needed to inhibit bacterial growth, but also decreases the chances of bacterial resistance. *Withania somnifera* was the only plant extracts that resulted in an increase in the monophenolase activity of tyrosinase. Four compounds typically present in plant extracts, namely coumarin, quercetin, withaferin and winthanone, were docked into the active site of tyrosinase enzyme to determine the interaction with active site residues. Mouse melanocytes (B16F10) treated with *Hypericum revolutum*, *Withania somnifera* (leaves) and *Terminalia prunoides* showed an increase in total melanin content as compared to untreated cells at 12 µg/ml, 12 µg/ml and 150 µg/ml respectively.

Considering both the antibacterial activity and the stimulatory effect of the treatment on melanin production, *Hypericum revolutum* and *Withania somnifera* (leaves) could be considered as potential plants for further studies for PMH.

Keywords: Antibacterial, Progressive macular hypomelanosis, cytotoxicity, phytochemical screening, synergy, melanin production

1. Introduction

Progressive macular hypomelanosis (PMH) was described by Halder as a disease identified by its symmetrically distributed hypopigmented spots mostly found on the trunk and back (Halder and Rodney, 2013). Consequently, these white patches on the skin occur due to a decreased level of melanin in the skin, known as melanogenesis (Olsson and Juhlin, 2002, Middelkamp- Hup et al., 2007,). Westerhof *et al.* (2004) hypothesised that the decreased melanin production in progressive macular hypomelanosis is caused by an inhibitory factor secreted by *Propionibacterium acnes* (Westerhof et al., 2004). The assumption that *P. acnes* is the pathogenic factor in PMH was also supported by a study conducted by Relyveld (2006), which showed that when both antibacterial and anti-inflammatory treatments were combined with ultra violet irradiation, the antibacterial treatment was significantly superior for the decreasing of hypopigmented lesions in PMH patients (Relyveld et al., 2006, Relyveld et al., 2007). Therefore, eliminating *P. acnes* with topical antibacterial therapy, such as in acne, could improve re-pigmentation in patients with PMH" (Relyveld et al., 2006).

Plants are known for their antibacterial activity against several Gram positive bacteria, or more specifically against *P. acnes,* therefore, plant extracts could be a possible alternative treatment to antibiotics (Kubo et al., 1974, Hopp et al., 1975, Iwasa et al., 2001, Takahashi et al., 2004, Chomnawang et al., 2005, Viyoch et al., 2006, Kumar et al., 2007, Lim et al., 2007, Roopashree et al., 2008, Pothitirat et al., 2009, Yoon et al., 2009, Vijayalakshmi et al., 2011). Some pharmaceutical drugs such as isotretinoin and benzoyl peroxide, used to treat acne, have extensive side-effects. Furthermore, *P. acnes* has acquired resistance mediated by bacterial enzymes towards certain pharmaceuticals and antibiotics, such as erythromycin and tetracycline, resulting in the inactivation of the antibiotics (Mclane, 2001, Ross et al., 2001, Westerhof et al., 2004). Antibiotic efficiency towards bacterial resistance may be enhanced through structural changes to the aminoglycosides (Zhang et al., 2009). Zhang *et al.* (2009),

investigated the use of antibiotics in combination with other clinically used antibiotics and found that using the combination of antibiotics is a common practice in the treatment of bacterial infections (Neu et al., 1996, Zhang et al., 2009). Combination of antibiotics with other active compounds or plant extracts, may lead to a potential enhancement of the overall efficacy of the treatment, thereby, reducing the dose of antibiotics, or reducing the likelihood of bacteria developing drug resistance (Zhang et al., 2009). The antibacterial activity of plant extracts, either alone or in combination with the known drug, tetracycline, together with the plant extract's ability to stimulate melanogenesis, could possibly lead to a treatment that not only inhibits the bacterial growth, but also accelerates the production of melanin, decreasing the time for the lesions to fade (Jung et al., 2001, Nagata et al., 2004, Takeyama et al., 2004, Lee et al., 2005, Hata et al., 2006, Andersen, 2007, Moleephan, 2012).

The selection of plants for the present study was based on the plants' traditional uses. *Equisetum ramosissimum* Desf. subsp. *ramosissimum* (Equisetaceae) is traditionally used for its antibacterial potential and against skin infections (Kelmanson et al., 2000, Sathiyaraj et al., 2015). The *Euclea* genus, *Combretum molle* R. Br. Ex G. Don (Combretaceae) and *Momordica balsamina* L. (Curcurbitaceae), have been traditionally used for skin diseases (Fyhrquist et al., 2002, Geidam et al., 2004, Miller and Morris, 2004). The dried herb form of *Tephrosia purpurea* L. Pers. subsp. leptostachya (DC.) Brummitt var. *pubescens* Baker (Fabaceae) was reported for its effectiveness in the treatment of boils and pimples, which are caused by Gram positive bacteria (Lodhi et al., 2006). *Terminalia prunioides* M.A. Lawson (Combretaceae) have many traditional applications including treating bacterial infections (Gram-positive organisms), bilharzia, skin diseases etc. (Oliver-Bever, 1986, Neuwinger, 1996,). *Crotalaria sp.* and *Leucas sp.* are traditionally used for skin diseases by the Indians by means of using the powder of the leaves and root bark to make a paste, which is then applied to treat skin diseases (Ayyanar and Ignacimuthu, 2005, Chouhan and Singh, 2011).

Other plant extracts chosen based on their traditional uses for skin diseases and wound healing were *Ficus glumosa* Delile (Moraceae), *Ficus lutea* Vahl (Moraceae), *Ficus sur* Forssk. (Moraceae), *Pelargonium reniforme* Curtis (Geraniaceae), *Pelargonium sidoides* DC. (Geraniaceae), and *Rapanea melanophloeos* L. Mez (Myrsinaceae) (Hutchings, 1996, La Cock and Briers, 1992, Marwah et al., 2007, Orwa et al., 2009, Tiedtke and Marks, 2002). *Ficus religiosa* L. (Moraceae), *Hypericum revolutum* Vahl subsp. *revolutum* (Hypericaceae) and *Withania somnifera* L. Dunal (Solanaceae) have been reported for their use in leukoderma, another hypopigmentary disease (Capitanio et al., 1989, Prakash et al., 2002, Chandrasekar et al., 2010, Singh et al., 2010, Zofou et al., 2011, Basha et al., 2014,).

The only current treatment available for progressive macular hypomelanosis is the combination of antibiotics with UV radiation, unfortunately there were cases observed where the patients' white macules reoccurred after some time (Olsson and Juhlin, 2002, Relyveld et al., 2006, Perman et al., 2008, De Morais Cavalcanti et al., 2011). Additively, UV radiation used together with the antibiotics, provided some risks as it increases the possibility of skin cancer and causes premature aging of the skin, inflammation due to damaged keratinocytes, DNA breakage and the depletion of antioxidants in the cell or the production of reactive oxygen species (Wood et al., 1996, Gilchrest and Eller, 1999, Rhie et al., 2001, Pillai et al., 2005). For that reason, the objective of the current study was to identify plant extracts that could prevent or inhibit *P. acnes'*, and increase the monophenolase activity of tyrosinase and induce melanin production in cultured mouse melanocytes (B16F10). Four compounds commonly found in most of the plant extracts that has previously shown to induce melanin production, investigated in the present study, coumarin, quercetin, withaferin and winthanone, were docked into the active site of tyrosinase enzyme to determine the interaction. The lead plant extracts were also evaluated for their antibacterial activity when combined with Tetracycline (a known antibiotic), to optimise the concentrations of the plant

and drug necessary to have an optimal effect and to counteract antibiotic resistance. Many phytochemicals have been reported for their antibacterial activity and their inducing effect on melanin production. Therefore, the present study identified the major phytochemical groups present in the selected plants.

2. Methods and Materials

Tetracycline, theophylline, Pesto Blue and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and vitamin C were obtained from Sigma-Aldrich (Johannesburg, South Africa). Nutrient broth and cow brain and heart agar were purchased from Merck SA (Pty) Ltd (Johannesburg, South Africa). *Propionibacterium acnes* (ATCC 6919) was purchased from Anatech Company South Africa (Johannesburg, South Africa). The cell culture reagents, equipment and the B16-F10 mouse melanocyte cell line were purchased from Highveld Biological (Johannesburg, South Africa), Labotech (Johannesburg, South Africa) and The Scientific Group (Johannesburg, South Africa).

2.1 Preparation of plant extracts

The aerial parts (leaves and twigs) of eight plants were collected, from Lillydale village in the Mpumalanga province of South Africa and ten plants were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria before noon. The leaves and twigs were collected as they are mostly used in the traditional preparations and due to their sustainability; except for *Withania somnifera* where the fruit of the plant was also collected. The fruit (seed) of *Withania somnifera* is used in traditional preparations for vitiligo and to improve the texture and colour of human skin (Akber et al., 2011). A voucher herbarium specimen number of each plant given by H.G.W.J. Schweickerdt Herbarium is depicted in Table 1. Alcoholic extracts of each plant material were prepared by soaking the plant material in ethanol (1:1 w/v) for 48 hours, filtered, dried and stored at 4^0C until further usage. Ethanol

was chosen as the extract solvent, due to its acceptability by the pharmaceutical industries. The extract preparation of the plant extracts was done as described by Sharma *et al.* (2014).

Table 1: Phytochemical groups identified in the selected ethanol plant extracts

Plant extract	Tannins	Alkaloids	Saponins	Cardiac glycosides	Terpenes	Flavonoids	Phenolics
***Combretum molle* R. Br. ex G. Don**	+	+	+	+	+	+	+
***Crotlaria lanceolate* E. Mey. subsp. lanceolata**	-	-	-	+	-	-	-
***Euclea crispa* (Thunb.) Gürke**	+	+	+	+	-	+	+
***Equisetum ramosissimum* Desf. subsp. ramosissimum**	-	-	-	+	-	-	+
***Ficus glumosa* Delile**	+	+	+	+	-	-	-
***Ficus lutea* Vahl**	+	+	++	+	-	-	+
***Ficus religiosa* L.**	-	+	+	-	-	-	-
***Ficus sur* Forssk.**	+	+	+	+	+	+	+
***Hypericum revolutum* Vahl subsp. revolutum**	-	-	+	-	-	-	-
***Leucas martinicensis* (Jacq.)**	-	-	-	-	-	-	-
***Momordica balsamina* L.**	-	-	+	-	-	-	-
***Pelargonium reniforme* Curtis**	+	+	+++	+	-	-	+
***Pelargonium sidoides* DC.**	-	+	+++	+	-	-	+
***Rapanea melanophloeos* (L.) Mez**	+	+	+++	+	+	-	+
***Terminalia prunoides* M.A. Lawson**	+	-	-	+	-	+	+

***Withania somnifera* (leaves) (L.) Dunal**	-	+	+	+	+	-	+
***Withania somnifera* (fruit) (L.) Dunal**	-	+	+	+	+	-	-

+, Present; ++, Present in average quantities; +++, Present in high quantities; -, Absent

2.2 Phytochemical screening

The major phytochemical groups present in the eighteen plant extracts, at a concentration of 150000.00 µ/ml, were determined as specified by Mushtaq et al. (2014). The presence of tannins was determined through the addition of ferric chloride to the extracts (dissolved in water) and the observation of a brown precipitate. The formation of a yellow precipitate indicated the presence of alkaloids after HCl and Dragendroff reagent were added to the ethanol extracts re-dissolved in methanol (Mushtaq et al., 2014). Ethanol extracts were re-dissolved in water and formed a froth in the presence of saponins after the extracts were shaken. Cardiac glycosides were determined through the addition of glacial acetic acid, ferric chloride and concentrated H_2SO_4. The formation of a brown ring at the interface indicated the presence of cardiac glycosides. Chloroform together with glacial acetic acid and concentrated H_2SO_4 were added to the extract dissolved in water, to determine the presence of terpenes, which were indicated by the reddish brown interface. The appearance of a magenta red colour, after the addition of concentrated HCl and magnesium turnings, indicated the presence of flavonoids. Phenolics were determined through the addition of ferric chloride and identified by the colour change to blue or green (Mushtaq et al., 2014).

2.3 Antibacterial bioassay

The ethanol plant extracts were tested against *P. acnes* (ATCC 11827, ATCC 6919) by determining the minimum inhibitory concentration (MIC) values obtained through a broth micro dilution method (Mapunya et al., 2011). Bacterial cultures were grown on cow's brain and heart agar and incubated at 37℃ for 120 hours, thereafter the cultures were sub-cultured

on cow's brain heart agar and incubated at 37℃ for 72 hours, under anaerobic conditions (anaerobic jar containing an anaerocult). The sub-cultured bacteria were suspended in nutrient broth after incubation and the bacteria concentration was adjusted to 0.50 McFarland standard turbidity with an absorbance of 0.13 (1.5 x 108 CFU/ml) at 600 nm. A stock solution consisting of the plant extract (2000.00 µg in 100 µl dimethyl sulphoxide (DMSO) and 900 µl ddH2O) was prepared. Hundred millilitres of the samples and the positive control tetracycline (200.00 µg/ml) were added to the first wells of a sterile 96-well plate, already containing 100 ml broth. Threefold serial dilutions were made in broth to give concentrations of 500.00–3.90 and 50.00–0.30 µg/ml for the plant extracts and the positive control (tetracycline), respectively. The bacterial suspension (100 ml) was added to all the wells. The wells with 2.5% DMSO and bacterial suspension without samples served as the solvent and negative controls, respectively. The plates were incubated at 37℃ for 72 hours in an anaerobic environment. The MIC was visually determined after the addition of PestoBlue. The MIC was defined as the lowest concentration that inhibited bacterial growth (De Canha et al., 2013).

2.4 Cytotoxicity assay on B16-F10 cells

The cell culture was prepared as described by Lall *et al.* in 2015 with a few modifications (Lall et al., 2015). The mouse melanocyte (B16-F10) cells were cultured in Minimum Essential Eagle's Medium (MEM), containing 50.00 µg/ml gentamicin instead of 10.00 µg/ml streptomycin. B16-F10 cells were seeded into a 96-well plate (100 000 cells per well). The cell viability was conducted according to Sharma *et al.* in 2014. The positive control (actinomycin D) and the plant extracts (concentrations ranging from 400.00 to 3.13 µg/ml) were added to the cells and were incubated again at 37℃ for 72 hours. Following incubation, 50 µl of XTT (sodium 3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-*bis* (4-methoxy-6-nitro) benzene-sulfonic acid hydrate) reagent was added to the wells and incubated for 3

hours, after which the optical densities (OD) of the wells were measured at 450 nm using BIOTEK Power-wave XS multi well reader. The cell survival rate was assessed by comparing the absorbance of the cells with the plant samples to the control (medium with DMSO). The statistical program 'Graph Pad Prism 4' was used to analyse the 50% inhibitory concentration (IC_{50}) of the plant extracts.

2.5 Monophenolase activity of tyrosinase

A similar protocol was followed that has been previously published by Wangthong *et al*. in 2007. Extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 20 000 µg/ml. This treatment (selected plant extracts) solution was then diluted to 300.00 µg/ml in 50 mM potassium phosphate buffer (pH 6.5). Seventy microliters of each solution of different concentrations (1.60–200.00 µg/ml) was combined with 30 µl of monophenolase tyrosinase (333 Units/ml in phosphate buffer, pH 6.5) in triplicate in 96-well microtitre plates (Wangthong et al., 2007). After incubation at room temperature for 5 minutes, 110 µl of substrate (360.00 µg/ml) was added to each well. The optical densities of the wells were determined at 492 nm over a period of 30 min at room temperature with the BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa). The concentration necessary to inhibit 50% of the enzyme activity was determined by using GraphPad prism software.

2.6 Mouse melanocyte melanin production assay

The amount of melanin produced in B16-F10 melanoma cells, after the treatment with the different plant extracts, was determined by following the method of Hill (Matsuda, *et al.*, 2004). Briefly, cultured murine B16F10 melanocytes (passage number 6) were trypsinized (0.25% trypsin and 0.1% EDTA at 37°C for 5-10 min). Cells (2×10^4 cells/well in 1.9 ml of MEM) were inoculated into 24-well plates using a pipette (FALCON 353046, Becton Dickinson Labware, NJ, U.S.A.), and incubated for 24 hours at 37°C in the CO_2 incubator.

After 24-hour incubation, 100 µl of each sample solution was added to each well in duplicate, and the 24-well plate was incubated for 3 days at 37°C in the CO_2 incubator. Test sample (concentrations ranging between 3.13 and 500.00 µg/ml) and the positive control – theophylline (concentrations ranging between 15.63 and 500.00 µg/ml) were dissolved in DMSO. The final concentration of DMSO was 0.1%. In the control group, the final DMSO concentration was used instead of the sample solution. After incubation, the cultured medium was removed by a pipette, and assayed for extracellular melanin as follows: The cultured medium was centrifuged (900 g, 20 min at 4°C) to give a supernatant. One milliliter of a mixture of 0.40 M HEPES buffer (pH 6.8) and EtOH (9:1, v/v) was added to 1 ml of the supernatant. The OD at 475 nm of the resulting solution was measured, and the amount of extracellular melanin was determined. The remaining melanoma cells were digested by the addition of 400 ml of 1 N NaOH, washed with 100 ml of CMF-D-PBS and trypsinized (0.25% trypsin and 0.1% EDTA at 37°C for 5 to 10 min), and then left standing for 16 hours at room temperature. The OD at 475 nm of the resulting solution was measured, and the amount of intracellular melanin was determined. A melanin standard curve was obtained through measuring the optical density of melanin (purchased from Sigma) at a wide range of concentrations and were used to determine the melanin produced intracellularly and extracellularly. A linear curve ($y = 0.0083x - 0.0158$) that perfectly fits the captured data (R^2 value of 0.9971) were obtained on the basis of Beer's law.

2.7 Molecular docking analysis

Molecular docking was performed using the GOLD program. It uses a genetic algorithm which considers ligand conformational flexibility and partial protein flexibility i.e. side chain residues (Jones et al., 1997, Verdonk et al., 2003). The default docking parameters were employed for the docking study. It includes 100000 genetic operations on a population size of 100 individuals and mutation rate of 95. The crystal structure of mushroom tyrosinase

(isolated from *Agaricus bisporus*) was taken from the Protein Data Bank (PDB ID: 2Y9X) (Bernstein et al., 1977). It has a crystal structure resolution of 2.78 Å and contained an inhibitor; tropolone and two Cu^{2+} atoms in the active site. The structures of the small compounds were sketched using Chemdraw3D and minimized considering RMSD cut-off of 0.10 Å. The docking protocol was set by extracting and re-docking tropolone in the tyrosinase crystal structure with RMSD <1.00 Å. This was followed by docking of all compounds in the active site defined as 6 Å regions around the co-crystal ligand in the tyrosinase protein. Furthermore, all compounds were evaluated for possible molecular interactions with tyrosinase active site residues using PyMol Molecular Graphics System (Delano, 2002).

2.8 Statistical analysis

Statistical analysis of the results obtained in the respective experiments were analysed with GraphPad Prism to obtain the effective concentrations derived from a sigmoidal dose response curve. Each experiment was conducted in triplicates and repeated at least three times. Excel was used to generate the isobolograms and the CI values was determined by CompuSyn software using the Chou-Talalay method (Chou, 2010).

3. Results and Discussion

3.1 Phytochemical analysis

The major groups of secondary compounds were determined in the eighteen plants selected (Table 1). *Combretum molle, Euclea crispa, Ficus sur, Rapanea melanophloeos* and *Withania somnifera* contained most of the phytochemicals tested. *Leucas martinisensis* was not found to contain any phytochemicals, which could be due to too low concentration of compounds.

The phytochemical groups with many compounds, identified previously, for their antibacterial activity are alkaloids, terpenes, flavonoids, tannins and phenolics (, Kumar et

al., 2007, Pithayanukul et al., 2007, Kim et al., 2008, Roopashree et al., 2008, Smith et al., 2008, Pan et al., 2009, Kim et al., 2010). The extracts containing most of the aforementioned phytochemicals were *Combretum molle* and *Ficus sur* (Table 1). However, *Hypericum revolutum* and *Withania somnifera* (leaves) showed the best bacterial inhibition.

3.2 Antibacterial activity

Hypericum revolutum and *Withania somnifera* (leaves) had a minimum inhibitory concentration of 62.50 µg/ml and 31.25 µg/ml for *P. acnes* strains 11827 and 6919 respectively (Table 2). A possible explanation for *Hypericum revolutum*'s effective bacterial inhibition could be due to the presence of acylphloroglucinols that is known for its bactericidal effect against honeybee pathogens (Verotta, 2002). The concentration at which *Hypericum revolutum* showed antibacterial activity were similar to the concentration at which the extract resulted in 50% viable cells in the cytotoxicity assay (Table 2). The antibacterial activity of xanthones, also present in *Hypericum revolutum,* against other Gram positive bacteria, have been published (Cortez et al., 2002). Withanolide isolated from *Withania somnifera*, which is also responsible for *W. somnifera* cytotoxicity, have shown to have bactericidal activity (Gibson et al., 2012). However, *Withania somnifera* (fruit and leaves), only showed antibacterial activity at concentrations higher than the IC_{50} value obtained in the cytotoxicity assay (Table 2). *Withania somnifera* (fruit) exhibited a higher MIC value (250.00 µg/ml and 125.00 µg/ml for *P. acnes* strain 11827 and 6919 respectively) than the *Withania somnifera* (leaves). The reported isolation of withanolide from *Withania somnifera* included the whole plant, but it was not specified if it was during fruiting season, therefore the concentration of withanolides in the fruit may have been less or even absent (Gibson et al., 2012). The minimum inhibitory concentration (MIC) of the positive drug control (tetracycline) was determined to be 0.78 and 0.39 µg/ml for the *Propionibacterium acnes* strains 11827 and 6919 respectively. Pretorius et al. (2003) mentioned that "antibacterial,

Table 2: Antibacterial activity against *Propionibacterium acnes* and cytotoxicity of the selected ethanol plants extracts against B16F10 mouse melanocytes

Plant name	**Family**	**Herbarium specimen (voucher) no.**	**A**		**B (µg/ml)**
			11827 (µg/ml)	**6919 (µg/ml)**	
***Combretum molle* R. Br. ex G. Don**	Combretaceae	120569	250.00	250.00	289.46 ± 10.36
Crotalaria lanceolata* E. Mey. subsp. *lanceolata	Fabaceae	120557	NI[a]	NI[a]	40.34 ± 5.35
Equisetum ramosissimum* Desf. subsp. *ramosissimum	Equisetaceae	120525	NI[a]	NI[a]	104.50 ± 2.65
***Euclea crispa* (Thunb.) Gürke**	Ebenaceae	120536	250.00	300.00	>400
***Ficus glumosa* Delile**	Moraceae	122173	500.00	500.00	306.50 ± 20.70
***Ficus lutea* Vahl**	Moraceae	122171	500.00	500.00	914.50 ± 8.20
***Ficus religiosa* L.**	Moraceae	122175	NI[a]	NI[a]	365.30 ± 26.55
***Ficus sur* Forssk.**	Moraceae	122172	500.00	500.00	510.20 ± 15.90
***Hypericum revolutum* Vahl subsp. revolutum**	Hypericaceae	122174	62.50	31.25	56.26 ± 10.12
***Leucas martinicensis* (Jacq.)**	Lamiaceae	120559	NI[a]	NI[a]	>400
***Momordica balsamina* L.**	Cucurbitaceae	120572	300.00	125.00	7.94 ± 11.25
***Pelargonium reniforme* Curtis**	Geraniaceae	P092558	250.00	250.00	353.30 ± 18.35
***Pelargonium sidoides* DC.**	Geraniaceae	P092559	250.00	250.00	144.70 ± 25.78
***Rapanea melanophloeos* (L.) Mez**	Myrsinaceae	122170	500.00	500.00	294.70 ± 7.50
***Terminalia prunioides* M.A. Lawson**	Combretaceae	120537	NI[a]	NI[a]	302.80 ± 12.33
***Withania somnifera* (fruit) (L.) Dunal**	Solanaceae	122169	250.00	125.00	78.34 ± 4.80
***Withania somnifera* (leaves) (L.) Dunal**	Solanaceae	122169	62.50	31.25	48.53 ± 4.37
Tetracycline (C)	-	-	0.78	0.39	-
Actinomycin D (D)	-	-	-	-	9.32×10^{-3}

NI[a], No inhibition at the highest concentration tested; A, Treatments minimum inhibitory concentration (µg/ml) against *Propionibacterium acnes* strains 6919 and 11827; B, Positive control for antibacterial assay; C, Positive control for cytotoxicity assay

antifungal and antiviral properties have been associated with individual or collective groups of flavonoids in the past", the potency is, however, dependent on the concentration of flavonoids present as well as the extraction solvent (Pretorius, 2003).

Although *P. acnes* strains 11827 and 6919 belong to the same serotype (determined previously by an agglutination test), namely group I, they belong to two different biotype groups: B1 and B3 respectively. Both strains 11827 and 6919 contain galactose in their cell walls. There are five different biotypes (B1 to B5) for *P. acnes* strains determined by the fermentation of ribose, erythritol and sorbitol. *P. acnes* strain 11827 contains ribose, erythritol and sorbitol, while strain 6919 contains ribose and sorbitol, but not erythritol (Kishishita et al., 1979). Undoubtedly, different responses from the bacteria towards the treatments were expected. Several factors determine the sensitivity of a bacteria such as the experimental conditions, the growing conditions and density of the bacteria as well as their sensitivity towards reduction in cell numbers (Pankey and Sabath, 2004).

The plant extracts that showed insignificant or no antibacterial activity were excluded from the combinational study with tetracycline. All the plant extracts showed a significant decrease in minimum inhibitory concentrations when combined with tetracycline, but the initial MICs of tetracycline and the plant extracts needed to be taken into consideration to evaluate whether the combination had synergistic or merely an additive effect. When the antibacterial activity of the compounds tested together is equal to the sum of their separate antibacterial activity, there is no synergy between the compounds and is known as an additive effect. When the MIC of the interaction between the two compounds is better than the compounds alone, it is known as synergy. Antagonism occurs when the two compounds, together, nullify each other's activity, in other words the activity is less in combination (MIC is higher) than the two compound separately (Timbrell, 2000). Once the most active ratios have been identified, the combination index (CI value) was determined to ensure statistical verification

and quantitatively describe the synergy between the plant extract and tetracycline (Table 3 and 4).

Hypericum revolutum, showed the best antibacterial activity with the lowest concentration of tetracycline required. The MIC of *Hypericum revolutum* decreased from 62.50 µg/ml to 7.03 µg/ml and from 31.25 µg/ml to 7.03 µg/ml for *P. acnes* strain 11827 and 6919 respectively. The MIC of the positive control (tetracycline) decreased from 0.72 µg/ml and 0.39 µg/ml for *P. acnes* strain 11827 and 6919 respectively to 0.078 µg/ml. *Combretum molle, Euclea crispa, Hypericum revolutum, Momordica balsamina, Tephrosia purpurea,* and *Withania somnifera* (leaves) showed a significant drop in the MIC values for *P. acnes* strain 6919 and the concentration of tetracycline reduced from 0.39 µg/ml to 0.16 µg/ml, 0.16 µg/ml, 0.078 µg/ml, 156 µg/ml, 0.16 µg/ml, and 0.23 µg/ml respectively. Ultimately, only *Hypericum revolutum*, showed the most significant synergy.

The CI values were determined with the equation CI = $\frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}$ where (Dx)1 and (Dx)2 is the minimum inhibitory concentration of the compounds alone, while (D)1 and (D)2 is the minimum inhibitory concentration of each compound in combination as determined in CompuSyn software (Chou, 2010). Synergism is represented by a CI value smaller than one, an additive effect is when the CI value is equal to one and antagonism is portrayed when the CI value is larger than one (Chou and Talalay, 1984). Conditional formatting was used in Table 3 and 4 to indicate the ratios exhibiting synergy (values < 1), additivity (values = 1) or antagonism (values > 1) respectively. The darkest black colour indicates the ratio of the plant extract and tetracycline with the best synergy, while the white indicates the more antagonistic the ratio between the plant extract and tetracycline.

3.3 Cytotoxicity

Previous studies have shown that there might be a correlation between the cytotoxicity and the antibacterial activity of a compound, as was the case with fluoroquinolones – a broad

Table 3: Combination index (CI value) of the different ratios obtained in the combinational studies between the plant extracts collected from Lillydale village and tetracycline (drug) against *Propionibacterium acnes*

Plant: Drug	***Euclea crispa* (Thunb.) Gürke**		***Combretum molle* R. Br. ex G. Don**		***Momordica balsamina* L.**	
Ratio	**A**	**B**	**A**	**B**	**A**	**B**
9:1	1.025	0.446	2.051	0.455	1.976	1.026
8:2	1.801	0.842	1.801	0.851	1.767	0.449
7:3	2.576	0.617	1.289	9.941	2.547	0.321
6:4	1.676	0.407	1.676	6.544	1.663	0.418
5:5	2.064	0.505	2.064	16.235	2.053	0.514
4:6	2.451	0.604	1.226	9.691	2.442	0.611
3:7	1.420	0.702	1.420	22.454	1.416	0.708
2:8	0.806	0.803	1.613	12.838	1.611	0.807
1:9	0.903	0.902	0.903	0.902	1.805	0.903

A, *P. acnes* ATCC 11827; B, *P. acnes* ATCC 6919

Synergy Additivity Antagonism

Table 4: Combination index (CI value) of the different ratios obtained in the combinational studies between the plant extracts collected from the Manie van der Schijff Botanical Garden and tetracycline (drug) against *Propionibacterium acnes*

Plant:Drug	***Ficus glumosa* Delile**		***Ficus lutea* Vahl**		***Ficus sur* Forssk.**		***Hypericum revolutum* Vahl subsp. *revolutum***		***Pelargonium reniforme* Curtis**		***Pelargonium sidoides* DC.**		***Withania somnifera* (Fruit) (L.) Dunal**		***Withania somnifera* (Leaves) (L.) Dunal**	
Ratio	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
9:1	0.913	1.823	0.913	1.711	1.826	1.711	0.212	0.424	2.051	1.823	2.051	1.823	1.025	1.026	0.425	0.849
8:2	1.701	1.698	1.701	1.648	1.701	3.297	0.3	0.599	1.801	3.397	1.801	1.698	0.900	1.798	1.2	1.201
7:3	1.245	2.486	0.622	1.221	0.622	2.443	0.387	0.774	2.576	1.243	0.644	1.243	1.289	1.287	0.776	0.774
6:4	1.638	1.636	0.819	1.617	0.819	3.234	0.476	0.951	1.676	1.636	0.838	3.272	1.676	3.347	0.95	1.899
5:5	2.033	1.016	1.016	1.008	1.016	2.013	0.563	1.125	2.064	1.016	0.516	1.016	1.031	2.06	0.563	1.125
4:6	4.852	1.212	0.607	2.411	1.213	2.411	0.651	1.3	2.451	1.212	0.306	1.212	1.226	2.449	2.601	1.3
3:7	5.639	1.408	1.41	2.807	0.705	2.807	0.368	0.736	1.42	1.408	0.354	0.703	1.42	2.835	1.476	1.474
2:8	1.607	3.209	1.607	1.602	1.607	3.203	0.825	1.648	0.806	3.209	1.613	3.209	1.613	6.444	1.651	3.297
1:9	1.803	3.602	1.803	1.8	1.803	3.599	0.913	1.823	0.903	3.602	0.452	3.602	0.903	7.219	1.825	3.646

A, *P. acnes* ATCC 11827; B, *P. acnes* ATCC 6919

Synergy Additivity Antagonism

spectrum antibiotic (Suto et al., 1992). Cytotoxicity is one of the first and foremost steps in finding an active plant extract, as the cytotoxicity of the plant not only determines the concentration safe to use in treatments, but also narrows the range of concentrations necessary to test in experimental analysis as a hypothetical outcome could be predicted. Cytotoxicity is associated by the effective concentration of a compound, which leads to only 50% of the viable cells to be present – known as the EC_{50} value. The higher the EC_{50} value, the less toxic the plant extract and, therefore, is recommended to be safe to use in treatments. The EC_{50} values of *C. molle, E. crispa, F. lutea, F, sur* and *P. reniforme* were higher than their minimum inhibitory concentration (MIC) for *P. acnes*, therefore, the active concentrations of the aforementioned plants are safe to use in treatments to be performed on mouse melanocytes (Table 2). The EC_{50} values obtained for *C. lanceolata, E. ramosissimum, F. glumosa, F. religiosa, M. balsamina, P. sidoides, R. melanophloeos* and *W. somnifera* were lower that their MIC for *P. acnes* and are, therefore, cytotoxic. The aforementioned plants showed melanin inhibitory effects on mouse melanocytes, as discussed in the following sections, which could be due to the cytotoxic effect of these extracts. *Terminalia prunioides* had an EC_{50} value of 302.80 μg/ml, which is higher that the concentration required to significantly stimulate melanin production (Figure 2). Consequently, *T. prunioides* will be safe to use in potential treatments for hypopigmentation

3.4 Anti-tyrosinase activity and molecular docking

Aside from the bacterial aspect of the PMH disorder, the objective of this study was to determine which plant extracts could stimulate melanin production in B16-F10 mouse melanocytes and also increase the monophenolase activity (hydroxylation of tyrosine to 3,4-dihydroxyphenyalanine) of tyrosinase (Ben-Yosef et al., 2010). Of the eighteen plant extracts *Ficus glumosa, Ficus lutea, Ficus religiosa* and *Hypericum revolutum* showed no effect on the monophenolase activity of tyrosinase at the highest concentration tested (200.00 μg/ml).

Withania somnifera (leaves) and *Withania somnifera* (fruit) at a concentration of 100.00 µg/ml increased the monophenolase activity of tyrosinase (Table 5).

Table 5: Anti-tyrosinase activity of the selected plant ethanol extracts

Plant species	**IC_{50} (µg/ml) (Monophenolase activity)**	**% activity increase of enzyme after treatment (100 µg/ml)**
***Combretum molle* R. Br. ex G. Don**	126.2 ± 0.4	n/a
Crotalaria lanceolata* E. Mey. subsp. *lanceolata	5.964 ± 0.56	n/a
Equisetum ramosissimum* Desf. subsp. *ramosissimum	108.4 ± 1.2	n/a
***Euclea crispa* (Thunb.) Gürke**	191.2 ± 0.23	n/a
***Ficus glumosa* Delile**	>200	n/a
***Ficus lutea* Vahl**	>200	n/a
***Ficus religiosa* L.**	>200	n/a
***Ficus sur* Forssk.**	45.38 ± 1.12	n/a
***Hypericum revolutum* Vahl subsp. revolutum**	>200	n/a
***Leucas martinicensis* (Jacq.)**	877.7 ± 1.34	n/a
***Momordica balsamina* L.**	35.72 ± 0.42	n/a
***Pelargonium reniforme* Curtis**	73.63 ± 0.165	n/a
***Pelargonium sidoides* DC.**	64.9 ± 0.14	n/a
***Rapanea melanophloeos* (L.) Mez**	110.75 ± 0.84	n/a
***Terminalia prunioides* M.A. Lawson**	80.76 ± 0.51	n/a
***Withania somnifera* (fruit) (L.) Dunal**	n/a	**14%**
***Withania somnifera* (leaves) (L.) Dunal**	n/a	**13%**
Kojic acid	1.663 ± 0.45	n/a

n/a, not applicable as the extract either inhibited or activated the tyrosinase enzyme

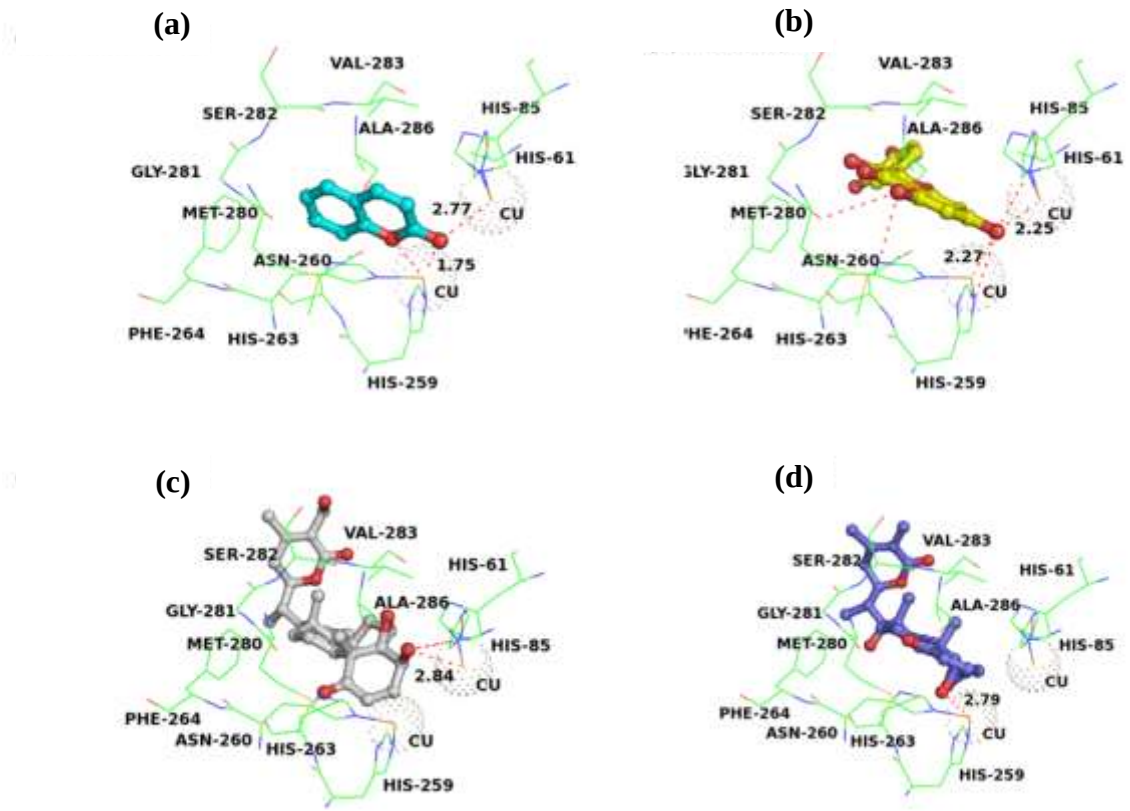

Figure 1: View of the tyrosinase protein and the interaction between a) coumarin, b) quercetin, c) withaferin and d) withanone with tyrosinase's active site.

Certain flavonoids, more specifically quercetin, coumarin (Benzopyrone that has a core structure of flavonoids), kaemferol and certain saponins, inhibits tyrosinase with their 3-hydroxylgroup chelating the copper ions, at the active site, necessary for the enzyme's activity. The inhibition of tyrosinase by quercetin and coumarin was verified through molecular docking (Figure 1). Coumarin and quercetin showed good interaction with docking fitness scores of 40.46 and 44.40 respectively. They showed interactions with two Cu^{2+} ions, with a van der Waals distance of < 2.70 Å. In addition to this, Quercetin was also observed to make H-bond interaction with residue His263 and Met280, which justified its high fitness score. However, if these 3-hydroxylgroup are bound and not available to react when dimerization occurred or when other compounds present in the plant extract interacted with the 3-hydroxylgroup, no inhibition of the enzyme could occur (Kubo and Kinst-Hori, 1999,

Xie et al., 2003, Zhang and Zhou, 2013). Additively, if quercetin and kaempferol structures contains 3-O-glycosides, no chelation could take place and tyrosinase inhibition do not occur (Kubo and Kinst-Hori, 1999).

Although flavonoids, more specifically quercetin, are present in *Withania somnifera* it does not infer that it will also inhibit tyrosinase activity. As mentioned earlier, it is important that the 3-hydroxyl group of quercetin is free to chelate the copper ions, which is not necessarily the case with the quercetin present in *Withania somnifera*. In a previous report it was mentioned that the tyrosinase activity was not affected by the addition of *Withania somnifera* (Nakajima et al., 2012). Conversely, *Withania somnifera* and its active compounds, withaferin and withanone, have shown to cause skin darkening, which could possibly be due to tyrosinase activation (Ali and Naaz, 2015). In the present study *Withania somnifera* activated the monophenolase activity of tyrosinase. During molecular docking analysis, withaferin and withanone showed poor docking fitness scores of -66.50 and -42.42 respectively. It signified the unfavourable binding into the active site of tyrosinase. They showed only single interactions to Cu^{2+} with large distance i.e. > 2.70 Å. In addition, none of residues were observed to be involved in H-bond interactions, which substantiated the poor fitness scores.

It was previously reported that the catalytic activity of tyrosinase was also stimulated by 3-hydroxyanthranilic acid (HHA) – an intermediate compound produced in the kynurenine pathway synthesising tryptophan (Rescigno et al., 1998). The kynurenine pathway occurs in plants to also produce pyridine alkaloids. This pathway might contain an explanation for the activation of tyrosinase by *Withania somnifera* (Ashihara, 2006). Another important pathway in plants is the shikimic acid pathway, which is responsible for the biosynthesis of L-phenylalanine and L-tyrosine – crucial aromatic amino acids that forms the substrates for the monophenolase activity of tyrosinase (Haslam, 1993).

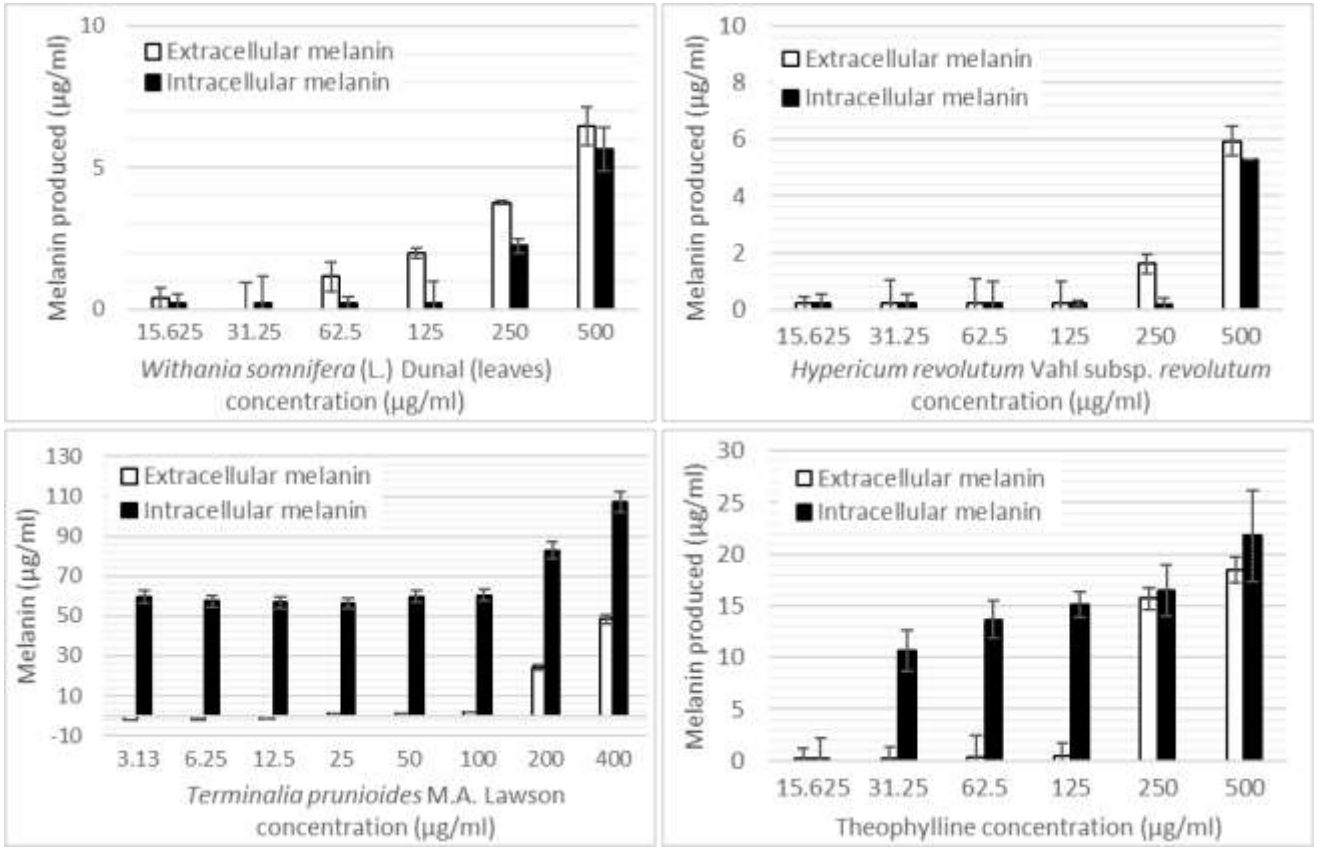

Figure 2: The melanin stimulating effect of the ethanol extracts of *Withania somnifera* (L.) Dunal (leaves), *Hypericum revolutum* Vahl subsp. *revolutum* and *Terminalia prunioides* M.A. Lawson as well as the positive control (Theophylline) on the concentration of intracellular melanin and extracellular melanin produced in B16F10 mouse melanocytes, where a zero melanin concentration represents the untreated cells.

3.5 Melanin production

An increase in tyrosinase activity can lead to induced melanin production in melanocytes. The concentration of pigments synthesised by the melanocytes was estimated spectrophotometrically and was depicted from a melanin standard curve. The absorbance increased as the concentration of extracellular and intracellular melanin present increased. Only three of the plant extracts tested increased both the extracellular and intracellular melanin concentration. Theophylline (an alkaloid) was used as the positive control. The ethanol extracts of *Hypericum revolutum*, *Terminalia prunioides* and *Withania somnifera* (leaves) increased the amount of extracellular and intracellular melanin (Figure 2). Cells

treated with 500.00 µg/ml *Hypericum revolutum* and *Withania somnifera* (leaves) produced approximately 12.00 µg/ml of melanin (6.00 µg/ml intracellularly and 6.00 µg/ml extracellularly), which was far less than the 40.00 µg/ml of melanin (22.00 µg/ml intracellularly and 18.00 µg/ml extracellularly) for the positive control (theophylline). *Hypericum revolutum* contains coumarins, which have been shown to induce melanin production (Matsuda et al., 2005).

Extracts containing withanone, as is the case with *Withania somnifera*, has shown to increase hair melanin in clinical trials conducted by Bone and Morgan in 1996 as published by Widodo et al. (2009). Withaferin A and quercetin, both found in *Withania somnifera*, stimulated melanin dispersion mediated by cyclic AMP, which leads to skin darkening (Novales, 1972, Ali and Meitei, 2011, Ali and Meitei, 2012,). However, both *Withania somnifera* and *Hypericum revolutum* stimulated melanin production only at higher concentrations than the EC_{50} values obtained for their cytotoxicity. The stimulation of melanin production could be due to a defence mechanism towards the cytotoxic effect of the plant extracts. Melanin is known for its radical scavenging properties (Riley, 1997). Therefore, if the cytotoxicity of the extracts is due to the presence of reactive oxygen species, then melanin production would be stimulated and the melanin particles would still be detected through spectrophotometry after the cells has been broken down.

Cells treated with 500.00 µg/ml *Terminalia prunioides* produced approximately 150.00 µg/ml of melanin (107.00 µg/ml intracellularly and 43.00 µg/ml extracellularly), which is a much higher concentration than the melanin produced in the positive control. No, active compounds, which stimulated melanogenesis, have been isolated from *Terminalia prunioides*, therefore the activation of melanin production through the treatment of *Terminalia prunioides* was reported for the first time.

The cells treated with *Rapanea melanophloeos* did not produce any extracellular melanin, but did, however, show a high absorbance for intracellular melanin (Figure 4). The high absorbance for intracellular melanin could be ascribed to the benzoquinones found in *Rapanea melanophloeos*. Gamma-L-glutaminyl-3-4-benzoquinone is a precursor in the production of melanin, therefore, the absorbance reading could have included both the melanin produced and the γ-L-glutaminyl-3-4-benzoquinone present (Prezioso et al., 1993). Consequently, the absorbance gave a false positive; possibly because γ-L-glutaminyl-3-4-benzoquinone was mostly present, there was no mature melanin which could move to the extracellular space.

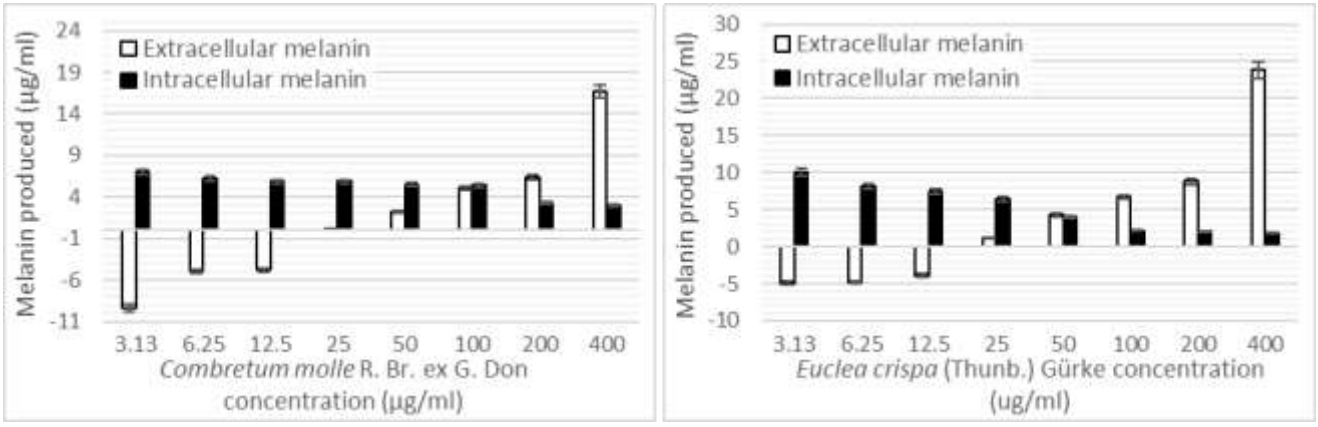

Figure 3: The effect of the ethanol extracts of *Combretum molle* R. Br. ex G. Don and *Euclea crispa* (Thunb.) Gürke on the concentration of intracellular melanin and extracellular melanin in B16F10 mouse melanocytes, where a zero melanin concentration represents the untreated cells.

Most of the plants had an insignificant effect on melanin production or inhibited the production of melanin in the melanocytes. As a result of the aforementioned process, it is noticeable that in most graphs, such as for *E. crispa, C. molle, M. balsamina, T. purpurea, C. lanceolate* and *L. martinicenses*, the intracellular melanin decreased with the increase of the plant concentration (Figures 3 & 5). Together with the decrease in intracellular melanin, the extracellular melanin increased with the increase in extract concentration for *E. crispa, C. molle* and *M. balsamina*. The increase in extracellular melanin could possibly mean that the

extract does not only stimulate melanin production, but at higher plant concentrations speeds up the melanogenesis process, leading to more melanin transferred.

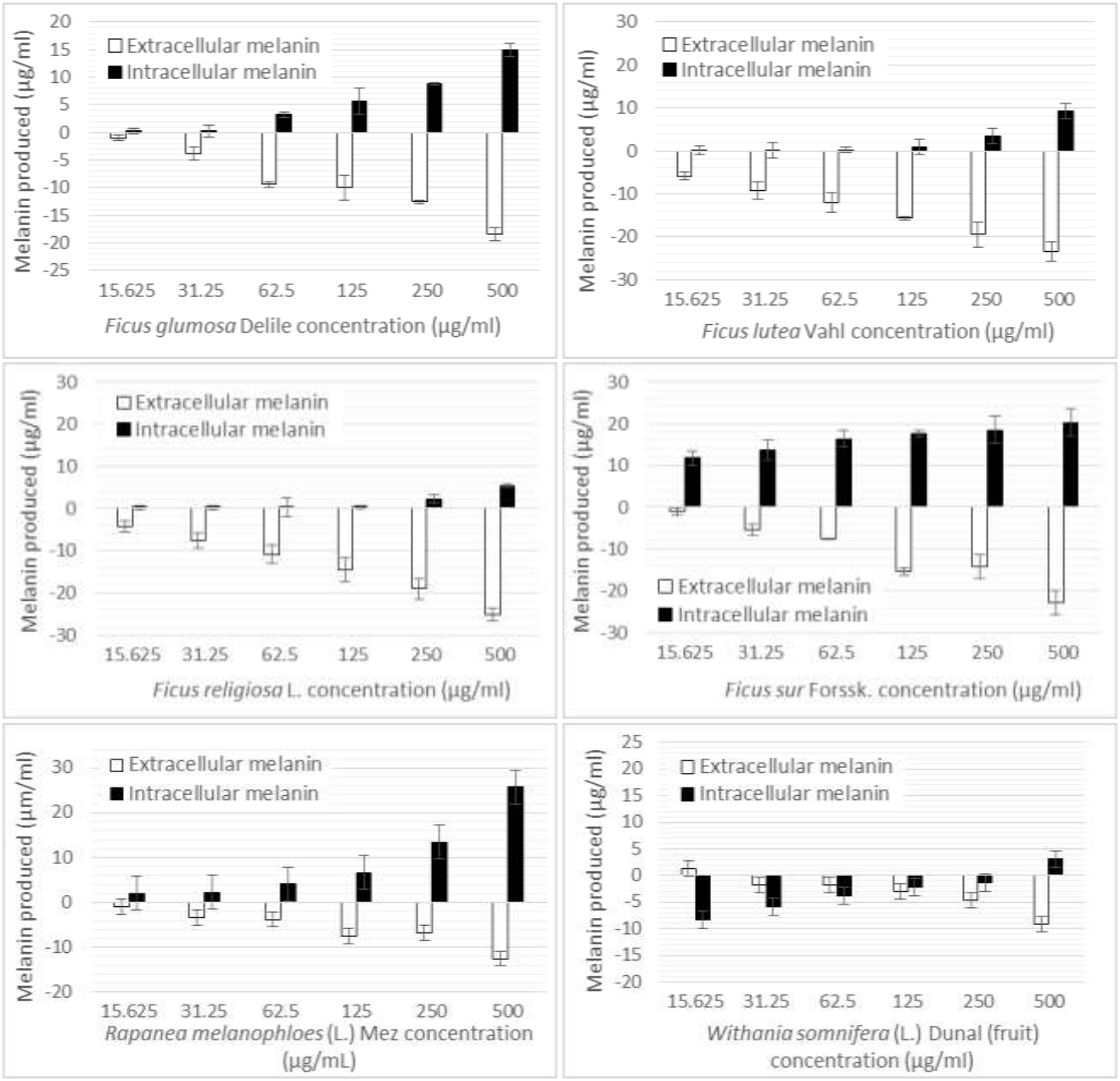

Figure 4: The effect of the ethanol extracts of *Ficus glumosa* Delile, *Ficus lutea* Vahl, *Ficus religiosa* L., *Ficus sur* Forssk., *Rapanea melanophloeos* (L.) Mez and *Withania somnifera* (L.) Dunal (fruit) on the concentration of intracellular melanin and extracellular melanin in B16F10 mouse melanocytes, where a zero melanin concentration represents the untreated cells.

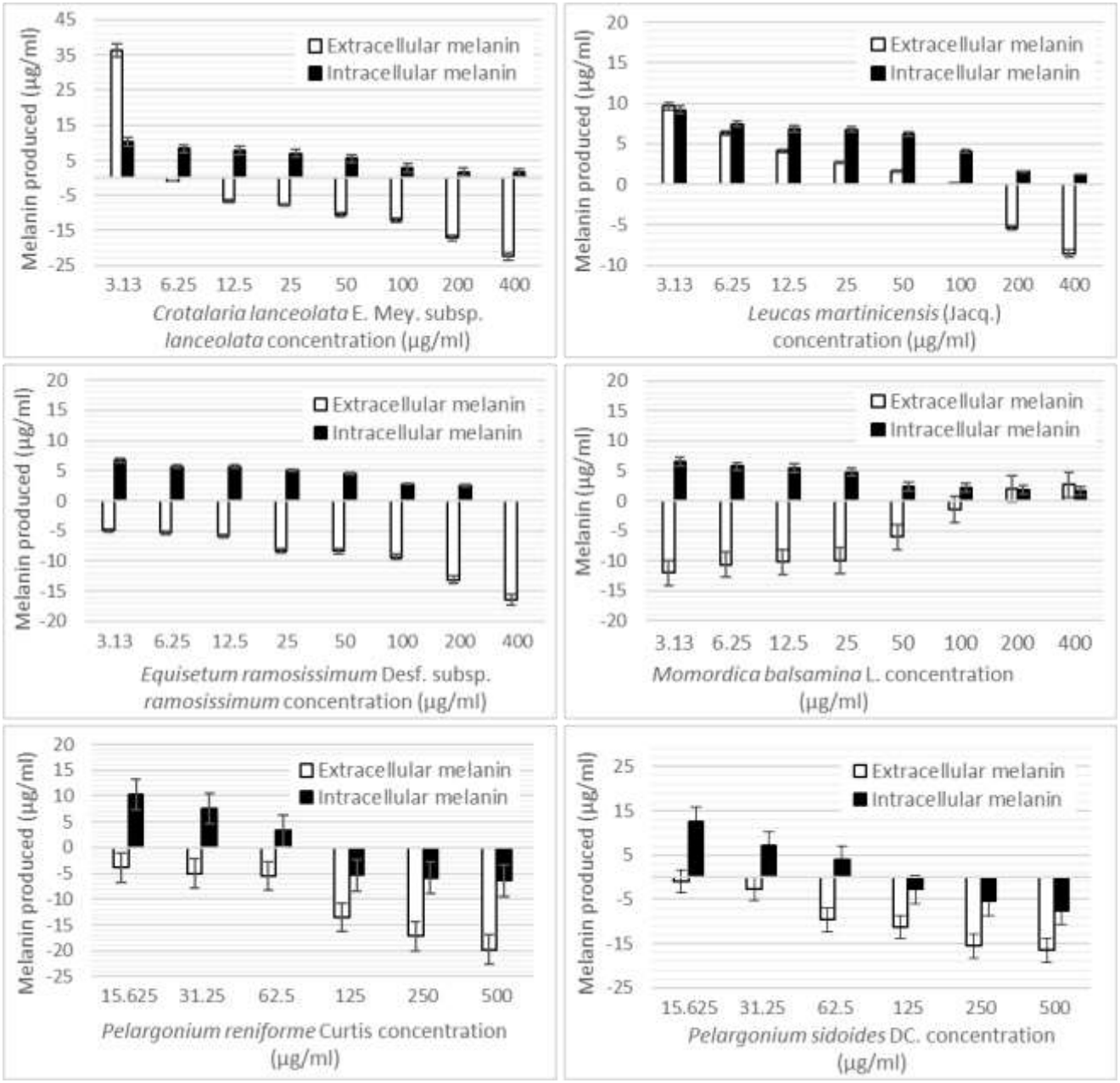

Figure 5: The effect of the ethanol extracts of *Crotalaria lanceolata* E. Mey. subsp. *lanceolata*, *Leucas martinicensis* (Jacq.), *Equisetum ramosissimum* Desf. subsp. *ramosissimum*, *Momordica balsamina* L., *Pelargonium reniforme* Curtis. and *Pelargonium sidoides* DC. on the concentration of intracellular melanin and extracellular melanin in B16F10 mouse melanocytes, where a zero melanin concentration represents the untreated cells.

4. Conclusion

Propionibacterium acnes was identified by Westerhof *et al.* in 2004 as the causative bacteria of progressive macular hypomelanosis (PMH) (Westerhof et al., 2004). The antibiotics currently used for PMH provide short term solutions, but through the investigation of antibacterial plants, an alternative – long term – solution may be identified. Both *Hypericum revolutum* and *Withania somnifera* are traditionally used for the hypopigmented disorder, Leukoderma, and for skin infections (Capitanio et al., 1989, Prakash et al., 2002, Singh et al., 2010, Zofou et al., 2011). *Terminalia prunioides* is traditionally used for bacterial infections and skin diseases (Neuwinger, 1996, Oliver-Bever, 1986). The aforementioned traditional uses led to the investigation of the selected plants for PMH. Although i*n vitro* studies acts mostly as peliminary studies, the results obtained provides a good determination of the plant extract's activity and could guide any future *in vivo* studies, which will strenghten the findings of active plants for the use for PMH. *Hypericum revolutum* and *W. somnifera* (leaves) were the most potent extracts against *P. acnes,* when combined with tetracycline (a known antibiotic). The significant synergy between *Hypericum revolutum* and tetracycline, decreases the chance of the strains becoming resistant and decreased *H. revolutum* and tetracycline's bioactivity below their cytotoxicity, potentially making it safer to use. *Hypericum revolutum, Terminalia prunioides* and *Withania somnifera* (leaves) were the only plant extract that increased the monophenolase activity of tyrosinase. During molecular docking analysis, it was concluded that small molecules like coumarin and quercetin have potential to inhibit the tyrosinase enzyme as they were buried deep and interacted with Cu^{2+} ions. However, larger molecules, such as withaferin and withanone A, are not supposed to inhibit the enzyme. Similarly, dimerization of single small molecules into larger molecules probed to make them inactive against the tyrosinase enzyme.

Withania somnifera (leaves) also showed an increase in the amount of extracellular and intracellular melanin. *Terminalia prunioides* stimulated melanin production even at the lowest concentration tested (3.00 μg/ml), therefore, was found to be active at concentrations lower than its cytotoxicity exhibited on the cells. As a result, *Hypericum revolutum, Terminalia prunioides* and *Withania somnifera* (leaves) has been identified as possible plants for PMH and further study would include using chromatography for the identification of the bioactive compounds, which could possibly be incorporated into formulation and used for progressive macular hypomelanosis.

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