The effect of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards on quorum sensing of bacteria associated with acne vulgaris

By

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Submitted in partial fulfilment of the requirements for the degree of

***PHILOSOPHIAE DOCTOR* (Medicinal Plant Sciences)**

in the

Department of Plant and Soil Sciences

of the

Faculty of Natural and Agricultural Sciences

UNIVERSITY OF PRETORIA

UNIVERSITEIT VAN PRETORIA
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January 2022

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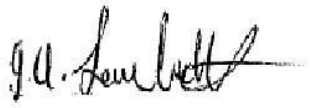
SIGNATURE: 

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Acknowledgements

This work is based on the research supported wholly by the National Research Foundation of South Africa (Grant Number: 121192). This work is further supported by the Department of Science and Innovation of the Republic of South Africa (DST/CON 0059/2019).

I would like to firstly acknowledge and thank my supervisor Professor Namrita Lall for her continued guidance and support. She has provided me with opportunities to develop my research skills and continues to motivate me to keep learning. Secondly, I would like to thank my husband, Edmund Kupke, for his love and support throughout this journey. It was not an easy journey with many lows, and he was there through it all, picking me up every time. I would also like to thank my parents, Hendrik and Christa for their love, advice and the sacrifices they made to help me on the path that I have chosen. To my dear friends, Sewes Alberts, Bianca Payne, Dr Marco de Canha and Dr Danielle Twilley, you have helped me in more ways you will ever know, and I am forever thankful for your guidance and love.

Lastly, I dedicate this thesis to my beloved Aunt Marietjie Collins, who passed away on 11 June 2021 at the age of 54 from arrhythmia. You have loved and supported me like a mother, and you have taught me so much about life. I will always strive to enjoy the small moments in life as you did. I miss your voice and your comforting hugs. Until we see each other again, I will miss you and love you every day.

"Life's battles don't always go to the stronger or faster man. But sooner or later, the man who wins is the man who thinks he can."

Napoleon Hill

Research Outputs - Appendix F

MANUSCRIPTS

- Lambrechts, IA, Lall, N. Traditional usage and biological activity of *Plectranthus madagascariensis* and its varieties: a review. Journal of Ethnopharmacology. 2020: 113663. Impact factor: 4.36

MANUSCRIPTS UNDER PREPARATION (Will be submitted for publication in 2022)

- Lambrechts, IA, Lall, N, Coenye T, Spittaels, KJ. *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards and its compounds targeting quorum sensing related to acne vulgaris. 2022. Journal Biomedicines. Impact factor: 6.081
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PATENTS

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- Lambrechts, IA (2018). An indigenous South African plant targeting inflammation and antibiotic resistance associated with acne vulgaris. African Traditional and Natural Product Medicine Conference. Limpopo, South Africa. Podium presentation
- Lambrechts, IA (2017). Potential of South African Plants and Essential Oils for Acne Vulgaris. Society of Cosmetic Chemists South Africa. Podium presentation

INTERNATIONAL CONFERENCES

- Lambrechts, IA (2021). An indigenous South African plant targeting antibiotic resistance and the pathogenic factors associated with acne vulgaris. 26th International Federation of Societies of Cosmetic Chemists Conference. Virtual Congress Platform. Poster presentation

- Lambrechts, IA (2021). A natural choice for antimicrobial protection using an indigenous South African plant for acne vulgaris. The 20th International Congress of the International Society for Ethnopharmacology. Virtual Congress Platform. Podium presentation

NATIONAL ACCOMPLISHMENTS

- South African Women in Science Awards (2018) - DST-Albertina Sisulu Fellowship (Doctoral) Award winner.
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- DST-NRF Innovation Doctoral Scholarship (2017 - 2019)
- UP Postgraduate Scholarship Doctoral Scholarship (2017 - 2019)

INTERNATIONAL ACCOMPLISHMENTS

- Dr. Willmar Schwabe Research Scholarship (2021) - Society for Medicinal Plant and Natural Product Research. Award winner. To be used for postdoctoral studies in 2022

Summary

The effect of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards on quorum sensing of bacteria associated with acne vulgaris

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The World Health Organization has raised concerns of antibiotic resistance threatening the global health care system and economy. Antibiotic resistance results from quorum sensing mechanisms that allow bacterial species to express genes associated with biofilm formation, protecting sessile cells from well-known antibiotic therapies. Increased antibiotic resistance is observed in maladies such as acne vulgaris, prosthetic implant-associated infections, and wounds due to the presence of pathogenic bacteria, including *Cutibacterium acnes* and *Staphylococcus epidermis*. However, controversy exists regarding the type of relationship between these bacteria and their role in these maladies. More research is required to understand better the pathogenic relationship of these bacteria and their role in the progression of antibiotic resistance. Furthermore, there is a need for new alternative therapies that target quorum sensing mechanisms and circumvent antibiotic resistance increasing the efficiency of treatments for acne vulgaris, prosthetic implant-associated infections and wounds.

This study investigated the quorum sensing relationship of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) in order to determine the combined virulent effect of these bacteria in a multispecies system under various growth conditions. Furthermore, this study investigated

the potential use of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards, an indigenous South African plant, its identified pure compounds and fractions for targeting quorum sensing mechanisms. The targeted mechanisms include the release of autoinducer-2 (AI-2), biofilm attachment and formation, lipase production, extracellular DNA release and potential wound healing activity. The potential use of nanoparticles synthesised from *P. aliciae* ethanolic extract and pure compounds to target biofilm attachment and formation inhibit the bacterial systems of interest and contribute to wound healing.

It was determined that *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) has a synergistic relationship. Under aerobic conditions, an increase in biofilm density, lipase and eDNA production was observed. This supported the hypothesis that *S. epidermidis* (ATCC® 35984) provides a safe environment for *C. acnes* (ATCC® 6919) to thrive under aerobic conditions. *Plectranthus aliciae*, its pure compounds and fractions, targeted the various quorum sensing mechanisms, including the release of AI-2, lipase inhibition, bacterial inhibition, biofilm attachment and formation, and eDNA production. Noteworthy wound healing activity was observed for *P. aliciae* ethanolic extract and the synthesised nanoparticles of rosmarinic acid.

This study is the first report of the synergistic quorum sensing relationship between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) in a multispecies system. This could be an indication that the pathogenic relationship between these bacteria is strain-specific and supports the hypothesis of the current study. To conclude, this is the first report of *P. aliciae*, its partitions and newly identified compound luteolin targeting quorum sensing processes of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) targeting AI-2, lipase, bacterial attachment and biofilm formation, inhibiting sessile bacteria within a mature biofilm, eDNA production and promoting wound closure that contributes to the novelty of the current project.

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APPENDIX A

List of abbreviations

ζ- potential	Zeta potential
%Inh	Percentage inhibition
AHL	Acyl-homoserine lactones
AI-2	AUTOINDUCER-2
AIP	Autoinducing peptides
ATCC	American Type Culture Collection
AuNP	Gold nanoparticle
$AUNP_{GA}$	Gum Arabic gold nanoparticles
$AUNP_{PAE}$	*Plectranthus aliciae* ethanolic extract gold nanoparticles
$AUNP_{RA}$	Rosmarinic acid gold nanoparticles
$AUNP_{TET}$	Tetracycline gold nanoparticles
CA-SE	*Cutibacterium acnes* and *Staphylococcus epidermidis* bacterial combination (1×10^6 CFU/mL)
BHI	Brain heart infusion
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
CTB	CellTiter-Blue®
CV	Crystal Violet
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
eDNA	External deoxyribonucleic acid
EPS	Extracellular polymeric substance
FBS	Foetal Bovine Serum
FTIR	Fourier-Transform Infrared Spectroscopy
GA	Gum Arabic
HaCat	Human keratinocyte cell line
$HAuCl_4.3H_2O$	Gold (III) chloride trihydrate salt

HMDS	Hexamethyldisilazane
IC_{50}	Fifty percent inhibitory concentration
Lu	Luteolin
MIC	Minimum Inhibitory Concentration
OD	Optical Density
PA_{EtOH}	*Plectranthus aliciae* ethanolic extract
QS	Quorum sensing
RA	Rosmarinic acid
RT	Room temperature
SD	Standard deviation
SEM	Scanning Electron Microscopy
SI	Selectivity Index
SLS	Sodium Lauryl Sulphate
TEM	Transmission Electron Microscopy
UPLC-QTOF	Ultra-Performance Liquid Chromatography - Quantitative Time of Flight
UV-Vis	Ultraviolet Visual Spectroscopy
WHO	World Health Organisation
XRD	X-Ray Powder Diffraction

Abstract

The World Health Organization has raised concerns of antibiotic resistance threatening the global health care system and economy. Antibiotic resistance results from quorum sensing mechanisms that allow bacterial species to express genes associated with biofilm formation, which protect sessile cells from well-known antibiotic therapies. Increased antibiotic resistance is observed in maladies such as acne vulgaris, prosthetic implant-associated infections, and wounds due to the presence of pathogenic bacteria, including *Cutibacterium acnes* and *Staphylococcus epidermis*. Since, controversy exists regarding the type of relationship between these bacteria and their role in these maladies, more research is required to understand the pathogenic relationship of these bacteria and their role in the progression of antibiotic resistance. Furthermore, there is a need for new alternative therapies that target quorum sensing mechanisms and circumvent antibiotic resistance increasing the efficiency of treatments for acne vulgaris, prosthetic implant-associated infections and wounds.

The study investigated the type of relationship between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984). The agar plate synergistic assay revealed that the bacteria do not have an antagonistic relationship under aerobic or anaerobic growth conditions. The multispecies bacterial biofilm systems were analysed using scanning electron microscopy, and the micrographs revealed that the bacteria were in an intermixed, cooperative relationship under both anaerobic and aerobic growth conditions. The single and multispecies bacterial systems were analysed for their quorum sensing activity. Studies on the release of AI-2 in single and multispecies systems revealed that after 72 h, no significant difference ($P > 0.05$) in extracellular AI-2 was observed for the different bacterial systems investigated. Earlier detection studies could potentially indicate a higher concentration of AI-2 production in a multispecies system since AI-2 is released at the start of the bacterial and biofilm growth phase. Antibacterial studies using antibiotic tetracycline revealed an increase in antibiotic resistance for the multispecies system under aerobic growth conditions compared to the single species systems for each bacteria alone. The multispecies systems grown under aerobic and anaerobic growth conditions revealed a statistically significant ($P < 0.001$) increase in the density of the mature biofilm. This data was supported by the significant ($P < 0.001$) increase in extracellular DNA (eDNA) levels present in the biofilm when grown under aerobic conditions with the multispecies bacterial system. Extracellular DNA is involved in the attachment of bacteria to a surface, which is required for

biofilm development, the transfer of mutated genes across the biofilm to other bacterial species and has a structural role. Under aerobic ($P < 0.05$) and anaerobic ($P < 0.001$) growth conditions, the multispecies bacterial system produced significantly more lipase secretion than the single bacterial systems. The increase in lipase production and release could potentially result in increased virulence and inflammatory responses in the host in the presence of a multispecies system under aerobic and anaerobic conditions. This data confirms that specific *C. acnes* and *S. epidermidis* strains could have a synergistic pathogenic relationship as a multispecies system promotes quorum sensing processes and contributes to antibiotic resistance in infections associated with these bacteria. This is the first report of the quorum sensing relationship of *C. acnes* and *S. epidermidis* as a multispecies bacterial system.

Furthermore, the study investigated the potential of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards, an endemic South African plant used to treat various maladies related to the skin and wounds, with a specific focus on targeting quorum sensing contributing to the prevention of antibiotic resistance associated with *C. acnes* and *S. epidermidis* infections. The ethanolic extract of *P. aliciae*, its hexane and ethyl acetate partitions and the compounds (luteolin and rosmarinic acid) were analysed for their potential in targeting various quorum sensing processes that contribute to antibiotic resistance associated with acne vulgaris, prosthetic implant-associated infections and wounds. The *P. aliciae* extract was analysed for the presence of sixteen known compounds using Ultra-Performance Liquid Chromatography - Quantitative Time of Flight. Five compounds, namely luteolin, protocatechuic acid, quercitrin, rosmarinic acid and syringic acid, were detected in high quantities. Luteolin was the only compound that displayed antibacterial activity against *C. acnes* (ATCC® 6919) with an MIC of 62.5 µg/mL and was selected for further studies together with rosmarinic acid. Although rosmarinic acid displayed no antibacterial activity against C. *acnes* (ATCC® 6919) at the highest concentration tested of 500 µg/mL, the compound is known to be present in the *Plectranthus* genus and has displayed anti-inflammatory activity. This is the first report of identifying luteolin in *P. aliciae*.

Plectranthus aliciae ethanolic extract, hexane and ethyl acetate partitions and luteolin displayed noteworthy antibacterial activity against *C. acnes* (ATCC® 6919) and moderate antibacterial activity against *S. epidermidis* (ATCC® 35984) and the multispecies systems grown under anaerobic and aerobic conditions. Although the *P. aliciae* ethanolic extract, hexane and ethyl acetate partitions, and luteolin displayed good antibacterial activity, the samples were more selective towards bacterial attachment and biofilm formation. The samples were unable to

eradicate the mature biofilms of the bacteria at their 2×MIC concentrations. However, they were able to penetrate the mature biofilm of *C. acnes* (ATCC® 6919) and inhibit the bacteria within, rendering the remaining bacteria metabolically inactive. Tetracycline and rosmarinic acid could not penetrate the biofilm and inhibit the *C. acnes* (ATCC® 6919) sessile bacteria using the artificial sebum model at their respective 2×MIC concentrations. Current acne therapies and research only focus on targeting the biofilm and not the ability of the samples to penetrate an established mature biofilm. The artificial sebum model simulates the interaction of the sample and the bacteria within the pilosebaceous unit under *in vitro* conditions. The *P. aliciae* ethanolic extract was found to be significantly ($P < 0.01$) more active towards targeting AI-2 production when compared to tetracycline. Noteworthy AI-2 inhibitory activity was observed for the hexane and ethyl acetate partitions and luteolin at sub-MIC values. Currently, no treatment on the market targets quorum sensing of *C. acnes* and *S. epidermidis* through AI-2 inhibition. At sub-MIC values, significant lipase inhibitory activity was observed for all the samples against the secretion of lipase enzyme, especially rosmarinic acid. This activity could be linked to the anti-inflammatory activity of the compound. However, under aerobic growth conditions in a multispecies system, the lipase inhibitory activity of rosmarinic acid was reduced. The selectivity of *P. aliciae,* its partitions and luteolin for bacterial attachment and biofilm formation could be attributed to their noteworthy eDNA inhibitory activity observed. The samples showed moderate toxicity towards HaCat cells, while luteolin exhibited a cytotoxic effect. The samples were tested for their wound closure activity, and *P. aliciae* ethanolic extract and its hexane and ethyl acetate liquid partitions displayed significant wound closure activity compared to the controls with a percentage wound closure of 13.6 ± 7.5%, 17.4 ± 7.3% and 19.3 ± 5.9% respectively. The *P. aliciae* ethanolic extract was selected for further preclinical and clinical studies and was formulated at 10% in a gel formulation with a final concentration of 600 µg/mL. The extract was confirmed to be non-mutagenic and has no *in vivo* irritancy when applied topically in its neat form or the gel formulation. *In vivo* efficacy studies confirmed that the formulated *P. aliciae* effectively reduced the number of comedones, blackheads, whiteheads, pustules, and micro cysts between 14 to 28 days of consecutive use twice a day. This is the first report of *P. aliciae* to treat various forms of non-inflammatory and inflammatory acne.

Gold nanoparticles of *P. aliciae,* rosmarinic acid and tetracycline were synthesised to determine if the activity of the samples could be improved. Although stable gold nanoparticles were formed, the samples had no improved antibacterial and inhibitory activity on bacterial attachment and biofilm formation. Nanoparticles of *P. aliciae* and rosmarinic acid were non-

toxic on HaCat cells, and statistically significant wound closure activity was observed for *P. aliciae* ($P < 0.001$) and rosmarinic acid ($P < 0.01$) nanoparticles. Wound closure was improved significantly ($P < 0.01$) when treated with rosmarinic acid nanoparticles as compared to the pure compound in its natural form, with a percentage wound closure activity of 24.4 ± 5.9% at 29.2 µg/mL. Therefore, synthesised gold nanoparticles of rosmarinic acid could potentially be considered for the development of a wound healing product. This is the first report of synthesised gold nanoparticles of rosmarinic acid for the treatment of wounds.

This study is also the first report of the synergistic quorum sensing relationship between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) in a multispecies system. This could be an indication that the pathogenic relationship between these bacteria is strain-specific and supports the hypothesis of the current study. To conclude, this is the first report of *P. aliciae*, its partitions and newly identified compound, luteolin, targeting quorum sensing processes of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) targeting AI-2, lipase, bacterial attachment and biofilm formation, inhibiting sessile bacteria within a mature biofilm, eDNA production and promoting wound closure that contributes to the novelty of the current project.

CHAPTER 1

General introduction and literature review on quorum sensing related to acne vulgaris

Chapter 1

This chapter forms part of the book "Medicinal Plants in Cosmetics, Health and Disease" by Prof Namrita Lall through the publisher Taylor and Francis, ISBN: 9780367622084.
Titled: Chapter 3: Phytomedicines targeting antibiotic resistance through quorum sensing and biofilm formation associated with acne vulgaris

Abstract

Acne affects 9% of the world's population, making it the eighth-most prevalent disease worldwide. Although not life-threatening, it has severe physiological implications, affecting the overall wellbeing of patients. The World Health Organization has raised awareness on the threat of antibiotic resistance to the global health system. Antibiotic resistance in bacteria is due to the ability of bacteria to form a biofilm. Quorum sensing is an inter-and-intraspecies form of communication through the release of signalling molecules. Once a critical concentration of the signalling molecules has been reached, it results in the expression and repression of target genes. This results in biofilm formation and the release of virulence factors. Acne-causing bacteria can release quorum-sensing molecules that could contribute to antibiotic resistance. This chapter discusses antibiotic resistance and quorum sensing targets associated with acne vulgaris. Furthermore, this chapter explores plants and their compounds to target antibiotic resistance associated with acne vulgaris. Among several plants and plant-derived compounds investigated, curcumin isolated from *Curcuma longa* and quercetin isolated from various plant species has shown significant antibacterial, autoinducer-2 and biofilm inhibitory activity against acne-causing bacteria. These compounds have the potential to be allies in targeting antibiotic resistance associated with acne vulgaris.

1. Introduction

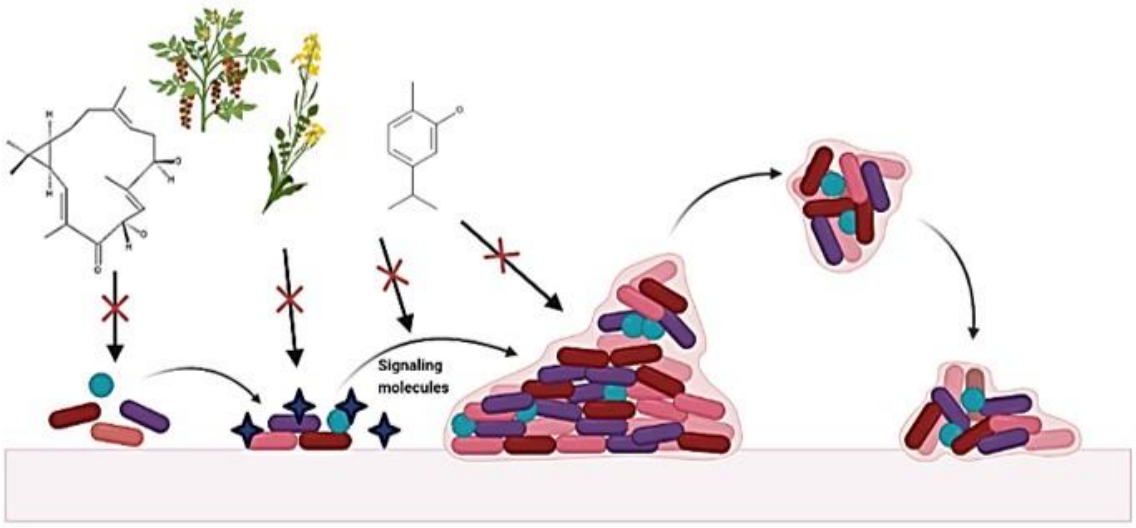

Figure 1. 1: Graphical abstract representing the content of the chapter.

1.1 Traditional medicine for skin diseases

Traditional medicine is used by nearly 80% of the world's population. In South Africa, approximately 45% of residents rely on traditional medicines each year as their main source of health care. This can be attributed to factors such as good accessibility to medicinal plants, affordability of traditional medicines compared to western medicines, extensive knowledge and expertise of ethnic groups and traditional healers on traditional medicines. Traditional medicine plays a significant role in managing skin disorders worldwide. In South Africa, rural communities use plants extensively for treating various skin maladies. Traditional healers in South Africa make use of indigenous plant species to treat various skin disorders and ailments such as burns, boils, rashes, wounds and acne. Treatment methods include herbal baths for treating rashes and itching skin. Traditional healers frequently use steam infused with medicinal plants to treat skin maladies such as acne vulgaris. There have been reported cases where traditional plant remedies have cleared acne after numerous months of unsuccessful treatment with western medicine. About 44.3% of the South African population had acne vulgaris in 2014. Of those experiencing acne, people having Fitzpatrick skin-types V-VI were most affected by the disease (Street et al., 2008; Williams et al., 2012; De Wet et al., 2013; Dlova et al., 2018). The pathogenesis of the disease, current treatments, side effects and potential alternative therapies have been discussed below.

1.2 Acne vulgaris, a global problem

Acne vulgaris affects about 9.4% of the world's population. Therefore, it is the eighth-most prevalent disease worldwide which is moderate to severe in 15-20% of patients. Acne vulgaris is commonly observed in the face, torso, and back, impacting the patient's visual appearance. While acne vulgaris not life-threatening, thus superficial disease can have detrimental effects on quality-of-life issues. For example, patients suffering from acne vulgaris are self-conscious about their appearance. This condition can affect one’s mental health, often spilling over into their daily lives and activities. Studies done by Yazici et al. (2004) reported the psychological impact of the disease on a patient's mental health. Patients who had acne had increased depression and anxiety levels compared to healthy patients (Yazici et al., 2004).

Furthermore, difficulties in academic and occupational fields have been observed with patients who have acne. Some people have shown a decreased career progression and an increase in unemployment since they are self-conscious about their appearance, thus affecting mood and productivity. Therefore, acne vulgaris should be acknowledged as a severe disorder (Gollnick, 2003; Tan and Bhate, 2015).

2. Acne vulgaris

Acne vulgaris is a disease related to the skin's pilosebaceous unit that includes the hair shaft, hair follicle and the sebaceous gland that produces sebum. Acne vulgaris predominantly occurs in the torso, back, neck and face. These areas are mostly affected due to the high amount of pilosebaceous units in these sebum-rich areas. These sebum-rich areas are more specifically found between the eyebrows known as the glabella, inside the ear on the sides of the nostrils and the cheeks, behind the ear and the manubrium that is the upper chest and back. Acne vulgaris is divided into four categories of severity: mild, moderate, moderately severe, and severe acne. (Kaur et al., 2005; Singh, Hatwar, and Nayak, 2011; Williams, Dellavalle, and Garner, 2012). James (2005) described the severities of acne as follows:

- Mild: Non-inflammatory blackheads and whiteheads, although some papules and pustules might be present (less than 10).
- Moderate: Between 10-40 inflamed papules, pustules and comedones present. Lesions can be found on the torso.

- Moderately severe: Between 40-100 papules, pustules and comedones present on the back torso and face. Up to five severe acne lesions such as nodules are present.
- Severe: Severe inflammation that includes cysts and nodules that are painful. Large pustules, papules and comedones present.

The types of non-inflammatory and inflammatory acne lesions are as follows (Table 1.2):

Table 1. 1: Types of non-inflammatory and inflammatory acne vulgaris (Singh, Hatwar and Nayak 2011).

TYPES	DESCRIPTION
Non-inflammatory acne	
Closed comedones (whiteheads)	Flesh or white-coloured papules less than 1 cm in diameter and slightly raised. No apparent follicular opening or associated erythema.
Open comedones (blackheads)	The follicular opening is dilated, and a black or dark-coloured comedone is visible.
Inflammatory acne	
Papule	Tender red blemishes can become severe forms of acne and cause scarring.
Pustule	Inflamed lesions with a puss-filled centre. Red and can become large.
Nodule	Firm lesions that are deeper in the follicle compared to pustules. Nodules are firm, painful lumps filled with puss.
Cyst	The most severe form of inflammatory acne that is difficult to treat and can be present for long periods of time. Scarring can easily occur. Cystic acne mainly develops from trying to pop inflamed papules, pustules and nodules.

2.1 Pathogenicity and microbiology of acne vulgaris

The pathogenesis of acne vulgaris is divided into four major etiological factors involved in the development of the disease (Burkhart and Burkhart, 2003):

- Hyperkeratinisation
- Increased sebum production
- The colonization and proliferation of *Cutibacterium acnes*
- Inflammation due to the immunological consequences of the microbiological factor

A range of theories about the sequence of these pathogenic factors and their contribution to the progression of acne vulgaris has not yet been fully answered. It is believed that follicular hyperkeratinisation (the abnormal shedding of the skin) is the first step in the pathogenic

pathway, blocking the pilosebaceous unit and leading to excess sebum accumulation. Comedone formation is a combined effect between the overproduction of keratinocytes and a reduction in the shedding of keratocytes due to the skin cells' inability to separate from one another. Comedones form when keratinocytes block the pilosebaceous unit resulting in the accumulation of sebum in the unit. Several factors contribute to hyperkeratinisation. These factors include the lack of linoleic acid in the sebum due to hyperseborrhea, resulting in abnormal keratinocyte differentiation. Cytokines such as cytokine interleukin-1 alpha (IL-1α) present in the follicle are believed to induce hyperkeratinisation. Furthermore, it was recently discovered that the acne-inducing bacteria, *Cutibacterium acnes* secrete a glycocalyx polymer that is a component of the bacterial biofilm. This secretion is incorporated in the sebum, which increases cohesion between the keratinocytes. This results in a blockage in the pilosebaceous unit and results in the formation of a comedone. It is, therefore, plausible that the initial step of acne pathogenesis could be the colonization and biofilm formation of *C. acnes* instead of hyperkeratinization (Pawin et al., 2004; Burkhart, and Morrell, 2009; Dessinioti and Katsambas, 2010).

Sebum production is hypothesized to be the second pathogenic factor in the pathway. Human sebum comprises several compounds that include cholesteryl esters, cholesterol, fatty acids, triglycerides, squalene, diglycerides, and wax esters. Androgens are a group of hormones present in males and females. These hormones can stimulate sebaceous secretion, and it is hypothesized that some acne patients can possess sebocytes that have an increased sensitivity to androgens. Sebum serves as a carbon and nutrient source for acne-causing bacteria such as *C. acnes*. The bacteria that proliferate in the pilosebaceous unit produce inflammatory and chemotactic mediators. For the bacteria to release these mediators, they attract neutrophils, which drive the inflammatory process (Pawin et al., 2004; Burkhart and Morrell, 2009; Bhate and Williams, 2013).

2.2 Microbiology of the skin and role in the pathogenesis of acne vulgaris

Sixty percent (60%) of the skin's microbiome involves three co-existing bacterial genera: *Corynebacterium*, *Staphylococci*, and the *Cutibacterium* (*Cutibacterium granulosum* and *Cutibacterium acnes*). The Gram-positive bacteria *Corynebacterium* and *Staphylococci* are aerobic bacteria that can be found in the epidermal (top) layer of the skin. The anaerobic diphtheroid *Cutibacterium* predominantly resides in the infra-infundibulum of the sebaceous follicle. Although all three genera have been isolated from the pilosebaceous unit, *Staphylococci*

and *Cutibacterium* species are predominantly isolated from acne lesions (Burkhart and Burkhart, 2003; Sanford and Gallo, 2013; Kumar et al., 2016).

The skin microenvironment is affected by several extrinsic and intrinsic factors, such as temperature, pH, sweat, sebum production and moisture, affecting how these microorganisms thrive. These microorganisms are not harmful to the skin. Theyplay an essential role in protecting the host and serve as the first line of defence against pathogens. Failure to control these resident microbes is linked to the onset of various skin diseases such as acne vulgaris. It is believed that microorganisms associated with the microflora of the skin co-exist to protect the host. However, change in this microbial balance due to changes in extrinsic and intrinsic factors contributes to various dermatological conditions. Microbial communities associated with the skin have become a subject of interest to understand cutaneous diseases better and develop novel therapies for these maladies (Sanford and Gallo, 2013).

Since *Staphylococcus epidermidis* and *C. acnes* are considered the primary cause of inflammatory acne and are predominantly isolated from acne lesions, the rest of the chapter will focus on these two bacteria.

Commensal bacteria, *S. epidermidis* and *C. acnes* play essential roles in protecting the skin from pathogenic strains of the same species or other pathogenic strains of microorganisms that form part of the skin microbiome in two ways, indirectly and directly. These commensal bacteria compete with pathogenic strains for resources such as space and nutrients, limiting the growth of harmful microorganism strains. Directly, commensal bacteria produce antimicrobial compounds called bacteriocins that directly restrict the growth of the pathogens. These proteinaceous factors inhibit the growth of closely related bacterial species but with no effect on the organism that produces them. *Staphylococcus epidermidis* is a skin commensal bacterium as well as an opportunistic pathogen, and it has been shown to cause serious infections in humans. The bacteria release several bacteriocins that inhibit the growth of various pathogens, including one within the same genus, *Staphylococcus aureus* (Sanford and Gallo, 2013).

Staphylococcus epidermidis produce *Enterococcus faecalis* surface proteins (Esp) that inhibit proteins involved in *S. aureus* biofilm production and growth. Serine protease enhances the antimicrobial activity of human beta-defensin-2 (hBD2) produced by keratinocytes due to inflammation but does not have antibacterial activity against Gram-positive bacteria alone. Furthermore, *S. epidermidis* releases antimicrobial molecules such as phenol-soluble modulins

(PSMs) that cause membrane leakage and targets mostly *Streptococcus pyogenes* and *S. aureus*. *Cutibacterium acnes*, another commensal skin bacterium, can protect the skin from pathogens such as methicillin-resistant *S. aureus* (MRSA). *Cutibacterium acnes* protects the skin from pathogens through fermenting glycerol, a product of sebum triglyceride hydrolysis, lowering the pH and inhibiting MRSA growth (Schröder and Harder, 1999; Sanford and Gallo, 2013).

Although these commensal bacteria protect the host and the skin, they can cause infection under certain conditions. *Cutibacterium acnes* is an opportunistic pathogen that plays an essential role in the advancement of inflammatory acne. These Gram-positive, non-motile, rod-shaped bacteria are aerotolerant, meaning they can grow under different oxygen tensions, although anaerobic conditions are preferred. *C. acnes* play a crucial function in acne vulgaris pathogenesis since enzymes produced by the bacterium aids in rupturing the follicular wall. *C. acnes* release three enzymes called hyaluronidases, lipases and proteases that contribute to the pathogenesis of the bacteria (Ingham, and Cunliffe, 1981; Falcocchio et al., 2006; Holland, Lee et al., 2010). Their role in acne vulgaris are as follows:

- The hyaluronidase enzyme produced by *C. acnes* breaks down intracellular substances within the wall of the pilosebaceous unit. This causes an increase in the permeability of the follicular epithelium and results in the diffusion of irritants into the dermis of the skin. Hyaluronidase acts as a spreading factor and increases the inflammatory response (Holland, Ingham, and Cunliffe, 1981).
- *Cutibacterium acnes* release proteases that stimulate cellular responses through the proteinase-activated receptor-2 (PAR-2) that mediates and contributes to inflammation associated with inflammatory acne (Lee et al., 2010).
- The bacteria release lipase enzymes, which hydrolyses sebum triacylglycerides into glycerol and free fatty acids like oleic and palmitic acid. These free fatty acids are chemotactic and irritate the sebaceous glands, contributing to the onset of inflammation (Falcocchio et al., 2006). Free fatty acids produced during sebum hydrolysis act as chemokines involved in producing pro-inflammatory cytokines. These cytokines include interleukin-1 beta (IL-1β), IL-6, IL-8, IL-12 and tumour necrosis factor-alpha (TNF-α), which subsequently elicits an inflammatory response that is associated with inflammatory acne (Burkhart and Burkhart, 2003; Kumar et al., 2016).

It has been hypothesized that *C. acnes* in other infections has been significantly underestimated due to the lack of understanding. *Cutibacterium acnes* is no longer only associated with acne vulgaris but also linked to various other diseases. For instance, *C. acnes* has been linked to biofilm-associated indwelling medical device infections, necro suppurative placentitis, and abortion in a Holstein cow (Ramanuj, Bachani, and Kothari, 2012).

Staphylococcus epidermidis is a facultative anaerobic bacterium of the skin that is harboured in acne lesions. Usually, *S. epidermidis* is a non-pathogenic bacterium of the human skin. However, it has been found to change into a pathogenic bacterium during a change in intrinsic factors, like an impaired immune system. During the onset of acne vulgaris, *S. epidermidis* protects itself from fatty acids that are bactericidal for the bacteria by releasing certain virulence factors. The virulence factor released by the bacteria is the fatty acid modifying enzyme (FAME) that esterifies these lipids to cholesterol which does not affect bacterial growth. Other virulence factors produced by the organism include delta-hemolysis and the lipase enzyme contributing to the inflammation seen in acne vulgaris (Kumar et al., 2016).

When evaluating acne lesions, Nishijima et al. (2000) found that when isolated alone, *C. acnes* and *S. epidermidis* were only associated with pustule formation in two out of thirty and one out of thirty acne lesions, respectively. *Cutibacterium acnes* and *S. epidermidis* was isolated together in 50% of acne lesions (15 out of 30). Twelve of these lesions were pustules, two papules and one a comedone. Although it is widely recognized that *C. acnes* is the only pathogenic bacteria involved in the development of acne vulgaris, *in vivo* studies have confirmed that *S. epidermidis* play a central role in inflammatory acne. Nishijima et al. (2000) reported that the bacteriology of acne lesions is not complicated and that acne lesions consist of only two bacteria, *C. acnes* and *S. epidermidis*. Failure to detect either one of these bacteria reflects a flaw in the methodology (Nishijima et al., 2000).

Studies done by Pathak et al. (2013) found that the population of *C. acnes* increased by 82%, while *S. epidermidis* increased by 70% in acne patients compared to the control, which consisted of non-acne patients (Pathak et al., 2013). In the test samples obtained from patients suffering from acne vulgaris, it was found that the microbial load increased simultaneously for both *C. acnes* and *S. epidermidis* (Packiavathy et al., 2012; Kumar et al., 2016). This increase in the bacterial load of both bacteria in acne lesions could indicate that these two bacteria are closely linked and could work together to develop and regulate inflammatory acne vulgaris.

3. Quorum sensing

Quorum sensing is a concentration-dependent communication mechanism between bacteria that results in the expression of genes involved in the social activities of these bacteria, such as the release of virulence factors, biofilm formation and motility. Microorganisms release chemical signalling molecules to communicate either with the same species (intraspecies) or different species (interspecies). Only once a specific concentration of the bacterial population and signalling molecule is reached in the environment does it result in gene expression. Microorganisms use this type of communication to differentiate self from non-self and as a measure to determine the population density in a specific environment. Bacteria use quorum sensing to detect the bacterial population density since individual bacteria cannot elicit a strong enough reaction compared to a large enough bacterial population to produce a more effective virulence response. These bacteria release signalling molecules called autoinducers to detect and respond to various environmental changes. Bacteria release molecules and autoinducers into the surrounding supernatant, where they can be detected. However, not all of these products can serve as a quorum-sensing signalling molecule (QSM). The QSM can be defined according to various criteria (Miller and Bassler, 2001; Winzer, Hardie, and Williams, 2002; Waters and Bassler, 2005):

- QSM production is stimulated because of physiological conditions, environmental factors or growth stages.
- QSMs accumulate in the biofilm's extracellular matrix, where bacteria receptors bind to them.
- A coordinated response is generated as a result of accumulating QSMs to a critical concentration threshold.
- The accumulation of the QSMs to a critical threshold concentration generates a combined response.
- The cellular response goes beyond the physiological changes needed for QSM to be metabolized or detoxified.

Bacteria have various receptors available to detect the accumulation of autoinducers, resulting in the alteration of gene expression. These activated genes encode either for symbiosis, biofilm

formation, virulence factor release, competence, motility, sporulation, antibiotic production, and conjugation (Miller and Bassler 2001; Waters and Bassler 2005).

Gram-negative and Gram-positive bacteria use quorum sensing to control various physiological activities. Acylated homoserine lactones (AHL) are used as autoinducers by Gram-negative bacteria, whereas Gram-positive bacteria use processed oligopeptide (AL) autoinducers during intraspecies communication (Bassler, 1999).

3.1 Gram-negative quorum-sensing

N-Acyl homoserine lactones (AHLs) are quorum sensing auto-inducers produced by Gram-negative bacteria. AHL is fatty acyl chains between four to eighteen carbons atoms long attached to an invariant homoserine lactone ring. Two regulatory proteins, namely LuxI and LuxR, are believed to control Gram-negative bacterial quorum sensing. LuxI AHL synthase enzymes biosynthesize AHL from S-adenosylmethionine (SAM) and an acylated acyl carrier protein (acyl-ACP). The membrane diffusible AHLs produced by LuxI bind to LuxR once a specific concentration is reached. The transcriptional activator protein LuxR promotes the transcription of the luxCDABE operon when bound to an autoinducer (Figure 1.2) (Bassler, 1999).

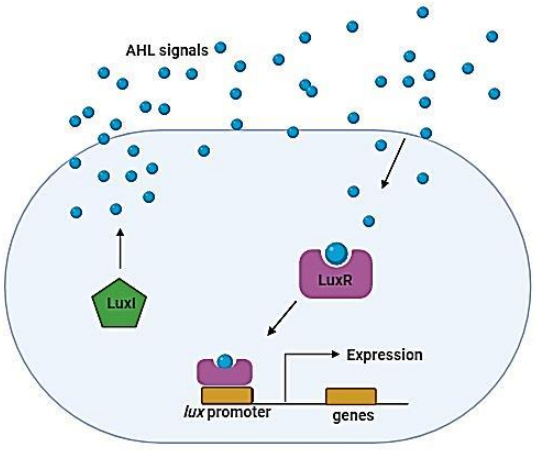

Figure 1. 2: Quorum sensing in Gram-negative bacteria. AHL: N-Acyl homoserine lactones. Figure adapted from (Bassler, 1999)

3.2 Gram-positive quorum-sensing

Gram-positive bacteria use a two-component communication system. Firstly, an adenosine triphosphate (ATP)-binding cassette known as ABC exporter protein transport processed AL molecules from the bacteria to the extracellular matrix. Once the peptide signalling molecules

reach a threshold concentration, the secreted peptide signals bind to a histidine kinase sensor protein on the bacterial membrane. Bassler (1999) reports that "autophosphorylation occurs on a conserved histidine residue, transferring the phosphoryl group to a cognate response regulator on a conserved aspartate residue". Following that, the phosphorylated response regulator binds to the *lux* promoters, suppressing or activating gene transcription (Figure 1.3) (Bassler, 1999).

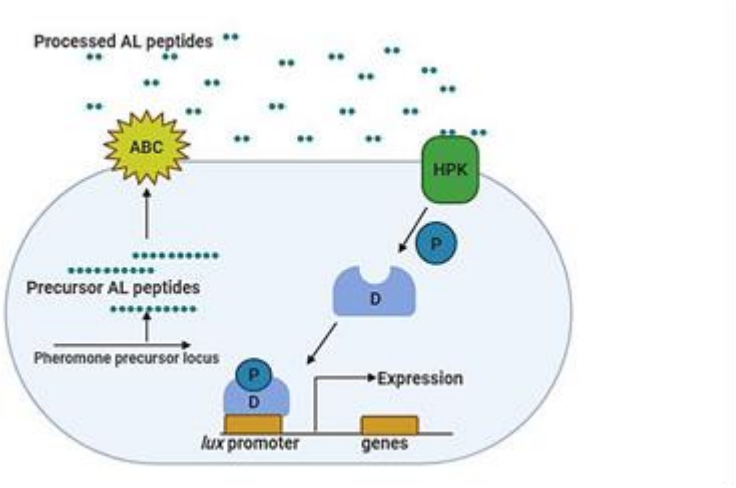

Figure 1. 3: Quorum sensing in Gram-positive bacteria. ABC: ABC exporter protein, HPK: histidine kinase, D: response regulator, P: phosphorylation. Figure adapted from (Bassler, 1999).

3.3 Interspecies quorum-sensing

Although many quorum-sensing signals are species-specific, interspecies communication has been studies intensively. The production and response to autoinducer-2 (AI-2) have been observed in various multispecies quorum sensing. During multispecies quorum sensing, one species of bacteria can produce AI-2 that affects the gene expression in another species of bacteria. This universal interspecies communication allows bacteria to change their behavior, and it affects social activities such as the release of virulent factors and biofilm formation between these different species. LuxS is a synthase enzyme widely conserved among Gram-positive and -negative bacteria and is required for AI-2 production. The AI-2 precursor, 5-dihydroxy-2,3-pentanedione (DPD), is produced from SAM through three enzymatic steps. S-adenosylmethionine serves as a methyl donor to form a toxic intermediate, S-adenosylhomocysteine (SAH). Subsequently, S-adenosylhomocysteine is hydrolyzed by 5'-methylthioadenosine/ *S*-adenosylhomocysteine nucleosidase (MTA/SAHase) also known as Pfs to yield S-ribosylhomocysteine (SRH) and adenine, reducing toxicity. The LuxS enzyme is

responsible for catalyzing the reaction of SRH to DPD. DPD is thought to rearrange further to produce AI-2. (Figure 1.4). The AI-2 sensor protein, LuxP, forms a LuxP-AI-2 complex that interacts with LuxQ, a hybrid sensor kinase that is located in the inner bacterial membrane to transfer the autoinducer signal. It is hypothesized that during low bacterial cell densities and therefore low AI-2 availability in *Vibrio harveyi*, LuxQ autophosphorylate results in the phosphorylation of the hybrid sensor kinase proteins LuxU and LuxO. The phosphorylation of LuxO prevents transcription of the *lux* operon. When a specific concentration of AI-2 is reached, the kinase proteins change to phosphatases resulting in dephosphorylation in the system and transcription of the *lux* operon commence. (Chen et al., 2002; Winans and Bassler. 2002; Winzer, Hardie, and Williams, 2002; Bassler and Miller, 2013; Pereira, Thompson, and Xavier, 2013).

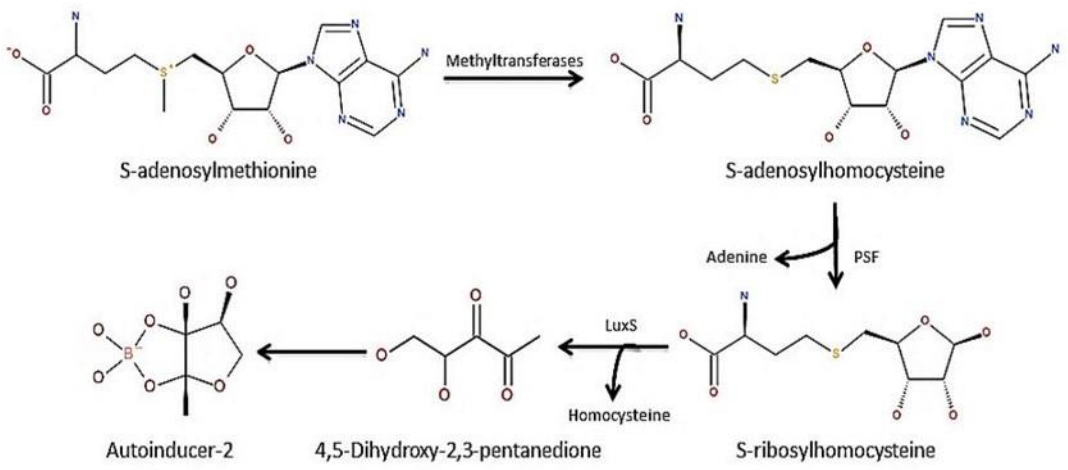

Figure 1. 4: The synthesis of autoinducer-2 (AI-2). Figure adapted from (Chen et al., 2002)

Quorum sensing can be disrupted by targeting various processes involved during the communication process. These mechanisms include (Kalia, 2013):

- Preventing the production of the signalling molecules
- Degrading the signalling molecule
- Interfering with the signal receptor

To date, few studies have been conducted on the quorum sensing mechanism of *C. acnes*. Studies done by Coenye et al. (2007) confirmed that sessile and planktonic *C. acnes* bacteria produce AI-2. However, AI-2 production was three times higher in mature biofilms than

planktonic cells. Considering these results, it was hypothesized that AI-2 could potentially contribute to the production of virulence factors as a result of virulence gene expression contributing to the pathogenesis of acne vulgaris (Coenye et al., 2007).

The quorum-sensing mechanism in *S. epidermidis* has been studied intensively. The accessory gene regulator (agr) quorum sensing mechanism controls modified peptides released as signals in *Staphylococci*. Previous genomic structures have confirmed the presence of the *luxS* homologs in the bacteria. Li et al. (2007) have confirmed that the LuxS/ AI-2 system regulates key *S. epidermidis* social activities and virulence factor release (Li et al., 2007).

4. Quorum sensing, biofilm formation and antibiotic resistance in acne vulgaris

4.1 Biofilm formation

In nature, most microorganisms do not exist as planktonic bacteria floating in a suspension but rather as a biofilm. Biofilms form when populations or communities of bacteria arrange in layers together with their decomposition and metabolic products and adhere to abiotic and biotic surfaces such as the wall of the pilosebaceous unit. After the bacteria have adhered to surfaces, they start to secrete polysaccharides in which these bacteria are encased. The extracellular polysaccharide is around 70% of the biofilm mass and is essential for biofilm architecture. The biofilm is comprised of four components: water, extracellular DNA (eDNA), extracellular polysaccharides and cellular byproducts. Extracellular DNA is produced due to quorum sensing and plays a role in biofilm formation, gene transfer, biofilm stabilization, and bacterial and biofilm attachment to a stratum. The extracellular polysaccharide is a glycocalyx polymer, which functions as a protective layer that prevents effective concentrations of antimicrobials from entering the microenvironment of the bacteria. Compared to planktonic bacteria, bacteria associated with a biofilm have altered growth and gene expression. It has been found that bacteria inside the biofilm is more resistant to antibiotics (50-500 times more) than planktonic bacteria not associated with a biofilm (Burkhart and Burkhart, 2003; Yarwood and Schlievert, 2003; Montanaro et al., 2011; Sandasi et al., 2011).

The formation of an established biofilm and distribution of the biofilm occurs in five stages, namely (Figure 1.5) (Unosson, 2015; Center for Biofilm Engineering: Montana State University, 2020):

- Reversible adherence of the planktonic bacteria to a substrate or surface.
- Irreversible adherence of biofilm, quorum sensing and formation of monolayer microcolonies and the production of the extracellular polysaccharide matrix.
- Mature biofilm formation starts and multilayer cell formation.
- Further maturation of the biofilm into a three-dimensional structure forming channels for the movement of nutrients.
- Biofilm reaches a critical mass and releases infectious planktonic bacteria and biofilm fragments to infect and colonize other substrata.

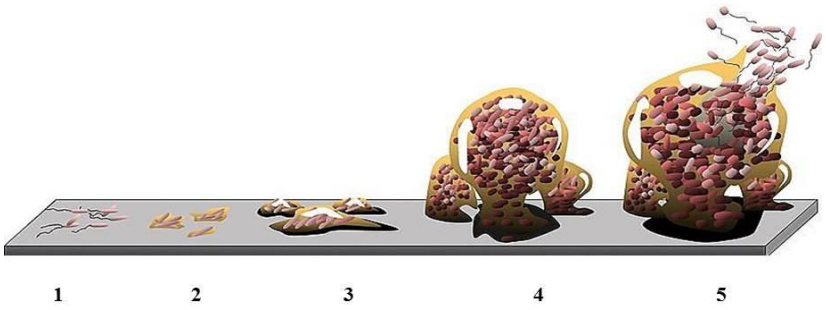

Figure 1. 5: The stages of biofilm formation (Monroe 2007).

There are several advantages for a microorganism to form a biofilm that includes (Burkhart and Burkhart, 2003; Sandasi et al., 2011):

- Gaining of new genetic material
- Metabolic cooperation
- Nutrient availability
- Environmental protection whereby the biofilm community is protected and less susceptible to antimicrobials.

Antibiotic resistance can be defined as when the minimum inhibitory concentration against the targeted bacteria is higher than the concentration of the active that is available *in vivo*. Therefore, antibiotic resistance is when antibiotics cannot inhibit the microorganisms they previously were able to inhibit. The antibiotic resistance observed in biofilms could be due to two mechanisms: a reduction in the diffusion of antibiotics through the biofilm and a reduction in bacterial

metabolism of the bacteria within the biofilm (Brandwein, Steinberg, and Meshner, 2016). Quorum sensing regulates genes involved in biofilm formation and dispersion; hence biofilm formation and quorum sensing are linked.

4.2 *Cutibacterium acnes* quorum sensing and biofilm formation

Quorum sensing in *C. acnes* is still relatively unexplored, however, Coenye, Peeters, and Nelis (2007) reported the presence of the PPA0450 gene that encodes a LuxS homolog in *Vibrio harveyi*. The researchers demonstrated the release of lipase and AI-2 by *C. acnes* that were three times higher in mature biofilms of the bacteria than in planktonic cultures. This finding suggests that AI-2 in *C. acnes* could contribute to the release of virulence factors and biofilm formation in the species (Coenye et al., 2007) -.

Antibacterial agents play a significant role in treating acne vulgaris. Azelaic acid, benzoyl peroxide, clindamycin, erythromycin, tetracycline, triclosan, and combinations of these drugs are examples of topical antibacterial agents used to treat acne vulgaris. However, recent studies have found an increase in antibiotic-resistant *C. acnes* strains in patients suffering from persistent acne vulgaris. The ability of *C. acnes* could contribute to antibiotic resistance reported in the disease. The sequenced genome of *C. acnes* revealed that the bacterial genome contains genes for biofilm formation. The genome of *C. acnes* has been found to contain genes coding to produce glucosyltransferases. The biofilm extracellular polysaccharide matrix is made up of dextran (glucan), which is formed by glucosyltransferases. In addition, several genes that encode for adhesion proteins and a LuxS involved in interspecies communication through AI-2 have been identified in the genome of *C. acnes* (Coenye et al., 2008).

The establishment of the *C. acnes* biofilm in the pilosebaceous unit has been confirmed in recent *in vivo* investigations. Results have shown that *C. acnes* biofilm formation is not exclusively associated with the anaerobic portions of the skin. In some cases, *C. acnes* reside in the stratum corneum of the epidermis, between 80-1900 µm deep. More mature biofilms can be found in the deeper layer of the epidermis. Jahns and Alexeyev (2014) identified four patterns of how *C. acnes* biofilms occur in the pilosebaceous unit (Jahns and Alexeyev, 2014):

- *Cutibacterium acnes* attachment to the pilosebaceous wall
- Attachment of the biofilm to the hair shaft
- Spreading of the biofilm over the entire lumen

- Biofilms are not attached to the pilosebaceous wall but occur in the center of the pilosebaceous unit.

4.3 *Staphylococcus epidermidis* quorum sensing and biofilm formation

The agr system is involved in quorum sensing in *Staphylococci* and increases bacterial pathogenicity and penetration into surrounding tissue. The agr system triggers the expression of accessory factors (Figure 1.6). There are two main components to the agr system. Firstly, a two-part signal transduction pathway detects the number of autoinducer peptides (AIP) in the environment. The AgrC and AgrA complex controls the signal transduction pathway. Once a critical concentration of these AIP is reached outside the bacterial cell wall it binds to AgrC to elicit a response. Upon binding with AIP in the external environment, AgrC activates the DNA binding protein, AgrA, binds to the agr intergenic region (agr-IR) and upregulates the expression of the promoters P2 for the transcription of RNAII and P3 for the transcription of RNAIII. Secondly, the agr system has an AI production side that includes AgrD and AgrB. RNAII encodes the genes *agrB*, *agrD*, *agrC* and *agrA*. The production of AI is under the regulation of genes *agrB* and *agrD.* The *agrD* gene encodes a seven to nine amino acid long AgrD autoinducer peptide (AIP precursor) trimmed by the enzyme AgrB, encoded by the *agrB* gene, into a ring structure. This structure contains thiolactone and is exported by the enzyme to the external environment (Vuong et al., 2003; Kong, Vuong, and Otto, 2006; Le and Otto, 2015).

RNAIII plays a role in the transcription of several virulent factors associated with the bacteria. RNAIII contains the *hld* gene that codes for the phenol-soluble modulin, delta toxin. These delta toxins play a role in the detachment of bacteria from the biofilm. This detachment could contribute to spreading the bacteria, establishing biofilms, and releasing virulence factors such as hemolysins, protease, and lipase enzymes associated with quorum sensing and various diseases. In addition, the RNAIII gene has been found to prevent the translation of the repressor of toxin protein (Rot) that results in the upregulation of enzymes such as lipase and protease involved in acne vulgaris (Vuong et al., 2003; Kong, Vuong, and Otto, 2006; Le and Otto, 2015).

Staphylococcus epidermidis virulence is under the LuxS/ AI-2 control. Several studies have investigated the role of AI-2 in *S. epidermidis* virulence and have confirmed that AI-2 acts as a signalling molecule in the species. However, the role of AI-2 in biofilm formation seems to be strain-specific. In contrasting studies, Xu et al. (2006) found that *S. epidermidis* strain 1457 does not form a mature biofilm compared to the luxS mutant strain that formed a biofilm while

confirming the formation of mature biofilm in the presence of AI-2 in *S. epidermidis* strain RP62A (ATCC 35984) (Xu et al., (2006). Studies have demonstrated that *Staphylococci* species harbouring the biofilm-associated protein (*bap*) gene can successfully attach to services and form a well-established mature biofilm. *Staphylococcus epidermidis* strain RP62A has been found to contain the Bap-homologous protein (*bhp*) gene that is upregulated in the presence of AI-2 to produce more biofilm compared to the wild type strain with no addition of AI-2 (Von Eiff, Peters, and Heilmann, 2002; Xue et al., 2015).

S. epidermidis uses biofilm formation as one of its main virulence mechanisms. The intracellular adhesion (icaA) gene encodes for the polysaccharide intracellular adhesion (PIA) molecule that regulates biofilm formation in *S. epidermidis*. PIA functions as an adhesion molecule for the bacteria to attach to the skin surface of the host. Several other adhesion factors are produced by *S. epidermidis*, including surface fibrinogen binding proteins, anchored proteins, autolysin proteins and poly-N-succinyl-glucosamine. An *in vivo* study done by Jahns and Alexeyev (2014) observed biofilm-like colonization of *Staphylococci* in areas close to the skin surface. This observation could indicate that *Staphylococci* not only form biofilms but could potentially contribute to antibiotic resistance in acne vulgaris (Jahns and Alexeyev, 2014).

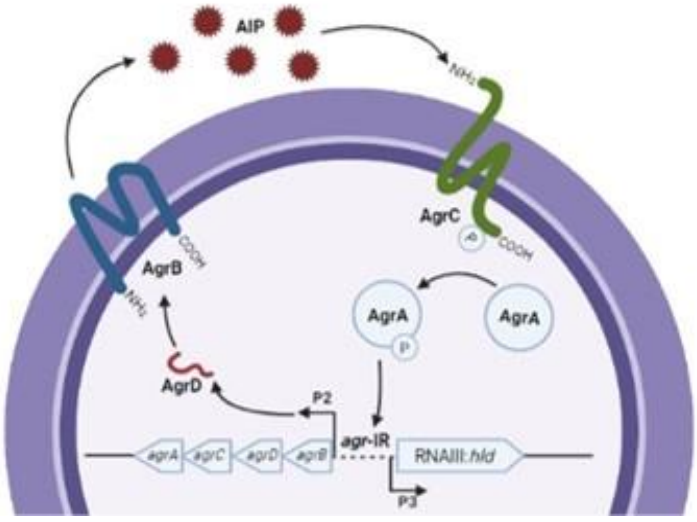

Figure 1. 6: The accessory gene regulator system in *Staphylococcus* species (Painter et al., 2014).

5. Plants and pure compounds targeting quorum sensing and biofilm formation

Current research focuses on plants and their compounds to inhibit bacterial growth, biofilm growth, and other virulence factors associated with acne vulgaris. However, there is a paucity of research using plants and plant-based compounds to target aspects of bacterial infections as a whole or against quorum sensing, which is hypothesized to be the root of numerous infections, including acne vulgaris. Furthermore, research on plants targeting acne-causing bacterial biofilms and quorum sensing is currently limited and more research in these areas is required (Table 1.3 and 1.4) (Figure 1.7 and 1.8) (Van Vuuren and Holl, 2017).

Figure 1. 7: Plants having quorum quenching and biofilm inhibitory activity against acne-causing bacteria. A, *Epimedium diphyllum*; B, *Malus pumila*; C, *Murtus communis*; D, *Polygonum cuspidatum*; E, *Rhodiola rosea*; F, *Origanum vulgare*; G, *Rosmarinus officinalis*; H, *Bauhinia kockiana* (Dessi, 2006; Qwert1234, 2016; Superior National Forest, 2010; Sehrg, 2011; Alpsdake, 2015; Friviere, 2019a, 2019b; Mokkie, 2020).

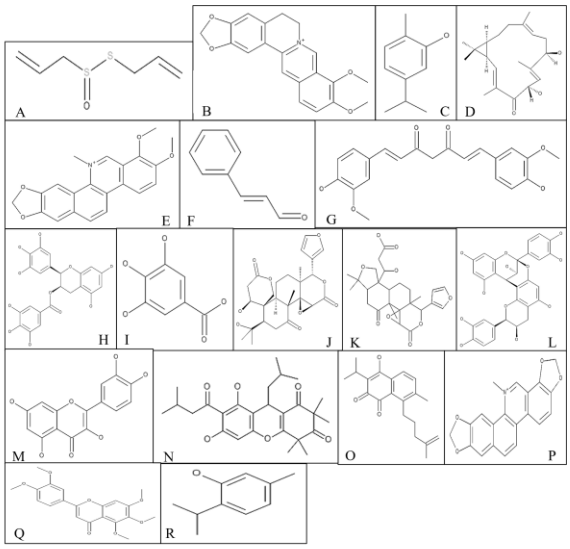

Figure 1. 8: Compounds with quorum quenching and biofilm inhibition potential against acne-causing bacteria. A, Allicin; B, Berberine; C, Carvacrol; D, Casbane diterpene; E, Chelerythrine; F, Cinnamaldehyde; G, Curcumin; H, Epigallocatechin gallate; I, Gallic acid; J, Ichangin; K, Isolimonic acid; L, Proanthocyanidin-A2; M, Quercetin; N, Rhodomyrtone; O, Salvipison; P, Sanguinarine; Q, Sinensetin; R, Thymol.

Table 1. 2*:* Quorum quenching and biofilm inhibitory potential of plants and their compounds against the *a*cne-causing bacteria *Cutibacterium acnes* and *Staphylococcus epidermidis.*

SCIENTIFIC NAMES AND PLANT PARTS USED	TRADITIONAL USE	ACTIVE COMPOUNDS AGAINST *C. ACNES*	QUORUM QUENCHING ACTIVITY	BIOFILM INHIBITORY ACTIVITY	OTHER TARGETS	REFERENCES
Epimedium brevicornum Plant	Impotence, numbness, rheumatic pain and seminal emission	Icariin	None reported	*E. brevicornum* extract inhibited *C. acnes* biofilm growth at sub-MIC values. Icariin inhibited 40-70% of *C. acnes* biofilm formation at a concentration between 0.01–0.08% (w/v).	*E. brevicornum* extract inhibited *C. acnes* planktonic growth at a MIC of 0.625% (w/v). Icariin displayed a MIC of 2.5% (w/v) against *C. acnes.*	(Yu et al., 2011; Coenye et al., 2012)
Malus pumila Fruit	Skincare and respiratory ailments such as coughs.	Polyphenols	None reported	The extract effectively inhibited *C. acnes* biofilm at a concentration of 0.05% (w/v).	Inhibited planktonic *C. acnes* at a MIC of 0.04% (w/v).	(Coenye et al., 2012; Korkmaz and Karakuş, 2015)
Myrtus communis Leaves and stems	Bleeding, conjunctivitis, diarrhea, epistaxis, excessive perspiration, hemorrhoids, headaches, inflammation, leucorrhoea, palpitation, peptic ulcers, pulmonary diseases, skin diseases and urethritis	5-acetoxy-4-hydroxy-4-isobutyl-2,2,6,6-tetramethylcyclohexan-1,3-dione and isomyrtucommulone-B	None reported	*M. communis* inhibited *C. acnes* biofilm formation at a concentration ranging between 0.03 to 0.0001% in a dose-dependent manner. At 0.001%, *M. communis* combined with 1000mg/L erythromycin and or 500 mg/L clindamycin significantly inhibited *C. acnes* biofilm formation.	Leaf extract inhibited *C. acnes* growth with a MIC of 7.8 µg/mL. Noteworthy antioxidant activity was observed for *M. communism* acetone leaf extract against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP).	(Alipour, Dashti, and Hosseinzadeh, 2014; Feuillolay et al., 2016; Abood, Addai, and Abood, 2016; Hamdy et al., 2017)
Polygonum cuspidatum	Traditionally used as an antipyretic,	Resveratrol	None reported	*P. cuspidatum* effectively inhibited *C. acnes* biofilm at	*P. cuspidatum* extract inhibited *C. acnes*	(Coenye et al., 2012; Wu et al., 2012; Peng

Rhizome	expectorant, analgesic and as a diuretic. It is also used to treat inflammation, skin burns and infection, jaundice and high cholesterol			0.5% (w/v). Resveratrol inhibited 80% of *C. acnes* biofilm at 0.32% (w/v). Resveratrol enhanced *S. epidermidis* biofilms 1.5 fold at a concentration of 100 μg/mL.	planktonic growth at a MIC of 0.625% (w/v). Resveratrol inhibited *C. acnes* planktonic bacteria at a MIC of 1.25% (w/v). Resveratol does not inhibit planktonic *S. epidermidis* at the highest concentration tested of 2 mg/mL.	et al., 2013; Morán et al., 2014; Ta and Arnason, 2016)
Rhodiola crenulata Root	Traditionally used to treat mountain sickness and lung damage.	Salidroside	None reported	*R. crenulat* effectively inhibited *C. acnes* biofilm at 0.5% (w/v). Salidroside inhibited *C. acnes* biofilms between 20 and 40% in a concentration range of 2.5-0.02% (w/v).	*R. crenulat* extract inhibited *C. acnes* planktonic growth at a MIC of 0.156% (w/v). No inhibition of *C. acnes* planktonic bacteria was observed for Salidroside at the highest concentration tested of 2.5% (w/v).	(Coenye et al., 2012; Lee et al., 2013)
Origanum vulgare L. Essential oil	Traditionally used to treat cutaneous skin infections and respiratory ailments and help promote appetite and as an antispasmodic treatment	Carvacrol and thymol	None reported	At 1/2MIC *O. vulgare* essential oil inhibited 35% of *S. epidermidis* biofilm formation and displayed a biofilm inhibitory concentration at 0.125% (v/v)	*O. vulgare* essential oil inhibited *S. epidermidis* planktonic bacteria at a MIC of 0.125% (v/v)	(Nostro et al., 2007; Vale-Silva et al., 2012)
Rosmarinus officinalis	Traditionally used to relieve flatulence, indigestion, pain,	Rosmarinic acid (phenolic compound)	None reported	Prevents *C. acnes* adhesion and has the potential for *C. acnes* biofilm disruption	Inhibits planktonic *C. acnes* at a MIC of 4 mg/mL	(Minaiyan et al., 2011; Tsai et al., 2013)

	inflammation, rheumatoid arthritis, relieve muscle spasms and is used as a choleretic treatment.			starting at 60 μg/mL		
Bauhinia acuruana Branches and fruit	Traditionally used to treat respiratory ailments such as tuberculosis, various skin disorders and urinary tract infections.	Traditionally used as a tonic, as a depurative and to treat diabetes	None reported	At a concentration of 4 mg/mL, 18.3% *S. epidermidis* biofilm formation occurred of a branch extract and 22.2% in the presence of the fruit extract.	Bactericidal against *S. epidermidis* at 1 mg/mL	(Trentin et al., 2011)

Table 1. 3: Quorum quenching and biofilm inhibitory potential of isolated compounds against the acne-causing bacteria *Cutibacterium acnes* and *Staphylococcus epidermidis*.

COMPOUNDS	PLANT ISOLATED FROM AND PLANT PART	QUORUM QUENCHING ACTIVITY	BIOFILM INHIBITORY ACTIVITY	OTHER TARGETS	REFERENCES
Allicin	*Allium sativum* L.	None reported	Inhibits more than 90% of *S. epidermidis* biofilm at 4 mg/mL. In combination with vancomycin at 4 mg/mL inhibited *S. epidermidis* biofilms in prosthetic joints of a rabbit model.	Inhibits methicillin-susceptible and methicillin-resistant strains of *S. epidermidis* at a MIC of 8 μg/mL	(Pérez-Giraldo et al., 2003; Zhai et al., 2014; Ta and Arnason, 2016)
Berberine	*Berberidacea*e and other species	None reported	Inhibits 50% of *S. epidermidis* biofilms at 30 and 45 μg/mL,	Berberine inhibited planktonic *S. epidermidis* at	(Wang et al., 2009; Ta

			strain-dependent.	MIC values of 64 μg/mL and 128 μg/mL depending on the strain	and Arnason, 2016)
Carvacrol	*Origanum vulgare* L. Essential oil	None reported	At 1/2 MIC carvacrol inhibited 29% of *S. epidermidis* biofilm formation and displayed a biofilm inhibitory concentration at 0.125% (v/v)	Carvacrol inhibited *S. epidermidis* planktonic bacteria at a MIC of 0.031% (v/v)	(Nostro et al., 2007)
Casbane diterpene	*Croton nepetaefolius*	None reported	*S. epidermidis* biofilm disruption at a concentration above 250 μg/mL	Inhibits planktonic S. epidermidis at concentrations above 62.4 μg/mL	(Carneiro et al., 2011)
Chelerythrine	Papaveraceae species	None reported	Inhibits 50% of *S. epidermidis* biofilms at 8.6 μM	Chelerythrine inhibited planktonic *S. epidermidis* at a MIC range of 16.3-32.6 μM	(Artini et al., 2012; Ta and Arnason, 2016)
Cinnamaldehyde	*Curcuma longa* Rhizome	Inhibit of 65% *Vibrio harveyi* AI-2-mediated bioluminescence at a concentration of 100 μM	In combination with curcumin, effective *S. epidermidis* biofilm formation inhibition was observed at a concentration of 15.62 μg/mL.	Inhibits *S. epidermidis* planktonic growth at 125 μg/mL. In combination with curcumin have a combined antibacterial effect against *S. epidermidis* at a MIC of 31.25 μg/mL.	(Brackman et al., 2008; Sharma et al., 2014; Ta and Arnason, 2016)
Curcumin	*Curcuma longa* L.	Inhibition of 88% *Vibrio harveyi* bioluminescence at 100 μg/mL	Inhibits *S. epidermidis* biofilm formation at a MIC of 25 μg/mL.	Curcumin significantly inhibited *C. acnes* bacteria after treatment with blue light at a concentration of 1.52 μM. Curcumin as a lipid vesicle significantly inhibited *C. acnes* growth at a concentration of 0.43 μg/mL.	(Sharma et al., 2014; Liu and Huang, 2013; Ta and Arnason, 2016; Q. Yu et al., 2016; Yang et al., 2018)

Epigallocatechin gallate	*Camellia sinensis*	Inhibition of AI-2 at sub-MIC concentrations inhibit biofilm formation of *Eikenella corrodens*	None reported	Targets sebum production through the AMPK-SREBP-1 pathway. Furthermore, it inhibits *C. acnes* bacteria *in vitro*	(Matsunaga et al., 2010; Yoon et al., 2013)
Gallic acid	Various species	None reported	Increase *S. epidermidis* biofilm by threefold at a concentration of 188 μg/mL.	Does not inhibit planktonic *S. epidermidis* at the highest concentration tested of 2 mg/mL.	(Morán et al., 2014; Ta and Arnason, 2016)
Isolimonic acid	*Citrus aurantium* L. Seeds	Inhibited 99.23% *Vibrio harveyi* AI-2-mediated bioluminescence at a concentration of 100 μg/mL	None reported	No activity reported against *C. acnes*	(Vikram et al., 2011; Ta and Arnason, 2016)
Ichangin	*Citrus aurantium* L. Seeds	Inhibited 90% *Vibrio harveyi* AI-2-mediated bioluminescence at a concentration of 100 μg/mL	None reported	No activity reported against *C. acnes*	(Vikram et al., 2011; Ta and Arnason, 2016)
Proanthocyanidins A2-phosphatidylCholine	*Aesculus hippocastanum*	None reported	Inhibits 50% of *S. epidermidis* biofilms at 7.6 μM	Does not inhibit planktonic bacteria of *S. epidermidis* at the highest concentration tested of 168.7 μM	(Artini et al., 2012)
Quercetin	Various species	Inhibited 75% *Vibrio harveyi* AI-2-mediated bioluminescence	None reported	Effectively inhibits *C. acnes* at a concentration less than or equal to 64 μg/mL.	(Lim, Kim, and Seo, 2007; Vikram et al., 2010; Ta and Arnason,

		at a concentration of 6.25 µg/mL			2016)
Rhodomyrtone	*Rhodomyrtus tomentosa* Leaves	None reported	Significant *C. acnes* biofilm inhibition at 1/16 MIC. At 4MIC and 8MIC 40-80% of viable *C. acnes* in a mature biofilm is inhibited.	Inhibits planktonic *C. acnes* at MIC values ranging between 0.125-0.5 µg/mL Significant *C. acnes* lipase and protease inhibition at ½MIC.	(Wunnoo, Saising, and Voravuthikunchai, 2017)
Salvipisone	*Salvia sclarea* L. Roots	None reported	Reduce *S. epidermidis* biofilm between 90 and 85 % at 37.5 µg/mL depending on the strain.	Inhibits *S. epidermidis* planktonic bacteria between 9.37 – 37.5 µg/mL depending on the strain.	(Kuźma et al., 2007; Walencka et al., 2007; Ta and Arnason, 2016)
Sinensetin	*Orthosiphon stamineus*	Significant *Vibrio harveyi* AI-2-mediated bioluminescence observed at 100 µg/mL	None reported	No activity reported against *C. acnes*	(Vikram et al., 2010)
Sanguinarine	*Papaveraceae* species	None reported	Inhibits 50% of *S. epidermidis* biofilms at 5 µM	Sanguinarine inhibited planktonic *S. epidermidis* at a MIC range of 8.5-17 µM	(Artini et al., 2012; Ta and Arnason, 2016)

6. Conclusion

Acne vulgaris is a disease that has been studied extensively. However, understanding quorum sensing and multispecies biofilm formation related to the disease remain largely elusive. Furthermore, although there are drugs available on the market and plant-based therapy research for acne vulgaris, these therapies target the pathogenic factors associated with acne vulgaris. Currently, there is no acne therapy on the market that targets quorum sensing and multispecies biofilms associated with antibiotic-resistant acne vulgaris. Research on plants that target quorum sensing, antibiotic resistance, and virulence factor release only focuses on one aspect of quorum sensing. More research in the field is required to investigate and better understand multispecies acne vulgaris, the bacterial interactions, the natural environments of these bacteria and the plants that have the potential to treat this disease.

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CHAPTER 2

Traditional usage and biological activity of *Plectranthus madagascariensis* and its varieties: a review

Chapter 2

This chapter has been accepted in the Journal of Ethnopharmacology
Impact factor: 4.36
Titled: Traditional usage and biological activity of *Plectranthus madagascariensis* and its varieties: a review

Abstract

Plectranthus madagascariensis (Pers.) Benth. is an indigenous aromatic South African plant species that are used to treat various dermatological and respiratory ailments. Three varieties of *P. madagascariensis* exist in South Africa, namely, *Plectranthus aliciae* (Codd) van Jaarsv. & T.J. Edwards, *Plectranthus ramosior* (Benth.) Van Jaarsv. and *Plectranthus madagascariensis* (Pers.) Benth var. *madagascariensis*. This article summarizes the documented ethnobotanical uses and research which has been conducted to date on the chemical constituents and biological effects of *P. madagascariensis* and its varieties. This review aimed to investigate and highlight the lack scientific reports of the potential activity of these varieties based on their traditional usage and to emphasise the need for further investigation of the benefits of *P. madagascariensis* and its varieties.Extensive database retrieval using platforms not limited to but including Google Scholar, ScienceDirect and PubMed, was performed by using keywords such as "*Plectranthus madagascariensis*" "*Plectranthus madagascariensis* var. aliciae", "*Plectranthus* aliciae", "*Plectranthus ramosior*", "*Plectranthus madagascariensis* var. ramosior" and "*Plectranthus hirtus"* In addition, relevant books and digital documentation were consulted to collect all available scientific literature to provide a comprehensive review. Several studies have reported on the traditional usage of *P. madagascariensis* for the treatment of diseases related to the respiratory system such as coughs, colds and asthma and dermatological disorders related to wounds and inflammation. No records were found on the traditional usage of *P. madagascariensis* varieties to treat other maladies, however, *P. ramosior* has been reported to be used as a toxin for fish. In literature, seven major phytochemical compounds have been identified in *P. madagascariensis*. Its extract and essential oil have been reported to have polyphenols, abietane diterpenes and abietane diterpenes with a quinone moiety as constituents. *Plectranthus madagascariensis* and its major phytochemicals have been reported to target various biological targets. The report on the antibacterial activity of *P.*

madagascariensis against tuberculosis and wound infections has been consistent and correlates with its documented traditional usage of the plant. Literature has been found on the antibacterial activity of *P. aliciae* targeting bacteria associated with wound infections and lung cancer cells. No further literature on the biological activity of the other *P. madagascariensis* varieties has been found. Other noteworthy biological activities reported in the literature of *P. madagascariensis* and identified phytochemicals include their activities against Alzheimer's disease and cancer, especially against breast cancer and this has not been linked to the traditional usage of the plant. *Plectranthus madagascariensis* and its compounds have shown to be effective in treating a range of maladies. Based on the extensive literature on this plant, it can be concluded that numerous *in vitro* pharmacological activities of *P. madagascariensis* have been reported. However, there is a lack of information available for this species with regards to its *in vivo* data including both pre-clinical and clinical studies. Since the extract of *P. madagascariensis* and its isolated compounds have displayed noteworthy anticancer potential, we recommend further investigation of pharmacokinetic studies to be included in future research.

1. Introduction

Plectranthus madagascariensis (Pers.) Benth. is a semi-succulent, aromatic, ground cover plant belonging to the Lamiaceae family. It grows to one meter in height and is widely distributed in KwaZulu-Natal and the Eastern Cape where it occurs on dry, rocky outcrops, forest margins and shaded subtropical thickets. The oppositely arranged oval leaves are distinctively variegated with white margins that are toothed at the edges (three to seven teeth). The leaves are slightly hairy above and below the blade that is between 35-40 mm long. The inflorescence is a 125 mm erect raceme with four to six flowers at each node. Between February and November small white, mauve or purple tubular flowers between seven and 18 mm long emerge. The small seeds are 1 mm in diameter. The genus largely relies on pollination by various fly species such the *Stenobasipteron* spp and bee species such as *Pachymelus limbatus* and *Amegilla caelestina*. Butterflies rarely pollinate *P. madagascariensis* and that the species is not dependent on this relationship (Van Jaarsveld and Edwards, 1997; Potgieter et al., 1999; Harrower, 2014; Latti, 2019; Random Harvest, 2020).

It is difficult to distinguish between *Plectranthus* species and its closely related species due to similarities in morphological features that can be identified between the various species. This has resulted in taxonomic difficulties related to the naming of various species belonging to the *Plectranthus* genera. In South Africa, *Plectranthus madagascariensis* (Pers.) Benth var. *madagascariensis*, syn. *Plectranthus hirtus* Benth. is classified as an indigenous South African plant and a variety of *P. madagascariensis* (Pers.) Benth from which *P. madagascariensis* 'Lynne' is a well-known cultivar. The endemic *Plectranthus aliciae* (Codd) van Jaarsv. & T.J. Edwards (syn. *Plectranthus madagascariensis* var. aliciae Codd) and *Plectranthus ramosior* (Benth.) Van Jaarsv., (syn. *Plectranthus madagascariensis* var. *ramosior* Benth its synonym *Plectranthus hadiensis* (Forssk.) Schweinf. ex Sprenger and *Plectranthus madagascariensis* (Pers.) Benth.) are considered varieties of *P. madagascariensis*. Although substantial literature on *Plectranthus madagascariensis* is available, articles documenting the differences and similarities amongst the three varieties of *P. madagascariensis* are in limited supply. Morphological similarities between these plants could contribute to misidentification of the plant varieties, resulting in inaccurate referencing *P. madagascariensis* (Codd, 1975; Foden and Potter, 2005a, 2005b; Lukhoba et al., 2006; Matlamela and Kamundi, 2006; The Plant List, 2012).

This study summarizes and reviews the documented ethnobotanical uses and research that had been conducted to date on the chemical constituents and biological effects of *P. madagascariensis* and its varieties.

1.1. Traditional medicinal uses

Rice et al. (2011) reported that *Plectranthus* species from southern Africa is used to treat 10 out of 13 disease hallmarks as reported by Cook (1995). Traditional use of *Plectranthus* species include treating diseases associated with the respiratory system, the central nervous system, the skin, the digestive system, the liver and is also used in the treatment of infections, pain, inflammation, fever and cancer (Matias et al., 2019c). Lukhoba et al. (2006) reported the traditional and ethnobotanical use of 62 *Plectranthus* species in Africa, America, Asia, Australasia and the pacific. The top three uses of *Plectranthus* species consists of digestive, respiratory, and skin diseases. However, other uses include pain, inflammation, infections and fevers, muscle contractions, neurological disorders and blood circulation. *Plectranthus* species are divided into two clades namely Clade 1 (formerly related to the *Coleus* genus) that are known for their medicinal potential and Clade 2 (*Plectranthus* Clade). Three hundred

Plectranthus species are found in Africa of which 50 can be found in southern Africa and Madagascar, 25% of which are from Clade 1. Twelve *Plectranthus* species are traditionally used in southern Africa. However, synonyms are highly prevalent in the genus making it extremely difficult to gather and combine information on the ethnobotanical use of the genus (Lukhoba et al., 2006; Rice et al., 2011). It is, therefore, possible that certain traditional knowledge has been lost or that information was incorrectly reported for a specific *Plectranthus* species. The possibility should not be excluded that reported traditional uses of *P. madagascariensis* are the only traditional usage of the species and the traditional usage of the synonymous species and their varieties should be taken into consideration.

Traditionally *P. madagascariensis* is used by the Zulu and Xhosa communities to treat respiratory and dermatological disorders. For respiratory problems such as coughs, colds and asthma, the whole plant including the roots are used and administered either as an enema or a decoction (plant material is boiled with water) and an infusion (boiling water is added to the plant material) that can be administered orally. Cutaneous wounds and scabies are treated with crushed leaves applied directly to the affected area (Hutchings et al., 1996; Rabe and van Staden, 1998; Rice et al., 2011; Pereira et al., 2015). Mahomoodally and Priyamka (2014) reported the traditional usage of *P. madagascariensis* as a popular folk medicine in Mauritius. The juice extract from the leaves are warmed with honey and lemon juice and administered orally twice a day to treat cough, flu, bronchitis and asthma. It is believed that the Egyptians made a perfume called Kyphi that is translated to "welcome to the gods" that contained *P. madagascariensis* and was used to induce hypnotic states (González-Minero and Bravo-Díaz, 2018). *Plectranthus hadiensis, a synonym of P. ramosior* is used as a fish poison (Rice et al., 2011).

Taking the top three traditional uses of *Plectranthus* species into consideration namely digestive, respiratory and skin disorders only two of these uses have been reported for *P. madagascariensis*. Although not reported as a traditional usage, further investigations of *P. madagascariensis* and its varieties on digestive disorders are recommended. Furthermore, *P. ramosior* previously classified as *P. madagascariensis* var. ramosior and a synonym for *P. hadiensis could potentially be traditionally used as a poison, therefore, toxicity studies are extremely important when conducting research on P. madagascariensis* and its varieties.

2. Methodology

Extensive database retrieval for ethnobotanical uses, biological activities reported for *P. madagascariensis*, its varieties and secondary metabolites using platforms not limited to but including Google Scholar, ScienceDirect and PubMed up to June 2020 was performed. In addition, several PhD and MSc dissertations were also reviewed. Many books were consulted on the traditional usage of *Plectranthus* species and *P. madagascariensis* in South Africa. The keywords used in the search engines regarding plants were "*Plectranthus madagascariensis*" "*Plectranthus madagascariensis* var. aliciae", "*Plectranthus* aliciae", "*Plectranthus ramosior*", "*Plectranthus madagascariensis* var. ramosior", "*Plectranthus hirtus"* and "*Coleus madagascariensis*". In addition, a separate search was performed on the secondary metabolites identified in *P. madagascariensis* using the keywords "*Plectranthus* compounds", "*Plectranthus madagascariensis* compounds", "rosmarinic acid", "coleon U", "coleon U quinone", "6β,7α-dihydroxyroyleanone", "7α-formyloxy-6β-hydroxyroyleanone" and "7α-acetoxy-6β hydroxyroyleanone". No criteria were excluded from the search engines.

The plant names were confirmed using the South African National Biodiversity Institute's Red List of South African Plants (http://redlist.sanbi.org/), PlantZAfrica (http://pza.sanbi.org/), The Plant List (www.theplantlist.org) and Kew science (https://mpns.science.kew.org/mpns-portal/).

3. Phytochemistry

Phytochemical studies on some *Plectranthus* species have confirmed high concentrations of di-and tri-terpenes in these plant species. Reports have indicated that *Plectranthus* species commonly contain abietane diterpenes of the coleon and royleanone type as well abietanoid quinone methides with significant cytotoxic and antiproliferative activity (Pereira et al., 2015; Matias et al., 2019a, 2019b).

Five major components have been isolated from the methanolic extract of *P. madagascariensis*, namely the polyphenol, rosmarinic acid (A) and the abietane diterpenes coleon U (B), 6β,7α-dihydroxyroyleanone (C), 7α-formyloxy-6β-hydroxyroyleanone (D) and 7α-acetoxy-6β hydroxyroyleanone (E) (Figure 1). The extract of *P. madagascariensis* also contained coleon U quinone (F) as the main component in addition to the above-mentioned

compounds. It is believed that coleon U quinone is the oxidised form of coleon U (Figure 1) (Garcia et al., 2019b; Kubínová et al., 2014; Matias et al., 2019a, 2019b).

The Lamiaceae family consists of various genera, such as *Mentha* (mint), *Ocimum* (basil) and *Salvia* (sage) which are known for their aromatic characteristics. The essential oil of *P. madagascariensis* is rich in 6,7-dehydroroyleanone (DHR) (Figure 1, G), an abietane diterpene with a quinone moiety (Pereira et al., 2015b; Sitarek et al., 2020a). Garcia et al. (2018) was successful in isolating and identifying DHR from *P. madagascariensis*. The leaves were subjected to hydrodistillation using a Clevenger apparatus and high-pressure extraction. The highest yield was confirmed for hydrodistillation with a yield of 18.55 ± 2.00% (% DHR weight/hydrodistillate extracts weight). DHR was isolated from the essential oil through flash chromatography with a yield of 20.4% (w/w). Nuclear magnetic resonance (NMR) was carried out and the NMR spectrum was compared with literature to structurally identify DHR.

A total oil yield of 0.1% v/w was isolated from the leaves of *P. madagascariensis* through hydrodistillation using a Clevenger apparatus (Ascensao et al., 1998). Gas chromatography-mass spectrometry (GC-MS) analysis done by Ascensao et al. (1998) determined that 86.6% of the essential oil from the leaves are composed of DHR.

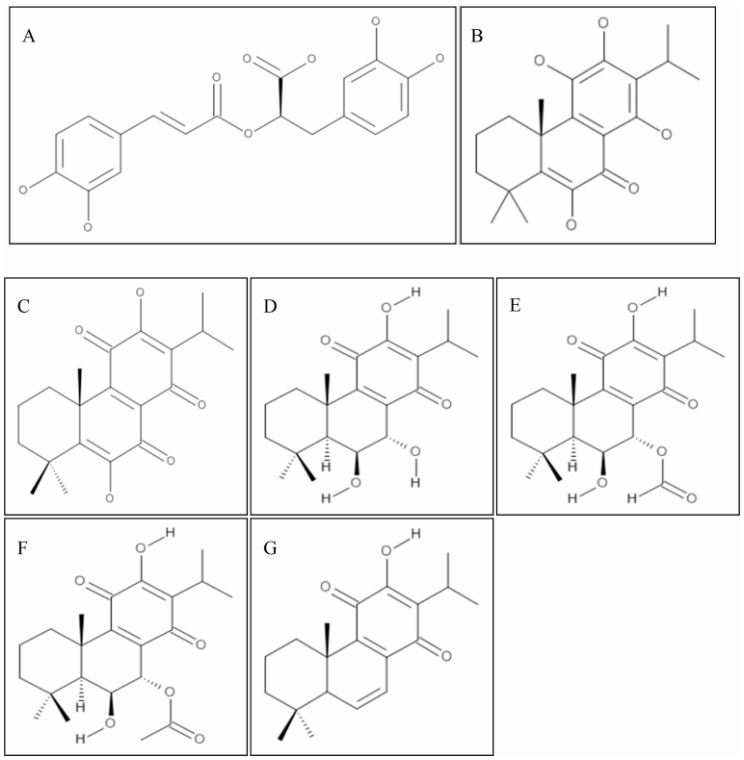

Figure 2. 1: Chemical structures of compounds isolated from *Plectranthus madagascariensis*. (A) Rosmarinic acid, (B) Coleon U, (C) 6β,7α-dihydroxyroyleanone, (D) 7α-formyloxy-6β-hydroxyroyleanone, (E) 7α-acetoxy-6β-hydroxyroyleanone, (F) Coleon U quinone, (G) 6,7-dehydroroyleanone.

4. Biological activity of the extracts and secondary metabolites

Plants from the Lamiaceae family are well known for their antibacterial activity and are a promising source for new antibacterial treatments. Several species from the *Plectranthus* genus have been confirmed to treat several diseases that are related to their use in traditional medicine. The biological activity of *P. madagascariensis* and its varieties are documented in the following section.

4.1 Antimicrobial and antimycobacterial activity

Several *Plectranthus* species including *P. madagascariensis* are used in traditional medicine to treat various respiratory diseases such as asthma, bronchitis and coughs. The *Plectranthus* species; *P. barbatus* and *P. bojeri* have demonstrated to be effective for treating pneumonia. Part of these effects could be due to the antibacterial activity of the Lamiaceae family and *Plectranthus* genus. *Plectranthus amboinicus* has previously been confirmed to have antibacterial activity against *Mycobacterium tuberculosis*, a bacteria associated with tuberculosis. (Lukhoba et al., 2006; Nguta et al., 2016; Rijo et al., 2010).

Pereira et al. (2015) and Matias et al. (2019a) investigated the antimicrobial activity of *P. madagascariensis* extract using various methods. The acetone extract was found to be significantly active against *Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* associated with skin infections, wounds and *Mycobacterium smegmatis*, a model bacterium for tuberculosis activity. Minimum inhibitory concentration (MIC) values of *P. madagascariensis* against *B. subtilis, M. smegmatis, S. aureus* and *S. epidermidis* was confirmed to be 3.91μg/mL, 31.25 μg/mL, 3.91 μg/mL and 7.81 μg/mL respectively. The positive controls vancomycin, amphotericin B and norfloxacin were included in the study, however, the MIC values were not provided in this article.

In a similar study, the acetone extracts of *P. madagascariensis* 'Lynne' and *P. aliciae* were tested to determine their MIC and minimum bactericidal concentration (MBC). The activity was determined against two Gram-positive bacteria, *Enterococcus faecalis* (ATCC 29212) and *S. aureus* (ATCC 25923). *Plectranthus madagascariensis* 'Lynne' displayed MIC values of 15.6 μg/mL and 125 μg/mL against *E. faecalis* and *S. aureus* respectively. On the other hand, *P. aliciae* had the same MIC value against *E. faecalis* but was slightly more active against *S. aureus* with a MIC of 62.5 μg/mL. Both extracts had a minimum bactericidal concentration (MBC) of 125 μg/mL against *S. aureus* and *P. aliciae* against *E. faecalis*. No MBC was observed for *P. madagascariensis* 'Lynne' against *E. faecalis* at the highest concentration tested (125 μg/mL) (Garcia et al., 2019a). An aqueous extract of *P. madagascariensis* prepared using microwave extraction was confirmed to have an MIC value of 40 μg/mL against *S. epidermidis* (Rijo et al., 2014b).

Furthermore, the acetone extract of *P. madagascariensis* was confirmed to inhibit the Gram-negative bacteria, *Pseudomonas syringae* and *Klebsiella pneumoniae* at MIC values of 40 μg/mL and 3.91 μg/mL respectively. The acetone extract did not inhibit the yeasts *Candida albicans* and *Saccharomyces cerevisiae* (Wellsow et al., 2006a; Matias et al., 2019b).

Kubínová et al. (2014) identified four compounds present in an extract of *P. madagascariensis* and investigated the antibacterial activity of these compounds. The four compounds identified were coleon U quinone, 7α-acetoxy-6β-hydroxyroyleanone, 6β,7α-dihydroxyroyleanone and rosmarinic acid. No antibacterial activity was found for rosmarinic acid against the Gram-positive bacteria *E. faecalis* (ATCC 29212) and *S. aureus* (ATCC 29213) and the Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Noteworthy antibacterial activity was reported for

coleon U quinone against *S. aureus* and *E. faecalis* with a MIC of 0.5 μg/mL and 8 μg/mL respectively. Coleon U has shown antibacterial activity against *B. subtilis* (IMI347329) and *P. syringae* (IMI347448) with MIC values of 3.13 μg/mL and 6.25 μg/mL respectively. The oxidised form of coleon U, coleon U quinone, inhibited the growth of *B. subtilis* at an MIC value of 25 μg/mL and *P. syringae* at an MIC value of 3.12 μg/mL (Wellsow et al., 2006b). Antimicrobial reports on coleon U isolated from *Plectranthus grandidentatus* displayed MIC values of 0.98 μg/mL and 31.25 μg/mL against MRSA and vancomycin-resistant *E. faecalis* respectively (González, 2015).

Kubínová et al. (2014) reported moderate antibacterial activity for 6β,7α dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone against *S. aureus* and *E. faecalis* with MIC values ranging between 16 μg/mL and 32 μg/mL. No antibacterial activity was found against the Gram-negative bacteria, *E. coli* and *P. aeruginosa*, for any of the compounds.

The antibacterial activity of compound; 7α-acetoxy-6β-hydroxyroyleanone was investigated against methicillin-sensitive *S. aureus* ATCC 25924, ATCC 43866 and ATCC 700699 (MSSA) and MRSA strains CIP 106760 and FFHB 29593. MIC values between 15.63 μg/mL and 31.25 μg/mL were observed against MSSA and 7.81 μg/mL against MRSA strains. Antibacterial activity against vancomycin-sensitive *E. faecalis* FFHB 427483, *E. casseliflavus* ATCC 49996, *E. faecium* FFHB 435628 and low-level vancomycin-resistant *E. faecalis* ATCC 51299 (VRE) at MIC values between 7.81 μg/mL and 15.63 μg/mL was observed (Rijo et al., 2014a).

The compounds 6β,7α-dihydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and 6,7-dehydroroyleanone were investigated for their anti-mycobacterial activity against two mycobacterial strains namely the *M. tuberculosis* strain H37Rv (ATCC 27294) and the MDR clinical isolate, strain 02TBDM039EP097. Furthermore, the cytotoxicity of the compounds on African green monkey kidney epithelial cells (Vero) and mouse embryonic fibroblast cells (3T3) was evaluated. Compound 7α-acetoxy-6β-hydroxyroyleanone exhibited noteworthy anti-mycobacterial activity against both bacterial strains with a MIC value of 25 μg/mL against H37Rv and 3.12 μg/mL against MDR. The compound 7α-acetoxy-6β-hydroxyroyleanone was found to be more active against the MDR-*M. tuberculosis* strain than the anti-tuberculosis drugs rifampicin and isoniazid with MIC values of 16 mg/ml and 4 mg/ml respectively. No anti-mycobacterial activity was observed for 6β,7α-

dihydroxyroyleanone and 6,7-dehydroroyleanone against H37Rv at the highest concentration tested of 25 μg/mL. However, 6β,7α-dihydroxyroyleanone inhibited MDR at a MIC of 12.5 μg/mL and 6,7-dehydroroyleanone at a MIC below 12.5 μg/mL. The study hypothesised that the noteworthy anti-mycobacterial activity against the MDR-*M. tuberculosis strain* was due to the 7α-AcO group at the B ring of the abovementioned compounds (Rijo et al., 2010b).

The cytotoxicity of 7α-acetoxy-6β-hydroxyroyleanone was confirmed against 3T3 and Vero cell lines. At a 50% growth inhibition concentration (GI_{50}) of 12.96 μg/mL and 12.80 μg/mL, 7α-acetoxy-6β-hydroxyroyleanone inhibited the growth of 3T3 and Vero cell lines respectively, revealing significant cytotoxicity. However, the compound was confirmed to be selective towards the MDR strain versus both cells lines, with selectivity indexes of 3.2 against 3T3 and 6.62 against Vero cell lines (Rijo et al., 2010b). The selectivity index is a ratio calculation to determine if a test substance is more targeted towards one biological target in comparison to another. A selectivity index is calculated by dividing the IC_{50} of one target with another. A selectivity index greater than one is an indication that the sample is more targeted towards the denominator biological target (Peña-Morán et al., 2016). Therefore, although 7α-acetoxy-6β-hydroxyroyleanone was significantly cytotoxic on the tested cell lines the compound is more targeted towards inhibiting the MDR strain.

The essential oil of *P. madagascariensis* was tested for its growth inhibitory potential against several Gram-positive and Gram-negative bacteria that included *Micrococcus* species, *B. subtilis*, *S. aureus* and *Yersinia enterocolitica*. The essential oil was confirmed to be most active towards the Gram-positive bacteria *S. aureus* and the *Micrococcus* sp. with inhibition zones of 7.5 mm and 12.5 mm respectively when 10 μL of the essential oil was tested using the agar-diffusion assay (Ascensao et al., 1998).

In a study on DHR isolated from the essential oil of *Tetradenia riparia*, DHR was confirmed to inhibit *M. tuberculosis* H37R together with antibiotic-resistant and susceptible isolates at an MIC value of 31.2 μg/mL. Cytotoxicity studies on murine macrophages confirmed that DHR was selective towards targeting the bacteria with a selectivity index of 7.9. The authors noted that DHR could be a potential anti-tuberculosis drug candidate (Baldin et al., 2018).

Lipid-based drug delivery systems such as phytosomes are attractive nanocarrier systems to enhance the delivery and availability of low-soluble drugs to the target site. These systems encapsulate both hydrophilic molecules (ones which dissolve in gastrointestinal fluids and

blood) and lipophilic molecules (ones that cross biological membranes) and prevent premature degradation in the body (Danaei et al., 2018a; Matias, 2016a). In a study by Matias (2016), phytosomes (PS) and chitosan-coated phytosomes (ChiPS) were prepared from the acetone extract of *P. madagascariensis*. Chitosan is a polysaccharide with proven bacteriostatic activity. Particles with a diameter of 191.3 ± 75.3 nm and 1082 ± 363 nm and polydispersity index (PDI) of 0.243 ± 0.18 and 0.22 ± 0.10 for PS and ChiPS were formed (Matias et al., 2015). The PDI is an indication of the size distribution of particles in a given sample. For pharmaceutical applications, a PDI of 0.3 and less is desired (Danaei et al., 2018). The particle size for PS fell within the ideal range for transdermal drug delivery systems. Matias (2016) set out to determine the *in vitro* antibacterial activity and cytotoxicity of PS and ChiPS and *in vivo* irritancy potential of ChiPS. At a concentration of 1 mg/mL, ChiPS displayed a zone of inhibition of 17 mm against *S. epidermidis* (ATCC 12228) that was comparable to the inhibition of the pure compound 6β, 7α-dihydroxyroyleanone, *P. madagascariensis* extract and the positive control vancomycin with inhibition diameters of 19 mm, 21 mm and 22 mm respectively. The chitosan control (not loaded with *P. madagascariensis* extract) displayed an inhibition zone of 8 mm. This indicates that the antibacterial activity of chitosan-coated phytosomes is increased with the addition of *P. madagascariensis* to concentrations comparable to the extract and positive control. The antibacterial activity of PS and ChiPS was tested against *S. aureus* (ATCC 25923), MRSA (CIP 106760) and *S. epidermidis* (ATCC 12228) using the broth microdilution assay against the extract and positive control vancomycin.The phytosomes (PS) displayed no increase in antibacterial activity compared to the extract. The chitosan coated phytosomes (ChiPS) displayed a fourfold increase in antibacterial activity against *S. aureus* (ATCC 25923). However, no control for chitosan phytosomes (not loaded with the extract) was included in this study. Therefore, it is unclear if the antibacterial activity observed was because of the synergistic activity of chitosan and *P. madagascariensis* as seen in the zone inhibition study or if the activity was due to the known antibacterial activity of chitosan.

4.2 Anti-cancer activity

Several *Plectranthus* species have demonstrated noteworthy anti-cancer and antitumor activity. *Plectranthus amboinicus, P. hadiensis* and *P. barbatus* are known to have cytotoxic effects on cancer cells. The *Plectranthus madagascariensis* acetone extract was investigated for its cytotoxic effect against several cancer cell lines. At a concentration of 15 µg/mL, the

acetone extract of *P. madagascariensis* inhibited 20.13% of breast cancer cell growth (MDA-MB-231). The IC_{50} of the acetone extract was determined to be 64.52 μg/mL and is considered to have low cytotoxic activity (Matias et al., 2019b).

The cytotoxic activity of the acetone extracts of *P. madagascariensis* 'Lynne' and *P. aliciae* against three types of cancer including human colon (HCT116), non-small cell lung carcinoma (NCI-H460) and breast adenocarcinoma (MCF-7) was determined. *Plectranthus madagascariensis* 'Lynne' displayed GI_{50} values of 3.47 ± 0.15 μg/mL and 5.39 ± 0.48 μg/mL against MCF-7 and NCI-H460 cell lines respectively and a GI_{50} value between 5 μg/mL and 10 μg/mL against HCT116. *Plectranthus aliciae* was active towards HCT116, MCF-7 and NCI-H460 between 15 μg/mL and 20 μg/mL (Garcia et al., 2019b). The acetone extracts of *P. madagascariensis* and *P. aliciae* are considered to have significant cytotoxic activity against HCT116, NCI-H460 and MCF-7 cell lines.

A study by Brito et al. (2018) on the cytotoxic activity of *P. madagascariensis* revealed that the aqueous extract inhibited cell growth at IC_{50} values of 1.7 ± 0.3 mg/mL against MCF-7 cells and 1.5 ± 0.2 mg/mL against human liver cancer cells (HepG2) cells and is considered to be non-cytotoxic.

Anti-cancer studies done by Matias et al. (2019b) determined that the two abietane diterpenes 6β,7α-dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone were effective and selective towards the lung cancer cell line NCI-H460 with cytostatic activity at 25.0 ± 2.0 μM and 3.1 ± 0.4 μM respectively. Matias et al. (2019b) hypothesized that the presence of lipophilic substituents at positions 6 and 7 of abietane diterpenes are required for cytotoxic activity.

The anticancer activity of 6β,7α-dihydroxyroyleanone, 7α-formyloxy-6β-hydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and coleon U isolated from *P. madagascariensis* against breast cancer cell lines (MDA-MB-231, MCF-7) and a colon cancer cell line (HCT116) was determined. Although none of the compounds affected the growth of MDA-MB-231, 6β,7α-dihydroxyroyleanone, 7α-formyloxy-6β-hydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and coleon U inhibited MCF-7 cells at a GI_{50} of 26.0 ± 0.6 μM, 7.9 ± 0.8 μM, 6.4 ± 0.4 μM and 5.5 ± 0.8 μM respectively. The positive control doxorubicin inhibited MCF-7 growth at a GI_{50} value of 0.16 ± 0.0018 μM. The compound 7α-formyloxy-6β-hydroxyroyleanone inhibited the growth of the colon cancer cell line HCT116 at a GI_{50} of 7.9

± 1.2 μM compared to the positive control doxorubicin with a GI_{50} of 0.125 ± 0.0013 μM (Matias et al., 2019d)

Anti-cancer studies by Sitarek et al. (2020) on human primary H7PX glioma cells found that 7α-acetoxy-6β-hydroxyroyleanone inhibited cell growth at an IC_{50} below 25 μg/mL. At the highest concentration of 100 μg/mL, 6β,7α-dihydroxyroyleanone inhibited 20% of H7PX glioma cell growth.

The compound 6,7-dehydroroyleanone isolated from the essential oil of *P. madagascariensis* was found to inhibit cell growth of human cervix carcinoma cell line (KB-3-1) at an IC_{50} value of 30 μM compared to the positive controls cryptophycin-52 with an IC_{50} value of 1.3×10^{-5} μM and griseofulvin with an IC_{50} value of 19 μM (Abdissa et al., 2017; Garcia et al., 2019c).

Rosmarinic acid was confirmed to inhibit the growth of MCF-7 and HepG2 (human liver cancer cell line) cells at IC_{50} values of 0.5 ± 0.1 mg/mL and 3.4 ± 0.9 mg/mL respectively (Brito et al., 2018).

The compound DHR induced apoptosis in primary H7PX glioma cells. Apoptosis is a form of cell death in which cells undergo morphological changes, resulting in cell death. Current cancer therapies such as chemo-and-radiotherapy induce apoptosis in cancer cells. In addition, DHR was found to induce G2/M cell cycle arrest and increase H2A.X phosphorylation (Sitarek et al., 2020b).

Anti-cancer studies conducted by Garcia et al. (2018) revealed the cytotoxic activity of DHR against human lymphoid leukaemia cells (MOLT-3) and the human tumour cell line HL-60. The IC_{50} value obtained for DHR cytotoxicity against MOLT-3 cells were 5.4 ± 0.3 μM and 4.46 μM against HL-60 cells as determined by Kusumoto et al. (2014). These results were compared to the cytotoxicity of a standard therapeutic drug, etoposide, with an IC_{50} value of 0.3 ± 0.1 μM and 0.4 ± 0.1 μM on MOLT-3 and HL-60 respectively. Moderate activity was observed against non-small-cell lung carcinoma cells A549, NCI-H460 and NCI-H460/R and with IC_{50} values of 30 μM, 14 μM and 11 μM respectively. In addition, DHR was tested for its cytotoxicity against human embryonal bronchial epithelial cells MRC-5 with an IC_{50} value of 24 μM, indicating that DHR is slightly more selective towards killing cancer cells without inducing toxic effects on normal cells. Furthermore, Garcia et al. (2018) set out to discover the mechanism of action by which DHR inhibit cell proliferation. They determined that DHR

does not target microtubules, but was able to evade P-glycoprotein mediated mechanisms of resistance at 5 μM compared to Dex-VER tested at 5 μM, a known P-glycoprotein inhibitor in NCI-H460/R cells. These results support the data reported by Sitarek et al. (2020) which confirmed the ability of DHR to activate caspases-3 and -9, triggering apoptosis and the release of pro-apoptotic factors involved in activating the apoptotic pathway.

Due to their chemical composition, DHR and 7α-acetoxy-6β-hydroxyroyleanone (Figure 1) have been identified as attractive lead compounds for derivatization (Isca et al., 2020). Derivatives of these compounds were investigated for enhanced cytotoxic activity and targeting the P-glycoprotein using molecular docking. P-glycoproteins is involved in the transfer of drugs out of the cell, enabling cancer cells to resist lethal doses of cytotoxic drugs resulting in multidrug-resistant cancer. The molecular docking study on the interaction between the above-mentioned compound derivatives and P-glycoprotein revealed that the presence of aromatic moieties increased the binding affinities of the compounds to the protein. The molecular docking studies confirmed that one benzoyloxy moiety is needed at position C-6 and chemical moieties smaller than unsubstituted benzoyl rings at position C-12 to effectively bind to the M-site of the protein pump (Isca et al., 2020b).

The use of gold for the synthesis of nanostructured materials is increasing. This is due to the relatively low toxicity to biological systems, optical properties, large service area-to-volume ratio and conformational flexibility of gold, making it ideal for nanoparticle formation (De Freitas et al., 2018).

Garcia et al. (2018) prepared hybrid nanoparticles to improve the cytotoxic activity and targeted delivery of DHR. Nanoparticles were prepared from a 20 μM acetone solution of DHR. In a 1:1 (v/v) ratio, the acetonic DHR solution was mixed with hybrid polymeric-gold nanoparticle solution and was stirred at 800 rpm for 24 h in the dark. The nanoparticles were characterized by dynamic light scattering (DLS), measuring the PDI and diameter of the nanoparticles. A PDI value of 0.1 and a particle diameter of 281.1 nm was detected, indicating narrow size distribution (PDI) ideal for pharmaceutical applications (Li and Mattei, 2019). On the other hand, the DHR-conjugated hybrid nanoparticles were characterized through atomic force microscopy (AFM) and were confirmed to be small spherical particles with a size of 19.05 ± 1.77 nm. HPLC-DAD determined a 98.57 ± 0.23% conjugation efficiency of the DHR nanosystem.

The anti-cancer potential of DHR hybrid nanoparticles was assessed on human non-small cell lung carcinoma (NCI-H460) and multidrug-resistant lung cancer cells (NCI-H460/R). Hybrid nanoparticles without DHR displayed no inhibition of the cells at the highest concentration tested of 50 μg/mL. However, DHR nanoparticles significantly increased the activity of DHR in both cells lines. DHR nanoparticles displayed an eightfold decrease in the IC_{50} against NCI-H460 from 4.10 ± 0.61 μg/mL to 0.53 ± 0.06 μg/mL and a fivefold decrease in the IC_{50} against and NCI-H460/R from 3.18 ± 0.32 μg/mL to 0.65 ± 0.18 μg/mL compared to DHR alone (Garcia et al., 2018b). Although anti-cancer activity was observed for the DHR nanoparticles, more research is required to optimize the size of the DHR nanoparticle to be effectively transported to the lungs.

4.3 Antioxidant activity

During inflammation, macrophages release pro-inflammatory factors such as free radicals and nitric oxide that can contribute to cellular damage of the tissue. Although controversy exists on testing plant extracts for their free-radical-scavenging activity, it is still a widely used practice. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is extensively used as a chemical assay to determine the potential free radical scavenging ability of a plant extract. However, it is suggested that the DPPH assay is supported by other antioxidant assays such as the ABTS (Ascorbic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Nitric oxide (NO) and FRAP (ferric-reducing antioxidant power) free-radical-scavenging assays (Lalhminghlui and Jagetia, 2018).

The antioxidant activity of *P. madagascariensis* acetone extract was screened to determine the free-radical-scavenging ability of the extract using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Matias et al. (2019a) concluded that a methanolic extract of *P. madagascariensis* obtained through ultrasound-assisted extraction and maceration at a tested concentration of 100 μg/mL scavenged 89.0% and 64.8% of the DPPH free radicals respectively. The antioxidant activity was due to high concentrations of polyphenols in the extract as determined by High-Performance Liquid Chromatography with a Diode Array Detector (HPLC-DAD). The acetone extract, on the other hand, scavenged less than 50% of the free radicals at 100 μg/mL. In a similar study, the methanol extract of *P. madagascariensis* scavenged 48.4 ± 1.23% of the DPPH free radicals at a concentration of 100 μg/mL (Andrade, 2016a). Studies performed by Rijo et al. (2014b) and Brito et al. (2018)

on the aqueous extract of *P. madagascariensis* produced similar DPPH free-radical scavenging activities with IC_{50} values of 41.66 μg/mL and 45.4 ± 2.2 μg/mL respectively. The antioxidant activity could be due to rosmarinic acid that is a major compound in the species that scavenged DPPH free-radicals at an IC_{50} value of 2.8 ± 0.1 μg/mL, as reported in an earlier study by Brito et al. (2018).

4.4 Anti-diabetic activity

Several enzymes play significant roles in the onset of diabetes mellitus. These enzymes include α-glucosidase, aldose reductase and phosphoenolpyruvate carboxykinase (PEPCK). The intestinal enzyme, α-glucosidase is a carbohydrate-hydrolase responsible for the release of α-glucose from large carbohydrates. Inhibition of α-glucosidase causes a decrease in α-linkage cleavage at the anomeric centre in a sugar molecule resulting in a reduction of glucose absorbance. Furthermore, α-glucosidase is associated with the onset of several diseases such as diabetes, cancer and viral infections. Studies have confirmed that the enzyme aldose reductase is linked to several diabetic complications. The enzyme affects the patient's sight by damaging the eye tissue and affecting the peripheral nervous system and organs such as the kidneys. Aldose reductase catalyses the reduction of glucose and aldehydes to sorbitol and is a key enzyme in the polyol pathway which contributes to various complications in diabetic patients. Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme in the gluconeogenesis metabolic pathway responsible for the sustained release of glucose during fasting. In patients with diabetes, PEPCK is overproduced resulting in the overexpression of the gluconeogenesis pathway in the liver and kidneys, resulting in hyperglycaemia. Glucose transporter type 4 (GLUT-4) is a glucose transporter protein and is regulated by insulin levels. In addition, insulin resistance has been linked to impaired GLUT-4 translocation in patients suffering from diabetes (Gómez-Valadés et al., 2006; Ha et al., 2012a; Kubínová et al., 2014b; Simmons, 2017).

Rosmarinic acid isolated from a *P. madagascariensis* methanolic extract was confirmed to inhibit α-glucosidase at an IC_{50} value of 33.0 ± 4.6 μM. This was comparable to quercetin previously confirmed to have an IC_{50} value of 26.7 μM (Ha et al., 2012b; Kubínová et al., 2014b). A study by Ha et al. (2012) reported the inhibitory activity of rosmarinic acid against aldose reductase with an IC_{50} value of 11.2 μM. *In vivo* studies conducted by Runtuwene et al. (2016) studied the effect of rosmarinic acid on insulin levels and the balance between

insulin and glucose levels in the blood of induced type-1 and type-2 diabetic rats. Runtuwene et al. (2016) confirmed rosmarinic acid was effective in reducing the expression of the PEPCK enzyme and increasing GLUT-4 protein expression, therefore reducing hyperglycemia and insulin sensitivity at a dose of 200 mg/kg. However, Runtuwene et al. (2016) suggested further *in vivo* studies in humans since the dosage of rosmarinic acid could differ in humans. This indicates the potential of rosmarinic acid as a potential treatment for diabetes mellitus and various allergy treatments.

4.5 Alzheimer's Disease

Alzheimer's disease (AD) is defined by Hase et al. (2018) as a "neurodegenerative disease-causing cognitive dysfunction such as memory impairment and disorientation". Various targets present themselves as potential treatments for AD. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are enzymes found in healthy brains. AChE and BuChE are responsible for controlling acetylcholine (ACh) levels in the brain. However, patients suffering from AD have demonstrated an increase in BuChE levels in their brain. Patients affected by AD experience altered levels of AChE and low levels of ACh. Therefore, AChE inhibitors have been proven to improve ACh levels and improve cholinergic neuron function that is damaged during AD progression. Furthermore, the accumulation of amyloid-β (Aβ) protein in the brain has been linked to the formation of neuritic plaques that contribute to AD. The most abundant Aβ peptides are Aβ 1–40 and Aβ1–42. Therefore, Aβ-peptides, AChE and BuChE have become attractive therapeutic strategies for AD (Kubínová et al., 2014b; Dos Santos et al., 2018; Hase et al., 2018b).

An aqueous extract from the aerial parts of *P. madagascariensis* 'Lynne' inhibited AChE at an IC_{50} value of 440 ± 80 µg/mL and alcohol dehydrogenase (ADH) at an IC_{50} value of 67±23 µg/mL. The inhibitory activity was said to be due to the presence of 83 µg/mg of rosmarinic acid in the extract. Rosmarinic acid inhibited AChE and ADH at IC_{50} values of 100 ± 30 µg/mL and 19 ± 4 µg/mL respectively (Brito et al., 2018b).

Kubínová et al. (2014), reported 6β,7α dihydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and coleon U quinone to have IC_{50} values of 287.3 ± 9.9 µM, 256.4 ± 5.9 µM and 290.7 ± 3.5 µM respectively against BuChE. This was comparable to the IC_{50} value of 168.0 ± 4.8 µM of the positive control, galantamine. The observed activity was hypothesized to be due to the presence of ester bonds in the compounds.

In vitro and *in vivo* studies by Hase et al. (2018) found that while the permeability of rosmarinic acid into the brain is low, the compound was able to reduce Aβ aggregation, by increasing monoamine secretion in 5-month-old female Tg2576 mice at 0.5% after 11 days of daily feeding. This study supported the *in vitro* results obtained by Ono et al. (2004) that confirmed the ability of rosmarinic acid to inhibit the accumulation of Aβ-peptides, the formation of Aβ-fibrils and destabilization of preformed Aβ-fibrils.

4.6 Anti-tyrosinase, anti-collagenase and anti-elastase activity

Tyrosinase is an enzyme that plays a key role in the production of melanin in the skin. High amounts of tyrosinase cause an overproduction of melanin, resulting in hyperpigmentation of the skin. A methanolic extract prepared from the fresh leaves of *P. madagascariensis* displayed an IC_{50} value of 23.99 µg/mL against the tyrosinase enzyme. The positive control, kojic acid, inhibited the tyrosinase enzyme at an IC_{50} value of 3.607 µg/mL. The researchers classified *P. madagascariensis* methanolic extract, as a noteworthy tyrosinase inhibitor (Etsassala, 2016).

Rosmarinic acid inhibited 47.87 ± 1.41% of the tyrosinase enzyme at a concentration of 50 µg/mL compared to the positive control, kojic acid, with an IC_{50} value of 5.71 µg/mL. The compounds 7α-acetoxy-6β-hydroxyroyleanone and 6β,7α-dihydroxyroyleanone inhibited 46.62 ± 4.74% and 14.73 ± 2.02% of the tyrosinase enzyme at 50 µg/mL, respectively. Anti-tyrosinase studies confirmed DHR inhibited 75.72 ± 3.61% of the enzyme at 50 µg/mL (Andrade, 2016b). Rosmarinic acid and 7α-acetoxy-6β-hydroxyroyleanone are considered moderate tyrosinase inhibitors.

Elastase and collagenase are the two main enzymes responsible for breaking down the two major components of connective tissue in the skin, elastin and collagen. The breakdown of elastin and collagen results in loss of skin elasticity resulting in sagging skin and the formation of wrinkles. The percentage elastase inhibitory activity of 7α-acetoxy-6β-hydroxyroyleanone and 6β,7α-dihydroxyroyleanone was 29.26 ± 2.75% and 39.16 ± 5.18% respectively for the two compounds when tested at 100 µg/mL. The positive control ursolic acid inhibited 69.85 ± 3.65% of the elastase enzyme at 100 µg/mL. At a concentration of 100 µg/mL rosmarinic acid exhibited 44.78 ± 4.53% inhibition of collagenase. The positive control epigallocatechin gallate inhibited 93.09 ± 5.27% of the enzyme activity at 100 µg/mL. At 100 µg/mL, the compounds 7α-acetoxy-6β-hydroxyroyleanone and 6β,7α-

dihydroxyroyleanone inhibited 33.45 ± 3.25% and 24.04 ± 3.02% of the collagenase enzyme respectively while DHR inhibited 38.33 ± 4.40% and 60.63 ± 9.68% of the elastase and collagenase enzymes respectively (Andrade, 2016b).

4.7 Targeting pain and inflammation

Plectranthus madagascariensis is used traditionally as an application for wounds and scabies, which are often painful and inflamed lesions. Inflammation is characterised by, swelling (edema), pain, redness, heat and tissue loss (Chen et al., 2018; Lukhoba et al., 2006). Several *Plectranthus* species including *P. amboinicus*, *P. barbatus* and *P. hadiensis* have been confirmed to target inflammation, suggesting that *P. madagascariensis* and its phytochemical constituents may have potential anti-inflammatory activity.

Several researchers have investigated the anti-inflammatory potential of rosmarinic acid using animal models. Lucarini et al. (2013) investigated the potential of rosmarinic acid isolated from *Rosmarinus officinalis* L. (Lamiaceae) targeting factors associated with inflammation on male Swiss albino mice. At an oral dosage of 40 mg/kg, rosmarinic acid reduced paw edema by 61% after three hours compared to the positive control, indomethacin which inhibited 53.83% of edema at 10 mg/kg. Rosmarinic acid was able to reduce 38.3% of formalin-induced pain in mice after 15-30 minutes at a dosage of 20 mg/kg compared to morphine that inhibited 55.8% of formalin-induced pain at a dosage of 4 mg/kg.

4.8 Antifeedant activity

Wellsow et al. (2006) determined the antifeedant activity of several *Plectranthus* species against *Spodoptera littoralis*, known by its three common names, the African cotton leafworm, the Mediterranean brocade and lastly the Egyptian cotton leafworm. *Plectranthus madagascariensis* acetone extract and isolated compound coleon U was determined to be phagostimulants. However, the oxidised form of coleon U, coleon U quinone, had significant antifeedant activity at 100 ppm with a 50% feeding index (FI_{50}) value of 91 ppm.

5. Toxicity

Before a substance is applied or administered *in vivo* for human safety and efficacy studies, it is important to determine the relative toxicity and safety of the test substance. Toxicity studies are performed in two ways, the first uses non-cancerous cell lines *in vitro* and the

second uses animal models such as mice, rats, brine shrimp or zebrafish (Arome and Chinedu, 2013; Twilley et al., 2020a).

The toxicity of *P. madagascariensis* 'Lynne' and *P. aliciae* acetone extracts were determined against *Artemia salina* 'brine shrimp' due to the ease and inexpensive nature of the assay. Toxic effects at 50% lethal concentration (LC_{50}) were observed at 91.7 µg/mL and 53.48 µg/mL for *P. madagascariensis* 'Lynne' and *P. aliciae* respectively. At 100 ppm, *P. madagascariensis* 'Lynne' displayed a mortality rate of 9.67 ± 0.93% and *P. aliciae* a mortality rate of 16.71 ± 1.01% (Garcia et al., 2019b).

Since *P. madagascariensis* and *P. aliciae* acetone extracts displayed LC_{50} values between 91.7 µg/mL and 53.48 µg/mL respectively this indicated an LD_{50} of more than 2500 mg/kg in mice. Therefore, although the samples were considered toxic with the brine shrimp toxicity assay, moderate toxicity was observed when tested in mice.

Rijo et al. (2014b) confirmed that an aqueous extract of *P. madagascariensis* had low toxicity towards the human keratinocyte (HaCaT) cell line at the highest concentration of 500 µg/mL after 24 hours. No toxicity data was found for *P. ramosior* or *P. madagascariensis* var. *madagascariensis*. Similar toxicity data was observed for the acetone extracts of *P. madagascariensis* and *P. aliciae* on brine shrimp. This could be an indication that the toxicity between the varieties is not significantly different. Toxicity studies on an aqueous extract *Plectranthus amboinicus* (Lour) Spreng at the highest tested concentration of 10 000 mg/kg revealed no acute toxicity in mice. Subacute toxicity studies revealed an increase in kidney function parameters which are related to the dosage. While the aqueous extract of *P. madagascariensis* appears safe to use, based on the IC_{50} data, caution should be taken at higher concentrations, particularly when ingested (Asiimwe et al., 2014).

Cytotoxicity studies on HaCaT cells compared the extract of *P. madagascariensis* and ChiPS to determine changes in the toxicity of the extract. It was confirmed that the cytotoxicity of *P. madagascariensis* was lowered when combined with chitosan in comparison with the extract alone with IC_{50} values of 85.87 µg/mL and 56.77 µg/mL respectively (Matias, 2016b).

In vivo acute and sub-chronic irritation studies were conducted on male hairless Sho® SCID mice to determine the irritancy potential of *P. madagascariensis* extract and ChiPS compared to a known skin irritant, Sodium Laureth Sulfate (SLS). Negligible irritation at 5% in

carboxymethyl cellulose hydrophilic gel was observed for the extract and ChiPS compared to 5% SLS that caused mild irritation (Matias, 2016b).

6. Discussion

Researchers regularly select plants based on their traditional usage to test the extracts and their secondary metabolites against the claimed disease or novel targets. Traditional knowledge directs researchers to find plant extracts and compounds with positive biological activity. This highlights the importance of preserving ethnomedicinal knowledge (Patwardhan, 2005; Twilley et al., 2020b).

Three varieties of *P. madagascariensis* are indigenous to South Africa, namely, *P. aliciae*, *P. ramosior* and *P. madagascariensis* var. *madagascariensis*. Lukhoba et al. (2006) reported the extensive use of synonyms in *Plectranthus* species that is evident from the species discussed in this review. The prevalence of synonyms of plant species can result in traditional knowledge being incorrectly documented. When investigating the top three traditional uses of *Plectranthus* species namely respiratory, skin and digestive disorders and comparing it to the traditional uses of *P. madagascariensis* only two of these disorders are documented. Although studies were conducted on the skin and respiratory disorders, no study investigated the use of *P. madagascariensis*, its varieties or isolated compounds on disorders of the digestive system. Since species in this genus have been used to treat digestive disorders, the possibility exists that *P. madagascariensis* could perform the same function. When investigating traditional usage of a species to determine its biological activity for an ethnobotanical evaluation, it is important consider the traditional uses of the synonymous plants and the varieties of these species. This is also where voucher specimen numbers and the inclusion of these voucher numbers are of utmost importance in scientific articles.

Due to the morphological similarities between the studied *Plectranthus* species, the possibility exists that *P. madagascariensis* and its varieties have not only been misidentified by researchers but also traditional health practitioners making use of these species. Therefore, *P. madagascariensis* and its varieties could potentially have different traditional uses than those currently documented. Various articles have discussed the difficulty of identifying *Plectranthus* species to the untrained eye due to morphological similarities and the lack of criteria to differentiate the species from one another. Evidence of misidentification could be found in an article by Matias et al. (2019b) referencing an image of *P. madagascariensis*

which lacks the characteristic variegated white leaf margins. The reviewed articles seldom reported the herbarium voucher specimen numbers. This could be problematic since vouchers serve as crucial supporting material for publications to ensure the reproducibility of the data. Vouchers are important for authenticating the taxonomy of a species, identifying geographical locations of the species and for ecological, environmental and genetic studies (Culley, 2013). For a genus such as *Plectranthus* where confusion between the species can easily occur due to morphological similarities, voucher specimens are crucial to ensure that the correct species is identified. Due to the lack of voucher specimens of *P. madagascariensis* and its varieties, it should be taken into consideration the misidentification of the species that could result in variable data between studies.

No reports of *P. madagascariensis* considered the synonyms and varieties of the species when conducting investigations of biological activities. Biological and phytochemical studies mostly focused only on *P. madagascariensis* and one study on *P. aliciae*. No study was found on *P. ramosior* or *P. madagascariensis* var. *madagascariensis* that could potentially give information on the similarities and differences in the biological activity of these varieties. Although *P. madagascariensis* is not considered toxic, *P. hadiensis, a synonym of P. ramosior* is used as a poison that could potentially be related to the toxicity observed in mice studies (Lukhoba et al., 2006).

Toxicity studies were only reported for the acetone extracts of *P. madagascariensis* and *P. aliciae* and the aqueous extract of *P. madagascariensis*. The acetone extracts of *P. madagascariensis* and *P. aliciae* were confirmed as being potentially toxic when ingested. However, the aqueous extract of *P. madagascariensis* was confirmed to have low toxicity towards HaCaT cells. No toxicity studies were reported on the compounds isolated from *P. madagascariensis* or the essential oil of the plant using HaCaT cells or animal models. Toxicity studies on plant extracts and secondary metabolites are useful to determine if further analysis of the samples should be conducted. Twilley et al. (2020) suggested that when the safety of a plant extract or secondary metabolite is being investigated the mutagenicity of the extract or compound should be determined. Mutagenicity refers to the potential of a test substance to cause permanent changes in genetic material and DNA damage in cells (Mortelmans and Zeiger, 2000a). No study investigated the mutagenic potential of *P. madagascariensis,* its varieties or the identified secondary metabolites that could provide useful information on the safety of the samples.

Cytotoxicity and toxicity studies were mostly performed on *P. madagascariensis* and *P. aliciae* acetone extracts. No cytotoxicity studies were reported for *P. ramosior* or *P. madagascariensis* var. *madagascariensis*. *Plectranthus madagascariensis* and *P. aliciae* were found to have similar significant cytotoxic activity against human breast (MCF-7) and human colon (HCT116) adenocarcinoma cells. Interestingly, compounds isolated from *P. madagascariensis* 6β,7α-dihydroxyroyleanone, 7α-formyloxy-6β-hydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone, rosmarinic acid and coleon U had moderate to significant cytotoxic activity against MCF-7, HCT116, human cervix carcinoma cell line (KB-3-1) and the human liver cancer cells (HepG2). Although these compounds have not yet been identified in *P. aliciae,* based on the similar activities between the extracts of these two species, the compounds of *P. madagascariensis,* could be present in *P. aliciae* and amongst the other varieties too. However, this is merely an assumption and the isolation and identification of compounds from the different varieties remains elusive. The secondary metabolites DHR, 6β,7α-dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone isolated from *P. madagascariensis* have been confirmed to be selective towards NCI-H460 lung carcinoma cells with moderate and significant cytotoxic effects respectively. DHR isolated from the essential oil of *P. madagascariensis* was confirmed to target multiple lung cancer cell lines including resistant NCI-H460 lung carcinoma cells. These compounds could be the reason for the significant cytotoxic activity of *P. madagascariensis* and *P. aliciae* against NCI-H460 lung carcinoma cells. None of the studies investigated the selectivity of the extracts or compounds for cancerous cell lines. Current cancer treatments not only target cancerous cells but also non-cancerous cells. Calculating the selectivity index could indicate if a sample is selective towards cancerous cells rather than non-cancerous cells (Peña-Morán et al., 2016).

Traditionally *P. madagascariensis* is used to treat respiratory ailments such as coughs, bronchitis and asthma (Huthings et al., 1996; Pereira et al., 2015; Rabe and van Staden, 1998; Rice et al., 2011). However, it is not clear whether these symptoms were confused with that of lung cancer and tuberculosis. No reports indicate the traditional usage of *P. madagascariensis* or the varieties thereof for lung cancer or tuberculosis. From these studies, it is clear that *P. madagascariensis* and its varieties could potentially be used for the treatment of lung cancer and tuberculosis or could have been used to treat the symptoms of these diseases without the knowledge of traditional users. However, no biological studies were found to investigate the plant extracts or compounds for asthma, or the bacteria

involved in the onset of bronchitis such as *Bordetella pertussis, Chlamydia pneumonia, Haemophilus influenza, Moraxella catarrhalis, Mycoplasma pneumonia* and *Streptococcus pneumonia (Sethi, 2020; Worrall, 2008)*. Furthermore, an aqueous root extract of *P. madagascariensis* and an extract prepared for the whole plant have been documented to be traditionally used for respiratory disorders. From the manuscripts reviewed herein, the aerial parts of the plants were used to conduct these studies and mostly extracted with acetone, ethanol or methanol. Several studies have confirmed that different plant parts have significant differences in biological activity. This is largely due to the fact that different secondary metabolites are present either exclusively in a certain plant part or are found in various concentrations in different plant parts. When traditional knowledge is adapted to confirm the activity *in vitro* or *in vivo* in biological studies it is important to include the ethnobotanical method in the studies to confirm and document the results. Several articles reviewed did not consider the traditional usage of *P. madagascariensis*. Therefore the traditional usage of *P. madagascariensis* for respiratory diseases and the way it is traditionally administered has not been fully explored, leaving a gap between traditional knowledge and scientific validation.

The use of nanosystems for the treatment of lung cancer and other respiratory diseases should not be overlooked. DHR nanoparticles were able to significantly decrease the IC_{50} value against NCI-H460 lung carcinoma cells (Garcia et al., 2018b). However, no other studies have investigated the potential of the *P. madagascariensis* extract, and compound, 6β,7α-dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone loaded nanoparticles for the treatment of lung cancer or other respiratory ailments.

The antibacterial activity of the Lamiaceae family can be attributed to phenolic secondary metabolites present in the extract and the essential oil of the plant. Antibiotic resistance has become a concern and the World Health Organisation has classified resistance as a global threat to human health. Antibiotic resistance occurs when bacteria become resistant to antibiotics resulting in longer treatment times and higher medical costs as a result of misuse of antibiotics (WHO, 2020). Antibiotic resistance is found in respiratory maladies such as tuberculosis and wound infections such as prosthetic implant-associated infections. As mentioned, *P. madagascariensis* is used mainly to treat respiratory ailments such as coughs. However, the cause of the symptom has not been reported in the literature and could be due to bacterial lung infections caused by *Haemophilus* species, *M. tuberculosis*, *K. pneumoniae*, *Streptococcus pneumoniae* and *S. aureus* (Speert, 2006). *Mycobacterium tuberculosis*

(MDR), resistant *K. pneumoniae,* and methicillin-resistant *S. aureus* (MRSA) strains are becoming more frequent and harder to treat with current antibiotics. The acetone extract of *P. madagascariensis* was found to be active against both Gram-positive and Gram-negative bacteria associated with respiratory diseases such as *M. smegmatis*, a model bacterium for *M. tuberculosis* research, MRSA and *K. pneumoniae*. In a similar study, the acetone extract of *P. aliciae* was confirmed to inhibit *S. aureus* growth at a lower concentration than the acetone extract *P. madagascariensis*. Although these species are morphologically similar, differences in antibacterial activity were observed. This could be due to a difference in the concentration of secondary metabolites, or the type of compounds present in the two species. No reports on the antibacterial activity of *P. ramosior* or *P. madagascariensis* var. *madagascariensis* were found and the anti-mycobacterial activity of *P. aliciae* is yet to be investigated. Traditionally *P. madagascariensis* plant material is prepared either as a decoction or an infusion when treating respiratory diseases. However, none of the studies investigated an aqueous extract for the inhibition of bacteria associated with lung infections or cytotoxicity against lung cancer cells. An aqueous extract of *P. madagascariensis* was confirmed to significantly inhibit *S. epidermis* that is associated with wound infections. Due to the significant antibacterial activity of the aqueous extract further studies of the extract on bacteria associated with respiratory infections could be beneficial. The noteworthy anti-mycobacterial activity of *P. madagascariensis* could be attributed to the abietane diterpenes 6β,7α-dihydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and 6,7-dehydroroyleanone present in the plant. Diterpenoids are known to have antimicrobial activity that is evident from the noteworthy anti-mycobacterial activity of the extracts against MDR *M. tuberculosis* due to the 7α-AcO group at the B ring.

Traditionally the crushed leaves of *P. madagascariensis* are used to treat wounds and scabies. Several factors are associated with wound healing as described by Robson (1997) as a series of event that includes “coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, epithelialization, contraction, and remodelling”. However, when there is an abnormality in the wound healing process, bacteria can cause infections, which are more difficult to treat especially when the bacteria are resistant to conventional treatment strategies. Bacteria that are associated with bacterial wound infections and nosocomial wound infections are normally *Staphylococcal* species such as *S. epidermidis* and *S. aureus*, and others such as *P. aeruginosa, E. faecalis* and *E. coli* (Parr et al., 1999). From the studies investigated only the acetone extracts of *P. madagascariensis* and *P. aliciae* were

investigated for their antibacterial activity against some of the bacteria associated with wound infections. Interestingly, only *P. aliciae* was bactericidal against *E. faecalis* and slightly more active against *S. aureus* than *P. madagascariensis*. However, none of the studies investigated the antibacterial activity of *P. madagascariensis* and *P. aliciae* on *E. coli* and *P. aeruginosa* and the extracts of *P. ramosior* or *P. madagascariensis* var. *madagascariensis* on any of the bacteria associated with wounds. Furthermore, only the aqueous extract of *P. madagascariensis* was tested for its antibacterial activity against *S. epidermidis*. More studies can be done on the antibacterial activity of these *Plectranthus* species on bacteria associated with wound infections. The essential oil of *P. madagascariensis* was confirmed to target only the Gram-positive bacterium *S. aureus* using the agar disc-diffusion assay. Several factors such as the bacterial species and type of antibiotic determine the sensitivity of the agar disc-diffusion assay. Studies have confirmed that although the agar disc-diffusion assay is consistent to the broth microdilution assay between 71% and 90% of the time, it cannot be used as a sole method to determine the antibacterial activity of a test substance (Dickert et al., 1981). Limitations of the agar-diffusion method include the inability to distinguish between the bactericidal and bacteriostatic activity of the test substance. Furthermore, although it is a fast and inexpensive method it is difficult to quantify the amount of the test substance that diffused into the agar medium and the MIC cannot be calculated (Balouiri et al., 2016). The biological variations that the microdilution assay takes into consideration should also be considered. An established microdilution method is available to determine the MIC value of essential oils as described by Kamatou et al. (2006) that should be considered when testing the antibacterial activity of essential oils. DHR, isolated from the essential oil of *P. madagascariensis* has been confirmed to have moderate antibacterial activity against *M. tuberculosis*, however, no tests were done on other Gram-positive or Gram-negative bacteria providing new opportunities for further research of this compound. Coleon U quinone isolated from *P. madagascariensis* was confirmed to inhibit *S. aureus* and *E. faecalis* that are associated with wound infections at noteworthy concentrations. Furthermore, coleon U and 7α-acetoxy-6β-hydroxyroyleanone displayed moderate to noteworthy antibacterial activity against resistant *S. aureus* and *E. faecalis*. Although the compounds 6β,7α-dihydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone displayed moderate antibacterial activity against Gram-positive bacteria, none of the compounds isolated from *P. madagascariensis* inhibited the Gram-negative bacteria *E. coli* and *P. aeruginosa*. Matias (2016) set out to develop phytosomes that are loaded with acetonic *P. madagascariensis*

extract and chitosan-coated phytosomes containing the acetonic *P. madagascariensis* extract. Agar disc-diffusion studies confirmed that the antibacterial activity of *P. madagascariensis* acetone extract was increased when the phytosomes was coated with chitosan compared to a chitosan-coated phytosomes without the extract. The antibacterial activity of the phytosomes was tested using the microdilution assay and was confirmed to have increased antibacterial activity and reduced toxicity and irritancy potential, however, the chitosan-coated phytosomes control was omitted from the study. When considering the methodologies used to identify the biological activities of the plant extract several articles failed to mention the activity of the positive and negative controls. This is vital information that should be included in biological assays and the reporting of such assays. The relevant controls and their results should be mentioned to ensure that the experiment can be repeated and controls are available to which the success of current and future assays can be measured (Choudhary, 2017).

Although some efforts have been made to develop nanoparticle delivery systems for *P. madagascariensis*, these studies are not complete with crucial information such as the activity of the controls which were missing. Furthermore, no delivery systems have been considered for the other *Plectranthus* species discussed in this review article. Rosmarinic acid is a major compound present in the Lamiaceae family and *Plectranthus* species including *P. madagascariensis*. Rosmarinic acid displayed no antibacterial activity on Gram-positive or Gram-negative bacteria. However, this secondary metabolite has been confirmed to target pain and reduce inflammation in *in vivo* mice studies that were comparable to that of the positive control, indomethacin. Considering the information on wound infections and traditional usage of *P. madagascariensis* for treatment, *P. madagascariensis, P. aliciae* and their isolated compounds have shown some noteworthy antibacterial activity, especially against Gram-positive bacteria associated with wound infections. Studies on the anti-inflammatory activity of the extracts, the potential of the extracts targeting pain, matrix synthesis and tissue remodelling is absent from the literature that can tie the traditional usage of the plant with its biological activity together. Therefore, future studies on the extracts, their compounds, and the delivery systems of these actives for wound remodelling are recommended. Furthermore, several articles have published on the anti-inflammatory potential of rosmarinic acid using animal models. The use of animal models are becoming more difficult due to ethical guidelines. Several *in vitro* anti-inflammatory models are available to determine the anti-inflammatory activity of reviewed *Plectranthus* species and their secondary metabolites. Flow cytometry is a rapid and useful tool to determine the anti-

inflammatory potential of several inflammatory markers simultaneously and should be considered when investigating the anti-inflammatory activity of *P. madagascariensis*, its varieties and secondary metabolites.

The release of free radicals has been associated with inflammation that can contribute to tissue damage; an increased inflammatory response can contribute to a range of diseases. Although the antioxidant activity of a natural product cannot be studied alone to confirm its activity, it can give some information on the free radical scavenging potential of a natural compound. A research article by Amorati and Valgimigli (2015) set out to discuss the advantages and limitations of current antioxidant assays. The DPPH antioxidant assay is the most utilised antioxidant method due to the inexpensive nature and ease of the assay. The endpoint of the assay is normally measured after 30 minutes as with the manuscripts reviewed. Amorati and Valgimigli (2015) acknowledged the potential of the assay, however, pointed out that there are flaws in the assay. Amorati and Valgimigli (2015) proposed a kinetic reading approach since the reactivity rate of natural products is different from one another. Through using the kinetic reading approach, the data will reflect the true rate constants of the actives instead of a single-point reading where this is not possible. Furthermore, solvents such as ethanol and methanol (polar protic solvents) are usually used to perform DPPH antioxidant assays. Studies have confirmed phenolic compounds react faster with DPPH when the compounds were dissolved in polar protic solvents resulting in a skewed data of the actual antioxidant capacity. Although noteworthy antioxidant activity was observed for *P. madagascariensis* and isolated compound, rosmarinic acid, it is not clear if the data presented is a true reflection of the free radical scavenging potential of the extract and compound and could further be explored using more sensitive and reliable antioxidant assays. *In vitro* cell-based antioxidant assays are more sensitive and comprehensive since compounds not necessarily involved in free radical scavenging but compounds that are involved in the antioxidant pathway and protecting cells against oxidative damage can be identified (Da Silva et al., 2016).

It was interesting to note that *P. madagascariensis* and its isolated compounds were found to be active against several enzymes involved in maladies such as diabetes, skin-hyperpigmentation and skin ageing not related to the reported traditional usage of the plant. Traditionally an aqueous extract from the aerial parts of several *Plectranthus* species is used to treat neurological disorders such as epilepsy, meningitis, convulsions, mental retardation

and depression (Lukhoba et al., 2006). The extract and secondary metabolites of *P. madagascariensis* has shown to target the enzymes related to the onset of Alzheimer's disease. Although the traditional usage of *Plectranthus* species for Alzheimer's disease has not been documented for *P. madagascariensis* or its varieties it does show potential to treat Alzheimer's disease and potentially other unexplored neurological disorders. The compounds 7α-acetoxy-6β-hydroxyroyleanone and 6β,7α-dihydroxyroyleanone showed moderate anti-elastase and collagenase activity associated with ageing. *Plectranthus madagascariensis* extracts displayed noteworthy tyrosinase, alcohol dehydrogenase and acetylcholinesterase activity. The compounds isolated from *P. madagascariensis* were confirmed to have noteworthy PEPCK, α-glucosidase, butyrylcholinesterase and acetylcholinesterase activity. However, no study focused on the enzyme inhibitory activity of *P. aliciae, P. ramosior* or *P. madagascariensis* var. *madagascariensis* and their secondary metabolites. These species have potential to target a range of enzymes related to cancer, asthma, diabetes and Alzheimer's diseases that are related to the traditional usage of the plant in some cases but have not been explored. Furthermore, the research articles reviewed did not explore the type of enzyme inhibition in any of the studies to confirm competitive, non-competitive or allosteric competitive inhibition of the extracts or secondary metabolites against the enzymes investigated. This is something that can further be explored to provide vital information when developing therapeutic agents for these maladies.

Lastly, the reviewed studies that isolated compounds from *P. madagascariensis* only focused on isolating, identifying and determining the biological activity of seven secondary metabolites (Figure 1). *Plectranthus* species are known to contain a wide range of compounds that have demonstrated noteworthy medical and economic potential. Although, some researchers have set out to investigate the phytochemicals of *Plectranthus* species this field is still largely unexplored (Abdel-Mogib et al., 2002). No studies were found on isolating and identifying compounds from the variety species. Studies on the phytochemical profiles and biological activities have revealed variety species either contain different secondary metabolites or the same compounds but in varying concentrations (Wang et al., 2017). This difference in phytochemical compounds between varieties could have different biological activities and should further be explored. Several isolation methods and equipment are available for rapid identification of compounds as discussed in an article by Altemimi et al. (2017). HPLC coupled with a mass spectrophotometer provides a rapid and accurate method

for the identification of compounds when pure compounds are unavailable and could be considered for the identification of more compounds in *P. madagascariensis* and its varieties.

7. Conclusion

Plectranthus species are known for their medicinal potential in treating various maladies. *Plectranthus madagascariensis* and its varieties have been used as a medicinal plant in South Africa for centuries, all sharing similar common names. However, research on these varieties indigenous and endemic to South Africa is largely unknown. Due to the morphological similarities between *P. madagascariensis* and its varieties, the possibility exists that researchers unknowingly test variety species rather than *P. madagascariensis.* Lukhoba et al. (2006) has also mentioned the difficulty in discriminating between morphological similar *Plectranthus* species and could contribute to discrepancies in data. Currently, there is a need for information on the morphological differences between these species to clearly distinguish between *P. madagascariensis* and its varieties. *Plectranthus madagascariensis* has a long history for being traditionally used in wound healing which is supported by the antibacterial and anti-inflammatory potential of the plant extract and secondary metabolites. Rosmarinic acid is a major compound of *P. madagascariensis*. The anti-inflammatory and antioxidant activity of rosmarinic acid could be linked to the traditional usage of *P. madagascariensis* for wound care. The ability of rosmarinic acid to reduce swelling and relieve pain could explain the traditional usage of *P. madagascariensis* in the treatment of skin ailments. Furthermore, an aqueous crude extract of *P. madagascariensis* displayed antibacterial activity towards bacteria associated with wounds and antioxidant activity in congruence with traditional preparation and usage of the plant. However, further mechanistic studies on wound healing and inflammatory studies remain elusive. In addition, no research on skin-related diseases such as acne vulgaris, scabies and eczema were observed. With the anti-inflammatory, antibacterial and wound healing activity of *P. madagascariensis* confirmed, other skin related diseases could be a potential target for *P. madagascariensis*. Regarding the traditional usage of *P. madagascariensis* treating respiratory ailments, compounds isolated from the extract has proven cytotoxicity towards lung cancer cells and target *M. tuberculosis*, a bacteria that is the cause of tuberculosis and is under the top ten causes of deaths worldwide according to the World Health Organization (2018). However, no research focused on the use of *P. madagascariensis* or its isolated compounds for the treatments of asthma or other respiratory-related diseases such as bronchitis that could be a potential target for further research. In

addition to rosmarinic acid, six major compounds namely coleon U, 6β,7α dihydroxyroyleanone, 7α-formyloxy-6β-hydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone, coleon U quinone, and 6,7-dehydroroyleanone (isolated from the essential oil) have been proven *in vitro* to have noteworthy antibacterial, anti-cancer, anti-diabetic potential and targeting Alzheimer's disease. These isolated compounds of *P. madagascariensis* have proven to have the potential to be developed into therapeutics targeting these life-threatening diseases. However, no *in vivo* human studies are available for these disease targets, opening a door for further human studies. Several researchers have set out to incorporate *P. madagascariensis* extracts and compounds in nano-delivery-systems, however, the biological activity of these systems is largely unanswered. These delivery systems could contribute to the development the pharmaceutical industry and improve public health, but more research in this area is required. The phytochemical and biological data reviewed suggest the potential use of *P. madagascariensis* and its varieties in the cosmeceutical and pharmaceutical fields. Lastly, toxicity and mutagenic studies are lacking for the species and their secondary metabolites to determine safety of the samples.

This review examines the potential use of *P. madagascariensis* and its varieties that are indigenous to South Africa based on its traditional usage and scientific validation studies to target various diseases. Additionally, this review highlights research opportunities for these species to ultimately develop safe and useful products with medicinal and economic potential.

8. Acknowledgements

This work is based on the research supported wholly by the National Research Foundation of South Africa (Grant Number: 121192).

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CHAPTER 3

Research questions, hypotheses, aims and objectives

Chapter 3

1. Introduction and motivation for the study

Acne vulgaris is an inflammatory disease of the pilosebaceous unit with four pathogenic factors that contribute to its onset. One of the major pathogenic contributors is the colonisation of pathogenic bacteria such as *Cutibacterium acnes* and *Staphylococcus epidermidis* in the sebaceous gland. These bacteria use sebum as a carbon energy source through the release of lipases that hydrolyse sebum triglycerides into glycerol and free fatty acids that contribute to both inflammation and abnormal keratinisation associated with the disease. These bacteria are not only associated with the skin but have been implicated in invasive infections of indwelling medical devices. Furthermore, the widespread use of antibiotics in the treatment of acne and implant infections has become a concern due to a rise in antibiotic resistance associated with these maladies. The World Health Organisation (WHO) (2020) describes antibiotic resistance as having a major threat to the economy and public health. Antibiotic resistance leads to longer treatment times (3-6 months), increased medical costs and a rise in mortalities worldwide. Several studies have confirmed the antibiotic resistance of *C. acnes* and *S. epidermidis* on several commonly used antibiotics used in the treatment of acne vulgaris. Biofilm development associated with antibiotic resistance has been identified for both these bacteria *in vitro* and *in vivo* in acne, prosthetic implant-associated infections and in wounds (Achermann et al., 2014; Fournière et al., 2020; Shiono et al., 2016; World Health Organisation, 2020).

Biofilm development in *C. acnes* and *S. epidermidis* is a result of quorum sensing processes. Quorum sensing is a communication mechanism used by sessile cells to determine the population density of the bacteria at the infection site. Quorum sensing is regulated by the release of signalling molecules that results in gene expression in the bacterial population once a critical concentration has been reached. Therefore, quorum sensing is a method for bacteria to elicit a virulent response and successfully infect the host once there are enough bacteria to overcome the hosts immune response. The expression of several genes linked to biofilm development, the release of virulent factors such as lipases, increased motility, extracellular DNA production and release involved in bacterial adherence and biofilm structure are under quorum sensing control. Gram-negative bacteria communicate with Nacyl-homoserine lactones as signalling molecules and Gram-positive bacteria with the release of oligopeptides.

Autoinducer-2 (AI-2) is considered a universal signalling molecule in inter-species communication. Both *C. acnes* and *S. epidermidis* has been confirmed to produce AI-2 *in vitro* and have the ability to form biofilms and release several virulent factors associated with inflammation. Therefore, quorum sensing can be considered the first step in antibiotic resistance and the pathogenesis of *C. acnes* and *S. epidermidis* (Coenye et al., 2008).

Several bacteria form part of the normal skin microbiome with *C. acnes* and *S. epidermidis* being the most prominent commensal bacteria found on the skin. These commensal microorganisms live in a symbiotic relationship to maintain the skin barrier and protect the skin from pathogenic microorganisms. However, dysbiosis of these commensal bacteria can result in skin related maladies such as acne vulgaris. This imbalance in microorganisms has been linked to a shift in *C. acnes* phylotypes with *C. acnes* phylotype IA1 becoming more dominant compared to phylotypes IA2, IB, IC, II and III in acne affected skin. *C. acnes* phylotype IA1 has been confirmed to release more virulence factors such as lipases and have a higher biofilm forming ability compared to the other phylotypes. Furthermore, studies have confirmed that *C. acnes* type I bacteria were more commonly isolated from prosthetic implants than type II and type III (Dréno et al., 2020; Fernandez Sampedro et al., 2009; Fournière et al., 2020).

An increase in antibiotic resistance associated with acne vulgaris, prosthetic implant-associated infections and wounds due to quorum sensing mechanisms of *C. acnes* and *S. epidermidis*, served as motivation to investigate the potential role of these bacteria in a multispecies system. An endemic South African *Plectranthus* species, *Plectranthus aliciae* is traditionally used to treat skin maladies and wounds (Lambrechts and Lall, 2021). However, *in vitro* studies on this specie remain elusive. *Plectranthus aliciae*, it's partitions and identified compounds were selected for further studies targeting antibiotic resistance through targeting quorum sensing mechanisms of *C. acnes* and *S. epidermidis* in single and multispecies systems.

2. Research questions

The project is sub-divided into three parts with each one benefitting previous knowledge.

2.1 The relationship of *c*

The research questions relating to the relationship of *C. acnes* and *S. epidermidis* were as follows:

- Do the bacterial species *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) have an antagonistic relationship *in vitro*?
- When grown as a multispecies system do these bacteria have a higher antibiotic resistance compared to a single species system?
- When grown as a multispecies system do these bacteria form a thicker biofilm compared to a single species system?
- When grown as a multispecies system is more AI-2 produced compared to a single species system?
- When grown as a multispecies system is more lipase produced compared to a single species system?
- When grown as a multispecies biofilm is more extracellular DNA (eDNA) present compared to a single species biofilm?

2.2 Activity of *Plectranthus aliciae*, its partitions and compounds

The traditional usage *P. aliciae* and the antibacterial activity of the compounds present in the plant led to the following research questions:

- Does the ethanolic leaf extract of *P. aliciae* (PA_{EtOH}) have antibacterial activity against *C. acnes* and *S. epidermidis*?
- Do the partitions of the PA_{EtOH} have antibacterial activity individually or does it have a combined antibacterial effect as a whole extract?
- Are there compounds present in the PA_{EtOH} that have not been identified before?
- Do these compounds have antibacterial activity against *C. acnes* and *S. epidermidis* in single and multispecies systems?
- Does PA_{EtOH}, its partitions and compounds target biofilm formation and antibiotic resistance by preventing attachment and formation of *C. acnes* and *S. epidermidis* biofilms in single and multispecies systems?
- Does PA_{EtOH}, its partitions and compounds target antibiotic resistance through eradication of a mature biofilm of *C. acnes* and *S. epidermidis* in single and multispecies systems or are these samples able to penetrate the mature biofilm and inhibit the bacteria within?

- Can PA_{EtOH}, its partitions and compounds target inter- and intra-species communication by targeting AI-2 production in *C. acnes* and *S. epidermidis* in single and multispecies systems?
- Can PA_{EtOH}, its partitions and compounds target virulence factors of *C. acnes* and *S. epidermidis* such as lipase enzymes?
- What is the effect of PA_{EtOH}, its partitions and compounds on wound healing?
- Is PA_{EtOH} harmful to human skin cells and does it have a mutagenic effect?
- Is PA_{EtOH} irritating to human skin, and can it potentially be used in a formulation for treating various types of non-inflammatory and inflammatory acne?

2.3 Activity of gold nanoparticles (AuNPs)

AuNPs formed from plant extracts and other compounds provide an alternative mode of bioactivity and delivery of the actives to the target site. Nanoparticles formed with plant extracts have shown significant antimicrobial and antioxidant activity *in vitro*. This information has led to the following research questions:

- Can the antibacterial activity of PA_{EtOH}, rosmarinic acid and tetracycline be enhanced by the synthesis of AuNPs?
- Do the AuNPs have an enhanced bacterial attachment
- Do the AuNPs have an enhanced wound healing potential compared to their sample controls?
- Do the AuNPs have a lowered cytotoxicity to their sample controls?

3. Aims and objectives

The aims and objectives of the project were divided into three sections. Firstly, to identify and confirm the relationship between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) as model bacterial strains related to antibiotic resistance as single and multispecies systems. Secondly, to determine the potential of *P. aliciae* to target antibiotic resistance that is associated with the model bacteria as individual pathogens and as a multispecies pathogenic system. Lastly, attempt to improve the activity of *P. aliciae*, an identified compounds and a commonly used acne antibiotic, tetracycline, by synthesising gold nanoparticles.

Several studies have confirmed the co-existence of *C. acnes* and *S. epidermidis* in acne lesions and implant related infections *in vivo*. However, controversy exists about the type of relationship between these bacteria. Furthermore, there is no information available on the effect these bacteria have on one another in terms of quorum sensing molecule production and release, virulence factor release and biofilm development. Therefore, the objectives were as follows:

- Determine if *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) have a synergistic or antagonistic relationship.
- View the bacterial biofilm structure as single and multispecies biofilms to determine the spatial distribution of the bacteria in the biofilm.
- Determine the release of AI-2 in single and multispecies bacterial growth conditions.
- Determine the release of lipase as a virulent factor in single and multispecies bacterial growth conditions.
- Determine the effect of multispecies growth conditions on biofilm production using the biofilm crystal violet staining assay.
- Determine the release of eDNA in single and multispecies biofilms.

To determine the antimicrobial effect of *P. aliciae* on skin related pathogens and targeting factors related to quorum sensing and antibiotic resistance, the objectives of the study were to:

- Identify active partitions and compounds present in PA_{EtOH}.
- Determine the antibacterial activity of PA_{EtOH}, its partitions and compounds on *C. acnes* and *S. epidermidis* as single and multispecies bacterial systems.
- Determine the bacterial adhesion and biofilm formation inhibitory activity of PA_{EtOH}, its partitions and compounds on *C. acnes* and *S. epidermidis* as single and multispecies biofilms.
- Determine the biofilm eradication potential of PA_{EtOH}, its partitions and compounds on *C. acnes* and *S. epidermidis* as single and multispecies biofilms.
- Determine the potential of PA_{EtOH}, its partitions and compounds to inhibit bacteria within the *C. acnes* biofilm using an artificial sebum model.

- Determine the effect of PA_{EtOH}, its partitions and compounds on *C. acnes* and *S. epidermidis* as single and multispecies systems on AI-2 at sub-MIC concentrations.
- Determine the effect of PA_{EtOH}, its partitions and compounds on *C. acnes* and *S. epidermidis* as single and multispecies systems on the lipase enzyme at sub-MIC concentrations.
- Determine the inhibitory activity of PA_{EtOH}, its partitions and compounds on eDNA eradication in *C. acnes* and *S. epidermidis* single and multispecies biofilms.
- Determine the cytotoxicity of PA_{EtOH}, its partitions and compounds on a human keratinocyte cell line (HaCat).
- Determine the wound healing potential of PA_{EtOH}, its partitions and compounds using human keratinocytes (HaCat) at sub-MIC concentrations.
- Determine the mutagenic potential of PA_{EtOH} using the Ames test.
- Determine the *in vivo* irritancy potential of a formulation containing PA_{EtOH}.
- Determine the *in vivo* activity of PA_{EtOH} on volunteers affected by various degrees of acne vulgaris.

Nanotechnology has in recent years become an area of interest for improving drug bioactivity, availability and delivery to a target site. The objectives for forming AuNPs with the samples were:

- To form AuNPs with the selected samples and determine their physical characteristics.
- Determine if there is an improvement in the antibacterial activity of the selected AuNPs samples.
- Determine the cytotoxicity of the selected AuNPs on HaCat cells.
- Determine if there is an improvement in the wound healing activity of the selected AuNPs samples.

4. Structure of the thesis

The content of each chapter is as follows:

Chapter 1: General introduction and literature review on quorum sensing related to acne vulgaris

This chapter provides background on quorum sensing in various bacteria and its role in *C. acnes* and *S. epidermidis*. Furthermore, the chapter explores the role of these bacteria and their quorum sensing mechanisms in antibiotic-resistant acne vulgaris. Previous research on plants targeting quorum sensing and antibiotic resistance is also discussed.

Chapter 2: Traditional usage and biological activity of *Plectranthus madagascariensis* and its varieties: a review

Plectranthus aliciae (Codd) Van Jaarsv. & T.J. Edwards was previously classified as *Plectranthus madagascariensis* var. aliciae Codd. This chapter explores the traditional and pharmacological activity of these species.

Chapter 3: Research questions, hypotheses, aims and objectives

Chapter 4: *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards and its compounds targeting quorum sensing related to acne vulgaris and *in vivo* acne studies

This research study explores the quorum sensing relationship of *C. acnes* (ATCC® 6919) and *S. epidermis* (ATCC® 35984). This study investigated the potential synergistic relationship of these strains to cause an increased virulent response and potentially an increased in antibiotic resistance. Furthermore, the chapter discusses the biological activity of the ethanolic extract of *P. aliciae*, its partitions and identified pure compounds targeting quorum sensing and its related mechanisms in single and multispecies *C. acnes* and *S. epidermidis* systems. Lastly, the chapter describes the *in vivo* safety and efficacy of *P. aliciae* formulated in a gel formulation to treat acne vulgaris.

Chapter 5: Gold nanoparticles of *Plectranthus aliciae*, rosmarinic acid and tetracycline for wound healing and antibacterial activity against *Cutibacterium acnes* and *Staphylococcus epidermidis*

This chapter discusses the biological activity of synthesised gold nanoparticles from *Plectranthus aliciae*, the pure compound rosmarinic acid and the antibiotic tetracycline. The chapter includes antibacterial activity, inhibitory activity of bacterial adhesion and biofilm formation, cytotoxicity, and wound healing potential.

Chapter 6: Concluding remarks, impact, novelty, and future recommendations

5. Methodology and thesis layout

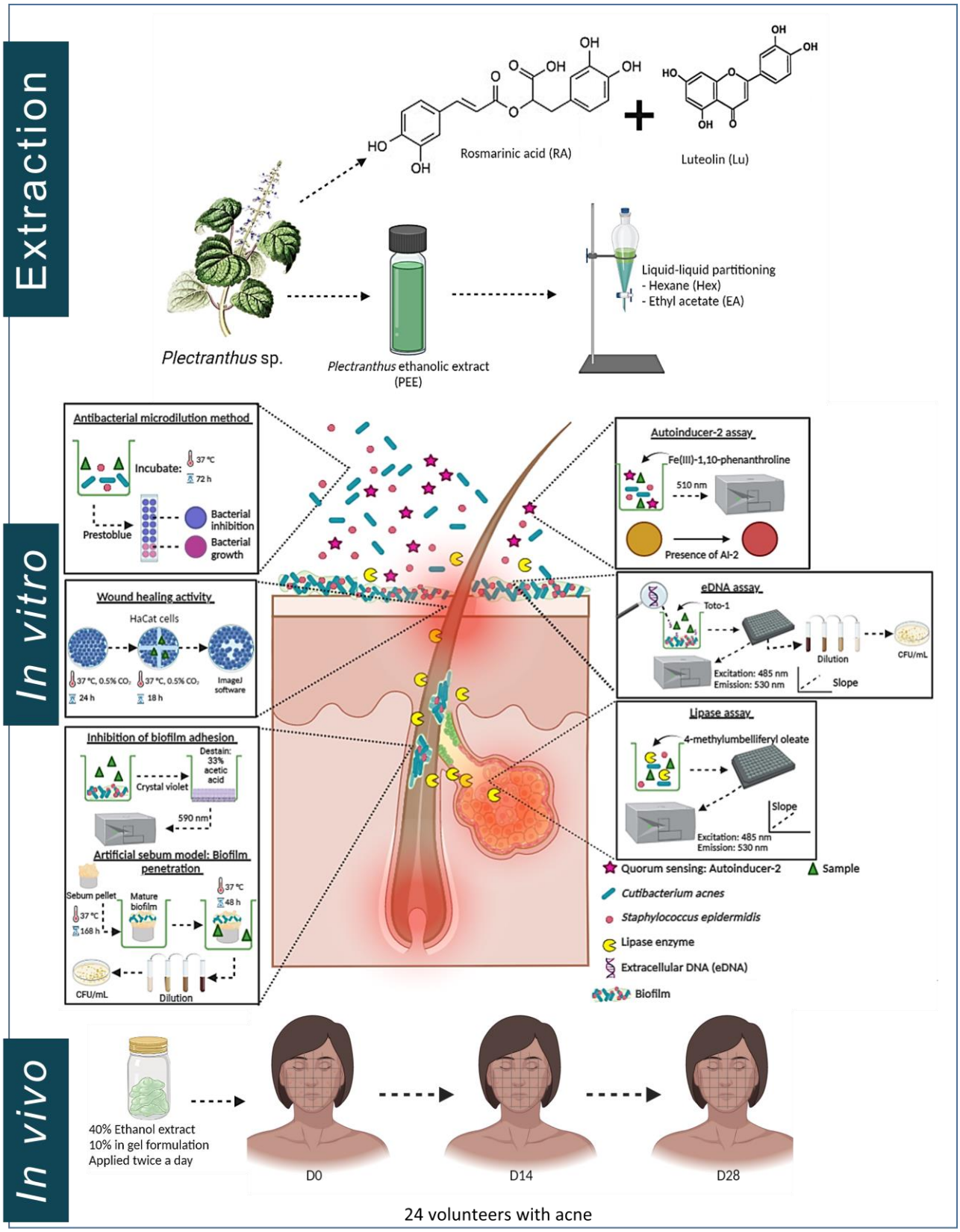

Figure 3. 1: Experimental design and thesis layout.

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CHAPTER 4

Plectranthus aliciae (Codd) Van Jaarsv. & T.J. Edwards and its compounds targeting quorum sensing related to acne vulgaris and *in vivo* acne studies

Chapter 4

This chapter will be submitted to the
Journal Biomedicines. Impact factor: 6.081
In November 2022
Titled: *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards and its compounds targeting quorum sensing related to acne vulgaris and *in vivo* acne studies

Abstract

Within a bacterial community, bacteria communicate through the release of chemical signalling molecules called autoinducers. Autoinducer-2 is known as a universal signalling molecule for interspecies communication. These signalling molecules are released through a quorum sensing mechanism which regulates the bacteria's gene expression only once an optimal concentration of bacterial cells have been reached. This form of communication ensures that the bacterial community work as a unit to elicit a response which could be detrimental for the host. Quorum sensing has been linked to the release of virulent factors such as lipase enzymes that trigger an inflammatory response but, most importantly, increases the release of extracellular DNA (eDNA) involved in biofilm development. A biofilm is a protective layer produced by bacteria to protect the community from external agents such as antibiotics, contributing to antibiotic resistance. Antibiotic resistance has become a global concern that renders current therapies ineffective, resulting in longer treatment times and in some cases, death. Antibiotic resistance associated with *Cutibacterium acnes* and *Staphylococcus epidermidis* is currently observed in maladies such as acne vulgaris, prosthetic implant-associated infections and wounds. Although both *C. acnes* and *S. epidermidis* are commensal bacteria and form part of the normal flora of the skin, researchers have confirmed that both these Gram-positive bacteria are opportunistic bacteria under quorum sensing control, resulting in maladies that are painful and difficult to treat. Current therapies do not target all the quorum sensing factors that cause inflammation and antibiotic resistance in acne vulgaris, prosthetic implant-associated infections and wounds. Furthermore, the relationship between *C. acnes* and *S. epidermidis* in the skin as opportunistic bacteria is not yet fully understood, and controversy exists with regards to the relationship of these bacteria in a multispecies system.

The relationship between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) was determined as synergystic, using the agar plate assay and scanning electron microscopy. As a

multispecies system grown under aerobic conditions, the bacteria formed a significantly denser mature biofilm ($P < 0.001$), produced more eDNA ($P < 0.001$), and produced more lipase enzyme ($P < 0.05$) when compared to the single species systems. Furthermore, a slight increase in AI-2 was observed. This data supports the hypothesis that *S. epidermidis* potentially provides a safe environment for the anaerobic *C. acnes* under aerobic environments. The ethanolic extract of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards, an endemic South African plant, and its hexane and ethyl acetate partitions displayed the most promising results in targeting the factors associated with quorum sensing and wound healing. Significant wound closure activity was observed for *P. aliciae* ($P < 0.05$) and its hexane ($P < 0.01$) and ethyl acetate ($P < 0.01$) partitions. This is the first report which identified the compound luteolin in the ethanolic extract of *P. aliciae*. *In vivo* studies confirmed the anti-acne activity of the *P. aliciae* extract against various acne vulgaris lesions, including micro cysts. This study is also the first to report the effect of *P. aliciae*, its partitions, and identified compounds against quorum sensing between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984).

1. Introduction

Traditional medicine plays a crucial part in the daily lives of South Africans. It is estimated that around 27 million South Africans rely on traditional medicine as their primary source of health care. In the year 2000, the World Health Organisation (WHO) African Regional Committee committed to improving the health of all on the African continent by promoting and optimising traditional medicines to reduce morbidity and death related to various maladies. This has put an emphasis on discovering new alternative medicines and therapies from indigenous and endemic plants to treat diseases that are a concern not only to the African continent but also globally (Street et al., 2008; Mothibe and Sibanda, 2019).

The Lamiaceae family is a group of aromatic plants that are traditionally and commercially used for their medicinal properties. Species of the *Plectranthus* genus have displayed promising activity against various maladies and are widely used in traditional medicine for their wide range of medicinal properties. However, this genus is largely unexplored, and more research is required on species from this genus to develop commercially safe and effective products. *Plectranthus aliciae* is an endemic South African *Plectranthus* species traditionally used to treat skin-related maladies such as scabies and wounds. Studies have

confirmed the antibacterial activity of *P. aliciae* and its compounds for treating inflammation and wounds. However, more research on this underexplored plant is required to confirm these claims (Lambrechts and Lall, 2021).

Much like humans, bacteria can communicate with one another to regulate social activities within a community. Bacterial species can communicate with bacteria of the same species (intraspecies communication) or bacteria from other species (interspecies communication). This form of communication is known as quorum sensing. Bacteria release signalling molecules called autoinducers to regulate gene expression. This form of communication assists bacteria to determine the bacterial population within the community and only once a specific autoinducer concentration is present and the bacterial population is large enough, will it elicit a response. A response by one bacterium will be unproductive compared to a community of bacteria and will not have a large enough response to evade potential threats. This form of communication helps a consortium of bacteria to synchronise a response through gene expression within the community. At high cell density, genes that favour the survival of the community are expressed. Intraspecific communication between Gram-positive bacteria occurs through the release of autoinducing peptides (AIP) and for Gram-negative bacteria, acyl-homoserine lactones (AHL). In addition, the discovery of autoinducer-2 (AI-2) in the bacteria *Vibrio harveyi* sparked the idea of interspecies communication. AI-2 is considered a universal "language" between different bacterial species as a result of quorum sensing. Several bacterial social activities have been linked to the release of AI-2, such as biofilm formation, the release of virulent factors and increased bacterial mobility in various bacterial species. Genes (*lux*S) required to produce AI-2 have been discovered in thousands of Gram-positive and Gram-negative bacterial species. Researchers have discovered that bacteria can use quorum sensing and AI-2 intraspecies communication to cause disease in humans and plants (Miller and Bassler, 2001; Waters and Bassler, 2005).

The microbiome of the skin is a complex system of various bacteria. These bacteria are mostly harmless and protect the skin from various pathogens that could cause disease. The microbiome consists mainly of *Brevibacterium*, *Corynebacterium*, *Cutibacterium, Micrococcus and Staphylococcus* species. It is believed that these bacteria colonise the skin in a mutualistic relationship and form part of the skin barrier function, protecting the skin from pathogens. Of these species, the commensal bacteria *Cutibacterium acnes* and *Staphylococcus epidermidis* are believed to be the primary colonisers of the skin. However,

dysbiosis of these commensal bacteria can occur due to a shift in conditions, which are more favourable for pathogenic bacterial strains, resulting in skin-related maladies such as acne vulgaris (Fournière et al., 2020).

Acne vulgaris is an inflammatory disease of the pilosebaceous unit. Four major pathophysiological factors are associated with the onset of inflammatory acne. The first of these factors is an increase in sebum production, secondly hyper keratinisation, thirdly, the proliferation of acne-causing bacteria *C. acnes* and *S. epidermidis* and lastly inflammation. Clinical studies have identified both *C. acnes* and *S. epidermidis* in acne lesions of patients suffering from the disease. These studies have confirmed a loss in *C. acnes* phylotype diversity in acne lesions, especially a predominance of phylotype IA1 resulting in acne lesions. *C. acnes* type IA1 has been linked to increased biofilm development and the release of virulent factors such as lipase enzymes, factors believed to be under quorum sensing control. Lipase enzymes hydrolyse sebum triglycerides into glycerol and free fatty acids that serve as a nutrient source for the bacteria and contribute to inflammation, respectively. *Staphylococcus epidermidis* is considered a skin commensal bacterium but has been characterised as an opportunistic pathogen that can enter the bloodstream and has been implicated in prosthetic implant-associated infections. As with *C. acnes*, *S. epidermidis* has been linked to the production of several virulence factors such as lipase enzyme release and biofilm formation (Fournière et al., 2020).

In nature, bacteria rarely occur in a single species system or a planktonic state but rather as multispecies biofilm communities. Studies have reported an increase in *S. epidermidis* by 70% and *C. acnes* by 80% in acne lesions. *In vivo* studies have identified both *C. acnes* and *S. epidermidis* in acne lesions alone and as a multispecies system, indicating that both these bacteria contribute to acne development. Nakase et al. (2014) reported an increase in antibiotic-resistant *C. acnes* and *S. epidermidis* associated with acne lesions.

In addition to *C. acnes* and *S. epidermidis* being implicated in acne vulgaris, they have also been linked to the pathophysiology of indwelling medical devices such as cardiac implants, breast implants, prosthetic joints, ocular and neurological implants. Removal and treatment of these infections can be highly invasive, often needing more surgery and some costly antibiotics. Infection results from either contamination of the wound or the implant before or during surgery (Platsidaki and Dessinioti, 2018a).

Biofilms consist of a community of bacteria of either the same species or various bacterial species in a protective extracellular polymeric substance (EPS) attached to a substratum. Biofilm formation is activated through quorum sensing signalling genes. Biofilms serve as a protective layer for the bacteria from unfavourable changes in the external environment, host immune cells and antibiotics. Antibiotic resistance has become a concern to the WHO since antibiotics cannot penetrate this protective layer preventing them from killing the bacteria within, rendering these medications ineffective. This can result in longer treatment times for various diseases that can be extremely expensive or result in death. Biofilm formation is a three-step process, firstly the adsorption of macro and micro molecules to a surface, secondly the attachment of the bacteria to the surface and the release of EPS and lastly, the formation of bacterial colonies and the maturation of the biofilm. The EPS consists of water, external deoxyribonucleic acid (eDNA), proteins and exopolysaccharides. Extracellular DNA serves as one of the major components of the biofilm matrix and is released through an autolysis mechanism. Apart from being an integral structural component of the bacterial biofilm, eDNA is involved in the initial attachment of the biofilm to a surface and serves as a nutrient source for bacterial growth and horizontal gene transfer involved in the uptake and transfer of resistant genes. Biofilm formation has been linked to antibiotic resistance in several diseases, including acne, chronic wounds and prosthetic implant-associated infections (Finkel and Kolter, 2001; Steinberger and Holden, 2005; Montanaro et al., 2011; Sharma et al., 2019).

Although several studies have focused on the role of *C. acnes* in acne vulgaris, studies on the role of *S. epidermidis* are still in their infancy. Controversy exists about the role of *S. epidermidis* in acne vulgaris and its relationship with *C. acnes* as commensal and pathogenic bacteria. Furthermore, little is known about quorum sensing and the presence and abundance of virulent factors such as lipase and eDNA in single and multispecies systems of *C. acnes* and *S. epidermidis*. This information can be helpful in better understanding biofilm structures of these species either as single or multispecies communities associated with acne, wounds, and prosthetic implants. The results obtained in this study provide information focusing on the potential relationship of *C. acnes* and *S. epidermidis*, the quorum sensing inhibitory effect and antibiotic resistance inhibitory effect of *P. aliciae*, its partitions and identified pure compounds.

2. Materials and methods

2.1 Agar plate synergistic assay between Cutibacterium acnes and Staphylococcus epidermidis

Cutibacterium acnes and *S. epidermidis* were grown as a multispecies community on Brain Heart Infusion (BHI) agar plates to determine if the bacteria have a synergistic or antagonistic relationship. The method, as described by Christensen et al. (2016), was followed with slight modifications. These modifications included growing *C. acnes* American Type Culture Collection (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) bacteria in anaerobic and aerobic conditions on BHI agar. Briefly, 72 h cultures of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) were inoculated n BHI broth to a concentration of 6×10^8 CFU/mL (OD_{600nm} 0.4). To a BHI agar plate, 150 µL of the individual bacteria was added and spread evenly across the plate to form a lawn of bacteria. The plates were allowed to dry for 15 min in a laminar flow cabinet, thereafter 10 µL of the test bacteria, the growth media and bacterial control was added as a single drop to the plate and allowed to dry in the laminar flow cabinet. The agar plate containing the *C. acnes* lawn was incubated anaerobically, and the plate containing the *S. epidermidis* lawn was incubated aerobically for 72 h at 37 ºC. After 72 h, the plates were analysed visually for bacterial growth and zones of inhibition.

2.2 Scanning electron microscopy (SEM)

Biofilm development of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) alone and in combination were visualised using scanning electron microscopy (SEM). This was performed to visualise the spatial distribution of bacteria when grown in a multispecies community and to determine the type of relationship among them.

The frosted side of microscopy slides were cut with a glass cutter in quarters and sterilised at 121 ºC for 15 min. The sterilised glass slides were transferred to a 24 well plate at a 45º angle with sterile forceps. A bacterial suspension (OD_{600nm} 0.2) for each bacteria was prepared in BHI broth. To each well, 2 mL of bacteria was added, and the plates were incubated anaerobically for 72 h at 37 ºC.

The slides were prepared for SEM evaluation according to the method of the Laboratory of Microscopy and Microanalysis (Document ID: SEM chemical prep) at the University of Pretoria, South Africa and Sambrook and Russel (2001). In brief, the slides were washed with

0.075 M sodium phosphate buffer (pH 7.4) and the cells were fixed to the slides with 2.5% Glutaraldehyde/ Formaldehyde solution for 1 h. The fixative solution was then removed, and the slides were washed three times for 15 min with sodium phosphate buffer. After washing, the buffer was removed, and 1% Osmium Tetroxide was added to post-fix the cells. The fixative solution was removed after 1 h, and the slides were rewashed three times for 15 min with sodium phosphate buffer. The buffer was removed, and the cells were serially dehydrated with 30%, 50%, 70%, 90% ethanol and three times with 100% ethanol, each for 15 min. The final wash step with 100% ethanol was left for 30 min. The ethanol was removed, and a 50:50 mixture of Hexamethyldisilazane (HMDS) and 100% ethanol was added to the slides and left for 1 h completely covered. The ethanol: HMDS solution was then removed, and HMDS was added and left for a further 1 h, completely covered. After 1 h, the HMDS was removed, and fresh HMDS was added to the slides, and the 24-well plate was left open in a fume hood to dry overnight. Dried slides were then sputter-coated with carbon before observing the bacteria with a Zeiss Crossbeam 540 FEG scanning electron microscope at an acceleration voltage of 1000 kV.

2.3 Plant material extraction

Plectranthus aliciae plant material was collected from the Manie van der Schijff Botanical Garden at the University of Pretoria with the help of the garden curator Mr Jason Sampson. Further taxonomical species identification was made with the help of Ms. Magda Nel from the H. G. W. J. Schweickerdt Herbarium at the University of Pretoria, and a voucher specimen number was deposited (PRU 122336).

Leaves and soft twigs of *P. aliciae* were collected, making sure to remove hard wooden twigs. After collection, the plant material was rinsed with distilled water and allowed to air dry for 1 h before it was dried at 40 ºC in an oven for approximately 48 h or until dry. The dried material was ground with a 0.22 mm sieve using an IKA MF 10 Basic grinder to a fine powder. The ground material was extracted using 99% ethanol (1:50 ratio of plant material to ethanol) for 72 h on a shaker. The extracted material was filtered using vacuum filtration and Whatman® 1.0 filter paper. The extract was subjected to reduced pressure using a Buchi-R200 Rotavapor until dry. To ensure that the *P. aliciae* ethanolic extract (PA_{EtOH}) was completely dry, it was dried to complete dryness at 40 ºC for 24 h in an oven. The extract was stored at room temperature until further use.

2.4 Ultra-Performance Liquid Chromatography - Quantitative Time of Flight (UPLC-QTOF) analysis.

UPLC-QTOF was used to quantify nineteen pure compounds purchased from Sigma Aldrich in PA_{EtOH}. The method, as described by Wooding et al. (2017), was used utilizing a Waters Synapt G2 QTOF system. Each compound was analysed at five concentrations (0.025 µg/mL, 0.25 µg/mL, 2.5 µg/mL, 25 µg/mL, 250 µg/mL) and a standard curve plotted to quantify the compounds and major fractions present in PA_{EtOH}.

2.5 Liquid-liquid partitioning

The crude ethanolic extract of *P. aliciae* was subjected to liquid-liquid separation to obtain four partitions as described by Kishore et al. (2018) with slight modifications. The extract (16.67g) was dissolved in 350 mL distilled water (dH_2O). The aqueous layer was successively extracted three times in 400 mL of hexane, ethyl acetate and n-Butanol (saturated). Saline water was added to improve the separation between the aqueous and ethyl acetate layers. The organic layers were dried with sodium sulphate (Na_2SO_4) and filtered twice through Whatman® 1.0 filter paper, dried at 40 ºC and stored at room temperature until further use.

2.6 Antibacterial activity

The antibacterial activity of PA_{EtOH}, partitions and identified compounds were tested against *C. acnes* (ATCC® 6919). In addition, PA_{EtOH}, partitions and compounds that showed an inhibitory effect against *C. acnes* was selected for further testing against *S. epidermidis* (ATCC® 35984) and a combination of *C. acnes* - *S. epidermidis* (6 x 10^6 CFU/mL; CA-SE). As described by de Canha et al. (2020), the microdilution method was followed with slight modifications. These modifications included testing a multispecies system of CA-SE instead of a single species system at 6 x 10^6 CFU/mL. Briefly, samples were dissolved in dimethyl sulfoxide (DMSO) and sonicated for 1 h at 35 ºC. In a sterile 96-well plate, 100 µL of the sample was combined with 100 µL of BHI broth and serially diluted. BHI broth was inoculated with 72 h old bacterial cultures grown on BHI agar at 37 ºC under aerobic (*S. epidermidis*) and anaerobic (*C. acnes*) growth conditions. The inoculated BHI broth had a final bacterial concentration of 6 x 10^6 CFU/mL. To all the wells of the 96-well plate, 100 µL of the bacterial culture solution was added. The final concentrations of the samples and

tetracycline, the positive control ranged between 500-3.91 µg/mL and 50-0.39 µg/mL, respectively. The plates were incubated at 37 ºC for 72 h aerobically (*S. epidermidis* and CA-SE) and anaerobically (*C. acnes* and CA-SE) in an Anaerocult® jar with Anaerocult A®. A DMSO (2.5% v/v) vehicle control, a growth media control without bacteria, and a bacterial control for each bacteria and CA-SE were included in the study. After 72 h, 20 µL of PrestoBlue® reagent was added to all the wells, and the plates were incubated for 1 h. The minimum inhibitory concentration (MIC) was determined visually through a colour change from blue to pink in the presence of metabolically active bacteria.

2.7 Inhibition of biofilm formation and adhesion

The method for the adhesion of *C. acnes* biofilms was followed as described by Coenye et al. (2007) with slight modifications. BHI broth was inoculated with 72 h bacterial cultures of *C. acnes* (ATCC® 6919), *S. epidermidis* (ATCC® 35984) and CA-SE (OD_{600nm} of 0.2; diluted 1:50). To the wells of a 96-well plate, all samples, including tetracycline, were serially diluted in BHI broth to concentrations ranging between 500-3.91 µg/mL and 50-0.39 µg/mL, respectively. Bacterial and broth controls were included in the study that served as 100% and 0% adhesion controls, respectively. The plates were incubated at 37 ºC for 72 h aerobically (*S. epidermidis* and CA-SE) and anaerobically (*C. acnes* and CA-SE) in an Anaerocult® jar with Anaerocult A® for 72 h. A DMSO (2.5% v/v) vehicle control, a growth media control without bacteria, and a bacterial control for each bacteria and CA-SE were included. Following incubation, the BHI was removed, and the plates were washed once with 200 µL distilled water (dH_2O) aspirated and allowed to air dry. The biofilm was fixed by adding 200 µL of 99% methanol for 15 min. After fixation, the methanol was removed and the plates were allowed to air dry. A 0.5% crystal violet (CV) solution was added to the wells and allowed to incubate for 20 min. After staining, the plates were washed with distilled water until the water ran clear and allowed to air dry. The biofilms were then destained with 210 µL of 33% acetic acid and allowed to shake at 150 rpm for 20 min on a Labcon platform shaker. The destained samples were transferred (100 µL) to clean 96-well plates, and the optical density was measured at 590 nm using a Perkin Elmer VICTOR Nivo microplate reader. The fifty percent inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 4.0 software.

2.8 Eradication of a mature biofilm

Studies were done to determine if the samples can eradicate a mature, fully formed *C. acnes*, *S. epidermidis* and CA-SE biofilms. The samples were tested at their respective 2×MIC concentrations due to the maturity and difficulty of eradicating a mature biofilm. The method, as described by Brackman et al. (2014) was followed, with slight modifications. Biofilms were grown in 96-well flat-bottom plates for the various bacteria in BHI broth (OD_{600nm} 0.2; diluted 1:50; 200 µL per well) for 72 h at 37 ºC under aerobic (*S. epidermidis* and CA-SE) and anaerobic (*C. acnes* and CA-SE) growth conditions. After 72 h, the plates were washed twice with PBS, and 200 µL of sterile BHI broth was added. A mature biofilm was allowed to form for 72 h where after the broth was carefully removed and 200 µL of the samples at their respective concentrations diluted in BHI broth added to the relevant wells. The plates were incubated for a further 48 h under aerobic and anaerobic growth conditions for the various bacteria and bacterial combinations. A 2.5% v/v DMSO, bacterial and broth control were included in the study. After 48 h, the plates were oven-dried, and the biofilm was fixed with 200 µL of 99% methanol for 15 min. The plates were washed twice with 200 µL of dH_2O, oven-dried and 200 µL of 99% methanol added again for 15 min and left to dry. The plates were stained with 0.5% crystal CV for 20 min and destained with 33% acetic acid for 15 min while shaking (150 rpm). The destained samples were transferred (100 µL) to clean 96-well plates, and the optical density was measured at 590 nm using a Perkin Elmer VICTOR Nivo microplate reader.

2.9 Artificial sebum model

Studies on the antibacterial activity of selected samples on *C. acnes* (ATCC® 6919) using the artificial sebum model was conducted as described by Spittaels and Coenye (2018). The sebum pellets were produced by combining tripalmitin (2 g), palmitic acid (300 mg), cholesterol (100 mg), tocopherol acetate (one drop), triolein (0.55 mL), jojoba oil (1.437 mL) and squalene (0.877 mL) and heated to 85 ˚C until a homogeneous oil was obtained which was then autoclaved. The warm oil (1 mL) was then mixed with 1 mL of double concentrated Reinforced Clostridial Agar (RCA) and 1 mL Reinforced Clostridial Media (RCM). The warm artificial sebum mixture (50 µL) was then added to autoclaved medical-grade silicone discs (diameter: 6 mm, thickness: 4 mm). The sebum pellets were allowed to dry in a sterile petri dish and stored at 5 ˚C until use.

Cutibacterium acnes were cultured on an RCA plate and incubated for 72 h at 37 ˚C anaerobically. An overnight culture of the bacteria was made by inoculating RCM with the bacteria. The liquid culture was then further incubated anaerobically at 37 ºC overnight. The bacterial suspension was prepared by centrifuging the overnight *C. acnes* culture for 5 min at 2 200 rcf, whereafter the supernatant was discarded, and the pellet resuspended in 2.5 mL phosphate-buffered saline (PBS; pH 7.4). The washing step was done two more times to remove the remaining RCM. The bacteria were resuspended in PBS to 5×10^6 CFU/mL (OD_{590nm} 0.05). The pellets were transferred to the inner wells of a sterile 48-well plate using sterile forceps. To the outer wells of the 48-well plate, 500 µL of sterile Milli-Q water was added. The bacterial suspension (800 µL) was added to each pellet, and the plates were incubated at 37 ˚C for 15 min. After incubation, the bacterial suspension was removed, and the pellets were washed once with 800 µL of PBS, whereafter the PBS was removed, and the pellets were incubated anaerobically at 37 ˚C for 7 days.

The samples were prepared by dissolving 2 mg of sample in 100 µL of DMSO and 900 µL of PBS. The samples were tested at a concentration equal to their 2×MIC *in vitro C. acnes* (ATCC® 6919) MIC concentration and were prepared by diluting it further in PBS. To the 7-day old pellets, 800 µL of the sample was added, and the pellets were incubated at 37 ˚C for 48 h anaerobically. A DMSO (2.5% v/v) vehicle control, a growth media control without bacteria, and bacterial control were included.

After 48 h the pellets were disrupted by adding each pellet to a sterile 5 mL Eppendorf tube® containing 2 mL of PBS and vortexing the tubes for 5 min followed by a 5 min sonication. This disruption was repeated twice to disrupt the pellet. Each pellet solution (200 µL) was transferred to the top row of a 96-well plate and diluted tenfold in physiological saline (PS; 0.9% NaCl). The concentrations of 1×10^{-3} to 1×10^{-7} CFU/mL was plated on RCA and incubated for 72 h anaerobically at 37 ºC. After 72 h the colonies were counted in order to determine the colony-forming units (CFU) recovered.

The presence of metabolically active bacteria was measured using the CellTiter-Blue® (CTB) (2.1 ml CTB stock in 10.5 mL PS) assay. To 48 h treated pellets, 1 mL of diluted CTB was added, and the plate was incubated at 37 ˚C for 2 h while shaking at 600 rpm on a temperature-controlled shaker. To a 96-well plate, 100 µl of the CTB samples were added, and the fluorescence was measured at an excitation/emission wavelength of 560/590 nm using an EnVision multilabel reader (Perkin Elmer).

The CFU/pellet was calculated using the following equation:

$$\text{CFU/Pellet} = \text{Colony counts x } (2\text{x}10^{n+2})$$

where n is the bacterial dilution concentration.

The percentage inhibitions of the samples were calculated using the following equation:

$$\%\text{Inhibition} = \frac{\left(\frac{\text{CFU control}}{\text{Pellet}} - \frac{\text{CFU sample}}{\text{Pellet}}\right)}{\frac{\text{CFU control}}{\text{pellet}}} \text{ x } 100$$

where the $\text{CFU/pellet}_{\text{control}}$ is the CFU counted per pellet for the vehicle control, and the $\text{CFU/pellet}_{\text{sample}}$ is the CFU counted per pellet for the sample.

The equation used to determine the percentage inhibition of bacterial metabolism was calculated using the following equation:

$$\%\text{Inhibition} = \frac{(\text{Flouresence}_{\text{control}} - \text{Flouresence}_{\text{sample}})}{\text{Flouresence}_{\text{control}}} \text{ x } 100$$

where the $\text{Fluorescence}_{\text{control}}$ is the fluorescence of the vehicle control, and the $\text{Fluorescence}_{\text{sample}}$ is the fluorescence of the sample.

2.10 Autoinducer-2 (AI-2) inhibition

The method described by Wattanavanitchakorn et al. (2014) for the detection of AI-2 was followed. Slight modifications were made to test samples using this model. Briefly, 72 h bacterial cultures of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) were used to inoculate BHI broth to a concentration of 6.0×10^8 CFU/mL (OD_{600nm} 0.4). The stock bacterial concentration, OD_{600nm} 0.4, was selected to ensure the detection of AI-2. In a sterile 96-well plate, each tested sample and the positive control tetracycline were dissolved in DMSO and BHI broth to obtain a final concentration equal to 0.5×MIC and a 2.5% v/v DMSO concentration. To all the wells of the 96-well plate, 100 µL of the bacterial culture solutions for *C. acnes*, *S. epidermidis* and CA-SE was added. A DMSO (2.5% v/v) vehicle control (100% AI-2 production control), a growth media control without bacteria (0% AI-2 production) and a bacterial control for each bacterium and combination of bacteria was included in the study. Colour controls for each test sample, including the vehicle control containing only BHI broth was included in the study. The plates were incubated at 37 ºC for

72 h aerobically (*S. epidermidis* and CA-SE) and anaerobically (*C. acnes* and CA-SE) in an Anaerocult® jar with Anaerocult®. After 72 h, the plates were subjected to centrifugation at 4 500 rpm for 12 min using a Hermle Z306 Universal Centrifuge. The supernatant of each sample (40 µL) was transferred to a clean 96-well plate for the detection of AI-2 production and inhibition. A solution of 10 mM 1,10-Phenanthroline and 3.32 mM Fe(III) (Ferric ammonium sulphate) in distilled water and adjusted to a pH of 2 was made. To the supernatant, 40 µL of the 1,10-Phenanthroline/ Fe(III) solution was added, and the plates were left to develop for 5 min where after 120 µL of distilled water was added, and the plates read at 510 nm using a Perkin Elmer VICTOR Nivo microplate reader. Bacterial viability was confirmed by adding 10 µL PrestoBlue® to 100 µL of the supernatant.

The percentage inhibitions were calculated using the following equation:

$$\%\text{Inhibition} = \frac{(\text{Absorbacne}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \text{ x } 100$$

where the $\text{Absorbance}_{\text{control}}$ is the (absorbance of the vehicle control) - (absorbance of the broth blank) and the $\text{Absorbance}_{\text{sample}}$ is the (absorbance of the sample) - (the absorbance of the sample control blank).

2.11 Bacterial lipase inhibition assay

The lipase inhibition assay was performed by directly measuring the presence of the enzyme in the supernatant of the various bacteria. The method as described by Coenye et al. (2007) and Spittaels and Coenye (2018) was followed. Briefly, 72 h bacterial cultures of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) were used to inoculate BHI broth to a concentration of 6.0×10^8 CFU/mL (OD_{600nm} 0.4). The stock bacterial concentration, OD_{600nm} 0.4, was selected to ensure the detection of lipase. In a sterile 96-well plate, each tested sample and the positive control tetracycline were dissolved in DMSO and BHI broth to obtain a final concentration equal to its 0.5×MIC and a 2.5% DMSO v/v concentration. To all the wells of the 96-well plate, 100 µL of the bacterial culture solutions for *C. acnes*, *S. epidermidis* and CA-SE was added. A DMSO (2.5% v/v) vehicle control (100% lipase production control), a growth media control without bacteria (0% lipase production) and a bacterial control for each bacterium and combination of bacteria was included in the study. The plates were incubated at 37 ºC for 72 h aerobically (*S. epidermidis* and CA-SE) and anaerobically (*C. acnes* and CA-SE) in an Anaerocult® jar with Anaerocult®. The lipase

released into the growth media of the bacterial cultures was measured after 72 h. The supernatant was collected by centrifuging the plates at 4 500 rpm for 12 min using a Hermle Z306 Universal Centrifuge. The supernatant (100 µL) was transferred to sterile untreated Nunc™ F96 MicroWell™ black polystyrene 96-well plates (Thermo Fisher Scientific™). A 4-methylumbelliferyl (4-MU) oleate substrate (Sigma Aldrich™) was prepared in DMSO (0.2 mg/mL), and 100 µL was added to each of the wells. The fluorescence was measured using a Perkin Elmer VICTOR Nivo microplate reader (excitation: 355 nm/emission: 460 nm) at 37 ˚C for 30 min. Bacterial viability was confirmed by adding 10 µL PrestoBlue® to 100 µL of the supernatant.

The percentage inhibitions were calculated using the following equation:

$$\%\text{Inhibition} = \frac{(\text{Slope}_{\text{control}} - \text{Slope}_{\text{sample}})}{\text{Slope}_{\text{control}}} \text{ x } 100$$

The $\text{Slope}_{\text{control}}$ is the average slope of the vehicle control fluorescence, and the $\text{Slope}_{\text{sample}}$ is the average slope of the sample fluorescence.

2.12 Quantification of extracellular DNA (eDNA) in bacterial biofilms

The methods described by Brackman et al. (2016) and Ravaioli et al. (2020) were used to detect and quantify eDNA with slight modifications. Samples for eDNA analysis were selected based on their selectivity towards inhibition of biofilm formation and adhesion compared to their antibacterial potential. Samples with a selectivity index (SI) larger than one were selected for eDNA studies. Briefly, biofilms were formed in the presence of the selected samples at their respective 50% biofilm adhesion inhibitory concentrations (IC_{50}) as previously described in sterile untreated Nunc™ F96 MicroWell™ black polystyrene 96-well plates (Thermo Fisher Scientific). A 2.5% v/v DMSO control, bacterial (100% eDNA control) and broth control (0% eDNA control) were included. The plates were incubated for 72 h at 37 ˚C under anaerobic and aerobic growth conditions, respectively, as previously described. After incubation, the broth was carefully removed and transferred to a clean 96-well plate for viability studies with 20 µL of PrestoBlue® reagent, incubated for 1 h at 37 ˚C. The black 96-well plates were washed once with nuclease-free PBS (pH 7.4). After washing, 100 µL of PBS was added to all the wells, and the biofilm was disrupted with vigorous agitation. An equal volume of 2 µM Toto-1® was added to the wells and incubated in the dark

for 20 min. Toto-1 dye was selected based on its selectivity for staining eDNA (Okshevsky and Meyer, 2014). The fluorescence was measured using a Perkin Elmer VICTOR Nivo microplate reader (excitation: 490 nm /emission: 530/30 nm). The eDNA concentration was normalised to the number of viable bacterial cells determined by plate counting on BHI agar plates, as Brackman et al. (2016) previously described.

The percentage inhibitions were calculated using the following equation:

$$\%\text{Inhibition} = \frac{(\text{Normalised fluorescence}_{\text{control}} - \text{Normalised fluorescence}_{\text{sample}})}{\text{Normalised fluorescence}_{\text{control}}} \text{ x } 100$$

where the Normalised $\text{fluorescence}_{\text{control}}$ is the (normalised fluorescence of the vehicle control) - (normalised fluorescence of the broth blank), and the Normalised $\text{fluorescence}_{\text{sample}}$ is the (normalised fluorescence of the sample) - (normalised fluorescence of the broth blank).

2.13 Cell culture

The human keratinocyte cell line (HaCat) was donated by Dr. Lester Davids from the Department of Human Biology, University of Cape Town" (Lall et al., 2019). The HaCat cells was gifted to Dr Davids by from Professor Fusenig, Germany from cells collected from the skin biopsy of a 62 year old male patient (Boukamp et al., 1988; Zwane et al., 2012). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL fungizone, and 10% heat-inactivated fetal bovine serum (FBS) at 37 ºC, 5% CO_2 until a confluent monolayer formed. The cells were detached from the flask with Trypsin-EDTA solution for 2 min and deactivated with fresh DMEM.

2.13.1 Cytotoxicity assay

The cytotoxicity of the samples, partitions and selected pure compounds was determined on HaCat cells. After detachment, the cells were seeded (1.0 x 10^5 cells/mL; 100 µL) in the centre wells of a sterile 96-well plate. To the outer wells of the plate, sterile DMEM (200 µL) was added. The plate was incubated for 24 h at 37 ºC, 5% CO_2 for cell attachment and reach a confluent monolayer. Stock solutions of the samples were prepared to a starting concentration of 40 mg/mL in DMSO. The samples were further diluted in DMEM and added in triplicate (100 µL) to the relevant wells of the 96-well plate. The final concentrations of the samples ranged between 400 – 12.5 µg/mL. The final concentrations of the positive control

actinomycin D ranged between 0.05-3.91 × 10^{-4} µg/mL. A media and cells control, 1% DMSO control and PB media control without cells were included in the study. The plates were incubated for 72 h at 37 ºC and 5% CO_2. Presto Blue® (20 µL) was added to all the wells, and the fluorescence was measured using a Perkin Elmer VICTOR Nivo microplate reader (excitation: 560 nm /emission: 590 nm) (Lall et al., 2019). The IC_{50} was calculated using GraphPad Prism 4.0 software.

2.13.2 Scratch wound healing assay

The scratch assay was conducted as described by Suarez-Arnedo et al. (2020) with slight modifications. To the wells of a 48-well plate, 500 µL of HaCat cells were seeded to a concentration of 7.5 x 10^4 cells per well in DMEM supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL fungizone, and 10% FBS. The plates were incubated for 24 h at 37 ºC, 5% CO_2 until a confluent monolayer formed. A cross was made from top to bottom and left to right using a sterile 1000 µL tip in each well. The media was carefully removed, and the wells were washed once with sterile Phosphate Buffered Saline (PBS), after which 1125 µL of fresh DMEM was added. Pictures of the untreated scratched wells containing the cells were taken under a ZEISS Primovert microscope at a 4x magnification (phase 0) that served as the pre-treatment control (0 h). The samples were added to the wells of the 48-well plate to a final volume of 1500 µL and a final sample concentration related to their respective *C. acnes* MIC concentrations. A 0.25% v/v DMSO control with cells and media control without cells were included in the experiment. The plates were then incubated at 37 ºC, 5% CO_2 for 18 as determined from preliminary studies as the ideal time before complete closure of the cell control and negative control scratches. Complete scratch closure was observed at the tested cell concentration of 7.5 x 10^4 cells per well after 24 h during the preliminary studies. Only partial wound closure was observed after 18 h which allowed for observing and analysing the scratch closure compared to the controls. After incubation, a picture was taken of each well (Nikon camera D90). The percentage wound closure was calculated using ImageJ and GraphPad Prism 4 software by comparing the sample scratch closure to the cell and vehicle control scratch closure. The viability of the cells for each sample was determined by first removing 1300 µL of the sample from the wells and adding 20 µL of PrestoBlue® reagent to each of the wells. The fluorescence was measured using a Perkin Elmer VICTOR Nivo microplate reader (excitation: 560 nm and emission: 590 nm).

2.14 Mutagenicity

The mutagenicity of PA_{EtOH} was conducted as described by Madikizela and McGaw. (2019) using the Ames test. Briefly, *Salmonella typhimurium* strains TA 98, and TA 100 strains were grown for 16 h at 37 ºC in nutrient broth with constant shaking. After incubation, the bacteria were mixed with top-agar containing histidine-biotin, test solution that contained either PA_{EtOH} (concentration range 5 000 µg/mL, 500 µg/mL and 50 µg/mL) dissolved in 10% v/v DMSO, the positive control 4-nitroquinoline-1-oxide (4-NQO) (tested at 2 µg/mL), 10% v/v DMSO and distilled water that served as the negative controls and lastly phosphate buffer. The top-agar mixture was poured over minimal agar plates and incubated at 37 ºC for 48 h. After incubation, the number of revertant colonies was counted on each plate using a plate counter. Each sample was tested in triplicate.

2.15 Heavy metal and microbial analysis of *Plectranthus aliciae* ethanolic extract

Heavy metal and microbial analysis were carried out for PA_{EtOH} by Swift Silliker (Pty) Ltd t/a Mérieux NutriSciences. The sample was prepared to a 600 µg/mL concentration in 4% ethanol solution with sterile dH_2O.

Heavy metal analysis was conducted according to the MP 1288 rev 14 2018 method for the presence of arsenic, cadmium, mercury, and lead (Appendix B). Microbial analysis was conducted according to the SWJM 35 method for Total Microbial Activity (TMA) and SWJM 50 for the detection of yeast and mould (Appendix C).

2.16 *In vivo* clinical studies

2.16.1 Formulation of *Plectranthus aliciae* ethanolic extract

The PA_{EtOH} was developed into a gel formulation with the assistance of Prof. Heidi Rolfes and Ms. Deveshnee Moodley of the Department of Chemical Engineering, Faculty of Engineering, Built Environment and Information Technology at the University of Pretoria, South Africa. The formulation developed by Prof. Heidi Rolfes and Ms. Deveshnee Moodley is described in Table 4.1.

Table 4. 1: Gel formulation for *Plectranthus aliciae* ethanolic extract *in vivo* studies.

Function	INCI name	Quantity (%)

Diluent/solvent	Aqua	86.0
Chelating agent	Tetrasodium EDTA	0.10
Humectant	Propylene Glycol	2.23
Thickening Agent	Carbomer	1.12
pH Buffer	Sodium Hydroxide	QS until pH 5.5
Active Ingredient	*P. aliciae* ethanolic extract	10.00
Preservative	Propylene Glycol(and) Diazolidinyl Urea (and) Methylparaben (and) Propylparaben	2 drops

The PA_{EtOH} was prepared by dissolving 300 mg of dry extract in 50 mL of a 40% ethanol solution in dH_2O. The final gel formulation contained 10% of the stock solution with a final sample concentration of 600 µg/mL and a final ethanol concentration of 4%.

2.16.2 Irritancy studies of *Plectranthus aliciae*

The PA_{EtOH} neat (final concentration 6 mg/mL) and in a gel formulation (final concentration 600 µg/mL; 10% in gel formulation) was submitted for skin patch irritancy studies at the Photobiology Laboratory at Sefako Makgatho Health Sciences University, South Africa (Appendix D). The study was conducted according to the MREC/H/48/2014: CR protocol (ethical clearance MREC/H/48/2014: CR approved)[1]. A set sample volume was applied to the inner forearm of 20 volunteers in a randomised blind layout; five of the participants had sensitive skin. At the end of the study, 18 volunteers fully completed the study. The samples were applied to the skin using aluminium Finn Chambers on Scanpore ® tape. The sample and controls were applied at the same positions on the forearm on day 1 (zero hours) and after 24 h. The test sites remained closed for 46 h where after the test site was left uncovered. After application, the test sites were analysed for irritancy at 0, 24, 48, 72 and 96 h. Sodium lauryl sulphate (SLS; 1%) served as the positive control and dH_2O as the negative control. The test sites were analysed in a double-blind manner firstly through visually scoring the irritancy based on a visual scoring scale and secondly using a Minolta Cr400 Chromameter, measuring colour on the red/green axis.

[1] Training received on an identical irritancy protocol at Future Cosmetics.

2.16.3 Acne efficacy studies of *Plectranthus aliciae* in a finished formulation

Plectranthus aliciae ethanolic extract in the finished gel formulation (10%) was submitted for acne efficacy studies at Future Cosmetics, Pretoria, South Africa (Appendix E). The gel formulation without the sample served as the placebo control. The study was conducted on 24 volunteers between the ages of 19 to 39 (average: 28.5) with Fitzpatrick skin types IV or V. The sample size was calculated by Future Cosmetics as explained in Appendix E using a non-probability sampling method. Since the sample size was small, "a parametric test, the (unequal or equal variances) t-test for comparison of two sets of data" was used as discussed in Appendix E. The study was conducted according to the FCAG014 test protocol[2]. Briefly, the faces of the volunteers were cleaned with make-up remover and allowed to dry. The face of each volunteer was demarcated according to a specified grid. Colour photographs were taken of each volunteer of the front, right and left facing (Figure 4.1). An operator trained each volunteer to apply a set volume of the test product and placebo to the right or left-hand side of their face in a randomised blind manner. The volunteers were instructed to apply the test products as trained twice a day consecutively for 28 days. The volunteers returned after 14 and 28 days for evaluation, and colour photographs were taken on each day as described. Two independent trained evaluators performed a physical count on the different acne lesions on day one (baseline), day 14 and day 28 for each volunteer.

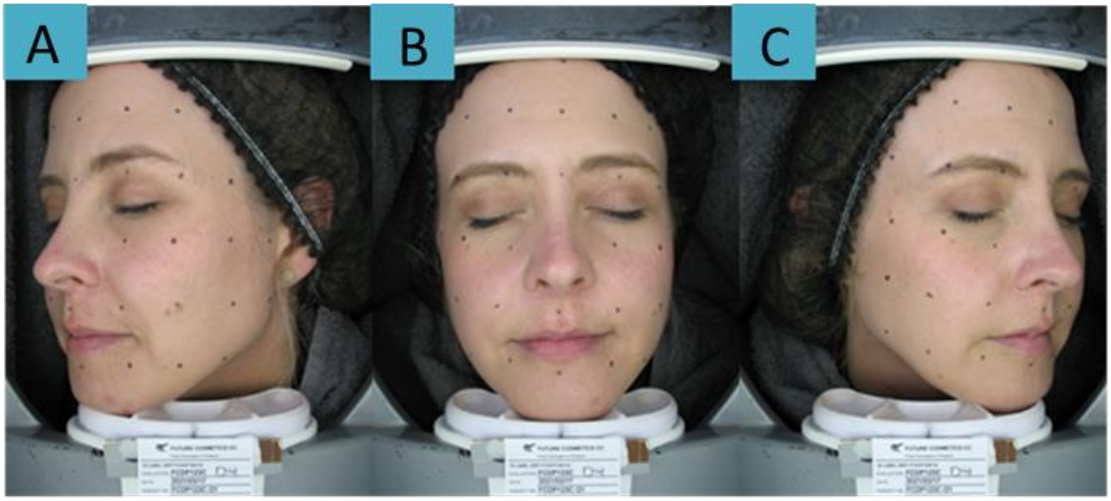

Figure 4. 1: Colour photographs representing photographs taken of volunteers depicting the demarcated grids used for counting of acne lesions. A: left facing, B: front facing, C: right facing.

[2] Training received on acne efficacy protocol FCAG014 at Future Cosmetics. Ethics number can be provided on request.

2.17 Statistical analysis

All *in vitro* biological experiments were performed in triplicate (n = 3) and in three independent experiments to ensure reproducible results, unless otherwise stated. The 50% inhibitory concentration (IC_{50}) and statistical analyses were conducted using GraphPad Prism 4 software. The statistical significance of the results was determined with One-way Analysis of Variance (ANOVA) and Tukey's multiple comparison test, unless otherwise stated.

3. Results and discussion

The relationship between bacteria in multispecies communities is a complex and ongoing science, not yet understood. Various intrinsic and extrinsic environmental factors play a role in this relationship. Only a few studies have investigated the relationship of *S. epidermidis* and *C. acnes* and fully understanding the relationship and the role of quorum sensing between these two bacteria is still in its infancy. Both pathogenic strains of *C. acnes* and *S. epidermidis* have been associated with acne vulgaris and prosthetic implant-associated infections either alone or in combination (Nishijima et al., 2000; Niazi et al., 2010; Moon et al., 2012; Portillo et al., 2013; Flurin et al., 2019; Fournière et al., 2020).

Researchers have suggested that pathogenic multispecies systems could contribute to increased antibiotic resistance and the inflammatory response in acne vulgaris and other maladies (O'Neill and Gallo, 2018). In an article by Fournière et al. (2020), the researchers looked at the relationship of commensal and opportunistic *C. acnes* and *S. epidermidis* species from a unique angle. The researchers noted that the relationship between *C. acnes* and *S. epidermidis* is still unclear. However, they suggested that pathogenic strains of these bacteria could have a synergistic-pathogenic relationship and have an antagonistic relationship with their commensal counterparts. Kumar et al. (2016) suggested that the *S. epidermidis* biofilm could potentially provide a favourable anaerobic environment for *C. acnes* to flourish in aerobic environments. The relationship between skin microorganisms is not fully understood. As commensal bacteria, *C. acnes* and *S. epidermidis* have a mutualistic relationship to protect the host from external pathogens. Therefore, leaving the question, if certain strains of these bacteria could potentially have a synergistic relationship in causing disease. Another question to consider is that in the case of an antagonistic relationship between the bacteria or a host defence mechanism from one or the other bacteria, what implications does this pose to the host (Nishijima et al., 2000; Fournière et al., 2020)?

Several acne therapies are currently used for the treatment of acne vulgaris. Most of these therapies have only been investigated to target *C. acnes* and no other bacteria such as *Staphylococcus* sp. that could contribute to the progression of inflammatory acne, wounds, and prosthetic implant-associated infections. Isotretinoin is widely used for the treatment of acne vulgaris by preventing the overproduction of sebum. However, acne vulgaris returns to 26 to 48% of patients, and other side effects include congenital disabilities, dry skin, liver damage and sensitivity to the sun that can result in hyperpigmentation, to name but a few. Topical and oral antibiotics are another widely used treatment for acne vulgaris, wounds and prosthetic implant-associated infections. These antibiotics include clindamycin, erythromycin, and tetracycline. However, treatment times with antibiotics can vary between three to six months. Side effects of antibiotics include an increase of antibiotic resistance in the bacteria and negative impacts to gut microflora. It has been proven that antibiotics could permanently affect the gut microbiota, resulting in other diseases such as irritable bowel syndrome, Chron's disease, obesity, and diabetes. Antibiotics can also result in dysbiosis of the skin microflora that can result in other skin related infections. Researchers have suggested the use of probiotics to treat acne vulgaris and other skin related maladies. The use of fermented *Staphylococcus* sp. as a probiotic against skin-related maladies has been investigated to some extent. However, the relationship between these bacteria and the combined effect on the host is not yet fully understood (Wang et al., 2014; Christensen et al., 2016; Francino, 2016; O'Neill and Gallo, 2018).

There is a need for new acne, wound and prosthetic implant therapies that can target pathogenic bacteria associated with these maladies. There is also a need to better understand the relationship of the pathogenic bacteria associated with these bacterial infections or the effect an antagonistic relationship between these bacteria can have on the host.

3.1 Agar plate synergistic assay between *C. acnes* and *S. epidermidis*

As with all organisms, the relationship between bacteria can either be synergistic or antagonistic. Bacteria can produce toxins and antibacterial agents to control the growth of other bacteria in an environment, preserving scarce resources for their intraspecies community. Controversy exists for the multispecies relationship of *C. acnes* and *S. epidermidis* in various maladies. Although multi and single species relationships have been identified between the bacteria associated with acne lesions, prosthetic implant-associated

infections, and wounds, researchers have proposed an antagonistic relationship between the bacteria. These studies have proposed an inhibitory effect of either bacteria on the other (Christensen et al., 2016; Xu and Li, 2019; Nakamura et al., 2020). However, these studies on various strains of *C. acnes* and *S. epidermidis* do not consider the effect this interaction has on quorum sensing and the production and release of virulent factors which could be harmful to the host. Although one bacterial species or strain inhibits the other bacterial species or strain, investigating the release of virulent factors and the quorum sensing effect of the remaining species could provide valuable information if there is an increase in factors that might cause more harm to the host when these bacterial strains are exposed to one another. Xu and Li (2019) proposed that despite some researchers hypothesising the antagonistic relationship between *C. acnes* and *S. epidermidis* and the commensal role of *S. epidermidis*, that this bacterial relationship be further explored.

Researchers have proposed the synergistic relationship of these bacteria and that the interaction between these bacteria is strain-specific. Fournière et al. (2020) highlighted that different strains of not only different *C. acnes* and *S. epidermidis* species have an antagonistic or synergistic relationship but also bacteria of the same species. Therefore, the interaction of bacteria that cause acne vulgaris and prosthetic implant and wound infections is strain-specific. More research investigating the interaction of various bacterial species found in the skin and their quorum sensing interaction and virulence factor release is required.

Christensen et al. (2016) have briefly investigated the antagonistic relationship between various *S. epidermidis* and *C. acnes* bacterial species. Their findings were interesting, with some bacterial species having an antagonistic relationship to various degrees. However, their results have also revealed that some combinations of various bacterial species do not have an antagonistic relationship. Since an antagonistic relationship was not observed between all the *C. acnes* and *S. epidermidis* strains tested, this leads to the question of whether certain acne-causing and biofilm-forming strains of *C. acnes* and *S. epidermidis* could have a synergistic activity. Much controversy exists about the relationship between *C. acnes* and *S. epidermidis* (Wang et al., 2014).

Therefore, the first step was to determine if *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) has a synergistic or antagonistic relationship.

Christensen et al. (2016) have previously indicated that *C. acnes* strains of the IA-2 phylogroup have antibacterial activity against 62% of *S. epidermidis* strains. *Cutibacterium acnes* of the IA-1 phylogroup only displayed antibacterial activity against 32% of *S. epidermidis* strains. In the present study, no antagonistic relationship was observed between *C. acnes* (ATCC® 6919; phylotype IA-1 and ribotype RT1) and *S. epidermidis* (ATCC® 35984; RP62A) as indicated in Figures 4.2 and 4.3 (Schmidt et al., 2015). There were no inhibition zones between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984), indicating that these bacteria do not produce bacteriocins that could inhibit the other bacterial species of interest. Compared to the controls, no inhibition zones were observed under either anaerobic or aerobic growth conditions, and apparent bacterial growth was observed. The results in this study indicate a potential synergistic relationship between the bacteria. This synergistic activity between these bacteria is not fully understood.

Cutibacterium acnes type IA-1 is primarily associated with acne lesions, where all other phylotypes are associated mainly with healthy skin or other opportunistic infections (McLaughlin et al., 2019). Christensen et al. (2016) found no correlation between the antibacterial activity of *S. epidermidis* strains isolated either from acne lesions (pathogenic strains) or healthy skin (commensal strains) against *C. acnes*. It was concluded that the antagonistic and synergistic relationship of *C. acnes* and *S. epidermidis* is strain-specific. These results support one of the hypotheses of this study, that opportunistic and pathogenic strains of *C. acnes* and *S. epidermidis* could have a synergistic relationship in acne lesions and that this is strain-specific. However, this relationship's impact on the host and treating acne vulgaris, wound and prosthetic implant-associated infections are unclear.

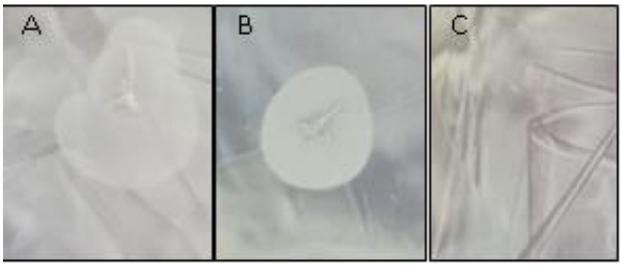

Figure 4. 2: Agar plate synergistic assay on a lawn of *Cutibacterium acnes* (ATCC® 6919) grown under anaerobic growth conditions. A: *Staphylococcus epidermidis* (ATCC® 35984) growth; B: *C. acnes* (ATCC® 6919) control; C: Broth control.

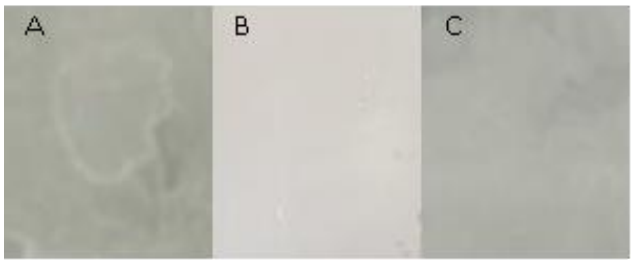

Figure 4. 3: Agar plate synergistic assay on a lawn of *Staphylococcus epidermidis* (ATCC®35984) grown under aerobic growth conditions. A: *Cutibacterium acnes* (ATCC® 6919) growth; B: *S. epidermidis* (ATCC® 35984) control; C: Broth control.

3.2 Scanning Electron Microscopy (SEM)

The spatial orientation of bacteria in a multispecies biofilm is an important factor influencing how these bacteria coordinate certain functions in the community. It has been hypothesised that the aerobic *S. epidermidis* could provide the anaerobic, aerotolerant *C. acnes* with the required environment to thrive under aerobic growth conditions (Kumar et al., 2016; Olsen et al., 2019). Studies have confirmed that although certain strains of *C. acnes* and *Staphylococcus aureus* have an antagonistic relationship, the biofilm of certain strains of *C. acnes* provides a safe environment for the aerobic *S. aureus* to thrive under anaerobic growth conditions. These studies were compared to their single-species biofilms, where under anaerobic growth conditions, single-species biofilms of *S. aureus* were not viable after 16 days, but in a multispecies biofilm with *C. acnes* viable *S. aureus* was isolated (Tyner and Patel, 2016; Fournière et al., 2020). The spatial orientation of the bacteria in a multispecies biofilm indicates the type of relationship between the bacteria, as seen in Figure 4.4. Quorum sensing and the release of various metabolites affect the interspecies interaction in a biofilm. There are five spatial orientations in a multispecies biofilm. Intermixing, bacterial layering and interspecific separation are spatial orientations that are forms of cooperative relationships. In cooperative relationships, mutualistic partners are near one another to exchange metabolites (Figure 4.4: A, B, C). Patchy patterning and certain forms of interspecific separation are associated with exploitation and competition for space and nutrients in a multispecies biofilm system. In these systems, a decrease in biomass of one or both bacterial species are observed (Figure 4.4: D, E, F) (Liu et al., 2016).

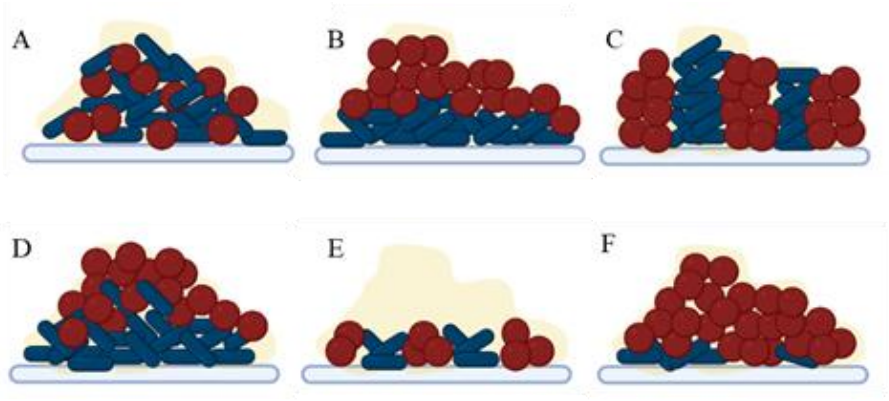

Figure 4. 4: Spatial orientations of bacteria in a multispecies biofilm and the interactions of the bacteria associated with these orientations. A: Intermixing – cooperative relationship; B: Bacterial layering – cooperative relationship; C: Interspecific separation - cooperative relationship; D: Layered patching patterning – exploitation; E: Interspecific separation -competition; F: Patch patterning – competition. The figures were adapted from Liu et al. (2016).

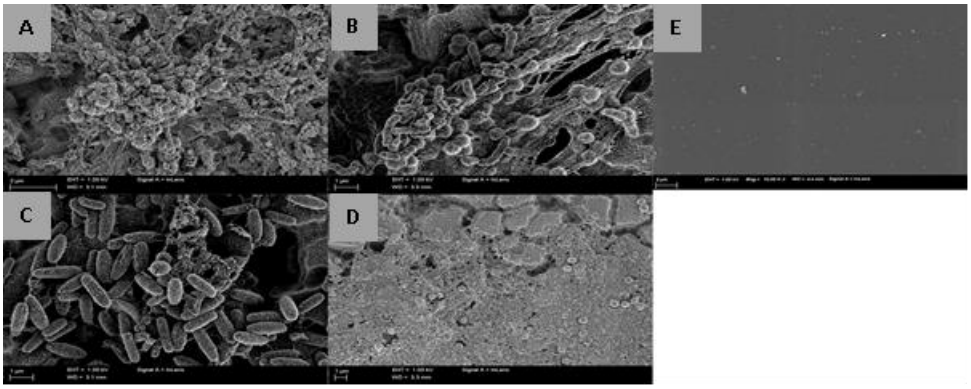

Figure 4. 5: Scanning Electron micrographs of A: a multispecies biofilm of *Cutibacterium acnes* (ATCC® 6919) and *Staphylococcus epidermidis* (ATCC® 35984) grown under anaerobic conditions; B: a multispecies biofilm of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) grown under anaerobic conditions; C: a single species biofilm of *C. acnes* (ATCC® 6919); D: a single species biofilm of *S. epidermidis* (ATCC® 35984) and E: the broth control.

The interspecies spatial orientation and relationship of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) in a multispecies biofilm were further investigated using SEM. There is no information available on the biofilm growth and the spatial orientation of these bacteria in a multispecies biofilm under various oxygen growth conditions. The spatial orientation and potential relationship of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) are shown in the SEM micrographs in Figure 4.5. Comparing the micrographs in Figure 4.5 to the spatial orientation and relationship of the bacteria in a multispecies biofilm as illustrated by Liu et al. (2016) in Figure 4.4, it can be observed that the multispecies biofilms under aerobic and anaerobic growth conditions are an intermixed, cooperative relationship. This synergistic relationship supports the data observed in the agar plate

synergistic assay and the hypothesis that synergistic and cooperative activity between *C. acnes* and *S. epidermidis* is strain-specific.

3.3 Identification of compounds using UPLC-QTOF and liquid-liquid partitioning

Plectranthus aliciae (Codd) Van Jaarsv. & T.J. Edwards is an endemic South African plant, previously a variety of *Plectranthus madagascariensis* (*P. madagascariensis* (Pers.) Benth. var. aliciae Codd). Seven compounds have been identified from *Plectranthus madagascariensis* that includes 7α-acetoxy-6β-hydroxyroyleanone, 6,7-dehydroroyleanone. 6β,7α-dihydroxyroyleanone, 7α-formyloxy-6β-hydroxyroyleanone coleon U, coleon U quinone and rosmarinic acid (Lambrechts and Lall, 2021). Luteolin (**1**) was identified as the major compound present in the crude extract of PA_{EtOH}. Luteolin has previously been identified to be present in *Plectranthus barbatus* and *Plectranthus amboinicus* (Gurgel et al., 2009; Porfírio et al., 2010). High amounts of luteolin (**8**), protocatechuic acid (**11**), quercitrin (**12**), rosmarinic acid (**13**) and syringic acid (**14**) were detected. Rosmarinic acid is known to be present in the family and this species in high concentrations (Gurgel et al., 2009; Porfírio et al., 2010). Trace amounts of caffeic acid (**1**), catechin (**2**), epicatechin (**3**), 4-hydroxybenzoic acid (**4**), isoquercitrin (**6**), isovitexin (**7**), vanillic acid (**15**), vitexin (**16**) (Table 4.2). Spectral data for the identified compounds are available in Appendix A. This is the first report of the presence of luteolin, quercitrin, syringic acid, caffeic acid, catechin, epicatechin, 4-hydroxybenzoic acid, isoquercitrin, isovitexin, vanillic acid and vitexin in PA_{EtOH}.

Four partitions were successfully collected from the crude extract of PA_{EtOH}. These partitions were from hexane, ethyl acetate, n-butanol and water. The yield for the hexane and ethyl acetate fractions that displayed antibacterial activity against *C. acnes* (ATCC® 6919) (Figure 4.6, Table 4.3) were 16.8% and 27.5%, respectively.

Table 4. 2: Quantification of major phyto-constituents present in the ethanolic extract of *Plectranthus aliciae* using Ultra Performance Liquid Chromatography – Quantitative Time of Flight.

Compounds		Quantification ($\mu g_{compound}/mg_{sample}$ injected ± SD[a])
1	Caffeic acid	0.91 ± 0.12
2	Catechin	0.31 ± 0.08

3	Epicatechin	0.37 ± 0.003
4	Ferulic acid	ND[b]
5	4-hydroxybenzoic acid	1.71 ± 0.31
6	Isoquercitrin	0.39 ± 0.03
7	Isovitexin	1.43 ±1.36
8	Luteolin	20.71 ± 7.24[c]
9	Orientin	ND[b]
10	Propyl gallate	0.013 ± 0.004
11	Protocatechuic acid	24.38 ± 6.20
12	Quercitrin	51.86 ± 16.03
13	Rosmarinic acid	24.09 ± 6.21
14	Syringic acid	28.26 ± 2.48
15	Vanillic acid	0.92 ± 0.29
16	Vitexin	1.43 ± 1.37

[a] SD: Standard deviation; [b] ND: Not detected; [c] 1x10^2

3.4 Antibacterial activity of *Plectranthus aliciae*, its pure compounds and partitions

Previous studies have confirmed the antibacterial activity of *Plectranthus* sp. against a range of bacteria associated with diseases such as tuberculosis, wounds, and various skin maladies such as acne vulgaris and eczema. The essential oils distilled from the genus have shown significant antibacterial activity and the potential to target antibiotic resistance in certain bacteria. The antibacterial activity of *Plectranthus* sp. could be attributed to their high diterpenoid, triterpenoid and flavonoid content (Rabe and van Staden, 1998; Urzúa et al., 2008; Galvão Rodrigues et al., 2013).

Much research has been conducted on the *Plectranthus* sp., *P. amboinicus, P. barbatus*, and *Plectranthus ornatus*. However, not much research has been done on *P. aliciae* or *P. madagascariensis* var. aliciae, a synonym of the species.

The MIC method to determine the antibacterial activity of anextract is a visual method widely used by researchers to determine the antibacterial activity of a plant extract and its pure compounds. Due to the narrow concentration range of a broth microdilution method, the

narrow hillslope of a sigmoidal dose-response curve could prevent the 50% inhibitory concentration to be calculated meaningfully. Therefore, determining the MIC through the broth microdilution method is an accepted method to determine the antibacterial activity of a sample according to the Clinical Laboratory Standards Institute (CLSI) (Clinical Laboratory Standards Institute, 2012; Van Vuuren and Holl, 2017). Standardisation for measuring the antibacterial activity and the criteria to determine the significant activity for plant extracts and their compounds are vital. Van Vuuren and Holl (2017) have proposed criteria to measure the antibacterial significance of plant extract, essential oil, and pure compound (Table 4.3).

Table 4. 3: Criteria for determining the significant antibacterial activity of plant extracts and their pure compounds (Van Vuuren and Holl, 2017). MIC: Minimum inhibitory concentration.

Natural product	MIC value (µg/mL)	Significance
Plant extract	< 100	Significant activity
	100	Strong activity
	≤ 160	Promising activity
	100-625	Moderate activity
Compounds	< 1	Significant activity
	16 - 4	Noteworthy activity
	100 - 64	Clinical relevance

The antibacterial activity of the five major compounds identified to be present in PA_{EtOH} and partitions were selected for antibacterial activity against *C. acnes* (ATCC® 6919). The antibacterial activity against *C. acnes* was performed first to determine if the samples could potentially be used to treat acne vulgaris, wounds and prosthetic implant-related infections. Luteolin displayed bacterial inhibition at an MIC of 62.5 µg/mL. The positive control tetracycline displayed an MIC value of 0.78 µg/mL. The hexane and ethyl acetate partitions had MIC values of 7.8 µg/mL and 15.6 µg/mL. The n-butanol and water partitions displayed no antibacterial activity at the highest concentration tested of 500 µg/mL.

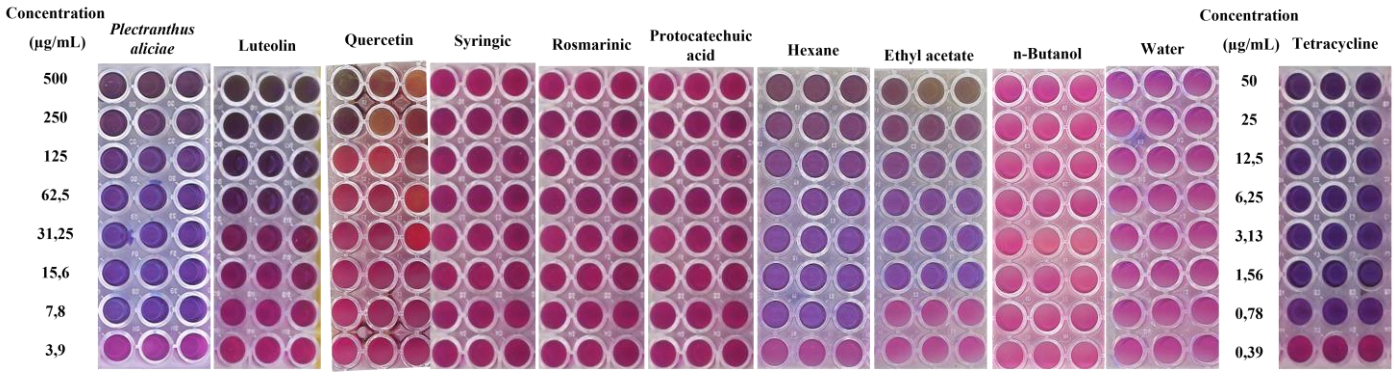

Figure 4. 6: Antibacterial activity of five major compounds identified to be present in the crude ethanolic extract of *Plectranthus aliciae* and the liquid partitions of the crude extract against *Cutibacterium acnes* (ATCC® 6919). The positive control was tetracycline. Pink is an indication of bacterial viability and growth and blue an indication of bacterial inhibition.

The pure compounds luteolin and rosmarinic acids as well as the hexane and ethyl acetate partitions were selected for further antibacterial, biofilm and quorum sensing activity. Although rosmarinic acid did not display antibacterial activity at the highest concentration tested of 400 µg/mL, it was selected for further studies due to the known availability of the compound in the Lamiaceae family and *Plectranthus* sp.

The selected samples were analysed for their bacterial inhibitory activity against *C. acnes* (ATCC® 6919), *S. epidermidis* (ATCC® 35984) and a combination of these bacteria grown under anaerobic and aerobic growth conditions.

Table 4. 4: Antibacterial and biofilm inhibitory concentration of *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions. The antibacterial activity was determined against *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984) and a combination of these bacteria under anaerobic and aerobic growth conditions. Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin; RA: rosmarinic acid; Tet: tetracycline.

SINGLE SPECIES SYSTEM					MULTISPECIES SYSTEM				
Strain	**Sample**	**MIC[a] (µg/mL)**	**Inhibition of bacterial adherence and biofilm formation (BF) (IC_{50}[b] ± SD[c])**	**SI[d]**	**Strain**	**Sample**	**MIC[a] (µg/mL)**	**Inhibition of bacterial adherence and biofilm formation (BF) (IC_{50}[b] ± SD[c])**	**SI[d]**
T C	**PA**	7.8	3.2 ± 2.0	2.4	*C. acne s* (AT	**PA**	500	25.7 ± 5.5	19.4

	Hex	7.8	2.3 ± 0.8	3.5		**Hex**	31.25	5.2 ± 1.2	6.0
	EA	15.6	8.9 ± 1.0	1.8		**EA**	125	13.2 ± 3.5	9.5
	Lu	62.5	33.1 ± 6.5	1.9		**Lu**	NI_{500}^{e}	NI_{500}^{e}	-
	RA	NI_{500}^{e}	NI_{500}^{e}	-		**RA**	NI_{500}^{e}	NI_{500}^{e}	-
	Tet	0.78	1.4 ± 0.2	0.6		**Tet**	0.78	0.9 ± 1.7	0.9
***S. epidermidis* (ATCC® 35984)**	**PA**	500	231.9 ± 13.2	2.2	***C. acnes* (ATCC® 6919) + *S. epidermidis* (ATCC® 35984) aerobic growth**	**PA**	500	147.1 ± 7.6	3.4
	Hex	125	27.2 ± 7.5	4.6		**Hex**	125	57.6 ± 1.3	2.2
	EA	250	106.6 ± 4.0	2.4		**EA**	250	98.4 ± 7.7	2.5
	Lu	NI_{500}^{e}	NI_{500}^{e}	-		**Lu**	NI_{500}^{e}	NI_{500}^{e}	-
	RA	NI_{500}^{e}	NI_{500}^{e}	-		**RA**	NI_{500}^{e}	NI_{500}^{e}	-
	Tet	0.78	1.6 ± 0.3	0.5		**Tet**	1.56	1.7 ± 0.74	0.9

[a] MIC: Minimum inhibitory concentration; [b] IC_{50}: 50% inhibitory concentration; [c] SD: Standard deviation; [d] SI: Selectivity index [SI=MIC/BF]; [e] NI_{500}: No inhibition at the highest concentration tested of 500 µg/mL.

According to the criteria proposed by Van Vuuren and Holl (2017) (Table 4.3), significant antibacterial activity was observed for all samples except for rosmarinic acid against *C. acnes* (ATCC® 6919) (Table 4.4). Promising antibacterial activity was observed for the hexane partition against *S. epidermidis* (ATCC® 35984), and aerobic CA-SE with MIC values of 125 µg/mL and strong activity was observed against anaerobic CA-SE with an MIC value of 31.25 µg/mL. The ethyl acetate partition had moderate antibacterial activity against *S. epidermidis* (ATCC® 35984) and aerobic CA-SE with an MIC value of 250 µg/mL and promising activity against anaerobic CA-SE with an MIC value of 125 µg/mL. Moderate antibacterial activity was observed for PA_{EtOH} against *S. epidermidis* (ATCC® 35984) and anaerobic and aerobic CA-SE with an MIC value of 500 µg/mL. The CA-SE aerobically grown multispecies system was more resistant to the antibiotic positive control tetracycline compared to the effect of tetracycline on the other bacterial systems. This could indicate that under aerobic growth conditions, certain species of *C. acnes* and *S. epidermidis* grown as a multispecies system could potentially have an increased antibiotic resistance against certain types of antibiotics. The increase in antibiotic resistance could indicate that these bacteria could potentially have a synergistic and cooperative relationship to cause disease and increase

antibiotic resistance. This data could potentially further support the hypothesis that *S. epidermidis* protects *C. acnes* under aerobic growth conditions (Kumar et al., 2016).

3.5 Inhibition of bacterial adhesion/biofilm formation and eradication of a mature biofilm

The biofilm produced by bacteria plays a crucial role in protecting the bacterial community from external factors. The biofilm protects the bacterial community from environmental changes such as pH or nutrient changes and antibacterial compounds such as antibiotics. Antibiotic resistance has become detrimental to the global population, resulting in prolonged and intensive treatment strategies. Therefore, there is an urgent need for alternative treatments targeting biofilm formation, adhesion, and eradicating a mature biofilm (Burkhart and Burkhart, 2007; Crouzet et al., 2014).

Current conventional acne therapies do not target the bacterial biofilm but are instead a cause of antibiotic resistance seen in many diseases such as acne vulgaris, wounds and prosthetic implant-associated infections. Current research on therapies targeting biofilm formation, bacterial adhesion or eradication of a mature biofilm do not consider multispecies biofilms associated with various infections. Instead, current research mainly focuses on a single species system of the bacteria associated with the disease of interest. Current acne research only focuses on targeting the *C. acnes* biofilm but do not consider other bacterial species that could potentially contribute to the disease and target a potential multispecies biofilm (Moore and Moore, 2021).

The ability of *C. acnes* (ATCC® 6919), *S. epidermidis* (ATCC® 35984) and anaerobically and aerobically grown CA-SE to form a mature biofilm was determined from three individual replicates consisting of a total of 144 wells per bacterial system. The study confirmed a significant increase in the production of a mature biofilm in the multispecies systems (CA-SE) under aerobic and anaerobic growth conditions compared to forming a mature biofilm of a single species system (Figure 4.7).

The increase in mature biofilm formation could indicate higher antibiotic resistance against various antibiotics or that the bacteria could be more resistant to other treatment strategies within a multispecies system. Therefore, in a multispecies infection of *C. acnes* and *S. epidermidis,* treatment strategies would likely have to be adapted, or new strategies developed to target the bacteria compared to a single species infection. Furthermore, it has

been hypothesised that biofilms could contribute to increased adhesion of keratinocytes to one another which blocks the pilosebaceous. This biological glue forms due to the glycocalyx polymer produced by biofilm-forming bacteria and sebum produced in the skin. Clumped keratinocytes that block the pilosebaceous unit contribute to the development of various forms of acne vulgaris. Therefore, the potential exists that when these bacteria are associated with the skin either as commensal or opportunistic bacteria, an increase in biofilm formation in a multispecies system could contribute to blockage of the pilosebaceous unit initiating acne progression (Burkhart and Burkhart, 2007).

Biofilm formation occurs in a series of events. The first of these steps is the attachment and adhesion of the bacteria to a surface, secondly microcolony formation, thirdly maturation of the biofilm and lastly, detachment and distribution to new infection sites (Crouzet et al., 2014). Table 4.4 indicates the potential of PA_{EtOH}, identified pure compounds and partitions targeting the adherence and formation of *C. acnes* (ATCC® 6919), *S. epidermidis* and CA-SE biofilms. A selectivity index (SI) was used to determine if the samples are more selective towards inhibiting the bacteria (antibacterial activity) or towards bacterial adherence and biofilm formation. A SI larger than one is an indication that the sample is more selective as an inhibitor of bacterial adherence and biofilm formation since the adhered biofilm inhibition concentration is lower than the antibacterial inhibition concentration (Oosthuizen et al., 2019). The PA_{EtOH}, hexane and ethyl acetate partitions were more selective towards targeting bacterial adherence and biofilm formation. The positive antibiotic control tetracycline was unable to inhibit bacterial adherence and biofilm formation. None of the samples, including tetracycline, could eradicate the established mature biofilm of all the bacterial species systems investigated at the highest concentration tested (2×MIC of each respective sample).

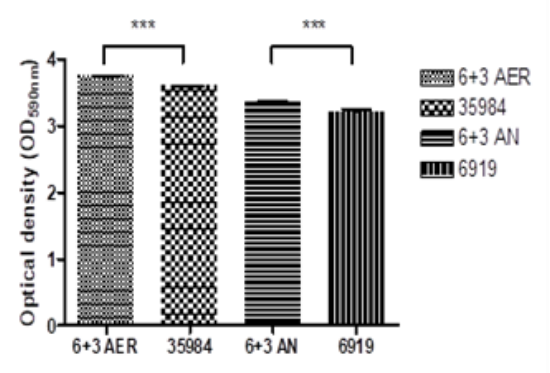

Figure 4. 7: Mature biofilms of *Staphylococcus epidermidis* (ATCC® 35984), *Cutibacterium acnes* (ATCC® 6919) and a combination of these bacteria under anaerobic (6+3 AN) and aerobic growth conditions (6+3

AER). One-way ANOVA with Tukey-Kramer multiple comparisons test from three individual replicates and a total of 144 wells per bacterial system. *p < 0.05, **p < 0.01, ***p < 0.001.

3.6 Artificial sebum model

Another mode of targeting antibiotic resistance is to penetrate the biofilm and inhibit the bacteria within.

The artificial sebum model was developed by Spittaels and Coenye (2018) to investigate the biofilm-forming activity of *C. acnes* in combination with a pellet that mimics the pilosebaceous unit and the sebum of the skin *in vitro*. This model was used to determine the ability of the samples to penetrate a mature *C. acnes* biofilm and either inhibit the bacteria within or have an effect on the bacteria's metabolism. The *C. acnes* biofilm in this model is grown under strained conditions ensuring an optimally resistant mature biofilm. The samples PA_{EtOH}, identified pure compounds and partitions were tested at their 2×MIC due to the maturity of the biofilm.

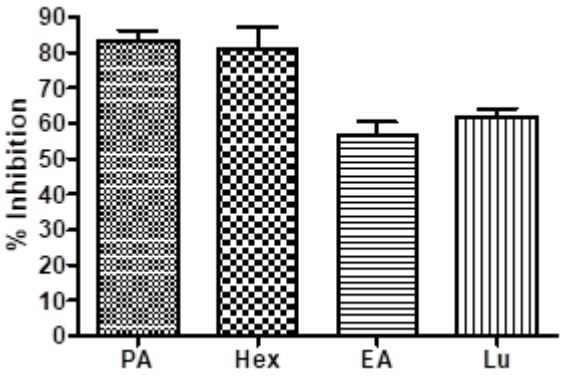

Figure 4. 8: Percentage inhibition of *Cutibacterium acnes* (ATCC®6919) using the artificial sebum model. PA: *Plectranthus aliciae* ethanolic extract; Hex: Hexane partition; EA: Ethyl acetate partition; Lu: Luteolin.

The samples PA_{EtOH}, hexane partition, ethyl acetate partition and luteolin displayed a percentage inhibition of 83.3 ± 6.9%, 80.8 ± 15.3%, 56.4 ± 9.6% and 61.9 ± 5.5%, respectively (Figure 4.8). Rosmarinic acid and tetracycline did not inhibit the sessile bacteria within the biofilm at their 2×MIC. Therefore, although PA_{EtOH}, the hexane and ethyl acetate partitions and luteolin were unable to eradicate a mature biofilm, the samples could penetrate a mature biofilm and inhibit the bacteria within, as confirmed by the artificial sebum model. Furthermore, PA_{EtOH}, hexane partition, ethyl acetate partition and luteolin resulted in a 28.3 ±

1.30%, 44.0 ± 9.7%, 10.9 ± 4.1% and 24.2 ± 8.7% reduction in metabolic activity of the remaining viable *C. acnes* bacteria in the mature biofilm, respectively (Figure 4.9).

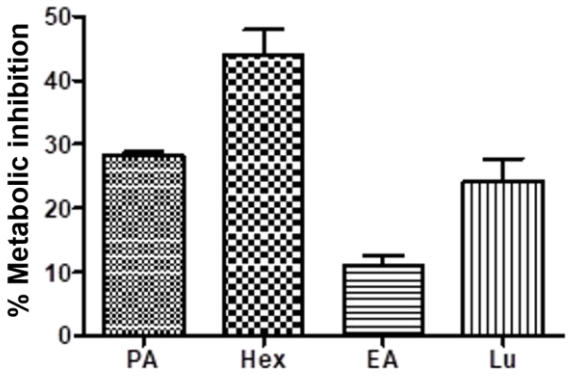

Figure 4. 9: Percentage inhibition of *Cutibacterium acnes* (ATCC® 6919) metabolic activity within a mature biofilm when treated with PA: *Plectranthus aliciae* ethanolic extract; Hex: Hexane partition; EA: Ethyl acetate partition and Lu: Luteolin. The study was conducted at the respective double minimum inhibitory concentrations of the samples against *C. acnes* (ATCC® 6919).

3.7 Targeting quorum sensing through autoinducer-2 inhibition

Quorum sensing and bacterial activities such as biofilm formation and the release of virulent factors are closely related. Quorum sensing is the cell-to-cell communication mechanism between bacteria which triggers a genetic response. This response is only triggered once a critical concentration of bacteria has been reached, since it is more sufficient for the bacteria to elicit a combined response than when there is only a small number of bacteria present. AI-2 is a universal signalling molecule used for interspecies communication in bacteria. *Cutibacterium acnes* and *S. epidermidis* are under AI-2 control to regulate the gene expression for the formation of biofilms and the release of virulent factors (Coenye et al., 2007a; Xue et al., 2015). Current research on quorum quenching activities of AI-2 uses *Vibrio harveyi* as a model bacterium to determine the AI-2 inhibitory ability of a sample. However, several limitations have been reported when using the Gram-negative *V. harveyi* for the detection of AI-2. Firstly *V. harveyi* can only detect AI-2 concentration at a minimum threshold of 100 nmol/mL^{-1} and is therefore not sensitive to lower AI-2 concentrations. Secondly, studies have determined the sensitivity of *V. harveyi* towards glucose present in several broths used in bacterial studies that could result in a false-positive result. Lastly, since *V. harveyi* is a Gram-negative bacterium, the production, regulation, and detection of AI-2 systems could be under different regulatory systems than that of Gram-positive bacteria (Turovskiy and Chikindas, 2006; Rickard et al., 2008). To date, no research could be found

on detecting AI-2 production of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) without the use of *V. harveyi*. The present study followed the colourimetric assay for detecting AI-2 in bacterial species as described by Wattanavanitchakorn et al. (2014). Furthermore, no research could be found on the AI-2 production of these bacterial species grown as a multispecies system under anaerobic and aerobic growth conditions. Currently, no therapy in the market targets AI-2 production as a quorum quenching active.

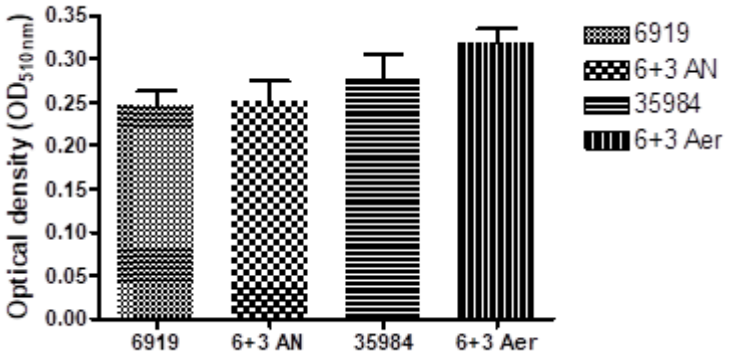

Figure 4. 10: Autoinducer-2 production after 72 h for *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984) and a combination of these species under anaerobic (6+3 AN) and aerobic (6+3 AER) growth conditions. One-way ANOVA with Tukey-Kramer multiple comparisons test n=5. *p < 0.05, **p < 0.01, ***p < 0.001.

Although not significant, a slight increase in AI-2 production was observed for the multispecies system under aerobic growth conditions (Figure 4.10). The AI-2 levels present in the various bacterial species and systems were only measured after 72 h. It would be interesting to measure the production of AI-2 and track the change in AI-2 production and usage in the initial stages of biofilm development. Tracking this data could result in significant differences in the results for the various bacterial species and systems investigated in the present study.

Table 4. 5: Percentage inhibition of autoinducer-2 (AI-2) for *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions at their respective half minimum inhibitory concentrations against *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984), and a combination of these bacteria under anaerobic and aerobic growth conditions. Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin; RA: rosmarinic acid; Tet: tetracycline. P-value: One way ANOVA Dunnets multiple comparison test against tetracycline n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.

SINGLE SPECIES SYSTEM				MULTISPECIES SYSTEM			
Strain	**Sample**	**AI-2 inhibition (%Inh[a] ±**	**P-value**	**Strain**	**Sample**	**AI-2 inhibition (%Inh ±**	**P-value**

		SD[b])				SD)	
***C. acnes* (ATCC® 61919)**	**PA**	82.8 ± 10.1	P < 0.01	***C. acnes* (ATCC® 6919) + *S. epidermidis* (ATCC® 35984) anaerobic growth**	**PA**	86.1 ± 2.2	P < 0.01
	Hex	27.5 ± 3.3	P < 0.01		**Hex**	85.4 ± 0.9	P < 0.01
	EA	58.0 ± 1.8	P < 0.01		**EA**	88.9 ± 3.2	P < 0.01
	Lu	6.4 ± 2.5	P > 0.05		**Lu**	22.6 ± 0.9	P < 0.01
	RA	NI_{500}[c]	P > 0.05		**RA**	19.9 ± 9.0	P < 0.01
	Tet	0.6 ± 8.2	-		**Tet**	3.8 ± 1.7	-
***S. epidermidis* (ATCC® 35984)**	**PA**	72.7 ± 1.6	P < 0.05	***C. acnes* (ATCC® 6919) + *S. epidermidis* (ATCC® 35984) aerobic growth**	**PA**	50.6 ± 9.3	P < 0.01
	Hex	66.7 ± 3.5	P > 0.05		**Hex**	78.2 ± 9.7	P < 0.01
	EA	71.4 ± 7.6	P < 0.05		**EA**	56.7 ± 1.4	P < 0.01
	Lu	46.7 ± 1.3	P > 0.05		**Lu**	NI_{500}[c]	P > 0.05
	RA	85.2 ± 14.8	P < 0.01		**RA**	50.5 ± 11.3	P < 0.01
	Tet	54.3 ± 0.9	-		**Tet**	4.08 ± 3.4	-

[a] %Inh: Percentage inhibition; [b] SD: Standard deviation; [c] NI_{500}: no inhibition at the highest concentration tested of 500 µg/mL.

The samples were tested for their quorum quenching activity by inhibiting AI-2 at 0.5×MIC values. Control studies confirmed no bacterial inhibition for the samples at their respective tested concentrations against the various bacterial systems. Evaluating the data in Table 4.5, $PA_{EtOH,}$ hexane, and ethyl acetate was significantly more active in inhibiting AI-2 of *C. acnes* (ATCC® 6919) and CA-SE under anaerobic and aerobic growth conditions to tetracycline. Significant inhibition was observed for $PA_{EtOH,}$ ethyl acetate partition, and rosmarinic acid of AI-2 released by *S. epidermidis* (ATCC® 35984) compared to tetracycline. Tetracycline was most active towards inhibiting AI-2 of *S. epidermidis* with a percentage inhibition of 54.3 ± 0.9% at 0.39 µg/mL.

Under aerobic growth conditions, no AI-2 inhibition was observed for rosmarinic acid and luteolin of *C. acnes* and aerobically grown CA-SE respectively at their 0.5×MIC. This data corresponds to the biofilm activity observed in Table 4.4, where no inhibitory activity of bacterial adherence and biofilm formation was observed for these samples against the respective bacteria. A study done by Geng et al. (2021) confirmed the quorum quenching activity of luteolin against the Gram-negative *Pseudomonas aeruginosa*. In the study by

Geng et al. (2021), luteolin reduced acetyl homoserine lactone accumulation by downregulating the quorum sensing gene transcription and could potentially have the same quorum quenching effect in the present study for AI-2.

Rosmarinic acid displayed significant AI-2 inhibitory activity towards AI-2 that is not linked to its inhibition of bacterial adherence and biofilm formation observed in Table 4.4. The AI-2 inhibitory activity observed against AI-2 could be related to other AI-2 targets, such as targeting virulent factors that are not related to bacterial adherence and biofilm formation. These targets include the inhibition of lipase enzymes that contribute to inflammation. Rosmarinic acid is a known anti-inflammatory compound that could target quorum sensing mechanisms (Bustanji et al., 2010).

This is the first report of the AI-2 activity of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) using the colourimetric method and the AI-2 production by these bacteria in a multispecies system. Furthermore, this is the first report of the quorum quenching activities of the tested samples in single and multispecies bacterial systems related to acne vulgaris, wounds, and prosthetic implant-associated infections. Currently, there are no known anti-acne active ingredients that target AI-2 quorum sensing in acne-causing bacteria.

3.8 Inhibition of quorum sensing factors causing virulence: lipase inhibition

Quorum sensing is linked to the production and secretion of several virulent factors that contribute to the pathogenicity of the bacteria. Lipase is an enzyme produced by both *C. acnes* and *S. epidermidis* and is involved in reducing sebum triglycerides to free fatty acids and glycerol. Glycerol serves as a nutrient source for the bacteria, and the free fatty acids aid the bacteria in attachment and colonisation in the pilosebaceous unit. Furthermore, the free fatty acids produced trigger an inflammatory response associated with acne vulgaris, prosthetic implant-associated infections, and wounds. Studies have confirmed that sessile bacterial cells involved in biofilm development produce more lipase enzymes than planktonic bacteria. Therefore, it is believed that *C. acnes* and *S. epidermidis* lipase is a virulent factor which is regulated by quorum sensing (Falcocchio et al., 2006; Coenye et al., 2007b; Li et al., 2007; Otto, 2014).

The lipase activity of *C. acnes, S. epidermidis,* and CA-SE grown anaerobically and aerobically was determined. Enzyme activity was measured quantitatively using the slope of the fluorescence time curves. CA-SE grown under aerobic growth conditions produced

significantly more lipase ($P < 0.05$) than *S. epidermidis* and *C. acnes* ($P < 0.001$) grown as single species systems (Figure 4.11). CA-SE anaerobically grown produced significantly more lipase than *C. acnes* grown as a single species system ($P < 0.001$). This could indicate that *C. acnes* and *S. epidermidis* have a combined virulence effect, especially under aerobic growth conditions and potentially under anaerobic growth conditions that contribute to inflammatory acne progression and increases treatment times associated with acne vulgaris, prosthetic implant-associated infections and wounds. Since lipase also indirectly contributes to the attachment and colonisation of biofilm-forming bacteria, the increased lipase production in the multispecies system could explain the significant increase in mature biofilm formation and antibiotic resistance observed in Figure 4.7 and Table 4.4.

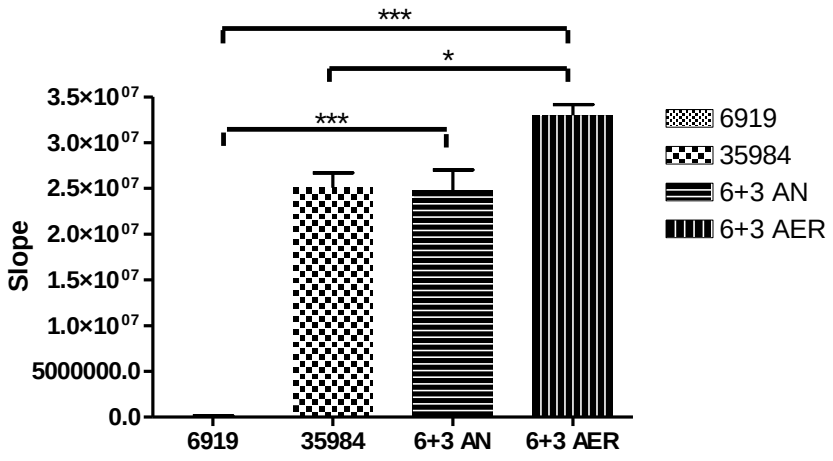

Figure 4. 11: Lipase production after 72 h for *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984) and a combination of these species under anaerobic (6+3 AN) and aerobic (6+3 AER) growth conditions. One-way ANOVA with Tukey-Kramer multiple comparisons test n=5. *p < 0.05, **p < 0.01, ***p < 0.001.

The samples were tested for their lipase inhibitory activity at 0.5×MIC values using the 4-methylumbelliferyl oleate fluorogenic assay. The percentage inhibition was determined from the slopes of the fluorogenic time curves compared to the slope of the DMSO control (2.5% v/v; 100% bacterial growth) for the respective bacterial systems. Control studies confirmed no bacterial inhibition by the samples at their respective tested concentrations against the various bacterial systems. Lipase inhibition was observed for the samples.

Rosmarinic acid and luteolin displayed an overall positive lipase inhibitory activity against all the bacterial systems tested. However, in the aerobically grown CA-SE multispecies system, lower lipase inhibition was observed for rosmarinic acid compared to the other bacterial systems. No lipase inhibition was observed for the antibiotic tetracycline against *C. acnes*

(ATCC® 6919) and CA-SE grown aerobically at 0.5×MIC. This could be an indication that under aerobic conditions *S. epidermidis* promotes the growth of *C. acnes*, contributing to an increased inflammatory response as observed with the lowered lipase inhibitory activity of rosmarinic acid. PA_{EtOH} displayed no lipase inhibition at the highest concentration of 3.9 µg/mL against *C. acnes*. However, PA_{EtOH} showed significant lipase inhibition for the other bacterial systems (Table 4.6). The significant lipase inhibitory activity of rosmarinic acid and luteolin could be due to the anti-inflammatory nature of these compounds. Studies have confirmed the lipase inhibitory activity of rosmarinic acid and luteolin, identified to be present in PA_{EtOH} (Table 4.2).

The varying lipase inhibitory effects could be due to the different environments each sample creates for the bacterial lipases released. Lipase enzymes are extracellular enzymes, and their activity is significantly affected by nutritional and physicochemical factors. These factors include temperature, pH, nitrogen and carbon sources, lipids and inorganics salts, all which are different for the samples tested and the manner in which the different bacterial systems investigated metabolise the samples (Shimura et al., 1994; Gupta et al., 2004; Bustanji et al., 2010; Devi et al., 2016).

Table 4. 6: Percentage inhibition of lipase for *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions at their respective half minimum inhibitory concentrations against *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984), and a combination of these bacteria under anaerobic and aerobic growth conditions. Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin; RA: rosmarinic acid; Tet: tetracycline.

SINGLE SPECIES SYSTEM			MULTISPECIES SYSTEM		
Strain	**Sample**	**Lipase inhibition (%Inh[a] ± SD[b])**	**Strain**	**Sample**	**Lipase inhibition (%Inh ± SD)**
***C. acnes* (ATCC® 61919)**	**PA**	NI[c]	***C. acnes* (ATCC® 6919) + *S. epidermidis* (ATCC® 35984) anaerobic growth**	**PA**	99.5 ± 1.1
	Hex	15.8 ± 1.1		**Hex**	95.6 ± 5.0
	EA	13.6 ± 6.7		**EA**	98.1 ±2.5
	Lu	97.5 ± 0.7		**Lu**	99.5 ± 0.1
	RA	100.0 ± 3.7		**RA**	63.0 ± 7.7
	Tet	NI		**Tet**	89.0 ± 7.0
S. epidermid	**PA**	98.2 ± 2.2	***C. acnes* (ATCC® 6919) + *S. epidermid***	**PA**	99.2 ± 1.5
	Hex	98.9 ± 0.6		**Hex**	94.1 ± 3.0
	EA	93.6 ± 8.9		**EA**	97.2 ± 1.6

	Lu	75.1 ± 1.4		**Lu**	97.5 ± 0.4
	RA	67.1 ± 3.1		**RA**	48.9 ± 6.4
	Tet	48.7 ± 3.9		**Tet**	NI

[a] %Inh: Percentage inhibition; [b] SD: Standard deviation; [c] NI: No inhibition at the highest concentration tested (0.5 × MIC)

3.9 Quantification of extracellular DNA in bacterial biofilms

Extracellular DNA (eDNA) has a crucial role in the attachment, structure, and transfer of genetic material in the bacterial biofilm (Montanaro et al., 2011). The biofilm protects sessile bacteria from external factors and treatments such as antibiotics, resulting in antibiotic resistance. The overexposure of bacteria such as *C. acnes* to antibiotics could result in mutations and selection pressure that allows these bacteria to multiply, rendering them more challenging to inhibit (Graber, 2021). Researchers have suggested the transfer of these mutated genes to other bacterial species that are in close contact with the bacterial species that have developed antibiotic resistance. Graber (2021) proposed that *C. acnes* bacterial strains resistant to antibiotics could transfer these mutated genes to other bacterial species that form part of the skin's microflora, such as *S. epidermidis*. This gene transfer could be due to eDNA in the bacterial biofilm in a multispecies bacterial system, contributing to multispecies antibiotic resistance. Studies have confirmed the role of eDNA in the initial attachment and biofilm formation of *C. acnes* and *S. epidermidis* (Montanaro et al., 2011; Okuda et al., 2018). Furthermore, eDNA is able chelate divalent cations that triggers gene expression increasing the pathogenicity and antibiotic resistance of bacteria. Targeting eDNA is a proposed method for targeting antibiotic resistance since targeting the eDNA can prevent bacterial attachment and biofilm formation, the dispersion of the biofilm, and degrade the biofilm, exposing sessile bacteria to antimicrobials (Okshevsky et al., 2015).

The Toto-1 eDNA detection assay was used to determine the relative quantities of eDNA in *C. acnes*, *S. epidermidis,* and CA-SE anaerobically and aerobically grown bacterial systems. Toto-1 is a stain that exclusively stains eDNA in bacterial biofilms and was selected to detect eDNA (Okshevsky and Meyer, 2014). The data revealed that under CA-SE aerobic growth conditions significantly ($P < 0.001$), more eDNA was produced compared to the other bacterial systems (Figure 4.12). This could indicate that under aerobic growth conditions, a multispecies system of *C. acnes* and *S. epidermidis* could result in an increase in antibiotic resistance and a more secure biofilm structure. This data supports the increased resistance

towards tetracycline in Table 4.4 and the increase in biofilm mass in Figure 4.7 for CA-SE grown under aerobic conditions.

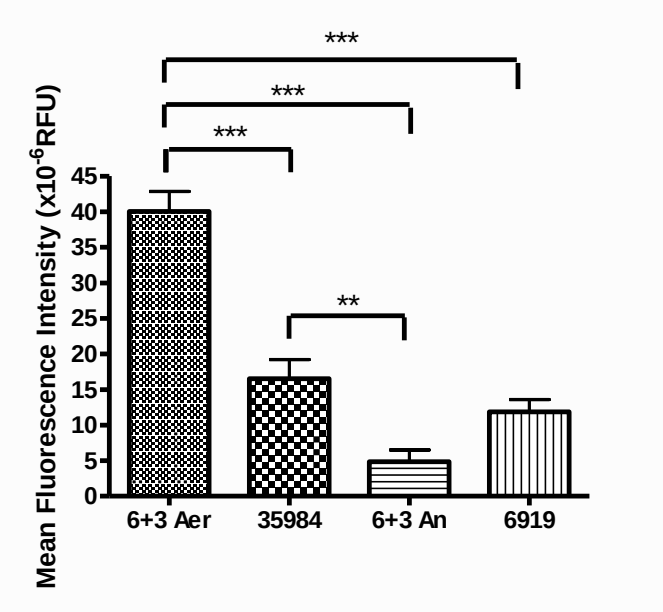

Figure 4. 12: Extracellular DNA production after 72 h for *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984) and a combination of these species under anaerobic (6+3 AN) and aerobic (6+3 AER) growth conditions. One-way ANOVA with Tukey-Kramer multiple comparisons test n=5. *p < 0.05, **p < 0.01, ***p < 0.001.

The eDNA fluorescence intensity was normalised to the number of colony-forming units determined through plate counting for each sample at their tested concentrations under various bacterial growth conditions described by Brackman et al. (2016). Samples targeting bacterial attachment and biofilm formation as indicated in Table 4.4 (SI > 1) were selected for eDNA analysis to determine if the selectivity of the samples towards bacterial attachment and biofilm formation could be due to the samples targeting eDNA secretion.

All of the selected samples inhibited eDNA associated with the various bacterial systems (Table 4.7). This indicated that these samples could be used to disrupt bacterial attachment and biofilm formation of the selected bacterial systems and could explain the selectivity of these samples towards bacterial attachment and biofilm formation in Table 4.4. Qian et al. (2020) identified the eDNA inhibitory activity of luteolin against single and multispecies systems of *Escherichia coli* and *Enterobacter cloacae*. Research confirmed that luteolin prevents eDNA production and reduces the antibiotic tolerance of bacterial biofilms. Previous studies have highlighted the potential of DNase targeting eDNA associated with bacterial biofilms, rendering them susceptible to antimicrobials. However, the high production cost of DNase motivates researchers to look for more affordable options to target eDNA, for which

there is currently no treatment on the market (Okshevsky et al., 2015). The samples tested in the present study could potentially be an affordable target for eDNA associated with *C. acnes* and *S. epidermidis* biofilms.

Table 4. 7: Percentage inhibition of extracellular DNA production for *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions at their respective 50% biofilm formation inhibitory concentrations against *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984), and a combination of these bacteria under anaerobic and aerobic growth conditions. Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin.

SINGLE SPECIES SYSTEM			MULTISPECIES SYSTEM		
Strain	**Sample**	**eDNA inhibition (%Inh[a] ± SD[b])**	**Strain**	**Sample**	**eDNA inhibition (%Inh ± SD)**
***C. acnes* (ATCC® 61919)**	**PA**	93.8 ± 7.9	***C. acnes* (ATCC® 6919) + *S. epidermidis* (ATCC® 35984) anaerobic growth**	**PA**	86.4 ± 8.2
	Hex	82.9 ± 0.8		**Hex**	80.1 ± 8.3
	EA	98.3 ± 0.9		**EA**	73.2 ± 4.6
	Lu	93.8 ± 8.3			
***S. epidermidis* (ATCC® 35984)**	**PA**	92.6 ± 1.8	**(ATCC® 6919) + *S. epidermidis* (ATCC® 35984) aerobic**	**PA**	96.6 ± 1.0
	Hex	81.4 ± 3.2		**Hex**	94.9 ± 2.2
	EA	95.1 ± 0.2		**EA**	88.7 ± 6.8

[a] %Inh: Percentage inhibition; [b] SD: Standard deviation

3.10 Cytotoxicity

The cytotoxicity of the samples was determined on HaCat cells to determine if the tested concentrations of the samples could have an effect on the cell viability and potentially be harmful to the skin. The SI values for the samples compared to their MIC values were calculated as follows

$$SI = \frac{\text{Cytotoxicity } IC_{50}}{\text{Antibacterial MIC}}$$

An SI value larger than one is an indication that the sample is more selective towards targeting the bacteria than the cells and could be applied topically without irritating the skin (Fontanay et al., 2008). The criteria for cytotoxicity of plant extracts and pure compounds against non-cancerous cell lines, as proposed by Kuete and Efferth (2015), was followed,

considering the SI values. Against *C. acnes*, the samples PA_{EtOH}, hexane, and ethyl acetate partitions were more selective towards targeting the bacteria than adversely affecting the viability of the HaCat cells. PA_{EtOH} displayed moderate toxicity, and the partitions were cytotoxic. At higher MIC values targeting *S. epidermidis* and CA-SE grown anaerobically and aerobically, the samples were more selective towards targeting the cells (Table 4.8).

Table 4. 8: Cytotoxicity of *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions on human keratinocyte cells (HaCat). Fifty percent inhibitory concentration (IC_{50}) ± Standard Deviation (SD). Selectivity index (SI) calculated from the minimum inhibitory concentrations (MIC) of *Cutibacterium acnes* (ATCC® 6919), *Staphylococcus epidermidis* (ATCC® 35984), and a combination of these bacteria under anaerobic and aerobic growth conditions. Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin; RA: rosmarinic acid; Tet: tetracycline. P-value: One-way ANOVA with Tukey-Kramer multiple comparisons test n=3. *p < 0.05, **p < 0.01, ***p < 0.001.

Sample	Cytotoxicity on HaCat cells (IC_{50}[b] ± SD[c])	P-value	Selectivity index			
			C. acnes (ATCC® 6919)	*S. epidermidis* (ATCC® 35984)	CA-SE Anaerobic	CA-SE Aaerobic
PA	109.9 ± 8.4	P < 0.001	14.0	0.2	0.2	0.2
Hex	63.5 ± 6.3	P < 0.001	8.1	0.5	2.0	0.5
EA	52.4 ± 5.6	P < 0.001	3.4	0.2	0.4	0.2
Lu	23.1 ± 1.3	P < 0.01	0.4	NI_{500}[a]	NI_{500}[a]	NI_{500}[a]
RA	222.1 ± 4.8	P < 0.001	NI_{500}[a]	NI_{500}[a]	NI_{500}[a]	NI_{500}[a]
Tet	185.4 ± 6.9	P < 0.001	237.7	237.7	237.7	118.8
ActD	0.0 3 ± 0.1					

[a] NI_{500}: No bacterial inhibition at the highest concentration tested of 500 µg/mL, [b] IC_{50}: 50% inhibitory concentration, [c] SD: Standard deviation

The goal of *in vitro* cytotoxicity studies was to determine the relative cytotoxicity of the samples. Although some samples displayed selectivity and moderate toxicity towards the cells, *in vivo* mechanisms within the skin are different from *in vitro* studies. Studies have found differences in *in vitro* and *in vivo* cytotoxicity studies, with *in vitro* studies serving as a guide for *in vivo* studies. Therefore, *in vivo* irritancy studies are vital to determine the true irritancy activity of the samples on various skin types to ensure that these actives are safe and used effectively (Garle et al., 1994; Nunzio et al., 2017).

3.11 Wound closure

Studies have confirmed the presence of bacterial biofilms in chronic wounds and are associated with wound development delayed wound healing (Singh et al., 2019). The samples were tested for their ability to promote wound closure at their respective antimicrobial concentrations against *C. acnes* (MIC concentrations). The samples PA_{EtOH}, hexane, and ethyl acetate partitions promoted 13.6 ± 7.5%, 17.4 ± 7.3%, and 19.3 ± 5.9 % wound closure, respectively. Compared to the 100% wound closure control (0.25% v/v DMSO), PA_{EtOH}, hexane, and ethyl acetate partitions significantly promoted wound closure, as displayed in Figures 4.13, 4.14 and Table 4.9. Although luteolin displayed a significant difference in wound closure activity compared to the control, the significance is due to a decrease in cell viability related to its cytotoxicity, as described in Table 4.8. There was a significant difference ($P < 0.01$) in cell viability for luteolin compared to the 0.25% v/v DMSO vehicle control. All other samples had no significant effect on cell viability ($P > 0.05$) at the tested concentrations compared to the vehicle control (Table 4.9). No significant improvement in wound closure was observed for rosmarinic acid or tetracycline at their tested concentrations when compared to the controls.

Plectranthus madagascariensis, of which *P. aliciae* has previously been considered a variety, is traditionally used to treat wounds. The anti-inflammatory and analgesic activity of rosmarinic acid could potentially contribute to the wound healing activity of PA_{EtOH}. (Lambrechts and Lall, 2021). Numerous *Plectranthus* species have demonstrated exceptional wound healing activity. The wound healing activity has been linked to the presence of thymol and a high concentration of magnesium, calcium and zinc that stimulates the proliferation of cells. Zinc is a cofactor in numerous enzyme systems, including zinc-dependant matrix-metalloproteinases that influence keratinocyte migration during wound healing. Thymol has previously been demonstrated to enhance cell growth and has anti-inflammatory activity. Studies have confirmed the inhibitory activity of thymol on the expression of proinflammatory cytokines IL-1β and IL-6 that contributes to impaired wound healing (Lansdown et al., 2007; Khorshid et al., 2010; Mirza et al., 2013; Alkafafy et al., 2014; García-Salinas et al., 2020; Kumar et al., 2020). Therefore, the activity of PA_{EtOH} and its hexane and ethyl acetate partitions to promote wound healing could directly be related to the wound healing activity of the genus.

Table 4. 9: Percentage wound closure of *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions on human keratinocyte cells (HaCat) at their respective *Cutibacterium acnes* minimum inhibitory concentrations Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin; RA: rosmarinic acid; Tet: tetracycline. Percentage wound closure normalised to 0.25% DMSO control. P-

value: One way ANOVA Dunnets multiple comparison test against tetracycline n = 3. *p < 0.05, **p < 0.01, ***p < 0.001. Compared to 0.25% v/v DMSO vehicle control.

Sample	Difference in the % wound closure compared to 0.25% v/v DMSO (%Closure ± SD[a])	%Viability of HaCat cells compared to 0.25%v/v DMSO	P-value
PA	13.6 ± 7.5	98.4 ± 6.6	P > 0.05
Hex	17.4 ± 7.3	99.8 ± 2.7	P > 0.05
EA	19.3 ± 5.9	98.9 ± 7.4	P > 0.05
Lu	NC[b]	63.2 ± 1.2	P < 0.01
RA	NC	98.2 ± 0.1	P > 0.05
Tet	2.3 ± 7.8	100.0 ± 1.8	P > 0.05

[a] SD: Standard deviation; [b] NC: No closure at the tested concentration

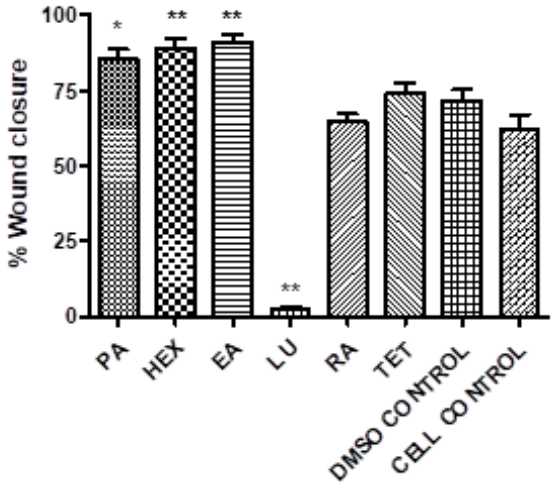

Figure 4. 13: Percentage wound closure of *Plectranthus aliciae* (PA) ethanolic extract, its pure compounds, and partitions on human keratinocyte cells (HaCat) at their respective *Cutibacterium acnes* minimum inhibitory concentrations Hex: hexane partition; EA: ethyl acetate partition; Lu: luteolin; RA: rosmarinic acid; Tet: tetracycline. Percentage wound closure normalised to 0.25% DMSO control. P-value: One way ANOVA Dunnets multiple comparison test against 0.25% v/v DMSO vehicle control n = 3. *p < 0.05, **p < 0.01, ***p < 0.001. Compared to 0.25% v/v DMSO vehicle control.

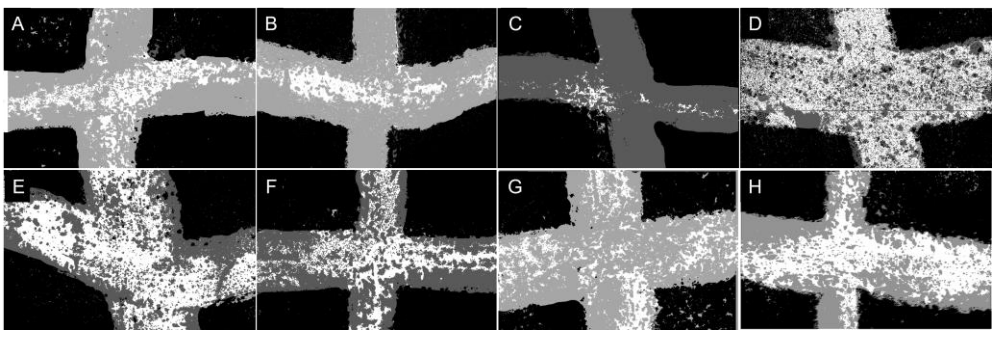

Figure 4. 14: Superimposed ImageJ images indicating the wound closure activity of A: *Plectranthus aliciae* ethanolic extract, B: hexane partition, C: ethyl acetate partition; D: luteolin; E: rosmarinic acid;

F: tetracycline; G: 0.25% v/v DMSO vehicle control; H: Cell control. A black background is an indication of wound closure. White indicates no cells present.

4. Development of a prototype from *Plectranthus aliciae* for acne vulgaris

4.1 Mutagenicity

Cytotoxicity and mutagenicity testing have become standard requirements to assess the safety of plant extracts during preclinical studies. Mutagens are chemicals or samples that can cause genetic mutations that could result in maladies such as cancer. Mutations occur when the cell attempts to replicate or repair its damaged DNA caused by the mutagen, and errors are introduced. The Ames test is widely used to detect gene mutations caused by samples and uses *Salmonella typhimurium* strains to detect mutagens in a minimal histidine growth medium. These bacterial strains are auxotrophic for histidine and require histidine to grow sufficiently. In the presence of a mutagen, bacteria that have undergone mutations and reverted to the wild type strain were able to synthesise histidine, while bacterial growth was being observed (Beedanagari et al., 2014; Gomes da Silva Dantas et al., 2020).

In this study, the Ames test was used to determine the mutagenic potential of PA_{EtOH} at three concentrations (5 000 µg/mL, 500 µg/mL and 50 µg/mL) using *S. typhimurium* T98 and T100 strains. The T98 strain was used to detect frameshift mutations and T100 to detect base-pair substitutions (Rossi et al., 2003). The number of revertant colonies that were observed for PA_{EtOH} and 10% v/v DMSO was lower than the negative water control and therefore the samples can be considered as non-mutagenic (Table 4.10). Samples are only considered mutagenic if there is a dose-dependent increase in the number of revertant colonies or if the number of revertant colonies are twice as much or equal to the number of revertant colonies for the negative control (Madikizela and McGaw, 2019; Rodríguez et al., 2012). The number of spontaneous revertants counted for PA_{EtOH}, and the negative and solvent control was in the normal limits for strains T98 and T100 (Mortelmans and Zeiger, 2000b). The positive control 4-Nitroquinoline N-oxide (4-NQO) resulted in 210 and 1065 revertant colonies in strains T98 and T100, respectively. This is in accordance with the results obtained for 4-NQO tested at a concentration of 2 µg/mL by Madikizela and McGaw (2019) with 235 ± 0.58 and 889.67 ± 0.33 revertant colonies of strains T98 and T100, respectively. No bacterial inhibitory activity was observed since the results were comparable to the negative and solvent

control, and no visible reduction in revertant colonies was observed between various concentrations of PA_{EtOH} tested at high and low concentrations.

Previous studies have confirmed the antimutagenic activity of species from the Lamiaceae family. This activity could be due to the presence of phenolic compounds with known free radical scavenging activity, preventing DNA mutations (Gomes da Silva Dantas et al., 2020). Rosmarinic acid is a phenolic compound abundantly present in the Lamiaceae family and has been identified to be present in *P. aliciae* (Table 4.2) (Lambrechts and Lall, 2021). This could explain the non-mutagenic activity of *P. aliciae* ethanolic extract.

Table 4. 10: The average number of revertant colonies of *Salmonella typhimurium* strains T98 and T100 as a result of *Plectranthus aliciae* ethanolic extract, the positive control 4-Nitroquinoline N-oxide, the negative water control and DMSO solvent control using the Ames test for the detection of mutagens. P-value: One way ANOVA Dunnets multiple comparison test against 4-Nitroquinoline N-oxide n = 3. *p < 0.05, **p < 0.01, ***p < 0.001. Compared to 0.25% v/v DMSO vehicle control.

Sample	The average number of revertants ± SD[a]		P-value	
	S. typhimurium T98	*S. typhimurium* T100	T98	T100
PA_{EtOH} (5 000 µg/mL)	13.0 ± 0.0	134.7 ± 4.0	P < 0.01	P < 0.01
PA_{EtOH} (500 µg/mL)	15.0 ± 1.0	141.0 ±1.0	P < 0.01	P < 0.01
PA_{EtOH} (50 µg/mL)	21.3 ± 1.5	116.3 ± 4.5	P < 0.01	P < 0.01
10% DMSO	20.3 ± 0.6	113.3 ± 0.6	P < 0.01	P < 0.01
Water	27.3 ± 1.2	158.0 ± 0.0	P < 0.01	P < 0.01
4-Nitroquinoline N-oxide[b] (0.74 µg/mL)	210.0 ± 4.4	1065.7 ± 1.5		

[a] SD: Standard deviation

4.2 Heavy metal and microbial analysis

Heavy metals and microbes are common contaminants of plant extracts that can have serious health complications. Heavy metals are dense metallic chemical elements that are toxic to humans at low concentrations. These metals can be found in water and soil exposed to mining activities or other environmental pollutants such as pesticides that contaminate plant material. Exposure to heavy metals such as arsenic, mercury, cadmium could have mutagenic activity and result in health complications such as cancer, neurological effects, organ damage, reproductive complications, gastrointestinal problems and could affect the central nervous system (Shaban et al., 2016). Microbials such as yeast and mould could contaminate plant material or extracts that are not correctly stored. These microbes can affect the activity of an

extract and cause opportunistic infections in immune-compromised patients (Street et al., 2008). Therefore, plant extracts used for cosmeceutical purposes must be evaluated for potential heavy metal and microbial contamination that could affect the end product's quality, safety, and efficacy.

The heavy metal (arsenic, cadmium, mercury, and lead), total microbial activity, and yeast and mould contamination of PA_{EtOH} were tested at Swift Silliker (Pty) Ltd t/a Mérieux NutriSciences. No heavy metal, microbial or yeast and mould contamination was observed for PA_{EtOH} (Appendix B and C).

4.3 Irritancy studies of *Plectranthus aliciae*

Irritancy studies were conducted at the Photobiology Laboratory at Sefako Makgatho Health Sciences University, South Africa, on the *P. aliciae* ethanolic extract alone (Appendix D, PT1333) and formulated at 10% in a gel formulation with a final PA_{EtOH} concentration of 600 µg/mL (Appendix D, PT 1433). The irritancy of the samples was compared to the positive control 1% SLS, and dH_2O served as the negative control. The mean visual score for 1% SLS in the extract irritancy studies was confirmed to be 2.76 and 0.58 for dH_2O. PA_{EtOH} had a mean visual score of 0.38. Furthermore, the irritancy studies of PA_{EtOH} formulated into a gel formulation was confirmed to have a mean visual score of 0.47 and for the positive and negative controls 2.01 and 0.61, respectively. Therefore, it can be concluded that the neat PA_{EtOH} and formulated PA_{EtOH} were non-irritants.

4.4 Acne efficacy studies of *Plectranthus aliciae* in a finished formulation

The *in vivo* acne efficacy of *P. aliciae* formulated at 10% in a gel formulation was conducted at Future Cosmetics (Pty) Ltd, South Africa (Appendix E). The study was conducted on 24 volunteers between the ages of 19 to 39 years having Fitzpatrick skin types IV and V. The gel formulation without 10% PA_{EtOH} served as the placebo control. Measurements were taken on day zero (baseline), day 14 and day 28. The participants were instructed to apply the sample consecutively twice a day for 14 to 28 days. Table 4.11 indicates the acne efficacy results of 10% PA_{EtOH} in a gel formulation.

Table 4. 11: *In vivo* acne efficacy studies of *Plectranthus aliciae* ethanolic extract tested at 10% in a gel formulation between 14 to 28 days on 24 volunteers.

Type of acne	Number of days until

	efficacy observed
Comedones	14 days
Blackheads	14 to 28 days
Whiteheads	14 to 28 days
Papules	NA[a]
Pustules	28 days
Micro cysts	14 to 28 days

[a]NA: No activity between 14 to 28 days

At 10% in a gel formulation, PA_{EtOH} was effective against various forms of acne vulgaris between 14 to 28 days, including severe forms of acne such as micro cysts. Cystic acne is associated with severe inflammation that results in hyperpigmentation and scarring (Goodman, 2009). *Plectranthus* species such as *Plectranthus barbatus* (synonym: *Coleus forskohlii*) have been confirmed to be effective in treating acne vulgaris (De Canha et al., 2020). *Plectranthus barbatus* is not indigenous to South Africa, but grows in parts of India and South America (Dorni et al., 2017). However, no product is currently on the market from an endemic South African *Plectranthus* species for the treatment of acne vulgaris, making *P. aliciae* an ideal candidate for commercialisation in South Africa and internationally.

5. Conclusion

This study confirmed that *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) could have a synergistic relationship and that the interactions between *C. acnes* and *S. epidermidis* associated with the skin are strain-specific. This data could support our hypothesis that *S. epidermidis* provides a favourable environment for *C. acnes* to thrive under aerobic conditions. Future studies can use fluorescence *in situ* hybridization (FISH) to visualise the three-dimensional structure and quantify *C. acnes* and *S. epidermidis* in the multispecies biofilm. This study confirmed that when grown in a multispecies system, the selected strains of *C. acnes* and *S. epidermidis* had a synergistic relationship, resulting in an increase in antibiotic resistance and virulence of the community. This synergistic activity was observed from a significant increase in lipase production that is associated with inflammation and a significant increase in the release of eDNA associated with biofilm development. The increased pathogenic response could result in increased antibiotic resistance under aerobic growth conditions. This supports the hypothesis of the current study and that of Kumar et al.

(2016) and Fournière et al. (2020) that bacterial synergistic activity is strain-specific and can have an increased pathogenic response.

Plectranthus aliciae, displayed promising results for targeting quorum sensing and biofilm formation associated with antibiotic resistance due to its hexane and partitions and identified compounds. More irritancy and mutagenicity studies are required for the partitions and identified compounds such as luteolin to ensure the safety of potential formulations with these as active ingredients. A formulation with *P. aliciae* ethanolic extract as the active ingredient was effective for the treatment of various forms of non-inflammatory and inflammatory acne. The anti-inflammatory and antibacterial activity of *P. aliciae* could be linked to its traditional use The anti-inflammatory, antibacterial and wound healing activity of *P. aliciae* could be due to the anti-inflammatory activity of the compounds present in the plant. Rosmarinic acid and luteolin were able to inhibit bacterial lipase enzymes involved in inflammation as well as potentially other inflammatory targets. Lastly, other compounds of *P. aliciae* that were not identified in this study could contribute to the observed activity. Future studies will attempt to identify other compounds present in *P. aliciae* with anti-inflammatory, antibacterial and wound healing activity using Ultra-high performance liquid chromatography-tandem mass spectrometry.

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CHAPTER 5

Gold nanoparticles of *Plectranthus aliciae*, rosmarinic acid and tetracycline for wound healing and antibacterial activity against *Cutibacterium acnes* and *Staphylococcus epidermidis*

Chapter 5

This chapter will be submitted to the special issue of Nanomaterials for Targeted Delivery of Therapeutic and Imaging Agents
In Frontiers in Cell and Developmental Biology Molecular and Cellular Pathology.
Impact factor: 6.684
Titled: Gold nanoparticles of *Plectranthus aliciae*, rosmarinic acid and tetracycline for wound healing and antibacterial activity against *Cutibacterium acnes* and *Staphylococcus epidermidis*

Abstract

Many researchers have become interested in green synthesised gold nanoparticles from plant extracts and their bioactive compounds to treat various maladies. Acne vulgaris is an inflammatory disease of the pilosebaceous unit caused by opportunistic bacteria (*Cutibacterium acnes* and *Staphylococcus epidermis*). These bacteria are not only associated with inflammatory acne but also with prosthetic implant-associated infections and wounds. Studies have hypothesised that these bacteria have a mutualistic relationship and act as a multispecies system to form biofilms under various conditions that contribute to increased antibiotic resistance. This study aimed to investigate the antibacterial and wound healing potential of green synthesised gold nanoparticles (AuNPs) from an endemic South African plant, *Plectranthus aliciae* ($AuNP_{PAE}$), its major compound rosmarinic acid ($AuNP_{RA}$), and a widely used antibiotic, tetracycline ($AuNP_{TET}$). Synthesised gold nanoparticles were successfully formed and characterised using ultraviolet-visible spectroscopy (UV-Vis), dynamic light scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), zeta potential (ζ-potential), high-resolution transmission electron microscopy (HRTEM), selected area electron diffraction (SAED) and investigated for stability under various biological conditions. The biosynthesised gold nanoparticles had no improved antibacterial or biofilm adhesion inhibition activity at their highest concentrations tested of 25% v/v. The biosynthesised AuNPs displayed no cytotoxicity against the human keratinocyte (HaCat) cell line. Gold nanoparticles formed with rosmarinic acid significantly increased wound closure compared to the rosmarinic acid compound tested alone. This study concluded that green synthesised gold nanoparticles of rosmarinic acid can be used for treating of wounds.

1. Introduction

Acne vulgaris is a disease of the pilosebaceous unit with a severity that can range from mild, which includes open and closed comedones, blackheads, whiteheads; moderate, which includes pustules and papules, or severe, characterised by the presence of nodules and cysts. Moderate to severe forms of acne can result in scarring and post-inflammatory hyperpigmentation. Patients suffering from acne vulgaris have confirmed that the disease affects their quality of life both socially and psychologically. Acne vulgaris develops when there is an abnormal shedding of the skin that blocks the pilosebaceous unit. Overproduction of sebum in the pilosebaceous unit results in skin distention and acts as a carbon and nutrient source for acne-causing bacteria. Acne-causing bacteria proliferate in the sebum releasing various pathogenic enzymes that trigger an inflammatory response resulting in scarring and post-inflammatory hyperpigmentation (Alkhawaja et al., 2020)

The skin microbiome consists of various bacterial species protecting the skin and the host from the external environment and other pathogenic bacteria. *Cutibacterium acnes* (anaerobic, aerotolerant) and *Staphylococcus epidermis* (aerobic) are Gram-positive bacteria that form part of the skin's normal microflora. These commensal bacteria are found abundantly in the skin and protect the host from pathogens. However, shifts in the external environment of the skin result in dysbiosis of these opportunistic commensal bacteria. This shift allows these opportunistic bacteria to flourish, causing skin-related maladies such as acne vulgaris. Both *C. acnes* and *S. epidermidis* have been isolated from acne lesions either alone or in combination. Pathogenic strains of *C. acnes* and *S. epidermidis* are not only associated with acne vulgaris but also with prosthetic implant-associated infections and wounds. Since both these bacteria have been isolated from acne lesions, prosthetic implants, and wounds, this suggests that certain *C. acnes* and *S. epidermidis* pathogenic strains could potentially have a mutualistic relationship and cause disease as a multispecies system (Nishijima et al., 2000; Niazi et al., 2010; Moon et al., 2012; Portillo et al., 2013; Flurin et al., 2019; Fournière et al., 2020).

Antibiotics are widely used for the treatment of acne vulgaris, prosthetic implant-associated infections, and wounds. However, antibiotic resistance is a growing concern for health care professionals. The World Health Organisation (WHO) has recognised antibiotic resistance as a threat to global human health and the world economy. Antibiotic resistance of *C. acnes* and *S. epidermidis* is a result of the bacteria forming a protective biofilm. The biofilm protects

these bacterial communities from their external environment, making them less susceptible and more resistant to mechanical forces and treatments such as antibiotics when compared with planktonic bacteria. The thick polymetric extracellular matrix of the biofilm structure prevents antibiotics from inhibiting the bacteria within (Moon et al., 2012; Alkhawaja et al., 2020; World Health Organisation, 2020; Jiang et al., 2021). There is a need for new treatments targeting resistant bacteria that cause acne vulgaris, prosthetic implant-associated infections, and wounds.

Antibiotic resistance observed in various diseases has forced scientists to study alternative treatments, including nanotechnology, as a potential solution to overcome bacterial resistance. Due to their small size, compared to proteins, and large surface-to-volume ratio, nanoparticles can accommodate many functional groups, making them ideal candidates for targeting resistant bacteria. Studies have shown that due to their size, nanoparticles capped with various compounds can pass through the bacterial envelope and inhibit bacteria. The biologically inert nature of gold and the increased biological activity of gold nanoparticles (AuNPs) makes this metal ideal for targeting antibiotic-resistant bacteria. AuNPs capped with various antibiotics and compounds for their antibacterial activity have undergone extensive research. Various factors play a role in the antibacterial activity of AuNPs. Research studies have revealed AuNPs with diameter sizes ranging between 2 - 52 nm to have noteworthy antibacterial activity. The surface charge of the AuNPs also plays a role in antibacterial activity, with positive surface charged AuNPs interacting with the negative electrostatic charges of the bacterial cell surface, inferring antibacterial activity. Furthermore, AuNPs capped with negatively charged functional groups have previously been shown to have no antibacterial activity (Zhao and Jiang, 2013; Li et al., 2014).

Plectranthus aliciae (Codd) Van Jaarsv. & T.J. Edwards previously classified as *Plectranthus madagascariensis* (Pers.) Benth. var. aliciae Codd is an endemic South African plant. Traditionally, the Zulu and Xhosa communities use this species to treat respiratory ailments such as coughs and asthma, ailments such as colds and flu, and skin-related maladies such as wounds and scabies. A study on the acetonic extract of *P. aliciae* confirmed that the species have antibacterial activity. *Plectranthus madagascariensis* displayed antibacterial activity against *S. epidermidis*, known to be associated with inflammatory acne and wounds. Several compounds have been isolated from *Plectranthus madagascariensis* and its varieties, such as *P. aliciae,* with antibacterial and potentially wound healing properties. Rosmarinic acid has

been isolated from *P. madagascariensis* and *P. aliciae*. This compound is abundant in the Lamiaceae family and the *Plectranthus* genus. It has shown analgesic and anti-inflammatory activity and could potentially contribute to wound healing (Lambrechts and Lall, 2021).

There are numerous acne products available to treat various severities of acne vulgaris. However, these treatments have severe side effects and long treatment times that could be harmful and discouraging. Treatments for mild to moderate acne include retinoids, benzoyl peroxide, salicylic acid, and isotretinoin. However, these treatments have several side effects that include contact dermatitis that can further harm the skin, increased photosensitivity, cause mental health issues, hearing loss, congenital disabilities, and vestibular dysfunction that can cause nausea. More severe forms of inflammatory acne are treated with topical and oral antibiotics. Tetracycline is an antibiotic frequently used topically and orally to treat acne vulgaris and other infections such as wounds and implant-associated infections. Among other side effects such as tooth discolouration, contact dermatitis, and affecting the gut microflora, antibiotic resistance towards antibiotics such as tetracycline has become a concern to researchers and healthcare professionals worldwide. The World Health Organisation has recognised antibiotic resistance as a global threat to public health and the economy. Antibiotic resistance can increase already lengthy treatment times that can be expensive and result in death in severe cases (Portillo et al., 2013; Foti et al., 2015; Oudenhoven et al., 2015; World Health Organisation, 2020). Nanoparticles and AuNPs as a potential delivery mechanism for *P. aliciae,* rosmarinic acid, and tetracycline could potentially provide a safe and effective alternative to current treatments.

2. Materials and methods

2.1 Synthesis of gold nanoparticles (AuNPs)

2.1.1 Synthesis of *Plectranthus aliciae* ethanolic extract AuNPs ($AuNP_{PAE}$)

In a 250 mL beaker, 100 mL of sterile distilled water (dH_2O) and 200 mg of *P. aliciae* ethanolic extract (PA_{EtOH}) were heated to 90 °C for approximately 5 min. The solution was then transferred to 50 mL falcon tubes and centrifuged for 5 min at 980 rpm to remove any sedimentary particles. The supernatant was removed, and to 50 mL of the solution, 165 mg of gum arabic (GA) was added. The solution was then heated to 60 °C with constant stirring and 8.25 mL of a 2.2 mM Gold (III) chloride trihydrate salt ($HAuCl_4.3H_2O$) solution was added

allowed to react for 30 min until nanoparticles were formed (solution becomes red wine colour). The solution was filtered through Whatman No. 1 filter paper, and the *P. aliciae* synthesised gold nanoparticle ($AuNP_{PAE}$) solution was stored at 2 °C until further use. The nano concentration was determined by drying 1 mL of $AuNP_{PAE}$ at 40 °C until dry and recording the weights (n = 3).

2.1.2 Synthesis of rosmarinic acid (RA) AuNPs ($AuNP_{RA}$)

Synthesised rosmarinic acid gold nanoparticles ($AuNP_{RA}$) were formed by dissolving 7.2 mg of rosmarinic acid (RA) in 100 mL sterile dH_2O at room temperature (RT). The pH of the solution was adjusted to 9 using a 1 M NaOH. To the solution, 15 mL of a 2.5 mM $HAuCl_4.3H_2O$ solution was added and stirred for 30 min until nanoparticles formed, indicated by a red wine colour change. The solution was filtered through Whatman No. 1 filter paper, and the formed nanoparticle solution was stored at 2 °C until further use. The nano concentration was determined by drying 1 mL of $AuNP_{PAE}$ at 40 °C until dry and recording the weights (n = 3).

2.1.3 Synthesis of tetracycline AuNPs ($AuNP_{TET}$)

Synthesised tetracycline gold nanoparticles ($AuNP_{TET}$) were synthesised by dissolving 60 mg of tetracycline in 60 mL of sterile dH_2O. The pH of the solution was adjusted to 11 with 1 M NaOH. With constant stirring, 40 mL of a 1 mM $HAuCl_4.3H_2O$ solution was added, and the solution was left to stir at RT for 24 h. After nanoparticle formation was observed, the solution was filtered through Whatman No. 1 filter paper, and the filtrate was stored at 2 °C until further use. The nano concentration was determined by drying 1 mL of $AuNP_{PAE}$ at 40 °C until dry and recording the weights (n = 3).

2.2 Characterisation of synthesised AuNPs

The formed AuNPs were analysed using various analytical instruments. The formation and stability of the AuNPs were analysed using an ultraviolet-visible (UV-Vis) BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, RSA). Fourier-transform infrared spectroscopy (FTIR; Perkin Elmer Spectrum 100) was used to determine the functional groups of compounds and extract bound to the AuNPs. The formation of stable AuNPs was determined using a Zetasizer Nano ZS (Malvern, United Kingdom) to measure the zeta (ζ) potential. The hydrodynamic diameter of the synthesised AuNPs was measured

using dynamic light scattering spectroscopy (DLS). The morphological features and d-spacing of the synthesised AuNPs were determined using high-resolution transmission electron microscopy (HRTEM) and selected area electron diffraction (SAED).

2.3 Stability studies of synthesised AuNPs

The stability of the formed AuNPs was tested in various solutions, including culture media and buffers used in the biological assays and other physiological environments, which the nanoparticles would encounter when used as a treatment. The stability studies were conducted in brain heart infusion broth (BHI; Oxoid Thermo Fisher;), Dulbecco's Modified Eagle Medium (DMEM; supplemented with 1% gentamicin and 10% fetal bovine serum), 0.5 % cysteine, 0.5 % bovine serum albumin (BSA), 5% sodium chloride (NaCl) and sterile dH_2O. The nanoparticles were tested in a 1:2 ratio of AuNP to the tested solution. The ultra-violet absorbance of the nanoparticles was measured at 0 h, 2 h, 24 h (1 day), 48 h (2 days), 72 h (3 days), and 168 h (7 days) at a wavelength range from 450 – 800 nm using a Perkin Elmer VICTOR Nivo microplate reader. The samples were stored at 37 °C between analyses to mimic physiological conditions.

2.4 Antibacterial activity

The antibacterial activity of the synthesised AuNPs was tested against *C. acnes* American Type Culture Collection 6919 (ATTCC 6919), *S. epidermidis* (ATCC 35984), and a combination of *C. acnes* and *S. epidermidis* (CA-SE). The microdilution methods described by Sathishkumar et al. (2016), Lall et al. (2019), and Da Silva et al. (2020), were followed with slight modifications. Briefly, 100 µL of the synthesised AuNPs were serially diluted in brain heart infusion (BHI) broth. BHI broth was inoculated with 72 h cultures of either *C. acnes*, *S. epidermidis*, or CA-SE to a final bacterial concentration of $1x10^6$ CFU/mL. The inoculated broth (100 µL) was added to the relevant wells of the samples and controls. The final concentrations of the liquid AuNP samples ranged between 0.78-25 % v/v. Oven-dried, 25% v/v equated to 777.5 ± 4.3 µg/mL for $AuNP_{PAE}$, 29.2 ± 3.8 µg/mL for $AuNP_{RA}$ and 172.5 ± 3.5 µg/mL for $AuNP_{TET}$. Tetracycline was included in the study as a control to measure the study's accuracy at a final concentration range of 0.39-50 µg/mL. The concentration of the sample controls for *P. aliciae* and rosmarinic acid that was not synthesised AuNPs ranged between 3.91-500 µg/mL. A 0.4 mM $HAuCl_4.3H_2O$ control solution was selected since it was the highest concentration used to synthesise the AuNPs.

Furthermore, a bacterial control, sterile dH_2O control, and broth control (without bacteria) were included. The plates were incubated for 72 h at 37 ºC under anaerobic conditions for *C. acnes*, aerobic conditions for *S. epidermidis*, and both anaerobic and aerobic conditions for the combination (CA-SE). After 72 h, 20 µL of PrestoBlue® reagent (Thermo Fisher) was added to all the wells and incubated for 60 min at 37 ºC. The viability of the bacteria was determined visually through a colour change from blue to pink in the presence of metabolically active bacteria, and the minimum inhibitory concentration (MIC) was recorded.

2.5 Inhibition of bacterial adhesion

The inhibitory effect of the synthesised AuNPs on bacterial adhesion was conducted as described by Coenye et al. (2007) with slight modifications. The microdilution method was followed as described in section 2.4. After 72 h of incubation, the supernatant was carefully removed, and the biofilms were washed once with sterile/ autoclaved dH_2O. The plates were oven-dried and the biofilm fixed with cold 99% methanol for 15 min. After removing the methanol, the plates were oven-dried again, and after the plates were dry, a 0.5% crystal violet (CV) solution was added to all the wells and incubated at room temperature for 20 min. The plates were then washed with dH_2O until the water was clear. The washed plates were dried, and the bound CV was dissolved using a 33% acetic acid solution for 20 min while shaking at 150 rpm. The destained biofilm solutions were transferred (100 µL) to clean 96-well plates. The optical density was measured at 590 nm using a Perkin Elmer VICTOR Nivo microplate reader. The 50% bacterial adhesion inhibition concentration was determined using GraphPad Prism 4.0 software.

2.6 Cytotoxicity

The human keratinocyte cell line (HaCat) was donated by Dr. Lester Davids from the Department of Human Biology, University of Cape Town" (Lall et al., 2019). The HaCat cells was gifted to Dr Davids by from Professor Fusenig, Germany from cells collected from the skin biopsy of a 62 year old male patient (Boukamp et al., 1988; Zwane et al., 2012). The cells were grown in T75 culture flasks in DMEM supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL=L fungizone, and 10% heat-inactivated fetal bovine serum (FBS) at 37 ºC, 5% CO_2 until a confluent monolayer formed. Trypsin-EDTA was used to detach the cells for no longer than 2 min and deactivated with fresh DMEM. The detached cells were seeded (1.0×10^5 cells/mL; 100 µL) in the centre wells of a sterile 96-well plate

and incubated for 24 h at 37 ºC, 5% CO_2. Stock solutions of the samples were prepared in 24-well plates in DMEM, and 100 µL of the sample dilutions were transferred to the 96-well plates containing the cells. The final concentrations of the liquid AuNP samples ranged between 0.78-25% v/v. Actinomycin D was included in the study as a control positive control with a final concentration range between 0.05-3.91 × 10^{-4} µg/mL. A 0.4 mM $HAuCl_4.3H_2O$ solution was included in the study as a maximum gold effect to equate for potential unreactive gold in the AuNPs. A 25% v/v sterile dH_2O to DMEM with cells control and media control without cells were also included in the experiment. The plates were incubated for 72 h at 37 ºC, 5% CO_2. After incubation, 20 µL of PrestoBlue® reagent was added, and the plates were incubated for 2 h. The fluorescence was measured at excitation: 560 nm and emission: 590 nm wavelengths using a Perkin Elmer VICTOR Nivo microplate reader.

2.7 Wound healing potential

The scratch assay was conducted as described by Suarez-Arnedo et al. (2020) with slight modifications. To the wells of a 48-well plate, 500 µL of HaCat cells were seeded to a concentration of 7.5 x 10^4 cells per well in DMEM supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL fungizone, and 10% FBS. The plates were incubated for 24 h at 37 ºC, 5% CO_2, until a confluent monolayer formed. A cross was made from top to bottom and left to right using a sterile 1 000 µL tip in each well. The media was carefully removed, and the wells were washed once with sterile Phosphate Buffered Saline (PBS), and 1125 µL of fresh DMEM was added. Pictures of the untreated scratched wells containing the cells were taken under a ZEISS Primovert microscope at a four times magnification (phase 0) that served as the pre-treatment control (0 h). The samples were added to the wells of the 48-well plate to a final volume of 1500 µL and a final sample concentration of 25% v/v. A 0.4 mM $HAuCl_4.3H_2O$ solution was included in the study as a maximum gold effect to equate for potential unreactive gold in the AuNPs. A 25% v/v sterile dH_2O control with cells and media control without cells were included in the experiment. The plates were then incubated at 37 ºC, 5% CO_2 for 18 h as determined from preliminary studies as the ideal time before complete closure of the cell control and negative control scratches. Complete scratch closure was observed at the tested cell concentration of 7.5 x 10^4 cells per well after 24 h during the preliminary studies. Only partial wound closure was observed after 18 h which allowed for observing and analysing the scratch closure compared to the controls. . After incubation, a picture was taken of each well (Nikon camera D90). The percentage wound closure was

calculated using ImageJ and GraphPad Prism 4 software by comparing the sample scratch closure to the cell and vehicle control scratch closure. . The viability of the cells for each sample was determined by first removing 1300 µL of the sample from the wells and adding 20 µL of PrestoBlue® reagent to each of the wells. The fluorescence was measured using a Perkin Elmer VICTOR Nivo microplate reader (excitation: 560 nm and emission: 590 nm wavelengths).

2.8 Statistical analysis

All *in vitro* biological experiments were performed in triplicate (n = 3) and in three independent experiments to ensure reproducible results. The 50% inhibitory concentration (IC_{50}) and statistical analyses were conducted using GraphPad Prism 4 software. The statistical significance of the results was determined with One-way Analysis of Variance (ANOVA) and Tukey's multiple comparison test.

3. Results and discussion

3.1 Ultraviolet-visible spectroscopy and stability of AuNPs

The stability of AuNPs in biological systems is vital to ensure optimal activity under various physiological conditions. The stability of the AuNPs was evaluated *in vitro* in various buffers and media to mimic physiological and biological environmental conditions. This information provides valuable information on how the synthesised nanoparticles react under various conditions (Figure 1).

UV-Vis spectroscopy is widely used to characterise AuNPs based on their surface plasmon resonance (SPR). According to Bindhu and Umadevi (2014), SPR is defined as the absorption band measured through UV-Vis spectroscopy due to free conduction electrons associated with AuNPs, that oscillate when exposed to light.

This method is used to determine the stability of the AuNPs in various biological solutions, and depending on the UV spectra, give information on the diameter and size distribution (Abdelhalim et al., 2012). Figure 1 shows UV–vis absorption spectra of $AuNP_{PAE}$, $AuNP_{RA,}$ and $AuNP_{TET}$ in various biological solutions. The absorbance intensity of the dH_2O water UV–vis spectra for $AuNP_{PAE}$ was around 539 nm and for $AuNP_{RA}$ and $AuNP_{TET}$ around 523 nm. Absorption peaks at low wavelengths indicate small spherical AuNP (Abdelhalim et al.,

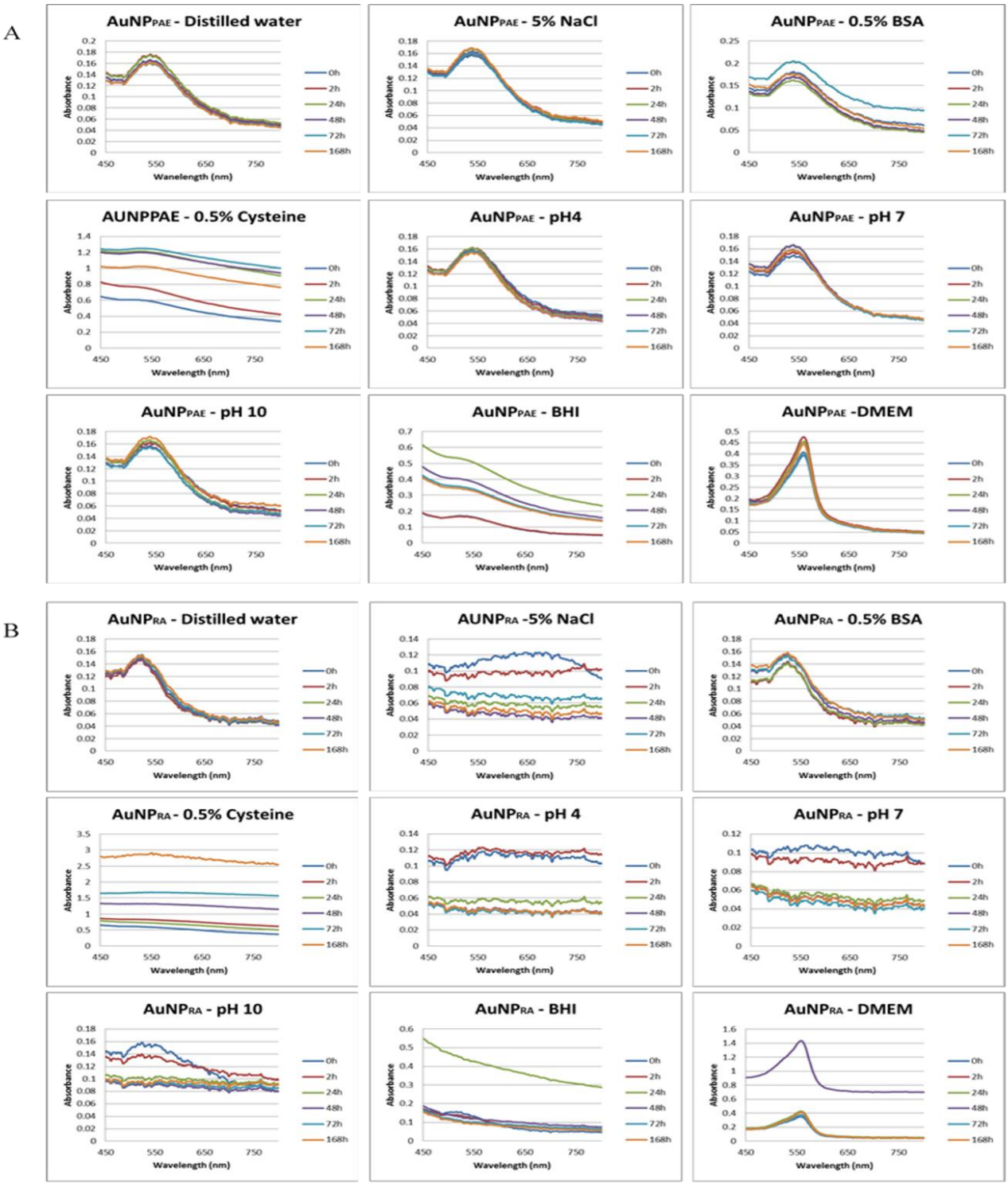
2012). The maximum absorption of $AuNP_{RA}$ could explain the small particle size observed using dynamic light scattering (DLS) analysis (section 3.5). Although $AuNP_{TET}$ had a low absorption maximum at 523 nm, the particle sizes of these nanoparticles were variable, as indicated by the large standard deviation observed with DLS analysis (section 3.5).

$AuNP_{PAE}$ was considered to be stable throughout the various biological solutions. Low stability was observed for $AuNP_{PAE}$ in a 0.5% cysteine solution and BHI broth. BHI broth has an overall pH of 7.4 ± 0.2 at room temperature; however, $AuNP_{PAE}$ was stable at pH of 7 in NaCl, an ingredient in BHI broth. The reduction in stability could be due to various factors that could neutralise the surface charge of the gold nanoparticles, causing aggregation. Studies have shown that glucose, an ingredient of BHI broth, could contribute to AuNP aggregation. AuNPs oxidise glucose to hydrogen peroxide and gluconic acid, causing a change in pH (Tangsong et al., 2011; Zeng et al., 2012). The change in pH could affect the stability of the AuNPs as observed in the UV-Vis spectral graphs for $AuNP_{RA}$ and $AuNP_{TET}$ (Figure 1.B, C). Another potential contributing factor to the low stability of the gold nanoparticles in BHI broth could be due to the presence of amino acids. Calf brain and pig heart are the main ingredients of BHI broth resulting in a media-rich in amino acids. A study performed on the aggregation of AuNPs in solutions containing certain amino acids showed that they contribute to the aggregation of AuNPs at a pH of 7. This could further explain the instability of the various AuNPs in the 0.5% cysteine solution (Zakaria et al., 2013).

$AuNP_{RA}$ was stable in dH_2O, 0.5% BSA, and DMEM, although an absorbance maxima shift was observed after 48 h. However, $AuNP_{RA}$ was unstable in various pH solutions and displayed minimal stability at a pH of 10 for up to 24 h before a shift in the absorbance maxima was observed. $AuNP_{TET}$ was stable for up to 72 h where after the stability of the AuNPs slightly decreased.

All AuNP samples were highly stable in DMEM used to maintain cell cultures for the cytotoxicity and wound healing assays. The stability of the AuNPs in DMEM could be due to the presence of 10% FBS in the media. A study on silver nanoparticles confirmed that DMEM, which has not been supplemented with 10% FBS, resulted in more unstable nanoparticles when compared to complete DMEM media. Studies have confirmed that certain proteins prevent aggregation of the AuNPs by forming a protein corona, contributing to abundant stable AuNPs. Furthermore, since FBS contains approximately 2.5 mg/mL of BSA

this could further explain the stability of the tested AuNPs in 0.5% BSA and DMEM (Liu et al., 2011; Perde-Schrepler et al., 2016; Yi et al., 2016; Soutar et al., 2019;).



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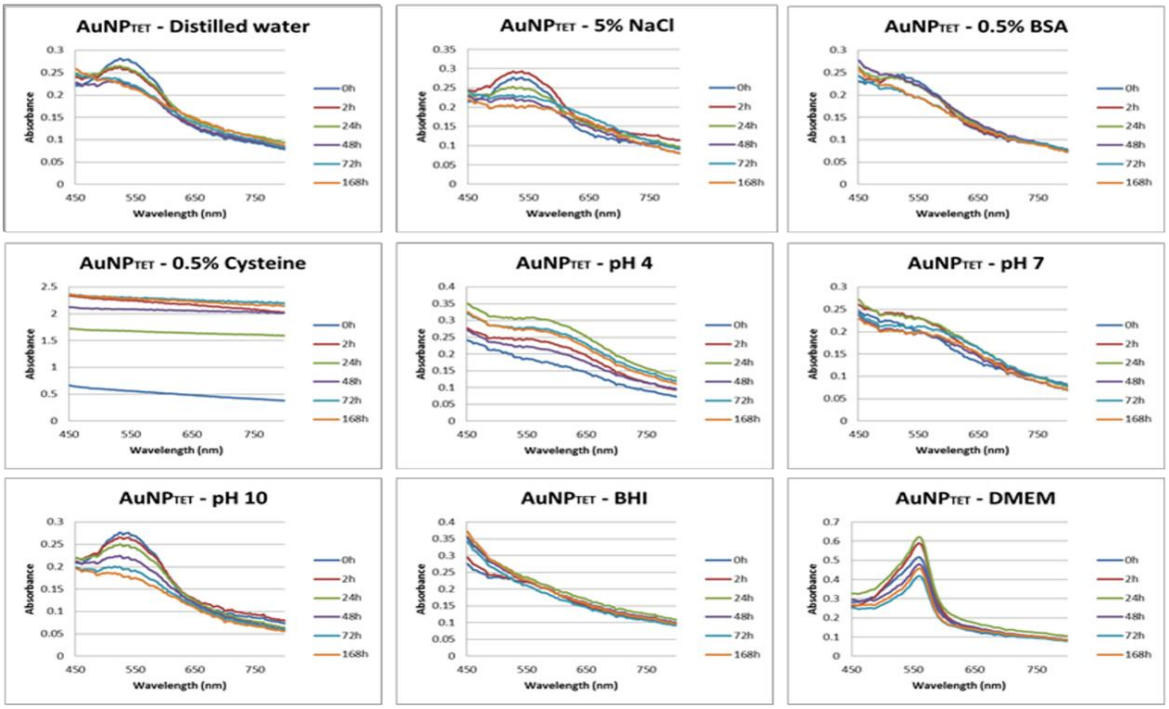

Figure 5. 1: Ultraviolet-visible spectroscopy (UV-Vis) and stability studies of biosynthesized gold nanoparticles (AuNPs) at various time intervals under various physiological conditions. (A) Biosynthesized *Plectranthus aliciae* AuNPs ($AuNP_{PAE}$); (B) biosynthesized rosmarinic acid AuNPs ($AuNP_{RA}$); (C) biosynthesized tetracycline AuNPs ($AuNP_{TET}$)in NaCl: sodium chloride; BSA: bovine serum albumin; BHI: brain heart infusion broth; DMEM: Dulbecco's Modified Eagle Medium.

3.2 Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR is used to detect functional groups corresponding to the plant extract present in synthesised AuNPs. The functional groups capping the AuNPs are observed as peaks in the spectra (Krithiga et al., 2015). The spectra of the synthesised AuNPs were compared to their respective controls that were not formulated into nanoparticles by reducing the gold salt. The peaks observed between 2360-2342 cm^{-1} in all figures are due to CO_2 in the atmosphere (Figure 2.A-C, Table 1).

Figure 2.A and Table 1 show the FTIR spectra of PA_{EtOH} and green synthesised AuNPs from *P. aliciae.* Strong absorption bands were observed at 3361 cm^{-1} and 3272 cm^{-1} that could be attributed to N-H stretching, 2075 cm^{-1} assigned to N=C=S stretching, and 1635 cm^{-1} and 1636 cm^{-1} attributed to C=C or N-H bending for PA_{EtOH} and $AuNP_{PAE}$, respectively. Rosmarinic acid was not a major capping agent during the formation of $AuNP_{PAE}$ due to the nature of the functional groups of PA_{EtOH} that capped the AuNPs (Chemistry LibreTexts, 2020; Merck, 2021).

Rosmarinic acid is a major compound of *Plectranthus* species, including *P. aliciae*. Major absorption bands for RA and $AuNP_{RA}$ were observed at 3302 $cm^{-1,}$ which could be assigned to O-H stretching, 1647 cm^{-1} and 1636 cm^{-1} assigned to C=C stretching for RA and $AuNP_{RA,}$ respectively. Minor peaks were observed at 1348 cm^{-1} and 1356 cm^{-1} assigned to O-H bending, 1259 cm^{-1} and 1253 cm^{-1} assigned to C-O stretching, 1152 cm^{-1} and 1150 cm^{-1} assigned to C-O stretching, and 669 cm^{-1} and 668 cm^{-1} assigned to C=C bending for RA and $AuNP_{RA,}$ respectively (Figure 2.B, Table 1). These infrared bands could be attributed to the functional groups observed in the chemical structure of rosmarinic acid (Figure 3) (Chemistry LibreTexts, 2020; Merck, 2021).

Tetracycline is an antibiotic used for the treatment of various bacterial infections, including acne vulgaris. Major peaks were observed for tetracycline (TET), and its formed AuNP ($AuNP_{TET}$) with the FTIR spectra at 3301 cm^{-1} and 3304 cm^{-1} that could be assigned to N-H stretching, 1641 cm^{-1} and 1635 cm^{-1} assigned to C=C or N-H bending. Minor peaks were observed at 1393 cm^{-1} and 1409 cm^{-1} assigned to O-H bending, 1252 cm^{-1} and 1255 cm^{-1} C-O stretching, 1161 cm^{-1} and 1176 cm^{-1} assigned to C-N stretching, 1060 cm^{-1} and 1065 cm^{-1} assigned to C-O stretching, and 669 cm^{-1} assigned to C=C bending for TET and $AuNP_{TET}$ respectively (Figure 2.C, Table 1). These infrared bands could be attributed to the functional groups observed in the chemical structure of tetracycline (Figure 4) (Chemistry LibreTexts, 2020; Merck, 2021).

Table 5. 1: Functional groups identified with Fourier Transform Infrared Spectroscopy.

Sample	Control		AuNP		Group (stretching)	Compound class	Reference
	WL (cm^{-1})	% T	WL (cm^{-1})	% T			
Plectranthus aliciae	3728	98.30	3727	98.15	O-H stretching	Free alcohol	(Chemistry LibreTexts, 2020; Merck, 2021)
	3361	80.49	3272	48.85	N-H stretching	Aliphatic primary amine	(Chemistry LibreTexts, 2020)
	2360	80.73	2360	87.08	O=C=O	Carbon dioxide	(Nodari and Ricciardi, 2019;

					stretching		Merck, 2021)
	2342	85.04	2342	89.61	O=C=O stretching	Carbon dioxide	(Nodari and Ricciardi, 2019; Merck, 2021)
	2075	98.49	2075	95.69	N=C=S stretching	isothiocyanate	(Chemistry LibreTexts, 2020; Merck, 2021)
	1635	83.37	1636	70.84	C=C OR N-H bending	Alkene OR amine	(Chemistry LibreTexts, 2020; Merck, 2021)
Rosmarinic acid	3772	95.82	3772	98.64	O-H stretching	Free alcohol	(Chemistry LibreTexts, 2020; Merck, 2021)
	3302	77.01	3302	48.77	O-H stretching	Carboxylic acid	(Chemistry LibreTexts, 2020; Merck, 2021)
	2360	70.61	2360	76.02	O=C=O stretching	Carbon dioxide	(Nodari and Ricciardi, 2019; Merck, 2021)
	2342	76.24	2342	80.97	O=C=O stretching	Carbon dioxide	(Nodari and Ricciardi, 2019; Merck, 2021)
	1647	71.90	1636	70.85	C=C stretching	alkene	(Merck, 2021)
	1348	46.25	1356	91.10	O-H bending	phenol	(Merck, 2021)
	1259	34.69	1253	90.95	C-O	aromatic ester	(Merck, 2021)

	1152	23.96	1150	90.92	C-O stretching	aliphatic ether	(Chemistry LibreTexts, 2020; Merck, 2021)
	669	52.34	668	39.41	C=C bending	alkene	(Merck, 2021)
Tetracycline	3728	91.06	3728	95.89	O-H stretching	free alcohol	(Chemistry LibreTexts, 2020)
	3705	91.90	3696	95.64	O-H stretching	Free alcohol	(Chemistry LibreTexts, 2020)
	3626	90.43	3623	85.22	O-H stretching	Free alcohol	(Chemistry LibreTexts, 2020)
	3301	82.30	3304	48.62	N-H stretching	aliphatic primary amine	(Chemistry LibreTexts, 2020; Merck, 2021)
	2361	57.06	2361	64.48	O=C=O stretching	Carbon dioxide	(Nodari and Ricciardi, 2019; Merck, 2021)
	2342	64.48	2342	71.77	O=C=O stretching	Carbon dioxide	(Nodari and Ricciardi, 2019; Merck, 2021)
	1641	75.89	1635	70.77	C=C or N-H bending	cyclic alkene or amine	(Merck, 2021)
	1409	90.79	1393	65.60	O-H bending	alcohol	(Chemistry LibreTexts, 2020; Merck, 2021)
	1252	90.72	1255	65.35	C-O stretching	aromatic ester or alkyl aryl ether	(Chemistry LibreTexts, 2020; Merck, 2021)

	1161	90.63	1176	65.45	C-N stretching	amine	(Chemistry LibreTexts, 2020; Merck, 2021)
	1060	90.32	1065	71.41	C-O stretching	primary alcohol	(Chemistry LibreTexts, 2020; Merck, 2021)
	669	53.99	669	37.81	C=C bending	alkene	(Merck, 2021)

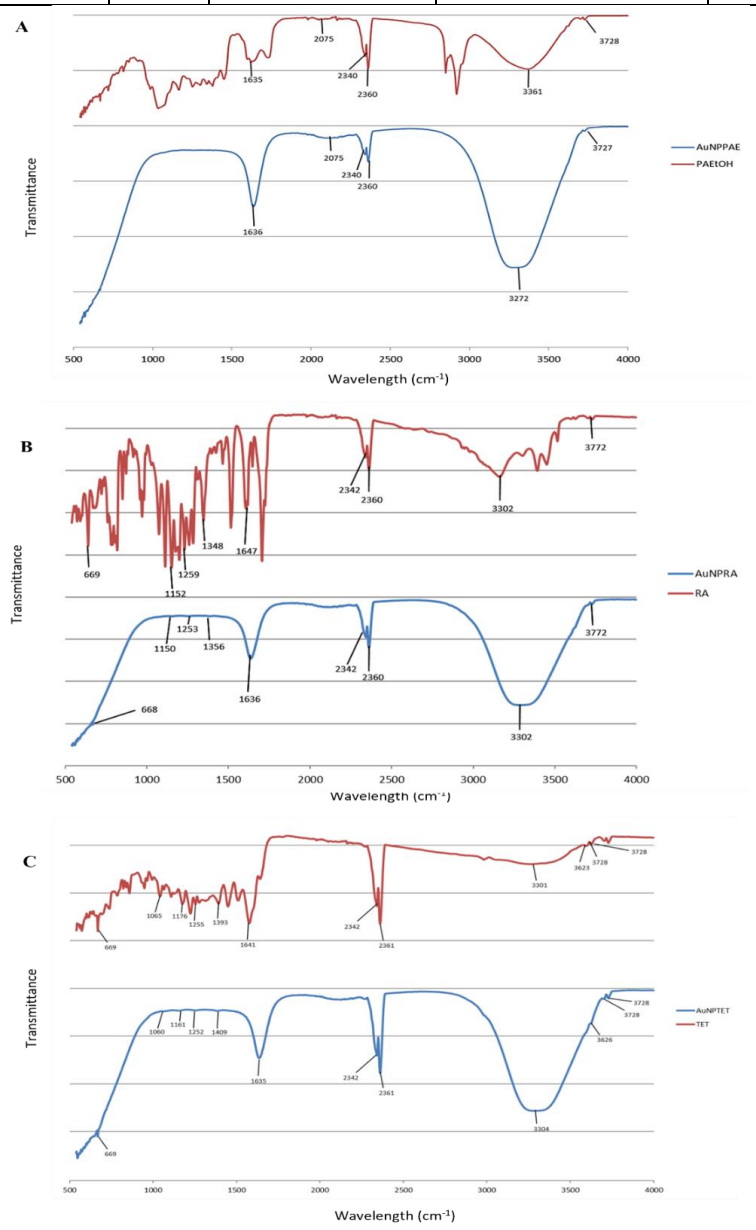

Figure 5. 2: Fourier Transform Infrared spectra for (A) *Plectranthus aliciae* ethanolic extract (PA_{EtOH}) and *Plectranthus aliciae* ethanolic extract gold nanoparticles ($AuNP_{PAE}$); (B) Rosmarinic acid (RA) and

rosmarinic acid gold nanoparticles ($AuNP_{RA}$); (C) Tetracycline (TET) and tetracycline gold nanoparticles ($AuNP_{TET}$).

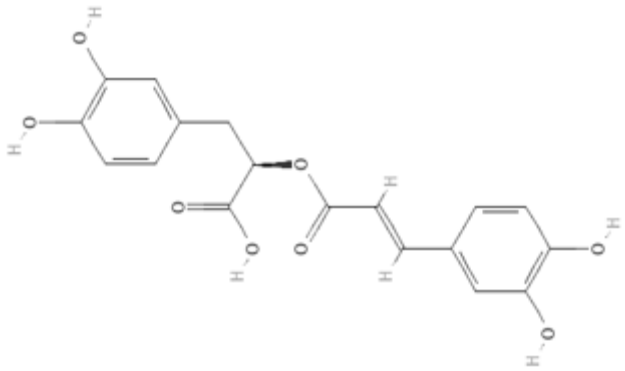

Figure 5. 3: The chemical structure of rosmarinic acid (National Center for Biotechnology Information, 2021).

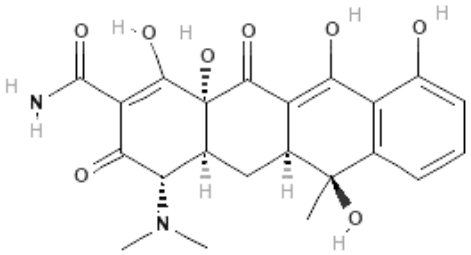

Figure 5. 4: The chemical structure of tetracycline (National Center for Biotechnology Information., 2021).

3.3 High-resolution transmission electron microscopy

HRTEM was conducted to obtain the SAED patterns, the lattice fringes, and the overall shape of the AuNPs. Due to limited sample availability, the estimated d-spacing values were calculated from the SAED pattern for all the samples using the d-spacing equation (Equation 1) and the lattice fringes observed from the HRTEM images using ImageJ software (Jeyadheepan, 2020; Le et al., 2021; NanoWorld, 2021).

$$d = \frac{1}{r}$$

Equation 1: D-spacing equation from the selected area electron diffraction (SAED) patterns, where r is the radius of the SAED ring (Jeyadheepan, 2020; NanoWorld, 2021).

Figure 5.A. shows the HRTEM images and SAED pattern of $AuNP_{PAE}$. Mainly spherically shaped nanoparticles were observed with some triangular shapes. Spherically shaped NPs

indicate good capping and stabilisation of the AuNP by the plant extracts components (Deokar and Ingale, 2016). The calculated d-spacing values from the SAED pattern for $AuNP_{PAE}$ were 2.40 Å and 1.25 Å. Calculating the lattice fringe spacing from the HRTEM image resulted in a 0.218 nm (2.18 Å) lattice fringe distance (Jeyadheepan, 2020). Bright circular rings of the SAED pattern and clear lattice fringes from the HRTEM images confirmed the crystalline nature of the $AuNP_{PAE}$.

Figure 5.B shows the HRTEM images and SAED pattern of $AuNP_{RA}$. The AuNPs were mainly spherically shaped, with some cluster formation observed. Clustering of the $AuNP_{RA}$ could be due to the functional groups capping the AuNPs. This clustering can be due to the acidic functional groups, such as carboxylic groups of rosmarinic acid (Le et al., 2021). The calculated d-spacing values from the SAED pattern for $AuNP_{RA}$ were 2.36 Å, 1.49 Å, and 1.27 Å. Calculating the lattice fringe spacing from the HRTEM image resulted in a 0.261 nm (2.61 Å) lattice fringe distance (Jeyadheepan, 2020). Bright circular rings of the SAED pattern and clear lattice fringes from the HRTEM images confirmed the crystalline nature of the $AuNP_{RA}$.

Figure 5.C shows the HRTEM images and SAED pattern of $AuNP_{TET}$. The AuNPs were mainly spherically shaped, with some cluster formation observed. Clustering of the $AuNP_{TET}$ could be due to the functional groups capping the AuNPs. This clustering can be due to tetracycline's basic amine functional groups (Le et al., 2021). The calculated d-spacing values from the SAED pattern for $AuNP_{TET}$ were 2.42 Å, 1.50 Å, and 1.28 Å. Calculating the lattice fringe spacing from the HRTEM image resulted in a 0.213 nm (2.13 Å) lattice fringe distance (Jeyadheepan, 2020). Bright circular rings of the SAED pattern and clear lattice fringes from the HRTEM images confirmed the crystalline nature of the $AuNP_{TET}$.

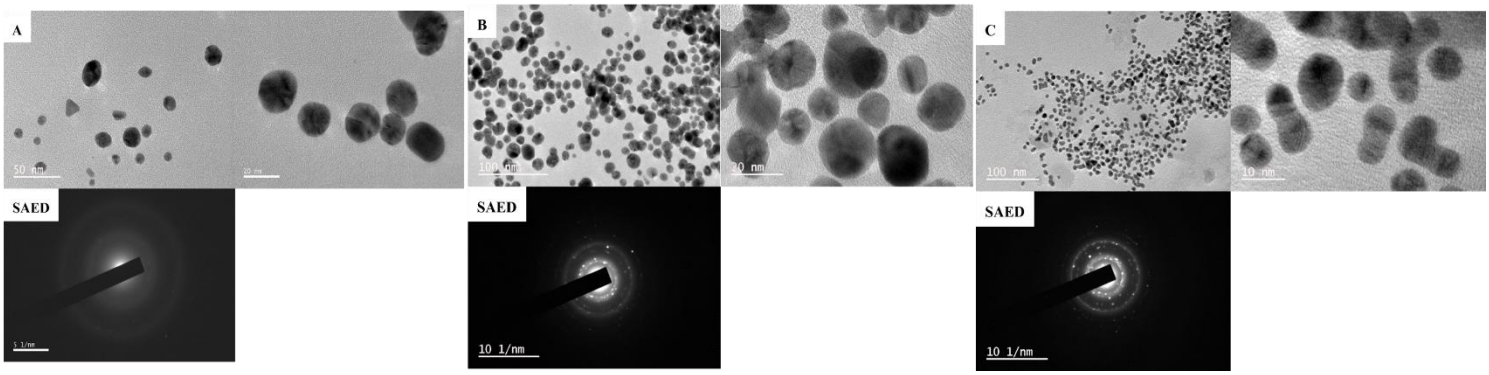

Figure 5. 5: High-resolution transmission electron microscopy (HRTEM) images depicting clustering, lattice fringes and selected area electron diffraction (SAED) patterns of biosynthesized gold nanoparticles

(AuNP). (A) biosynthesized *Plectranthus aliciae* AuNPs; (B) biosynthesized rosmarinic acid AuNPs; (C) biosynthesized tetracycline AuNPs.

3.4 Zeta (ζ) potential

Zeta potential is a measure of the surface charge of nanoparticles and indicates long-term stability of the nanoparticles in suspension. All the AuNPs in the present study had a negative zeta potential ranging from -18 to -40 mV. Salopek et al. (1992) discussed in detail the various ζ-potential values and how they relate to the stability of gold nanoparticles. The higher the negative value of the AuNPs, the higher the stability of the NPs, and less agglomeration and precipitation can be expected. The researchers discussed that a ζ-potential between the range of -16 to -30 mV is indicative of the beginning of dispersion, and a ζ-potential between -30 to -40 mV indicated medium stability. The AuNPs, $AuNP_{PAE}$, $AuNP_{RA}$ and $AuNP_{TET}$ had ζ-potential values of -18.07 ± 0.95 mV, -21.5 ± 2.66 mV and -39.83 ± 1.6 mV respectively. These results indicated that the AuNPs formed were stable. The colloidal stability of $AuNP_{RA}$ was in agreement with previously formed $AuNP_{RA}$ with a ζ-potential of -24.09 ± 3.97 mV. The researchers noted that the large negative value of $AuNP_{RA}$ is indicative of stable AuNPs (Lim and Park, 2018). Based on these results, the order of stability from highest to lowest stability of the AuNPs formed were $AuNP_{TET}$ > $AuNP_{RA}$ > $AuNP_{PAE}$. The higher stability of $AuNP_{TET}$ and $AuNP_{RA}$ could be due to the purity of the samples, where $AuNP_{PAE}$ were formed using the *P. aliciae* extract that contains a mixture of various compounds affecting the stability. Bogireddy et al. (2015) discussed the oxidation of polyhydroxy (-OH) groups that contribute to nanoparticle stabilisation through the presence of negatively charged carboxylic (–C=O) groups, which cap the surface of the Au through electrostatic interactions (Ahmed et al., 2016). The -OH groups of tetracycline and rosmarinic acid (Figures 3 and 4) could contribute to their higher stability. Furthermore, de Aragão et al. (2019) reported that nanoparticles formed at higher pHs were more likely to have higher ζ-potential values. Both $AuNP_{TET}$ and $AuNP_{RA}$ were formed at high pH values of 11 and 9, respectively that could explain their highly negative ζ-potential values. Although $AuNP_{PAE}$ was less stable than $AuNP_{TET}$ and $AuNP_{RA,}$ it still displayed monodispersed nanoparticles and is considered stable according to the UV-Vis analysis (Padalia et al., 2015; Sibuyi et al., 2021).

3.5 Dynamic light scattering (DLS) analysis

DLS is used to determine the hydrodynamic diameter of nanoparticles. The average diameter of the AuNPs, $AuNP_{PAE}$, $AuNP_{RA,}$ and $AuNP_{TET}$ were determined to be 71.26 ± 0.44 nm, 29.88 ± 3.30 nm, and 132.6 ± 99.5 nm, respectively. The larger diameter of $AuNP_{PAE}$ could potentially be due to the addition of gum arabic used as a stabilising agent.

3.6 Antibacterial activity and prevention of bacterial adhesion

Antibiotic resistance of *C. acnes* towards various antibiotics has become prevalent. From 1979 until 2000, an increase of 44% in antibiotic resistance has been observed for *C. acnes* towards antibiotics such as tetracycline, erythromycin, and clindamycin used to treat acne vulgaris. This could affect acne vulgaris and other diseases where *C. acnes* is a causative pathogen, increasing the chances of treatments being unsuccessful once these bacteria have adhered to a surface (Platsidaki and Dessinioti, 2018a). Biofilms develop through the initial adhesion of the bacteria to a surface, and once these bacteria face environmental challenges, they will disperse and attach to a new surface (Jiang et al., 2021). New treatments are required to overcome the mechanisms that result in antibiotic resistance. This includes preventing the formation and adhesion of bacterial biofilms to a surface and their inhibition. Alternative treatments could help prevent bacterial attachment and indirectly reduce antibiotic resistance, making it easier to treat various maladies and prevent the attachment and spread of antibiotic-resistant bacteria.

The antibacterial activity of the liquid AuNPs was investigated against *C. acnes* (ATCC 6919), *S. epidermidis* (ATCC 35984), and a combination of these bacteria (CA-SE). *Cutibacterium acnes* and *S. epidermidis* have been associated with acne vulgaris, prosthetic implant-associated infections, and wounds, either alone or in combination. These bacteria can form biofilms that contribute to antibiotic resistance. Biofilms produced by these bacteria act as a protective layer that prevents antibiotics from inhibiting their growth, contributing to antibiotic resistance. New therapies are required that can either inhibit the bacteria or prevent bacterial adhesion, preventing formation (Alkhawaja et al., 2020; Fournière et al., 2020; Jiang et al., 2021).

No antibacterial activity or bacterial adhesion inhibition was observed for $AuNP_{PAE}$ and $AuNP_{RA}$ at the highest concentration tested of 25% v/v against *C. acnes*, *S. epidermidis*, and CA-SE. This reduction in antibacterial activity could be due to the decrease in stability in BHI broth for these AuNPs, as seen in the stability studies (Figure 1). In comparison, the

control sample of PAE displayed antibacterial activity at an MIC value of 7.8 µg/mL against *C. acnes* (ATCC 6919) and 500 µg/mL against *S. epidermidis* (ATCC 35984) and anaerobically and aerobically grown CA-SE. However, rosmarinic acid as a control had no improved antibacterial and bacterial adhesion inhibition since the control displayed no activity at the highest concentration tested of 500 µg/mL against all the tested bacteria. The gold control displayed no antibacterial activity or bacterial adhesion inhibition at the highest concentration tested of 25% v/v.

Tetracycline is an antibiotic isolated from *Streptomyces aureofaciens*. It is an antibiotic used to treat various infections, including acne vulgaris (National Center for Biotechnology Information., 2021). A previous study performed by Djafari et al. (2016) investigated the antibacterial activity of $AuNP_{TET}$ against *Staphylococcus aureus* and *Escherichia coli*. The researchers found no antibacterial activity for $AuNP_{TET}$ against tetracycline-resistant bacterial species. Furthermore, the antibacterial activity of $AuNP_{TET}$ displayed a higher MIC value than the tetracycline control against all the bacterial species tested. The current study showed no antibacterial activity at the highest concentration of 25% v/v against *C. acnes* (ATCC 6919) and CA-SE grown aerobically. The antibacterial bacterial activity of $AuNP_{TET}$ was observed at an MIC value of 0.39% v/v (≈ 0.67 µg/mL) against *S. epidermidis* (ATCC 35984) and CA-SE grown anaerobically. The tetracycline control displayed MIC values of 1.56 µg/mL against aerobically grown CA-SE and 0.78 µg/mL against *C. acnes*, *S. epidermidis,* and anaerobically grown CA-SE. Although the antibacterial activity for $AuNP_{TET}$ was not significantly different from the tetracycline controls, noteworthy antibacterial activity was still observed for $AuNP_{TET}$ against *S. epidermidis* and anaerobically grown CA-SE. Compounds with an MIC value lower than 16 µg/mL are considered to have noteworthy antibacterial activity (Van Vuuren and Holl, 2017). $AuNP_{TET}$ prevented the adhesion of *C. acnes* and *S. epidermidis* at IC_{50} values of 0.50 ± 0.049% v/v (≈ 0.86 µg/mL) and 0.83 ± 0.013% v/v (≈ 1.43 µg/mL), respectively. Bacterial adhesion inhibition of $AuNP_{TET}$ was observed for anaerobically grown and aerobically grown CA-SE with IC_{50} values of 1.98 ± 2.25% v/v (≈ 3.42 µg/mL) and 12.47 ± 1.32% v/v (≈ 21.51 µg/mL), respectively. The tetracycline control prevented biofilm adhesion with IC_{50} values of 0.90 ± 0.53 µg/mL, 1.64 ± 0,28 µg/mL, 0.92 ± 0,23 µg/mL and 1.68 ± 0.74 µg/mL for *C. acnes*, *S. epidermidis*, anaerobically grown and aerobically grown CA-SE, respectively. The $HAuCl_4.3H_2O$ control showed no antibacterial activity or inhibitory bacterial adhesion activity.

3.7 Cytotoxicity

The cytotoxicity of the respective AuNPs was tested on the HaCat cell line. The cytotoxicity of the samples was tested to determine whether the samples could potentially be biocompatible with human keratinocytes considering that they would be topically applied. $AuNP_{PAE}$, $AuNP_{RA,}$ and the $HAuCl_4.3H_2O$ control displayed no cytotoxicity at the highest concentration tested of 25% v/v. $AuNP_{TET}$ had minimal cytotoxic activity at the highest concentration tested of 25% v/v (≈ 172.5 µg/mL) and inhibited 29.65 ± 4.74% of the HaCat cells. The tetracycline compound alone displayed an IC_{50} value of 185.4 ± 6.93 µg/mL. A reduction in the cytotoxicity was observed for the *P. aliciae* control with an IC_{50} value of 65.16 ± 7.30 µg/mL. Since no cytotoxicity was observed for the AuNPs, the samples were tested for their wound healing potential *in vitro* using HaCat cells.

3.8 Wound healing potential

The respective AuNPs were tested for their potential to increase wound closure. Since the samples displayed no antibacterial activity at the highest concentration tested, AuNPs were tested at their highest available concentration for *in vitro* testing of 25% v/v. Sterile 25% v/v dH_2O was added to seeded HaCat cells served as the 100% closure control since all the nanoparticles were synthesised in dH_2O. The percentage wound closure of the AuNPs compared to the dH_2O control is depicted in Figure 6. $AuNP_{PAE}$ displayed a significant percentage wound closure of 96.65 ± 1.03% ($P < 0.001$). PA_{EtOH} displayed significant wound closure at 7.8 µg/mL. No significant wound closure was observed for PA_{EtOH} and $AuNP_{PAE}$ (Figure 7). This could indicate that at a lower concentration, the wound closure activity of $AuNP_{PAE}$ could potentially decrease. $AuNP_{RA}$ displayed significant wound healing activity ($P < 0.01$) with a percentage wound closure of 85.88 ± 5.85% at 29.2 µg/mL. The rosmarinic acid control displayed no wound closure at 500 µg/mL. Therefore, the wound healing activity of rosmarinic acid was significantly increased ($P < 0.01$) when the AuNP was capped with the compound (Figure 7). No significant wound closure activity was observed for $AuNP_{TET}$ ($P > 0.05$); this could partially be due to the mild cytotoxic activity of the sample at 25% v/v.

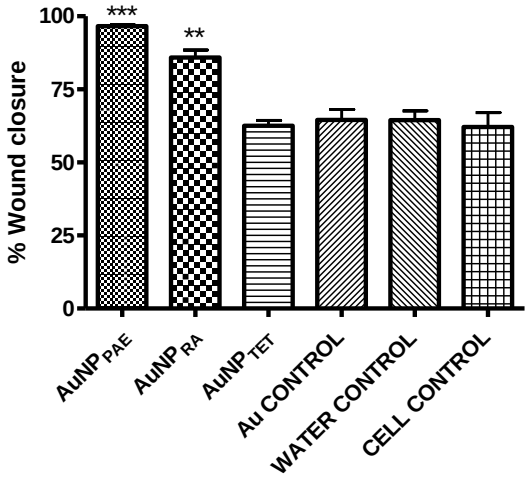

Figure 5. 6: Percentage wound closure at 25% v/v. $AuNP_{PAE}$: Gold nanoparticles formed from *Plectranthus aliciae* ethanolic extract; $AuNP_{RA}$: Gold nanoparticles formed from rosmarinic acid; $AuNP_{TET}$: Gold nanoparticles formed from tetracycline; Au control: unreacted $HAuCl_{4.3}H_2O$ control.

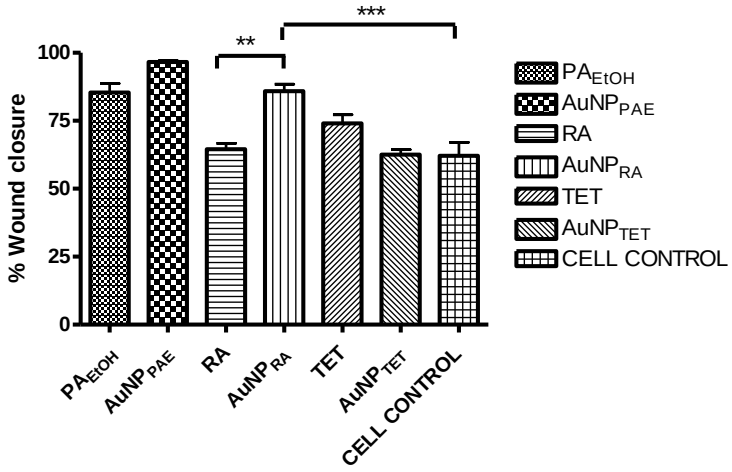

Figure 5. 7: Percentage wound closure comparison between gold nanoparticle samples and their respective controls. $AuNP_{PAE}$: Gold nanoparticles formed from *Plectranthus aliciae* ethanolic extract (PA_{EtOH}); $AuNP_{RA}$: Gold nanoparticles formed from rosmarinic acid (RA); $AuNP_{TET}$: Gold nanoparticles formed from tetracycline (TET).

4. Conclusion

This is the first report on the antibacterial and wound healing potential of biosynthesised AuNP of *P. aliciae*, rosmarinic acid, and tetracycline as potential treatments for acne vulgaris, prosthetic implant-associated infections, and wounds.

Cutibacterium acnes and *S. epidermidis* are opportunistic bacteria that have been associated with maladies such as acne vulgaris, prosthetic implant-associated infections, and wounds. It is hypothesised that these bacteria may exist in a mutually beneficial relationship and could

have a combined bacterial effect that results in the formation of a multispecies biofilm under certain conditions. Biofilm formation of *C. acnes* and *S. epidermidis* contributes to antibiotic resistance observed in acne vulgaris prosthetic implant-associated infections and wounds. Antibiotic resistance results in longer treatment times that could be life-threatening to the patient (Fournière et al., 2020; Jiang et al., 2021).

Green synthesised gold nanoparticles could hold the key to improved activity of plant extracts and their compounds. Gold nanoparticles from *P. aliciae*, rosmarinic acid, and tetracycline were successfully formed. Although the biosynthesised AuNPs had no improved antibacterial or biofilm adherence inhibition, significant wound healing activity was observed for $AuNP_{RA.}$ $AuNP_{RA}$ could potentially be a safe and effective nanocarrier for wound healing treatments due to its low cytotoxicity and significantly increased wound healing activity. Further studies could investigate the permeability of $AuNP_{RA}$ and improve the stability of the AuNP under various physiological conditions. This study reports the antibacterial and wound healing potential of green synthesised gold nanoparticles from *P. aliciae*, rosmarinic acid, and tetracycline.

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CHAPTER 6

Concluding remarks, impact, novelty, and future recommendations

Chapter 6

1. Concluding remarks and answering research questions

Plectranthus aliciae (Codd) Van Jaarsv. & T.J. Edwards is an endemic South African species. Despite its use in traditional medicine to treat various maladies, validation of these claims through scientific research is absent in the literature. This study focused on the elucidation of the effect of *P. aliciae* on quorum sensing mechanisms utilised by *Cutibacterium acnes* and *Staphylococcus epidermidis*, which contributed to the progression of acne vulgaris, prosthetic implant-associated infections and wounds. While several studies have identified the presence of *C. acnes* and *S. epidermidis* in acne lesions, implants and wounds, the type of multispecies relationship between these bacteria is still unknown. This study investigated the relationship of these bacteria in an attempt to answer the research questions as laid out in Chapter 3.

Scanning electron micrographs confirmed that *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) form an intermixed colony, with a cooperative relationship under both anaerobic and aerobic growth conditions. After investigating the effects of the bacteria as a multispecies system, there was a reduction in susceptibility to tetracycline treatment and an increase in antibiotic resistance under aerobic conditions when compared to the single species system. Furthermore, there was a significantly more dense biofilm for the multispecies system under anaerobic and aerobic growth conditions and a significant increase in extracellular DNA production under aerobic growth conditions. Extracellular DNA has an active role in the attachment of bacteria to a surface and aids in the transfer of mutated genes across the biofilm between species. Although no significant increase in AI-2 release was observed in the multispecies system compared to the single species systems, a slight increase in AI-2 production was observed under aerobic growth conditions. AI-2 release is the first step in the quorum sensing system, where bacteria use this signalling molecule to regulate gene expression. Measuring AI-2 levels over the duration of the 72 h incubation time rather than after 72 h, could result in a significant difference of AI-2 production in multispecies systems.

Lastly, the study confirmed that the multispecies system has a higher virulence potential under aerobic conditions, with a significant increase in lipase production observed. Increased

lipase enzyme production has been linked to increased attachment of bacterial biofilms and activation of the host inflammatory response. Researchers have hypothesised that the relationship between *C. acnes* and *S. epidermidis* is strain-specific. This study confirms that strains *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) have a synergistic relationship that could indicate a synergistic quorum sensing relationship between other strains of these bacteria. Further studies on the multispecies quorum sensing relationship of various *C. acnes* and *S. epidermidis* strains could contribute to better understanding the maladies these bacteria are involved with. Current studies investigating the relationship of *C. acnes* and *S. epidermidis* do not investigate the effect this relationship might have on the quorum sensing activity of the respective bacteria. Investigating the quorum sensing effect of these bacteria should be included in future studies for understanding the relationship and potential adverse outcomes from this bacterial relationship. This study is the first report of whether quorum sensing mechanisms exist between between *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) when the bacteria are grown in a multispecies system.

The ethanolic extract of *P. aliciae* (PA_{EtOH}), its hexane and ethyl acetate partitions, and two compounds, luteolin and rosmarinic acid were selected to investigate their activity against quorum sensing mechanisms that contribute to inflammation and antibiotic resistance associated with acne vulgaris, prosthetic implant-associated infections and wounds. This is the first report of the presence of luteolin in *P. aliciae*. Although several compounds were identified in the ethanolic extract of *P. aliciae,* luteolin was the only one that displayed significant antibacterial activity against *C. acnes* (ATCC® 6919) and was selected for further investigation. While rosmarinic acid displayed no antibacterial activity against *C. acnes* (ATCC® 6919), it was selected for further investigation due toits reported anti-inflammatory activity and its abundance in other the *Plectranthus* species and other species in the Lamiaceae family.

$PA_{EtOH,}$ hexane and ethyl acetate partitions and luteolin displayed noteworthy antibacterial activity against *C. acnes* (ATCC® 6919) and moderate antibacterial activity against *S. epidermidis* (ATCC® 35984) as well as the multispecies systems colonies grown under anaerobic and aerobic conditions. $PA_{EtOH,}$ hexane and ethyl acetate partitions and luteolin were more selective towards preventing bacterial attachment and biofilm formation than inhibiting the bacteria, even though noteworthy antibacterial activity was observed for these samples. Although these samples were unable to eradicate the mature biofilms of the bacteria,

they were able to penetrate the mature biofilm of *C. acnes* (ATCC® 6919) and inhibit the bacteria within, rendering them metabolically inactive. Tetracycline and rosmarinic acid did not penetrate the biofilm and inhibit the *C. acnes* (ATCC® 6919) sessile bacteria, which was determined using the artificial sebum model. The model simulates the interaction of the sample and the bacteria within the pilosebaceous unit *in vitro*. PA_{EtOH} was found to be significantly more active towards targeting AI-2 compared to tetracycline. Noteworthy AI-2 inhibitory activity was also observed for the hexane and ethyl acetate partitions and the compound luteolin. Significant lipase inhibitory activity was observed for all the samples, with rosmarinic acid showing the highest activity. This activity could be explained by its anti-inflammatory activity. However, under aerobic growth conditions in a multispecies system, the lipase inhibitory activity of rosmarinic acid was reduced.

Samples which showed selectivity for targeting bacterial attachment and biofilm formation were selected for biofilm eDNA inhibitory activity. The Toto-1 method was followed due to the stain specificity for eDNA. The fluoresce results for each sample were normalised to the number of living bacteria at the samples' tested concentrations as determined from plate counting experiments. The samples were able to inhibit eDNA in the bacterial biofilms and support the biofilm formation and attachment data explaining the susceptibility of the bacteria to antibacterial treatments. Inhibiting eDNA could reduce antibiotic resistance observed in acne vulgaris, prosthetic implant-associated infections and wounds.

Moderate cytotoxicity was observed against human keratinocytes (HaCat). PA_{EtOH} had the lowest cytotoxicity towards the cells and had no mutagenic activity based on the results from the Ames test with two *Salmonella typhimurium* strains (T98 and T100).

Wound closure potential of the samples was determined, and $PA_{EtOH,}$ hexane and ethyl acetate partitions significant wound closure activity compared to the controls. Heavy metal and microbial analyses confirmed that PA_{EtOH} had no heavy metal or microbial contaminants that could affect the sample's safety and efficacy. The formulated sample (10% in a gel formulation) and the neat extract were tested *in vivo* for their irritancy potential. The neat extract and formulated active were confirmed to be non- irritants and submitted for *in vivo* acne efficacy studies. The studies performed on 24 volunteers between the ages of 19 to 39 years having Fitzpatrick skin types IV and V. The formulated product effectively reduced the number of comedones, blackheads, whiteheads, pustules, and micro cysts between 14 to 28 days of consecutive use, twice a day.

Gold nanoparticles (AuNP) were synthesised from PA_{EtOH} ($AuNP_{PAE}$), rosmarinic acid ($AuNP_{RA}$) and tetracycline ($AuNP_{TET}$). Cytotoxicity studies confirmed that $AuNP_{PAE}$ and $AuNP_{RA}$ samples were non-toxic, while $AuNP_{TET}$ exhibited moderate toxicity was against HaCat cells. $AuNP_{RA}$ acid displayed significantly improved wound closure activity and reduced cytotoxicity compared to the compound alone and could potentially be considered a wound healing treatment. This is the first report of synthesised $AuNP_{RA}$ for the treatment of wounds.

The study supported the hypothesis that acne-associated phylotypes of *C. acnes* and *S. epidermidis* could work together as a multispecies system and increase the virulence of these bacteria in the progression of acne vulgaris and prosthetic implant-associated infections and wounds. Furthermore, the study supported the hypothesis that *P. aliciae*, traditionally used to treat skin maladies and wounds, could be useful for treating acne vulgaris and promoting acne lesions' closure and healing.

2. Significance of the project

Acne vulgaris is an inflammatory disease of the pilosebaceous unit that is mainly due to a dysbiosis of the skin microflora. Although not fatal, this disease affects the quality of life for millions of people of all ages. It has been linked to depression, anxiety, social withdrawal, and suicide. *Cutibacterium acnes* is mostly accepted to be responsible for the onset of acne vulgaris, however, researchers have hypothesised that additional opportunistic bacteria associated with the skin could contribute to inflammation. *Staphylococcus epidermidis* is a commensal bacterium of the skin, with many studies reporting the opportunistic nature of the bacterium and the presence of this bacterium in acne lesions. Controversy exists around the multispecies relationship of *C. acnes* and *S. epidermidis* in various maladies. The current project confirmed that the pathogenic relationship between *C. acnes* and *S. epidermidis* is strain-specific and that it can have various effects on the quorum sensing mechanisms of the bacteria (Miller and Bassler, 2001; Nakase et al., 2014; Christensen et al., 2016; Fournière et al., 2020) .

Furthermore, *C. acnes* and *S. epidermidis* are not only associated with the onset of acne vulgaris but also prosthetic implant-associated infections and wounds. These infections are life-threatening and could result in longer treatment times or even the patient's death. Antibiotic resistance of *C. acnes* and *S. epidermidis* towards current antibiotic treatments has

become a concern to medical professionals and researchers worldwide. Antibiotic resistance is a result of quorum sensing mechanisms in these bacteria (Miller and Bassler, 2001; Platsidaki and Dessinioti, 2018). Therefore, there is a need for new alternative therapies that can circumvent antibiotic resistance by targeting quorum sensing activities.

Plectranthus species are used in traditional medicine to treat a variety of maladies. This genus is mainly unexplored and provides an opportunity to isolate new active compounds and therapies that could be useful for treating acne vulgaris, prosthetic implant-associated infections and wounds (Lambrechts and Lall, 2021). The current study investigated the potential of *Plectranthus aliciae* to target quorum sensing mechanisms associated with single and multispecies *C. acnes* and *S. epidermidis* that could contribute to the onset of antibiotic resistance of these bacteria. Furthermore, the study investigated the activity of a compound, luteolin, that has never been isolated from *P. aliciae*. *Plectranthus aliciae*, its partitions and luteolin were effective in targeting various quorum sensing factors. Due to the positive *in vitro* activity of the *P. aliciae* ethanolic extract, *in vivo* studies confirmed the safety and efficacy of the formulated active for treating severe forms of acne vulgaris. The anti-acne activity of the extract could potentially be a result of the inhibitory activity of the extract towards the quorum sensing mechanisms of the bacteria associated with acne vulgaris.

3. The novelty of the project

- Investigating the synergistic multispecies relationship of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) through various quorum sensing mechanisms.
- Luteolin was identified as a compound of *P. aliciae*.
- Currently, no treatment on the market that targets AI-2 of *C. acnes* and *S. epidermidis*. *Plectranthus aliciae*, its liquid partitions and pure compounds were determined to target this communication mechanism.
- Tetracycline, a widely used antibiotic, is unable to penetrate the mature *C. acnes* biofilm. *Plectranthus aliciae*, its partitions and compound luteolin can penetrate the mature biofilm, inhibit the sessile bacteria within, and render the remaining bacteria metabolically inactive.
- The anti-acne activity of *P. aliciae* was confirmed through *in vivo* efficacy studies.
- *P. aliciae* has *in vitro* wound closure activity, potentially supporting the traditional use of the plant.

- Rosmarinic acid gold synthesised nanoparticles having improved wound closure activity compared to the compound control.

4. Future recommendations

4.1. Use fluorescence *in situ* hybridisation (FISH) to visualise the three-dimensional structure and quantify *C. acnes* and *S. epidermidis* in the multispecies biofilm.

4.2. Determine the release of *C. acnes* (ATCC® 6919) and *S. epidermidis* (ATCC® 35984) single and multispecies AI-2 over a period and not only as a single time point.

4.3. Develop an artificial sebum model for *S. epidermidis* (ATCC® 35984) and a multispecies system to model the interaction of these bacteria in the pilosebaceous unit and determine the ability of the samples to target the sessile cells within the biofilms of these bacterial systems.

4.4. Determine if the samples are preventing AI-2 production, disrupting the chemical structure, or inhibiting the bacterial uptake of the molecules by targeting the LuxS enzyme.

4.5. Mutagenicity studies of the liquid partitions, rosmarinic acid and luteolin.

4.6. Investigate the PA_{EtOH} for *in vivo* wound healing activity.

4.7. Investigate the permeability of $AuNP_{RA}$ and active compounds in the skin.

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Appendix A

Quantification of Pure Compounds Using Ultra-Performance Liquid Chromatography – Quantitative Time of Flight

Appendix A

The spectral data and spectra regarding the Ultra-Performance Liquid Chromatography-Quantitative Time of Flight (UPLC-QTOF) analysis of *P. aliciae* is provided in Table A, the negative mode of the spectral scan in Figure A1 and the postive mode of the spectral scan in Figure A2.

Table A. 1: Spectral data of the major secondary chemical compounds present in the ethanolic extract of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards using Ultra-Performance Liquid Chromatography- Quantitative Time of Flight.

Compounds		Mode	Calibration curve	R^2	LOD[a] (ppm)	LOQ[b] (ppm)
1	Caffeic acid	Negative	y = 91.19x + 28.86	0.999	< 0.25	< 0.25
2	Catechin	Negative	y = 16.02x – 9.72	0.995	< 0.25	< 0.25
3	Epicatechin	Negative	y = 20.79x – 15.33	0.999	< 0.25	< 0.25
4	Ferulic acid	Negative	y = 9.60x + 32.83	0.954	< 0.25	< 2.5
5	4-hydroxybenzoic acid	Negative	y = 14.13x + 13.03	0.996	< 0.25	< 2.5
6	Isoquesrcitrin	Negative	y = 91.16x – 51.37	0.998	< 0.25	< 0.25
7	Isovitexin	Positive	y = 106.56x – 97.81	0.995	< 0.25	< 0.25
8	Luteolin	Positive	y = 0.0063x + 0.306	0.933	< 2.5	< 2.5
9	Orientin	Positive	y = 0.032x + 0.262	0.981	< 0.25	< 0.25
10	Propyl gallate	Negative	y = 333.02x + 2.91	0.999	< 0.25	< 0.25
11	Protocatechuic acid	Negative	y = 59.07x + 10.43	0.999	< 2.5	< 2.5
12	Quercitrin	Positive	y = 0.164x + 0.291	0.956	< 2.5	< 2.5
13	Rosmarinic acid	Negative	y = 59.91x + 8.97	0.999	< 2.5	< 2.5
14	Syringic acid	Negative	y = 17.17x + 7.44	0.997	< 2.5	< 25
15	Vanillic acid	Negative	y = 0.89x + 1.11	0.983	< 25	< 25
16	Vitexin	Positive	y = 98.24x – 88.35	0.995	< 0.25	< 2.5

[a]Limit of Detection, [b]Limit of quantification.

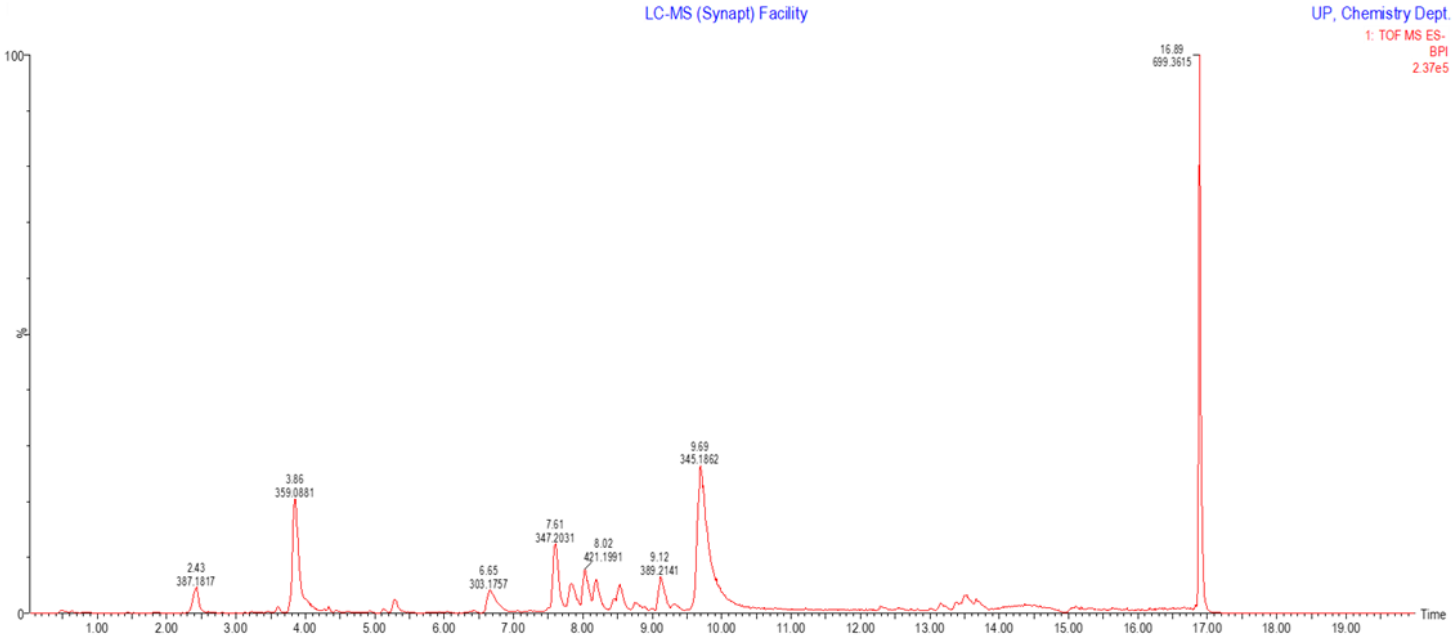

Figure A. 1: Liquid Chromatography-Quantitative Time of Flight spectra in negative mode of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards ethanolic extract.

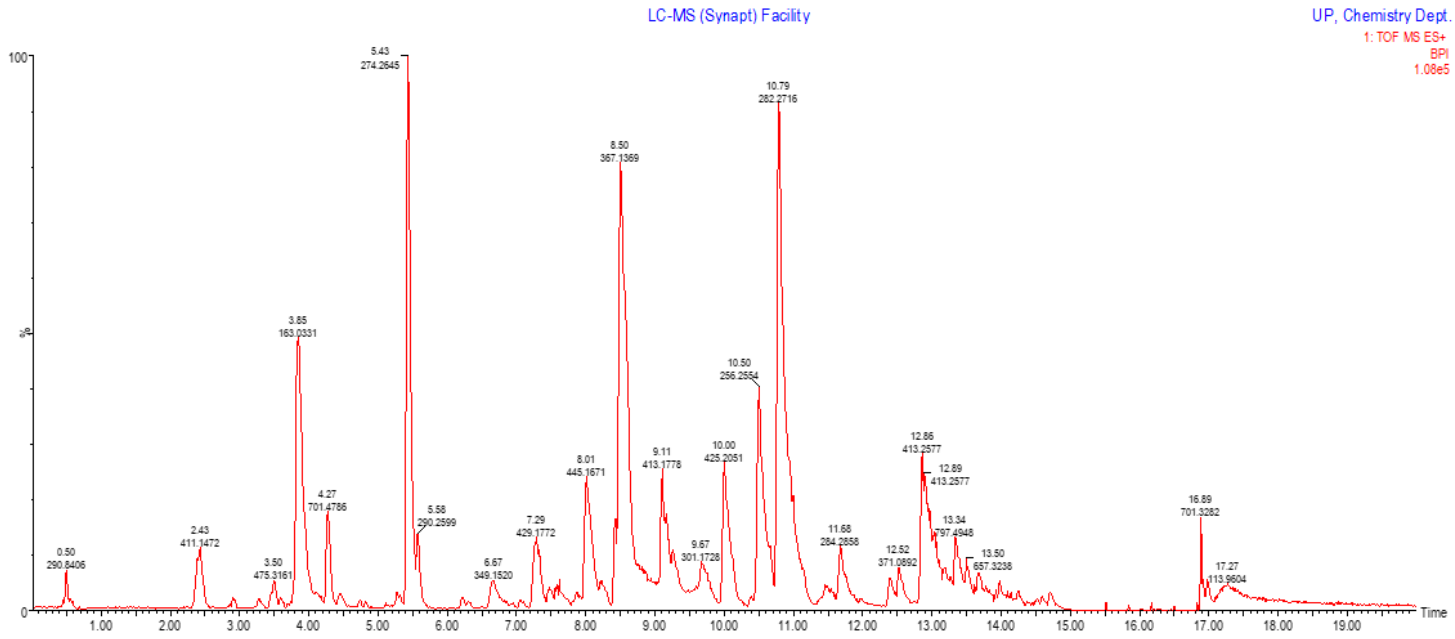

Figure A. 2: Liquid Chromatography-Quantitative Time of Flight spectra in positive mode of *Plectranthus aliciae* (Codd) Van Jaarsv. & T.J. Edwards ethanolic extract.

Appendix B

Heavy metal analysis of *Plectranthus aliciae* ethanolic extract

Appendix B

MERIEUX NutriSciences
CHELAB S.R.L.

LAB N° 0051

TEST REPORT N. 19/000002119

date of issue 03/01/2019

Customer ID 0068079/001

Messrs
SWIFT SILLIKER (PTY) LTD
7 WARRINGTON RD
CLAREMONT
7708
ZA

Sample information

Acceptance number	18.654338.0001
Delivered by	Fedex on 27/12/2018
Receiving Date	27/12/2018
Place of origin	SWIFT SILLIKER (PTY) LTD 7 WARRINGTON RD CLAREMONT 7708 ZA
Sample Description	SSGT 2398 PA (PM) UNIVERSITY OF PRETORIA

Sampling information

Sampled by	Customer

ANALYTICAL RESULTS

	Value/Uncertain	Unit of measure	LoQ	LoD	Start/end date of analysis	Op. units	Row
ON SAMPLE AS IT IS							1
ARSENIC Met.: MP 1288 rev 14 2018	n.d.	mg/kg	0,010	0,0067	31/12/2018--03/01/2019	02	2
CADMIUM Met.: MP 1288 rev 14 2018	n.d.	mg/kg	0,0050	0,0017	31/12/2018--03/01/2019	02	3
MERCURY Met.: MP 1288 rev 14 2018	n.d.	mg/kg	0,0050	0,0017	31/12/2018--03/01/2019	02	4
LEAD Met.: MP 1288 rev 14 2018	n.d.	mg/kg	0,0050	0,0017	31/12/2018--03/01/2019	02	5

Operative units

Unit 02 : Via Castellana Resana (TV)

Chemical responsible	Laboratory manager
Dott.ssa Barbara Scantamburlo Chimico Ordine dei chimici - Provincia di treviso Iscrizione n. A351	Dott. Sébastien Moulard
Num. certificato 18131956 emesso dall'ente certificatore ArubaPEC S.p.A. NG CA 3, ArubaPEC S.p.A., IT	Num. certificato 18132016 emesso dall'ente certificatore ArubaPEC S.p.A. NG CA 3, ArubaPEC S.p.A., IT

- The line marked by a star (*) is not accredited by Accredia, member of MLA. - If not otherwise specified, the uncertainty is extended and has been calculated with a coverage factor k=2 corresponding to a probability interval of about 95%. - LoD is the detection limit and identifies a confidence interval of zero with a probabilty interval of about 99%. - LoQ is the limit of quantification."n.d" is not detected and indicates a value inferior to the LoD. "traces (X)" means a value between LoD and LoQ, this value is indicative. "<x" or ">x" indicate inferior or superior to the measurement field of the test. - If not differently specified, the sums are calculated by lower bound criteria (L.B.). - Registration with the number 7 of the Regional List of the laboratories of the Regione Veneto which perform analyses as regards the procedures for the food safety in food industries, as reported in Annex A of DDR n°73 of 16th January 2008

CHELAB S.r.l. Socio Unico, Company subject to the direction and coordination of Mérieux NutriSciences Corporation
Head office: Via Fratta 25 31023 Resana, Italy Phone. + 39 0423.7177 / Fax + 39 0423.715058 www.merieuxnutrisciences.it
VAT nr. 01500900269, R.E.A Treviso n. 156079 Fully paid up € 103.480,00.

Appendix C

Total microbial activity and yeast and mould analysis of *Plectranthus aliciae* ethanolic extract

Appendix C

FINAL CERTIFICATE OF ANALYSIS

COA N°:	388565-1
Supersedes:	388565-0
COA Date:	02/05/2019
Page	1 / 4

Customer: University of Pretoria
Order N°: 0000598583
Client Reference N°:
Project N°: GT 18-030861

Analysed By Swift Silliker (Pty) Ltd t/a Mérieux NutriSciences
Constantia Office Park
526 16th Street
Midrand 1685
South Africa
Phone: +27 11 805 4310 Fax: +27 11 805 7930

Received from University of Pretoria
Office 3-39, Medicinal Plant Science,Department of Plant Science
Lynwood Road,Hatfield Pretoria
0002
Gauteng

To University of Pretoria
Namrita Lall
Office 3-39, Medicinal Plant Science,Department of Plant Science
Lynwood Road,Hatfield Pretoria
0002
Gauteng

SAMPLE DESCRIPTION

Lab Sample Ref #: GT 18-030861-001 **Sample N°:** 2 506 910 **Test Date:** 05/12/2018
Category Detail: Routine **Sample Condition:** SEALED **Date received:** 05/12/2018
Product Description:** Product: PA(PM)

ANALYTICAL RESULTS

Tests	Analysis Start Date	Results	Units	Limits *[Target value]*
☑ **TMA** METHOD: SWJM 35	05/12/2018	No Growth	cfu/ml	
☑ **Yeast & Mould** METHOD: SWJM 50	05/12/2018			
Yeast		No Growth	cfu/ml	
Mould		No Growth	cfu/ml	

Appendix D

Irritancy reports of *Plectranthus aliciae*

Appendix D

REPORT: SKIN IRRITANCY PATCH TEST

STUDY: Determination of Skin Irritancy Potential of Topically Applied Products.

Protocol MREC/H/48/2014: CR

Date: 6th June – 10th June 2016
Sponsor: University Pretoria

Product(s):

Sample C:	PM Plant Extract no 1	PT1333
Sample E:	BS Plant Extract no 2	PT1335

Conducted by the PHOTOBIOLOGY LABORATORY,
Sefako Makgatho Health Sciences University, South Africa

Contact details:

Physical Address:
Room N311 (3rd Floor)
Basic Medical Science Building
Sefako Makgatho Health Sciences University, 0204
South Africa
Tel: +2712 521 4673
Fax: +2712 521 3992

Postal Address:
P O Box 218
MEDUNSA
0204

e-mail: beverley.summers@smu.ac.za

IRRITANCY PATCH TEST SUMMARY

The test was conducted according to the Patch Test Protocol which follows this Summary.

METHOD

Twenty adult female volunteers were used, of whom five had sensitive skin. Signed consent forms were obtained from all subjects. Controls and products were applied to the inner forearm of each volunteer in a randomized blinded pattern at zero hours and repeated on the same position at 24hours.

Product(s) was/were tested according to the conditions as set out in Table 1 below, by using specially designed aluminium Finn Chambers on Scanpore ® tape for occluded sites or modified Hilltop chambers for unoccluded sites.

Products were tested neat or in a 10% concentration as set out in Table 1 below. Leave-on products are tested neat. Products which contain surfactants are tested diluted. The test sites were observed at 0, 24, 48, 72 and 96 hours after application. Test sites were covered with the chambers for the first 2 x 23 hours, thereafter the chambers were removed.

The protocol uses 1% sodium lauryl sulphate (SLS) solution as a positive control and de-ionised water as a negative control.

Colour assessments were performed in a “double blind” manner in 2 ways: visual score and an instrumental score using the Minolta Cr400 Chromameter using the a* value, which measures colour on the red/green axis.

RESULTS

Panellist no.7 did not attend from Days 3 to 5 (48hrs – 96hrs) of the study due to unforeseen circumstances.

Panellist no.8 did not attend Day 3 and 5 (48hrs and 96hrs) and Panellist no.15 did not attend Day 3 (48hrs) of the study due to unforeseen circumstances.

There were no adverse events during the study.

A summary of the product results is provided in the Table below:

Table 1:

Product	Maximum value (mean visual score + SD)	Time point (hrs)	Patch occluded (O) or non-occluded (N)	Neat (N) or Diluted (D)	Conclusion (irritancy category)
Sample C: PM Plant Extract no 1 PT1333	0.34	48	O	N	Non-irritant
Sample E: BS Plant Extract no 2 PT1335	0.32	72	O	N	Non-irritant
Sample I: Positive Control 1 % SLS	2.76	72	O	N	Irritant
Sample F: Negative Control De-ionised water	0.58	24	O	N	Non-irritant

Based on the visual scores the following products were Non-irritant:

Sample C:	PM Plant Extract no 1	PT1333
Sample E:	BS Plant Extract no 2	PT1335

_____ Date: _____

Beverley Summers B Pharm, MSc(Med), PhD
PROFESSOR & PHOTOBIOLOGY LABORATORY MANAGER

APPENDICES

Data sheets: Visual raw data and graphs
Chromameter raw data and graphs
Summary graphs of maximum irritancy value (mean + SD)

<u>*Foot Note:</u>

1. **This report provides the results of an Irritancy patch test. Products are referred to by the name submitted by the manufacturer. It is not within the scope of the report to comment individually on the appropriateness of the product names, or the claims inherent in those names.**

IRRITANCY PATCH TEST – PROTOCOL

1. INTRODUCTION

1.1 Aim / Objective
The objective of the study was to determine skin irritancy potential of topically applied products.

1.2 Test Site
Controls and product(s) were applied to the inner forearm of each volunteer.

1.3 Test Product(s)

The product(s) under investigation is/are listed in the Summary:

1.4 Ethics and Relevance
The study was conducted according to internationally recognised Good Clinical Practice Guidelines. Permission to conduct the study was covered under the protocol approval for project MREC/H/48/2014: CR of the Research, Ethics and Publications Committee of the Sefako Makgatho Health Sciences University.

1.5 Study Staff

Principal Investigator: Prof B. Summers
(012) 521 4673 beverley.summers@smu.ac.za

_____
Signature

_____
Date

Assistant Study Investigator 1: Lebogang Kgatuke
(012) 521 3027 lebogang.kgatuke@smu.ac.za

_____
Signature

_____
Date

Assistant Study Investigator 2: Lee-ann Raaff
(012) 521 3027 lee-ann.raaff@smu.ac.za

_____
Signature

_____
Date

Assistant Study Investigator 3: Caroline Moeletsi
(012) 521 4278 caroline.moeletsi@smu.ac.za

_____
Signature

_____
Date

Assistant Study Investigator 4: Marlize Lategan
(012) 521 4278 marlize.lategan@smu.ac.za

_____
Signature

_____

2. METHOD

The study was conducted as per protocol MREC/H/48/2014: CR, details of which follow.

2.1 Study Panel

Recruitment for the study began two weeks before the commencement of the study. Approximately 25 panellists were recruited according to the study requirements, to commence the study with a test panel of 20.

2.1.1 *Inclusion Criteria*

All panellists (were):

1. Females between 18-65 years of age.
2. In good general health (self reported/documented by a signed Health Questionnaire).
3. Willing and able to read and sign an informed consent form and to carry out all the procedures of the study.
4. Able to read and write in English.
5. Agreed to refrain from using creams, lotions, moisturizers, bath oils, additives or any other skin products on the test sites during the study.
6. Agreed to refrain from consuming hot or caffeine containing beverages or smoking for one hour prior to appointments when instrumental assessments were taken.
7. Agreed to comply with Study requirements.
8. Free of cuts or abrasions on the test sites at the beginning of the study.

2.1.2 *Exclusion Criteria*

The panellists had not/were not:

1. Participated in any clinical or consumer research study in the previous six weeks.
2. Diabetes, history of epilepsy, or severe asthma.
3. Currently using any medication on a regular basis such as anti-histamine, insulin, anti-inflammatory agents, cortisones, etc.
4. Pregnant, lactating or planning pregnancy (as determined by interview).
5. Documented allergies to study product components, soaps, latex or fragrances
6. Skin diseases or inflammatory skin condition including psoriasis, eczema, sunburn or excessive suntan, scars or uneven skin tones on the test sites.
7. Used any topical drugs at test sites.

2.2 Study Design and Conditions

Potential panellists attended a series of study briefings held during the week prior to the study. Panellists were verbally briefed and given the Study Instructions, Informed Consent forms (plus coloured duplicate copies of the latter).

Subject Eligibility Checklist and Consent forms were screened, by the Study Investigator (Prof. B Summers), for eligibility and completeness. Queries, omissions or potential problems on the forms were 'flagged' for clarification at the following visit.

The most suitable potential panellists were given appointments for the study week.

Cards were prepared with the panellist names on one side and unique study numbers on the other. The cards were retained at the study centre and left out on a table where panellists collected them on arrival, prior to signing the register. Panellists were given specifically timed appointments for the whole study.

2.3 Study Procedure

Test products were received from the sponsor.

Unless otherwise stated in the Summary, twenty adult female volunteers were used, of whom five had sensitive skins (as determined from the subject enrolment questionnaire). Signed consent forms were obtained from all subjects. Subjects with a history of clinical dermal abnormalities or who are taking or using medication which may affect test results through mediation of the inflammatory response were excluded.

Procedure

Controls and products were applied to the inner forearm of each volunteer in a randomized, blinded pattern at zero hours and repeated on the same position at 24 hours.

A measured volume of control or pieces of product was placed in the respective chamber and the chamber attached to the forearm.

Product(s) was/were tested according to the conditions as set out in Table 1 above by using specially designed aluminium Finn Chambers on Scanpore ® tape for occluded sites or modified Hilltop chambers for unoccluded sites.

Table 1 of the Summary indicates the specific test conditions for the product(s).
The patches were observed at 0, 24, 48, 72 and 96 hours after application. Patch areas were covered with the chambers for the first 2 x 23 hours, thereafter the chambers were removed.

The protocol uses 1% sodium lauryl sulphate (SLS) solution as a positive control and de-ionised water as a negative control. A measured amount of 0.02ml of sodium lauryl sulphate and de-ionised water was applied.

2.4 Panellist Assessment

The following visual rating system was used to classify the reactions:

Colour assessments were performed in a “double blind” manner in 2 ways:

I. a visual score where
0= no reaction
1= slight erythema
2= strong erythema confined to the immediate contact area
3= strong erythema spreading beyond the contact area Irritancy levels are classified by our laboratory, based on the visual scores, as follows:
product mean visual score + SD >1.5 - Product is irritant
product mean visual score +SD <=1.5 and >negative control – Product is of low irritancy potential.
Product mean visual score +SD <= negative control – Product is non-irritant

II. an instrumental score using the Minolta Cr400 chromameter using the a* value, which measures colour on the red/green axis.

2.5 Quality Control

2.5.1 *Study Control*
All studies are covered by detailed Standard Operating Procedures and conducted in accordance with the Declaration of Helsinki and the Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participation in South Africa.

2.5.2 *Checking Products*
Product formulation(s) was/were checked for acceptability by Prof. B Summers. Product(s) was/were then coded and diluted, where necessary.

2.5.3 *Proof Reading*
After data entry, the computer printout was checked in hardcopy against the raw data.

2.5.4 *Calibration of Instrument*
The Cr400 Chromameter is calibrated against a D65 white plate at the start of each study.

3. RESULTS AND DISCUSSION

3.1 Panellists

3.1.1 *Visits*
All panellists attended their daily visits, unless otherwise stated in the Summary.

3.1.2 *Adverse Events*
There were no adverse events during the study, unless listed in the Summary. In the case of adverse events, a report is submitted to Sefako Makgatho Health Sciences University Research and Ethics Committee.

3.2 Data

Data were collected at each visit as per the method described in Section 2.3 and 2.4 above. The data for the study accompany this report as Appendices as listed in the Summary.

The product(s) was/were tested on the volar forearms of 20 panellists. The graphical representations of the results (attached) show the data for the product(s), the negative control (de-ionised water) and the positive controls (1% SLS).

The raw numerical data and graphical representations of both the visual and chromametric assessments are attached.

3.2.1 Visual Scores

The maximum irritancy (as calculated by the mean visual score + one standard deviation of the visual score) for the products and the controls is tabulated below. The irritancy limit for the visual score + one standard deviation (SD) is 1.5 on a 0-3 scale.

3.2.2 *Chromameter*

The raw data for the Chromameter a* values are attached. The change in chroma a* values for each test site compared to baseline, is calculated as follows:
Delta a* = (Product a*time t – Product a*time 0) – (untreated a* time t – untreated a*time 0)

The delta a*values for all panellists for a given product at a given time point were averaged and plotted on the attached graphs.
Chromameter a*value data patterns supported the visual erythemal assessment scores

Please note:

The irritancy patch test should not be used as the basis for hypoallergenic claims because:

1. *An allergic reaction depends on a previous exposure to an allergen, formation of antibodies (which takes several days to weeks) and then a re-challenge at a subsequent time point. The irritancy patch test depends on 2x23 hour exposures on consecutive days – hence the interval between the exposures is not sufficient for antibody development.*
2. *The likelihood of an allergic response to a given ingredient is small (e.g. 1 in 100 to 1 in 1000 individuals). It is therefore very unlikely in a panel of only 20 people that an allergic reaction would be generated.*

Hypoallergenic claims should therefore be based on large study panels or the fact that the product contains ingredients which are of proven/documented hypoallergenicity.

Appendix E

Acne efficacy studies of *Plectranthus aliciae* in a finished formulation

Appendix E

FUTURE COSMETICS CC

From Concept to Product

ATT: PROFESSORS LALL AND MOODLEY

UNIVERSITY OF KWAZULU-NATAL
5TH FLOOR
WESTVILLE CAMPUS
UNIVERSITY ROAD
WESTVILLE

2018/05/28

Re: Report on In Vivo Acne Reduction Efficacy Testing: FCAG014

Dear Namrita and Indres,

Herewith the report on the Acne Reduction Testing Study performed for the following:

Product Name	Future Cosmetics Test Substance Ref:	Future Cosmetics Sequence Ref:
PM in Gel	FCAG014/5936	1
Placebo Gel	FCAG014/5937	2

Member: Heibrie le Roux
Registration Number: 2001/055088/23
Physical Address: 287 Sinovich Street, Grootfontein Country Estates,
Garsfontein Road, Pretoria East, South Africa,
Postal Address: PO Box 39675, Garsfontein East, South Africa, 0060
Tel: +27-12-811 0621 Fax: +27-12-811 0510 Mobile: +27-82-9613509
E-mail: heibrie@futurecosmetics.co.za

FUTURE COSMETICS CC

From Concept to Product

ATT: PROFESSORS LALL AND MOODLEY

UNIVERSITY OF KWAZULU-NATAL
5TH FLOOR
WESTVILLE CAMPUS
UNIVERSITY ROAD
WESTVILLE

2018/05/28

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Product Name	Future Cosmetics Test Substance Ref:	Future Cosmetics Sequence Ref:
PM in Gel	FCAG014/5936	1
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Postal Address: PO Box 39675, Garsfontein East, South Africa, 0060
Tel: +27-12-811 0621 Fax: +27-12-811 0510 Mobile: +27-82-9613509
E-mail: heibrie@futurecosmetics.co.za

2018/05/28

REPORT ON IN VIVO ACNE REDUCTION EFFICACY TESTING: REF: FCAG014

Responsible for study: Heibrie Le Roux
Managing Member

Study Technicians: Emily Mthunzi
Theresa Mafiri
Melita Manamela

Quality Control and Report Writing: Theresa Mafiri

Testing Facility: Future Cosmetics CC
287 Sinovich Street,
Grootfontein Country Estates
Pretoria East,
South Africa
0060

The following report is an accurate account of the test method used as described by the protocol (FCAG014) and the results obtained during the course of the above study, which was performed within the months prior to the date of this report.

Product Tested:

PM in Gel	FCAG014/5936
Placebo Gel	FCAG014/5937

Sponsor: **UNIVERSITY OF KWAZULU-NATAL**
5TH FLOOR
WESTVILLE CAMPUS
UNIVERSITY ROAD
WESTVILLE

Concentration of Products: Neat

Member: Heibrie le Roux
Registration Number: 2001/055088/23
Physical Address: 287 Sinovich Street, Grootfontein Country Estates,
Garsfontein Road, Pretoria East, South Africa,
Postal Address: PO Box 39675, Garsfontein East, South Africa, 0060
Tel: +27-12-811 0621 Fax: +27-12-811 0510 Mobile: +27-82-9613509
E-mail: heibrie@futurecosmetics.co.za

2018/05/28

REPORT ON IN VIVO ACNE REDUCTION EFFICACY TESTING: REF: FCAG014

Responsible for study: Heibrie Le Roux
Managing Member

Study Technicians: Emily Mthunzi
Theresa Mafiri
Melita Manamela

Quality Control and Report Writing: Theresa Mafiri

Testing Facility: Future Cosmetics CC
287 Sinovich Street,
Grootfontein Country Estates
Pretoria East,
South Africa
0060

The following report is an accurate account of the test method used as described by the protocol (FCAG014) and the results obtained during the course of the above study, which was performed within the months prior to the date of this report.

Product Tested:

PM in Gel	FCAG014/5936
Placebo Gel	FCAG014/5937

Sponsor: **UNIVERSITY OF KWAZULU-NATAL**
5TH FLOOR
WESTVILLE CAMPUS
UNIVERSITY ROAD
WESTVILLE

Concentration of Products: Neat

Member: Heibrie le Roux
Registration Number: 2001/055088/23
Physical Address: 287 Sinovich Street, Grootfontein Country Estates,
Garsfontein Road, Pretoria East, South Africa,
Postal Address: PO Box 39675, Garsfontein East, South Africa, 0060
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E-mail: heibrie@futurecosmetics.co.za

Objective:
The objective of the study was to determine the Acne Reduction efficacy of a test product on the face of human subjects.

Test Protocol:
The test protocol (FCAG014) is available on request.

Materials:
Standard Eye Make-up Remover
Sterile Cotton makeup remover pads
White Facial Tissue
Alcohol swabs

Camera:
VISIO FACE

Testing Conditions:
The study was carried out at an average ambient range temperature 20.5 °C to 22.8 °C with a relative humidity of 41.0 %RH and 46.7 %RH (See Appendix B: Testing Conditions).

Method:
A group of twenty-four (24) subjects between the ages of 19 to 39 were included and completed the full study (Appendix A: Subject Demographics). A couple of deviations were observed during the study (Appendix H: Deviation Form).

The subjects complied with the rules and specifications of the study and all results recorded were used in the noted calculations.

The procedure of testing was explained to them verbally and with the consent form and medical history were signed by each subject. Personal details and each subject's assessment of their own skin type were recorded (Appendix D: Medical History Form and Appendix C: The Consent Form).

Colour photographs were taken at each time interval to serve as a recording of the study conducted.

All subjects were instructed to rest for twenty (20) minutes before any testing was conducted.

The designated left or right side of the face was cleansed with a standard eye make-up remover and allowed to air dry for three (3) minutes.

The temperature and relative humidity were recorded during the time of the study (Appendix B: Testing Conditions).

The test sites were demarcated as per test sequence substance (Appendix E: Test Substance Sequence).

Two (2) trained evaluators performed a physical count on both test sites and recorded the scores.

An operator trained the test subjects in applying the test products evenly to the test sites, according to the test substance sequence (Appendix E: Test Substance Sequence).

Subjects were restricted from using any topical products or any medication not approved by the study sponsor for the duration of the study.

Subjects returned to the testing facility on twenty-eight (D28) days respectively after baseline readings.

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Garsfontein Road, Pretoria East, South Africa,
Postal Address: PO Box 39675, Garsfontein East, South Africa, 0060
Tel: +27-12-811 0621 Fax: +27-12-811 0510 Mobile: +27-82-9613509
E-mail: heibrie@futurecosmetics.co.za

The temperature and relative humidity were recorded during the time of the study (See Appendix B: Testing Conditions).

Research Design:

Sensory: Objective (Physical Count)
The primary objective sensory evaluation results were scored and rated by two (2) trained evaluators. Both inflammatory and retentional elements of acne were counted.

A research design can be defined as the basic plan that guides the data collection and analysis phases of the research project. It is the framework that specifies the type of information to be collected, the sources of data, and the data collection procedure (Kinnear & Taylor, 1996: p.129). The information needed to reach the aim of the current study is based on values that represent the Acne Reduction characteristics of human subjects and can thus be classified as interval data, since the quantitative data can be represented in a quantitative manner.

Data Sampling:
A sample can be defined as a subset of the whole population which is actually investigated by a researcher and whose characteristics will be generalised to the entire population (Bless & Higson-Smith, 1995). A sampling plan can be described as a design, scheme of action or procedure that specifies how the participants are to be selected in a survey study (Rosnow & Rosenthal, 1996). A distinction is made between probability and non-probability sampling. In this study use was made of a non-probability sampling method. A non-probability sampling method can be described as the selection of a population element to be part of the sample based in some part on the judgment of the researcher (Kinnear & Taylor, 1996). The researcher only included respondents who were 19 - 39 years of age. A sample of convenience was used in this study.

Statistical Data Analysis Procedure (to be performed on full studies)

Data Analysis:
The data was captured onto excel and converted to extended excel statistical tests in order to do the analysis. The data analysis had the following aims:

1. To first determine whether the data was distributed normally.
2. To determine whether significant differences existed between the test product and placebo regarding the Acne Reduction efficacy.

Since the sample was relatively small and consisted of a group of at least twenty-four (24) respondents, use was made of a parametric test, the (unequal or equal variances) t-test for comparison of two sets of data. The t-test is used to determine whether a given treatment had a significant effect on a population (Keller & Warrack, 2003). In the case of not normally distributed data the Wilcoxon Signed Rank Sum Test was be used.

The following statistical data analysis procedures were used:

Descriptive Analysis: Descriptive statistics are primarily aimed at describing the data.
Inferential statistics: Test hypotheses about differences in populations on the basis of measurements made on samples of subjects (Tabachnick & Fidell, 1996: p.9).

Member: Heibrie le Roux
Registration Number: 2001/055088/23
Physical Address: 287 Sinovich Street, Grootfontein Country Estates, Garsfontein Road, Pretoria East, South Africa,
Postal Address: PO Box 39675, Garsfontein East, South Africa, 0060
Tel: +27-12-811 0621 Fax: +27-12-811 0510 Mobile: +27-82-9613509
E-mail: heibrie@futurecosmetics.co.za

Statistically significant differences between variables are indicated by a significance value p. If the value of p is equal to or less than 0.05, it gives an indication that there is a statistically significant difference, at the 5% level of confidence.

Results:

Descriptive Analysis:

Descriptive Analysis entails the ordering and summarising of data by means of tabulation and the calculation of descriptive measures.

A Summary of the individual results recorded during the study for all test products, as well as all calculations are given in Appendix F:

Objective Sensory Evaluation (Physical Count)

TABLE F1: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (PUSTULES) FOR FCAG014/5936 (BL – D28)

TABLE F2: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (PUSTULES) FOR FCAG014/5937 (BL – D28)

TABLE F3: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (PAPULES) FOR FCAG014/5936 (BL – D28)

TABLE F4: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (PAPULES) FOR FCAG014/5937 (BL – D28)

TABLE F5: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (WHITEHEADS) FOR FCAG014/5936 (BL – D28)

TABLE F6: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (WHITEHEADS) FOR FCAG014/5937 (BL – D28)

TABLE F7: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (COMEDONES) FOR FCAG014/5936 (BL – D28)

TABLE F8: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (COMEDONES) FOR FCAG014/5937 (BL – D28)

TABLE F9: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (BLACKHEADS) FOR FCAG014/5936 (BL – D28)

TABLE F10: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (BLACKHEADS) FOR FCAG014/5937 (BL – D28)

TABLE F11: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (MICROCYSTS) FOR FCAG014/5936 (BL – D28)

TABLE F12: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION (MICROCYSTS) FOR FCAG014/5937 (BL – D28)

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Interferential Statistics:

Statistical Inference draws conclusions about the population from which the sample was drawn by using descriptive measures that has been calculated.

A Summary of the Statistical Analysis is given in Appendix G:
Objective Sensory (Physical Count)

TABLE G1: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR BASELINE – PUSTULES
TABLE G2: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D14 - PUSTULES
TABLE G3: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D28 - PUSTULES

TABLE G4: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR BASELINE - PAPULES
TABLE G5: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D14 – PAPULES
TABLE G6: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D28 – PAPULES

TABLE G7: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR BASELINE - WHITEHEADS
TABLE G8: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D14 – WHITEHEADS
TABLE G9: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D28 – WHITEHEADS

TABLE G10: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR BASELINE - COMEDONES
TABLE G11: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D14 - COMEDONES
TABLE G12: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D28 - COMEDONES

TABLE G13: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR BASELINE - BLACKHEADS
TABLE G14: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D14 – BLACKHEADS
TABLE G15: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D28 - BLACKHEADS

TABLE G16: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR BASELINE - MICROCYST
TABLE G17: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D14 - MICROCYST
TABLE G18: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 FOR D28 - MICROCYST

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Table A: Comparison of objective sensory (physical count, PUSTULES) values for FCAG014/5936 compared to FCAG014/5937 test sites:

Mean
(± Standard Deviation)

Time Interval	FCAG014/5936	Table Ref.	FCAG014/5937	Table Ref.	(Diff) p-Value	Table Ref.
BASELINE	0.08 (± 0.35)	F1	0.04 (± 0.20)	F2	0.000*	G1
D14 - BASELINE	0.04 (± 0.29)	F1	-0.02 (± 0.25)	F2	**0.020**	G2
D28 - BASELINE	-0.06 (± 0.24)	F1	0.00 (± 0.29)	F2	**0.013**	G3

Conclusion:

Objective sensory (physical count, PUSTULES)
A Wilcoxon Signed Rank Sum Test was performed to compare the test product treated and the placebo control treated sites at baseline (thus before application of test products) and there was a statistical significant difference was found between the two test sites on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.
**This is of no significance to test product efficacy*

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day fourteen (D14) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day twenty-eight (D28) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

It can therefore be concluded that the product FCAG014/5936 was only effective in reducing the number of pustules; after twenty-eight (28) days of consecutive use; when compared to the placebo control FCAG014/5937.

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Table B: Comparison of objective sensory (physical count, PAPULES) values for FCAG014/5936 compared to FCAG014/5937 test sites:

Mean
(± Standard Deviation)

Time Interval	FCAG014/5936	Table Ref.	FCAG014/5937	Table Ref.	(Diff) p-Value	Table Ref.
BASELINE	12.85 (± 17.31)	F3	12.50 (± 13.12)	F4	*0.008	G4
D14 - BASELINE	-2.81 (± 8.67)	F3	-2.23 (± 8.07)	F4	0.337	G5
D28 - BASELINE	-3.65 (± 12.08)	F3	-3.67 (± 12.97)	F4	0.496	G6

Conclusion:

Objective sensory (physical count, PAPULES)

A Wilcoxon Signed Rank Sum Test was performed to compare the test product treated and the placebo control treated sites at baseline (thus before application of test products) and a statistical significant difference was found between the two test sites on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.
**This is of no significance to test product efficacy*

A t-Test was performed to determine the treatment effect by comparing the average difference values at day fourteen (D14) of the test product treated and the placebo control treated sites and there was no statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937.

A t-Test was performed to determine the treatment effect by comparing the average difference values at day twenty-eight (D28) of the test product treated and the placebo control treated sites and there was no statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.

It can therefore not be concluded that the product FCAG014/5936 was not effective in reducing the number of papules; after twenty-eight (28) days of consecutive use twice a day; when compared to the placebo control FCAG014/5937.

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Table 3: Comparison of objective sensory (physical count, WHITEHEADS) values for FCAG014/5936 compared to FCAG014/5937 test sites:

Mean
(± Standard Deviation)

Time Interval	FCAG014/5936	Table Ref.	FCAG014/5937	Table Ref.	(Diff) p-Value	Table Ref.
BASELINE	8.29 (± 8.98)	F5	7.79 (± 8.89)	F6	*0.000	G7
D14 - BASELINE	-1.88 (± 7.63)	F5	0.85 (± 6.13)	F6	**0.007**	G8
D28 - BASELINE	-4.13 (± 7.03)	F5	-1.31 (± 7.84)	F6	**<0.001**	G9

Conclusions:

Objective sensory (physical count, WHITEHEADS)

A Wilcoxon Signed Rank Sum Test was performed to compare the test product treated and the placebo control treated sites at baseline (thus before application of test products) and already a statistical significant difference was found between the two test sites on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.
**This is of no significance to test product efficacy*

A t-Test was performed to determine the treatment effect by comparing the average difference values at day fourteen (D14) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day twenty-eight (D28) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

It can therefore be concluded that the product FCAG014/5936 was effective in reducing the number of whiteheads; after fourteen (14) to twenty-eight (28) days of consecutive use; when compared to the placebo control FCAG014/5937.

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Table 4: Comparison of objective sensory (physical count, COMEDONES) values for FCAG014/5936 compared to FCAG014/5937 test sites:

Mean
(± Standard Deviation)

Time Interval	FCAG014/5936	Table Ref.	FCAG014/5937	Table Ref.	(Diff) p-Value	Table Ref.
BASELINE	1.19 (± 1.95)	F7	1.08 (± 1.27)	F8	*0.000	G10
D14 - BASELINE	-0.52 (± 1.65)	F7	-0.19 (± 1.25)	F8	**<0.001**	G11
D28 - BASELINE	-0.63 (± 2.00)	F7	-0.67 (± 1.21)	F8	0.453	G12

Conclusion:

Objective sensory (physical count, COMEDONES)

A Wilcoxon Signed Rank Sum Test was performed to compare the test product treated and the placebo control treated sites at baseline (thus before application of test products) and already a statistical significant difference was found between the two test sites on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.
**This is of no significance to test product efficacy*.

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day fourteen (D14) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

A t-Test was performed to determine the treatment effect by comparing the average difference values at day twenty-eight (D28) of the test product treated and the placebo control treated sites and there was no statistical significance difference on a 5% level of confidence.

It can therefore be concluded that the product FCAG014/5936 was only effective in reducing the number of comedones; after fourteen (14) days of consecutive use; when compared to the placebo control FCAG014/5937.

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Table 5: Comparison of objective sensory (physical count, BLACKHEADS) values for FCAG014/5936 compared to FCAG014/5937 test sites:

Mean
(± Standard Deviation)

Time Interval	FCAG014/5936	Table Ref.	FCAG014/5937	Table Ref.	(Diff) p-Value	Table Ref.
BASELINE	3.44 (± 4.63)	F9	3.04 (± 4.69)	F10	*0.000	G13
D14 - BASELINE	-0.65 (± 2.74)	F9	-0.06 (± 4.73)	F10	**<0.001**	G14
D28 - BASELINE	-1.25 (± 3.34)	F9	-0.35 (± 3.09)	F10	**<0.001**	G15

Conclusions:

Objective sensory (physical count, BLACKHEADS)

A Wilcoxon Signed Rank Sum Test was performed to compare the test product treated and the placebo control treated sites at baseline (thus before application of test products) and already a statistical significant difference was found between the two test sites on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.
**This is of no significance to test product efficacy.*

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day fourteen (D14) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day twenty-eight (D28) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

It can therefore be concluded that the product FCAG014/5936 was effective in reducing the number of blackheads; after fourteen (14) twenty-eight (28) days of consecutive use; when compared to the placebo control FCAG014/5937.

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Table 6: Comparison of objective sensory (physical count, MICROCYSTS) values for FCAG014/5936 compared to FCAG014/5937 test sites:

Mean
(± Standard Deviation)

Time Interval	FCAG014/5936	Table Ref.	FCAG014/5937	Table Ref.	(Diff) p-Value	Table Ref.
BASELINE	0.23 (± 0.69)	F11	0.08 (± 0.35)	F12	*0.000	G16
D14 - BASELINE	-0.10 (± 0.59)	F11	0.00 (± 0.21)	F12	**0.010**	G17
D28 - BASELINE	-0.08 (± 0.61)	F11	-0.02 (± 0.33)	F12	**0.013**	G18

Conclusions:
Objective sensory (physical count, MICROCYSTS)

A Wilcoxon Signed Rank Sum Test was performed to compare the test product treated and the placebo control treated sites at baseline (thus before application of test products) and already a statistical significant difference was found between the two test sites on a 5% level of confidence. Test product FCAG014/5936 tested significantly higher than control FCAG014/5937.
**This is of no significance to test product efficacy.*

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day fourteen (D14) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

A Wilcoxon Signed Rank Sum Test was performed to determine the treatment effect by comparing the average difference values at day twenty-eight (D28) of the test product treated and the placebo control treated sites and there was a statistical significance difference on a 5% level of confidence. Test product FCAG014/5936 tested significantly lower than control FCAG014/5937

It can therefore be concluded that the product FCAG014/5936 was effective in reducing the number of microcyst; after fourteen (14) to twenty-eight (28) days of consecutive use; when compared to the placebo control FCAG014/5937.

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FUTURE COSMETICS CC

From Concept to Product

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
STUDY NUMBER: FCAG014

APPENDIX A: SUBJECT DEMOGRAPHICS

DISTRIBUTION OF TEST SUBJECT DEMOGRAPHICS WHICH WAS INCLUDED INTO THE CALCULATIONS:		TEST SUBJECT DROP-OUT AND ELIMINATION FROM CALCULATIONS:	
TOTAL: n =	24	TEST SUBJ REF	REASON FOR EXCLUSION
AVERAGE AGE:	28.5		
MIN AGE:	19		
MAX AGE:	39		
STUDY AGE RANGE :	19 to 39		
FEMALE	100%		
MALE	0		
ETHNIC BLACK	100%		
CAUCASIAN	0%		
UNDER 18: n =	0		
18 to 20: n =	2		
21 to 30: n =	16		
31 to 40: n =	6		
41 to 50: n =	0		
51 to 71: n =	0		

TEST REFERENCE	SUBJECT NUMBER	SKIN TYPE (Fritzpatrick)	AGE	GENDER	ETHNIC CLASSIFICATION
FCAG014	/01	IV	29	FEMALE	ETHNIC BLACK
FCAG014	/02	V	26	FEMALE	ETHNIC BLACK
FCAG014	/04	V	25	FEMALE	ETHNIC BLACK
FCAG014	/05	V	28	FEMALE	ETHNIC BLACK
FCAG014	/06	IV	29	FEMALE	ETHNIC BLACK
FCAG014	/07	V	30	FEMALE	ETHNIC BLACK
FCAG014	/08	IV	24	FEMALE	ETHNIC BLACK
FCAG014	/09	IV	20	FEMALE	ETHNIC BLACK
FCAG014	/10	V	39	FEMALE	ETHNIC BLACK
FCAG014	/11	IV	34	FEMALE	ETHNIC BLACK
FCAG014	/12	V	35	FEMALE	ETHNIC BLACK
FCAG014	/13	V	23	FEMALE	ETHNIC BLACK
FCAG014	/14	IV	19	FEMALE	ETHNIC BLACK
FCAG014	/16	IV	27	FEMALE	ETHNIC BLACK
FCAG014	/17	V	27	FEMALE	ETHNIC BLACK
FCAG014	/18	IV	26	FEMALE	ETHNIC BLACK
FCAG014	/19	V	36	FEMALE	ETHNIC BLACK
FCAG014	/20	V	29	FEMALE	ETHNIC BLACK
FCAG014	/21	IV	35	FEMALE	ETHNIC BLACK
FCAG014	/22	IV	35	FEMALE	ETHNIC BLACK
FCAG014	/23	IV	29	FEMALE	ETHNIC BLACK
FCAG014	/24	IV	22	FEMALE	ETHNIC BLACK
FCAG014	/25	V	28	FEMALE	ETHNIC BLACK
FCAG014	/27	V	28	FEMALE	ETHNIC BLACK

FUTURE COSMETICS CC

From Concept to Product

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
STUDY NUMBER: FCAG014

APPENDIX B: TESTING CONDITIONS

DISTRIBUTION OF TESTING CONDITIONS:

DISTRIBUTION OF TESTING CONDITIONS:		TEMPERATURE Degrees Celsius (20-22C) (Mean)			HUMIDITY (40%- 50%) (Mean)		
TIME INTERVAL		BASELINE	DAY14(D14)	DAY 28 (D28)	BASELINE	DAY14(D14)	DAY 28 (D28)
MEAN		22.29	21.16	21.48	46.11	44.04	44.07
TOTAL MEAN			21.65			44.74	
MIN VALUE			20.50			41.00	
MAX VALUE			22.80			46.70	
TEMPERATURE RANGE		20.50	to	22.80	41.00	to	46.70
TEST REFERENCE	SUBJECT NUMBER	TEMPERATURE (Degrees Celsius 20-22C)			HUMIDITY (40%- 50%)		
		BASELINE	DAY14(D14)	DAY 28 (D28)	BASELINE	DAY14(D14)	DAY 28 (D28)
FCAG014	/01	22.5	20.5	20.9	45.8	44.5	43.2
FCAG014	/02	22.5	20.5	20.9	45.8	44.5	43.2
FCAG014	/04	22.5	20.8	20.9	45.9	44.5	43.2
FCAG014	/05	22.7	20.8	20.9	46.4	44.5	43.5
FCAG014	/06	22.7	20.8	21.1	46.4	44.4	43.5
FCAG014	/07	22.8	20.8	21.2	46.7	44.4	43.5
FCAG014	/08	21.4	21.0	21.2	45.9	44.4	43.5
FCAG014	/09	21.9	21.0	21.3	46.3	44.3	43.6
FCAG014	/10	21.9	21.0	21.3	46.3	44.3	43.6
FCAG014	/11	22.0	21.1	21.4	46.5	44.3	43.6
FCAG014	/12	22.0	21.1	21.4	46.5	44.3	44.1
FCAG014	/13	22.1	21.1	21.5	46.3	44.2	44.1
FCAG014	/14	22.1	21.2	21.5	46.3	44.2	44.3
FCAG014	/16	22.2	21.2	21.6	46.3	44.1	44.3
FCAG014	/17	22.2	21.2	21.6	46.2	44.1	44.4
FCAG014	/18	22.2	21.3	21.7	46.2	44.1	44.4
FCAG014	/19	22.3	21.3	21.7	46.2	44.0	44.5
FCAG014	/20	22.3	21.4	21.8	46.0	44.0	44.5
FCAG014	/21	22.3	21.4	21.8	46.0	44.0	44.6
FCAG014	/22	22.3	21.5	21.8	46.0	43.9	44.6
FCAG014	/23	22.5	21.5	21.9	45.8	43.9	44.6
FCAG014	/24	22.5	21.6	21.9	45.8	43.7	44.7
FCAG014	/25	22.5	21.6	21.9	45.8	43.7	44.7
FCAG014	/26	22.4	21.7	21.9	45.7	43.7	44.7
FCAG014	/27	22.4	21.7	22.0	45.7	41.0	44.8

FUTURE COSMETICS CC

From Concept to Product

APPENDIX C: INFORMED CONSENT FORM: ACNE REDUCTION

PROTOCOL NO:	FCAG014	SUBJECT NO: *To be assigned by admission staff*	FULL NAME & SURNAME:	ID/PASSPORT NO:	3.1
				NATIONALITY:	3.2

1. INTRODUCTION: Before agreeing to participate in this study, it is important that you read & understand the following explanation and procedures. You have the right to withdraw from this study at any time. No guarantees or assurances can be given to the results of this study.

2. BACKGROUND: You have been invited to participate in this study designed to evaluate the performance of one/more products to be tested for its efficacy to reduce acne. You must be at least **18 years of age**, in **good health** and **willing** to follow study guidelines/rules (including medication restrictions). In addition if you have any reactions, allergies to cosmetics/ preservatives/fragrance or have any condition that might interfere with the study or use any medication that might affect results or if you are pregnant/breastfeeding, you cannot participate in this study.

The minimum amount of volunteers required for this study is 24

Please sign if agree | QC CHECK

3. RULES TO COMPLY WITH:

PLEASE READ THE FOLLOWING STATEMENTS CAREFULLY AND SIGN:

Please reply to each Block Yes/No/OK *Volunteer Signature Agreement*

I have complied with the "non-smoking an hour before the test" rule.			3.2
I am not currently busy with any other study or have not been doing a similar study in the last four (4) weeks.			3.4
I am not on cortisone medication.			3.5
I agree to refrain from using any other creams in the morning AND evening except for the cream supplied.			3.6
I do not have/or are aware of any skin disease.			3.7
I am not being treated for asthma.			3.8
I am not receiving systemic or topical drugs or medication , which can influence the study results.			3.9
I have used skin care products in the past			3.10
I have used sunscreen products in the past.			3.11
I have adhered to comply to a three (3) days washout before testing commence			3.12
I agree to have my face shaved if too many vellus hair			3.13
I agree not to make any changes to my laundry soap/softener for the duration of the test .			3.14
I full understand above statements.			3.15
I agree to wear one (1) shade of foundation for the duration of the study. *Please sign if agree* _____			
I agree to apply the foundation at least once a day in the morning and would wear it for at least 8 hours per day for the duration of the study. *Please sign if agree* _____			
I agree to wear the supplied products every day for 42 days. *Please sign if agree* _____			

4. DURATION OF EVALUATION AND PROCEDURES: Baseline (DAY 0): You will be asked to read and sign the informed consent form (Appendix C) and your medical history (Appendix D) will be reviewed to determine if you qualify for the study. If you meet the inclusion criteria, you must sign an attendance register. The test sites will be examined for hair, bruises, scarring etc. If you qualify a battery of tests will be performed, with test product application at the test facility. You will receive a flow chart which would indicate at which time intervals you need to return to the testing facility for further evaluations. The approximate time for procedure is 2 hours. **DAY 14** and **DAY 28**: You will be asked to sign attendance register. Relevant test procedures to be performed. Approximate time for procedures is 2 hours.

Please sign if agree | QC CHECK

5. RISK & UNFORESEEN RISKS: The test products are intended to come in contact with skin, there might be irritation/allergic or other positive reactions to the test sites, the test sites will be marked for the entire study. Most of these reactions are rapidly reversible. There may be unknown risks, in extreme rare cases, blistering may occur which might cause blemishes/ scarring.

Please sign if agree | QC CHECK | 3.16

6. COMPENSATION FOR INJURY: In the unlikely event that medical treatment is required due to a severe reaction linked to the participation of this test, appropriate and reasonable medical treatment will be provided at our discretion when it is deemed necessary. Provision of such care is not an admission of legal liability or responsibility for the condition being treated. Extended medical care will not be provided.

Please sign if agree | QC CHECK

7. BENEFITS: Participation in this study is voluntary, you are not an employee of the company and will not be compensated at month end. You will only be paid after the completion of the entire study as communicated to you by the study leader, the money will be transferred into your account after the testing has been completed. If the study leader concludes it would be best to discontinue your participation due to a severe reaction, you will be paid in full. If you are dismissed for not obeying rules and following instructions, you will not be paid.

Please sign if agree | QC CHECK | 3.17

8. CONFIDENTIALITY: Reports prepared by Future Cosmetics CC will utilize descriptive information only. Confidentiality of any information you provide will be maintained to the maximum extent possible. You are not allowed to convey any confidential information to any person on the test procedures or products or companies tested.

Please sign if agree | QC CHECK

9. EMERGENCY CONTACT: During the study, should you experience any medical problems, suffer a research-related injury or have questions about the study, please contact the study investigator on **0608 932 934 / 012 811 0621**

10. VOLUNTARY PARTICIPATION AND WITHDRAWAL: Your participation in this study is entirely voluntary. You may decide not to participate or may withdraw from the study at any time, and if you choose to do so, you will be instructed to state your reason. Your participation in this study may be ended without your consent and without compensation (payment) for the following reasons: if you fail to follow directions/rules for participating in the study, if it is discovered that you do not meet study requirements. Your participation in this study may be ended without your consent with full or partial compensation (at discretion of the study leader) for the following reasons: If it appears to be medically harmful to you or other administrative reasons. Please confirm that you have read, understood and will comply to the following statement: **"Please do not partake in this study if you are immune-compromised (HIV + or have contracted AIDS)**

11. CONSENT: I have carefully read and understand this informed consent. The test has been explained to my satisfaction and I agree to participate in the study as outlined above. I have been given the opportunity to discuss all aspects of the study and to ask questions. I am at least 18 years old, in good health and freely give my consent to serve as a volunteer in this study. If I have any questions regarding my rights as a research participant I may call the study investigator. We at Future Cosmetics reserves the right at anytime to request a quality check, by using a voice over recognition at any time during or after a study.

" 3.18

SIGNATURE: **FULL NAME:** _____ **ADDRESS:** _____ **DATE:** _____

" 3.19

(Please take note of the payment rules)

Please note that this form must be signed off by our designated study technician before you can be enrolled into the study

FUTURE COSMETICS CC

From Concept to Product

APPENDIX D:

MEDICAL HISTORY FORM for PROTOCOL NO:	FCAG014	SUBJECT NO:		NAME & SURNAME:		AGE:	3.20
		SEX: Male	Female	RACE: Caucasian	Asian	Indian	Black - SA	Black - Nigeria

If any of the following questions is yes, please talk to the study technician at this station.

yes	no	Do you have any hair/marks/scars/broken or chapped skin on your arms	3.22	yes	no	Do you use any bath oils, skincare lotion or other cosmetic products on the face?	3.21
yes	no	Are you currently pregnant or breastfeeding?	3.24	yes	no	Have you ever experienced a phototoxic/photo allergenic responses/a photosensitive skin disease (reaction to sun)?	3.23
yes	no	Do you currently have or do you have a history of psoriasis / eczema?	3.26	yes	no	Have you had any allergic, sensitive reactions to skin products ?	3.25
yes	no	Do you have any medical conditions, which you are currently under a physician's care for?	3.26	yes	no	Do you make use of a sunbed? If yes, when was your last tanning session?	3.27

Please specify if YES to any of above was mentioned? _____ Please specify if YES to any of above was mentioned? _____

WHAT SKIN CARE PRODUCTS DO YOU USE? *Please specify* 3.29

WHAT SOAPS/PRODUCTS HAVE YOU USED IN THE LAST FOUR (4) WEEKS? *Please specify*

PLEASE INDICATE WHICH MEDICATION HAVE YOU USED WITHIN THE LAST WEEK (W), LAST MONTH (M) or LAST 3 MONTHS (3M)

Allergy Injection	Allergy medication	Antihistamines	Analgesics	Anti-anxiety	Anti-seizure	Antibiotics	Asthma medication
Antidepressants	Anti-diarrhea	Anti-hypertension	Appetite suppressants	Arthritis medication	Anti-fungal	Blood thinners	Cough/ Cold medicine
Cortisone injections	Cortisone	Diuretics	Diabetic medicine	Eye drops	Gout medication	Headache relief	Heart medication
Hormones	Itch relief	Immuno suppressive	Laxative	Muscle ache relief	Prostate medication	Premenstrual relief	Pain relievers
Sleep aid products	Steroids	Thyroid medication	Tranquilizers	Ulcer medication	Contraceptive		

IF ANY ITEMS MARKED, PLEASE LIST SPECIFIC NAMES OR LIST ANY MEDICATION TAKEN WITHIN THE LAST WEEK NOT LISTED ABOVE: 3.30

SKIN CONDITION:	Oily (cheeks, forehead, chin)	Normal/Combination (oily T panel)	Dry, Scaly	Sensitive	Known to have positive reactions to any cosmetics

HAVE YOU EVER BEEN TREATED FOR ANY OF THE FOLLOWING:							3.31
Eczema	Hair Loss/ Thinning	Keratosis	Acne	Body Fungus	Chronic Dry Skin	Cold Sore	Dandruff
			Psoriasis	Seborrhea	Skin irritation	Sensitive skin	Warts
PRODUCT SENSITIVITES:	Deodorants	Soaps	Eye Cosmetics	Facial Cosmetics	Fragrances	Moisturisers	Sunscreen Products	3.32
CANCER:	Skin	OTHER, (please specify)					
If you have marked any of the above, please specify							3.33

	Score1	Score 1.5	Score 2	Score 3	Score 4	Score 5	
YOUR NATURAL COLOUR OF UNTANNED SKIN?	Reddish-white	White Beige/ Milky	Light brown	Brown	Dark Brown	Black	
NATURAL HAIR COLOUR ?	Red, light brown	Blonde, light brown	Light Brown	Medium Brown	Brownish Black	Black	
NATURAL EYE COLOUR?	Light-blue/green/grey		Light brown	Brown	Dark Brown	Black	AVG
HOW MANY FRECKLES DO YOU HAVE?	Many	Few	Some		None	Pigmented	#####
WHICH BEST DESCRIBE YOUR GENETIC HERITAGE/NATURAL SKIN?	Rose/Pink White Skin Celtic (English, Irish)	Milk White Light skin European	White/light brown Dark skin European	White/Olive skin Mediterranean	Tanned Brown skin Middle Eastern, Indian, Asian, Hispanic, Coloured/Mix	Dark Brown/black African, black	3.34

Please note that this form must be signed off by our designated study technician before you can be enrolled into the study 3.35

FUTURE COSMETICS CC

From Concept to Product

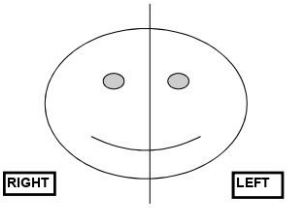
INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
STUDY NUMBER: FCAG014

TEST SUBSTANCE SEQUENCE - FACE

TEST REFERENCE:	PRODUCT REFERENCE:	PRODUCT NAME:	NO:
FCAG014	/5936	PM in Gel	1
FCAG014	/5937	Placebo Gel	2

APPLICATION AMOUNT: 0.6 grams
TEST SUB SITE: HALF-FACE

TEST REFERENCE	SUBJECT NUMBER	RIGHT HALF FACE	LEFT HALF FACE
FCAG014	/01	1	2
FCAG014	/02	1	2
FCAG014	/04	2	1
FCAG014	/05	2	1
FCAG014	/06	1	2
FCAG014	/07	2	1
FCAG014	/08	1	2
FCAG014	/09	2	1
FCAG014	/10	2	1
FCAG014	/11	1	2
FCAG014	/12	2	1
FCAG014	/13	2	1
FCAG014	/14	2	1
FCAG014	/16	1	2
FCAG014	/17	1	2
FCAG014	/18	1	2
FCAG014	/19	2	1
FCAG014	/20	1	2
FCAG014	/21	2	1
FCAG014	/22	1	2
FCAG014	/23	2	1
FCAG014	/24	1	2
FCAG014	/25	1	2
FCAG014	/27	1	2



SAMPLE PREP _____
DATE: _____

DATE: _____

APPENDIX F:

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
TEST SITE: HALF FACE
PARAMETER: PUSTULES
EVALUATION TYPE: OBJECTIVE SENSORY
STUDY NUMBER: FCAG014

TABLE F1: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5936

	Baseline (BL)		AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL		AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL
AVERAGE	0.08		0.13	0.04	0.06		0.02	-0.06	-0.06
STANDARD DEVIATION	0.35		0.33	0.29	0.38		0.14	0.24	0.32
QTY OF DATA POINTS	144								
QTY OF TEST SUBJECTS	48								

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	AVERAGE INCREASE RELATIVE TO CONTROL	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)	AVERAGE INCREASE RELATIVE TO CONTROL
/01	RF	1	1	1.0	1	1.0	0.0	0.0	0	0.0	-1.0	-1.0
/02	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/04	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/05	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/06	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/07	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/08	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/09	LF	1	0	0.0	1	1.0	1.0	1.0	0	0.0	0.0	0.0
/10	LF	1	0	0.0	1	1.0	1.0	1.0	0	0.0	0.0	0.0
/11	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/12	LF	1	0	0.0	0	0.0	0.0	-1.0	0	0.0	0.0	0.0
/13	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/14	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/16	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	-1.0
/17	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/18	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/19	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/20	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/21	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/22	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/23	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/24	RF	1	1	1.0	1	1.0	0.0	1.0	0	0.0	-1.0	0.0
/25	RF	1	0	0.0	1	1.0	1.0	1.0	0	0.0	0.0	-1.0
/27	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/01	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/02	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/04	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/05	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/06	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/07	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/08	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/09	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/10	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/11	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/12	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/13	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/14	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/16	RF	1	0	0.0	0	0.0	0.0	1.0	0	0.0	0.0	1.0
/17	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/18	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/19	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/20	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/21	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/22	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/23	LF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/24	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
/25	RF	1	2	2.0	1	1.0	-1.0	-1.0	1	1.0	-1.0	-1.0
/27	RF	1	0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
TEST SITE:	HALF FACE
PARAMETER:	PUSTULES
EVALUATION TYPE:	OBJECTIVE SENSORY
STUDY NUMBER:	FCAG014

TABLE F2: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5937

	Baseline (BL)	AVERAGE	Difference	AVERAGE	Difference
AVERAGE	0.04	0.02	-0.02	0.04	0.00
STANDARD DEVIATION	0.20	0.14	0.25	0.20	0.29
QTY OF DATA POINTS	144				
QTY OF TEST SUBJECTS	48				

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)
/01	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/02	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/04	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/05	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/06	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/07	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/08	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/09	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/10	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/11	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/12	RF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/13	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/14	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/16	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/17	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/18	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/19	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/20	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/21	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/22	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/23	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/24	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/25	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/27	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/01	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/02	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/04	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/05	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/06	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/07	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/08	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/09	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/10	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/11	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/12	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/13	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/14	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/16	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/17	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/18	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/19	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/20	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/21	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/22	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/23	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/24	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/25	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/27	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
TEST SITE:	HALF FACE
PARAMETER:	PAPULES
EVALUATION TYPE:	OBJECTIVE SENSORY
STUDY NUMBER:	FCAG014

TABLE F3: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5936

	Baseline (BL)	AVERAGE	DIFF	AVERAGE DECREASE RELATIVE TO CONTROL	AVERAGE	DIFFE	AVERAGE DECREASE RELATIVE TO CONTROL
AVERAGE	12.85	10.04	-2.81	-0.58	9.21	-3.65	0.02
STANDARD DEVIATION	17.31	18.72	8.67	9.56	10.38	12.08	13.35
QTY OF DATA POINTS	144						
QTY OF TEST SUBJECTS	48						

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	AVERAGE INCREASE RELATIVE TO CONTROL	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)	AVERAGE INCREASE RELATIVE TO CONTROL
/01	RF	1	15	15.00	11	11.00	-4.00	-1.00	3	3.0	-12.0	-13.0
/02	RF	1	1	1.00	5	5.00	4.00	4.00	0	0.0	-1.0	-2.0
/04	LF	1	8	8.00	3	3.00	-5.00	-12.00	1	1.0	-7.0	-10.0
/05	LF	1	2	2.00	0	0.00	-2.00	-1.00	0	0.0	-2.0	-1.0
/06	RF	1	8	8.00	9	9.00	1.00	2.00	27	27.0	19.0	14.0
/07	LF	1	1	1.00	2	2.00	1.00	2.00	0	0.0	-1.0	2.0
/08	RF	1	3	3.00	2	2.00	-1.00	3.00	0	0.0	-3.0	2.0
/09	LF	1	10	10.00	11	11.00	1.00	-1.00	6	6.0	-4.0	8.0
/10	LF	1	2	2.00	3	3.00	1.00	9.00	6	6.0	4.0	9.0
/11	RF	1	2	2.00	5	5.00	3.00	2.00	1	1.0	-1.0	-4.0
/12	LF	1	1	1.00	0	0.00	-1.00	-1.00	0	0.0	-1.0	-1.0
/13	LF	1	7	7.00	3	3.00	-4.00	1.00	1	1.0	-6.0	-2.0
/14	LF	1	8	8.00	7	7.00	-1.00	-2.00	9	9.0	1.0	7.0
/16	RF	1	0	0.00	3	3.00	3.00	3.00	5	5.0	5.0	2.0
/17	RF	1	3	3.00	2	2.00	-1.00	3.00	1	1.0	-2.0	2.0
/18	RF	1	2	2.00	1	1.00	-1.00	-1.00	1	1.0	-1.0	1.0
/19	LF	1	1	1.00	1	1.00	0.00	1.00	3	3.0	2.0	4.0
/20	RF	1	13	13.00	16	16.00	3.00	8.00	12	12.0	-1.0	17.0
/21	LF	1	4	4.00	1	1.00	-3.00	2.00	0	0.0	-4.0	1.0
/22	RF	1	5	5.00	2	2.00	-3.00	-3.00	3	3.0	-2.0	-4.0
/23	LF	1	2	2.00	1	1.00	-1.00	0.00	1	1.0	-1.0	1.0
/24	RF	1	22	22.00	11	11.00	-11.00	2.00	32	32.0	10.0	17.0
/25	RF	1	52	52.00	55	55.00	3.00	18.00	30	30.0	-22.0	22.0
/27	RF	1	8	8.00	4	4.00	-4.00	8.00	8	8.0	0.0	12.0
/01	RF	1	6	6.00	13	13.00	7.00	9.00	6	6.0	0.0	1.0
/02	RF	1	2	2.00	9	9.00	7.00	3.00	2	2.0	0.0	-6.0
/04	LF	1	27	27.00	19	19.00	-8.00	-20.00	25	25.0	-2.0	-35.0
/05	LF	1	5	5.00	8	8.00	3.00	2.00	5	5.0	0.0	-3.0
/06	RF	1	19	19.00	12	12.00	-7.00	2.00	21	21.0	2.0	17.0
/07	LF	1	7	7.00	4	4.00	-3.00	3.00	6	6.0	-1.0	-9.0
/08	RF	1	22	22.00	4	4.00	-18.00	-5.00	9	9.0	-13.0	-6.0
/09	LF	1	13	13.00	9	9.00	-4.00	16.00	10	10.0	-3.0	14.0
/10	LF	1	45	45.00	7	7.00	-38.00	-36.00	9	9.0	-36.0	-33.0
/11	RF	1	25	25.00	11	11.00	-14.00	-12.00	29	29.0	4.0	-35.0
/12	LF	1	10	10.00	6	6.00	-4.00	10.00	10	10.0	0.0	10.0
/13	LF	1	14	14.00	3	3.00	-11.00	-10.00	4	4.0	-10.0	-7.0
/14	LF	1	18	18.00	10	10.00	-8.00	-14.00	18	18.0	0.0	1.0
/16	RF	1	7	7.00	3	3.00	-4.00	2.00	10	10.0	3.0	3.0
/17	RF	1	5	5.00	6	6.00	1.00	1.00	2	2.0	-3.0	-5.0
/18	RF	1	8	8.00	3	3.00	-5.00	-4.00	8	8.0	0.0	-1.0
/19	LF	1	5	5.00	0	0.00	-5.00	-8.00	6	6.0	1.0	-4.0
/20	RF	1	39	39.00	25	25.00	-14.00	-25.00	16	16.0	-23.0	2.0
/21	LF	1	7	7.00	5	5.00	-2.00	-4.00	2	2.0	-5.0	-2.0
/22	RF	1	2	2.00	12	12.00	10.00	12.00	6	6.0	4.0	5.0
/23	LF	1	5	5.00	4	4.00	-1.00	1.00	4	4.0	-1.0	4.0
/24	RF	1	32	32.00	14	14.00	-18.00	7.00	38	38.0	6.0	28.0
/25	RF	1	99	99.00	122	122.00	23.00	-2.00	36	36.0	-63.0	-33.0
/27	RF	1	15	15.00	15	15.00	0.00	-2.00	10	10.0	-5.0	11.0

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
TEST SITE:	HALF FACE
PARAMETER:	PAPULES
EVALUATION TYPE:	OBJECTIVE SENSORY
STUDY NUMBER:	FCAG014

TABLE F4: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5937

	Baseline (BL)	AVERAGE	Difference	AVERAGE	Difference
AVERAGE	12.50	10.27	-2.23	8.83	-3.67
STANDARD DEVIATION	13.12	14.16	8.07	9.49	12.97
QTY OF DATA POINTS	144.0				
QTY OF TEST SUBJECTS	48				

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)
/01	LF	2	8.0	**8.00**	5.0	**5.00**	**-3.00**	9.0	**9.00**	**1.00**
/02	LF	2	0.0	**0.00**	0.0	**0.00**	**0.00**	1.0	**1.00**	**1.00**
/04	RF	2	7.0	**7.00**	14.0	**14.00**	**7.00**	10.0	**10.00**	**3.00**
/05	RF	2	2.0	**2.00**	1.0	**1.00**	**-1.00**	1.0	**1.00**	**-1.00**
/06	LF	2	7.0	**7.00**	6.0	**6.00**	**-1.00**	12.0	**12.00**	**5.00**
/07	RF	2	3.0	**3.00**	2.0	**2.00**	**-1.00**	0.0	**0.00**	**-3.00**
/08	LF	2	10.0	**10.00**	6.0	**6.00**	**-4.00**	5.0	**5.00**	**-5.00**
/09	RF	2	15.0	**15.00**	17.0	**17.00**	**2.00**	3.0	**3.00**	**-12.00**
/10	RF	2	11.0	**11.00**	3.0	**3.00**	**-8.00**	6.0	**6.00**	**-5.00**
/11	LF	2	6.0	**6.00**	7.0	**7.00**	**1.00**	9.0	**9.00**	**3.00**
/12	RF	2	0.0	**0.00**	0.0	**0.00**	**0.00**	0.0	**0.00**	**0.00**
/13	RF	2	6.0	**6.00**	1.0	**1.00**	**-5.00**	2.0	**2.00**	**-4.00**
/14	RF	2	9.0	**9.00**	10.0	**10.00**	**1.00**	3.0	**3.00**	**-6.00**
/16	LF	2	1.0	**1.00**	1.0	**1.00**	**0.00**	4.0	**4.00**	**3.00**
/17	LF	2	6.0	**6.00**	2.0	**2.00**	**-4.00**	2.0	**2.00**	**-4.00**
/18	LF	2	5.0	**5.00**	5.0	**5.00**	**0.00**	3.0	**3.00**	**-2.00**
/19	RF	2	4.0	**4.00**	3.0	**3.00**	**-1.00**	2.0	**2.00**	**-2.00**
/20	LF	2	33.0	**33.00**	28.0	**28.00**	**-5.00**	15.0	**15.00**	**-18.00**
/21	RF	2	6.0	**6.00**	1.0	**1.00**	**-5.00**	1.0	**1.00**	**-5.00**
/22	LF	2	0.0	**0.00**	0.0	**0.00**	**0.00**	2.0	**2.00**	**2.00**
/23	RF	2	3.0	**3.00**	2.0	**2.00**	**-1.00**	1.0	**1.00**	**-2.00**
/24	LF	2	20.0	**20.00**	7.0	**7.00**	**-13.00**	13.0	**13.00**	**-7.00**
/25	LF	2	60.0	**60.00**	45.0	**45.00**	**-15.00**	16.0	**16.00**	**-44.00**
/27	LF	2	19.0	**19.00**	7.0	**7.00**	**-12.00**	7.0	**7.00**	**-12.00**
/01	LF	2	9.0	**9.00**	7.0	**7.00**	**-2.00**	8.0	**8.00**	**-1.00**
/02	LF	2	2.0	**2.00**	6.0	**6.00**	**4.00**	8.0	**8.00**	**6.00**
/04	RF	2	3.0	**3.00**	15.0	**15.00**	**12.00**	36.0	**36.00**	**33.00**
/05	RF	2	3.0	**3.00**	4.0	**4.00**	**1.00**	6.0	**6.00**	**3.00**
/06	LF	2	29.0	**29.00**	20.0	**20.00**	**-9.00**	14.0	**14.00**	**-15.00**
/07	RF	2	16.0	**16.00**	10.0	**10.00**	**-6.00**	24.0	**24.00**	**8.00**
/08	LF	2	16.0	**16.00**	3.0	**3.00**	**-13.00**	9.0	**9.00**	**-7.00**
/09	RF	2	24.0	**24.00**	4.0	**4.00**	**-20.00**	7.0	**7.00**	**-17.00**
/10	RF	2	16.0	**16.00**	14.0	**14.00**	**-2.00**	13.0	**13.00**	**-3.00**
/11	LF	2	14.0	**14.00**	12.0	**12.00**	**-2.00**	53.0	**53.00**	**39.00**
/12	RF	2	16.0	**16.00**	2.0	**2.00**	**-14.00**	6.0	**6.00**	**-10.00**
/13	RF	2	10.0	**10.00**	9.0	**9.00**	**-1.00**	7.0	**7.00**	**-3.00**
/14	RF	2	8.0	**8.00**	14.0	**14.00**	**6.00**	7.0	**7.00**	**-1.00**
/16	LF	2	6.0	**6.00**	0.0	**0.00**	**-6.00**	6.0	**6.00**	**0.00**
/17	LF	2	11.0	**11.00**	11.0	**11.00**	**0.00**	13.0	**13.00**	**2.00**
/18	LF	2	9.0	**9.00**	8.0	**8.00**	**-1.00**	10.0	**10.00**	**1.00**
/19	RF	2	0.0	**0.00**	3.0	**3.00**	**3.00**	5.0	**5.00**	**5.00**
/20	LF	2	38.0	**38.00**	49.0	**49.00**	**11.00**	13.0	**13.00**	**-25.00**
/21	RF	2	5.0	**5.00**	7.0	**7.00**	**2.00**	2.0	**2.00**	**-3.00**
/22	LF	2	4.0	**4.00**	2.0	**2.00**	**-2.00**	3.0	**3.00**	**-1.00**
/23	RF	2	10.0	**10.00**	8.0	**8.00**	**-2.00**	5.0	**5.00**	**-5.00**
/24	LF	2	37.0	**37.00**	12.0	**12.00**	**-25.00**	15.0	**15.00**	**-22.00**
/25	LF	2	52.0	**52.00**	77.0	**77.00**	**25.00**	22.0	**22.00**	**-30.00**
/27	LF	2	21.0	**21.00**	23.0	**23.00**	**2.00**	5.0	**5.00**	**-16.00**

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
TEST SITE:	HALF FACE
PARAMETER:	WHITEHEADS
EVALUATION TYPE:	OBJECTIVE SENSORY
STUDY NUMBER:	FCAG014

TABLE F5: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5936

	Baseline (BL)		AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL		AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL
AVERAGE	8.29		6.42	-1.88	-7.30		4.17	-4.13	-8.47
STANDARD DEVIATION	8.98		8.64	7.63	10.55		4.08	7.03	12.85
QTY OF DATA POINTS	144								
QTY OF TEST SUBJECTS	48								

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	AVERAGE INCREASE RELATIVE TO CONTROL	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)	AVERAGE INCREASE RELATIVE TO CONTROL
/01	RF	1	4	4.00	0	0.00	-4.00	-4.00	6	6.00	2.00	2.50
/02	RF	1	1	1.00	1	1.00	0.00	-5.00	0	0.00	-1.00	-1.00
/04	LF	1	4	4.00	0	0.00	-4.00	-2.50	1	1.00	-3.00	-6.00
/05	LF	1	0	0.00	0	0.00	0.00	1.50	1	1.00	1.00	4.00
/06	RF	1	2	2.00	4	4.00	2.00	-1.50	4	4.00	2.00	1.50
/07	LF	1	0	0.00	0	0.00	0.00	2.50	0	0.00	0.00	-2.50
/08	RF	1	1	1.00	1	1.00	0.00	4.50	0	0.00	-1.00	6.00
/09	LF	1	17	17.00	9	9.00	-8.00	-10.50	7	7.00	-10.00	-12.00
/10	LF	1	2	2.00	12	12.00	10.00	11.50	1	1.00	-1.00	3.00
/11	RF	1	4	4.00	0	0.00	-4.00	1.50	2	2.00	-2.00	5.50
/12	LF	1	1	1.00	0	0.00	-1.00	-1.50	0	0.00	-1.00	-0.50
/13	LF	1	1	1.00	1	1.00	0.00	0.00	0	0.00	-1.00	-3.50
/14	LF	1	4	4.00	3	3.00	-1.00	-14.50	1	1.00	-3.00	8.50
/16	RF	1	1	1.00	2	2.00	1.00	1.50	2	2.00	1.00	-4.00
/17	RF	1	0	0.00	1	1.00	1.00	6.50	0	0.00	0.00	2.00
/18	RF	1	1	1.00	0	0.00	-1.00	-3.00	0	0.00	-1.00	-4.50
/19	LF	1	4	4.00	9	9.00	5.00	-8.00	5	5.00	1.00	2.50
/20	RF	1	25	25.00	3	3.00	-22.00	-23.00	2	2.00	-23.00	-16.00
/21	LF	1	3	3.00	0	0.00	-3.00	-8.50	3	3.00	0.00	1.50
/22	RF	1	0	0.00	0	0.00	0.00	-2.50	1	1.00	1.00	1.00
/23	LF	1	1	1.00	1	1.00	0.00	-1.00	3	3.00	2.00	1.50
/24	RF	1	3	3.00	7	7.00	4.00	5.00	3	3.00	0.00	-14.00
/25	RF	1	10	10.00	5	5.00	-5.00	-2.00	6	6.00	-4.00	12.50
/27	RF	1	2	2.00	13	13.00	11.00	13.50	5	5.00	3.00	5.50
/01	RF	1	8	8.00	6	6.00	-2.00	-0.50	9	9.00	1.00	2.50
/02	RF	1	6	6.00	7	7.00	1.00	1.50	1	1.00	-5.00	-4.50
/04	LF	1	14	14.00	9	9.00	-5.00	-5.50	10	10.00	-4.00	-4.50
/05	LF	1	3	3.00	0	0.00	-3.00	-4.50	0	0.00	-3.00	-4.50
/06	RF	1	15	15.00	12	12.00	-3.00	-5.50	16	16.00	1.00	-1.50
/07	LF	1	7	7.00	1	1.00	-6.00	-9.50	1	1.00	-6.00	-9.50
/08	RF	1	20	20.00	4	4.00	-16.00	-20.50	9	9.00	-11.00	-15.50
/09	LF	1	29	29.00	17	17.00	-12.00	-17.50	15	15.00	-14.00	-19.50
/10	LF	1	5	5.00	5	5.00	0.00	-6.50	1	1.00	-4.00	-10.50
/11	RF	1	20	20.00	3	3.00	-17.00	-24.50	8	8.00	-12.00	-19.50
/12	LF	1	1	1.00	3	3.00	2.00	-6.50	1	1.00	0.00	-8.50
/13	LF	1	7	7.00	2	2.00	-5.00	-14.50	1	1.00	-6.00	-15.50
/14	LF	1	11	11.00	18	18.00	7.00	-3.50	8	8.00	-3.00	-13.50
/16	RF	1	7	7.00	15	15.00	8.00	-3.50	12	12.00	5.00	-6.50
/17	RF	1	17	17.00	2	2.00	-15.00	-27.50	9	9.00	-8.00	-20.50
/18	RF	1	6	6.00	0	0.00	-6.00	-19.50	2	2.00	-4.00	-17.50
/19	LF	1	11	11.00	18	18.00	7.00	-7.50	4	4.00	-7.00	-21.50
/20	RF	1	34	34.00	16	16.00	-18.00	-33.50	11	11.00	-23.00	-38.50
/21	LF	1	5	5.00	9	9.00	4.00	-12.50	4	4.00	-1.00	-17.50
/22	RF	1	5	5.00	1	1.00	-4.00	-21.50	4	4.00	-1.00	-18.50
/23	LF	1	2	2.00	1	1.00	-1.00	-19.50	5	5.00	3.00	-15.50
/24	RF	1	26	26.00	32	32.00	6.00	-13.50	5	5.00	-21.00	-40.50
/25	RF	1	26	26.00	43	43.00	17.00	-3.50	5	5.00	-21.00	-41.50
/27	RF	1	22	22.00	12	12.00	-10.00	-31.50	6	6.00	-16.00	-37.50

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
TEST SITE: HALF FACE
PARAMETER: WHITEHEADS
EVALUATION TYPE: OBJECTIVE SENSORY
STUDY NUMBER: FCAG014

TABLE F6: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5937

	Baseline (BL)	AVERAGE	Difference	AVERAGE	Difference
AVERAGE	7.79	8.65	0.85	6.48	-1.31
STANDARD DEVIATION	8.89	10.12	6.13	7.13	7.84
QTY OF DATA POINTS	144				
QTY OF TEST SUBJECTS	48				

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)
/01	LF	2	1	1.00	5	5.00	4.00	2	2.00	1.00
/02	LF	2	0	0.00	4	4.00	4.00	0	0.00	0.00
/04	RF	2	2	2.00	2	2.00	0.00	1	1.00	-1.00
/05	RF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/06	LF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/07	RF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/08	LF	2	0	0.00	3	3.00	3.00	0	0.00	0.00
/09	RF	2	12	12.00	16	16.00	4.00	22	22.00	10.00
/10	RF	2	1	1.00	6	6.00	5.00	1	1.00	0.00
/11	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/12	RF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/13	RF	2	3	3.00	4	4.00	1.00	3	3.00	0.00
/14	RF	2	6	6.00	15	15.00	9.00	0	0.00	-6.00
/16	LF	2	0	0.00	0	0.00	0.00	4	4.00	4.00
/17	LF	2	1	1.00	0	0.00	-1.00	2	2.00	1.00
/18	LF	2	0	0.00	1	1.00	1.00	1	1.00	1.00
/19	RF	2	15	15.00	27	27.00	12.00	15	15.00	0.00
/20	LF	2	8	8.00	6	6.00	-2.00	3	3.00	-5.00
/21	RF	2	3	3.00	2	2.00	-1.00	1	1.00	-2.00
/22	LF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/23	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/24	LF	2	2	2.00	8	8.00	6.00	24	24.00	22.00
/25	LF	2	11	11.00	9	9.00	-2.00	5	5.00	-6.00
/27	LF	2	6	6.00	10	10.00	4.00	16	16.00	10.00
/01	LF	2	8	8.00	4	4.00	-4.00	6	6.00	-2.00
/02	LF	2	4	4.00	10	10.00	6.00	4	4.00	0.00
/04	RF	2	7	7.00	4	4.00	-3.00	14	14.00	7.00
/05	RF	2	7	7.00	4	4.00	-3.00	2	2.00	-5.00
/06	LF	2	5	5.00	11	11.00	6.00	6	6.00	1.00
/07	RF	2	6	6.00	2	2.00	-4.00	12	12.00	6.00
/08	LF	2	17	17.00	5	5.00	-12.00	3	3.00	-14.00
/09	RF	2	28	28.00	29	29.00	1.00	22	22.00	-6.00
/10	RF	2	11	11.00	3	3.00	-8.00	3	3.00	-8.00
/11	LF	2	19	19.00	8	8.00	-11.00	3	3.00	-16.00
/12	RF	2	3	3.00	5	5.00	2.00	3	3.00	0.00
/13	RF	2	8	8.00	7	7.00	-1.00	13	13.00	5.00
/14	RF	2	28	28.00	46	46.00	18.00	11	11.00	-17.00
/16	LF	2	4	4.00	3	3.00	-1.00	10	10.00	6.00
/17	LF	2	14	14.00	4	4.00	-10.00	9	9.00	-5.00
/18	LF	2	1	1.00	4	4.00	3.00	7	7.00	6.00
/19	RF	2	18	18.00	32	32.00	14.00	15	15.00	-3.00
/20	LF	2	22	22.00	26	26.00	4.00	13	13.00	-9.00
/21	RF	2	4	4.00	16	16.00	12.00	3	3.00	-1.00
/22	LF	2	4	4.00	8	8.00	4.00	4	4.00	0.00
/23	RF	2	1	1.00	3	3.00	2.00	2	2.00	1.00
/24	LF	2	20	20.00	12	12.00	-8.00	26	26.00	6.00
/25	LF	2	32	32.00	28	28.00	-4.00	5	5.00	-27.00
/27	LF	2	29	29.00	20	20.00	-9.00	14	14.00	-15.00

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
TEST SITE: HALF FACE
PARAMETER: COMEDONES
EVALUATION TYPE: OBJECTIVE SENSORY
STUDY NUMBER: FCAG014

TABLE F7: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5936

	Baseline (BL)	AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL	AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL
AVERAGE	1.19	0.67	-0.52	-6.18	0.56	-0.63	-6.79
STANDARD DEVIATION	1.95	0.93	1.65	8.15	0.85	2.00	9.16
QTY OF DATA POINTS	144						
QTY OF TEST SUBJECTS	48						

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	AVERAGE INCREASE RELATIVE TO CONTROL	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)	AVERAGE INCREASE RELATIVE TO CONTROL
/01	RF	1	0	0.00	2	2.00	2.00	2.00	0	0.00	0.00	0.50
/02	RF	1	1	1.00	0	0.00	-1.00	1.00	1	1.00	0.00	2.00
/04	LF	1	2	2.00	2	2.00	0.00	-1.00	4	4.00	2.00	2.50
/05	LF	1	1	1.00	1	1.00	0.00	0.50	0	0.00	-1.00	0.50
/06	RF	1	0	0.00	2	2.00	2.00	3.00	1	1.00	1.00	3.00
/07	LF	1	4	4.00	0	0.00	-4.00	-5.00	1	1.00	-3.00	-4.00
/08	RF	1	0	0.00	1	1.00	1.00	3.00	0	0.00	0.00	2.50
/09	LF	1	1	1.00	0	0.00	-1.00	0.00	0	0.00	-1.00	0.00
/10	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.50
/11	RF	1	0	0.00	1	1.00	1.00	1.50	0	0.00	0.00	0.50
/12	LF	1	0	0.00	0	0.00	0.00	-1.00	0	0.00	0.00	1.00
/13	LF	1	0	0.00	0	0.00	0.00	1.00	0	0.00	0.00	0.50
/14	LF	1	0	0.00	2	2.00	2.00	1.50	1	1.00	1.00	1.50
/16	RF	1	0	0.00	0	0.00	0.00	-1.00	1	1.00	1.00	1.50
/17	RF	1	0	0.00	0	0.00	0.00	-0.50	2	2.00	2.00	2.50
/18	RF	1	0	0.00	2	2.00	2.00	1.50	3	3.00	3.00	3.50
/19	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	-1.00
/20	RF	1	3	3.00	0	0.00	-3.00	-2.50	0	0.00	-3.00	-2.50
/21	LF	1	1	1.00	0	0.00	-1.00	-1.00	0	0.00	-1.00	-1.00
/22	RF	1	0	0.00	0	0.00	0.00	0.50	1	1.00	1.00	1.00
/23	LF	1	0	0.00	0	0.00	0.00	1.00	0	0.00	0.00	1.00
/24	RF	1	2	2.00	1	1.00	-1.00	-0.50	0	0.00	-2.00	0.50
/25	RF	1	0	0.00	0	0.00	0.00	-1.50	1	1.00	1.00	1.00
/27	RF	1	0	0.00	0	0.00	0.00	1.00	0	0.00	0.00	-0.50
/01	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	-1.50
/02	RF	1	1	1.00	1	1.00	0.00	-1.00	1	1.00	0.00	-2.50
/04	LF	1	10	10.00	4	4.00	-6.00	-8.00	1	1.00	-9.00	-12.50
/05	LF	1	2	2.00	1	1.00	-1.00	-4.00	0	0.00	-2.00	-6.50
/06	RF	1	0	0.00	0	0.00	0.00	-4.00	1	1.00	1.00	-4.50
/07	LF	1	6	6.00	2	2.00	-4.00	-9.00	2	2.00	-4.00	-10.50
/08	RF	1	0	0.00	0	0.00	0.00	-6.00	0	0.00	0.00	-7.50
/09	LF	1	3	3.00	0	0.00	-3.00	-10.00	0	0.00	-3.00	-11.50
/10	LF	1	0	0.00	0	0.00	0.00	-8.00	0	0.00	0.00	-9.50
/11	RF	1	1	1.00	0	0.00	-1.00	-10.00	0	0.00	-1.00	-11.50
/12	LF	1	0	0.00	1	1.00	1.00	-9.00	1	1.00	1.00	-10.50
/13	LF	1	5	5.00	2	2.00	-3.00	-14.00	1	1.00	-4.00	-16.50
/14	LF	1	1	1.00	0	0.00	-1.00	-13.00	0	0.00	-1.00	-14.50
/16	RF	1	1	1.00	2	2.00	1.00	-12.00	1	1.00	0.00	-14.50
/17	RF	1	0	0.00	0	0.00	0.00	-14.00	1	1.00	1.00	-14.50
/18	RF	1	0	0.00	0	0.00	0.00	-15.00	1	1.00	1.00	-15.50
/19	LF	1	3	3.00	1	1.00	-2.00	-18.00	1	1.00	-2.00	-19.50
/20	RF	1	1	1.00	1	1.00	0.00	-17.00	0	0.00	-1.00	-19.50
/21	LF	1	0	0.00	0	0.00	0.00	-18.00	0	0.00	0.00	-19.50
/22	RF	1	0	0.00	0	0.00	0.00	-19.00	0	0.00	0.00	-20.50
/23	LF	1	1	1.00	2	2.00	1.00	-19.00	0	0.00	-1.00	-22.50
/24	RF	1	2	2.00	0	0.00	-2.00	-23.00	0	0.00	-2.00	-24.50
/25	RF	1	1	1.00	0	0.00	-1.00	-23.00	0	0.00	-1.00	-24.50
/27	RF	1	4	4.00	1	1.00	-3.00	-26.00	0	0.00	-4.00	-28.50

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
TEST SITE:	HALF FACE
PARAMETER:	COMEDONES
EVALUATION TYPE:	OBJECTIVE SENSORY
STUDY NUMBER:	FCAG014

TABLE F8: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5937

	Baseline (BL)	AVERAGE	Difference	AVERAGE	Difference
AVERAGE	1.08	0.90	-0.19	0.42	-0.67
STANDARD DEVIATION	1.27	1.28	1.25	0.94	1.21
QTY OF DATA POINTS	144				
QTY OF TEST SUBJECTS	48				

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)
/01	LF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/02	LF	2	2	2.00	0	0.00	-2.00	0	0.00	-2.00
/04	RF	2	5	5.00	5	5.00	0.00	4	4.00	-1.00
/05	RF	2	2	2.00	0	0.00	-2.00	0	0.00	-2.00
/06	LF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/07	RF	2	0	0.00	1	1.00	1.00	1	1.00	1.00
/08	LF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/09	RF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/10	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/11	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/12	RF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/13	RF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/14	RF	2	1	1.00	2	2.00	1.00	1	1.00	0.00
/16	LF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/17	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/18	LF	2	0	0.00	2	2.00	2.00	0	0.00	0.00
/19	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/20	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/21	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/22	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/23	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/24	LF	2	2	2.00	1	1.00	-1.00	1	1.00	-1.00
/25	LF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/27	LF	2	1	1.00	1	1.00	0.00	2	2.00	1.00
/01	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/02	LF	2	2	2.00	0	0.00	-2.00	0	0.00	-2.00
/04	RF	2	0	0.00	2	2.00	2.00	0	0.00	0.00
/05	RF	2	1	1.00	2	2.00	1.00	0	0.00	-1.00
/06	LF	2	3	3.00	1	1.00	-2.00	0	0.00	-3.00
/07	RF	2	3	3.00	4	4.00	1.00	4	4.00	1.00
/08	LF	2	4	4.00	0	0.00	-4.00	0	0.00	-4.00
/09	RF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/10	RF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/11	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/12	RF	2	2	2.00	3	3.00	1.00	0	0.00	-2.00
/13	RF	2	2	2.00	0	0.00	-2.00	0	0.00	-2.00
/14	RF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/16	LF	2	1	1.00	2	2.00	1.00	0	0.00	-1.00
/17	LF	2	1	1.00	2	2.00	1.00	0	0.00	-1.00
/18	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/19	RF	2	0	0.00	0	0.00	0.00	2	2.00	2.00
/20	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/21	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/22	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/23	RF	2	2	2.00	0	0.00	-2.00	0	0.00	-2.00
/24	LF	2	5	5.00	5	5.00	0.00	1	1.00	-4.00
/25	LF	2	0	0.00	2	2.00	2.00	0	0.00	0.00
/27	LF	2	2	2.00	0	0.00	-2.00	2	2.00	0.00

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
TEST SITE: HALF FACE
PARAMETER: BLACK HEADS
EVALUATION TYPE: OBJECTIVE SENSORY
STUDY NUMBER: FCAG014

TABLE F9: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5936

	Baseline (BL)	AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL	AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL
AVERAGE	3.44	2.79	-0.65	-6.61	2.19	-1.25	-6.82
STANDARD DEVIATION	4.63	3.44	2.74	8.58	3.81	3.34	9.54
QTY OF DATA POINTS	144						
QTY OF TEST SUBJECTS	48						

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE / EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 / EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	AVERAGE INCREASE RELATIVE TO CONTROL	READING AT D28 / EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)	AVERAGE INCREASE RELATIVE TO CONTROL
/01	RF	1	0	0.00	0	0.00	0.00	2.50	0	0.00	0.00	3.00
/02	RF	1	2	2.00	0	0.00	-2.00	-1.00	0	0.00	-2.00	-1.00
/04	LF	1	0	0.00	0	0.00	0.00	1.50	0	0.00	0.00	1.00
/05	LF	1	0	0.00	0	0.00	0.00	1.50	0	0.00	0.00	2.50
/06	RF	1	0	0.00	0	0.00	0.00	1.00	5	5.00	5.00	7.50
/07	LF	1	17	17.00	6	6.00	-11.00	2.00	16	16.00	-1.00	-4.50
/08	RF	1	0	0.00	1	1.00	1.00	-0.50	0	0.00	0.00	2.50
/09	LF	1	1	1.00	0	0.00	-1.00	-3.50	0	0.00	-1.00	-3.50
/10	LF	1	1	1.00	0	0.00	-1.00	0.00	0	0.00	-1.00	1.00
/11	RF	1	3	3.00	2	2.00	-1.00	-1.50	0	0.00	-3.00	-4.00
/12	LF	1	3	3.00	4	4.00	1.00	-1.00	2	2.00	-1.00	-2.50
/13	LF	1	3	3.00	5	5.00	2.00	4.00	0	0.00	-3.00	-1.50
/14	LF	1	2	2.00	3	3.00	1.00	-4.00	2	2.00	0.00	-1.50
/16	RF	1	1	1.00	1	1.00	0.00	0.00	1	1.00	0.00	0.50
/17	RF	1	1	1.00	0	0.00	-1.00	-1.00	1	1.00	0.00	0.50
/18	RF	1	0	0.00	0	0.00	0.00	-2.50	0	0.00	0.00	0.00
/19	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	-0.50
/20	RF	1	1	1.00	0	0.00	-1.00	2.50	0	0.00	-1.00	-3.00
/21	LF	1	0	0.00	0	0.00	0.00	1.00	0	0.00	0.00	1.00
/22	RF	1	0	0.00	1	1.00	1.00	1.50	2	2.00	2.00	3.00
/23	LF	1	0	0.00	0	0.00	0.00	0.00	2	2.00	2.00	1.50
/24	RF	1	0	0.00	1	1.00	1.00	0.50	3	3.00	3.00	3.50
/25	RF	1	2	2.00	4	4.00	2.00	-11.00	2	2.00	0.00	1.00
/27	RF	1	0	0.00	0	0.00	0.00	0.50	0	0.00	0.00	1.00
/01	RF	1	0	0.00	5	5.00	5.00	4.50	1	1.00	1.00	1.00
/02	RF	1	1	1.00	1	1.00	0.00	-1.50	2	2.00	1.00	0.00
/04	LF	1	9	9.00	4	4.00	-5.00	-7.50	0	0.00	-9.00	-11.00
/05	LF	1	4	4.00	2	2.00	-2.00	-5.50	1	1.00	-3.00	-6.00
/06	RF	1	6	6.00	4	4.00	-2.00	-6.50	3	3.00	-3.00	-7.00
/07	LF	1	16	16.00	16	16.00	0.00	-5.50	19	19.00	3.00	-2.00
/08	RF	1	2	2.00	3	3.00	1.00	-5.50	0	0.00	-2.00	-8.00
/09	LF	1	2	2.00	4	4.00	2.00	-5.50	1	1.00	-1.00	-8.00
/10	LF	1	5	5.00	2	2.00	-3.00	-11.50	2	2.00	-3.00	-11.00
/11	RF	1	14	14.00	10	10.00	-4.00	-13.50	3	3.00	-11.00	-20.00
/12	LF	1	5	5.00	6	6.00	1.00	-9.50	6	6.00	1.00	-9.00
/13	LF	1	14	14.00	5	5.00	-9.00	-20.50	7	7.00	-7.00	-18.00
/14	LF	1	9	9.00	5	5.00	-4.00	-16.50	10	10.00	1.00	-11.00
/16	RF	1	2	2.00	1	1.00	-1.00	-14.50	1	1.00	-1.00	-14.00
/17	RF	1	1	1.00	5	5.00	4.00	-10.50	1	1.00	0.00	-14.00
/18	RF	1	5	5.00	3	3.00	-2.00	-17.50	0	0.00	-5.00	-20.00
/19	LF	1	4	4.00	5	5.00	1.00	-15.50	1	1.00	-3.00	-19.00
/20	RF	1	5	5.00	3	3.00	-2.00	-19.50	2	2.00	-3.00	-20.00
/21	LF	1	1	1.00	3	3.00	2.00	-16.50	1	1.00	0.00	-18.00
/22	RF	1	2	2.00	0	0.00	-2.00	-21.50	2	2.00	0.00	-19.00
/23	LF	1	2	2.00	0	0.00	-2.00	-22.50	2	2.00	0.00	-20.00
/24	RF	1	0	0.00	2	2.00	2.00	-19.50	2	2.00	2.00	-19.00
/25	RF	1	15	15.00	14	14.00	-1.00	-23.50	2	2.00	-13.00	-35.00
/27	RF	1	4	4.00	3	3.00	-1.00	-24.50	0	0.00	-4.00	-27.00

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
TEST SITE: HALF FACE
PARAMETER: BLACK HEADS
EVALUATION TYPE: OBJECTIVE SENSORY
STUDY NUMBER: FCAG014

TABLE F10: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5937

	Baseline (BL)	AVERAGE	Difference	AVERAGE	Difference
AVERAGE	3.04	2.98	-0.06	2.69	-0.35
STANDARD DEVIATION	4.69	4.72	4.73	4.89	3.09
QTY OF DATA POINTS	144				
QTY OF TEST SUBJECTS	48				

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)
/01	LF	2	4	4.00	0	0.00	-4.00	0	0.00	-4.00
/02	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/04	RF	2	3	3.00	0	0.00	-3.00	0	0.00	-3.00
/05	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/06	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/07	RF	2	11	11.00	6	6.00	-5.00	25	25.00	14.00
/08	LF	2	0	0.00	2	2.00	2.00	0	0.00	0.00
/09	RF	2	0	0.00	1	1.00	1.00	2	2.00	2.00
/10	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/11	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/12	RF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/13	RF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/14	RF	2	1	1.00	3	3.00	2.00	2	2.00	1.00
/16	LF	2	3	3.00	2	2.00	-1.00	3	3.00	0.00
/17	LF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/18	LF	2	0	0.00	2	2.00	2.00	1	1.00	1.00
/19	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/20	LF	2	4	4.00	0	0.00	-4.00	8	8.00	4.00
/21	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/22	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/23	RF	2	0	0.00	0	0.00	0.00	0	0.00	0.00
/24	LF	2	0	0.00	1	1.00	1.00	0	0.00	0.00
/25	LF	2	4	4.00	13	13.00	9.00	5	5.00	1.00
/27	LF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/01	LF	2	2	2.00	1	1.00	-1.00	0	0.00	-2.00
/02	LF	2	1	1.00	0	0.00	-1.00	0	0.00	-1.00
/04	RF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/05	RF	2	5	5.00	2	2.00	-3.00	0	0.00	-5.00
/06	LF	2	6	6.00	4	4.00	-2.00	1	1.00	-5.00
/07	RF	2	28	28.00	7	7.00	-21.00	21	21.00	-7.00
/08	LF	2	8	8.00	9	9.00	1.00	3	3.00	-5.00
/09	RF	2	1	1.00	5	5.00	4.00	4	4.00	3.00
/10	RF	2	4	4.00	2	2.00	-2.00	0	0.00	-4.00
/11	LF	2	7	7.00	8	8.00	1.00	8	8.00	1.00
/12	RF	2	1	1.00	5	5.00	4.00	3	3.00	2.00
/13	RF	2	7	7.00	3	3.00	-4.00	5	5.00	-2.00
/14	RF	2	3	3.00	11	11.00	8.00	5	5.00	2.00
/16	LF	2	5	5.00	6	6.00	1.00	4	4.00	-1.00
/17	LF	2	3	3.00	3	3.00	0.00	2	2.00	-1.00
/18	LF	2	1	1.00	4	4.00	3.00	0	0.00	-1.00
/19	RF	2	1	1.00	1	1.00	0.00	2	2.00	1.00
/20	LF	2	8	8.00	5	5.00	-3.00	8	8.00	0.00
/21	RF	2	3	3.00	1	1.00	-2.00	1	1.00	-2.00
/22	LF	2	4	4.00	3	3.00	-1.00	1	1.00	-3.00
/23	RF	2	0	0.00	0	0.00	0.00	1	1.00	1.00
/24	LF	2	1	1.00	1	1.00	0.00	0	0.00	-1.00
/25	LF	2	10	10.00	27	27.00	17.00	7	7.00	-3.00
/27	LF	2	5	5.00	4	4.00	-1.00	2	2.00	-3.00

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
TEST SITE: HALF FACE
PARAMETER: MICROCYST
EVALUATION TYPE: OBJECTIVE SENSORY
STUDY NUMBER: FCAG014

TABLE F11: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5936

	Baseline (BL)	AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL	AVERAGE	Difference	AVERAGE DECREASE RELATIVE TO CONTROL
AVERAGE	0.23	0.13	-0.10	-6.60	0.15	-0.08	-6.32
STANDARD DEVIATION	0.69	0.33	0.59	8.17	0.46	0.61	7.96
QTY OF DATA POINTS	144						
QTY OF TEST SUBJECTS	48						

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	AVERAGE INCREASE RELATIVE TO CONTROL	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)	AVERAGE INCREASE RELATIVE TO CONTROL
/01	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/02	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/04	LF	1	3	3.00	1	1.00	-2.00	-1.50	2	2.00	-1.00	-0.50
/05	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/06	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/07	LF	1	1	1.00	0	0.00	-1.00	-1.00	0	0.00	-1.00	-1.50
/08	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/09	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/10	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/11	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/12	LF	1	0	0.00	0	0.00	0.00	0.00	1	1.00	1.00	1.00
/13	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/14	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/16	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/17	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/18	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/19	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.50
/20	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/21	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/22	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/23	LF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/24	RF	1	1	1.00	1	1.00	0.00	0.00	1	1.00	0.00	0.00
/25	RF	1	0	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
/27	RF	1	0	0.00	0	0.00	0.00	-0.50	0	0.00	0.00	0.00
/01	RF	1	0	0.00	0	0.00	0.00	-1.50	0	0.00	0.00	-1.00
/02	RF	1	0	0.00	1	1.00	1.00	-1.50	0	0.00	0.00	-2.00
/04	LF	1	3	3.00	0	0.00	-3.00	-6.50	0	0.00	-3.00	-6.00
/05	LF	1	0	0.00	0	0.00	0.00	-4.50	0	0.00	0.00	-4.00
/06	RF	1	0	0.00	0	0.00	0.00	-5.50	0	0.00	0.00	-5.00
/07	LF	1	1	1.00	1	1.00	0.00	-6.50	0	0.00	-1.00	-7.00
/08	RF	1	0	0.00	0	0.00	0.00	-7.50	0	0.00	0.00	-7.00
/09	LF	1	0	0.00	0	0.00	0.00	-8.50	0	0.00	0.00	-8.00
/10	LF	1	0	0.00	0	0.00	0.00	-9.50	0	0.00	0.00	-9.00
/11	RF	1	0	0.00	0	0.00	0.00	-10.50	0	0.00	0.00	-10.00
/12	LF	1	0	0.00	0	0.00	0.00	-11.50	0	0.00	0.00	-11.00
/13	LF	1	0	0.00	0	0.00	0.00	-12.50	0	0.00	0.00	-12.00
/14	LF	1	0	0.00	0	0.00	0.00	-13.50	0	0.00	0.00	-13.00
/16	RF	1	0	0.00	0	0.00	0.00	-14.50	0	0.00	0.00	-14.00
/17	RF	1	0	0.00	0	0.00	0.00	-15.50	0	0.00	0.00	-15.00
/18	RF	1	0	0.00	0	0.00	0.00	-16.50	0	0.00	0.00	-16.00
/19	LF	1	2	2.00	1	1.00	-1.00	-18.50	1	1.00	-1.00	-18.00
/20	RF	1	0	0.00	0	0.00	0.00	-18.50	0	0.00	0.00	-18.00
/21	LF	1	0	0.00	0	0.00	0.00	-19.50	0	0.00	0.00	-19.00
/22	RF	1	0	0.00	0	0.00	0.00	-20.50	0	0.00	0.00	-20.00
/23	LF	1	0	0.00	0	0.00	0.00	-21.50	0	0.00	0.00	-21.00
/24	RF	1	0	0.00	1	1.00	1.00	-21.50	2	2.00	2.00	-20.00
/25	RF	1	0	0.00	0	0.00	0.00	-23.50	0	0.00	0.00	-23.00
/27	RF	1	0	0.00	0	0.00	0.00	-24.50	0	0.00	0.00	-24.00

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
TEST SITE:	HALF FACE
PARAMETER:	MICROCYST
EVALUATION TYPE:	OBJECTIVE SENSORY
STUDY NUMBER:	FCAG014

TABLE F12: DESCRIPTIVE ANALYSIS OF OBJECTIVE SENSORY EVALUATION VALUES FOR FCAG014/5937

	Baseline (BL)		AVERAGE	Difference		AVERAGE	Difference
AVERAGE	0.08		0.08	0.00		0.06	-0.02
STANDARD DEVIATION	0.35		0.35	0.21		0.24	0.33
QTY OF DATA POINTS	144.0						
QTY OF TEST SUBJECTS	48						

SUBJECT NUMBER	TEST PRODUCT	TEST SITE	READING AT BASELINE EVALUATOR	AVERAGE Baseline (BL)	READING AT D14 EVALUATOR	AVERAGE DAY 14	Difference (D14-BL)	READING AT D28 EVALUATOR	AVERAGE DAY 28	Difference (D28-BL)
/01	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/02	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/04	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/05	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/06	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/07	RF	2	0	**0.00**	0	**0.00**	**0.00**	1	**1.00**	**1.00**
/08	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/09	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/10	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/11	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/12	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/13	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/14	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/16	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/17	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/18	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/19	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/20	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/21	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/22	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/23	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/24	LF	2	2	**2.00**	2	**2.00**	**0.00**	1	**1.00**	**-1.00**
/25	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/27	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/01	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/02	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/04	RF	2	1	**1.00**	0	**0.00**	**-1.00**	0	**0.00**	**-1.00**
/05	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/06	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/07	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/08	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/09	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/10	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/11	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/12	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/13	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/14	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/16	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/17	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/18	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/19	RF	2	1	**1.00**	1	**1.00**	**0.00**	0	**0.00**	**-1.00**
/20	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/21	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/22	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/23	RF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/24	LF	2	0	**0.00**	0	**0.00**	**0.00**	1	**1.00**	**1.00**
/25	LF	2	0	**0.00**	0	**0.00**	**0.00**	0	**0.00**	**0.00**
/27	LF	2	0	**0.00**	1	**1.00**	**1.00**	0	**0.00**	**0.00**

APPENDIX G:

FUTURE COSMETICS CC

From Concept to Product

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	PUSTULES

Table G1: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG013/5936 VS PLACEBO CONTROL FCAG013/5937 for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG013/5936 and FCAG013/5937 for BASELINE

H1 -There is a significant difference between FCAG013/5936 and FCAG013/5937 for BASELINE

Chi-Squared Test of Normality

	Column 1
Mean	0.0000
Standard deviation	0.0000
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	46
(-1 < z <= 0)	0.341345	16.38456	0
(0 < z <= 1)	0.341345	16.38456	0
(z > 1)	0.158655	7.61544	0

chi-stat	233.8565651
df	1
p-value	0.00000000
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	**Column 1 - Column 2**
T+	**310**
T-	**0**
Observations (for test)	**46**
z Stat	**-5.905164497**
P(Z<=z) one-tail	**1.76148E-09**
z Critical one-tail	**1.6449**
P(Z<=z) two-tail	**3.52295E-09**
z Critical two-tail	**1.96**

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG013/5936 and FCAG013/5937 for BASELINE

Table G2: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG013/5936 VS PLACEBO CONTROL FCAG013/5937 for DAY14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG013/5936 and FCAG013/5937 for DAY14

H1 -There is a significant difference between FCAG013/5936 and FCAG013/5937 for DAY14

Chi-Squared Test of Normality

	Column 1
Mean	0
Standard deviation	0
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	2
(-1 < z <= 0)	0.341345	16.38456	40
(0 < z <= 1)	0.341345	16.38456	0
(z > 1)	0.158655	7.61544	5

chi-stat	55.46096537
df	1
p-value	9.53334E-14
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	**Column 1 - Column 2**
T+	**1102.5**
T-	**-3**
Observations (for test)	**46**
z Stat	**-2.055001081**
P(Z<=z) one-tail	**0.01993945**
z Critical one-tail	**1.6449**
P(Z<=z) two-tail	**0.0398789**
z Critical two-tail	**1.96**

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG013/5936 and FCAG013/5937 for DAY14

Table G3: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG013/5936 VS PLACEBO CONTROL FCAG013/5937 for DAY28

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG013/5936 and FCAG013/5937 for DAY28

H1 -There is a significant difference between FCAG013/5936 and FCAG013/5937 for DAY28

Chi-Squared Test of Normality

	Column 1
Mean	-0.0625
Standard deviation	0.3200
Observations	48.0000

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	-0.382490026	4
(-1 < z <= 0)	0.341345	-0.0625	0
(0 < z <= 1)	0.341345	0.257490026	43
(z > 1)	0.158655	2.257490026	1

chi-squared Stat	67.08245372
df	1
p-value	2.60386E-16
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	**Column 1 - Column 2**
T+	**1166**
T-	**-10**
Observations (for test)	**48**
z Stat	**-2.239556624**
P(Z<=z) one-tail	**0.012559861**
z Critical one-tail	**1.6449**
P(Z<=z) two-tail	**0.025119721**
z Critical two-tail	**1.96**

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG013/5936 and FCAG013/5937 for DAY28

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	PAPULES

Table G4: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

Chi-Squared Test of Normality

	Column 1		
Mean	0.3542		
Standard deviation	10.5981		
Observations	48		
Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	3
(-1 < z <= 0)	0.341345	16.38456	25
(0 < z <= 1)	0.341345	16.38456	16
(z > 1)	0.158655	7.61544	4
chi-stat	9.052939218		
df	1		
p-value	0.00262273		
chi-squared Critical	3.8415		

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	770
T-	-351
Observations (for test)	48
z Stat	-2.430801194
P(Z<=z) one-tail	0.007532739
z Critical one-tail	1.6449
P(Z<=z) two-tail	0.015065478
z Critical two-tail	1.96

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	PAPULES

Table G5: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D14

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

Chi-Squared Test of Normality

	Column 1		
Mean	-1		
Standard deviation	10		
Observations	48		
Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	6
(-1 < z <= 0)	0.341345	16.38456	14
(0 < z <= 1)	0.341345	16.38456	22
(z > 1)	0.158655	7.61544	6
chi-stat	2.956964403		
df	1		
p-value	0.085508337		
chi-squared Critical	3.8415		

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	-2.8125	-2.229166667
Variance	75.09175532	65.2016844
Observations	48	48
Pearson Correlation	0.349397874	
Hypothesized Mean Differenc	0	
df	47	
t Stat	-0.422737773	
P(T<=t) one-tail	0.337207249	
t Critical one-tail	1.677926722	
P(T<=t) two-tail	0.674414499	
t Critical two-tail	2.011740514	

H0 - data is normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D14

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	PAPULES

Table G6: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D28

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

Chi-Squared Test of Normality

	Column 1		
Mean	0.0208		
Standard deviation	13.3472		
Observations	48.0000		
Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	-13.32639458	4
(-1 < z <= 0)	0.341345	0.020833333	17
(0 < z <= 1)	0.341345	13.36806124	20
(z > 1)	0.158655	15.36806124	7
chi-squared Stat	2.5870768		
df	1		
p-value	0.107739013		
chi-squared Critical	3.8415		

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	-3.645833333	-3.666666667
Variance	145.9782801	168.2269504
Observations	48	48
Pearson Correlation	0.434108361	
Hypothesized Mean Differenc	0	
df	47	
t Stat	0.010814048	
P(T<=t) one-tail	0.495708789	
t Critical one-tail	1.677926722	
P(T<=t) two-tail	0.991417579	
t Critical two-tail	2.011740514	

H0 - data is normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	WHITEHEAD

Table G7: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

Chi-Squared Test of Normality

	Column 1
Mean	0.5000
Standard deviation	5.3832
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	6
(-1 < z <= 0)	0.341345	16.38456	14
(0 < z <= 1)	0.341345	16.38456	23
(z > 1)	0.158655	7.61544	5

chi-stat	4.25901862
df	1
p-value	0.03904247
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	966
T-	-136
Observations (for test)	48
z Stat	-4.635958396
P(Z<=z) one-tail	1.77644E-06
z Critical one-tail	1.6449
P(Z<=z) two-tail	3.55287E-06
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	WHITEHEAD

Table G8: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D14

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

Chi-Squared Test of Normality

	Column 1
Mean	-3
Standard deviation	7
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	5
(-1 < z <= 0)	0.341345	16.38456	19
(0 < z <= 1)	0.341345	16.38456	18
(z > 1)	0.158655	7.61544	6

chi-stat	1.817695814
df	1
p-value	0.177587795
chi-squared Critical	3.8415

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	-1.875	0.854166667
Variance	58.28191489	37.61657801
Observations	48	48
Pearson Correlation	0.434356872	
Hypothesized Mean Differenc	0	
df	47	
t Stat	-2.544431903	
P(T<=t) one-tail	0.007145295	
t Critical one-tail	1.677926722	
P(T<=t) two-tail	0.01429059	
t Critical two-tail	2.011740514	

H0 - data is normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	WHITEHEAD

Table G9: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D28

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

Chi-Squared Test of Normality

	Column 1
Mean	-2.8125
Standard deviation	7.6622
Observations	48.0000

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	-10.47466527	8
(-1 < z <= 0)	0.341345	-2.8125	8
(0 < z <= 1)	0.341345	4.849665268	30
(z > 1)	0.158655	6.849665268	2

chi-squared Stat	19.76510762
df	1
p-value	8.75668E-06
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	711
T-	-351
Observations (for test)	48
z Stat	-9.34372329
P(Z<=z) one-tail	4.65024E-21
z Critical one-tail	1.6449
P(Z<=z) two-tail	9.30047E-21
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	COMEDONE

Table G10: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

Chi-Squared Test of Normality

	Column 1
Mean	0.1042
Standard deviation	2.1757
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	4
(-1 < z <= 0)	0.341345	16.38456	33
(0 < z <= 1)	0.341345	16.38456	5
(z > 1)	0.158655	7.61544	6

chi-stat	26.81907333
df	1
p-value	0.00000022
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	473
T-	-171
Observations (for test)	48
z Stat	-4.276979316
P(Z<=z) one-tail	9.47232E-06
z Critical one-tail	1.6449
P(Z<=z) two-tail	1.89446E-05
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	COMEDONE

Table G11: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D14

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

Chi-Squared Test of Normality

	Column 1
Mean	0
Standard deviation	2
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	5
(-1 < z <= 0)	0.341345	16.38456	12
(0 < z <= 1)	0.341345	16.38456	25
(z > 1)	0.158655	7.61544	6

chi-stat	6.944473707
df	1
p-value	0.008407857
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	615
T-	-153
Observations (for test)	48
z Stat	-5.21872329
P(Z<=z) one-tail	9.00803E-08
z Critical one-tail	1.6449
P(Z<=z) two-tail	1.80161E-07
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	COMEDONE

Table G12: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D28

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

Chi-Squared Test of Normality

	Column 1
Mean	0.0417
Standard deviation	2.4141
Observations	48.0000

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	-2.372461371	6
(-1 < z <= 0)	0.341345	0.041666667	20
(0 < z <= 1)	0.341345	2.455794705	17
(z > 1)	0.158655	4.455794705	5

chi-squared Stat	2.061828095
df	1
p-value	0.151028887
chi-squared Critical	3.8415

t-Test: Paired Two Sample for Means

	Variable 1	Variable 2
Mean	-0.625	-0.666666667
Variance	3.984042553	1.460992908
Observations	48	48
Pearson Correlation	-0.079370408	
Hypothesized Mean Difference	0	
df	47	
t Stat	0.119577392	
P(T<=t) one-tail	0.452663803	
t Critical one-tail	1.677926722	
P(T<=t) two-tail	0.905327606	
t Critical two-tail	2.011740514	

H0 - data is normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	BLACKHEAD

Table G13: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

Chi-Squared Test of Normality

	Column 1
Mean	0.3958
Standard deviation	3.5773
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	3
(-1 < z <= 0)	0.341345	16.38456	25
(0 < z <= 1)	0.341345	16.38456	12
(z > 1)	0.158655	7.61544	8

chi-stat	8.520219823
df	1
p-value	0.00351222
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	770
T-	-136
Observations (for test)	48
z Stat	-4.635958396
P(Z<=z) one-tail	1.77644E-06
z Critical one-tail	1.6449
P(Z<=z) two-tail	3.55287E-06
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	BLACKHEAD

Table G14: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D14

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

Chi-Squared Test of Normality

	Column 1
Mean	-1
Standard deviation	5
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	4
(-1 < z <= 0)	0.341345	16.38456	18
(0 < z <= 1)	0.341345	16.38456	24
(z > 1)	0.158655	7.61544	2

chi-stat	9.556006669
df	1
p-value	0.001992962
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	648
T-	-253
Observations (for test)	48
z Stat	-7.302056624
P(Z<=z) one-tail	1.41701E-13
z Critical one-tail	1.6449
P(Z<=z) two-tail	2.83402E-13
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	BLACKHEAD

Table G15: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D28

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

Chi-Squared Test of Normality

	Column 1
Mean	-0.8958
Standard deviation	4.3723
Observations	48.0000

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	-5.268084243	4
(-1 < z <= 0)	0.341345	-0.895833333	20
(0 < z <= 1)	0.341345	3.476417576	21
(z > 1)	0.158655	5.476417576	3

chi-squared Stat	6.611616411
df	1
p-value	0.010131566
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	680
T-	-300
Observations (for test)	48
z Stat	-8.28122329
P(Z<=z) one-tail	6.09582E-17
z Critical one-tail	1.6449
P(Z<=z) two-tail	1.21916E-16
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	MICROCYST

Table G16: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for BASELINE

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

Chi-Squared Test of Normality

	Column 1
Mean	0.1458
Standard deviation	0.5831
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	1
(-1 < z <= 0)	0.341345	16.38456	42
(0 < z <= 1)	0.341345	16.38456	0
(z > 1)	0.158655	7.61544	5

chi-stat	63.07645234
df	1
p-value	0.00000000
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	230
T-	-1
Observations (for test)	48
z Stat	-6.02059187
P(Z<=z) one-tail	8.68902E-10
z Critical one-tail	1.6449
P(Z<=z) two-tail	1.7378E-09
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for BASELINE

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	MICROCYST

Table G17: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D14

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D14

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

Chi-Squared Test of Normality

	Column 1
Mean	0
Standard deviation	1
Observations	48

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	7.61544	5
(-1 < z <= 0)	0.341345	16.38456	0
(0 < z <= 1)	0.341345	16.38456	41
(z > 1)	0.158655	7.61544	2

chi-stat	58.40464405
df	1
p-value	2.13387E-14
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	95
T-	-15
Observations (for test)	48
z Stat	-2.34372329
P(Z<=z) one-tail	0.009546165
z Critical one-tail	1.6449
P(Z<=z) two-tail	0.01909233
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D14

INITIATION DATE:	2018/04/04
COMPLETION DATE:	2018/05/02
STUDY NUMBER:	FCAG014
EVALUATION:	OBJECTIVE SENSORY EVALUATION
PARAMETER:	MICROCYST

Table G18: STATISTICAL ANALYSIS OF OBJECTIVE SENSORY EVALUATION FOR FCAG014/5936 VS PLACEBO CONTROL FCAG014/5937 for D28

H0 - data is normally distributed

H1 - data is not normally distributed

H0 -There is no significant difference between FCAG014/5936 and FCAG014/5937 for D28

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

Chi-Squared Test of Normality

	Column 1
Mean	-0.0625
Standard deviation	0.5221
Observations	48.0000

Intervals	Probability	Expected	Observed
(z <= -1)	0.158655	-0.584617212	4
(-1 < z <= 0)	0.341345	-0.0625	0
(0 < z <= 1)	0.341345	0.459617212	41
(z > 1)	0.158655	2.459617212	3

chi-squared Stat	57.87939534
df	1
p-value	2.78691E-14
chi-squared Critical	3.8415

Wilcoxon Signed Rank Sum Test

Difference	Column 1 - Column 2
T+	141
T-	-10
Observations (for test)	48
z Stat	-2.239556624
P(Z<=z) one-tail	0.012559861
z Critical one-tail	1.6449
P(Z<=z) two-tail	0.025119721
z Critical two-tail	1.96

H1 - data is not normally distributed

H1 -There is a significant difference between FCAG014/5936 and FCAG014/5937 for D28

APPENDIX H:

FUTURE COSMETICS CC

From Concept to Product

INITIATION DATE: 2018/04/04
COMPLETION DATE: 2018/05/02
STUDY NUMBER: FCAG014

DEVIATION FORM (REF:FCAG014)	
DEVIATION FROM:	**NOTE:**
SUBJECT 03,15 and 26	Lost to follow up

Appendix F

Publications and Awards

Appendix F

F1 Manuscript published in South African Journal of Botany

Journal of Ethnopharmacology 269 (2021) 113663

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Review

Traditional usage and biological activity of *Plectranthus madagascariensis* and its varieties: A review

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ARTICLE INFO

Keywords:
Plectranthus madagascariensis
Plectranthus madagascariensis var. aliciae
Plectranthus aliciae
Plectranthus madagascariensis var. madagascariensis
Plectranthus hirtus
Lamiaceae
Ethnobotanical use
Phytochemicals
Biological activity

ABSTRACT

Ethnopharmacological relevance: *Plectranthus madagascariensis* (Pers.) Benth. is an indigenous aromatic South African plant species that are traditionally used to treat various dermatological and respiratory ailments.
Aim of the study: Three varieties of *P. madagascariensis* exist in South Africa, namely, *Plectranthus aliciae* (Codd) van Jaarsv. & T.J. Edwards, *Plectranthus ramosior* (Benth.) Van Jaarsv. and *Plectranthus madagascariensis* (Pers.) Benth var. *madagascariensis*. This article summarizes the documented ethnobotanical uses and research which has been conducted to date on the chemical constituents and biological effects of *P. madagascariensis* and its varieties. This review aimed to investigate and highlight the lack scientific reports of the potential activity of these varieties based on their traditional usage and to emphasise the need for further investigation of the benefits of *P. madagascariensis* and its varieties.
Materials and methods: Extensive database retrieval using platforms not limited to but including Google Scholar, ScienceDirect and PubMed, was performed by using keywords such as "*Plectranthus madagascariensis*" "*Plectranthus madagascariensis* var. aliciae", "*Plectranthus* aliciae", "*Plectranthus ramosior*", "*Plectranthus madagascariensis* var. ramosior" and "*Plectranthus hirtus*" In addition, relevant books and digital documentation were consulted to collect all available scientific literature to provide a comprehensive review.
Results: Several studies have reported the traditional usage of *P. madagascariensis* for the treatment of diseases related to the respiratory system such as coughs, colds and asthma as well as dermatological disorders associated with wounds and inflammation. Whilst there are no reports on the traditional usage of *P. madagascariensis* varieties to treat other maladies, several other species within the genus are used in other traditional practices. *Plectranthus ramosior* is used as a toxin for fishing. In literature, seven major phytochemical compounds have been identified from *P. madagascariensis*. Its extract and essential oil contain polyphenols, abietane diterpenes and abietane diterpenes with a quinone moiety. The extracts and major chemical constituents of *P. madagascariensis* and its major phytochemicals have reported activity against several biological targets. Reports relating to the antibacterial activity of *P. madagascariensis* against microbes associated with tuberculosis and wound infections has been consistent and correlates with the documented traditional usage of the plant. Literature reported on the antibacterial activity of *P. aliciae* targeting bacteria associated with wound infections and lung cancer cells. No further literature reports of the biological activity of the other *P. madagascariensis* varieties have been found. Other noteworthy biological activities reported in the literature of *P. madagascariensis* and its compounds include their activities against targets of Alzheimer's disease and breast cancer, in particular. This activity is not related to the traditional usage of the plant.
Conclusion: *Plectranthus madagascariensis* and its compounds have been proven to be effective in treating a range of maladies. Based on the extensive literature on this plant, it can be concluded that numerous *in vitro* pharmacological activities of *P. madagascariensis* have been reported. However, there is a lack of information available for this species with regards to its *in vivo* data including both pre-clinical and clinical studies. Since the extract of *P. madagascariensis* and its isolated compounds have displayed noteworthy anticancer potential, we recommend further investigation of pharmacokinetic studies to be included in future research.

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https://doi.org/10.1016/j.jep.2020.113663
Received 9 July 2020; Received in revised form 28 November 2020; Accepted 30 November 2020
Available online 3 December 2020

F2 Patent: Anti-Acne Pharmaceutical Composition

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 June 2020 (18.06.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/121187 A1

(51) International Patent Classification:
A61K 36/53 (2006.01) *A61K 31/352* (2006.01)
A61K 8/00 (2006.01) *A61P 17/10* (2006.01)
A61K 9/00 (2006.01)

(21) International Application Number:
PCT/IB2019/060604

(22) International Filing Date:
10 December 2019 (10.12.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2018/08307 10 December 2018 (10.12.2018) ZA

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

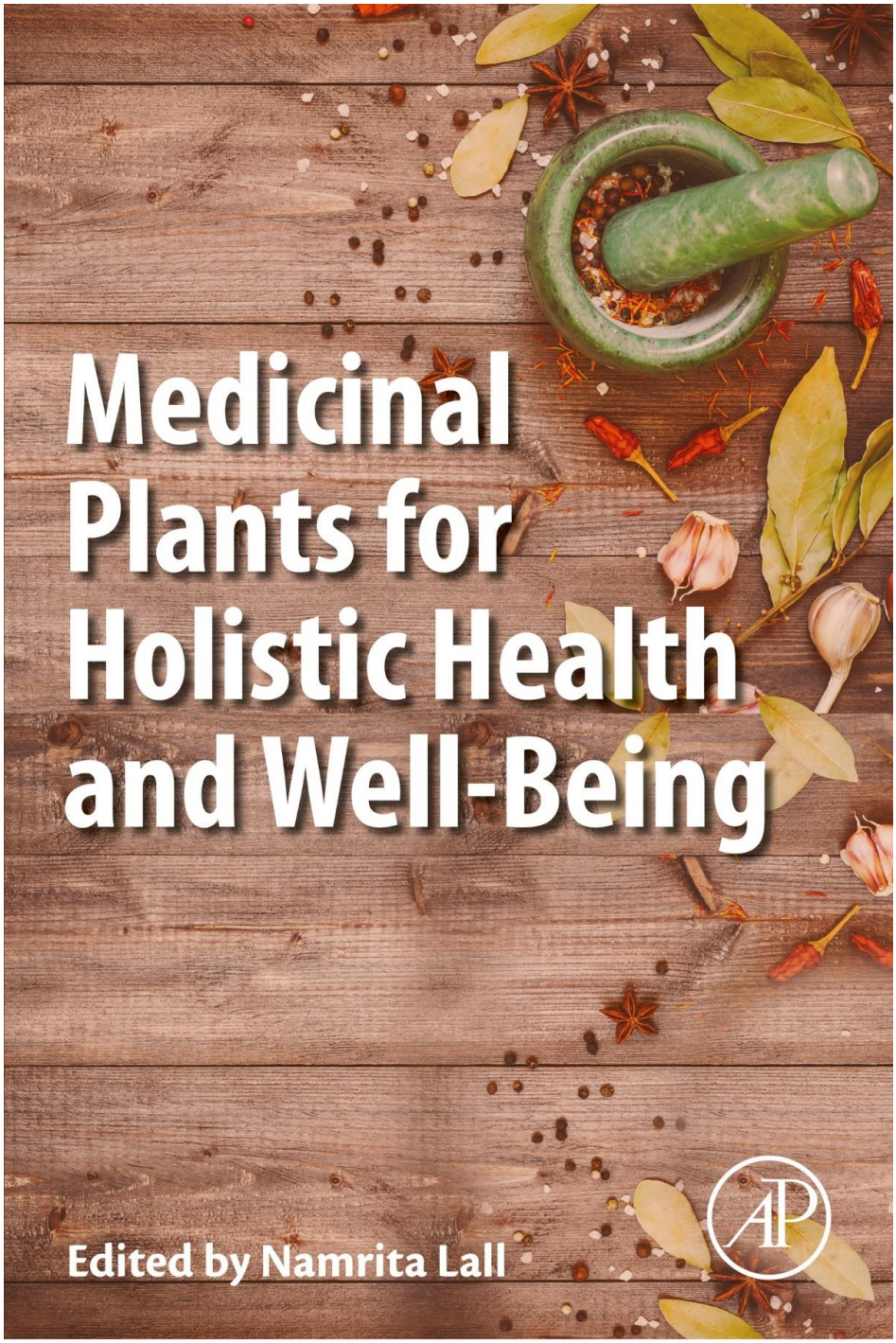
WO 2020/121187 A1

(54) **Title:** ANTI-ACNE PHARMACEUTICAL COMPOSITIONS

(57) **Abstract:** The invention provides a pharmaceutical composition suitable for topical application. The pharmaceutical composition comprises an extract of *Plectranthus aliciae* and a pharmaceutically acceptable carrier. The invention extends to provide a pharmaceutical composition for the treatment of acne, a pharmaceutical gel composition, a substance or composition comprising an extract of *Plectranthus aliciae*, a use of a *Plectranthus aliciae* extract, and various methods of preventing or treating acne.

F3 Book chapter in Medicinal Plants for Holistic and Well-being

A chapter was published on medicinal plants to treat acne vulgaris in Medicinal Plants for Holistic and Well-being (2018).



Chapter 1

Traditional Medicine: The Ancient Roots of Modern Practice

Anna-Mari Reid, Carel B. Oosthuizen, Bianca D. Fibrich, Danielle Twilley, Isa Anina Lambrechts, Marco Nuno de Canha, Sunelle Rademan, Namrita Lall

University of Pretoria, Pretoria, South Africa

Chapter Outline

Chapter 4

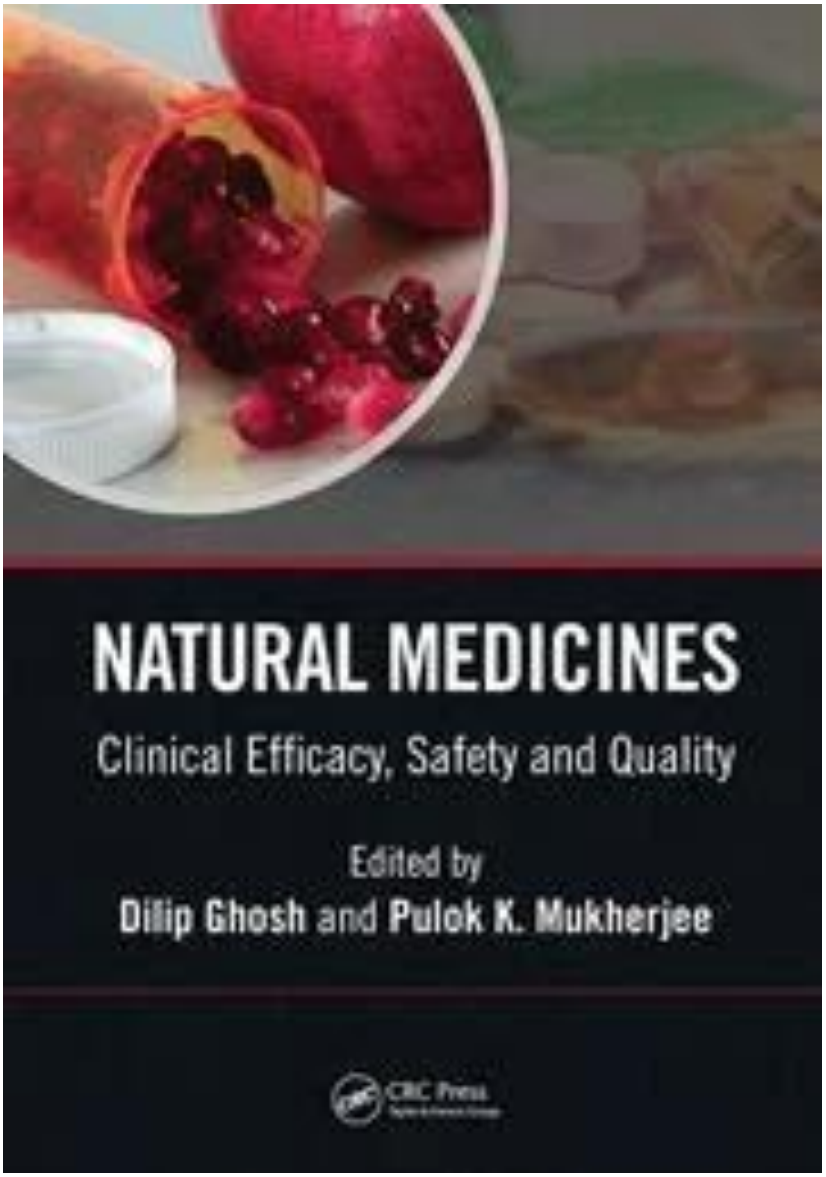
Exploiting Medicinal Plants as Possible Treatments for Acne Vulgaris

Isa Anina Lambrechts, Marco Nuno de Canha, Namrita Lall
University of Pretoria, Pretoria, South Africa

Chapter Outline

F4 Book Chapter: Natural Medicines: Clinical Efficacy, Safety and Quality

A chapter was published on green nanotechnology and medicinal plants to target skin ageing Natural Medicines: Clinical Efficacy, Safety and Quality (2019).



21 Nanotechnology and Anti-Ageing Skin Care

B. Fibrich, I.A. Lambrechts and N. Lall

CONTENTS

F5 Book Chapters: Underexplored Medicinal Plants from Sub-Saharan Africa

Various chapters were published on indigenous South African medicinal plants for their ethnobotanical use in Underexplored Medicinal Plants from Sub-Saharan Africa (2020).

CHAPTER

32

Ocimum labiatum

Isa A. Lambrechts, Namrita Lall

Department of Plant and Soil Sciences, University of Pretoria, Pretoria, Gauteng, South Africa

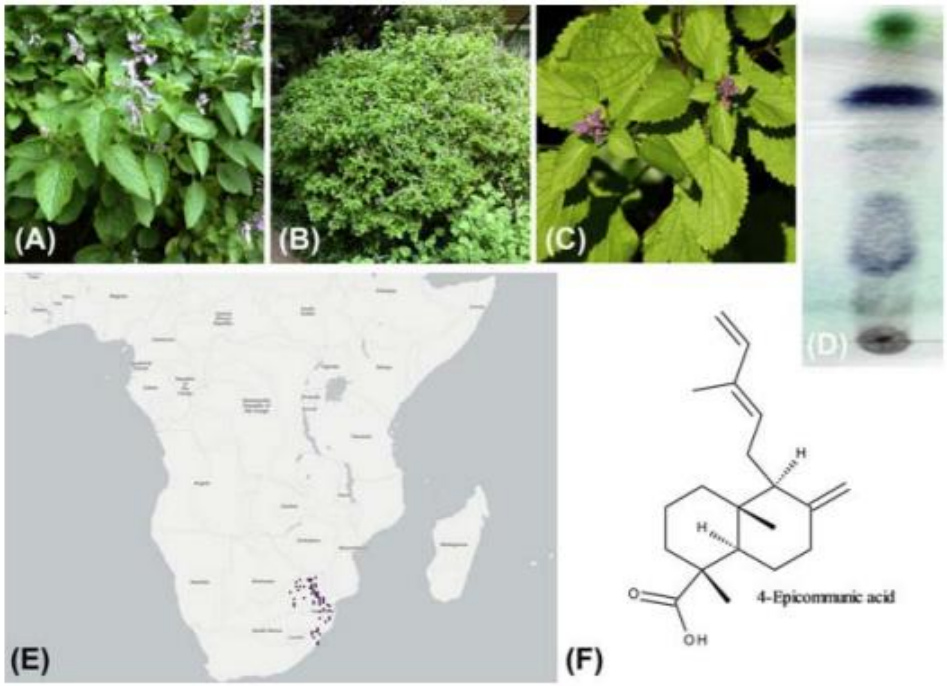


FIGURE 32 Aerial plant parts of *Ocimum labiatum* (JMK, 2015a) (A), bigger plant of *O. labiatum* (JMK, 2015b) (B), closer view of the flowers of *O. labiatum* (Dinkum, 2011) (C), TLC chromatogram, fingerprint of *O. labiatum* extract (D), distribution of *O. labiatum* in sub-Saharan Africa (GBIF, 2017) (E), chemical structure of 4-epicommunic acid (F).

CHAPTER

35

Plectranthus ecklonii

Isa A. Lambrechts, Namrita Lall

Department of Plant and Soil Sciences, University of Pretoria, Pretoria, Gauteng, South Africa

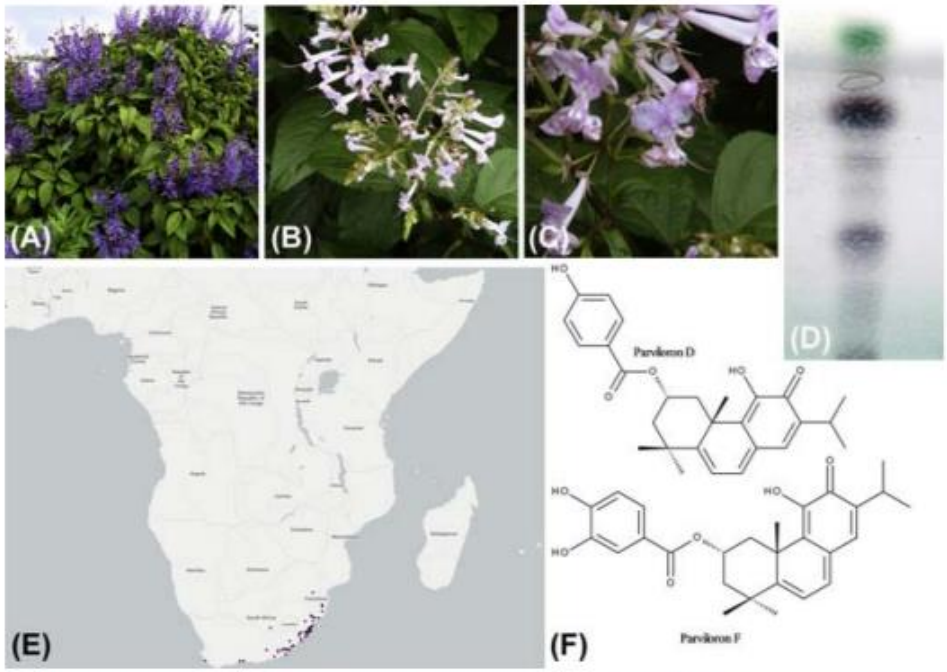


FIGURE 35 Aerial plant parts of *Plectranthus ecklonii* (Wildflowernursery, 2018) (A), flowers of *P. ecklonii* (JMK. 2013a) (B), closer view of the flowers (JMK, 2013b) (C), TLC chromatogram, fingerprint of *P. ecklonii* extract (D), distribution of *P. ecklonii* in sub-Saharan Africa (GBIF, 2018) (E), chemical structures of parviloron D and F (F).

CHAPTER

36

Plectranthus neochilus

Isa A. Lambrechts, Namrita Lall

Department of Plant and Soil Sciences, University of Pretoria, Pretoria, Gauteng, South Africa

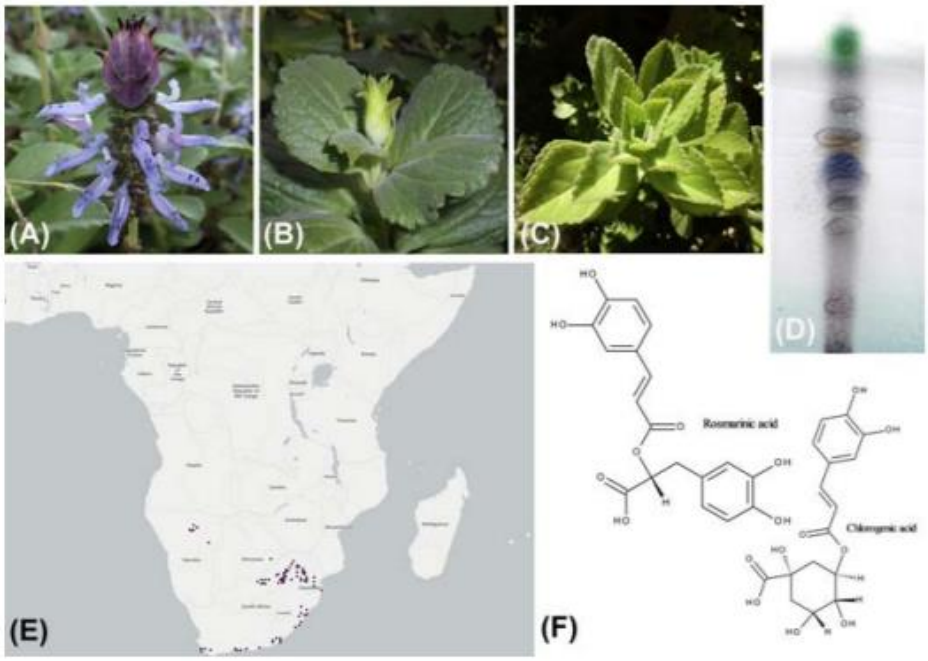
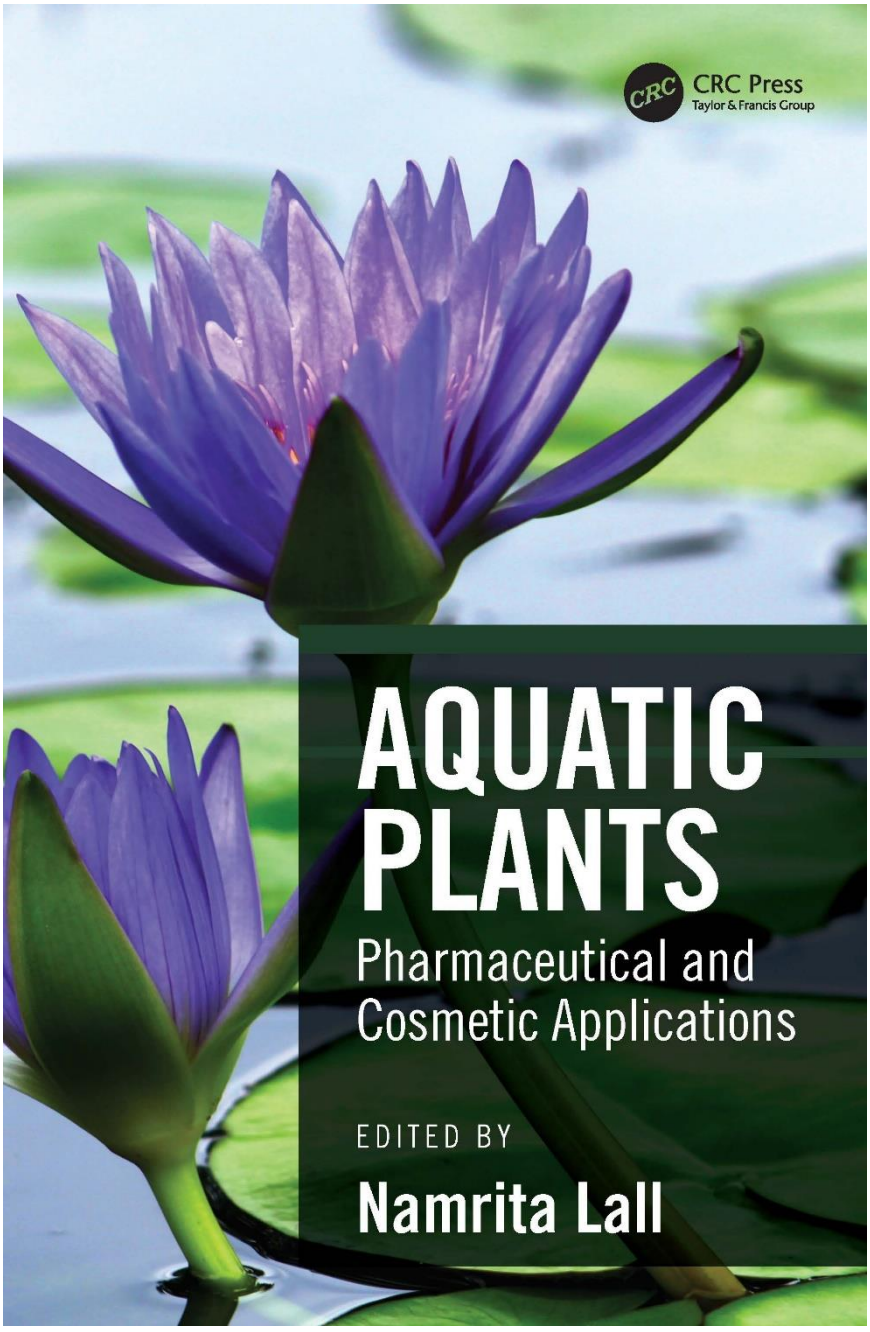


FIGURE 36 Flower of *Plectranthus neochilus* (Kenraiz, 2017) (A), closer view of the leaves of *P. neochilus* (Salicyna, 2017) (B), leaves of *P. neochilus* (JMK, 2012) (C), TLC chromatogram, fingerprint of *P. neochilus* extract (D), distribution of *P. neochilus* in sub-Saharan Africa (GBIF, 2018) (E), chemical structures of rosmarinic acid and chlorogenic acid (F).

F6 Book Chapter: Aquatic Plants: Pharmaceutical and Cosmetic Applications

A chapter was published on aquatic and wetland plants native to Europe for their ethnobotanical use in Aquatic Plants: Pharmaceutical and Cosmetic Applications (2020).



5 Aquatic Plants Native to Europe

Isa A. Lambrechts and Lydia Gibango
University of Pretoria

Antonios Chrysargyris and Nikolaos Tzortzakis
Cyprus University of Technology

Namrita Lall
University of Pretoria
University of Missouri
JSS Academy of Higher Education and Research

CONTENTS

F7 Book Chapter: Medicinal Plants in Cosmetics, Health and Disease

A chapter will be published on medicinal plants to target quorum sensing of bacteria associated with acne vulgaris in Medicinal Plants in Cosmetics, Health and Disease (2022). This chapter forms part of Chapter 1 in this thesis.

Chapter 3. Phytomedicines targeting antibiotic resistance through quorum sensing and biofilm formation associated with acne vulgaris

Isa A. Lambrechts (0000-0002-3349-5033) [1] and Namrita Lall (0000-0002-3242-3476) [1, 2, 3, 4]

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[4] *Senior Research Fellow, Bio-Tech R& D Institute, University of West Indies, Kingston, Jamaica*

Acne affects 9% of the world's population, making it the 8th most prevalent disease worldwide. Although not life-threatening, it has severe physiological implications, affecting the overall wellbeing of the patient. The World Health Organisation has raised awareness on the threat of antibiotic resistance to the global health system. Antibiotic resistance in bacteria is due to the ability of bacteria to form a biofilm. Quorum sensing is an inter-and-intraspecies form of communication through the release of signalling molecules. Once a critical concentration of the signalling molecules has been reached, it results in the expression and repression of target genes. This results in biofilm formation and the release of virulence factors. Acne causing bacteria can release quorum-sensing molecules that could contribute to antibiotic resistance. This chapter discusses antibiotic resistance and quorum sensing targets associated with acne vulgaris. Furthermore, this chapter explores plants and their compounds to target antibiotic resistance associated with acne vulgaris. Among several plants and plant-derived compounds investigated, curcumin isolated from *Curcuma longa* and quercetin isolated from various plant species has shown significant antibacterial, autoinducer-2 and biofilm inhibitory activity against acne- causing bacteria. These compounds have the potential to be allies in targeting antibiotic resistance associated with acne vulgaris.

F8 26th International Federation of Societies of Cosmetic Chemists Conference 2021

An indigenous South African plant targeting antibiotic resistance and the pathogenic factors associated with acne vulgaris

Lambrechts, I.A.[1], Lall, N [1,2,3]

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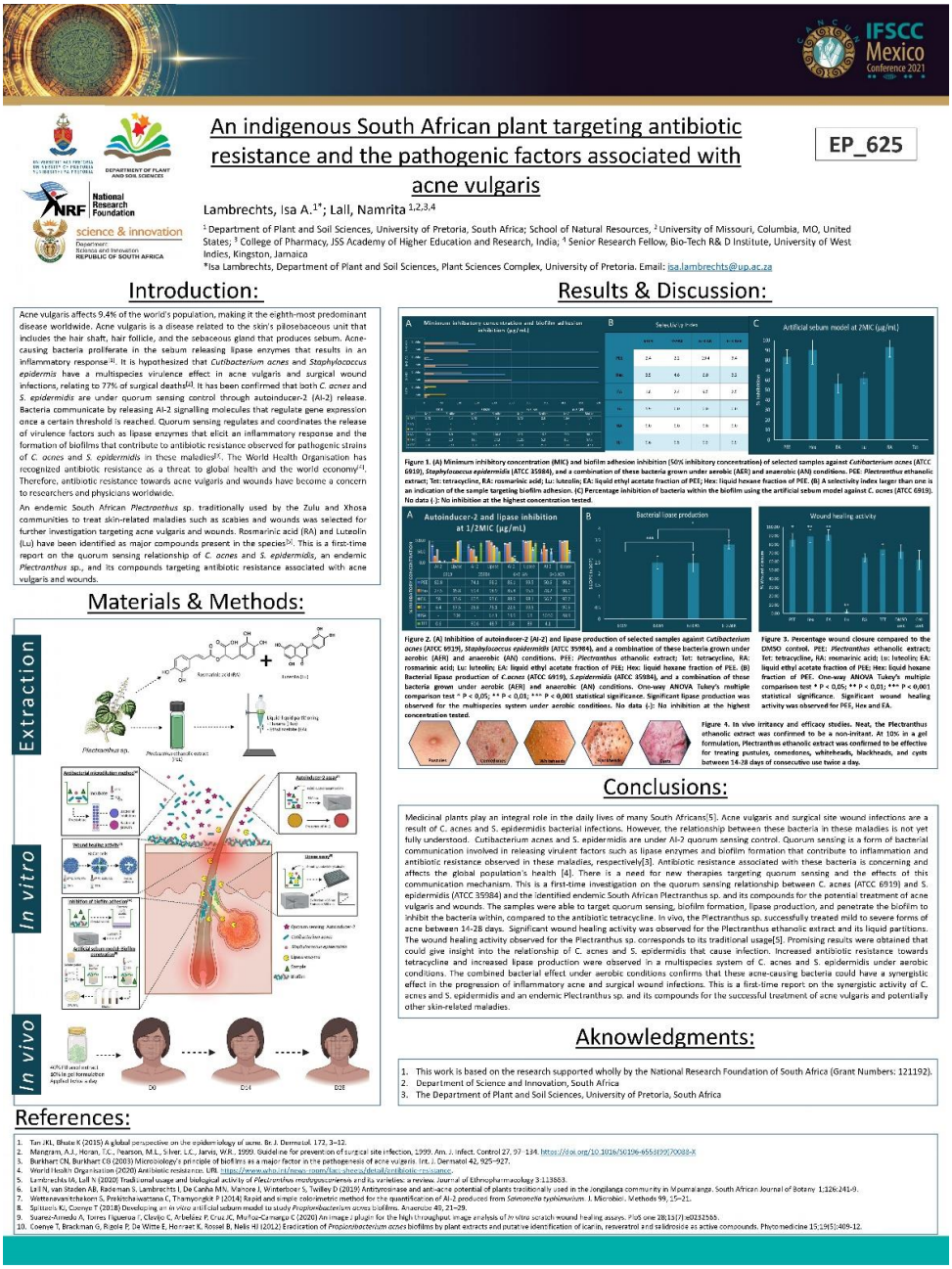

A natural choice for antimicrobial protection using an indigenous South African plant for acne vulgaris

Lambrechts, I.A.[1], Lall, N [1,2,3]

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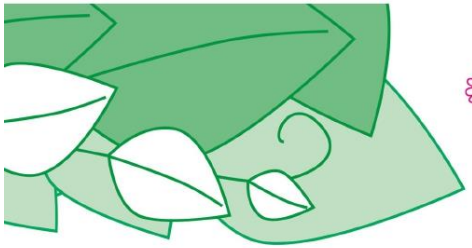
Acne vulgaris (AV) is a chronic inflammatory disease of the pilosebaceous follicle caused by the Gram-positive bacteria, *Cutibacterium acnes* and *Staphylococcus epidermidis*. The disease affects approximately 9.4% of the world population, making it the 8th most prevalent disease worldwide. In nature, microorganisms rarely exist as planktonic microorganism suspensions but rather as a complex biofilm. These biofilms, consisting of consortia of species, contribute to antibiotic resistance seen in AV. The Lamiaceae family are aromatic plants known for their antibacterial properties, and traditional healers in southern Africa use them to treat skin diseases. The ethanolic semi-pure fraction from the leaves of plants belonging to the Lamiaceae family was tested against *C. acnes* (ATCC 6919) to determine the antibacterial, prevention of biofilm formation and quorum sensing inhibition potential. *In vitro* studies revealed that acne-causing bacteria have a virulence effect through the increased production of the quorum-sensing molecule autoinducer-2 (AI-2). This signalling molecule contributes to the formation of biofilms and the release of virulence factors such as lipase that is associated with inflammation. The inhibition potential of the sample on biofilm formation and AI-2 release was determined. The semi-pure fraction was tested against inflammatory enzymes such as lipase, matrix metalloproteinase-9 (MMP-9) and Cyclooxygenases (COX), which have been identified to contribute to inflammatory acne. The semi-pure fraction was confirmed to prevent biofilm formation and the release of AI-2, preventing quorum sensing. Enzymatic studies revealed that the semi-pure fraction was effective against various inflammatory enzymes associated with AV. In vivo studies confirmed that the semi-pure fraction was not irritating to the skin and formulated at 10% in a gel formulation was effective against acne after fourteen to twenty-eight days of consecutive use twice a day. These data suggest the potential of this South African plant for the treatment of acne vulgaris.



20th International Congress of the International Society for Ethnopharmacology

Virtual Congress

18-20 April 2021

www.ethnopharmacology2021.org

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CERTIFICATE OF ATTENDANCE

This is to certify that:

Isa Lambrechts

Has attended the
20th International Congress of the International Society for Ethnopharmacology
that was held online,
as a Virtual Congress, between 18-20 of April 2021

On behalf of the Organising Committee,

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Assistant Professor
Department of Pharmacognosy-Pharmacology
Aristotle University of Thessaloniki, Greece

Lazari Diamanto
Associate Professor
Department of Pharmacognosy-Pharmacology
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An indigenous South African plant targeting antibiotic resistance and the pathogenic factors associated with acne vulgaris

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Acne vulgaris is a chronic inflammatory disease of the pilosebaceous follicle caused by the Gram-positive bacteria, *Cutibacterium acnes*. The disease affects approximately 9.4% of the world population, making it the 8th most prevalent disease worldwide. Therefore, acne vulgaris should be acknowledged as a severe disorder. Current acne therapies are damaging to the skin and antibiotic resistance associated with the disease has become a concern worldwide. There are four major etiological factors involved in the development of inflammatory acne, with the biofilm forming *C. acnes* contributing to inflammation and antibiotic resistance seen in the disease.

In modern society, medicinal plants are growing in popularity as natural alternatives to synthetic chemicals. Natural products and their derivatives are used in more than 50% of all drugs clinically used in the world with 25% being from higher plants.[1] The Lamiaceae family are aromatic plants known for their antibacterial properties and is used by traditional healers in southern Africa for treating skin diseases. This far, little research has been conducted on the potential of Lamiaceae plants that are indigenous to South Africa regarding acne vulgaris and targeting antibiotic resistance associated with acne vulgaris.

The ethanolic semi-pure fraction from the leaves of plants belonging to the Lamiaceae family was tested against *C. acnes* (ATCC 6919) to determine the antibacterial, prevention of biofilm formation and quorum sensing inhibition potential. The aforementioned factors have contributed to antibiotic resistance seen in the disease. Inflammation associated with acne vulgaris contributes to the formation of permanent scars and pigmentation. Several enzymes have been identified to contribute to inflammatory acne. These enzymes are Cyclooxygenase-2 (COX-2), lipase and matrix metalloproteinase-9 (MMP-9). Nitric oxide is a free radical that is associated with inflammatory acne. The antioxidant capacity of the active semi-pure fraction was measured and found to have a high free radical scavenging activity. *In vitro*

studies revealed that the semi-pure fraction was effective against various enzymes involved in inflammatory acne and antibiotic resistance associated with the disease. *In vivo* studies confirmed that the semi-pure fraction was not irritating to the skin and formulated at 10% in a gel formulation was effective against acne after fourteen to twenty-eight days of consecutive use twice a day.

These data suggest the potential of South African plants for the treatment of acne vulgaris.

REFERENCES:

1. Gurib-Fakim, A. Molecular aspects of Medicine. 2006, 27, 1-93.

An indigenous South African plant targeting inflammation and antibiotic resistance associated with acne vulgaris

Lambrechts, I.A.[1], Lall, N [1,2,3]

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Acne vulgaris is a chronic inflammatory disease of the pilosebaceous follicle caused by the Gram-positive bacteria, *Propionibacterium acnes.* The disease affects approximately 9.4% of the world population, making it the 8th most prevalent disease worldwide. Therefore, acne vulgaris should be acknowledged as a severe disorder. Current acne therapies are damaging to the skin and antibiotic resistance associated with the disease has become a concern worldwide. There are four major etiological factors involved in the development of inflammatory acne, with the biofilm forming *P. acnes* contributing to inflammation and antibiotic resistance seen in the disease.

In modern society, medicinal plants are growing in popularity as natural alternatives to synthetic chemicals. Natural products and their derivatives are used in more than 50% of all drugs clinically used in the world with 25% being from higher plants.[1] The Lamiaceae family are aromatic plants known for their antibacterial properties and is used by traditional healers in southern Africa for treating skin diseases. This far, little research has been conducted on the potential of Lamiaceae plants that are indigenous to South Africa regarding acne vulgaris and targeting antibiotic resistance associated with acne vulgaris.

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These data suggest the potential of South African plants for the treatment of acne vulgaris.

REFERENCES:

1. Gurib-Fakim, A. *Molecular aspects of Medicine*. 2006, *27*, 1-93.

Potential of South African plants and essential oils for acne vulgaris and skin hyper-higmentation

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[3] *College of Pharmacy, JSS Academy of Higher Education and Research, India*

Acne vulgaris is a chronic inflammatory disease of the pilosebaceous follicle caused by the Gram-positive bacteria, *Propionibacterium acnes.* The disease affects approximately 9.4% of the world population, making it the 8th most prevalent disease worldwide. Although acne vulgaris is a superficial and not life-threatening disease, it can severely detriment the patients quality of life, affecting social, emotional and physiological functions. Therefore, acne vulgaris should be acknowledged as a severe disorder. Current acne therapies are damaging to the skin, and antibiotic resistance associated with the disease has become a concern worldwide. There are four major etiological factors involved in the development of inflammatory acne, with the biofilm-forming *P. acnes* contributing to inflammation and antibiotic resistance seen in the disease.

In modern society, medicinal plants are growing in popularity as natural alternatives to synthetic chemicals. Natural products and their derivatives are used in more than 50% of all drugs clinically used in the world, with 25% being from higher plants. The potential of southern African aromatic plants has rarely been investigated with respect to acne vulgaris and its pathogenesis as a whole. Essential oils have been used for centuries for their antibacterial properties and are used in the pharmaceutical, cosmetic, food and medical industries as preservatives, disinfectants, and scents. The Lamiaceae family are aromatic plants known for their antibacterial properties, and traditional healers in southern Africa use them to treat skin diseases. So far, little research has been conducted on the potential of Lamiaceae plants indigenous to South Africa regarding acne vulgaris and PIH.

Ethanolic extracts and the essential oils of leaves from plants belonging to the Lamiaceae family were tested against *P. acnes* (ATCC 11827), their Cyclooxygenase -2 (COX-2) and tyrosinase inhibitory activity was determined. DPPH and nitric oxide radical scavenging

assays were done to determine the antioxidant capacity of the extracts. Cytotoxicity tests were done against a human keratinocyte (HaCaT) cell line. Both plant extracts and essential oils from the Lamiaceae family are effective against various factors involved in the pathogenesis of acne vulgaris.

The ethanolic extract of sample PM was found to have significant antibacterial potential against *P. acnes* with a minimum inhibitory concentration (MIC) of 7.8 μg/mL, comparable to the positive control tetracycline (MIC 0.78 μg/mL). PM also inhibited biofilm growth at a 50% inhibitory concentration (IC_{50}) of 6.65 ± 1.3 μg/mL. The essential oils of the selected Lamiaceae species tested for their antibacterial activity were found to inhibit P. acnes at lower concentrations than tea tree oil. Tea tree oil is widely known for its antibacterial properties and its use in treating acne vulgaris. PM moderately inhibited the inflammatory COX-2 enzyme (IC_{50} of 44 μg/mL). Further, the toxicity of the extracts was investigated and was found to have low levels of cytotoxicity (IC_{50} >100 μg/mL) for most of the plant species.

Current acne therapies are harmful to the skin, disrupting the skin barrier function leaving it dry and red and irritated. There is a need for combination products that effectively control acne vulgaris, which can hydrate the skin. Recently one semi-pure fraction of an indigenous plant LS and in combination with another one, HO, have been formulated as an alternative acne therapy. LS aqueous extract has been found to be a non-irritant and passed a 24-hour hydration test at 8%. The ethanolic extract of LS in an SPF base was effective in reducing the papule count after 28 days and blackheads after 14-28 days during *in vivo* studies. Hiliminate, a combination of LS and HO, has been indicated to assist in controlling acne lesions, including open and closed comedones after 14 days and more severe forms of inflammatory acne such as papules and pustules after 28 days during *in vivo* studies. The plant ingredients LH and HO (ethanolic extracts) activity could be attributed to their antibacterial and antioxidant potential, which assists in reducing acne vulgaris. L_sO_2H, is a combination of LS and a plant native to South Africa, SJ (water extracts), that can naturally hydrate the skin after 24 hours, preventing and treating scars associated with acne vulgaris.

These data suggest the potential of South African plants and their essential oils for treating acne vulgaris.

Society for Medicinal Plant and Natural Product Research

Gesellschaft für Arzneipflanzen- und Naturstoff-Forschung e.V.

Dr. Willmar Schwabe Research Scholarship

With the aim to support young scientists in a research attachment to a leading research institution, the **Dr. Willmar Schwabe Research Scholarship 2021** (10.000 EUR) is.bestowed to

Isa Anina Lambrechts

to perform studies on *Plectranthus* genus fermented extracts to treat skin-related infections such as acne vulgaris, wound and prosthesis-related infections.

Professor Judith Rollinger, President of the GA

MINISTRY: SCIENCE AND TECHNOLOGY
REPUBLIC OF SOUTH AFRICA

South African Women in Science Awards
2018

ALBERTINA SISULU DOCTORAL FELLOWSHIP

presented to

Isa Anina Lambrechts

in recognition of her outstanding academic and research ability

Ms Mmamoloko Kubayi-Ngubane, MP
Minister of Science and Technology