IN VITRO INVESTIGATION OF *ROSA RUBIGINOSA* (ROSEHIP) OIL AND ESSENTIAL OIL COMBINATIONS FOR THE TOPICAL TREATMENT OF ACNE

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

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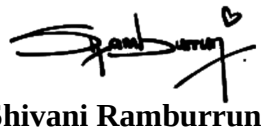
DECLARATION

I, Shivani Ramburrun, hereby declare that this dissertation is my own work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted in fulfilment of the degree of Master of Pharmacy in the Faculty of Health Sciences, at the University of the Witwatersrand, Parktown, Johannesburg, South Africa. This work has not been submitted before for any other degree or examination at this or any other university.



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ABSTRACT

Acne vulgaris is the eighth most prevalent disease worldwide. *Cutibacterium acnes* overgrowth and inflammation are responsible for acne pathogenesis. Essential oils are one of the leading natural products used for dermatological applications, including acne. Essential oils are combined with fixed oils to reduce irritation and toxicity, however, the effect of fixed oils on essential oil bio-activity requires further investigation. This study aimed to investigate the *in vitro* antimicrobial, toxicity, anti-inflammatory, and associated cytotoxicity, as well as the skin absorption and retention properties of *Rosa rubiginosa* (rosehip) fixed oil independently and in combination with essential oils to treat the pathophysiological mechanisms involved in acne.

For the antimicrobial activity, the minimum inhibitory concentration (MIC) was determined using the broth microdilution assay. The fractional inhibitory concentration index (ΣFIC) was calculated to evaluate the antimicrobial interactions of the oil combinations. *Rosa rubiginosa* fixed oil combined with *Cinnamomum zeylanicum* (true cinnamon) essential oil displayed antimicrobial synergy (mean MIC = 0.42 mg/mL, mean ΣFIC = 0.45) against three acne-inducing reference strains (*C. acnes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*). *Rosa rubiginosa* fixed oil combined with either *Eugenia caryophyllata* (clove), *Kunzea ericoides* (kanuka), *Melaleuca quinquenervia* (niaouli), *Melissa officinalis* (lemon balm), *Rosa damascena* (Damask rose), or *Styrax benzoin* (gum benjamin) essential oil displayed antimicrobial additive interactions. *Rosa rubiginosa* fixed oil combined with *Santalum austrocaledonicum* (New Caledonia sandalwood) essential oil displayed antimicrobial antagonism. These eight oil combinations were further analysed in various ratios. The ΣFIC for the various ratios were expressed graphically on isobolograms, which revealed that most of the antimicrobial effects were attributed to the essential oils rather than *R. rubiginosa* fixed oil.

When the toxicity was evaluated using the brine shrimp lethality assay, *R. rubiginosa* fixed oil was found to be non-toxic with a percentage mortality of 0.35-1.64% after 24 hrs and 0.74-2.00% after 48 hrs, across all concentrations in the range of 0.03-1.00 mg/mL. For the combinations, *R. rubiginosa* fixed oil combined with *K. ericoides* essential oil at 0.25 mg/mL produced the greatest reduction in toxicity, where the percentage mortality decreased significantly from 100% (*K. ericoides* essential oil independently) to less than 3% (1:1 oil combination) at 24 hrs (p-value = 0.04) and 48 hrs (p-value = 0.03).

For the anti-inflammatory and associated cytotoxicity studies, nitric oxide inhibition was investigated in lipopolysaccharide (LPS) induced RAW 264.7 murine macrophages treated with *R. rubiginosa* fixed oil and the eight essential oils. Simultaneous evaluation of cell viability (MTT assay) was used to confirm the absence of cytotoxicity of the test samples. *Rosa rubiginosa* fixed oil reduced LPS induced nitrite production by 18.75%, and the combination of *R. rubiginosa* fixed oil with *S. benzoin* essential oil reduced nitrite production by 33.80%, relative to the LPS control. Cytotoxicity in A549 human epithelial cells was ascertained by measuring cell viability, using a dual-staining method. The combination of *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil showed the best non-cytotoxicity in A549 epithelial cells relative to the cell culture medium.

Skin absorption (via diffusion) and retention of topically applied *R. rubiginosa* fixed oil and the eight essential oils were studied using excised porcine skin. Fourier Transform Infrared spectroscopy (FTIR) was used to obtain the qualitative infrared profiles depicting the vibration transitions of *R. rubiginosa* fixed oil and the essential oils. Differences in the wavenumber positions and peak intensities (percentage transmittance) of the oils at various concentrations, were noted. The combinations of *R. rubiginosa* fixed oil with either *C. zeylanicum*, *E. caryophyllata*, *S. benzoin*, or *R. damascena* essential oil, were extracted from the skin after 24 hrs and qualitatively detected using FTIR. This suggested that FTIR could be a suitable method for detection and qualitative analysis of *R. rubiginosa* fixed oil and essential oils within the concentration range of 1.25-100.00% v/v.

This study revealed that *R. rubiginosa* fixed oil (containing 41.93% of linoleic, 35.73% of linolenic, and 15.45% of oleic acids) with *S. benzoin* essential oil (containing 72.20% of dimethyl phthalate, 7.20% of benzyl acetate, and 5.70% of ethyl cinnamate) is an effective inhibitor of LPS induced nitrite production (as a parameter of anti-inflammatory activity). *Rosa rubiginosa* fixed oil with *S. benzoin* essential oil is a promising non-cytotoxic oil combination, able to absorb into and be retained within the skin when topically applied, which may be used to potentially treat the pathophysiological mechanisms involved in acne vulgaris. Future experimental analysis entails quantitative permeation studies of *R. rubiginosa* fixed oil and essential oils across an *in vitro* and/ or *ex vivo* skin model, to further support the use of these oils and combinations for treating acne pathogenesis.

DEDICATION

To the pursuit of happiness and the will of God.

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LIST OF PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Publication draft (abstract in Appendix A)

Ramburrun, S., Orchard, A., du Toit, L., and van Vuuren, S. (2022). *In vitro* bio-activity of *Rosa rubiginosa* (rosehip) oil combined with commercial essential oils for acne vulgaris. (In preparation).

Presentations (Appendix B)

1. Ramburrun, S., Orchard, A., and van Vuuren, S. FameLab 2021 WITS Preliminary Heat – University of the Witwatersrand, Johannesburg, South Africa, 6 May 2021. (Oral presentation).

2. Ramburrun, S., Orchard, A., and van Vuuren, S. FameLab 2021 WITS Final Heat – University of the Witwatersrand, Johannesburg, South Africa, 7 May 2021. (Oral presentation). **Awarded First Prize**.

3. Ramburrun, S., Orchard, A., and van Vuuren, S. *In vitro* antimicrobial, toxicity, cytotoxicity, and anti-inflammatory properties of *Rosa rubiginosa* (rosehip) fixed oil and essential oil combinations for acne treatment. Indigenous Plant Use Forum (IPUF) 2021 Virtual Conference – Johannesburg, South Africa, 5-7 July 2021. (Oral presentation). **Awarded Third Best Paper Presentation by a Young Scientist**.

4. Ramburrun, S., Orchard, A., and van Vuuren, S. *In vitro* investigation of *Rosa rubiginosa* (rosehip) oil and essential oil combinations for acne treatment. Cross-Faculty Postgraduate Symposium 2021 – University of the Witwatersrand, Johannesburg, South Africa, 26-30 July 2021. (Oral presentation).

5. Ramburrun, S., Orchard, A., and van Vuuren, S. FameLab 2021 South Africa Semi-Final – Johannesburg, South Africa, 28 September 2021. (Oral presentation).

6. Ramburrun, S., Orchard, A., and van Vuuren, S. FameLab 2021 South Africa Final – Johannesburg, South Africa, 6 October 2021. (Oral presentation).

ETHICS DECLARATION

I hereby confirm that the following study titled ‘Antimicrobial activity’ has received a waiver from ethics clearance from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with ethics waiver certificate reference number: W-CBP-200326-02 (Appendix C).

I hereby confirm that the following studies titled ‘Brine shrimp toxicity’ and ‘Skin absorption and retention’ have received a waiver from ethics clearance from the Animal Research Ethics Committee of the University of the Witwatersrand with ethics waiver certificate reference number: Waiver 09-04-2020-O (Appendix D).

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LIST OF ABBREVIATIONS

% Mortality	Percentage mortality
%	Percent
®	Registered trademark symbol
°C	Degrees Celsius
µg/mL	Micrograms per millilitre
µL	Microlitre
µM	Micromolar
ATCC	American Type Culture Collection
BSLA	Brine shrimp lethality assay
CC	Close corporation
CFU/mL	Colony forming units per millilitre
cm	Centimetres
cm^2	Centimetres squared
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
ePIA	Exopolysaccharide intercellular adhesion
FAME	Fatty acid modifying enzymes
FBS	Foetal bovine serum
FnBP	Fibronectin binding proteins
FTIR	Fourier Transform Infrared spectroscopy
g	Grams
GC-MS	Gas chromatography coupled to mass spectrometry
HIV	Human immunodeficiency virus
hr	Hour
hrs	Hours
iNOS	Inducible nitric oxide synthase
INT	*ρ*-iodonitrotetrazolium
kg	Kilograms
LPS	Lipopolysaccharide
mg/mL	Milligrams per millilitre
MIC	Minimum inhibitory concentration

min	Minutes
mL	Millilitre
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
ng/mL	Nanograms per millilitre
nm	Nanometre
NO	Nitric oxide
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PI	Propidium iodide
Pvt. Ltd.	Private limited company
rpm	Revolutions per minute
SARs	Structure-activity relationships
ΣFIC	Fractional inhibitory concentration index
TEWL	Transepidermal water loss
TGB	Thioglycolate broth
™	Trademark symbol
TNF-α	Tumour necrosis factor α
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
U/mL	Units per millilitre
USA	United States of America
V	Volts
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1
General introduction

1.1. Physical and psychosocial impact of acne

Acne is the eighth most prevalent disease worldwide (Zaenglein, 2018), appearing as pimples and dark spots on the skin throughout adolescence and frequently continuing into adulthood (Sutaria *et al.*, 2020). Acne vulgaris, a chronic inflammatory condition of the pilosebaceous unit, affects the sebaceous gland-rich skin of the face, upper arms, chest, and back (Sutaria *et al.*, 2020). This typical, cosmetic, and often self-limiting disease, may be exacerbated by bacterial infection, prompting comedones to form painful papules, pustules, nodules, and cysts. Enlarged, traumatised acne lesions develop fibrosis and depressed, hypertrophic or keloidal scarring (Sutaria *et al.*, 2020), post-inflammatory hyperpigmented macules, and erythema (Zaenglein, 2018). Since various skin features are typical indicators of health involved in attraction between sexes (Mescher, 2018), individuals with acne and consequent scarring may suffer from poor facial aesthetics and disfigurement. This may instigate negative perceptions (Zaenglein, 2018), stigmatisation and embarrassment which contribute to profound and lifelong psychosocial complications such as diminished self-esteem, social withdrawal, anxiety, and depression (Sutaria *et al.*, 2020). Adults with severe acne are also at a higher risk of an inferior quality of life than those without acne (Zaenglein, 2018).

1.2. Acne vulgaris pathogenesis

The pilosebaceous unit, consisting of a hair follicle and its associated sebaceous gland, secretes sebum (a complex mixture of lipids that are hydrolysed by bacterial enzymes after secretion) to maintain the *stratum corneum* and hair shafts (Mescher, 2018). These holocrine sebaceous glands become overly active during puberty and are involved in the pathogenesis of acne vulgaris (Mescher, 2018). Hyperkeratinisation and excessive holocrine sebum secretion, prompted by the surge of testosterone in males, and ovarian and adrenal androgens in females during puberty, lead to obstruction of the follicular ducts. The obstruction of the follicular ducts creates an ideal growth environment for three bacterial species most liable to induce acne vulgaris, including *Cutibacterium acnes* (formerly known as *Propionibacterium acnes*),

Staphylococcus epidermidis and *Staphylococcus aureus* (Kumar *et al.*, 2016). Activity of the normal skin commensal, *C. acnes*, causes proliferation within the accumulated sebum and produces localised inflammation and neutrophil infiltration (Mescher, 2018). Moreover, *S. epidermidis* induces inflammation and epidermal hyperproliferation (Sutaria *et al.*, 2020), while *S. aureus* causes tissue damage and deeper infection (Kumar *et al.*, 2016). The resulting distended follicle creates a small white, tender lesion known as a comedone (Mescher, 2018). Acne vulgaris pathogenesis is multifactorial, however, with several risk factors that include a family history of severe acne, polycystic ovary syndrome (PCOS), metabolic syndrome, and rare genetic conditions (example: Apert's syndrome) (Zaenglein, 2018).

1.2.1. *Cutibacterium acnes*

Cutibacterium acnes, ubiquitously present within the sebaceous follicles, is an opportunistic pathogen that promotes the progression of inflammatory acne vulgaris. *Cutibacterium acnes* (Gram-positive, aerotolerant anaerobic, non-motile bacillus) possesses degradative secretory enzymes. These enzymes aggravate the rupturing of the follicular walls in blocked hair follicles. *Cutibacterium acnes* colonisation of the pilosebaceous unit is considered as one of the key factors contributing to the inflammatory response of the skin in acne vulgaris (Dréno *et al.*, 2018, Jusuf *et al.*, 2020). *Cutibacterium acnes* affects keratinocytes and macrophages. Protein extracts, including peptidoglycan, lipoteichoic acid, cytosolic proteins, and membrane proteins (Jugeau *et al.*, 2005), of *C. acnes* stimulate macrophage expression of toll-like receptor 2 (TLR-2) *in vitro* (Kim *et al.*, 2002, Beylot *et al.*, 2014). *Cutibacterium acnes* also stimulates the production of pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) (Lee *et al.*, 2010), thereby further instigating inflammatory acne. The notable virulence genes involved in the pathogenesis of acne are camp5, GehA lipase, tly, sialidases, neuraminidases, endoglycoceramidases, lipases, and haemolysins (Kumar *et al.*, 2016). Moreover, porphyrins, hyaluronate lyase, cardiolipin synthetase, and calcineurin-like phosphoesterase contribute to the acne process by destroying healthy tissue. The bacterium also possesses several proteins associated with cell invasion making it highly immunoreactive, thereby increasing virulence (Kumar *et al.*, 2016).

1.2.2. *Staphylococcus epidermidis*

Staphylococcus epidermidis (Gram-positive, non-motile, facultative anaerobe), which produces virulent factors such as lipase and haemolysin delta, is also found in abundance in inflammatory acne vulgaris lesions (Kumar *et al.*, 2016, Jusuf *et al.*, 2020). It is usually a non-pathogenic resident of the skin, however, may become infectious due to extrinsic factors such as an immune system deficiency (Kumar *et al.*, 2016). *Staphylococcus epidermidis* possesses fatty acid modifying enzymes (FAMEs) which esterifies the fatty acids in the skin to cholesterol, as well as several adhesion factors (surface anchored proteins, fibrinogen binding protein, autolysin protein, exopolysaccharide intercellular adhesion (ePIA), and poly-N-succinyl-glucosamine) for its attachment to the skin surface (Kumar *et al.*, 2016). Additionally, *S. epidermidis* may form biofilms fostering antibiotic resistant genes, which horizontally transfer to other micro-organisms. Amongst various virulence factors of *S. epidermidis*, ePIA is responsible for biofilm formation and is secreted, protecting the bacterium against major components of the innate immune defence. This biofilm also provides favourable anaerobic conditions for *C. acnes* proliferation (Kumar *et al.*, 2016).

1.2.3. *Staphylococcus aureus*

Staphylococcus aureus (Gram-positive, non-motile, facultative anaerobe) is the most prominent member of the skin microbiota (Kumar *et al.*, 2016). *Staphylococcus aureus* may be pathogenic in many skin infections such as folliculitis and impetigo, and co-exist with other microbes in acne lesions (Kumar *et al.*, 2016). The pathogen invades the host cell by producing an extracellular matrix and serum binding proteins, such as adhesins and fibronectin binding proteins FnBP-A and FnBP-B. These factors connect the pathogen to cellular integrins of the host cell. The enzyme staphylokinase binds to defensins, rendering them unable to act against the pathogen, and the enzyme aureolysin binds and cleaves human cathelicidin LL-37, thereby enhancing the protection of the pathogen against the immune system. Once internalised, *S. aureus* plagues the skin and produces various extracellular enzymes such as proteases, lipases, hyaluronidases, and collagenase, that damages tissue and results in the intrusion of the pathogen into the deeper tissues. The prevalence and resistance patterns of *S. aureus* in the oropharynx of individuals with acne are higher compared to those without acne, which may indicate that the bacterium is associated with acne (Kumar *et al.*, 2016).

1.3. Causes of acne vulgaris

Endocrine conditions (PCOS and pregnancy); genetic factors (percentage of branched fatty acids in sebum); medicines (steroids, lithium, and anticonvulsants); occlusive wear (shoulder pads, headbands, backpacks, and underwire brassieres); and excessive exposure to sunlight may cause acne (Sutaria *et al.*, 2020). Moreover, foods with a high glycaemic index (dairy products and junk food) stimulate insulin-like growth factor one, triggering hyperkeratinisation and comedone formation. Severe anxiety and anger (which stimulate stress hormones), premenstrual hormonal fluctuations, and topical oil-based cosmetics, may exacerbate acne (Sutaria *et al.*, 2020). Acne vulgaris is clinically diagnosed, and although a multitude of grading scales are employed (because interobserver reliability and systems between studies vary greatly), a standard universal grading scale has not been established. The documentation of severity (clear, almost clear, mild, moderate, or severe) commonly guides treatment (Zaenglein, 2018). Figure 1.1 illustrates a simplified general scale which may be used to grade acne (Sutaria *et al.*, 2020):

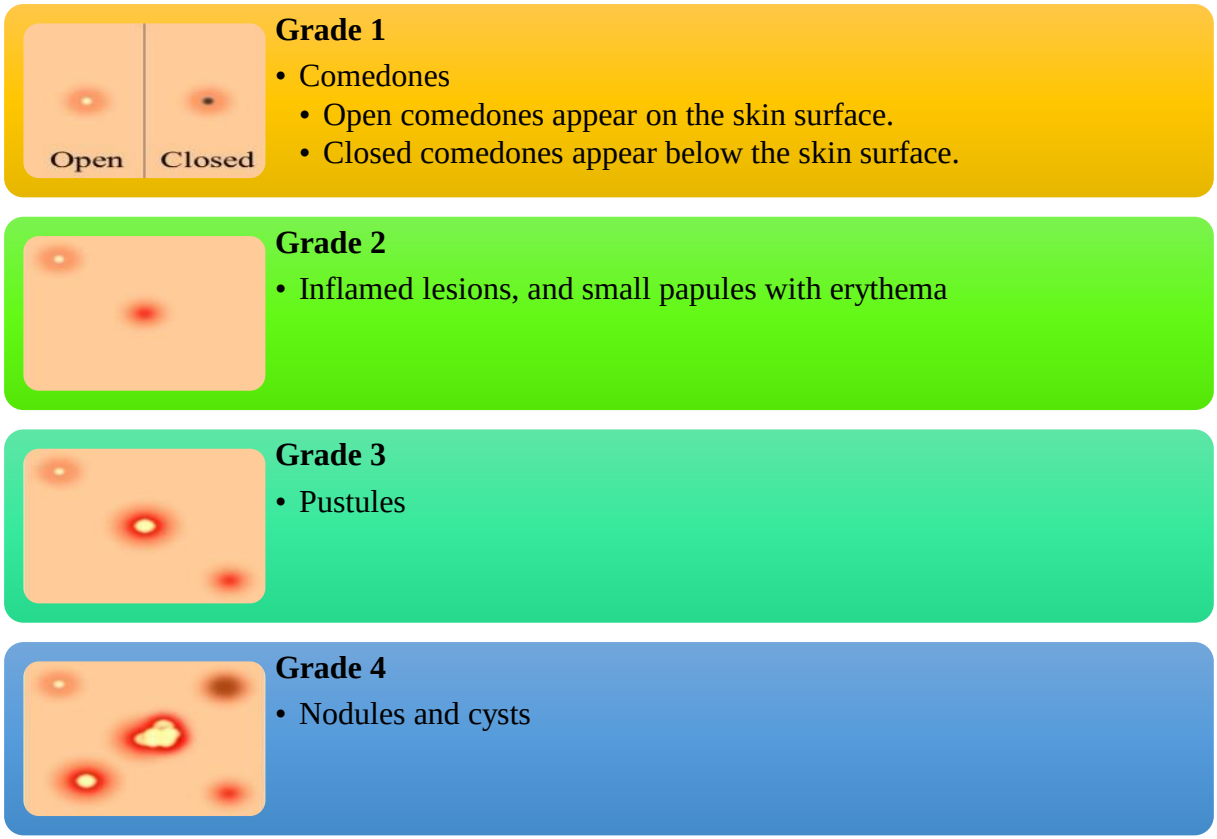

Figure 1.1. Simplified acne grading scale adapted from Sutaria *et al.* (2020) and Origimm Biotechnology (2020).

1.4. Current acne treatment

Anti-acne treatment consists of various topical and systemic preparations intended to reduce one or more of the following pathophysiological mechanisms of the disease (Haider and Shaw, 2004):

- Androgen-mediated sebum production
- Abnormal keratinisation
- Bacterial proliferation
- Inflammation

Current pharmacological treatments for acne and their limitations including adverse effects and contra-indications, based on the literature reviews carried out by Haider and Shaw (2004) and Marson and Baldwin (2019a, 2019b), are presented in Table 1.1.

Table 1.1. Current pharmacological acne treatments and their limitations adapted from Haider and Shaw (2004) and Marson and Baldwin (2019a, 2019b).

Pharmacological acne treatments	Limitations i.e. adverse effects, contra-indications
Topical	
Antibacterial: • Benzoyl peroxide • Clindamycin • Erythromycin • Tetracyclines • Azelaic acid	Broad: Cutaneous irritation i.e. erythema, peeling, dryness, and burning; bacterial resistance. Specific: • Benzoyl peroxide – irritant dermatitis, bleaching of hair and fabrics.
Retinoids: • Adapalene • Tazarotene • Tretinoin	Broad: Cutaneous irritation i.e. erythema, peeling, oedema, pruritis, and burning/ stinging; increased sensitivity to sunlight; teratogenicity.
Systemic	
Antibiotics: • Tetracyclines • Minocycline • Doxycycline • Macrolides • Trimethoprim/ sulfamethoxazole • Erythromycin	Broad: Gastrointestinal disturbances; hypersensitivity leading to anaphylaxis; candidiasis; antibiotic resistance. Specific: • Tetracyclines – gastrointestinal dyspepsia, vaginal candidiasis (females), photosensitivity, enamel hypoplasia and yellowish discolouration of the forming teeth (children under ten years of age).

Pharmacological acne treatments	Limitations i.e. adverse effects, contra-indications
	• Minocycline – vertigo, dizziness, ataxia, bluish discolouration of the skin, drug-induced lupus, auto-immune hepatitis, and pigment deposition in the skin and mucous membranes. • Trimethoprim/ sulfamethoxazole – mild photosensitivity, life-threatening drug eruptions (Stevens-Johnson syndrome), toxic epidermal necrolysis, haematologic disorders (neutropenia, aplastic anaemias, and thrombocytopenia), and human immunodeficiency virus (HIV) patients are at higher risk of adverse effects.
Hormonal: • Oral contraceptives containing oestrogen • Spironolactone • Flutamide • Cyproterone acetate	Broad: Tolerated in females only; weight gain, breast tenderness, breakthrough bleeding, moodiness, venous thromboembolism, stroke, myocardial infarction; increased risk of cardiovascular disease, breast and ovarian cancers; contra-indications include smoking, migraine headaches with aura, and hypertension. Specific: • Spironolactone – menstrual irregularities, breast enlargement, fatigue, dizziness, headache, diuresis (concern for hyperkalaemia), and gynaecomastia (males).
Isotretinoin • Vitamin A metabolites	Broad: Dry lips, skin and eyes; decreased night vision; headache; epistaxis; backache; benign intracranial hypertension, elevation in liver enzymes and serum lipid indices, especially triglycerides; musculoskeletal complaints; teratogenicity; depression and suicide.

Procedural therapies (comedone extraction, chemical peels, laser or light treatment) have been used to complement pharmacological acne treatments (Taub, 2007), however, procedural therapies may be expensive with limited availability. Although procedural therapies have demonstrated efficacy, treatment is limited by frequent and lengthy maintenance procedures; and lacks long-term follow-up (Taub, 2007). Over-the-counter cosmetic therapies containing charcoal, zinc, or essential oils, are available in the form of cleansers, masks, gels, or creams (Hammer, 2015). However, many of these cosmetic treatments lack scientific or clinical evidence proving their efficacy. Although the general prognosis of acne is positive with a combination of topical and systemic treatments (Taub, 2007, Marson and Baldwin, 2019b),

harsh adverse effects causing discomfort, complicated medication regimens, and expensive remedies lower compliance. Since most antibiotic acne treatments are used for both their antimicrobial and anti-inflammatory effects (Haider and Shaw, 2004), the rise in antimicrobial resistance further restricts treatment of inflammatory acne vulgaris. Therefore, the importance of research and development strategies for discovering alternative agents to treat acne, such as essential oils, is emphasised (Orchard *et al.*, 2018a).

1.5. Essential oils for acne treatment

Essential oils are prevalent natural, volatile products with dermatological use being one of their leading applications. The aromatherapeutic literature reviewed by Orchard and van Vuuren (2017) identified that essential oils were predominantly used to treat infections, followed by inflammatory skin concerns and then general skin homeostasis. Forty-nine of the 98 (50%) essential oils proposed for dermatological application were endorsed particularly for acne treatment (Orchard and van Vuuren, 2017). Research conducted by Orchard *et al*. (2017, 2018a, 2019) studied 59 commercial essential oils, of which 27 oils inhibited the growth of two acne-inducing micro-organisms, and 17 oils inhibited the growth of three acne-inducing micro-organisms.

1.6. Fixed oils in dermatology

Essential oils are rarely applied undiluted, directly onto the skin due to potent skin irritancy (Trattner *et al.*, 2008). Hence, essential oils are combined within a base prior to application as a means of dilution to subsequently reduce toxicity and skin irritation. Fixed oils are the commonly used base in aromatherapy, believed to reduce the evaporation and enhance the cutaneous absorption of essential oils (Orchard and van Vuuren, 2019). Fixed oils may also augment therapeutic efficacy of essential oils by moisturising, nourishing, and healing the skin (Michalak, 2018, Michalak and Glinka, 2018). In a recent review by Orchard and van Vuuren (2019), several studies revealed that the majority of fixed oils exhibited moderate to poor antimicrobial activity, however, an enhanced healing rate was observed due to anti-oxidant or anti-inflammatory properties, or improved collagen formation. Thus, it appears that fixed oils do not always contribute towards wound healing via antimicrobial activity, but rather as anti-oxidants, anti-inflammatories or as tissue growth inducers (Orchard and van Vuuren, 2019).

Fixed oils contribute a necessary role in dermatological application, however, studies supporting the dermatological application of fixed oils are rare. Hence, fixed oils (such as *Rosa rubiginosa* (rosehip) fixed oil) require further investigation concerning their interactions with essential oils for dermatological application.

1.7. Potential of various *Rosa* species

A study conducted by Yi *et al.* (2007) explored the anti-oxidant and antimicrobial properties of several *Rosa* extracts (*Rosa nutkana* (Nootka rose), *Rosa pisocarpa* (cluster rose) and *Rosa woodsia* (Woods' rose)) from wild British Columbia populations, and reported that all extracts displayed promising anti-oxidant activity correlated with antimicrobial activity. Antimicrobial activity of the extracts was determined using the disc diffusion method. The growth of Gram-positive bacteria (*Enterococcus faecalis*, *Bacillus subtilis*, and *S. aureus* – including a methicillin-resistant strain) was inhibited by the extracts (Yi *et al.*, 2007). The methanolic leaf extract (Živković *et al.*, 2015) and powdered rosehips (Ghendov-Moşanu *et al.*, 2018) of *Rosa canina* (dog rose) (a powerful inhibitor of *Pseudomonas aeruginosa*, *Leishmania monocytogenes*, *Escherichia coli*, and *S. aureus*), and polyphenol obtained from *Rosa damascena* (Damask rose) (a potent tyrosinase inhibitor capable of reducing hyperpigmentation) may be beneficially employed in cosmeceutical formulations (Dorni *et al.*, 2017). *Rosa damascena* essential oil also demonstrated noteworthy antimicrobial properties against the acne-inducing micro-organisms, *C. acnes*, *S. epidermidis* and *S. aureus* (Orchard *et al*., 2018a, 2018b). The ethanol and chloroform extracts (Mahboubi, 2016), and essential oil (Akram *et al.*, 2020) of *R. damascena* have also displayed anti-inflammatory effects in animal models. Literature reveals that both the fixed and essential oils of various *Rosa* species may be used for the topical therapy of several skin diseases as numerous preparations displayed promising anti-oxidative, antimicrobial, and anti-inflammatory activities (Chrubasik *et al.*, 2008, de Santana *et al.*, 2016, Dorni *et al.*, 2017).

1.8. *Rosa rubiginosa* (rosehip) fixed oil

Rosa rubiginosa (also known as *Rosa eglanteria* or *Rosa mosqueta*) contains substantial concentrations of trans retinoic acid, exhibits regenerative properties, and is commonly applied in cosmetics and medical clinics to support the regeneration of scarred skin (de Santana *et al.*,

2016). *Rosa rubiginosa* oil is a fixed oil comprising of high compositions of unsaturated fatty acids, encompassing linoleic (18:2 ω-6) (41.93% ±1.20), linolenic (18:3 ω-3) (35.73% ±2.50), and oleic (18:1 ω-9) (15.45% ±0.46) acids, and as such, may offer relatively high protection against inflammation (Lin *et al.*, 2018). Furthermore, linoleic, linolenic, and oleic acids are naturally present within the *stratum corneum* with linoleic acid being the most abundant polyunsaturated fatty acid (Lin *et al.*, 2018). Lin *et al.* (2018) speculated that the linoleic acid content in the skin may support the passage of fixed oils (such as *R. rubiginosa* fixed oil) through the epidermal layers. Clinical studies by van der Walt (2016) found beneficial results including improved skin moisture levels, antiwrinkle and visco-elastic properties of *R. rubiginosa* fixed oil. Van der Walt (2016) concluded that *R. rubiginosa* fixed oil may be considered as a potential, valuable cosmetic constituent for the enhancement of healthy skin aesthetics. Despite the belief that fixed oils enhance the absorption of diluted essential oils through the skin (Orchard and van Vuuren, 2019), supportive studies are lacking.

1.9. Fixed and essential oil combinations

Contrary to copious laymen literature favouring the application of plant oils for acne treatment, scientific research concerning essential oils as a treatment for acne vulgaris is limited (Orchard *et al.*, 2018a). Research concerning the use of fixed oils is explored to an even lesser extent, although both fixed and essential oils are frequently used in combination for skin preparations (Orchard and van Vuuren, 2019). A study by Orchard *et al.* (2019) reported that fixed oils positively influence essential oil toxicity without antagonising antimicrobial activity. Many of the essential oils were toxic at 24 and 48 hrs, even at a low concentration of 1.00 mg/mL. Several essential oils exhibited high toxicity levels, including *Cymbopogon citratus* (lemon grass), *Syzygium aromaticum* (clove), and *Thymus vulgaris* (thyme), with 50% or less of the fixed oils reducing their toxicity. Synergism and reduced essential oil toxicity after 48 hrs (referred to as quenching) were frequently noticed. *Aloe vera* (aloe vera) macerate oil and *Simmondsia chinensis* (jojoba) liquid wax were observed to be the fixed oils most commonly involved in increased antimicrobial activity and decreased toxicity. *Hypericum perforatum* (St John's wort), *Calendula officinalis* (calendula), and *Persea americana* (avocado) fixed oils also improved antimicrobial activity of several essential oils (Orchard *et al.*, 2019). Fixed oils may possibly be used in dermatology to lower the toxicity of products that irritate the skin, however, research examining *R. rubiginosa* fixed oil combined with essential oils is lacking.

1.10. Skin absorption and retention

The *stratum corneum* is the primary skin barrier and acts as the rate-limiting factor impeding the diffusion of hydrophilic molecules (World Health Organization, 2006, Petrilli and Lopez, 2018). For topical delivery, the active ingredient needs to permeate the *stratum corneum* to reach the viable epidermis and dermis, which are the layers of the skin afflicted by acne vulgaris (Thiboutot and Del Rosso, 2013, Wortsman *et al.*, 2014). Topical delivery of the active ingredient is facilitated by three major routes (Figure 1.2): transcellular, inter-cellular and appendageal permeation. The transcellular route concerns the diffusion of substances through corneocyte membranes, whereas the inter-cellular route concerns the diffusion of substances through the lipid matrix, in between packed corneocytes. The appendageal route is aided by hair follicles, and sebaceous and sweat glands (World Health Organization, 2006). Typically, small lipophilic and hydrophilic molecules can passively permeate the *stratum corneum* or traverse the epidermis via the shunts of hair shafts and sweat glands (Goyal *et al.*, 2016). The key challenge for topical delivery is the balance between the permeation of the active ingredient through the *stratum corneum*, and subsequent accumulation thereof in the skin, to ensure an appropriate therapeutic concentration (Petrilli and Lopez, 2018).

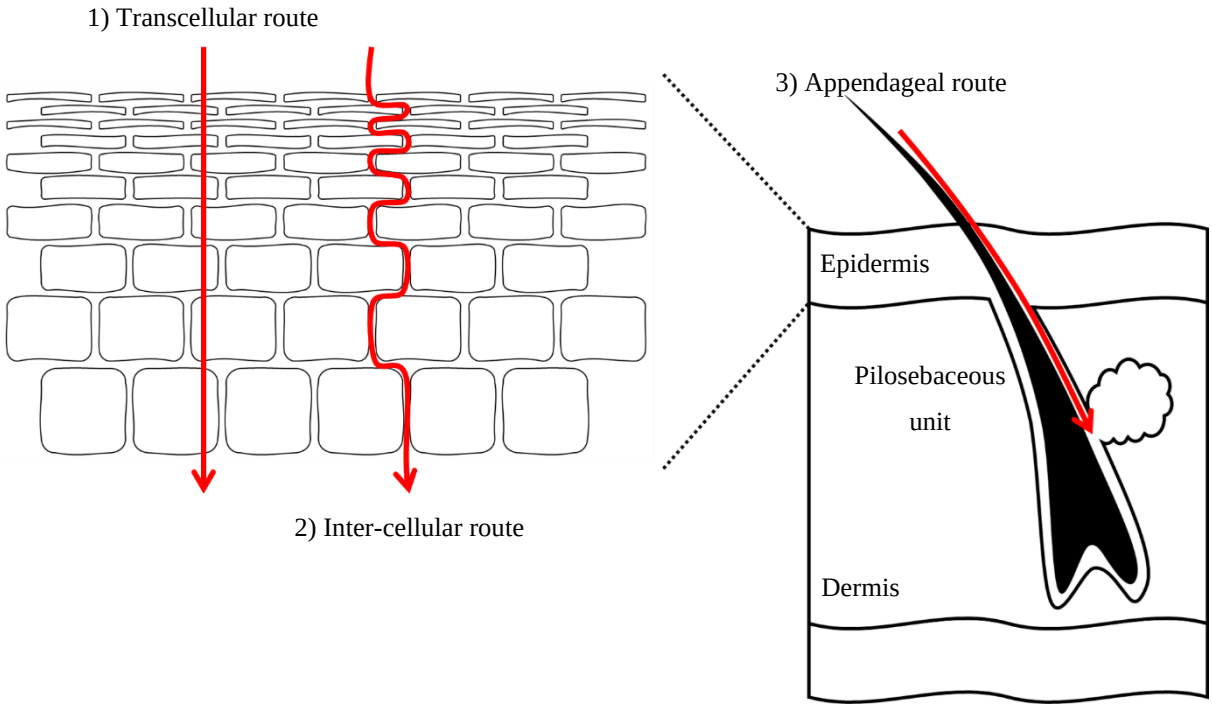

Figure 1.2. Three major routes of topical delivery adapted from Schneider-Rauber *et al.* (2020).

1.11. Skin absorption and retention of fixed and essential oils

Research concerning topically applied fixed and essential oils in various animal models are limited to transdermal permeation enhancement (Jiang *et al.*, 2017, Lin *et al.*, 2018). As such, research studying skin absorption and retention of fixed and essential oils as active ingredients requires further analysis. Despite the belief that fixed oils improve the absorption of diluted essential oils through the skin, inadequate reports of fixed oil skin absorption exist (Orchard and van Vuuren, 2019). The skin constantly maintains its barrier function to primarily protect the body against noxious stimuli (irritants) (Lin *et al.*, 2018). Topical applications of essential oils to treat acne require permeation through the numerous layers of the skin to reach the viable epidermis, yet skin absorption studies of essential oils are lacking. The transport of a molecule through the *stratum corneum* is generally achieved via diffusion, however, the hydrophilic epidermal layers and damage to the *stratum corneum* (as with acne vulgaris) hinder lipophilic molecular permeation (van der Walt, 2016). The chemical composition of fixed and essential oils also influences skin absorption (Tisserand and Young, 2013, van der Walt, 2016). Thus, investigations concerning the effect of fixed and essential oil combination therapy on skin absorption necessitates additional studies.

1.12. Rationale and motivation for this study

Aesthetic skin-care products are a billion-dollar industry (Statista, 2020) with acne presenting as one of the most popular cosmetic uses for essential oils (Orchard *et al.*, 2018a). With the global trend towards the use of naturally-occurring products (Statista, 2020), fixed and essential oil combinations offer an alternative and viable approach to discovering modern agents for anti-acne therapy while conventional chemistry faces challenges in the discovery of new antimicrobials. Noting that fixed oils contribute a crucial aspect in dermatology, further research concerning their interaction with essential oils are necessary (Orchard and van Vuuren, 2019). Despite the limited literature profiling different forms (extracts, powders, or essential oils) of various *Rosa* species inciting notable antimicrobial and anti-inflammatory activities, there are no current studies concerning *R. rubiginosa* fixed oil as a carrier for essential oils. Neither is there any acne research of this fixed oil in combination with essential oils. Hence, this study is the first to investigate the influence of *R. rubiginosa* fixed oil in combination with commercial essential oils.

1.13. Aim and objectives

This study aimed to investigate the *in vitro* antimicrobial, toxicity, anti-inflammatory, and associated cytotoxicity, as well as the skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil independently and in combination with commercial essential oils to find promising oil combinations for the potential and efficient topical treatment of the pathophysiological mechanisms of acne vulgaris. This was achieved by the following objectives:

Objective 1: To determine the antimicrobial activity of *R. rubiginosa* fixed oil independently and in combination with a selection of essential oils against the reference strains of three acne-inducing pathogens (*C. acnes*, *S. epidermidis* and *S. aureus*) using the broth microdilution assay. The antimicrobial interactions were then evaluated by calculating the fractional inhibitory concentration index (ΣFIC), and interactions (synergy, additive interaction, or antagonism) were analysed using isobolograms.

Objective 2: To screen the toxicity of *R. rubiginosa* fixed oil independently and in combination with the essential oils (whereby selection was based on those combinations displaying antimicrobial synergy, additive interactions, or antagonism) using the brine shrimp lethality assay (BSLA).

Objective 3: To investigate the anti-inflammatory activity and cytotoxicity of *R. rubiginosa* fixed oil independently and in combination with the essential oils whereby selection was based on those combinations displaying antimicrobial synergy, additive interactions, or antagonism. The lipopolysaccharide (LPS) induced inflammatory pathway in a RAW 264.7 murine macrophage model was used, and cell viability was simultaneously evaluated using the MTT assay. The cytotoxicity was also assessed in A549 epithelial cells.

Objective 4: To detect porcine skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil independently and in combination with the essential oils (whereby selection was based on those combinations displaying antimicrobial synergy, additive interactions, or antagonism) using qualitative Fourier Transform Infrared spectroscopy (FTIR) analysis.

CHAPTER 2

Antimicrobial activity

2.1. Introduction

Antimicrobial susceptibility testing is essential for verifying the susceptibility of antimicrobial products to facilitate treatment decisions (Jorgensen and Ferraro, 2009). Dilution techniques, used to ascertain the minimum inhibitory concentration (MIC) of antimicrobials, are the most used antimicrobial susceptibility testing techniques. The MIC is defined as "*the lowest concentration of an antimicrobial agent (in mg/mL) that, under defined in vitro conditions, prevents the appearance of visible growth of a micro-organism within a defined period of time*" (EUCAST, 2003). The broth microdilution used in the current study is one of the most prevalent methods used to assess the antimicrobial activities of plant oils (Kalemba and Kunicka, 2003, Tan and Lim, 2015, Golus *et al.*, 2016).

In vitro combined antimicrobial susceptibility testing is commonly performed to evaluate the interaction between antimicrobial products (Huang *et al.*, 2019). The fractional inhibitory concentration index (ΣFIC) is applied to isobologram analysis to determine the interaction between antimicrobial products. Of the various methods used to evaluate the interaction between antimicrobial products, isobologram analysis has been mathematically proven and is frequently used to determine the interaction (Huang *et al.*, 2019).

This chapter focuses on the antimicrobial efficacy of *R. rubiginosa* fixed oil independently and in combination with a selection of 30 commercial essential oils against the reference strains of three acne-inducing pathogens, using the broth microdilution assay to ascertain the MIC. The antimicrobial interactions of the 1:1 oil combinations, as determined by calculating the ΣFIC, and varied ratio combinations of the 1:1 oil combinations displaying either synergy, additive interactions, or antagonism, were further explored.

2.2. Materials and methods

2.2.1. Oil selection, procurement, and sample preparation

Rosa rubiginosa fixed oil was donated by Wynand Gericke (Rosehip Farm, Gauteng, South Africa) and its certificate of analysis was supplied (Appendix E). Thirty commercial essential oils based on the research conducted by Orchard *et al*. (2017, 2018a, 2019) (Appendix F), identifying oils with noteworthy antimicrobial activity against at least two of the three acne-inducing micro-organisms, were selected for combination studies. The essential oils were obtained from international flavour and fragrance industries: Prana Monde CC, Scatters Oils, Escentia Products, Aromatics International, Windrose CC, and Meadowbank Essential Oils. The chemical profiles of the selected essential oils have been formerly investigated, using gas chromatography coupled to mass spectrometry (GC-MS), and reported (van Vuuren *et al.*, 2014, Orchard *et al.*, 2017, 2018a, 2019). Appendix F provides a list of the selected essential oils including a summary of the chemical profiles and mean antimicrobial activities previously reported.

Samples were prepared by first weighing and then solubilising the oils in absolute acetone (MK Chemicals) to obtain a starting concentration of 32.00 mg/mL, using Equation 2.1:

$$\text{Volume of acetone required } (\mu\text{L}) = \frac{\text{Weight of oil (mg)} \times 1000}{32.00 \text{ mg/mL}}$$

Equation 2.1.

The prepared oil samples were stored in tightly sealed amber bottles at approximately 4°C until further analysis (van Vuuren *et al.*, 2019).

Table 2.1 lists the negative and positive controls used in the current study and the purpose of each for antimicrobial investigation.

Table 2.1. Controls used in the antimicrobial investigation.

Sample	Type of control	Purpose
Thioglycolate broth with *C. acnes*	Culture control	Bacterial culture medium to ensure viability.
Tryptone Soya broth with either *S. epidermidis* or *S. aureus*	Culture control	
Sterile water in acetone (32.00 mg/mL)	Negative control	To ensure antimicrobial activity was not because of the solvent used for the samples.
Ciprofloxacin (0.01 mg/mL)	Positive control	Broad-spectrum antibiotic to ensure susceptibility of the reference strains.
Benzyl peroxide (5% w/v)	Positive control	Anti-acne agent to ensure susceptibility of the reference strains to a conventional antimicrobial.
Erythromycin (0.01 mg/mL)	Positive control	

2.2.2. Culture preparation

Reference strains of *C. acnes* (ATCC® 11827™), *S. epidermidis* (ATCC® 12228™) and *S. aureus* (ATCC® 25923™) were obtained from the American Type Culture Collection (ATCC) via Davies Diagnostics (Pty) Ltd. This selection was based on their acne relevance (Kim *et al.*, 2008, Dryden, 2010, Kumar *et al.*, 2016). *Cutibacterium acnes* was inoculated into Thioglycolate broth (TGB) (Oxoid) and incubated under anaerobic conditions using a CO_2 (5.00%) incubator (Heal Force HF90 CO_2 Incubator) for seven days at 37°C. *Staphylococcus epidermidis* and *S. aureus* were grown in Tryptone Soya broth (TSB) (Oxoid) for 24 hrs at 37°C. All reference strains were streaked onto Tryptone Soya agar (TSA) (Oxoid) plates and incubated (Memmert Incubator IN260) aerobically for 24 hrs at 37°C to confirm purity. The absence of growth on the streak plates for *C. acnes* indicated purity as no growth should be present for aerobic conditions for the anaerobe. An ethics waiver certificate for the use of these micro-organisms was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical), reference number: W-CBP-200326-02 (Appendix C).

2.2.3. Minimum inhibitory concentration (MIC) assay

Rosa rubiginosa fixed oil and the selected essential oils were tested independently, and then in 1:1 combinations (fixed oil: essential oil) using the broth microdilution assay (Orchard *et al.*, 2018a). The microtitre plates were aseptically prepared by adding 100 µL of broth (TGB for *C. acnes* or TSB for *S. epidermidis* and *S. aureus*) into each of the 96 wells. The test samples

(100 µL of *R. rubiginosa* fixed oil or essential oil, or 50 µL of *R. rubiginosa* fixed oil and 50 µL of essential oil for combinations) were pipetted into the first row, then serially diluted to achieve sample concentrations of 8.00, 4.00, 2.00, 1.00, 0.50, 0.25, 0.125, and 0.063 mg/mL. Each sample and combination were tested in quadruplicate and repeated where discrepancies were found ($n \geq 4$). Broth with test organism (100 µL) was included as a culture control. The broad-spectrum antibiotic, ciprofloxacin (Sigma-Aldrich®), was included as a positive control (100 µL of 0.01 mg/mL). Benzyl peroxide (5% w/v) and erythromycin (0.01 mg/mL) (Sigma-Aldrich®) were also included as positive controls (100 µL). Sterile water in acetone was included as a negative control (100 µL of 32.00 mg/mL). An approximate inoculum concentration of 1×10^6 colony forming units (CFU)/mL was prepared for each micro-organism and 100 µL added to each well. The microtitre plates were then sealed with sterile adhesive sealing film and incubated at the respective incubation conditions.

After incubation, 40 µL of 0.04% w/v *ρ*-iodonitrotetrazolium violet solution (INT) (Sigma-Aldrich®) was added to each well, where a colour change from clear to pink or purple indicated microbial growth. The MIC was taken as the lowest concentration displaying no colour change. An MIC value of ≤ 1.00 mg/mL was considered noteworthy (Orchard *et al.*, 2017) and an MIC value of > 1.00 - ≤ 2.00 mg/mL was considered moderate (Orchard *et al.*, 2019). The values were recorded, and the mean MIC with standard deviation was calculated for each sample using Microsoft Excel Office 365 (Version 2202) software. The ΣFIC was then calculated for the oil combinations.

2.2.4. Fractional inhibitory concentration index (ΣFIC)

The ΣFIC, adapted from the review by van Vuuren and Viljoen (2011), was calculated according to Equation 2.2:

$$\text{Division factor} = \frac{\text{MIC of oil combination}}{2}$$

$$\text{FIC(i)} = \frac{\text{Division factor}}{\text{MIC of } \textit{R. rubiginosa} \text{ fixed oil independently}}$$

$$\text{FIC(ii)} = \frac{\text{Division factor}}{\text{MIC of essential oil independently}}$$

$$\Sigma\text{FIC} = \text{FIC(i)} + \text{FIC(ii)}$$

Equation 2.2.

The ΣFIC per oil combination was interpreted as ≤ 0.5 indicating synergy; > 0.5 - ≤ 1.0 as additive interaction; > 1.0 - ≤ 4.0 indicating indifference; and > 4.0 as antagonism (van Vuuren and Viljoen, 2011).

The values were recorded, and the mean ΣFIC was calculated for each sample against all three reference strains using Microsoft Excel Office 365 software. Attention was given to the combinations that displayed synergy, additive interactions, or antagonism against the three reference strains, which were then further investigated at various ratios using an isobologram. The large number of indifferent interactions, which were of lesser interest, were not further investigated.

2.2.5. Varied ratio combinations

The same method for the 1:1 combination in Section 2.2.3. was used, however, the concentration of the combinations varied in ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. The MIC values for the various ratios were recorded (Appendix G), and the ΣFIC was expressed graphically on an isobologram using GraphPad Prism® (Version 5) software. The lines connecting the two axes indicated the individual doses, and the isobolograms were interpreted by examining the data points of the ratios. Data points falling beneath and inclusive of the 0.5:0.5-line are represented as synergy, indicating that the combined effect produced an overall superior antimicrobial effect. Data points between the 0.5:0.5 and 1:1-lines displayed additive interaction, indicating that the combined effect produced an overall improved antimicrobial effect. Data points above the 1:1-line and below and inclusive of the 4:4-line exhibited indifference. Data points above the 4:4-line signified antagonism, indicating that the combined effect produced an overall inferior antimicrobial effect (van Vuuren and Viljoen, 2011).

2.3. Results and discussion

2.3.1. Independent oils

The mean MIC values (n ≥ 4) of *R. rubiginosa* fixed oil independently are presented in Table 2.2. The antimicrobial activity of *R. rubiginosa* fixed oil is reported for the first time in this study. *Rosa rubiginosa* fixed oil displayed moderate antimicrobial activity against *C. acnes* and poor antimicrobial activity against *S. epidermidis* and *S. aureus* (mean MIC = 3.33 mg/mL against all three reference strains). This was expected as fixed oils typically do not exhibit antimicrobial activity (Orchard *et al.*, 2019, Orchard and van Vuuren, 2019).

Table 2.2. Mean independent MIC values (n ≥ 4) with standard deviation of *R. rubiginosa* fixed oil against the three reference strains.

	Mean independent MIC values		
Sample	***C. acnes* (ATCC® 11827™)**	***S. epidermidis* (ATCC® 12228™)**	***S. aureus* (ATCC® 25923™)**
Rosa rubiginosa L. (rosehip)	2.00 mg/mL ±0.00	4.00 mg/mL ±0.00	4.00 mg/mL ±0.00
Benzoyl peroxide (5.00% w/v)	0.78 mg/mL ±0.00	1.56 mg/mL ±0.00	3.13 mg/mL ±0.00
Ciprofloxacin (0.01 mg/mL)	0.625 µg/mL ±0.00	0.625 µg/mL ±0.00	0.625 µg/mL ±0.00
Erythromycin (0.01 mg/mL)	0.039 µg/mL ±0.00	1.25 µg/mL ±0.00	1.25 µg/mL ±0.00
Sterile water in acetone	2.00 mg/mL ±0.00	4.00 mg/mL ±0.00	4.00 mg/mL ±0.00
Culture control	> 8.00 mg/mL ±0.00	> 8.00 mg/mL ±0.00	> 8.00 mg/mL ±0.00

The mean MIC values (n ≥ 4) of the independent essential oils against the three reference strains are presented in Table 2.3. The selection of the 30 commercial essential oils was based on previous research conducted by Orchard *et al*. (2017, 2018a, 2019), thus most of the antimicrobial activity of the independent essential oils tested in the present study was similar to that of the previous research. As such, attention was given to the antimicrobial activity of *R. rubiginosa* fixed oil combined with the essential oils rather than that of the essential oils independently.

Of the 30 essential oils studied, fewer of the oils demonstrated noteworthy antimicrobial activity against the acne-inducing reference strains (*C. acnes*, *S. epidermidis*, and *S. aureus*) compared to the previous studies by Orchard *et al.* (2017, 2018a, 2019). The differences in noteworthy antimicrobial activity may be attributed to variations in the chemotype (Appendix F) of the essential oils. Composition may differ between plants of the same species depending on the environment or location in which the plants were grown, harvest season, part of the plants harvested, the process of essential oil extraction, storage, and degradation. Alternatively, composition may alter due to exposure of the essential oils to light, oxygen, extreme temperatures, and various storage conditions over time (Gonçalves *et al.*, 2007, Kırmızıbekmez *et al.*, 2009, Riahi *et al.*, 2013).

Table 2.3. Mean MIC values ($n \geq 4$) with standard deviation of the essential oils independently, and in 1:1 combination with *R. rubiginosa* fixed oil, with the respective ΣFIC, against the three reference strains.

	Mean MIC values (mg/mL) and ΣFIC									
	***C. acnes* (ATCC® 11827™)**			***S. epidermidis* (ATCC® 12228™)**			***S. aureus* (ATCC® 25923™)**			
Sample	**Independent MIC[a]**	**Combination MIC[b]**	**ΣFIC[c]**	**Independent MIC**	**Combination MIC**	**ΣFIC**	**Independent MIC**	**Combination MIC**	**ΣFIC**	**Mean ΣFIC[d]**
Cinnamomum zeylanicum Blume (true cinnamon)[e]	**0.19 ±0.06[f]**	**0.25 ±0.00**	*0.73*[g]	**1.00 ±0.00**	**0.50 ±0.00**	***0.31***[h]	**1.00 ±0.00**	**0.50 ±0.00**	***0.31***	***0.45***
Citrus aurantium var. *amara* L. (bitter orange)	**0.88 ±0.22**	1.50 ±0.50	1.23	3.00 ±1.00	3.00 ±1.00	*0.88*	2.00 ±0.00	4.00 ±0.00	1.50	1.20
Commiphora molmol (Engl.) Engl. ex Tschirch (myrrh)	**0.75 ±0.25**	**1.00 ±0.00**	*0.92*	3.00 ±1.00	3.00 ±1.00	*0.88*	2.00 ±0.00	4.00 ±0.00	1.50	1.10
Cymbopogon citratus (DC.) Stapf (lemon grass)	**0.44 ±0.11**	**1.00 ±0.00**	1.39	1.50 ±0.50	2.00 ±0.00	*0.92*	**1.00 ±0.00**	2.00 ±0.00	1.25	1.19
Cymbopogon martini (Roxb.) W.Watson (palmarosa)	**0.38 ±0.13**	**1.00 ±0.00**	1.58	1.50 ±0.50	2.00 ±0.00	*0.92*	**1.00 ±0.00**	2.00 ±0.00	1.25	1.25
Cymbopogon nardus (L.) Rendle (citronella grass)	**1.00 ±0.00**	**1.00 ±0.00**	*0.75*	3.00 ±1.00	3.00 ±1.00	*0.88*	2.00 ±0.00	4.00 ±0.00	1.50	1.04
Eugenia caryophyllata Thunb. (clove)	**0.50 ±0.00**	**1.00 ±0.00**	1.25	2.00 ±0.00	2.00 ±0.00	*0.75*	2.00 ±0.00	2.00 ±0.00	*0.75*	*0.92*
Helichrysum italicum (Roth) G.Don (Italian strawflower)	**0.38 ±0.13**	1.50 ±0.50	2.38	3.00 ±1.00	4.00 ±0.00	1.17	4.00 ±0.00	4.00 ±0.00	1.00	1.51
Hyssopus officinalis L. (hyssop)	**0.50 ±0.00**	**1.00 ±0.00**	1.25	2.00 ±0.00	3.00 ±1.00	1.13	2.00 ±0.00	4.00 ±0.00	1.50	1.29
Kunzea ericoides (A.Rich.) Joy Thomps. (kanuka)	**1.00 ±0.00**	**1.00 ±0.00**	*0.75*	4.00 ±0.00	4.00 ±0.00	1.00	4.00 ±0.00	4.00 ±0.00	1.00	*0.92*
Laurus nobilis L. (bay laurel)	**1.00 ±0.00**	**1.00 ±0.00**	*0.75*	3.00 ±1.00	4.00 ±0.00	1.17	3.00 ±1.00	4.00 ±0.00	1.17	1.03
Leptospermum petersonii F.M.Bailey (lemon-scented tea tree)	**0.44 ±0.11**	**1.00 ±0.00**	1.39	1.50 ±0.50	2.00 ±0.00	*0.92*	**1.00 ±0.00**	2.00 ±0.00	1.25	1.19

Mean MIC values (mg/mL) and ΣFIC										
	***C. acnes* (ATCC® 11827™)**			***S. epidermidis* (ATCC® 12228™)**			***S. aureus* (ATCC® 25923™)**			
Sample	**Independent MIC[a]**	**Combination MIC[b]**	**ΣFIC[c]**	**Independent MIC**	**Combination MIC**	**ΣFIC**	**Independent MIC**	**Combination MIC**	**ΣFIC**	**Mean ΣFIC[d]**
Leptospermum scoparium J.R.Forst. & G.Forst. (manuka)	**0.38 ±0.13**	**0.88 ±0.22**	1.39	1.25 ±0.43	2.00 ±0.00	1.05	**1.00 ±0.00**	2.00 ±0.00	1.25	1.23
Litsea cubeba (Lour.) Pers. (may chang)	**0.38 ±0.13**	**0.75 ±0.25**	1.19	**1.00 ±0.00**	2.00 ±0.00	1.25	1.50 ±0.50	2.00 ±0.00	*0.92*	1.12
Matricaria recutita L. (chamomile)	**0.44 ±0.11**	**1.00 ±0.00**	1.39	2.00 ±0.00	4.00 ±0.00	1.50	3.00 ±1.00	4.00 ±0.00	1.17	1.35
Melaleuca alternifolia (Maiden & Betche) Cheel (tea tree)	**0.63 ±0.22**	1.25 ±0.43	1.31	2.00 ±0.00	4.00 ±0.00	1.50	2.00 ±0.00	3.00 ±1.00	1.13	1.31
Melaleuca quinquenervia (Cav.) S.T.Blake (niaouli)	**0.75 ±0.25**	**0.75 ±0.25**	*0.69*	2.00 ±0.00	4.00 ±0.00	1.50	2.00 ±0.00	2.00 ±0.00	*0.75*	*0.98*
Melissa officinalis L. (lemon balm)	**1.00 ±0.00**	**1.00 ±0.00**	*0.75*	2.00 ±0.00	3.00 ±1.00	1.13	2.00 ±0.00	2.00 ±0.00	*0.75*	*0.88*
Nardostachys grandiflora DC. (spikenard)	**0.31 ±0.11**	**1.00 ±0.00**	1.85	2.00 ±0.00	2.00 ±0.00	*0.75*	2.00 ±0.00	2.00 ±0.00	*0.75*	1.12
Ocimum sanctum L. (holy basil)	**0.31 ±0.11**	**1.00 ±0.00**	1.85	2.00 ±0.00	2.00 ±0.00	*0.75*	2.00 ±0.00	2.00 ±0.00	*0.75*	1.12
Origanum majorana L. (marjoram)	**0.75 ±0.25**	1.50 ±0.50	1.38	2.00 ±0.00	3.00 ±1.00	1.13	2.00 ±0.00	2.00 ±0.00	*0.75*	1.08
Origanum vulgare L. (oregano)	**0.31 ±0.11**	**0.75 ±0.25**	1.39	**1.00 ±0.00**	2.00 ±0.00	1.25	**1.00 ±0.00**	2.00 ±0.00	1.25	1.30
Pelargonium graveolens L'Hér. (sweet scented geranium)	**0.44 ±0.11**	**1.00 ±0.00**	1.39	2.00 ±0.00	3.00 ±1.00	1.13	2.00 ±0.00	2.50 ±0.87	*0.94*	1.15
Pistacia lentiscus L. (mastic tree)	**0.75 ±0.25**	**1.00 ±0.00**	*0.92*	3.00 ±1.00	4.00 ±0.00	1.17	2.00 ±0.00	4.00 ±0.00	1.50	1.19
Pogostemon cablin (Blanco) Benth. (patchouli)	**0.50 ±0.00**	**1.00 ±0.00**	1.25	2.00 ±0.00	3.00 ±1.00	1.13	1.50 ±0.50	4.00 ±0.00	1.83	1.40
Rosa damascena Herrm. (Damask rose)	**0.75 ±0.25**	**1.00 ±0.00**	*0.92*	2.00 ±0.00	2.00 ±0.00	*0.75*	2.00 ±0.00	2.00 ±0.00	*0.75*	*0.81*
Santalum austrocaledonicum Vieill.	**0.125 ±0.00**	**0.25 ±0.00**	1.06	**0.17 ±0.06**	**1.00 ±0.00**	3.13	**0.125 ±0.00**	**1.00 ±0.00**	4.13	2.77

Mean MIC values (mg/mL) and ΣFIC										
	***C. acnes* (ATCC® 11827™)**			***S. epidermidis* (ATCC® 12228™)**			***S. aureus* (ATCC® 25923™)**			
Sample	**Independent MIC[a]**	**Combination MIC[b]**	**ΣFIC[c]**	**Independent MIC**	**Combination MIC**	**ΣFIC**	**Independent MIC**	**Combination MIC**	**ΣFIC**	**Mean ΣFIC[d]**
(New Caledonia sandalwood) *Styrax benzoin* Dryand. (gum benjamin tree)	**0.50 ±0.00**	**0.75 ±0.25**	*0.94*	3.00 ±1.00	4.00 ±0.00	1.17	3.00 ±1.00	3.00 ±1.00	*0.88*	*0.99*
Thymus vulgaris L. (thyme)	**0.38 ±0.13**	**1.00 ±0.00**	1.58	**1.00 ±0.00**	2.00 ±0.00	1.25	**1.00 ±0.00**	2.00 ±0.00	1.25	1.36
Vetiveria zizanioides (L.) Nash (vetiver grass)	**0.125 ±0.00**	**0.44 ±0.11**	1.86	**0.50 ±0.00**	2.00 ±0.00	2.25	**0.50 ±0.00**	1.50 ±0.50	1.69	1.93

[a] Values for MIC of essential oils independently; [b] values for MIC of *R. rubiginosa* fixed oil combined with essential oils; [c] where the MIC of *R. rubiginosa* fixed oil used to calculate the ΣFIC was 2.00 mg/mL against *C. acnes* and 4.00 mg/mL against *S. epidermidis* and *S. aureus*; [d] overall mean ΣFIC of the three reference strains; [e] essential oils combined with *R. rubiginosa* fixed oil selected for further investigation (grey highlight); [f] noteworthy activity (MIC ≤ 1.00 mg/mL, bold); [g] additive interaction (ΣFIC > 0.5 - ≤ 1.0, italic); and [h] synergy (ΣFIC ≤ 0.5, bold, italic).

All 30 of the independent essential oils showed noteworthy antimicrobial activity against *C. acnes*. Six essential oils including *Cinnamomum zeylanicum* (true cinnamon), *Litsea cubeba* (may chang), *Origanum vulgare* (oregano), *Santalum austrocaledonicum* (New Caledonia sandalwood), *T. vulgaris*, and *Vetiveria zizanioides* (vetiver grass), displayed noteworthy antimicrobial activity against *S. epidermidis*. Nine essential oils including *C. zeylanicum*, *C. citratus*, *Cymbopogon martini* (palmarosa), *Leptospermum petersonii* (lemon-scented tea tree), *Leptospermum scoparium* (manuka), *O. vulgare*, *S. austrocaledonicum*, *T. vulgaris*, *and V. zizanioides*, exhibited noteworthy antimicrobial activity against *S. aureus*. Five of the independent essential oils demonstrated noteworthy antimicrobial activity against all three reference strains: *S. austrocaledonicum* (mean MIC = 0.14 mg/mL), *V. zizanioides* (mean MIC = 0.38 mg/mL), *C. zeylanicum* (mean MIC = 0.73 mg/mL), *O. vulgare* (mean MIC = 0.77 mg/mL), and *T. vulgaris* (mean MIC = 0.79 mg/mL). *Santalum austrocaledonicum* essential oil presented the best antimicrobial activity overall.

Essential oil research focusing on acne-related conditions is mostly limited to *Santalum album* (East Indian sandalwood) essential oil. Several reviews have discussed the antimicrobial activity of *S. album* essential oil against *C. acnes*, *S. epidermidis*, and *S aureus* (Moy *et al.*, 2012, Moy and Levenson, 2017, Winkelman, 2018). Similar to *S. album* essential oil (Kumar *et al.*, 2015), antimicrobial activity could be attributed to the major chemical constituents α-santalol (42.80%) and β-santalol (18.60%) present in *S. austrocaledonicum* essential oil. The current study supports the antimicrobial activity of *S. austrocaledonicum* essential oil against acne-inducing reference strains where previous studies were lacking.

A study by Kurrimboccus *et al.* (2021) investigated the antimicrobial activity of *V. zizanioides* essential oil against *C. acnes* (MIC = 0.25 mg/mL ±0.00), *S. epidermidis* (MIC = 0.10 mg/mL ±0.02), and *S. aureus* (MIC = 0.17 mg/mL ±0.09). These noteworthy MIC values are similar to the antimicrobial activity of *V. zizanioides* essential oil in the present study, as the same chemotype was tested in both studies. Thus, the major chemical constituents (khusenic acid (15.90%), khusimol (7.90%), isovalencenol (7.40%), α-vetivone (3.70%), vetiselinenol (3.32%), and β-vetivone (2.70%)) could contribute to the observed noteworthy antimicrobial effect. In addition, a previous study by Champagnat *et al.* (2007) presented noteworthy antimicrobial activity (MIC = 0.60 mg/mL) for *V. zizanioides* essential oil against *S. aureus*, where khusimol (13.30%) and vetiselinenol (7.80%) were identified as the major constituents of the essential oil.

The essential oil of *C. zeylanicum* demonstrated antimicrobial activity against *C. acnes* (MIC = 0.016% v/v) (Zu *et al.*, 2010), *S. epidermidis* (MIC = 12.50% v/v), and *S. aureus* (MIC = 6.30% v/v) (Shabani *et al.*, 2016). In another study, *C. zeylanicum* essential oil exhibited noteworthy antimicrobial activity against *S. aureus* (MIC = 0.22 mg/mL) (Teles *et al.*, 2019). The previously reported potent MIC values correlate to the noteworthy antimicrobial activity of *C. zeylanicum* essential oil in the present study. The major chemical constituents, including cinnamaldehyde (61.50%), β-caryophyllene (6.80%), and cinnamyl acetate (6.50%), were present in *C. zeylanicum* essential oil in the current study. Similarly, the chemical constituents, cinnamaldehyde (46.30%), β-caryophyllene (8.26%), and cinnamyl acetate (7.54%), were present in the essential oil in the study by Teles *et al.* (2019). The chemical constituent, α-copaene (16.35%), was also reported as a major constituent of *C. zeylanicum* essential oil (Teles *et al.*, 2019). Thus, there is a possibility that a combination of chemical constituents could be responsible for antimicrobial activity.

Similar to the current study, *O. vulgare* essential oil displayed noteworthy antimicrobial activity against *C. acnes* (MIC = 0.34 mg/mL), *S. epidermidis* (MIC = 0.67 mg/mL) (Taleb *et al.*, 2018), and *S. aureus* (MIC = 0.17 mg/mL) (Teles *et al.*, 2019). Of interest, *O. vulgare* essential oil contained carvacrol (60.40%), γ-terpinene (7.90%), ρ-cymene (7.00%), and thymol (5.70%) in the current study. However, *O. vulgare* essential oil contained thymol (99.44%), ρ-cymene (0.20%), γ-terpinene (0.03%), and no carvacrol, in the study by Taleb *et al.* (2018). Furthermore, *O. vulgare* essential oil contained cis-ρ-menth-2-en-1-ol (33.88%), linalyl acetate (13.90%), and ρ-cymene (8.29%) in the study by Teles *et al.* (2019). Thereby suggesting that the antimicrobial activity of different *O. vulgare* essential oil chemotypes could be conferred by a combination of various chemical constituents or minor constituents (de Martino *et al.*, 2009).

Similar to the current study, *T. vulgaris* essential oil exhibited antimicrobial activity against *C. acnes* (MIC = 0.65 mg/mL), and *S. epidermidis* (MIC = 1.30 mg/mL) (Taleb *et al.*, 2018). Compared to the current study, *T. vulgaris* essential oil displayed better antimicrobial activity against *S. aureus* (MIC = 0.02-0.17 mg/mL) (Lemos *et al.*, 2017). The major chemical constituents of *T. vulgaris* essential oil in the current study were thymol (43.30%), ρ-cymene (19.20%), and γ-terpinene (11.20%). The same major chemical constituents, thymol (72.08%), ρ-cymene (25.18%), and γ-terpinene (2.52%), were reported by Taleb *et al.* (2018) for *T. vulgaris* essential oil. Although *T. vulgaris* essential oil also contained the major constituents

thymol, cymene, and terpinene, antimicrobial activity was attributed to thymol (MIC = 0.25 mg/mL), carvacrol (MIC = 0.25 mg/mL), and linalool (MIC = 2.00 mg/mL) against *S. aureus* (Lemos *et al.*, 2017). This affirms that antimicrobial activity could be conferred by minor chemical constituents, such as linalool, in addition to the major constituents.

2.3.2. Oil combinations (1:1)

The mean MIC values ($n \geq 4$) of *R. rubiginosa* fixed oil in 1:1 combination with the essential oils, and the ΣFIC and mean ΣFIC for each combination against the three reference strains, are presented in Table 2.3. When combined with *R. rubiginosa* fixed oil in 1:1 ratio, *C. zeylanicum* and *S. austrocaledonicum* essential oils maintained noteworthy antimicrobial activity against the three reference strains (combined mean MIC = 0.42 and 0.75 mg/mL, respectively). This may be due to the strong presence of cinnamaldehyde (61.50%) and eugenol (3.70%) in *C. zeylanicum* essential oil (Marchese *et al.*, 2017); and *α*-santalol (42.80%) and β-santalol (18.60%) in *S. austrocaledonicum* essential oil (Kumar *et al.*, 2015). The antimicrobial activity of *V. zizanioides*, *O. vulgare*, and *T. vulgaris* essential oils against the three reference strains was weaker (MIC = 0.44-2.00 mg/mL) when combined in a 1:1 ratio with *R. rubiginosa* fixed oil. Each of these oil combinations, however, maintained noteworthy antimicrobial activity against *C. acnes*. Another study performed by Orchard *et al.* (2019) investigated six fixed oils in 1:1 combinations with 23 essential oils, including most of the essential oils tested in the present study. Similar to the present study, the antimicrobial activity of the majority of the 1:1 oil combinations was slightly decreased against the three acne-inducing reference strains (*C. acnes*, *S. epidermidis*, and *S. aureus*) as compared to the independent essential oils (Orchard *et al.*, 2019).

Rosa rubiginosa fixed oil combined in a 1:1 ratio with *C. zeylanicum* essential oil displayed synergism against *S. epidermidis* and *S. aureus*. Ten combinations exhibited additive interactions against *C. acnes* including *R. rubiginosa* combined in a 1:1 ratio with the essential oils of *C. zeylanicum*, *Commiphora molmol* (myrrh), *Cymbopogon nardus* (citronella grass), *Kunzea ericoides* (kanuka), *Laurus nobilis* (bay laurel), *Melaleuca quinquenervia* (niaouli), *Melissa officinalis* (lemon balm), *Pistacia lentiscus* (mastic tree), *R. damascena*, and *Styrax benzoin* (gum benjamin). Ten combinations demonstrated additive interactions against *S. epidermidis* including *R. rubiginosa* combined in a 1:1 ratio with the essential oils of *Citrus aurantium* (bitter orange), *C. molmol*, *C. citratus*, *C. martini*, *C. nardus*, *Eugenia caryophyllata*

(clove), *L. petersonii*, *Nardostachys grandiflora* (spikenard), *Ocimum sanctum* (holy basil), and *R. damascena*. Ten combinations displayed additive interactions against *S. aureus* including *R. rubiginosa* combined in a 1:1 ratio with the essential oils of *E. caryophyllata*, *L. cubeba*, *M. quinquenervia*, *M. officinalis*, *N. grandiflora*, *O. sanctum*, *Origanum majorana* (marjoram), *Pelargonium graveolens* (sweet scented geranium), *R. damascena*, and *S. benzoin*. *Rosa rubiginosa* fixed oil combined in a 1:1 ratio with *S. austrocaledonicum* essential oil displayed antagonism against *S. aureus*. An overview of the interactions is given in Figure 2.1.

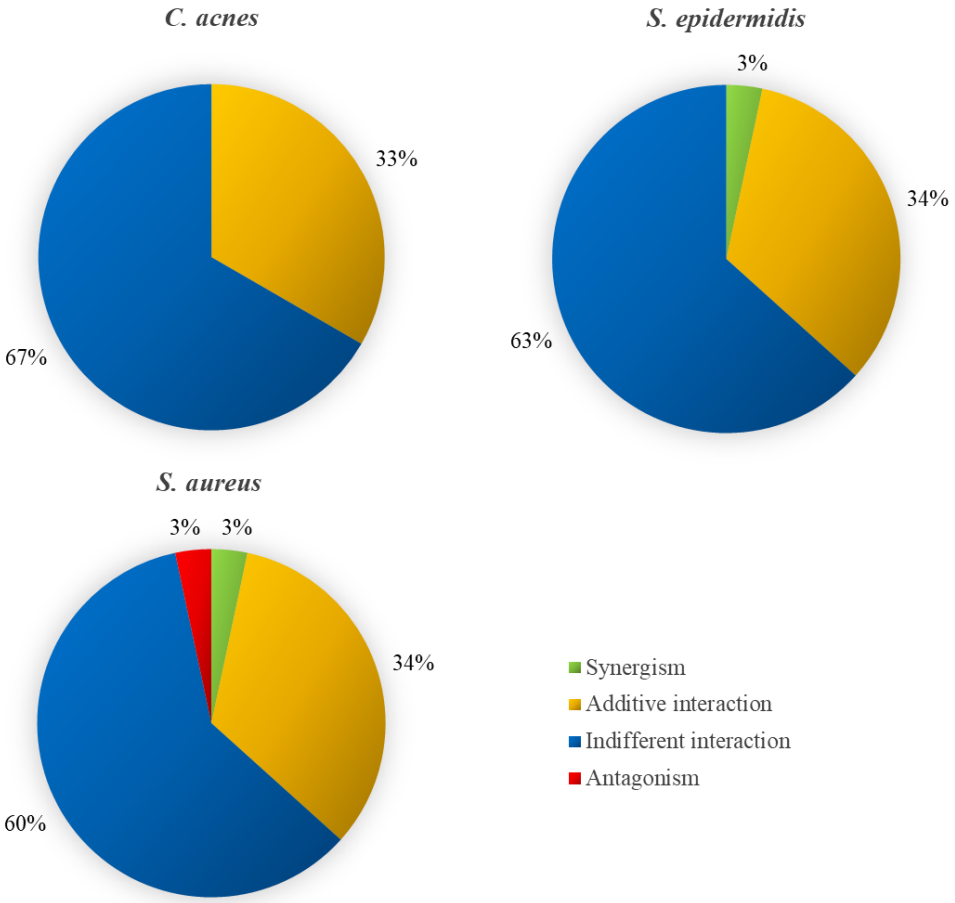

Figure 2.1. Antimicrobial interactions of *R. rubiginosa* fixed oil in 1:1 combination with essential oils against all three reference strains.

The 1:1 combination of *R. rubiginosa* fixed oil with *C. zeylanicum* essential oil displayed additive interaction (ΣFIC = 0.73) against *C. acnes*; and synergy (ΣFIC = 0.31) against *S. epidermidis* and *S. aureus*. This combination lowered the mean MIC of *C. zeylanicum* essential oil from 0.73 mg/mL (*C. zeylanicum* essential oil independently) to 0.42 mg/mL (1:1 combination), exhibiting an overall synergistic interaction (mean ΣFIC = 0.45) across all three

reference strains. *Cinnamomum zeylanicum* essential oil combined in a 1:1 ratio with other fixed oils produced mostly additive and indifferent interactions against the three acne-inducing reference strains (Orchard *et al.*, 2019). Thus, the favourable antimicrobial synergy of *R. rubiginosa* fixed oil in 1:1 combination with *C. zeylanicum* essential oil is ideal for treating the bacterial pathogenesis of acne vulgaris.

Six combinations (*R. rubiginosa* fixed oil with either *E. caryophyllata, K. ericoides, M. quinquenervia, M. officinalis, R. damascena,* or *S. benzoin* essential oil) displayed mean additive interactions against all three acne-inducing reference strains (mean ΣFIC = 0.81-0.99). *Eugenia caryophyllata, K. ericoides, M. quinquenervia, M. officinalis,* or *S. benzoin* essential oil combined in 1:1 ratios with other fixed oils produced mostly indifferent interactions against the three acne-inducing reference strains (Orchard *et al.*, 2019). The antimicrobial activity of *E. caryophyllata* essential oil was reported against multiple strains of *S. epidermidis* and *S. aureus* in a review by Chaieb *et al.* (2007). When tested against *C. acnes* and *S. aureus, E. caryophyllata* essential oil showed better antimicrobial activity than conventionally used antibiotics, thereby suggesting that this essential oil could be considered for use in treating skin and soft tissue infection (Subramanian *et al.*, 2021). Studies investigating the antimicrobial activity of *K. ericoides* essential oil are limited. A study by van Vuuren *et al.* (2014) reported overall poor antimicrobial activity of *K. ericoides* essential oil against the three acne-inducing micro-organisms (*C. acnes, S. epidermidis,* and *S. aureus*). However, the favourable antimicrobial additive interaction of *R. rubiginosa* fixed oil in 1:1 combination with *K. ericoides* essential oil in the present study, suggests the potential of this oil combination to elicit antimicrobial activity at higher concentrations. *Melaleuca quinquenervia* essential oil showed promising antimicrobial activity against *C. acnes* and *S. epidermidis* when formulated in a topical anti-acne treatment (Shakeel *et al.*, 2021). In addition, promising antimicrobial activity of *M. quinquenervia* essential oil against *S. aureus* has been reported in previous studies (Wilkinson and Cavanagh, 2005, Siddique *et al.*, 2018). Research studying *M. officinalis* is mostly limited to extracts, where topical application to acne lesions resulted in improved skin condition (Teymouri and Teimouri, 2019). In contrast to the present study, *M. officinalis* essential oil exhibited noteworthy antimicrobial activity against *S. epidermidis* (MIC = 1.00 mg/mL) (Alizadeh Behbahani and Shahidi, 2019) and *S. aureus* (MIC = 0.12 mg/mL) (Ehsani *et al.*, 2017). *Rosa rubiginosa* fixed oil combined with *R. damascena* essential oil in a 1:1 ratio showed additive interaction against each of the three acne-inducing reference strains. Furthermore, the antimicrobial activity of *R. damascena* essential oil has been studied

extensively, with several reviews reporting potent antimicrobial activity of this essential oil against *C. acnes*, *S. epidermidis*, and *S. aureus* (Mahboubi, 2016, Nayebi *et al.*, 2017, Akram *et al.*, 2020). The antimicrobial activity of *S. benzoin* essential oil against the acne-inducing micro-organisms is limited, however, topical application of *S. benzoin* essential oil to treat acne has been described in several reviews (Magin *et al.*, 2006, Fox *et al.*, 2016). Thus, the favourable antimicrobial additive interactions of *R. rubiginosa* fixed oil in 1:1 combination with either *E. caryophyllata, K. ericoides, M. quinquenervia, M. officinalis, R. damascena,* or *S. benzoin* essential oil are advantageous for treating the bacterial pathogenesis of acne vulgaris.

Rosa rubiginosa fixed oil in 1:1 combination with *S. austrocaledonicum* essential oil displayed indifference against *C. acnes* and *S. epidermidis* (ΣFIC = 1.06 and 3.13, respectively), and antagonism (ΣFIC = 4.13) against *S. aureus*. These results indicate that *R. rubiginosa* fixed oil diluted the effects of *S. austrocaledonicum* essential oil, increasing its mean MIC from 0.14 mg/mL (*S. austrocaledonicum* essential oil independently) to 0.75 mg/mL when combined in a 1:1 ratio. Despite this, the oil combination exhibited noteworthy antimicrobial activity against all three reference strains. Such activity may be attributed to the high content of sesquiterpenes such as santalol, as established with several *Santalum* species (Jirovetz *et al.*, 2006, Han *et al.*, 2017a). Thus, the noteworthy antimicrobial activity of *R. rubiginosa* fixed oil in 1:1 combination with *S. austrocaledonicum* essential oil could be considered for treating the bacterial pathogenesis of acne vulgaris.

2.3.3. Varied ratio oil combinations and isobologram analysis

Oil combinations displaying antimicrobial synergy (*R. rubiginosa* fixed oil combined with *C. zeylanicum* essential oil), or an overall antimicrobial additive interaction (*R. rubiginosa* fixed oil with either *E. caryophyllata, K. ericoides, M. quinquenervia, M. officinalis, R. damascena,* or *S. benzoin* essential oil), or antimicrobial antagonism (*R. rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil), were further investigated in various ratios. Figures 2.2-2.9 depict the contributory effect of each of the varied rations and Tables G1-G8 (Appendix G) provide the exact oil concentrations for each combination represented as data points labelled 1-9 on the isobolograms.

The various ratio combinations of *R. rubiginosa* fixed oil with *C. zeylanicum* essential oil indicated mostly additive interactions against *C. acnes* (Figure 2.2). When there was an increased proportion of the fixed oil, this interaction shifted toward indifference against *C. acnes*. Of interest, a synergistic effect was observed only with the 1:1 combination against *S. epidermidis* and *S. aureus*. Therefore, suggesting this to be the optimal ratio for the *R. rubiginosa* fixed oil with *C. zeylanicum* essential oil combination. Except for the 1:1 combination, all other various ratio combinations displayed additive interactions against *S. epidermidis* and *S. aureus*. The main contribution to antimicrobial activity was provided by *C. zeylanicum* essential oil.

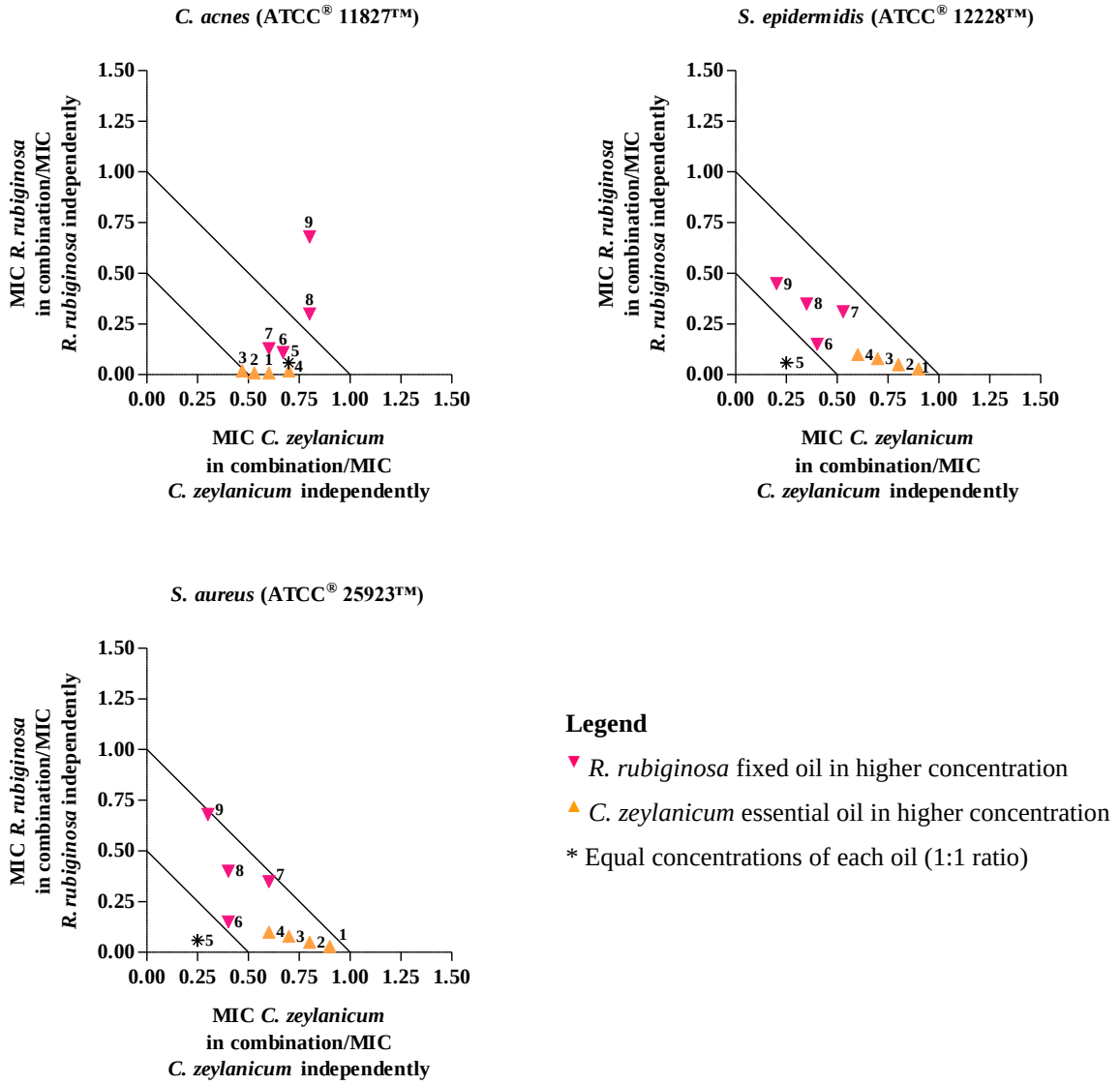

Figure 2.2. Isobologram representation of *R. rubiginosa* fixed oil in combination with *C. zeylanicum* essential oil against the three reference strains.

The various ratio combinations including the 1:1 combination of *R. rubiginosa* fixed oil with *E. caryophyllata* essential oil showed mostly indifference against *C. acnes* (Figure 2.3). When there was an increased proportion of the essential oil, this interaction shifted towards an additive interaction against *C. acnes*. Most of the interactions were additive when tested against *S. epidermidis* and *S aureus*. Higher concentrations of *R. rubiginosa* fixed oil shifted the data points towards mostly indifference against *S. epidermidis* and *S. aureus*. Thus, the antimicrobial activity of this combination depended on the essential oil.

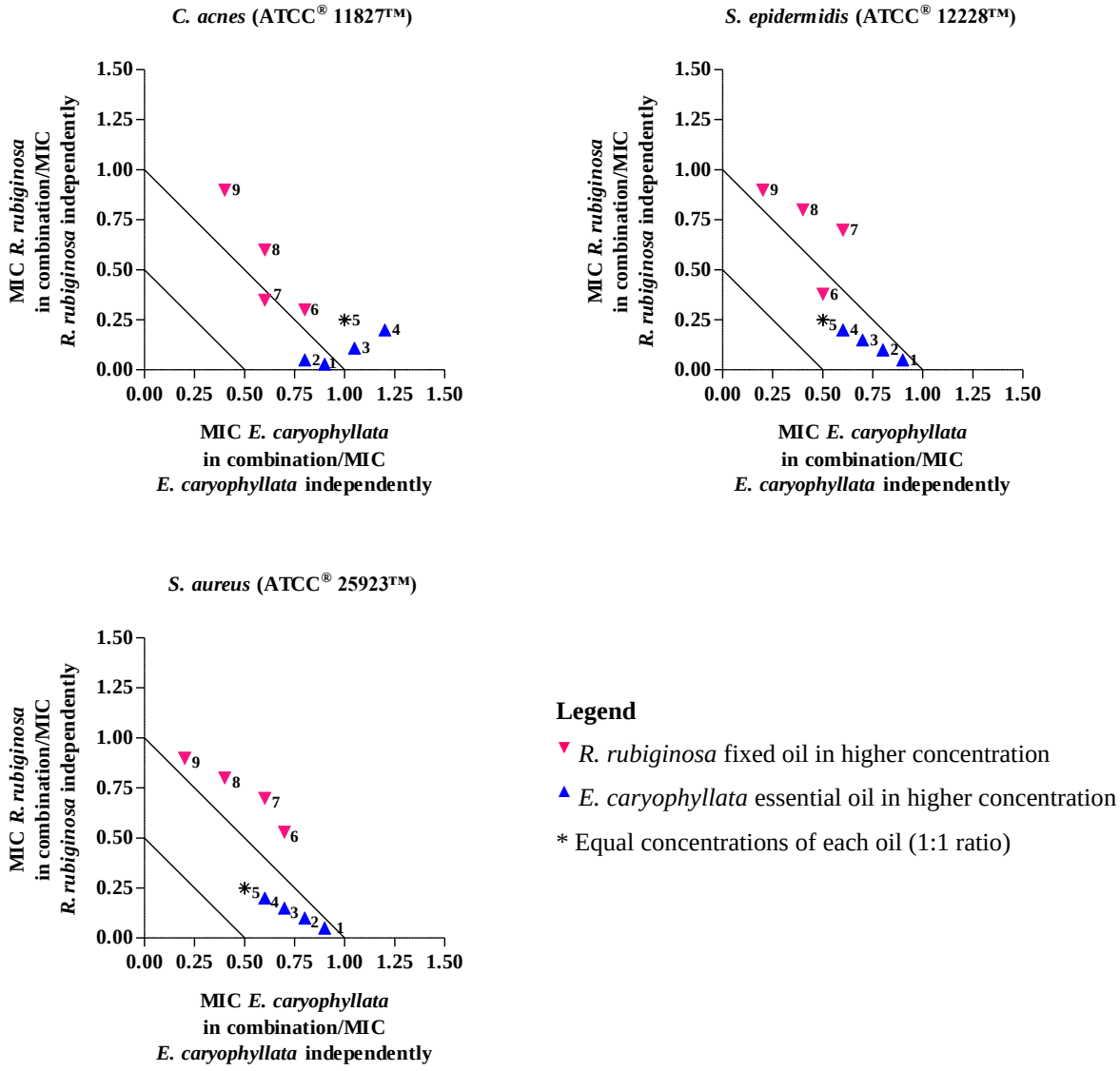

Figure 2.3. Isobologram representation of *R. rubiginosa* fixed oil in combination with *E. caryophyllata* essential oil against the three reference strains.

The various ratio combinations of *R. rubiginosa* fixed oil with *K. ericoides* essential oil (Figure 2.4) indicated additive interactions across all three reference strains. In this case, both oils appeared to contribute equally to the overall additive antimicrobial effect.

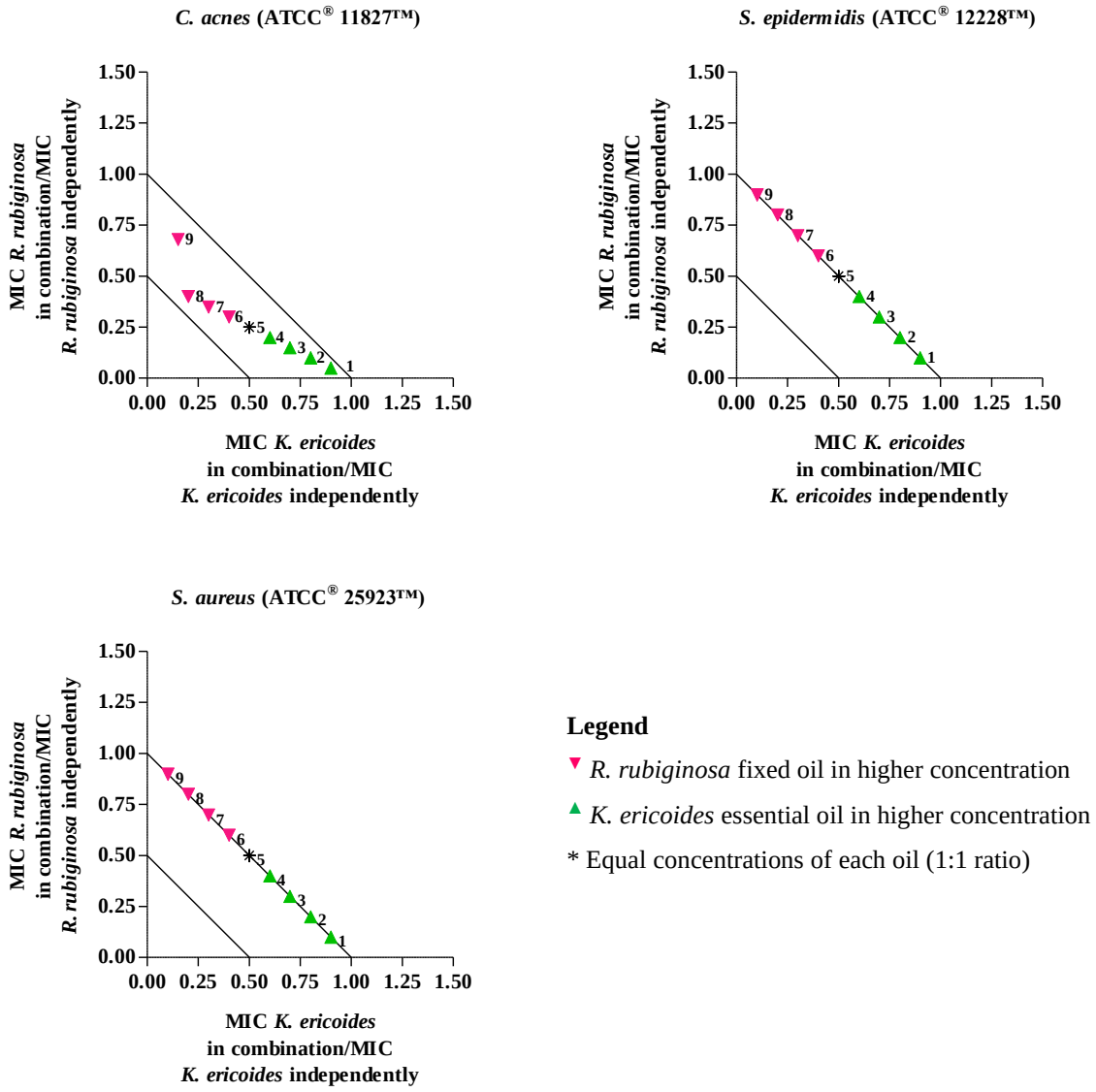

Figure 2.4. Isobologram representation of *R. rubiginosa* fixed oil in combination with *K. ericoides* essential oil against the three reference strains.

The various ratio combinations of *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil displayed additive interactions against *C. acnes* and most of the interactions against *S. aureus* (Figure 2.5). However, against *S. epidermidis*, the combination largely produced an indifferent effect. Overall, the antimicrobial effect was exerted by *M. quinquenervia* essential oil when in higher concentrations. Thus, the antimicrobial activity of this combination depended on the essential oil.

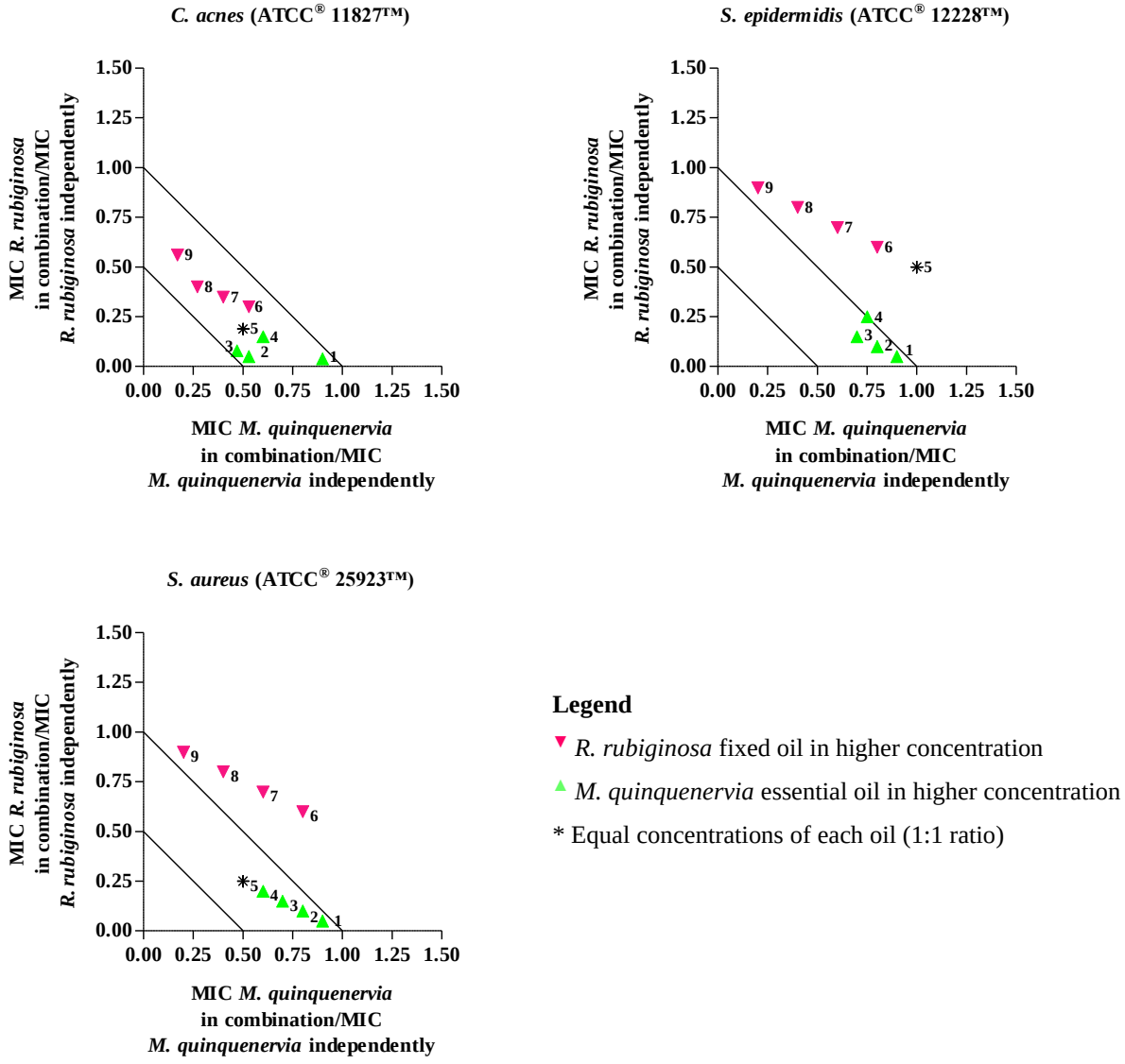

Figure 2.5. Isobologram representation of *R. rubiginosa* fixed oil in combination with *M. quinquenervia* essential oil against the three reference strains.

The various ratio combinations of *R. rubiginosa* fixed oil with *M. officinalis* essential oil indicated mostly additive interactions against *C. acnes* and *S. aureus* (Figure 2.6). However, against *S. epidermidis*, the combination largely produced an indifferent effect, where the antimicrobial effect was primarily displayed by *M. officinalis* essential oil when in higher concentrations. Increasing amounts of *R. rubiginosa* fixed oil mostly shifted the interactions toward indifference thereby confirming the contributory antimicrobial effect of *M. officinalis* essential oil. Thus, the antimicrobial activity of this combination depended on the essential oil.

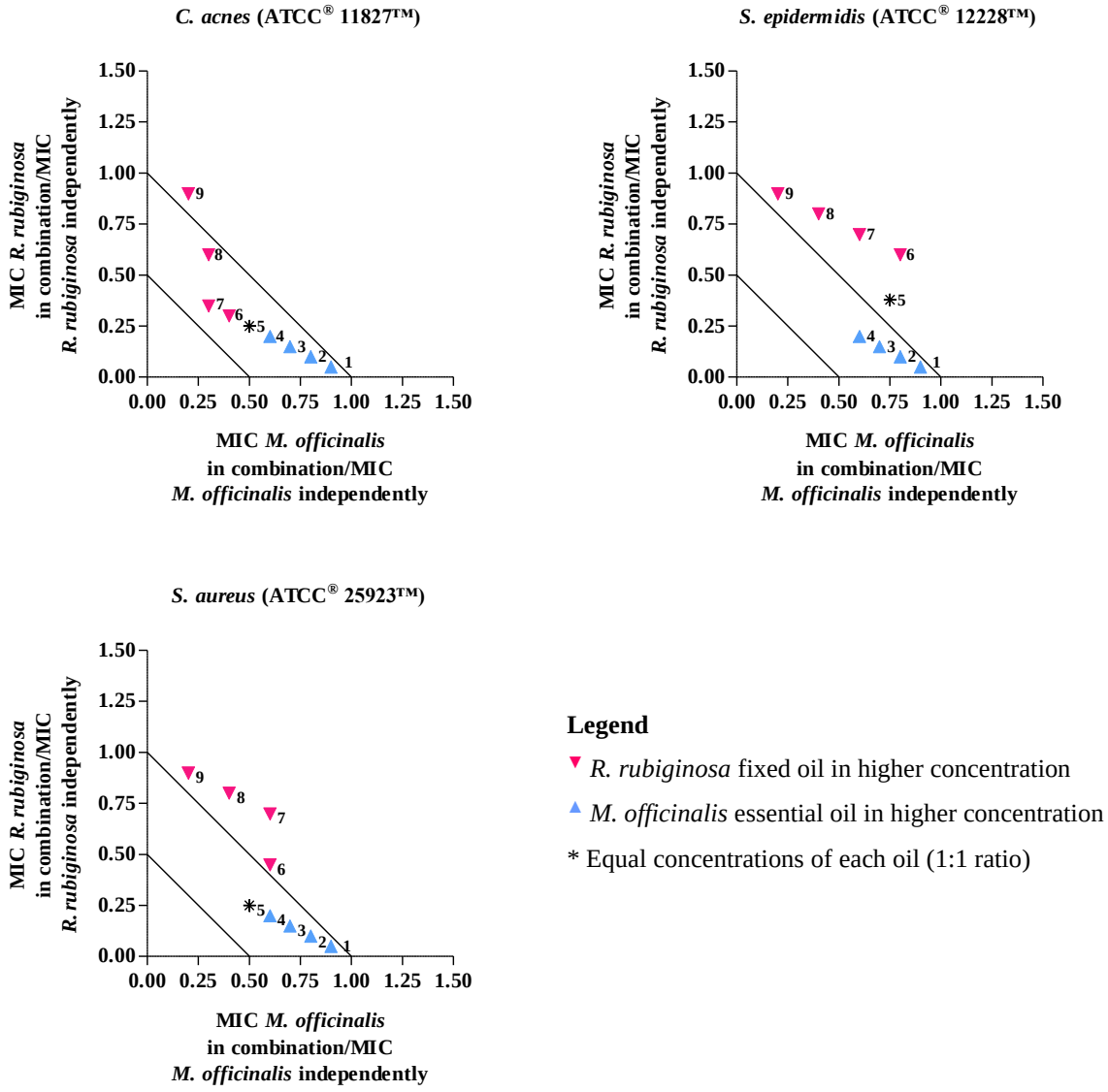

Figure 2.6. Isobologram representation of *R. rubiginosa* fixed oil in combination with *M. officinalis* essential oil against the three reference strains.

The various ratio combinations of *R. rubiginosa* fixed oil with *R. damascena* essential oil displayed mostly additive interactions across the three reference strains (Figure 2.7). The large contributory antimicrobial effect of *R. damascena* essential oil when used in 1:1 combination or higher concentrations is evident in the plots of *S. aureus* and *S. epidermidis*, whereas increased proportions of *R. rubiginosa* fixed oil provided an indifferent effect. Thus, the antimicrobial activity of this combination depended mostly on the essential oil.

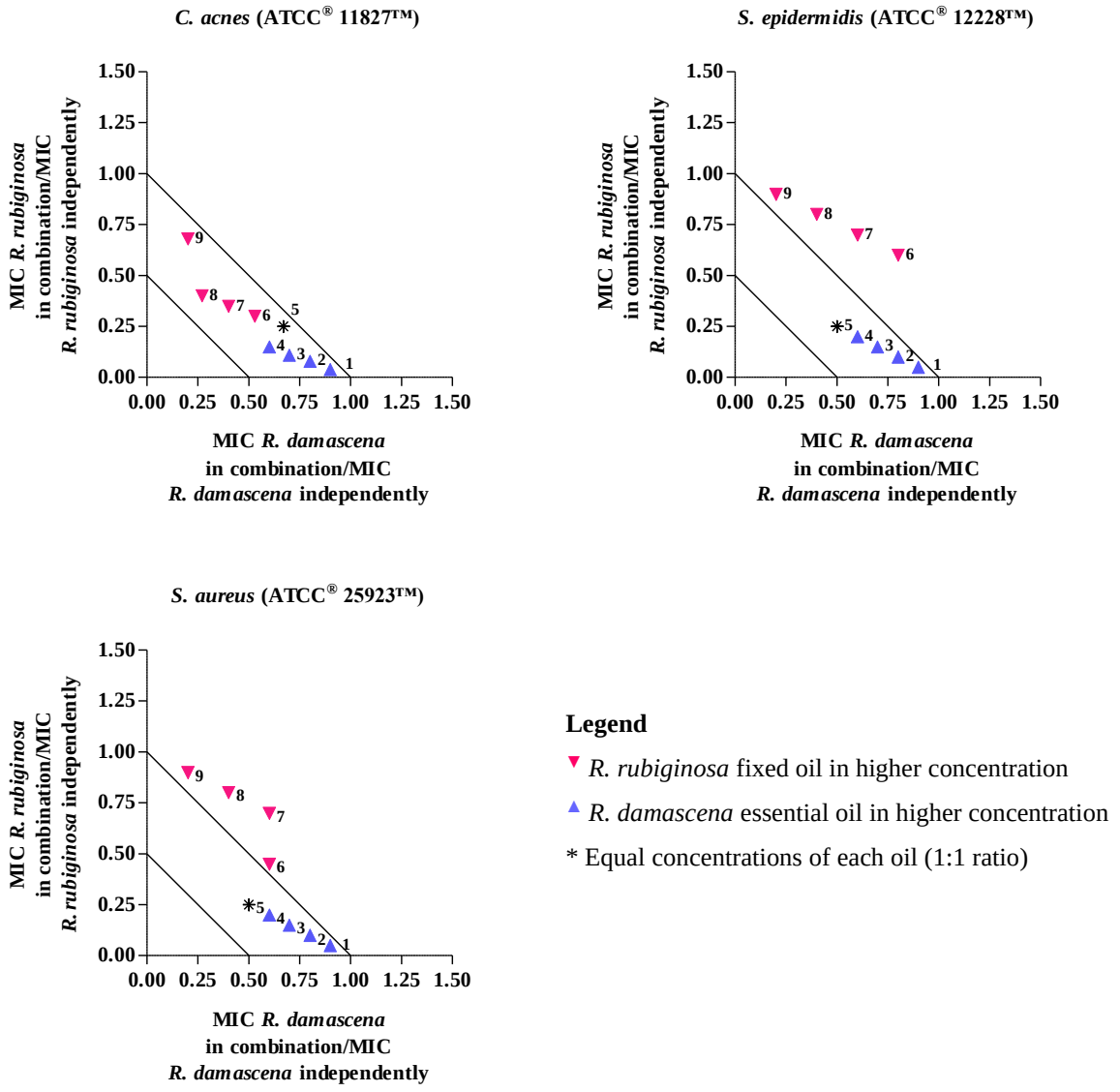

Figure 2.7. Isobologram representation of *R. rubiginosa* fixed oil in combination with *R. damascena* essential oil against the three reference strains.

The various ratio combinations of *R. rubiginosa* fixed oil with *S. benzoin* essential oil displayed mostly additive interactions against *C. acnes* and *S. aureus*, whereas mostly indifference was shown against *S. epidermidis* (Figure 2.8). The contribution to antimicrobial activity of *S. benzoin* essential oil when in higher concentration exceeded that of *R. rubiginosa* fixed oil, whereas increased proportions of the fixed oil largely provided an indifferent effect. Thus, the antimicrobial activity of this combination depended mostly on the essential oil.

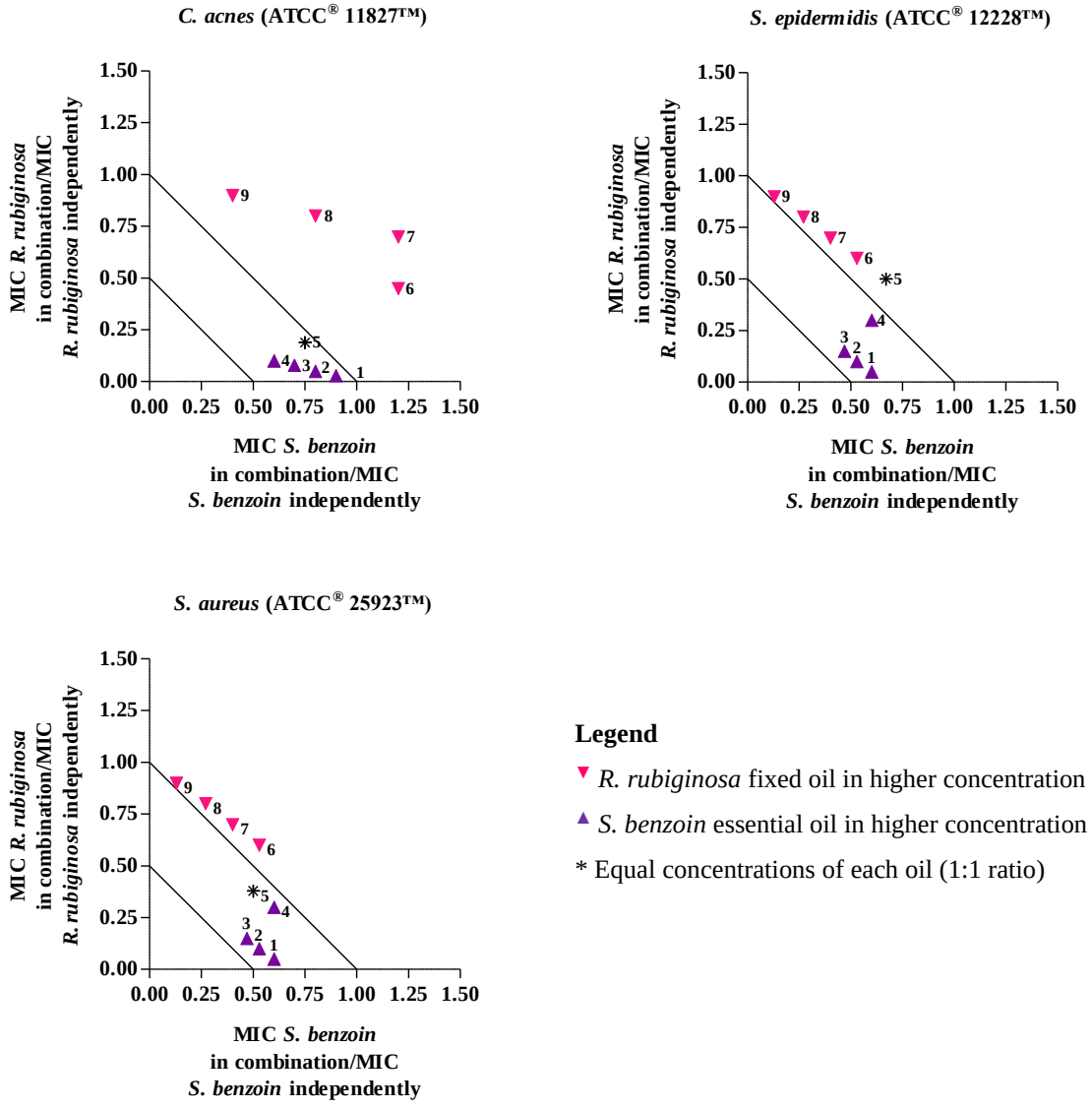

Figure 2.8. Isobologram representation of *R. rubiginosa* fixed oil in combination with *S. benzoin* essential oil against the three reference strains.

The various ratio combinations of *R. rubiginosa* fixed oil with *S. austrocaledonicum* essential oil displayed mostly additive interactions against *C. acnes*, mostly indifference against *S. epidermidis*, and mostly antagonism against *S. aureus* (Figure 2.9). The antimicrobial effect was predominantly exerted by *S. austrocaledonicum* essential oil when in higher concentration in the combination. Increased proportions of *R. rubiginosa* fixed oil shifted the interactions toward antagonism against *S. aureus*. Thus, the antimicrobial activity of this combination depended on the essential oil.

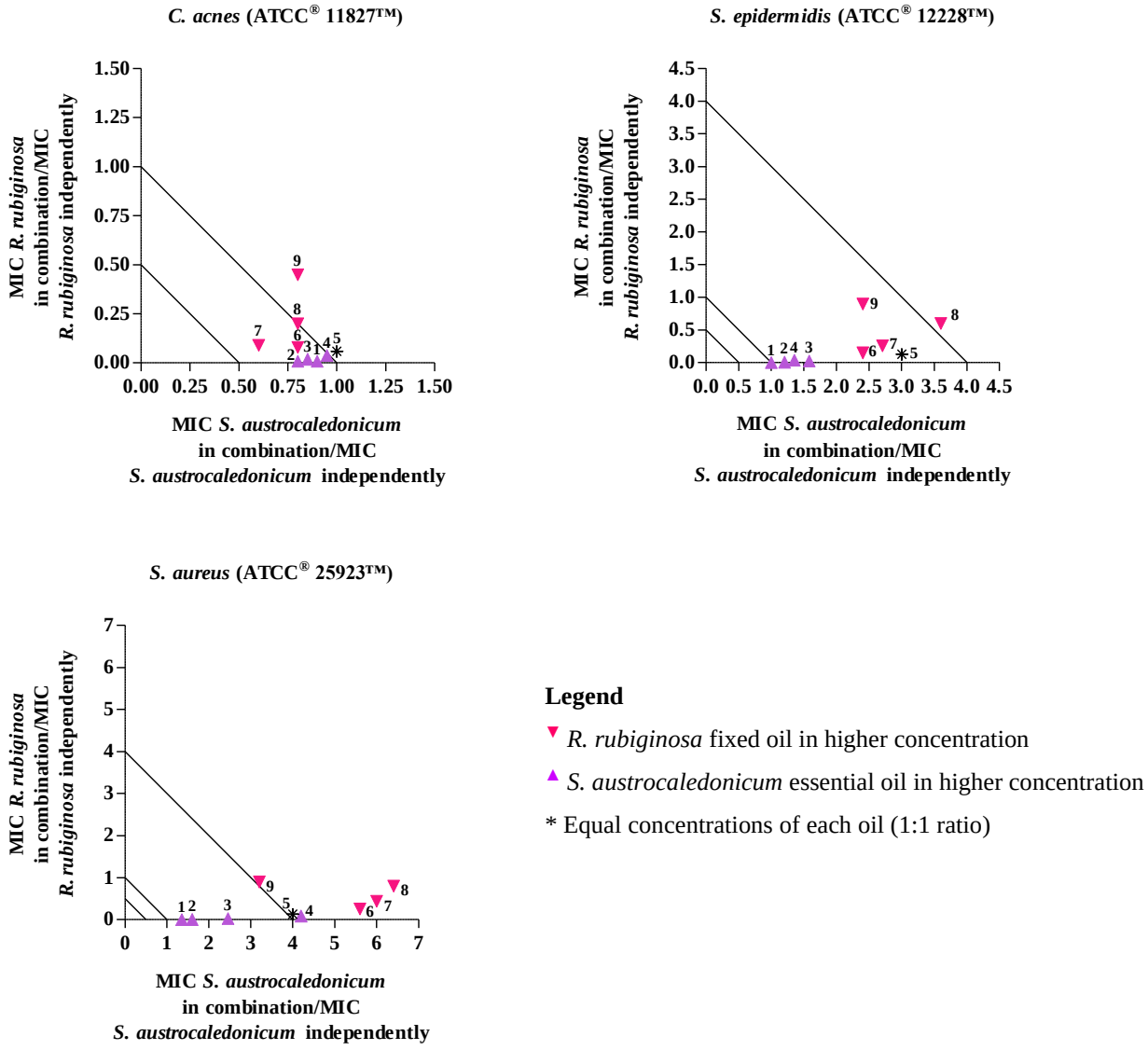

Figure 2.9. Isobologram representation of *R. rubiginosa* fixed oil in combination with *S. austrocaledonicum* essential oil against the three reference strains.

In this study, most of the antimicrobial effects were attributed to the essential oils rather than *R. rubiginosa* fixed oil, as depicted in the isobolograms. Research examining the interactions between fixed and essential oils is limited (Orchard *et al.*, 2019). The present study serves as an initial step in the complex process of determining the interactions of fixed and essential oil combinations. Thus, further investigations are necessary to elucidate the structure-activity relationships (SARs) of fixed and essential oil interactions to determine the mechanisms of antimicrobial action.

2.4. Overview

- *Rosa rubiginosa* fixed oil displayed poor antimicrobial activity overall against the three reference strains (mean MIC = 3.33 mg/mL).
- Five of the essential oils (*S. austrocaledonicum* (mean MIC = 0.14 mg/mL), *V. zizanioides* (mean MIC = 0.38 mg/mL), *C. zeylanicum* (mean MIC = 0.73 mg/mL), *O. vulgare* (mean MIC = 0.77 mg/mL), and *T. vulgaris* (mean MIC = 0.79 mg/mL)) displayed noteworthy antimicrobial activity against all three reference strains.
- When combined with *R. rubiginosa* fixed oil in a 1:1 ratio, *C. zeylanicum* (combined mean MIC = 0.42 mg/mL) and *S. austrocaledonicum* (combined mean MIC = 0.75 mg/mL) essential oils exhibited noteworthy antimicrobial activity against the three reference strains.
- *Rosa rubiginosa* fixed oil combined with *C. zeylanicum* essential oil exhibited antimicrobial synergism (ΣFIC = 0.31) against *S. epidermidis* and *S. aureus*, where the MIC of the independent essential oil decreased from 1.00 mg/mL (*C. zeylanicum* essential oil independently) to 0.50 mg/mL when in 1:1 combination with *R. rubiginosa* fixed oil.
- Six combinations (*R. rubiginosa* fixed oil with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, or *S. benzoin* essential oil) displayed mean additive interactions against all three acne-inducing reference strains (mean ΣFIC = 0.81-0.99).
- *Rosa rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil exhibited antimicrobial antagonism (ΣFIC = 4.13) against *S. aureus*.
- The majority of the favourable interactions as determined by the isobologram analysis were attributed to the contribution of essential oils rather than *R. rubiginosa* fixed oil.

- *Rosa rubiginosa* fixed oil and essential oils displaying antimicrobial synergy (*R. rubiginosa* fixed oil combined with *C. zeylanicum* essential oil), or an overall antimicrobial additive interaction (*R. rubiginosa* fixed oil combined with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, or *S. benzoin* essential oil), or antimicrobial antagonism (*R. rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil), were further investigated for brine shrimp toxicity (Chapter 3).

CHAPTER 3

Brine shrimp toxicity

3.1. Introduction

Toxicology is defined as the study of harmful interactions between chemical substances and living systems (Tisserand and Young, 2013). Although fixed oils are believed to reduce essential oil toxicity, toxicity reports of these fixed oils are scarce, with only one study (Orchard *et al.*, 2019) to date exhibiting the effect of fixed oils in lowering the toxicity of essential oils (Orchard and van Vuuren, 2019). Research conducted by Orchard *et al.* (2019) concluded that fixed oils are able to decrease essential oil toxicity without antagonising antimicrobial action, thus fixed oils may be used as a diluent for essential oils to reduce the toxicity of topical dermatological products that irritate the skin. The toxicity of the essential oil may be affected by interactions between the chemical constituents of the fixed and essential oils, thus determining these interactions are challenging (Tisserand and Young, 2013).

Due to its simplicity, efficiency and reproducibility, the brine shrimp lethality assay (BSLA) is a favoured assay used as a preliminary screening tool for testing toxicity (Sarah *et al.*, 2017). The *Artemia franciscana* species of brine shrimp is frequently used in the toxicological evaluation of natural medicines (Ocaranza-Joya *et al.*, 2019). The BSLA is a viable alternative to mammalian (such as mouse) toxicity bioassays, which are expensive and subjected to strict ethical considerations (Ruebhart *et al.*, 2008). A lack of brine shrimp toxicity may indicate safety for topical treatment (Barillot and Cock, 2021).

This chapter focuses on the BSLA toxicity screening of *R. rubiginosa* fixed oil independently and in 1:1 combinations with the eight essential oils identified as having interest due to either synergistic, additive, or antagonistic antimicrobial interaction (Chapter 2). Varied ratios of *R. rubiginosa* fixed oil with the essential oils were further explored to determine if toxicity could be reduced depending on the ratio at which *R. rubiginosa* fixed oil and the essential oil were combined.

3.2. Materials and methods

3.2.1. Oil selection and sample preparation

Rosa rubiginosa fixed oil and essential oils displaying antimicrobial synergy (*R. rubiginosa* fixed oil combined with *C. zeylanicum* essential oil), or an overall antimicrobial additive interaction (*R. rubiginosa* fixed oil combined with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, or *S. benzoin* essential oil), or antimicrobial antagonism (*R. rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil) as identified in Chapter 2, were selected for this study to determine BSLA toxicity of the oils.

Samples were prepared by first weighing and then solubilising the oils in 2.00% v/v dimethyl sulfoxide (DMSO) (Sigma-Aldrich®) to obtain a stock concentration of 20.00 mg/mL (Equation 3.1).

$$\text{Volume of 2.00\% DMSO required } (\mu L) = \frac{\text{Weight of oil (mg)} \times 1000}{20.00 \text{ mg/mL}}$$

Equation 3.1.

The stock concentration was then further diluted to 2.00 mg/mL using 2.00% v/v DMSO. The prepared oil samples were stored in tightly sealed amber bottles at approximately 4°C until further analysis (van Vuuren *et al.*, 2019).

Table 3.1 lists the negative and positive controls used in the current study and the purpose of each for the BSLA.

Table 3.1. Controls used in the BSLA.

Sample	Type of control	Purpose
Saltwater (32.00 mg/mL)	Negative control	Artificial sea water to ensure the growth and survival of brine shrimp by mimicking their natural environment.
Sterile water in 2.00% v/v DMSO (2.00 mg/mL)	Negative control	To ensure toxicity was not because of the solvent used for the samples.
Potassium dichromate (1.60 mg/mL)	Positive control	Toxic compound to ensure brine shrimp death.

3.2.2. Brine shrimp lethality assay (BSLA)

Rosa rubiginosa fixed oil and the eight essential oils were tested independently and then in 1:1 combination (fixed oil: essential oil) for toxicity using the BSLA (Orchard *et al.*, 2019). Artificial sea water was prepared by dissolving 16 g of Tropic Marin® sea salt in 500 mL of sterile, distilled water. Dried *A. franciscana* (brine shrimp) eggs (Ocean Nutrition®) (0.5 g) were added to the saltwater and aerated with a rotary pump (Kiho). A constant source of warmth and light was provided with a lamp (220-240 V). The eggs were incubated for 18-24 hrs at ambient temperature. Microtitre plates were prepared for toxicity evaluation by adding 400 µL of saltwater containing 40-60 live brine shrimp to each of the 48 wells. An ethics waiver certificate for the use of brine shrimp was obtained from the University of the Witwatersrand Animal Research Ethics Committee, reference number: Waiver 09-04-2020-O (Appendix D).

The test samples, at a starting concentration of 2.00 mg/mL, were serially diluted to achieve sample concentrations of 1.00, 0.50, 0.25, 0.125, 0.063, and 0.031 mg/mL. The test samples (400 µL of *R. rubiginosa* fixed oil or essential oil; or 200 µL of *R. rubiginosa* fixed oil and 200 µL of essential oil for combinations) were then added to each well, tested in triplicate, and repeated where discrepancies were found ($n \geq 3$). Saltwater (400 µL of 32.00 mg/mL) and 400 µL of sterile water in 2.00% v/v DMSO were included as negative controls. Potassium dichromate (Sigma-Aldrich®) was used as a positive control at a concentration of 1.60 mg/mL (400 µL). The plates were viewed after 24 and 48 hrs under a light microscope (Olympus) at 40 × magnification and the dead brine shrimp counted. Thereafter, a lethal dose (50 µL) of 100.00% acetic acid (SAAR Chem-Trade Pvt. Ltd.) was added to each well and a final count of dead brine shrimp was taken (Orchard *et al.*, 2019). The percentage mortality was calculated (Equation 3.2).

$$\% \text{ Mortality} = \frac{\text{dead shrimp at 24 hrs (or 48 hrs) (before acetic acid) - dead shrimp at 0 hrs}}{\text{dead shrimp (after acetic acid)}} \times 100$$

Equation 3.2.

The mean percentage mortality and standard deviation were calculated for each sample using Microsoft Excel Office 365 (Version 2202) software. Biological toxicity was considered for a percentage mortality of ≥ 50% (Bussmann *et al.*, 2011). The mean percentage mortality results were depicted graphically using GraphPad Prism® (Version 5) software.

The *p*-value of *R. rubiginosa* fixed oil in 1:1 combination with the essential oils was determined by performing a T-Test using Microsoft Excel Office 365 software. A $p \leq 0.05$ was considered significant (Greenland *et al.*, 2016), where toxicity reduction was due to *R. rubiginosa* fixed oil quenching the negative effect as opposed to the decrease in toxicity being due to the dilution of the essential oil concentration by the fixed oil. A $p > 0.05$ was considered non-significant (Greenland *et al.*, 2016), where the decreased toxicity was attributed to the dilution of the oil combination instead.

3.2.3. Varied ratio combinations

The same method for the 1:1 combination in Section 3.2.2 was used, however, the concentration of the combinations varied in ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. The mean percentage mortality for the various ratios were recorded.

3.3. Results and discussion

3.3.1. Independent oils

The mean percentage mortality ($n \geq 3$) for *R. rubiginosa* fixed oil and the eight essential oils independently is reported in Table 3.2. This study reports the first BSLA data on the toxicity of *R. rubiginosa* fixed oil. *Rosa rubiginosa* fixed oil was found to be non-toxic, with a percentage mortality of 0.35-1.64% after 24 hrs and 0.74-2.00% after 48 hrs, across all concentrations in the range of 0.03-1.00 mg/mL. A study by Orchard *et al.* (2019) explored the toxicity of six fixed oils most frequently cited for dermatological applications (including, *A. vera*, *C. officinalis*, *H. perforatum*, *P. americana*, *Prunus armeniaca* (apricot kernel), and *S. chinensis*). The lowest percentage mortality after 48 hrs was observed for *P. armeniaca* (10.38%) (Orchard *et al.*, 2019). In comparison, *R. rubiginosa* fixed oil is far less toxic and thus more favourable for dermatological use. The high concentrations of unsaturated fatty acids (linoleic (18:2 ω-6) (41.93% ±1.20), linolenic (18:3 ω-3) (35.73% ±2.50), and oleic (18:1 ω-9) (15.45% ±0.46) acids) may be responsible for the observed non-toxic effect of *R. rubiginosa* fixed oil. An animal study by Bushita *et al.* (2018) concluded that consumption of α-linolenic acid enriched diacylglycerol oil – constituting of linoleic, linolenic and oleic acids – was unlikely to produce toxic effects. Moreover, animal studies by Priyadarshini *et al.* (2012) and

Raj *et al.* (2014) revealed that α-linolenic acid (18:3 ω-3) and linoleic acid (18:2 ω-6) respectively, were able to reduce the toxicity of or illicit protective effects against drug-induced toxicity.

Table 3.2. Mean % mortality ($n \geq 3$) with standard deviation of *R. rubiginosa* fixed oil and the essential oils independently after 24 and 48 hrs.

Sample	Concentration (mg/mL)	Mean independent % mortality[a]	
		24 hrs	48 hrs
Rosa rubiginosa L. (rosehip)	1.00	***0.35 ±0.35***[b]	***0.74 ±0.74***
	0.50	***1.64 ±1.64***	***1.64 ±1.64***
	0.25	***0.62 ±0.03***	***1.70 ±1.04***
	0.13	***1.04 ±0.35***	***1.65 ±0.35***
	0.06	***0.98 ±0.42***	***2.00 ±0.13***
	0.03	***0.63 ±0.63***	***1.01 ±1.01***
Cinnamomum zeylanicum Blume (true cinnamon)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	100.00 ±0.00	100.00 ±0.00
	0.06	*80.43 ±2.21*[c]	*89.80 ±0.17*
	0.03	***6.32 ±2.43***	***18.78 ±0.73***
Eugenia caryophyllata Thunb. (clove)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	100.00 ±0.00	100.00 ±0.00
	0.06	*97.03 ±1.35*	100.00 ±0.00
	0.03	***1.11 ±1.57***	***5.60 ±1.98***
Kunzea ericoides (A.Rich.) Joy Thomps. (kanuka)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	100.00 ±0.00	100.00 ±0.00
	0.06	*55.53 ±1.76*	*71.78 ±1.98*
	0.03	***5.14 ±1.87***	***14.81 ±3.43***
Melaleuca quinquenervia (Cav.) S.T.Blake (niaouli)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	*92.86 ±1.21*	*98.78 ±0.87*
	0.06	*56.17 ±1.71*	*82.19 ±0.86*
	0.03	***32.26 ±2.39***	*68.74 ±1.48*
Melissa officinalis L. (lemon balm)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	100.00 ±0.00	100.00 ±0.00
	0.06	*99.39 ±0.86*	100.00 ±0.00
	0.03	***16.88 ±2.94***	***33.08 ±6.15***
Rosa damascena Herrm. (Damask rose)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	*98.57 ±1.05*	100.00 ±0.00
	0.06	*54.72 ±1.09*	*68.60 ±1.15*
	0.03	***1.25 ±0.89***	***2.40 ±0.75***
Styrax benzoin Dryand. (gum benjamin tree)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	100.00 ±0.00	100.00 ±0.00

Sample	Concentration (mg/mL)	Mean independent % mortality[a]	
		24 hrs	48 hrs
	0.06	*96.25 ±2.70*	100.00 ±0.00
	0.03	***26.27 ±0.64***	***43.75 ±1.48***
Santalum austrocaledonicum Vieill. (New Caledonia sandalwood)	1.00	100.00 ±0.00	100.00 ±0.00
	0.50	100.00 ±0.00	100.00 ±0.00
	0.25	100.00 ±0.00	100.00 ±0.00
	0.13	100.00 ±0.00	100.00 ±0.00
	0.06	100.00 ±0.00	100.00 ±0.00
	0.03	100.00 ±0.00	100.00 ±0.00
Potassium dichromate	1.60	100.00 ±0.00	100.00 ±0.00
Sterile water in 2.00% v/v DMSO	1.00	0.66 ±0.05	1.43 ±0.72
Saltwater	32.00	1.43 ±0.20	2.11 ±0.49

[a] Percentage mortality of ≥ 50% is considered toxic, and < 50% is considered non-toxic; [b] non-toxic (bold, italic); and [c] reduced toxicity (italic).

Independently, all eight essential oils were found to be highly toxic at concentrations between 0.06-1.00 mg/mL after 24 and 48 hrs. Further dilution to a concentration of 0.03 mg/mL rendered all the essential oils non-toxic after 24 and 48 hrs, except for *M. quinquenervia* and *S. austrocaledonicum* essential oils. At a concentration of 0.03 mg/mL, *M. quinquenervia* essential oil remained toxic after 48 hrs, whereas *S. austrocaledonicum* essential oil remained toxic at all tested concentrations. Similar toxicity results for *C. zeylanicum*, *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, and *S. benzoin* essential oils, were observed by Orchard *et al.* (2019). Several previous studies also reported high toxicity of *C. zeylanicum* and *E. caryophyllata* essential oils against *Artemia salina* (brine shrimp) where toxic concentrations ranged from 0.03 µg/mL to 0.60 mg/mL (Sharififar *et al.*, 2009, Bajracharya and Tuladhar, 2011, Ewais *et al.*, 2014, Cansian *et al.*, 2017). Moreover, a study by Sharopov *et al.* (2013) reported high toxicity of *M. officinalis* essential oil against *A. salina* at a concentration of 21.80 µg/mL. Compared to the present study, a similar toxicity result for *R. damascena* essential oil was observed by Leigh-de Rapper *et al.* (2021), where the percentage mortality was 97.32% ±2.34 at a concentration of 1.00 mg/mL. The present study supports toxicity of *S. austrocaledonicum* essential oil in brine shrimp, where previous studies were limited to *S. album* essential oil (Orchard *et al.*, 2019). Overall, *S. austrocaledonicum* essential oil was the most toxic essential oil investigated in this study.

3.3.2. Oil combinations (1:1)

The mean percentage mortality (n ≥ 3) of *R. rubiginosa* fixed oil and the essential oils independently and in 1:1 combination (fixed oil: essential oil) at six serially diluted concentrations, after 24 hrs and 48 hrs, were investigated (Figures 3.1-.3.8). The combination of *R. rubiginosa* fixed oil with *C. zeylanicum* essential oil was toxic at most of the concentrations tested after 24 hrs (Figure 3.1a) and 48 hrs (Figure 3.1b). At 0.063 mg/mL and 0.031 mg/mL (absolute points in Figure 3.1), toxicity of this oil combination was reduced to non-toxic levels because of the dilution of the combination ($p > 0.05$).

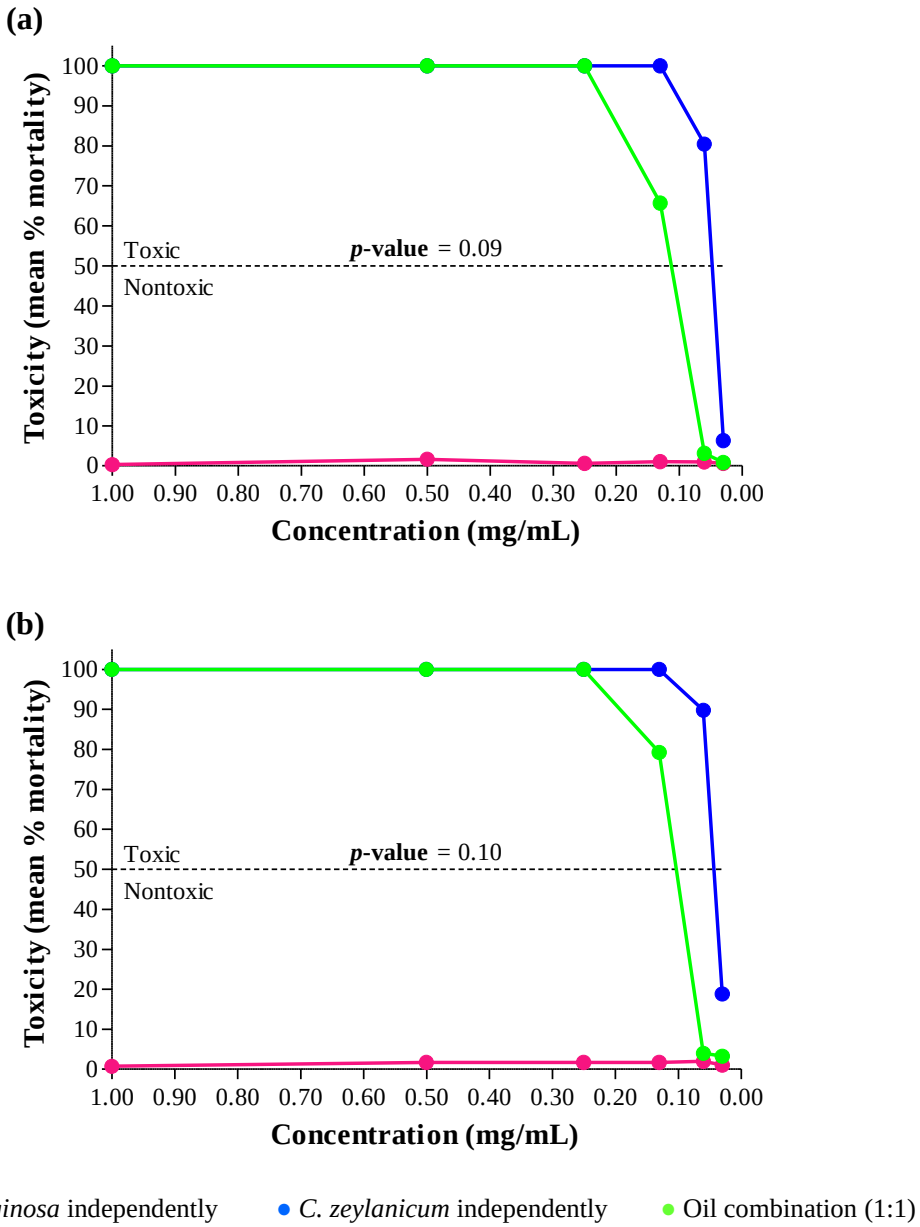

Figure 3.1. Mean % mortality for *R. rubiginosa* fixed oil and *C. zeylanicum* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *E. caryophyllata* essential oil was toxic at most of the concentrations tested after 24 hrs (Figure 3.2a) and 48 hrs (Figure 3.2b). At 0.125 mg/mL (24 hrs) and 0.063 mg/mL (48 hrs) and subsequent dilutions (absolute points in Figure 3.2), toxicity of this oil combination was reduced to non-toxic levels because of the dilution of the combination ($p > 0.05$).

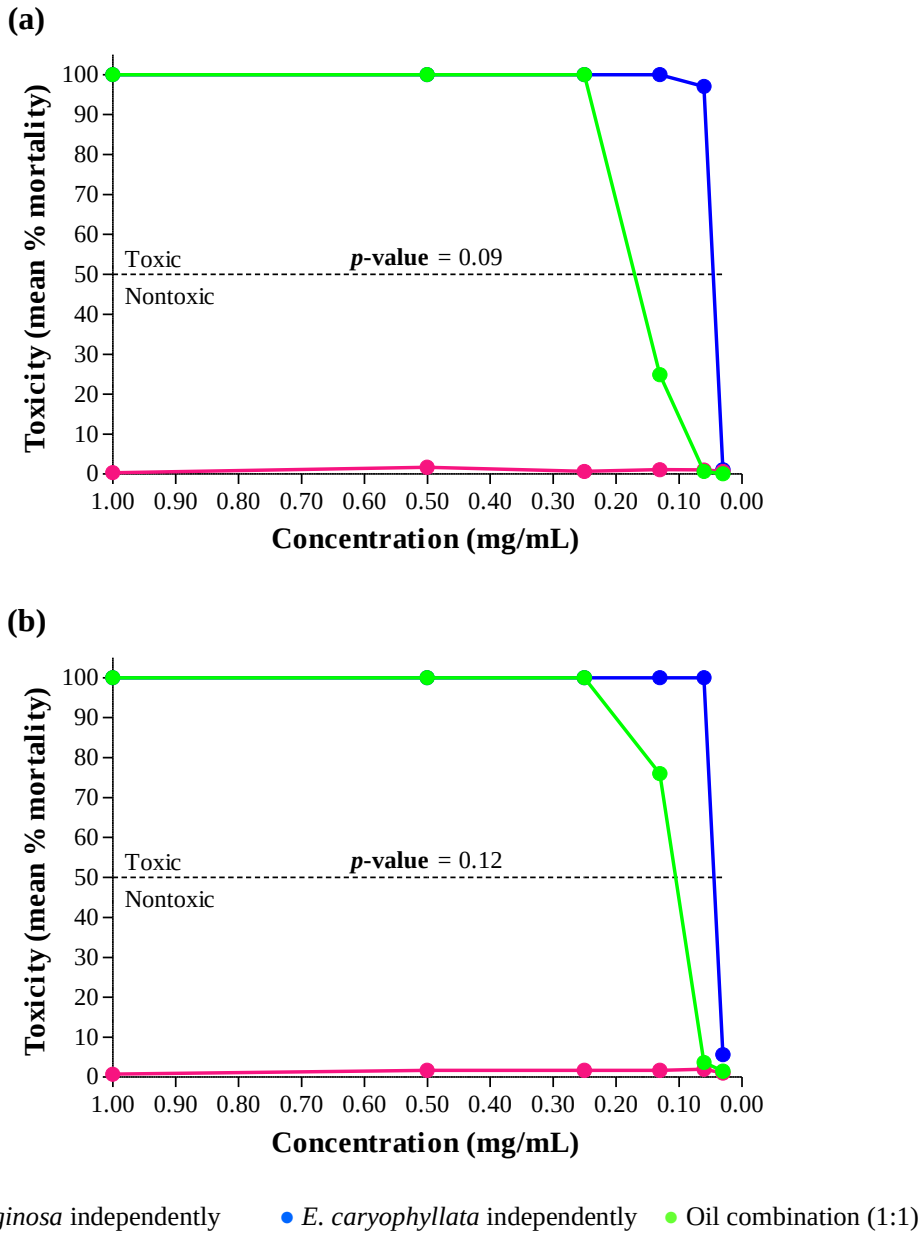

Figure 3.2. Mean % mortality for *R. rubiginosa* fixed oil and *E. caryophyllata* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *K. ericoides* essential oil was non-toxic at most of the concentrations tested after 24 hrs (Figure 3.3a) and 48 hrs (Figure 3.3b). At 0.25 mg/mL and subsequent dilutions (absolute points in Figure 3.3), toxicity of this oil combination was reduced to non-toxic levels because of *R. rubiginosa* fixed oil ($p \leq 0.05$). Therefore, the combination of *R. rubiginosa* fixed oil with *K. ericoides* essential oil may be a favourable non-toxic combination at concentrations of 0.25 mg/mL or less for further investigation of its topical application in treating acne.

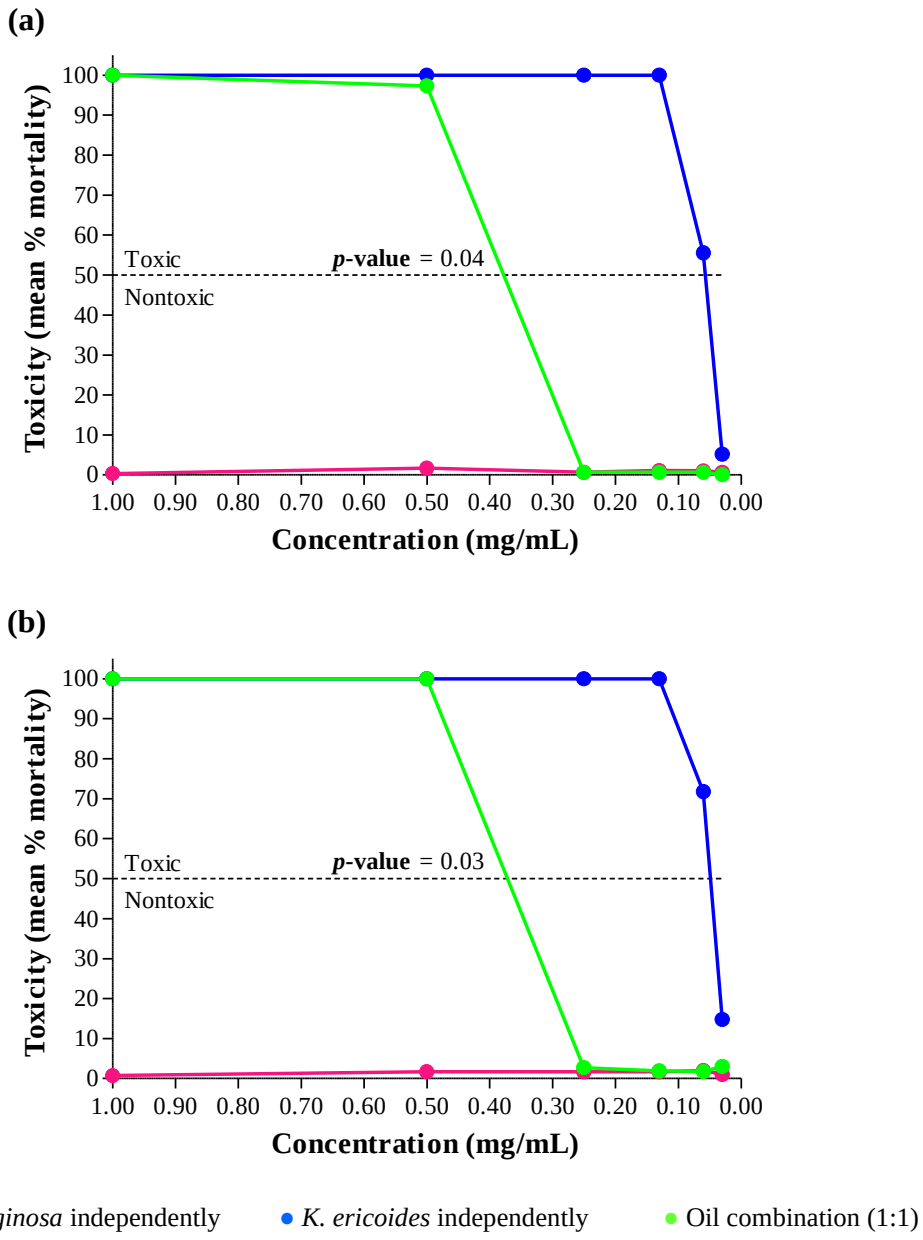

Figure 3.3. Mean % mortality for *R. rubiginosa* fixed oil and *K. ericoides* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil was non-toxic at most of the concentrations tested after 24 hrs (Figure 3.4a) and 48 hrs (Figure 3.4b). At 0.25 mg/mL (24 hrs) and 0.125 mg/mL (48 hrs) and subsequent dilutions (absolute points in Figure 3.4), toxicity of this oil combination was reduced to non-toxic levels because of *R. rubiginosa* fixed oil ($p \leq 0.05$). Therefore, the combination of *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil may be a favourable non-toxic combination at concentrations of 0.125-0.25 mg/mL or less for further investigation of its topical application in treating acne.

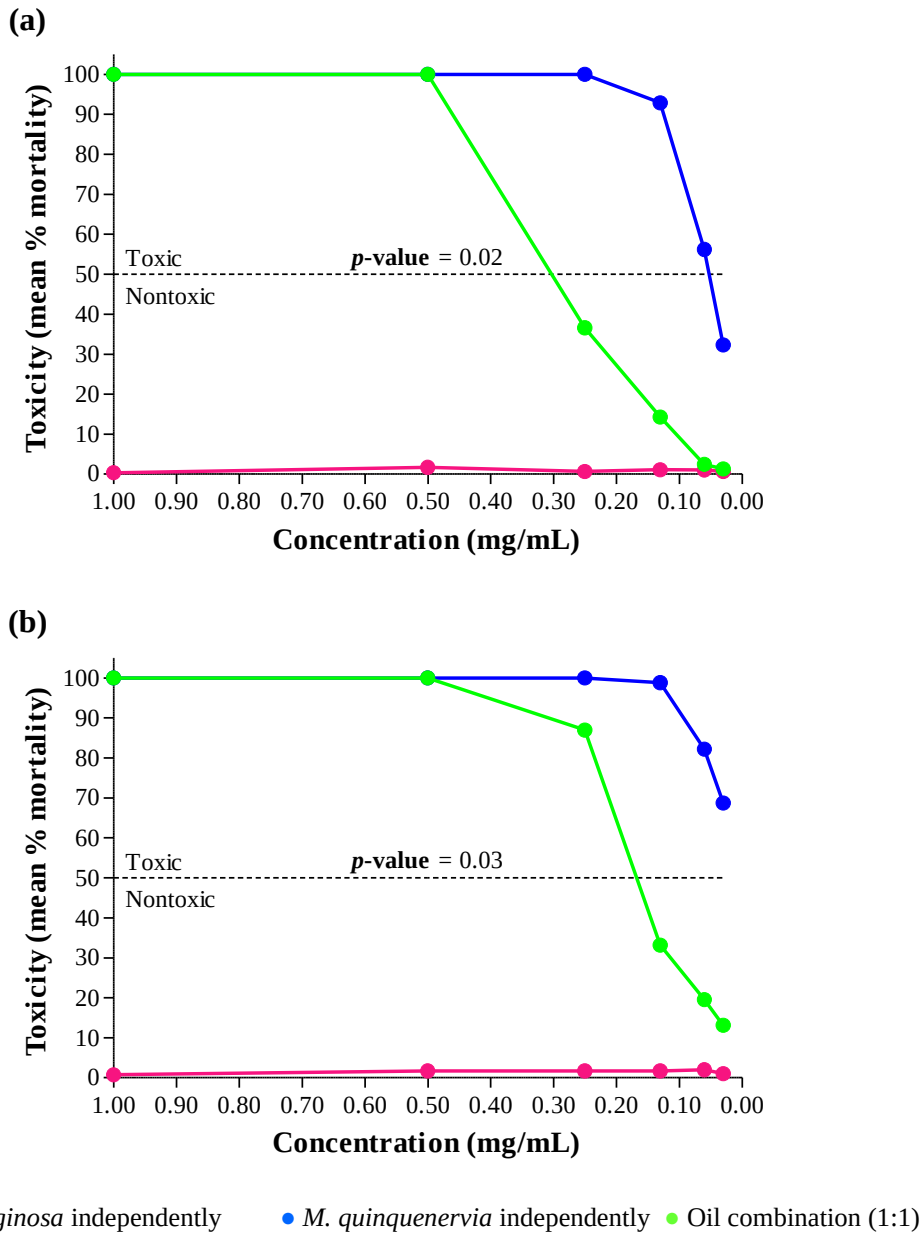

Figure 3.4. Mean % mortality for *R. rubiginosa* fixed oil and *M. quinquenervia* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *M. officinalis* essential oil was non-toxic at the lower concentrations tested after 24 hrs (Figure 3.5a) and 48 hrs (Figure 3.5b). At 0.125 mg/mL and subsequent dilutions (absolute points in Figure 3.5), toxicity of this oil combination was reduced to non-toxic levels because of the dilution of the combination ($p > 0.05$) after 24 hrs (Figure 3.5a). At the same concentration after 48 hrs (Figure 3.5b), this oil combination was reduced to non-toxic levels because of *R. rubiginosa* fixed oil ($p \leq 0.05$). Therefore, the combination of *R. rubiginosa* fixed oil with *M. officinalis* essential oil may be a favourable non-toxic combination at concentrations of 0.125 mg/mL or less for further investigation of its topical application in treating acne.

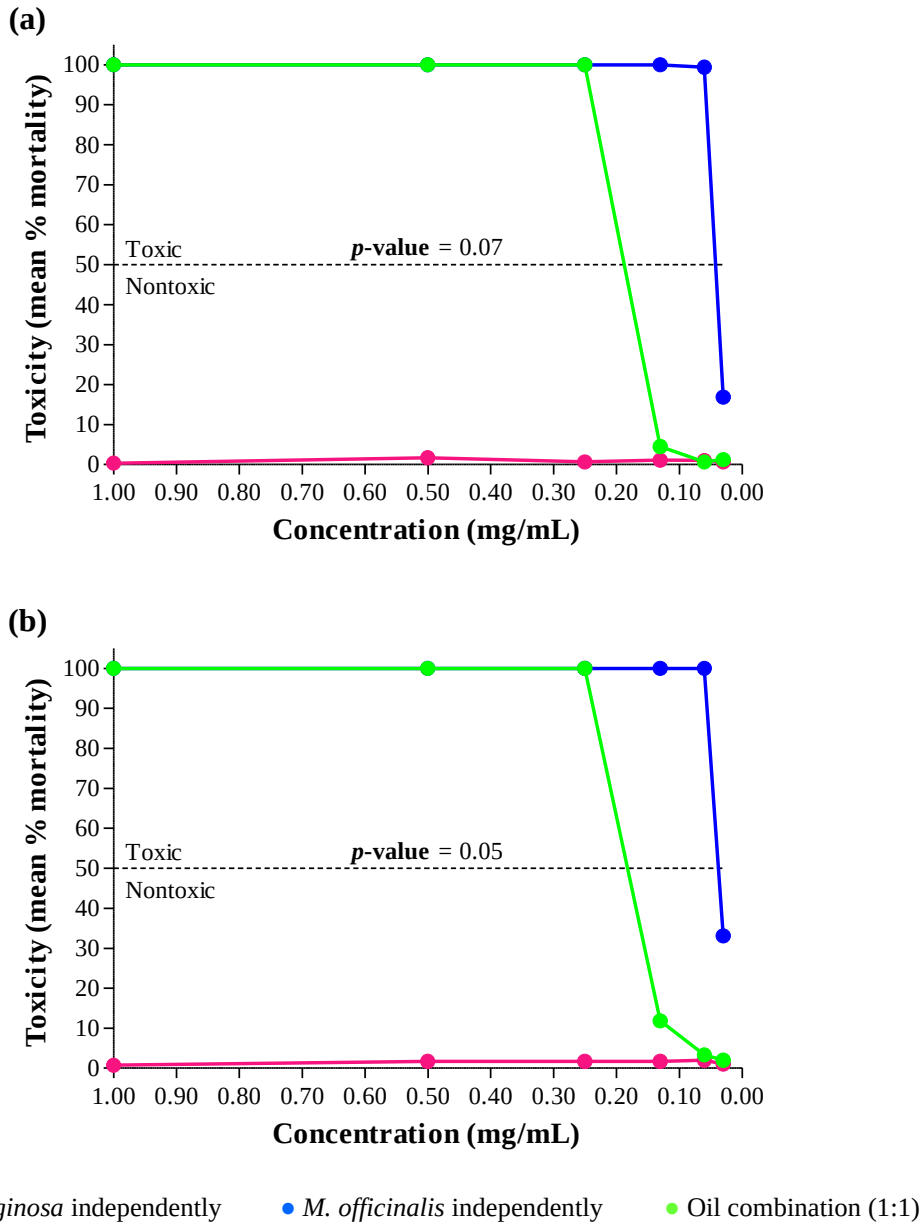

Figure 3.5. Mean % mortality for *R. rubiginosa* fixed oil and *M. officinalis* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *R. damascena* essential oil was non-toxic at the lower concentrations tested after 24 hrs (Figure 3.6a) and 48 hrs (Figure 3.6b). At 0.125 mg/mL and subsequent dilutions (absolute points in Figure 3.6), toxicity of this oil combination was reduced to non-toxic levels because of the dilution of the combination ($p > 0.05$). The combination of *R. rubiginosa* fixed oil with *R. damascena* essential oil at concentrations of 0.125 mg/mL or less has potential for treating acne.

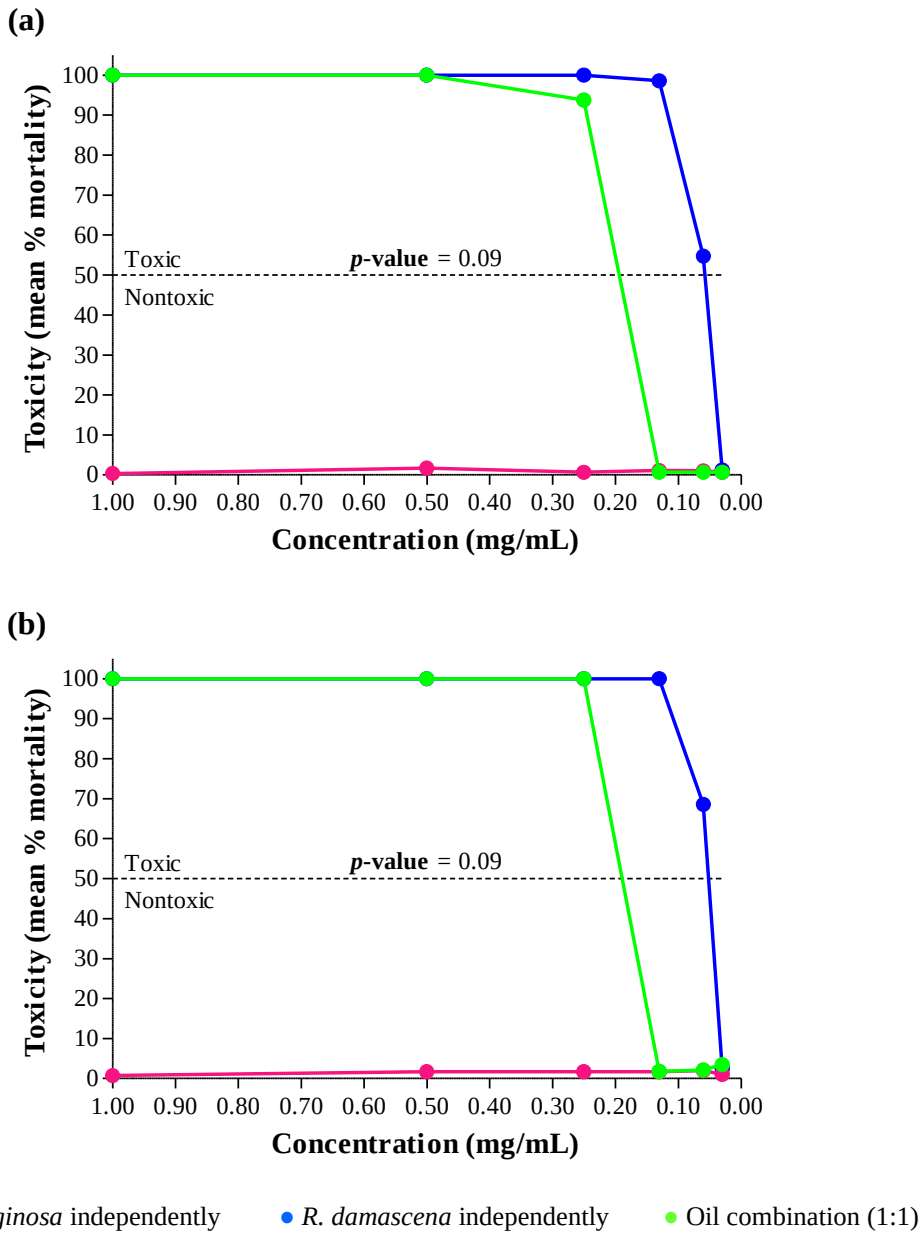

Figure 3.6. Mean % mortality for *R. rubiginosa* fixed oil and *R. damascena* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *S. benzoin* essential oil was toxic at most of the concentrations tested after 24 hrs (Figure 3.7a) and 48 hrs (Figure 3.7b). At 0.125 mg/mL and subsequent dilutions (absolute points in Figure 3.7), toxicity of this oil combination was reduced to non-toxic levels because of the dilution of the combination ($p > 0.05$) after 24 hrs (Figure 3.7a). However, this oil combination was reduced to non-toxic levels only at 0.063 mg/mL and 0.031 mg/mL after 48 hrs (Figure 3.7b) because of *R. rubiginosa* fixed oil ($p \leq 0.05$).

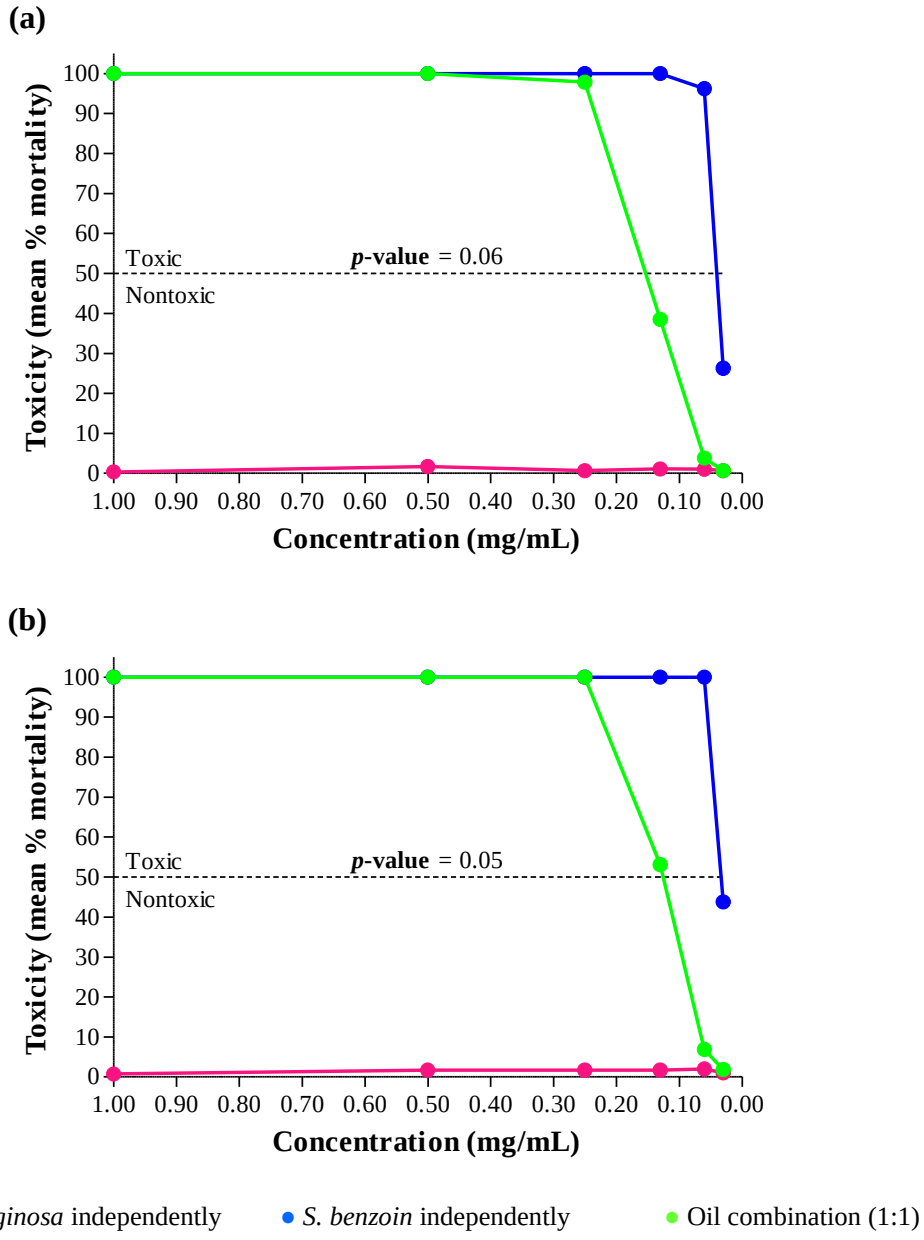

Figure 3.7. Mean % mortality for *R. rubiginosa* fixed oil and *S. benzoin* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

The combination of *R. rubiginosa* fixed oil with *S. austrocaledonicum* essential oil was toxic at all except one of the concentrations tested after 24 hrs (Figure 3.8a) and 48 hrs (Figure 3.8b). At 0.031 mg/mL (absolute points in Figure 3.8), toxicity of this oil combination was reduced to a non-toxic level after 24 hrs (Figure 3.8a). The combination was toxic at the same concentration (0.031 mg/mL) after 48 hrs (Figure 3.8b), however, showed lower toxicity than *S. austrocaledonicum* essential oil when tested independently.

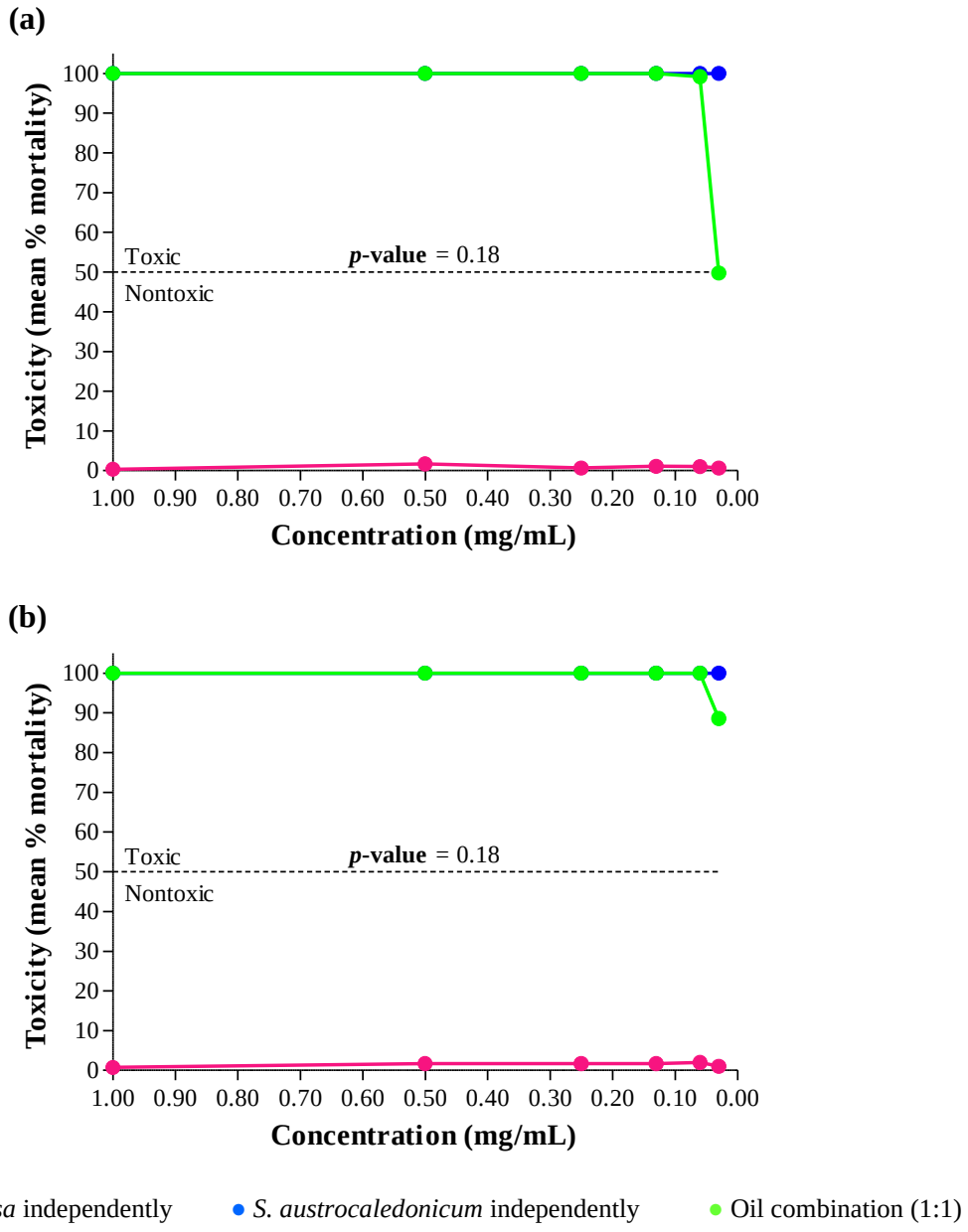

Figure 3.8. Mean % mortality for *R. rubiginosa* fixed oil and *S. austrocaledonicum* essential oil independently and in 1:1 combination after **(a)** 24 hrs and **(b)** 48 hrs.

Compared to the independent essential oils, the toxicity of all eight *R. rubiginosa* fixed oil with essential oil combinations was reduced at concentrations ranging between 0.03-0.25 mg/mL. The greatest toxicity reduction was observed for the *R. rubiginosa* fixed oil with *K. ericoides* essential oil combination (Figure 3.3) at 0.25 mg/mL, where the percentage mortality decreased significantly from 100% (*K. ericoides* essential oil independently) to less than 3% (1:1 combination) at 24 hrs (p-value = 0.04) and 48 hrs (p-value = 0.03). This was followed by the *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil combination (Figure 3.4), where the percentage mortality at 0.25 mg/mL was reduced significantly from 100% to 36.56% at 24 hrs (p-value = 0.02), and at 0.13 mg/mL was reduced from 98.78% to 33.14% at 48 hrs (p-value = 0.03), respectively. "Quenching" of toxicity (Orchard *et al.*, 2019) in terms of the present study, refers to the chemical interaction of *R. rubiginosa* fixed oil combined with the essential oils to illicit a significant decrease in overall toxicity. Thus, *R. rubiginosa* fixed oil quenched the toxicity of *K. ericoides* and *M. quinquenervia* essential oils after 24 and 48 hrs.

Rosa rubiginosa fixed oil lowered the toxicity of *M. officinalis* and *S. benzoin* essential oils after 48 hrs as the results for these combinations was statistically significant ($p \leq 0.05$). The decreased toxicity noted for the *R. rubiginosa* fixed oil combinations with either *C. zeylanicum*, *E. caryophyllata*, *R. damascena*, or *S. austrocaledonicum* essential oil may have been due to the decreased concentration of the respective essential oils in combination rather than attributed to the influence of *R. rubiginosa* fixed oil, as the overall results were statistically non-significant ($p > 0.05$). Since *R. rubiginosa* fixed oil was combined with the essential oils in a 1:1 ratio, diluting the essential oils in a higher concentration of *R. rubiginosa* fixed oil may possibly lower the toxicity of the oil combinations further (Orchard *et al.*, 2019). Therefore, the combinations of *R. rubiginosa* fixed oil with either *C. zeylanicum*, *E. caryophyllata*, *M. officinalis*, *R. damascena*, *S. benzoin*, or *S. austrocaledonicum* essential oil require substantial dilution of the essential oils in the fixed oil to negate the toxic effects of the essential oils.

3.3.3. Varied ratio oil combinations

The mean varied ratio combination percentage mortality ($n \geq 3$) of *R. rubiginosa* fixed oil combined with the eight essential oils (Table 3.3) was investigated to determine whether diluting the essential oils in a higher concentration of *R. rubiginosa* fixed oil may possibly lower the toxicity of the essential oils further than the 1:1 ratio oil combinations.

Table 3.3. Mean varied ratio combination % mortality (n ≥ 3) with standard deviation of *R. rubiginosa* fixed oil in combination with the eight essential oils after 24 and 48 hrs.

Sample	Volume ratio	Mean varied ratio combination % mortality[a]	
	Essential oil: *R. rubiginosa* fixed oil	**24 hrs**	**48 hrs**
Cinnamomum zeylanicum Blume (true cinnamon)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	100.00 ±0.00	100.00 ±0.00
	3:7	100.00 ±0.00	100.00 ±0.00
	2:8	100.00 ±0.00	100.00 ±0.00
	1:9	*90.28 ±1.45*[b]	100.00 ±0.00
Eugenia caryophyllata Thunb. (clove)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	100.00 ±0.00	100.00 ±0.00
	3:7	100.00 ±0.00	100.00 ±0.00
	2:8	*77.71%±2.31*	100.00 ±0.00
	1:9	*72.14%±2.81*	100.00 ±0.00
Kunzea ericoides (A.Rich.) Joy Thomps. (kanuka)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	*85.88 ±0.68*	100.00 ±0.00
	3:7	*50.07 ±0.92*	*82.44 ±0.57*
	2:8	***23.28 ±1.72***[c]	***47.39 ±1.88***
	1:9	***0.71 ±1.00***	***8.33 ±1.49***
Melaleuca quinquenervia (Cav.) S.T.Blake (niaouli)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	*88.40 ±0.81*	100.00 ±0.00
	3:7	*74.84 ±0.70*	*94.81 ±0.99*
	2:8	*60.61 ±4.10*	*86.20 ±2.33*
	1:9	***42.64 ±3.73***	*71.65 ±0.90*
Melissa officinalis L. (lemon balm)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	100.00 ±0.00	100.00 ±0.00
	3:7	*96.83 ±1.62*	100.00 ±0.00
	2:8	*89.04 ±2.16*	100.00 ±0.00
	1:9	*79.67 ±0.59*	100.00 ±0.00
Rosa damascena Herrm. (Damask rose)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	100.00 ±0.00	100.00 ±0.00

Sample	Volume ratio	Mean varied ratio combination % mortality[a]	
	Essential oil: *R. rubiginosa* fixed oil	**24 hrs**	**48 hrs**
	3:7	100.00 ±0.00	100.00 ±0.00
	2:8	*91.06 ±0.75*	100.00 ±0.00
	1:9	*61.88 ±2.68*	*80.29 ±0.69*
Styrax benzoin Dryand. (gum benjamin tree)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	*83.26 ±1.14*	100.00 ±0.00
	3:7	*63.67 ±3.03*	*82.35 ±0.76*
	2:8	***43.47 ±1.13***	*61.46 ±0.88*
	1:9	***19.95 ±1.42***	***39.90 ±2.85***
Santalum austrocaledonicum Vieill. (New Caledonia sandalwood)	9:1	100.00 ±0.00	100.00 ±0.00
	8:2	100.00 ±0.00	100.00 ±0.00
	7:3	100.00 ±0.00	100.00 ±0.00
	6:4	100.00 ±0.00	100.00 ±0.00
	5:5	100.00 ±0.00	100.00 ±0.00
	4:6	100.00 ±0.00	100.00 ±0.00
	3:7	*90.23 ±0.55*	100.00 ±0.00
	2:8	*82.86 ±1.26*	*99.35 ±0.92*
	1:9	*71.19 ±1.51*	*95.33 ±0.78*

[a] Percentage mortality of ≥ 50% is considered toxic, and < 50% is considered non-toxic; [b] reduced toxicity (italic); and [c] non-toxic (bold, italic).

Varied ratio combinations of *R. rubiginosa* fixed oil with the eight essential oils displayed a general trend in decreased percentage mortality when minimal quantities of essential oil were present in each combination. This was expected, as the purpose of the fixed oil is to dilute the essential oil (Orchard and van Vuuren, 2019). The toxicity of three of the eight essential oils, *K. ericoides*, *M. quinquenervia*, and *S. benzoin*, were reduced to non-toxic levels through combination with *R. rubiginosa* fixed oil. At a 1:9 ratio (essential oil: fixed oil), *K. ericoides* essential oil toxicity resulted in the greatest decrease with a percentage mortality of 0.71% and 8.33% at 24 and 48 hrs, respectively. At the same ratio, this was followed by *S. benzoin* essential oil (percentage mortality of 19.95% at 24 hrs and 39.90% at 48 hrs) and *M. quinquenervia* essential oil (percentage mortality of 42.64% at 24 hrs). After the 1:9 ratio (essential oil: fixed oil), the least toxic *R. rubiginosa* fixed oil and essential oil combination was demonstrated by the 2:8 ratio of *K. ericoides* essential oil (percentage mortality of 23.28% at 24 hrs and 47.39% at 48 hrs) and *S. benzoin* essential oil (percentage mortality of 43.47% at 24 hrs). This study demonstrated that despite dilution with high proportions of non-toxic *R. rubiginosa* fixed oil, the toxicity of essential oils is challenging to relegate as even the smallest of quantities can elicit brine shrimp death.

3.4. Overview

- *Rosa rubiginosa* fixed oil was found to be non-toxic, with a percentage mortality of 0.35-1.64% after 24 hrs and 0.74-2.00% after 48 hrs, across all concentrations in the range of 0.03-1.00 mg/mL.
- All eight essential oils were found to be highly toxic at concentrations between 0.06-1.00 mg/mL after 24 and 48 hrs.
- Further dilution to a concentration of 0.03 mg/mL rendered all the essential oils non-toxic after 24 and 48 hrs, except for *M. quinquenervia* and *S. austrocaledonicum* essential oils.
- *Santalum austrocaledonicum* essential oil remained toxic at all tested dilutions (mean % mortality = 100.00%).
- The toxicity of all eight essential oils in combination with *R. rubiginosa* fixed oil was reduced at concentrations between 0.03-0.25 mg/mL.
- The greatest reduction in toxicity was observed for *R. rubiginosa* fixed oil combined with *K. ericoides* essential oil at 0.25 mg/mL, where the percentage mortality decreased significantly from 100% (*K. ericoides* essential oil independently) to less than 3% (1:1 combination) at 24 hrs (p-value = 0.04) and 48 hrs (p-value = 0.03).
- The toxicity of three of the eight essential oils, *K. ericoides*, *M. quinquenervia*, and *S. benzoin*, was reduced to non-toxic levels when diluted in higher concentrations of *R. rubiginosa* fixed oil.
- At a 1:9 ratio (essential oil: fixed oil), *K. ericoides* essential oil displayed the best decrease in toxicity with a percentage mortality of 0.71% and 8.33% at 24 and 48 hrs, respectively.
- While the BSLA served as a beneficial tool to identify toxicity, a more sensitive distinction of activity includes testing using cell lines. Hence, *R. rubiginosa* fixed oil and the eight essential oils (*C. zeylanicum*, *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, *S. benzoin*, and *S. austrocaledonicum*) were further investigated for anti-inflammatory activity and associated cytotoxicity (Chapter 4).

CHAPTER 4

Anti-inflammatory study and associated cytotoxicity

4.1. Introduction

The murine macrophage cell line, RAW 264.7, is a well characterised and popular model to investigate the anti-inflammatory potential of natural products. RAW 264.7 macrophages behave in a similar manner to human macrophages when exposed to lipopolysaccharide (LPS), which induces signal pathways and gene expression (de Canha *et al.*, 2020). One of the most prominent effects of LPS exposure is overexpression of the enzyme, inducible nitric oxide synthase (iNOS), with concomitant nitric oxide (NO) formation (Ma *et al.*, 2018, de Canha *et al.*, 2020). Inducible nitric oxide synthase – an inflammatory gene product associated with acne vulgaris – catalyses L-arginine, thereby producing various highly reactive oxidants such as NO (Martinez *et al.*, 2008, de Canha *et al.*, 2020). Nitric oxide produced via the iNOS pathway is associated with many physiological processes of inflammation and disease, and is an important inflammatory target concerning acne vulgaris (Lin *et al.*, 2003, de Canha *et al.*, 2020).

Cytotoxicity, defined as cell death by endogenous and/ or exogenous effects (Istifli and Ila, 2019), is one of the key indicators for *in vitro* biological evaluation studies (Aslantürk, 2018). Cell viability and/ or cell proliferation (increased number of cells because of cell growth and cell division) rates are important indicators of cell health (Aslantürk, 2018). As chemical substances may affect cell health and metabolism, *in vitro* cell viability and cytotoxicity assays with cultured cells are commonly used for cytotoxicity screening of pharmaceutical and organic products (Aslantürk, 2018). Cytotoxicity assays are used to test the ability of cells to continue proliferating in the presence of test samples over a specific period. In recent years, there has been growing interest concerning the application of cytotoxicity assays (Aslantürk, 2018), as determining the cytotoxic potential (number of viable cells and/ or dead cells) and harmful effects are imperative when investigating possible new therapies or developing new products to treat an ailment.

This chapter focuses on nitrite production as a parameter of macrophage activation and anti-inflammatory activity, as well as associated cytotoxicity (examined in RAW 264.7 macrophages and A549 epithelial cells, respectively) of *R. rubiginosa* fixed oil and the eight essential oils, independently and in 1:1 combination. Decreased or lack of nitrite production could be attributed to high cytotoxicity of the oils resulting in declined cell viability. Therefore, the anti-inflammatory response was considered together with the cytotoxicity to assess whether decreased nitrite production resulted from low cell viability.

4.2. Materials and methods

The anti-inflammatory study, MTT assay, and the cytotoxicity screening protocol were performed by BioAssaix *in vitro* screening services (under the guidance of Professor Maryna van de Venter and team at Nelson Mandela University, Gqeberha). The data analysis was self-performed.

4.2.1. Oil selection

Rosa rubiginosa fixed oil and essential oils displaying antimicrobial synergy (*R. rubiginosa* fixed oil combined with *C. zeylanicum* essential oil), or an overall antimicrobial additive interaction (*R. rubiginosa* fixed oil combined with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, or *S. benzoin* essential oil), or antimicrobial antagonism (*R. rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil) as identified in Chapter 2, were selected for this study to determine the anti-inflammatory activity and associated cytotoxicity of the oils. The toxicity of *R. rubiginosa* fixed oil and the eight essential oils in the brine shrimp lethality assay (BSLA) (Chapter 3) was compared to the cytotoxicity in A549 epithelial cells.

4.2.2. Materials and procurement

RAW 264.7 murine macrophages and A549 human epithelial cells were purchased from Cellonex (South Africa). Lipopolysaccharide, Griess reagent, aminoguanidine, Bis-benzamide H 33342 trihydrochloride (Hoechst), and propidium iodide (PI), were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA). RPMI 1640, DMEM low glucose, and foetal bovine

serum (FBS), were purchased from GE Healthcare Life Sciences (Logan, Utah, USA). Phosphate-buffered saline (PBS), with and without Ca^{2+} and Mg^{2+}, and trypsin, were purchased from Lonza (Wakersville, Maryland, USA).

4.2.3. Sample preparation

Rosa rubiginosa fixed oil and the eight essential oils, independently and in 1:1 combination, were prepared in absolute dimethyl sulfoxide (DMSO) to a stock concentration of 10.00% v/v, and further diluted in RPMI complete culture medium to a concentration of 0.02% v/v. Table 4.1 lists the negative and positive controls used in the current study and the purpose of each for the anti-inflammatory screening, MTT assay, and cytotoxicity screening.

Table 4.1. Controls used in the anti-inflammatory, MTT, and cytotoxicity assays.

Assay	Sample	Type of control	Purpose
Anti-inflammatory screening and MTT assay	RPMI complete medium	Culture control	Cell culture medium to ensure macrophage growth.
	Lipopolysaccharide (1.00 µg/mL of LPS in RPMI complete medium)	Negative control	Endotoxin to induce NO formation and elicit an inflammatory response from the macrophages.
	Aminoguanidine (100 µM)	Positive control	Anti-inflammatory agent to inhibit NO formation and elicit an anti-inflammatory response from the macrophages.
Cytotoxicity screening	DMEM supplemented with 10.00% v/v FBS	Culture control	Cell culture medium to ensure epithelial cell growth.
	Melphalan (40 µM)	Positive control	Cytotoxic agent to ensure epithelial cell death.

4.2.4. Anti-inflammatory screening and MTT assay

Nitric oxide inhibition was investigated using the LPS induced inflammatory pathway in a RAW 264.7 murine macrophage model (Leigh-de Rapper *et al.*, 2021). Changes in NO production were determined by measuring the concentration of nitrite (NO_2^-) in the culture medium. Nitrite production as a parameter of macrophage activation and anti-inflammatory activity of *R. rubiginosa* fixed oil and the eight essential oils, independently and in 1:1 combination, was assessed. Simultaneous evaluation of cell viability (3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay) was used to confirm the absence of cytotoxicity of the test samples.

RAW 264.7 macrophages were cultured in RPMI 1640 supplemented with 10.00% v/v FBS in a humidified, 5.00% CO_2 incubator at 37°C. RAW 264.7 macrophages were then seeded into 96-well microtitre plates at a density of 1×10^5 cells per well and allowed to attach overnight. Spent culture medium was replaced with 50 µL of test sample, or RPMI complete medium (as a culture control; or for the LPS induced inflammation control), or aminoguanidine. Samples were tested in quadruplicate (n = 4). To assess nitrite production as a parameter of macrophage activation and anti-inflammatory activity, 50 µL of 1.00 µg/mL LPS in RPMI complete medium was added to all wells except for the culture control, which received 50 µL RPMI complete medium. Final concentrations were therefore 0.01% for the test samples, 500.00 ng/mL for the LPS control and 100 µM for the aminoguanidine positive control. Thereafter, RAW 264.7 macrophages were incubated for 24 hrs. To quantify NO production, 50 µL of the spent culture medium was transferred to a new 96-well plate, and then 50 µL of Griess reagent was added. The microtitre plates were incubated at room temperature for 10 min before absorbance was measured at 540 nm, using a BioTek® PowerWave XS spectrophotometer (Winooski, Vermont, USA), and the results expressed relative to the LPS control. A standard curve, using sodium nitrite dissolved in culture medium, was used to determine the concentration of NO for each test sample.

The ability of the test samples to supress LPS induced nitrite production was compared to three controls: the cell culture medium, LPS, and aminoguanidine. Increased nitrite production (compared to the cell culture medium and LPS control) generally related to lower anti-inflammatory activity of the test samples, whereas decreased nitrite production (compared to the cell culture medium and aminoguanidine), indicated anti-inflammatory activity. Decreased nitrite production of the studied test samples, however, did not necessarily indicate an increased propensity of the sample to reduce inflammation. In some instances, decreased or lack of nitrite production was attributed to the high cytotoxicity of the test sample resulting in declined cell viability. As such, sufficient nitrite production was not induced in low cell numbers. Therefore, the anti-inflammatory response should be considered together with the relative cell viability. Thus, simultaneous evaluation of cell viability, using the MTT assay, was used to confirm the absence of cytotoxicity of the test samples as a contributory factor (Leigh-de Rapper *et al.*, 2021). This was done by replacing the remaining medium in each well with medium containing

0.50 mg/mL MTT, and further incubation for 30 min at 37°C. Samples were tested in quadruplicate (n = 4). Thereafter, MTT was removed and 100 µL of DMSO was added to each well to solubilise the formazan crystals.

4.2.5. Cytotoxicity screening

In addition to the cell viability MTT assay in RAW 264.7 murine macrophages, the cytotoxicity of *R. rubiginosa* fixed oil and the eight essential oils, independently and in 1:1 combination, was assessed in A549 epithelial cells. A549 epithelial cells were cultured in DMEM supplemented with 10.00% v/v FBS in a humidified, 5.00% CO_2 incubator at 37°C. Epithelial cells (A549) were then seeded into 96-well microtitre plates at a density of approximately 4000 cells per well, using a volume of 100 µL in each well. The microtitre plates were incubated for 24 hrs prior to addition of the test samples, to allow for cell attachment. These were treated with 0.01% of test sample diluted in culture medium. The cell culture medium was used as a culture control and melphalan (40 µM), diluted in culture medium, was used as a positive control. One hundred microlitre aliquots of the diluted test samples or melphalan were used to treat A549 epithelial cells in fresh medium. Samples were tested in quadruplicate (n = 4). The microtitre plates were then incubated for a further 48 hrs. Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5.00 µg/mL) and incubated for 20 min at room temperature. Thereafter, A549 epithelial cells were stained with PI (100.00 µg/mL) to enumerate the proportion of dead cells within the population. A549 epithelial cells were imaged immediately after the addition of PI, using the ImageXpress Micro XLS Widefield microscope (Molecular Devices, San Jose, California, USA) with a 10 × Plan Fluor Objective, and 4′,6-diamidino-2-phenylindole (DAPI) and Texas Red™ filter cubes. Nine image sites were acquired per well, representing approximately 75% of the 0.32 cm^2 growth area of the well. Results were expressed as percentage of the cell culture medium control. The cytotoxicity of the test samples was compared to two controls: the cell culture medium and melphalan. A percentage cell viability closer to that of the cell culture medium was considered non-cytotoxic and a percentage cell viability closer to that of melphalan was considered cytotoxic.

4.3. Results and discussion

4.3.1. Anti-inflammatory screening and MTT assay

The LPS induced inflammatory pathway in a RAW 264.7 murine macrophage model was used as a parameter of macrophage activation to assess the anti-inflammatory activity of *R. rubiginosa* fixed oil and the eight essential oils, independently and in 1:1 combination, after 24 hrs (Figure 4.1). To confirm the absence of cytotoxicity as a contributory factor, cell viability was assessed using the MTT assay (Figure 4.2).

Rosa rubiginosa fixed oil reduced LPS induced nitrite production by 18.75% compared to the LPS control. This anti-inflammatory effect may have been due to the high cell viability of *R. rubiginosa* fixed oil (9.23% higher than the LPS control), which was similar to macrophages treated with aminoguanidine – a potent inhibitor of LPS induced iNOS (Zha *et al.*, 2011). A study, investigating *R. canina* powder, showed that *R. canina* powder inhibited inflammatory NO in RAW 264.7 macrophages without impairing cell viability (Schwager *et al.*, 2011). The high content of polyunsaturated fatty acids present in *R. canina* fixed oil (linoleic, linolenic, and oleic acids) are known to protect against inflammation (Chrubasik *et al.*, 2008, Lin *et al.*, 2018). Furthermore, an early study by Shabykin and Godorazhi (1967) proposed promising therapeutic potential of *R. canina* fixed oil in inflammatory dermatological conditions. This was reiterated in later literature by several authors (Chrubasik *et al.*, 2008, Mármol *et al.*, 2017, Lin *et al.*, 2018). A similar mechanism may explain the observation noted in the current study where *R. rubiginosa* fixed oil inhibited nitrite production in RAW 264.7 macrophages, thereby encouraging macrophages to proliferate (increase in the number of cells because of cell growth and cell division) in the presence of reduced inflammation. *Rosa rubiginosa* fixed oil is most commonly utilised in skin cosmetics for its regenerative and anti-inflammatory properties due to the high concentrations of polyunsaturated fatty acids (linoleic (18:2 ω-6) (41.93% ±1.20), linolenic (18:3 ω-3) (35.73% ±2.50), and oleic (18:1 ω-9) (15.45% ±0.46) acids) (de Santana *et al.*, 2016, Lin *et al.*, 2018).

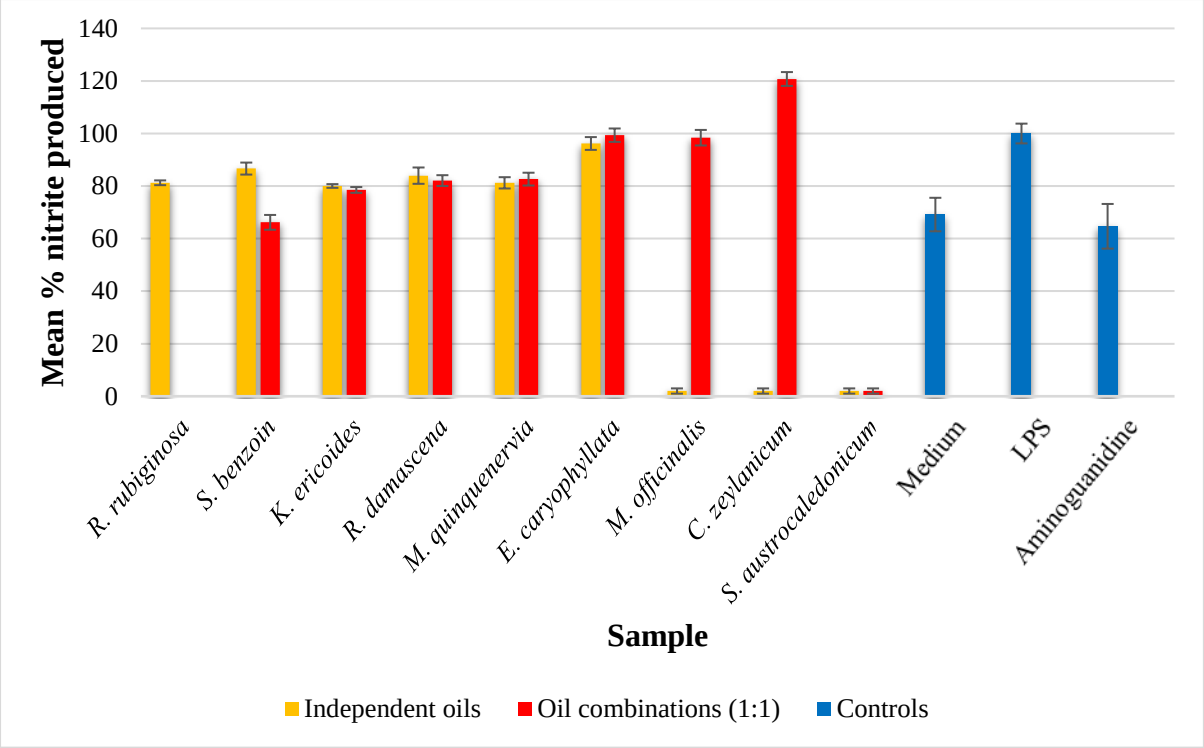

Figure 4.1. Mean % nitrite produced (n = 4) as a parameter of macrophage activation and anti-inflammatory response relative to % LPS activated for test samples in RAW 264.7 macrophages after 24 hrs.

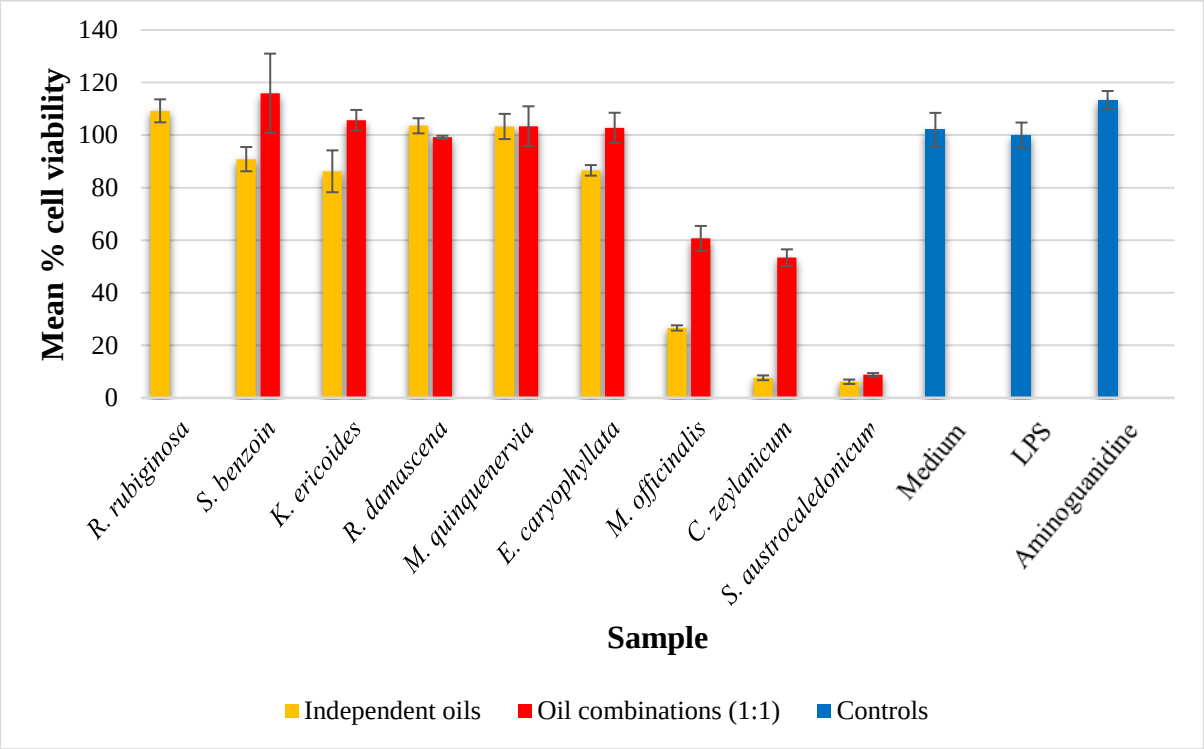

Figure 4.2. Mean % cell viability (n = 4) measuring cytotoxicity (as per the MTT assay) relative to % LPS activated for test samples in RAW 264.7 macrophages after 24 hrs.

Styrax benzoin essential oil was able to decrease nitrite production by 13.34% compared to the LPS control. Although *S. benzoin* resins are commonly used in products to treat skin diseases such as irritated, dry, or inflamed skin (Sharif *et al.*, 2016), scientific evidence supporting anti-inflammatory activity, especially of *S. benzoin* essential oil, is lacking. As shown in Figure 4.1, the combination of *R. rubiginosa* fixed oil and *S. benzoin* essential oil reduced nitrite production by 33.80% compared to the LPS control – representing the best improvement in nitrite inhibition as a parameter of anti-inflammatory activity of the oil combination compared to the oils tested independently. This may be attributed to a combinatory anti-inflammatory effect conferred by *R. rubiginosa* fixed oil and *S. benzoin* essential oil. Furthermore, *R. rubiginosa* fixed oil in combination with *S. benzoin* essential oil produced the best improvement in cell viability, increasing RAW 264.7 macrophage proliferation by 2.56% compared to the positive control (Figure 4.2). This combination greatly reduced nitrite production, which may have provided RAW 264.7 macrophages an opportunity to proliferate.

The three essential oils (*K. ericoides*, *R. damascena*, and *M. quinquenervia*) inhibiting nitrite production the most, decreased nitrite production by 19.97%, 16.03%, and 18.77% respectively, compared to the LPS control. Research by Chen *et al.* (2016) revealed that *K. ericoides* essential oil inhibited LPS induced inflammation in THP-1 human monocytes and was non-cytotoxic. As such, *K. ericoides* essential oil may be effective in topical epidermal products due to its anti-inflammatory activity and absence of adverse allergic reaction resulting from cytokine release (Chen *et al.*, 2016). Animal studies have reported potent anti-inflammatory activity of *R. damascena* (Latifi *et al.*, 2015, Fatemi *et al.*, 2020) and *M. quinquenervia* (Acha *et al.*, 2019, Cilingir-Kaya and Gurler, 2021) essential oils, yet cell studies using the LPS induced inflammatory pathway in RAW 264.7 macrophages are limited.

Rosa rubiginosa fixed oil combined in 1:1 ratio with *K. ericoides*, and *R. damascena* essential oils slightly increased the ability of the oil combinations to supress LPS induced nitrite production compared to the independent essential oils. The combination of *R. rubiginosa* fixed oil and *K. ericoides* essential oil improved cell viability by 19.45%, while that of the *R. rubiginosa* fixed oil and *R. damascena* essential oil combination slightly decreased by 4.39%, compared to the essential oils tested independently. *Rosa rubiginosa* fixed oil combined in a 1:1 ratio with *M. quinquenervia* essential oil slightly decreased the ability of the oil combination to supress LPS induced nitrite production compared to the independent essential oils, which may be a result of a dilution effect.

Eugenia caryophyllata essential oil slightly reduced LPS induced nitrite production by 3.80% compared to the LPS control. In contrast, a study using *E. caryophyllata* essential oil by Lang *et al.* (2019) described a significant increase in nitrite production, both in the absence or presence of LPS induction in RAW 264.7 macrophages. Lang *et al.* (2019) suggested that incubation time may influence iNOS regulation, however, both the current study and the study by Lang *et al.* (2019) pre-treated RAW 264.7 macrophages for 24 hrs. Anti-inflammatory activity of *E. caryophyllata* essential oil has been observed in human dermal fibroblasts (HDF3CGF) (Han and Parker, 2017b), and in several animal models (Öztürk and Özbek, 2005, Humbal *et al.*, 2019). *Rosa rubiginosa* fixed oil combined in a 1:1 ratio with *E. caryophyllata* essential oil slightly decreased the ability of the oil combination to supress LPS induced nitrite production compared to the independent essential oils, which may be a result of a dilution effect.

Decreased nitrite production of the studied test samples, however, did not necessarily indicate an increased propensity of the sample to reduce inflammation. In some instances, decreased or lack of nitrite production was attributed to the high toxicity of the test sample resulting in declined cell viability. As such, sufficient nitrite production was not induced in low cell numbers. Therefore, the anti-inflammatory response (Figure 4.1) should be considered together with the relative cell viability (Figure 4.2). This was specifically noted with the anti-inflammatory response and cell viability of the independent essential oils: *M. officinalis, C. zeylanicum*, and *S. austrocaledonicum.* These three essential oils lacked nitrite production as the cell viability of the respective samples were very low at 26.59%, 7.62%, and 6.12%, respectively.

Several animal studies have reported promising anti-inflammatory activity of *M. officinalis* essential oil (Bounihi *et al.*, 2013), yet cell studies using the LPS induced inflammatory pathway in RAW 264.7 macrophages are limited. Cell studies concerning *C. zeylanicum*, using the LPS induced inflammatory pathway in RAW 264.7 macrophages, are limited to bark extracts. Goyal *et al.* (2018) found that *C. zeylanicum* bark extracts inhibited the growth of RAW 264.7 macrophages and suggested that this may be due to the presence of different phytochemicals. Another study by Gunawardena *et al.* (2015) found that *C. zeylanicum* bark extracts demonstrated considerable anti-inflammatory activity by suppressing NO production in RAW 264.7 macrophages, however, the author suggested that non-cytotoxic therapeutic concentrations be achieved by using advanced delivery methods (for example:

microencapsulation) so that *C. zeylanicum* may be considered for use as an anti-inflammatory agent. *Santalum austrocaledonicum* essential oil is conventionally used to treat inflammation (Kumar *et al.*, 2015, Page *et al.*, 2018). The current study supports the anti-inflammatory activity of *S. austrocaledonicum* essential oil where previous studies were lacking.

The combinations of *R. rubiginosa* fixed oil with *M. officinalis*, or *C. zeylanicum* essential oils in a 1:1 ratio, improved cell viability by 34.09% and 45.75% respectively, compared to the independent essential oils. As a result of improved cell viability, an LPS induced inflammatory response became apparent, however, nitrite production of these oil combinations surged. This may indicate that the essential oils were still highly cytotoxic and induced inflammation in RAW 264.7 macrophages, as evidenced by the combination of *R. rubiginosa* fixed oil with the essential oil, *C. zeylanicum*. *Rosa rubiginosa* fixed oil combined with *C. zeylanicum* essential oil increased nitrite production by 20.75% compared to the LPS control – hence eliciting an inflammatory response within RAW 264.7 macrophages. *Rosa rubiginosa* fixed oil combined with *M. officinalis* essential oil produced an inflammatory response similar to that of the LPS control. The combination of *R. rubiginosa* fixed oil and *S. austrocaledonicum* essential oil remained highly cytotoxic which resulted in poor cell viability (8.76%) and an inadequate cellular response of nitrite production.

4.3.2. Cytotoxicity screening

The cytotoxicity results in A549 epithelial cells are represented in Figure 4.3. The cell viability (as a percentage of the cell culture medium) was determined after 48 hrs exposure to *R. rubiginosa* fixed oil and the eight essential oils, independently and in 1:1 combination. Compared to the cell culture medium (100% cell viability), melphalan was cytotoxic by four-fold – reducing A549 epithelial cell viability to 23.72%. Compared to the non-toxic observation of *R. rubiginosa* fixed oil (percentage mortality = 0.74-2.00%, after 48 hrs) in the BSLA, *R. rubiginosa* fixed oil could be considered non-cytotoxic as cell viability (98.56%) was similar to that of the cell culture medium. The current study is one of the first to test *R. rubiginosa* fixed oil in A549 epithelial cells.

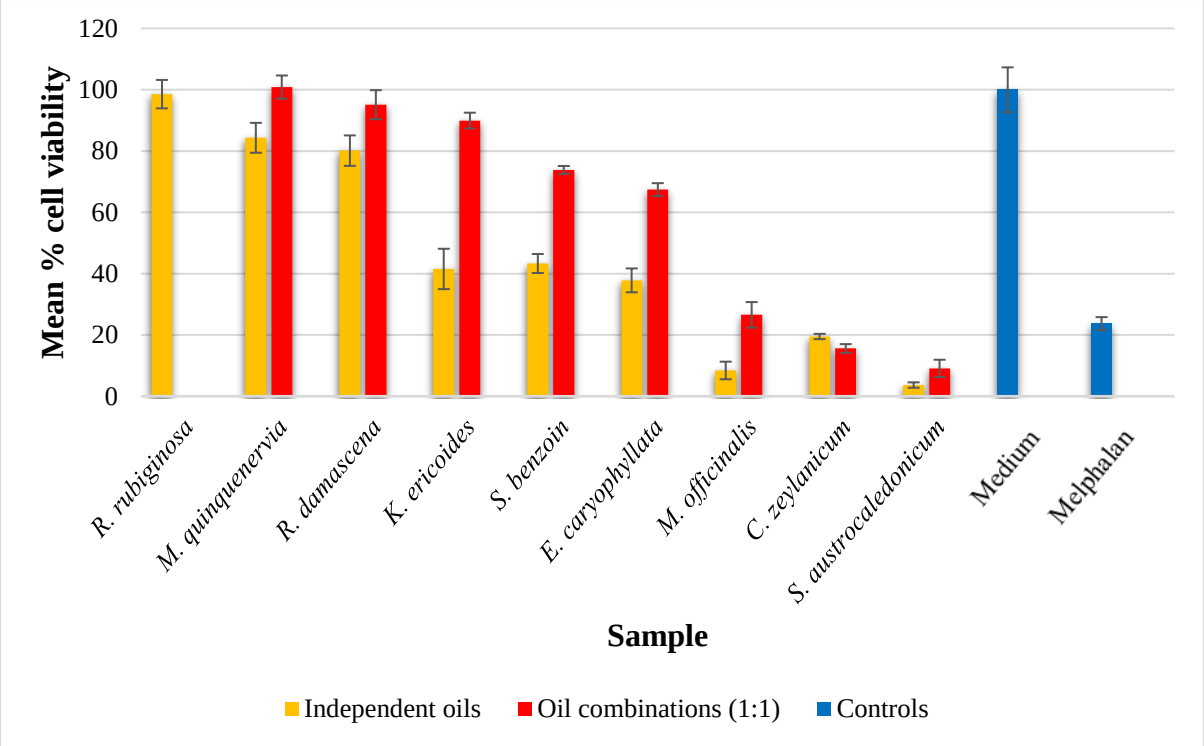

Figure 4.3. Mean % cell viability (n = 4) measuring cytotoxicity (as per the cytotoxicity protocol) relative to the medium control for test samples in A549 epithelial cells after 48 hrs.

The least cytotoxic essential oil was that of *M. quinquenervia* (84.32% cell viability) followed by *R. damascena* (80.13% cell viability). Previous cytotoxicity studies investigating *M. quinquenervia* essential oil are lacking. In contrast, *R. damascena* essential oil demonstrated strong cytotoxicity in A549 epithelial cells in a study by Zu *et al.* (2010), using the MTT assay. *Kunzea ericoides*, *S. benzoin*, and *E. caryophyllata* essential oils were moderately cytotoxic compared to the controls. Other cytotoxicity studies investigating *K. ericoides* and *S. benzoin* essential oils are deficient and require investigation. Research by Kouidhi *et al.* (2010) revealed that RAW 264.7 macrophages were more vulnerable to *E. caryophyllata* essential oil compared to A549 epithelial cells, using the MTT assay. This emphasises the need for cytotoxicity screening in various cell lines to identify different interactions between cell types. The most cytotoxic essential oils of those that were tested, displaying greater cytotoxicity than melphalan, were that of *M. officinalis* (8.42% cell viability), *C. zeylanicum* (19.49% cell viability), and *S. austrocaledonicum* (3.66% cell viability). A study by de Sousa *et al.* (2004) confirmed potent cytotoxic and anti-proliferative effects of *M. officinalis* essential oil in various cell lines, including A549 epithelial cells. Zu *et al.* (2010) confirmed potent cytotoxicity *of C. zeylanicum* essential oil in A549 epithelial cells. *Santalum austrocaledonicum* essential oil was revealed to be cytotoxic in multiple cell lines, including MCF-7 and MCF-10A epithelial cells

(Ortiz *et al.*, 2016). The current study supports the cytotoxicity of *S. austrocaledonicum* essential oil in A549 epithelial cells where previous studies were lacking. Despite demonstrating toxicity in the BSLA and cytotoxicity in various cell lines, *S. austrocaledonicum* essential oil is conventionally used to treat inflammation (Kumar *et al.*, 2015, Page *et al.*, 2018).

The 1:1 combinations of *R. rubiginosa* fixed oil with *M. quinquenervia*, and *R. damascena* essential oils, decreased cytotoxicity further resulting in a profile similar to that of the cell culture medium. In this case, *R. rubiginosa* fixed oil combined with *M. quinquenervia* essential oil was non-cytotoxic and could support A549 epithelial cell growth. *Rosa rubiginosa* fixed oil combined with *M. quinquenervia* essential oil also exhibited the second best toxicity reduction observed in the BSLA (Chapter 3). Compared to the essential oils independently, the 1:1 combinations of *R. rubiginosa* fixed oil with *K. ericoides*, *S. benzoin*, and *E. caryophyllata* essential oils reduced cytotoxicity by 48.41%, 30.52%, and 29.57%, respectively. In comparison, *R. rubiginosa* fixed oil combined with *K. ericoides* essential oil displayed the greatest toxicity reduction in the BSLA. Additionally, *R. rubiginosa* fixed oil combined with either *S. benzoin* or *M. officinalis* essential oil significantly lowered toxicity in the BSLA. Despite the 1:1 combinations of *R. rubiginosa* fixed oil with *M. officinalis*, and *S. austrocaledonicum* essential oils lowering cytotoxicity, the oil combinations maintained a cytotoxic profile similar to melphalan.

Except for *R. rubiginosa* fixed oil combined with *C. zeylanicum* essential oil which slightly increased cytotoxicity, this study showed that *R. rubiginosa* fixed oil combined with essential oils could substantially reduce the cytotoxic nature of most oil combinations, and in some instances confer the combinations with a cell proliferative effect (increased number of cells because of cell growth and cell division). This effect may be mediated by the ability of *R. rubiginosa* fixed oil, and its high content of polyunsaturated fatty acids, to inhibit essential oil induced inflammation and cell death (Chrubasik *et al.*, 2008, Lin *et al.*, 2018). The reduced concentration of essential oil in the 1:1 oil combination compared to the essential oil independently may have contributed to improved cell viability in addition to the effect of *R. rubiginosa* fixed oil. A similar observation was noted in the BSLA where reduced toxicity was attributed to the dilution of the essential oil in combination with *R. rubiginosa* fixed oil. *Rosa rubiginosa* fixed oil was combined with the essential oils in a 1:1 ratio, so diluting the essential oils in a higher concentration of *R. rubiginosa* fixed oil may possibly lower the cytotoxicity of the essential oils further (Orchard *et al.*, 2019).

4.4. Overview

- *Rosa rubiginosa* fixed oil reduced LPS induced nitrite production (as a parameter of anti-inflammatory activity) by 18.75% compared to the LPS control.
- *Rosa rubiginosa* fixed oil could be considered non-cytotoxic as A549 epithelial cell viability (98.56%) was similar to that of the cell culture medium.
- Of the independent essential oils, *K. ericoides* essential oil decreased nitrite production the most by 19.97% compared to the LPS control.
- Of the independent essential oils, the least cytotoxic essential oil was that of *M. quinquenervia* (84.32% cell viability).
- The combination of *R. rubiginosa* fixed oil and *S. benzoin* essential oil reduced nitrite production the most by 33.80% compared to the LPS control.
- The combination of *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil showed the best non-cytotoxicity (cell viability of 1.01-fold compared to the cell culture medium) and the potential to support A549 epithelial cell growth.
- When combined with *R. rubiginosa* fixed oil, *M. officinalis*, *C. zeylanicum*, and *S. austrocaledonicum* essential oils remained highly cytotoxic akin to melphalan in A549 epithelial cells.
- *Rosa rubiginosa* fixed oil combined with essential oils could substantially reduce the cytotoxic nature of most oil combinations.
- *Rosa rubiginosa* fixed oil and the eight essential oils (*C. zeylanicum*, *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, *S. benzoin*, and *S. austrocaledonicum*) were further investigated for skin absorption and retention properties (Chapter 5).

CHAPTER 5

Skin absorption and retention

5.1. Introduction

Static diffusion cells, especially Franz diffusion cell systems, have evolved into one of the most important research methodologies for the assessment of *in vitro* skin permeation pertaining to conventional drugs and formulations (Ng *et al.*, 2010). However, owing to the volatile nature of essential oils (Orchard and van Vuuren, 2017), essential oil test samples may evaporate from the skin before detection and analysis. Thus, an experiment was adapted to study *in vitro* skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil and essential oils in the current study.

Fourier Transform Infrared spectroscopy (FTIR) provides a characteristic signature of the chemical or biochemical substances present in a sample by featuring their molecular vibrations (stretching, bending, and torsions of the chemical bonds) (Lupoi *et al.*, 2015). Therefore, the FTIR spectrum represents a molecular fingerprint of the sample (Lucarini *et al.*, 2020). Multiple studies using FTIR to quantitatively analyse the main functional groups and fingerprints of various fixed oils including *Olea europaea* (olive), *Azadirachta indica* (neem), *Linum usitatissimum* (flaxseed), *Sesamum indicum* (sesame), *Vitis vinifera* (grape seed), and *Ziziphus spina Christi* (Christ's Thorn Jujube) fixed oils have demonstrated the successful use of FTIR combined with chemometric analysis to be a simple, rapid, economic, and accurate tool for characterising fixed oils (Allam and Hamed, 2007, Elzey *et al.*, 2016, Ozulku *et al.*, 2017, Lucarini *et al.*, 2020, Abubaker *et al.*, 2021). Similarly, various essential oils (including several *Cinnamomum* species, *Salvia officinalis*, *S. album*, *R. damascena*, and *Mentha spicata* essential oils) and chemical constituents thereof (such as cinnamaldehyde, eugenol, and menthol) have been successfully characterised using FTIR (Li *et al.*, 2013, Ciko *et al.*, 2016, Bisht *et al.*, 2021, Cebi *et al.*, 2021a, Cebi *et al.*, 2021b, Taylan *et al.*, 2021).

This chapter focuses on the determination of skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil independently and in 1:1 combination with essential oils, within porcine skin, via detection using qualitative FTIR analysis as a possible preliminary screening tool for *in vitro* and/ or *ex vivo* skin permeation studies.

5.2. Materials and methods

5.2.1. Oil selection and sample preparation

Rosa rubiginosa fixed oil and essential oils displaying antimicrobial synergy (*R. rubiginosa* fixed oil combined with *C. zeylanicum* essential oil), or an overall antimicrobial additive interaction (*R. rubiginosa* fixed oil combined with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, or *S. benzoin* essential oil), or antimicrobial antagonism (*R. rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil) as identified in Chapter 2, were selected for this study to determine the skin absorption and retention properties of the oils.

Known concentrations for *R. rubiginosa* fixed oil and the eight essential oils independently (100% of *R. rubiginosa* fixed oil or essential oil) and in 1:1 combination (50% of *R. rubiginosa* fixed oil and 50% of essential oil) were prepared by first measuring, and then solubilising the oils within absolute ethanol (MK Chemicals) to obtain a stock concentration of 20.00% v/v. The stock concentration was then further diluted to 5.00% and 1.25% v/v (most dilute concentration for all oil test samples to be clearly detected by FTIR) using absolute ethanol. The prepared oil sample sets were stored in tightly sealed amber bottles at approximately 4°C until further analysis (van Vuuren *et al.*, 2019). The same method was used to prepare sets of samples of known concentration for cinnamaldehyde and eugenol (Sigma-Aldrich®). The essential oil constituents, cinnamaldehyde and eugenol, have previously been shown to be absorbed by the skin in numerous studies (Hotchkiss, 1998, Bickers *et al.*, 2005, Schmitt *et al.*, 2010a), thus were included as positive controls. Table 5.1 lists the negative and positive controls, and standard solutions used in the current study and the purpose of each for the skin absorption and retention experiment.

Table 5.1. Controls used in the skin absorption and retention experiment.

Sample	Type of control	Purpose
Untreated skin samples	Negative control	Blank reference.
Cinnamaldehyde (≥ 95.00% purity, undiluted)	Positive control	To ensure the integrity of skin samples to absorb and retain oil.
Eugenol (99.00% purity, undiluted)	Positive control	
Rosa rubiginosa fixed oil and the eight essential oils independently and in 1:1 combination (undiluted; 5.00% and 1.25% v/v in absolute ethanol)	Standard solution	Comparator infrared profiles depicting the vibration transitions (reference samples) to confirm skin absorption and retention properties of the test samples.
Cinnamaldehyde and eugenol independently (undiluted; 5.00% and 1.25% v/v in absolute ethanol)	Standard solution	

5.2.2. Porcine ear skin preparation

Skin from animals is commonly considered as an alternative to human skin, as animal skin may be easily obtained, freshly excised prior to skin permeation studies, and presents less variability (Todo, 2017, Kerimoğlu and Şahbaz, 2018). A review by Neupane *et al.* (2020) proposed that porcine skin is generally preferred due to its structural similarity to human skin concerning the thickness of the *stratum corneum* and viable epidermis, stratified, keratinising epithelium, and hair growth density. Therefore, freshly excised porcine skin is routinely used to assess skin permeation (Neupane *et al.*, 2020).

Porcine ears (harvested from 22-week-old male pigs with an average weight of 81.04 kg), intact with full skin and hair, were obtained from the local abattoir, Lynca Meats™ (Gauteng, South Africa). Full thickness porcine skin was freshly excised from the outer part of the pig's ear, and the hair and subcutaneous fat was removed aseptically using surgical scissors and a scalpel (Lucca *et al.*, 2015, Kreutz *et al.*, 2018). The skin was cut into 2 cm × 2 cm (4 cm^2) sized squares (to fit small 25 mL glass ointment jars) and were randomised. Porcine ear skin samples were wrapped in aluminium foil to maintain the tissue integrity and stored in a freezer at -20°C until further use (not exceeding 30 days) (Kreutz *et al.*, 2018). An ethics waiver certificate for the use of porcine skin was obtained from the University of the Witwatersrand Animal Research Ethics Committee, reference number: Waiver 09-04-2020-O (Appendix D).

5.2.3. Skin absorption and retention experiments and FTIR analysis

The skin absorption and retention experiments were adapted from the skin penetration experiments by Schwarz *et al.* (2013). Prior to the experiment, porcine ear skin samples were washed and conditioned in phosphate-buffered saline (PBS) solution, pH 7.4, at ambient temperature (Lai *et al.*, 2007). To remove excess moisture, the skin samples were blotted dry, and were then placed in small glass ointment jars. The inner surface (dermis) of the skin sample was in contact with the base of the jar and the outer skin surface (epidermis) was exposed for the application of the oil test samples. Oil test samples (100 µL of undiluted *R. rubiginosa* fixed oil or undiluted essential oil, or 50 µL of undiluted *R. rubiginosa* fixed oil and 50 µL of undiluted essential oil for combinations) were applied to the epidermis and tested in triplicate and repeated where discrepancies were found ($n \geq 3$). The incised edges of the skin sample were carefully avoided when applying the oil test samples to ensure that the absorption and retention of the oil test samples occurred via the epidermis only, and not via the edges or dermis of the skin sample. Untreated skin samples were included as a blank reference (negative control), and cinnamaldehyde and eugenol (100 µL, undiluted) were included as positive controls. The glass ointment jars were sealed to limit evaporation of the essential oils from the skin and to prevent the skin samples from drying out, and incubated (Memmert Incubator IN260) at 37°C for 24 hrs.

After 24 hrs of incubation, the skin samples were removed from the glass ointment jars, and the skin surface was blotted dry using tissue paper to remove remaining oil that was not absorbed and retained within the skin. The skin samples were then placed in 400 µL of absolute methanol (minimal amount of methanol that was required to fully submerge the skin samples) which was added as a solvent to extract the oil test samples that were absorbed and retained within the skin. Methanol (ACE Chemicals) was used as the solvent as a study by Truong *et al.* (2019) recommended that methanol was the optimal solvent (among the solvents tested) for the extraction of bioactive compounds. Methanol produced the highest extraction yield and highest content of phytochemical constituents (phenolics, alkaloids, flavonoids, and terpenoids), suggesting that highly polar solvents support extraction efficiency (Truong *et al.*, 2019). The skin samples were macerated and then left undisturbed for extraction at ambient temperature for a further 24 hrs.

After 24 hrs of extraction in methanol, the solvent was removed and placed in Eppendorf tubes for centrifugation at 3500 rpm for 10 min (Jarzabek, 2013) using a mini centrifuge LLG-uniCFUGE 5 (LLG-Labware) to separate skin particles from the solvent. The supernatant was collected, vortexed and then scanned within the 4000-650 cm^{-1} wavelength range using a Spectrum™ 100 FTIR Spectrometer (PerkinElmer®). The peak positions and intensities (percentage transmittance) were determined using the PerkinElmer® Spectrum (Version 10.4.1) software. Fourier Transform Infrared spectra averaging 20 scans were obtained for each oil test sample, the controls, and for the respective sets of samples of known concentration. The replicates ($n \geq 3$) for each oil test sample and the controls were scanned, and a mean spectrum was calculated using Microsoft Excel Office 365 (Version 2202) software and compared to the spectra of the respective sets of known concentration. The background spectrum of air was subtracted from the spectra of the tested samples.

5.3. Results and discussion

Qualitative FTIR analysis was used to detect the skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil independently and in 1:1 combination with each of the eight essential oils, within the porcine skin. The chemical profiles of the eight essential oils have been formerly investigated, using gas chromatography coupled to mass spectrometry (GC-MS), and reported (van Vuuren *et al.*, 2014, Orchard *et al.*, 2017, 2018a, 2019). Appendix F provides a list including the eight essential oils and a summary of the chemical profiles previously reported. Because the oil test samples were composed of mixtures of numerous chemical constituents and the concentrations of each chemical constituent for each oil varied, chemical analysis of these constituents was not quantified in the current study. Instead, qualitative infrared profiles depicting the vibration transitions of *R. rubiginosa* fixed oil and the eight essential oils (independently and in 1:1 combination), were obtained using FTIR (Figures 5.1-5.11). Differences were noted in the wavenumber positions and peak intensities (percentage transmittance) of the oils at various concentrations.

5.3.1. *Rosa rubiginosa* fixed oil independently

Figure 5.1 displays the mean FTIR spectrum (n ≥ 3) of *R. rubiginosa* fixed oil extracted from the skin, compared to the infrared profile depicting the vibration transitions of *R. rubiginosa* fixed oil at three known concentrations (100%, 5%, and 1.25% v/v). The infrared profile depicting the vibration transitions of the set of known concentrations was then used to identify whether the extraction solvent from the skin contained *R. rubiginosa* fixed oil. The same process was applied for all the oil samples tested in Figures 5.3-5.11.

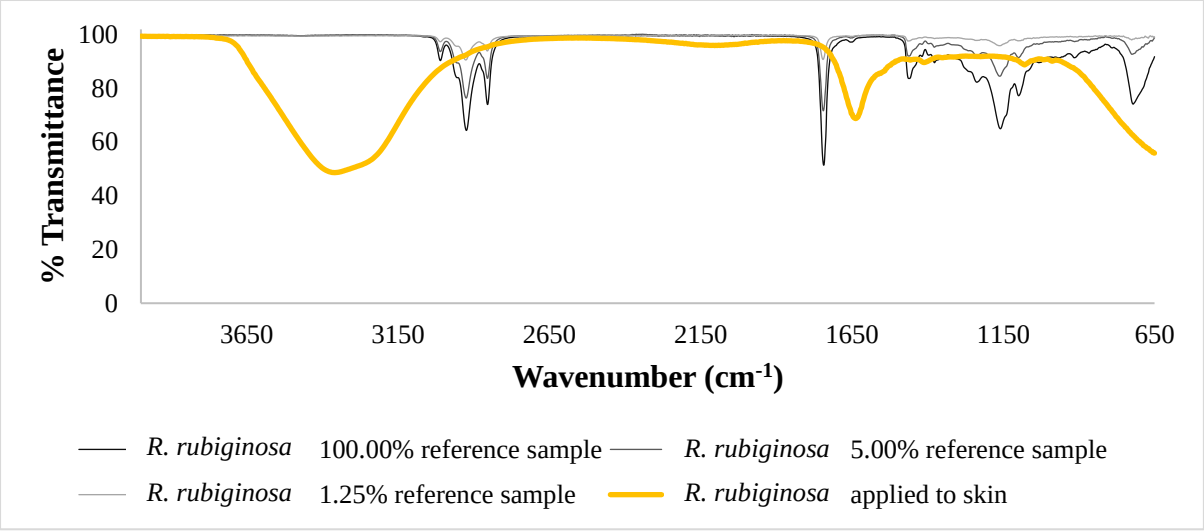

Figure 5.1. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of *R. rubiginosa* fixed oil extracted from the skin, compared to the spectra of the respective set of known concentrations (v/v).

The infrared profile depicting the vibration transitions of *R. rubiginosa* fixed oil was not detected in the extraction solvent from the skin samples (Figure 5.1), thereby indicating that *R. rubiginosa* fixed oil was not absorbed and retained within the skin. In comparison, an *in vivo* study by Patzelt *et al.* (2012) demonstrated that various fixed oils mostly remain at the surface of the skin, without deep penetration into the *stratum corneum*, when topically applied. Moreover, Bos and Meinardi (2000) argued that the molecular weight of a compound must be < 500 Dalton to permeate the *stratum corneum* and allow skin absorption ("500 Dalton Rule"). As such, the skin permeation of high molecular weight molecules remains a challenge (Goyal *et al.*, 2016, Petrilli and Lopez, 2018). *Rosa rubiginosa* fixed oil contains high concentrations of multiple unsaturated fatty acids (linoleic (18:2 ω-6) (41.93% ±1.20), linolenic (18:3 ω-3) (35.73% ±2.50), and oleic (18:1 ω-9) (15.45% ±0.46) acids) which may contribute to an overall

high molecular weight (> 500 Dalton) that may hinder skin absorption. The topical application of *R. rubiginosa* fixed oil may rather offer an occlusive effect forming a protective skin barrier to retain moisture and lower transepidermal water loss (TEWL), as do various other fixed oils (Patzelt *et al.*, 2012, Lin *et al.*, 2018).

Figure 5.2 displays the mean FTIR spectrum (n ≥ 3) of the untreated skin samples in the solvent (methanol). This infrared profile depicting the vibration transitions served as a reference since a similar spectrum was observed for the oil test samples which were not detected in the extraction solvent from the skin (as was the case with *R. rubiginosa* fixed oil). According to Cal (2006), the skin absorption of an essential oil diluted in a substance with lower permeability, will be reduced, indicating that essential oils diluted in a fixed oil (such as *R. rubiginosa* fixed oil) are absorbed slower than undiluted essential oils (Cal, 2006). However, the occlusion of fixed oils may prevent essential oil evaporation, thereby possibly enhancing the skin permeation of essential oils by increasing contact time with the skin (Tisserand and Young, 2013).

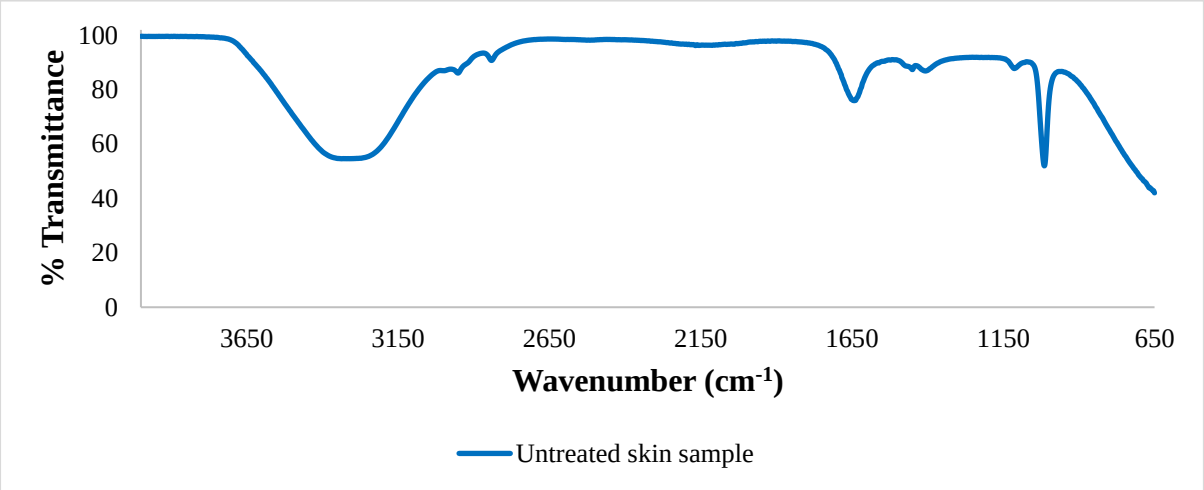

Figure 5.2. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of the untreated skin samples in methanol (negative control).

The infrared profiles depicting the vibration transitions of cinnamaldehyde (Figure 5.3a) and eugenol (Figure 5.3b) were detected in the extraction solvent from the skin samples, thereby indicating that cinnamaldehyde and eugenol were absorbed and retained within the skin. Several studies have described potent skin absorption of cinnamaldehyde (Hotchkiss, 1998, Bickers *et al.*, 2005), and eugenol (Hotchkiss, 1998, Schmitt *et al.*, 2010a, Kamatou *et al.*,

2012). Cinnamaldehyde, the major chemical constituent of *C. zeylanicum* essential oil, has a low molecular weight of approximately 132.16 Dalton (National Center for Biotechnology Information, 2022b). Eugenol, the major chemical constituent of *E. caryophyllata* essential oil, also has a low molecular weight of approximately 164.20 Dalton (National Center for Biotechnology Information, 2022a). The low molecular weights (< 500 Dalton) of cinnamaldehyde and eugenol may contribute to *stratum corneum* permeation and skin absorption, as per the "500 Dalton Rule" (Bos and Meinardi, 2000).

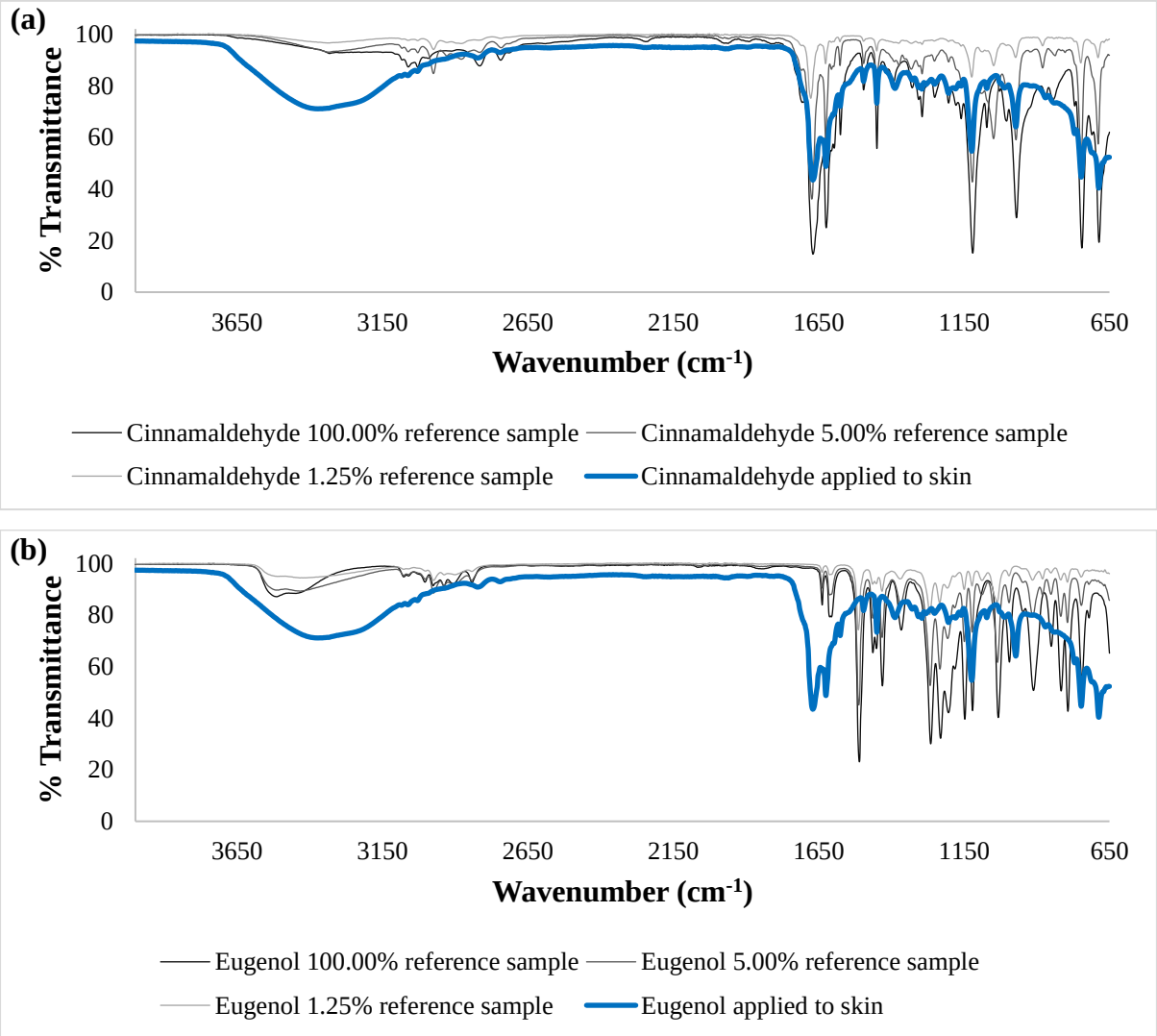

Figure 5.3. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** cinnamaldehyde and **(b)** eugenol extracted from the skin (positive controls), compared to the spectra of the respective sets of known concentration of the compounds (v/v).

5.3.2. Detected oils independently and in 1:1 combination

The infrared profile depicting the vibration transitions of *C. zeylanicum* essential oil was detected in the extraction solvent from the skin samples, when tested independently (Figure 5.4a) and in combination with *R. rubiginosa* fixed oil (Figure 5.4b), thereby indicating that *C. zeylanicum* essential oil was absorbed and retained within the skin.

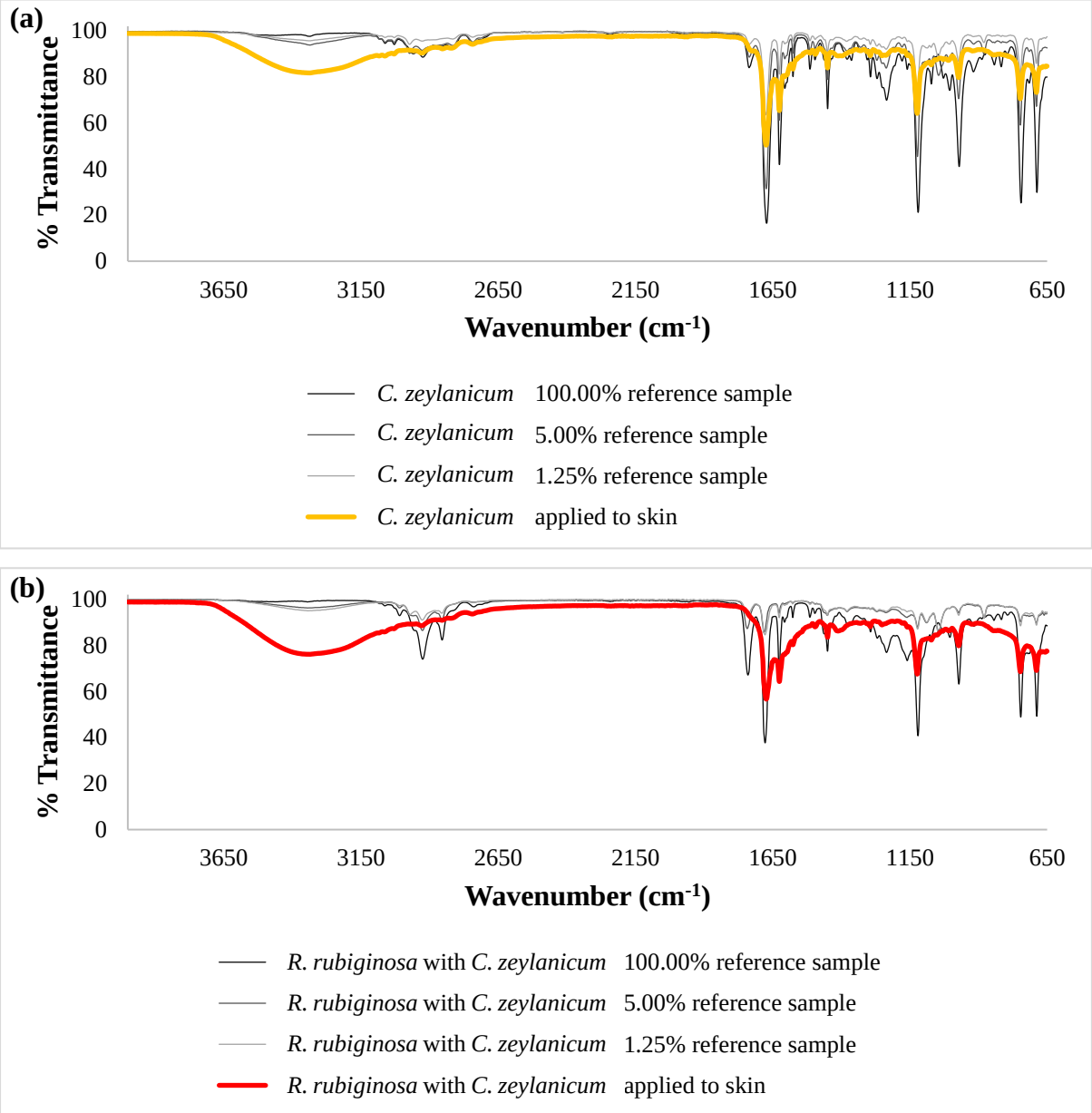

Figure 5.4. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *C. zeylanicum* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *C. zeylanicum* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

Multiple reviews have discussed *C. zeylanicum* essential oil as a potent skin permeation enhancer typically incorporated in cosmetic and pharmaceutical formulations (Caliskan and Karakus, 2020, Amra *et al.*, 2021, Kashyap *et al.*, 2021). However, research studying the skin absorption of *C. zeylanicum* essential oil as an active ingredient is lacking. The essential oil of *C. zeylanicum* was visually observed to be less viscous than *R. rubiginosa* fixed oil. Moreover, *C. zeylanicum* essential oil contains a mixture of low molecular weight (< 500 Dalton) chemical constituents (cinnamaldehyde (61.50%), β-caryophyllene (6.80%), cinnamyl acetate (6.50%), eugenol (3.70%), and β-phellandrene (3.70%)) which may more readily absorb into and be retained within the skin. Since fixed oils are the commonly used base in aromatherapy, believed to reduce the evaporation and enhance the cutaneous absorption of essential oils (Orchard and van Vuuren, 2019), *R. rubiginosa* fixed oil may reduce the evaporation of *C. zeylanicum* essential oil when in combination. Thus, increasing skin retention of *C. zeylanicum* essential oil to potentially treat the pathogenesis of acne vulgaris when topically applied.

The infrared profile depicting the vibration transitions of *E. caryophyllata* essential oil was detected in the extraction solvent from the skin samples, when tested independently (Figure 5.5a) and in combination with *R. rubiginosa* fixed oil (Figure 5.5b), thereby indicating that *E. caryophyllata* essential oil was absorbed and retained within the skin. Multiple reviews have discussed research studying *E. caryophyllata* essential oil as a potent skin permeation enhancer, typically incorporated in cosmetic and pharmaceutical formulations (Jiang *et al.*, 2017, Caliskan and Karakus, 2020, Amra *et al.*, 2021). However, research studying the skin absorption of *E. caryophyllata* essential oil as an active ingredient is lacking. Similar to *C. zeylanicum* essential oil, the essential oil of *E. caryophyllata* was visually observed to be less viscous than *R. rubiginosa* fixed oil. Moreover, *E. caryophyllata* essential oil contains a mixture of low molecular weight (< 500 Dalton) chemical constituents (eugenol (70.70%), eugenyl acetate (19.20%), and caryophyllene (8.10%)) which may absorb into and be retained within the skin. *Rosa rubiginosa* fixed oil may also reduce the evaporation of *E. caryophyllata* essential oil when in combination. Thus, increasing skin retention of *E. caryophyllata* essential oil to potentially treat the pathogenesis of acne vulgaris when topically applied.

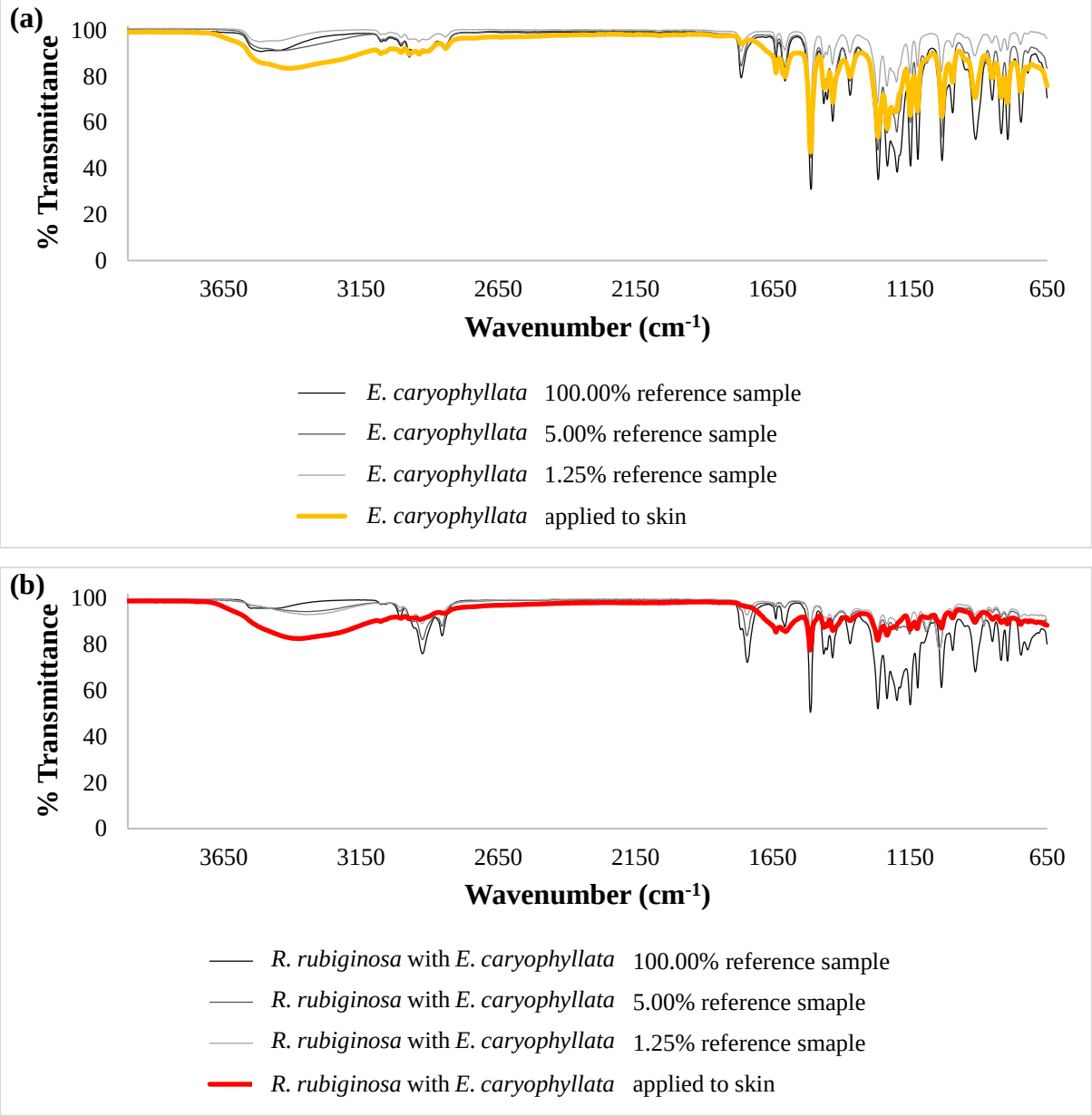

Figure 5.5. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *E. caryophyllata* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *E. caryophyllata* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

The infrared profile depicting the vibration transitions of *S. benzoin* essential oil was detected in the extraction solvent from the skin samples, when tested independently (Figure 5.6a) and in combination with *R. rubiginosa* fixed oil (Figure 5.6b), thereby indicating that *S. benzoin* essential oil was absorbed and retained within the skin.

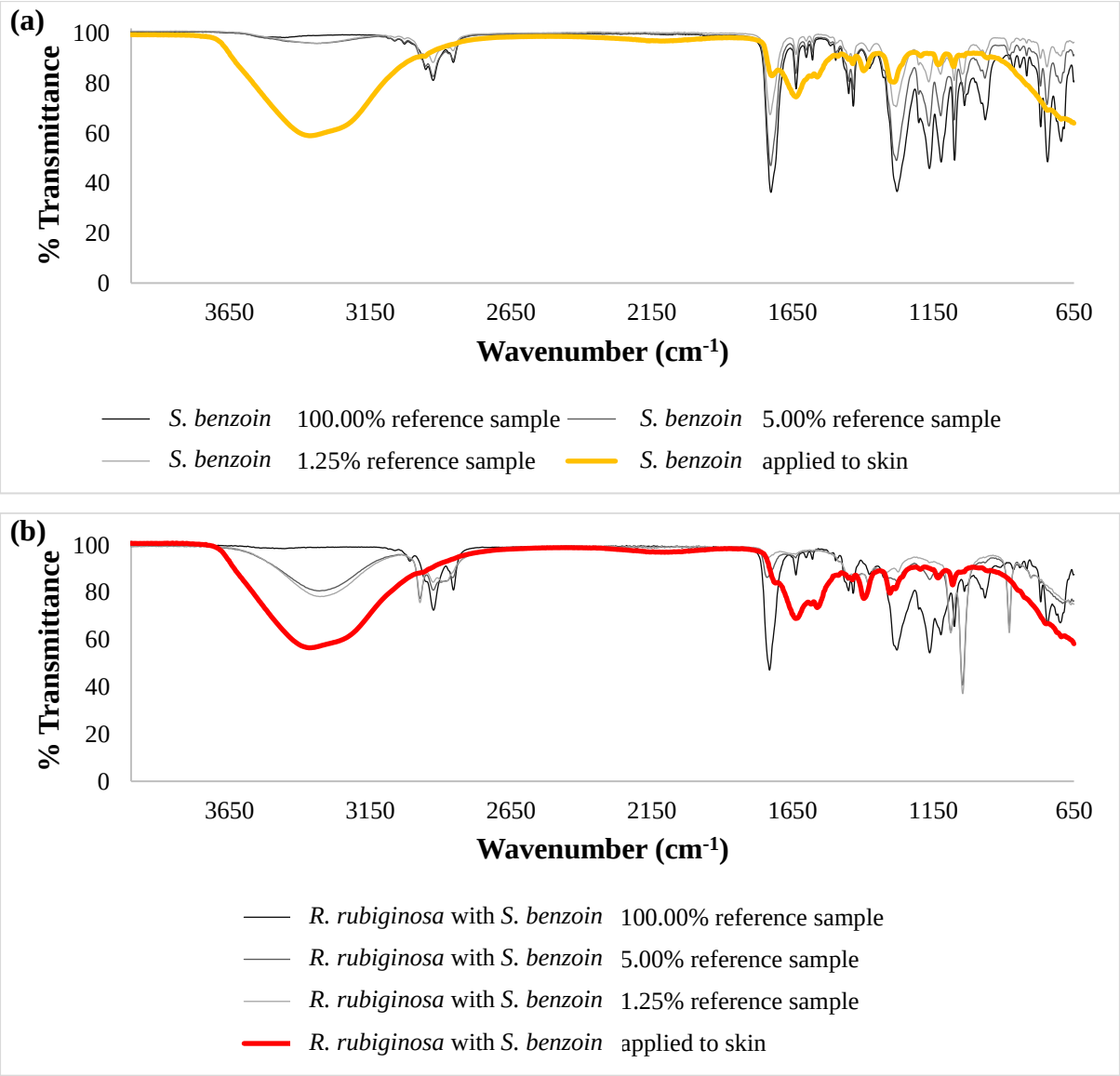

Figure 5.6. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *S. benzoin* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *S. benzoin* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

The skin applications of *S. benzoin* are mostly limited to the resin and gum (Manvi and Sharma, 2017) and although *in vitro* and *in vivo* skin absorption of various chemical constituents of *S. benzoin* have been previously reported (Sheppard-Hanger, 1996), research concerning the skin absorption and retention properties of *S. benzoin* essential oil is lacking. Similar to *C. zeylanicum* and *E. caryophyllata* essential oils, the essential oil of *S. benzoin* was visually observed to be less viscous than *R. rubiginosa* fixed oil. Moreover, *S. benzoin* essential oil contains a mixture of low molecular weight (< 500 Dalton) chemical constituents (dimethyl phthalate (72.20%), benzyl acetate (7.20%), ethyl cinnamate (5.70%)) which may absorb into and be retained within the skin. *Rosa rubiginosa* fixed oil may also reduce the evaporation of *S. benzoin* essential oil when in combination, thus increasing the skin retention of *S. benzoin* essential oil to potentially treat the pathogenesis of acne vulgaris when topically applied.

The infrared profile depicting the vibration transitions of *R. damascena* essential oil was minimally detected in the solvent from the skin samples (as indicated by the dotted lines in Figure 5.7), when tested independently (Figure 5.7a) and in combination with *R. rubiginosa* fixed oil (Figure 5.7b); thereby indicating that *R. damascena* essential oil was minimally absorbed and retained within the skin. A recent review by Amra *et al.* (2021) discussed the use of *R. damascena* essential oil as a skin permeation enhancer. However, Niazi *et al.* (2017) suggested that measuring the skin permeation and absorption of *R. damascena* essential oil requires further investigation. Research by Schmitt *et al.* (2010a) affirmed that co-operative interactions between the chemical constituents of *R. damascena* essential oil may enhance skin permeation, as the skin permeation of the chemical constituents was promoted when applied in *R. damascena* essential oil. Furthermore, in another study concerning *R. damascena* essential oil, Schmitt *et al.* (2010b) highlighted that since essential oils are volatile mixtures, skin retention may last only for several hours. The essential oil of *R. damascena* was visually observed to be the least viscous of the oil test samples. Moreover, *R. damascena* essential oil contains a mixture of low molecular weight (< 500 Dalton) chemical constituents (citronellol (29.90%), phenelyl alcohol (22.40%), geraniol (20.80%), and nerol (10.20%)) which may absorb into and be retained within the skin, albeit to a lesser extent than *C. zeylanicum* and *E. caryophyllata* essential oils tested in this study. This suggests that *R. damascena* essential oil may require further dilution in *R. rubiginosa* fixed oil to improve skin retention and effectively treat the pathogenesis of acne vulgaris when topically applied.

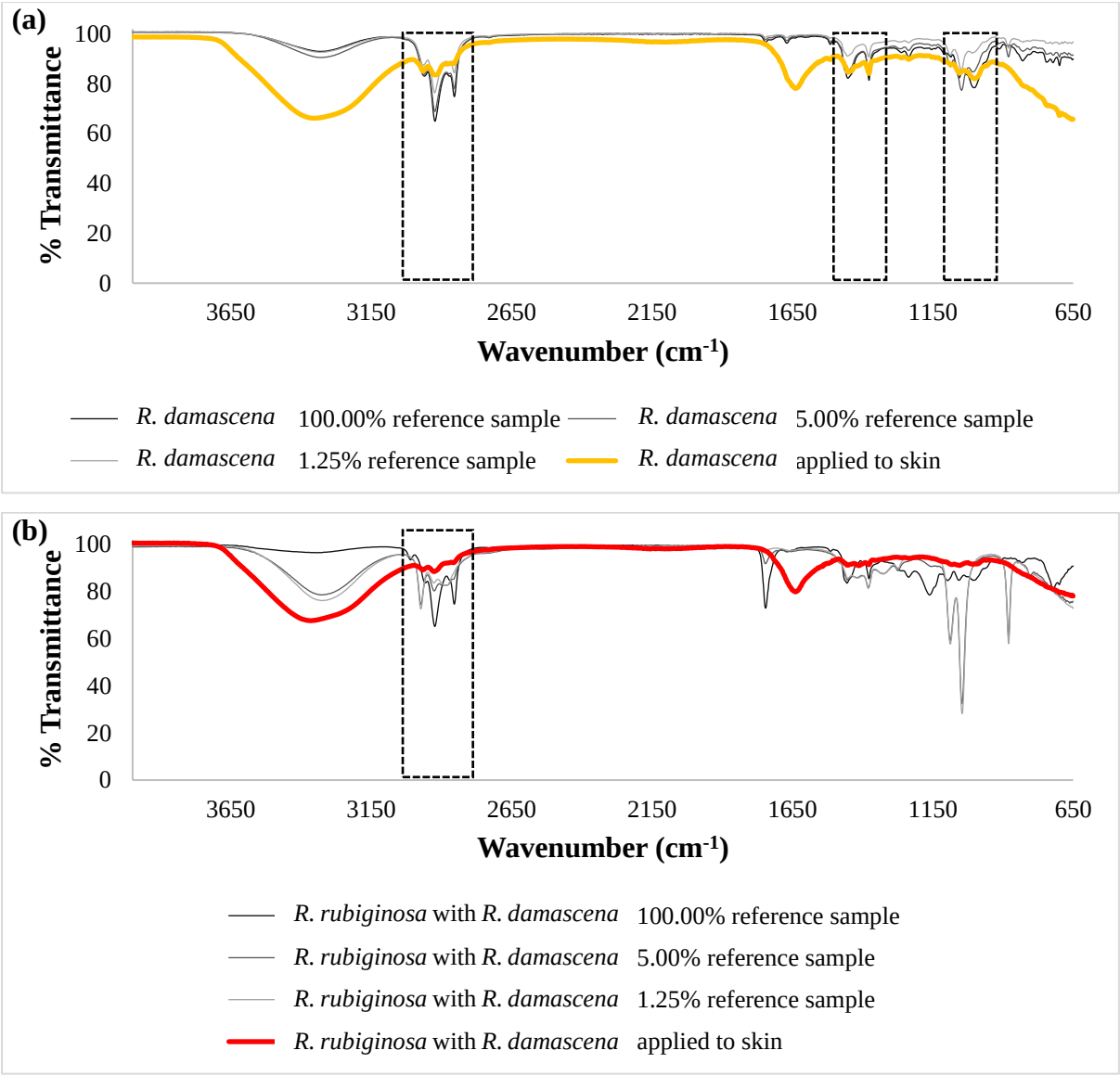

Figure 5.7. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *R. damascena* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *R. damascena* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

5.3.3. Undetected oils independently and in 1:1 combinations

The infrared profile depicting the vibration transitions of *K. ericoides* essential oil was not detected in the extraction solvent from the skin samples, when tested independently (Figure 5.8a) and in combination with *R. rubiginosa* fixed oil (Figure 5.8b), thereby indicating that *K. ericoides* essential oil was not absorbed and retained within the skin.

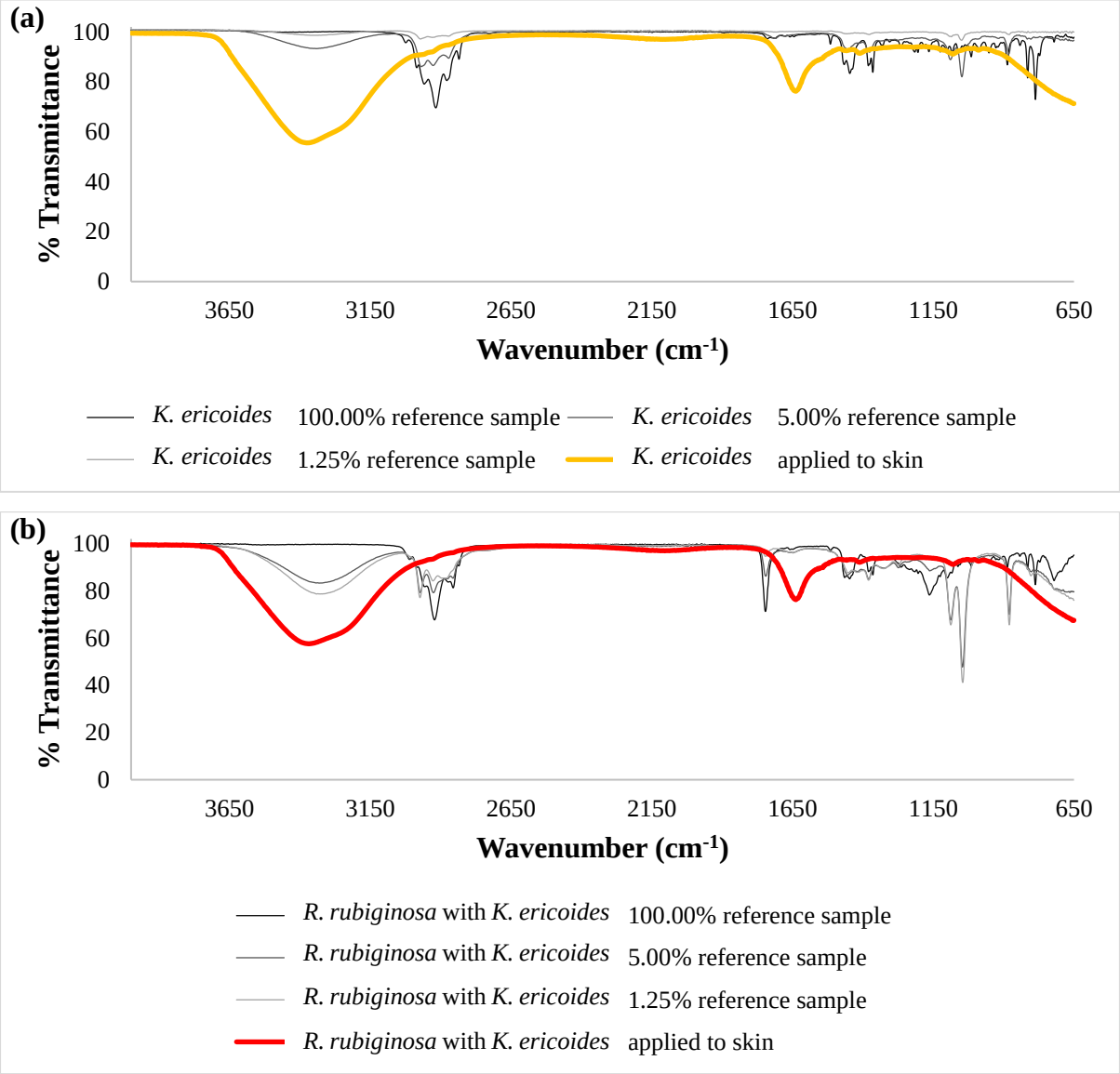

Figure 5.8. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *K. ericoides* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *K. ericoides* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

Reviews by Essien *et al.* (2019) and more recently Kashyap *et al.* (2021), examining *K. ericoides* essential oil, lacked discussion concerning skin absorption. The essential oil of *K. ericoides* was visually observed to be less viscous than *C. zeylanicum* and *E. caryophyllata* essential oils. Moreover, *K. ericoides* essential oil contains a mixture of low molecular weight (< 500 Dalton) chemical constituents (α-pinene (26.20-46.70%) and ρ-cymene (5.80-19.10%)) which may absorb into the skin, however, not be retained within the skin. This suggests that *K. ericoides* essential oil may require further dilution in *R. rubiginosa* fixed oil to improve skin retention and effectively treat the pathogenesis of acne vulgaris when topically applied.

The infrared profile depicting the vibration transitions of *M. quinquenervia* essential oil was not detected in the extraction solvent from the skin samples, when tested independently (Figure 5.9a) and in combination with *R. rubiginosa* fixed oil (Figure 5.9b), thereby indicating that *M. quinquenervia* essential oil was not absorbed and retained within the skin. Multiple reviews have discussed research studying *M. quinquenervia* essential oil as a potent skin permeation enhancer, typically incorporated in cosmetic and pharmaceutical formulations (Herman and Herman, 2015, Das and Ahmed, 2017, Jiang *et al.*, 2017). However, research studying the skin absorption of *M. quinquenervia* essential oil as an active ingredient is lacking. Similar to *K. ericoides* essential oil, the essential oil of *M. quinquenervia* was visually observed to be less viscous than *C. zeylanicum* and *E. caryophyllata* essential oils. Moreover, *M. quinquenervia* essential oil contains a mixture of low molecular weight (< 500 Dalton) chemical constituents (linalyl acetate (49.80%) and linalool (30.10%)) which may absorb into the skin, however, not be retained within the skin. The potent transdermal skin permeation enhancement properties (Herman and Herman, 2015, Das and Ahmed, 2017, Jiang *et al.*, 2017) may suggest that *M. quinquenervia* essential oil permeated through the skin, without being retained. This suggests that *M. quinquenervia* essential oil may require further dilution in *R. rubiginosa* fixed oil to improve skin retention and effectively treat the pathogenesis of acne vulgaris when topically applied.

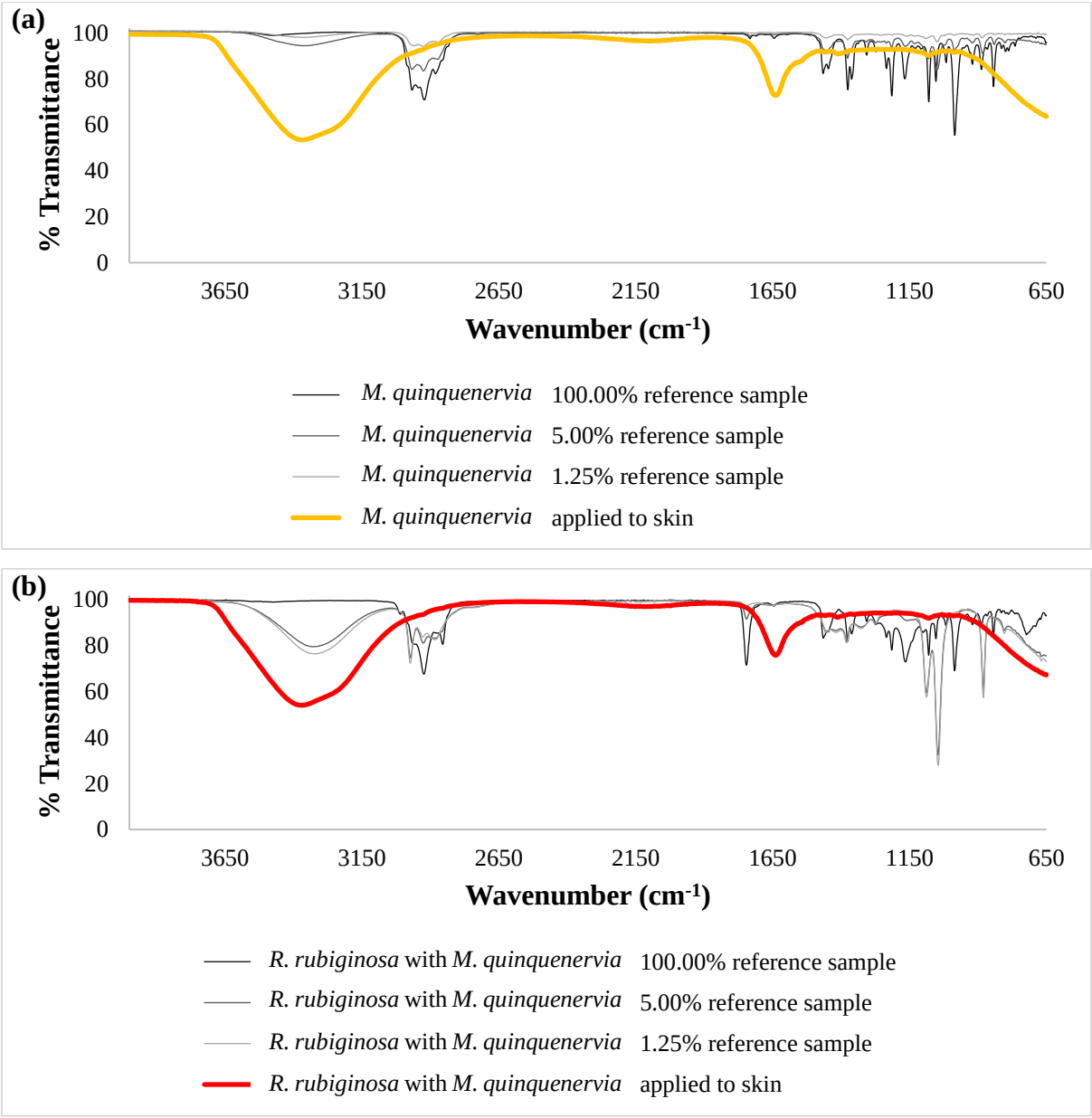

Figure 5.9. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *M. quinquenervia* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *M. quinquenervia* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

The infrared profile depicting the vibration transitions of *M. officinalis* essential oil was not detected in the extraction solvent from the skin samples, when tested independently (Figure 5.10a) and in combination with *R. rubiginosa* fixed oil (Figure 5.10b), thereby indicating that *M. officinalis* essential oil was not absorbed and retained within the skin.

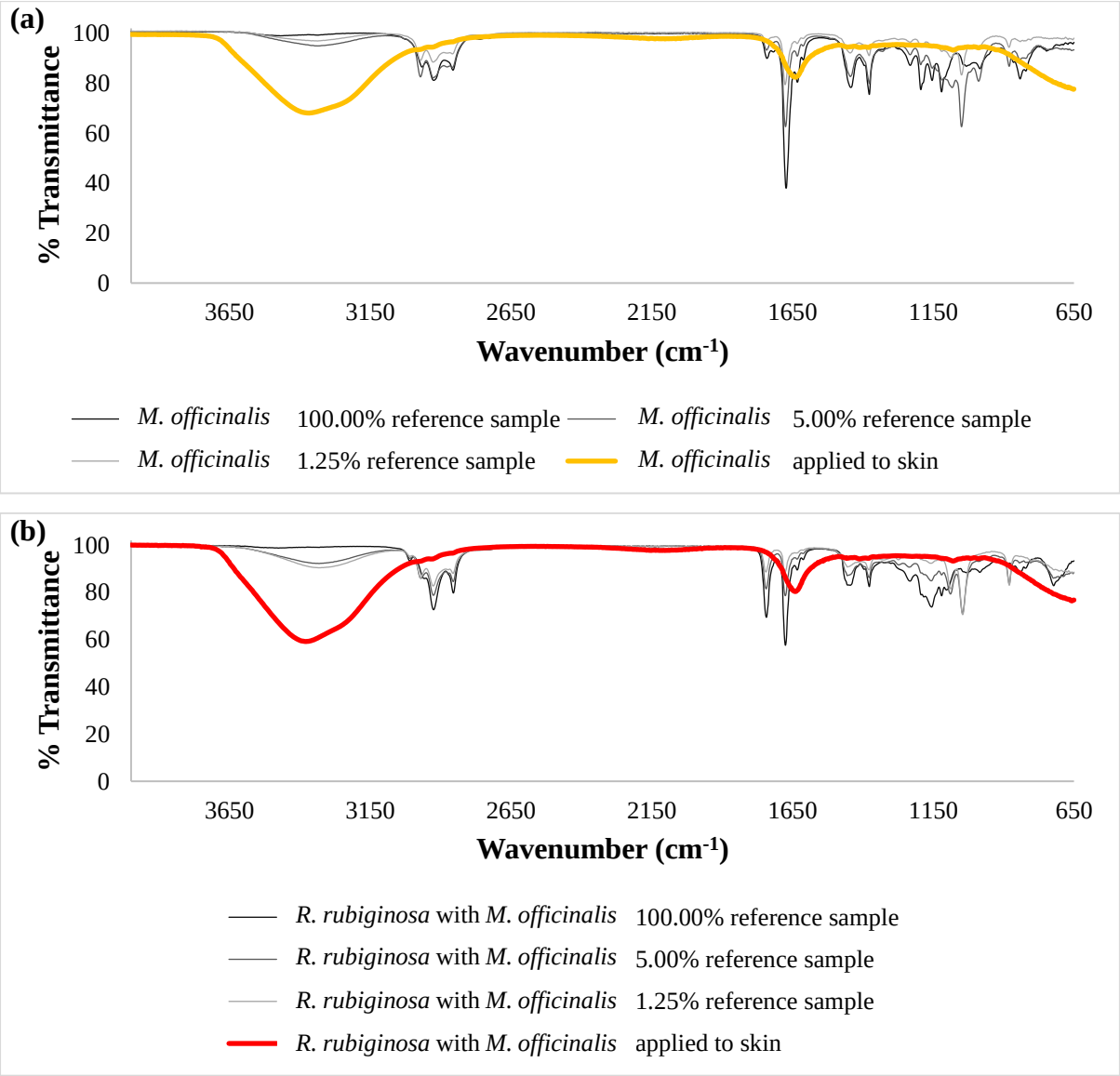

Figure 5.10. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *M. officinalis* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *M. officinalis* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

A review by Wojtunik-Kulesza *et al.* (2019) examining *M. officinalis* essential oil, lacked discussion concerning skin absorption. Earlier studies by de Sousa *et al.* (2004) confirmed potent cytotoxic and anti-proliferative effects of *M. officinalis* essential oil in various animal and human cell lines. Moreover, the current study also revealed high cytotoxicity of *M. officinalis* essential oil, tested independently and in combination with *R. rubiginosa* fixed oil, in both murine macrophages and human epithelial cells (as discussed in Chapter 4). Research testing the topical application of *M. officinalis* essential oil is lacking, possibly due to the high cytotoxicity of the essential oil. Studies conducted by Ramanauskienė *et al.* (2015) and Dimitris *et al.* (2020) are limited to extracts, and aimed to either formulate *M. officinalis* extracts as a pharmaceutical preparation for skin delivery, or to isolate chemical constituents responsible for bio-activity on the skin. The essential oil of *M. officinalis* was visually observed to be as viscous as *R. rubiginosa* fixed oil. Moreover, *M. officinalis* essential oil contains a mixture of multiple (albeit low molecular weight) chemical constituents (citronellal (35.60%), terpinyl acetate (14.20%), geranial (8.40%), α-terpineol (7.30%), cis-geraniol (7.20%), and neral (7.10%)) which may contribute to an overall high molecular weight (> 500 Dalton) that may hinder skin absorption. This suggests that the topical application of *M. officinalis* essential oil diluted in *R. rubiginosa* fixed oil may rather offer an occlusive effect forming a protective skin barrier to retain moisture and lower TEWL to effectively treat the pathogenesis of acne vulgaris.

The infrared profile depicting the vibration transitions of *S. austrocaledonicum* essential oil was not detected in the extraction solvent from the skin samples, when tested independently (Figure 5.11a) and in combination with *R. rubiginosa* fixed oil (Figure 5.11b), thereby indicating that *S. austrocaledonicum* essential oil was not absorbed and retained within the skin. Although *S. austrocaledonicum* essential oil has been used topically to treat skin infections (Page *et al.*, 2018), studies on the skin absorption and retention properties of *S. austrocaledonicum* essential oil is lacking. In a review by de Groot and Schmidt (2017), over 30 publications and several case reports reported allergic contact dermatitis attributed to sandalwood oil (botanical source unspecified). Moreover, *S. austrocaledonicum* essential oil was found to be highly toxic and cytotoxic (as discussed in Chapters 3 and 4, respectively). Thus, the incidence of allergic contact dermatitis and the high toxicity and cytotoxicity of *S. austrocaledonicum* essential oil may possibly contribute to the lack of studies examining the skin permeation properties of this essential oil.

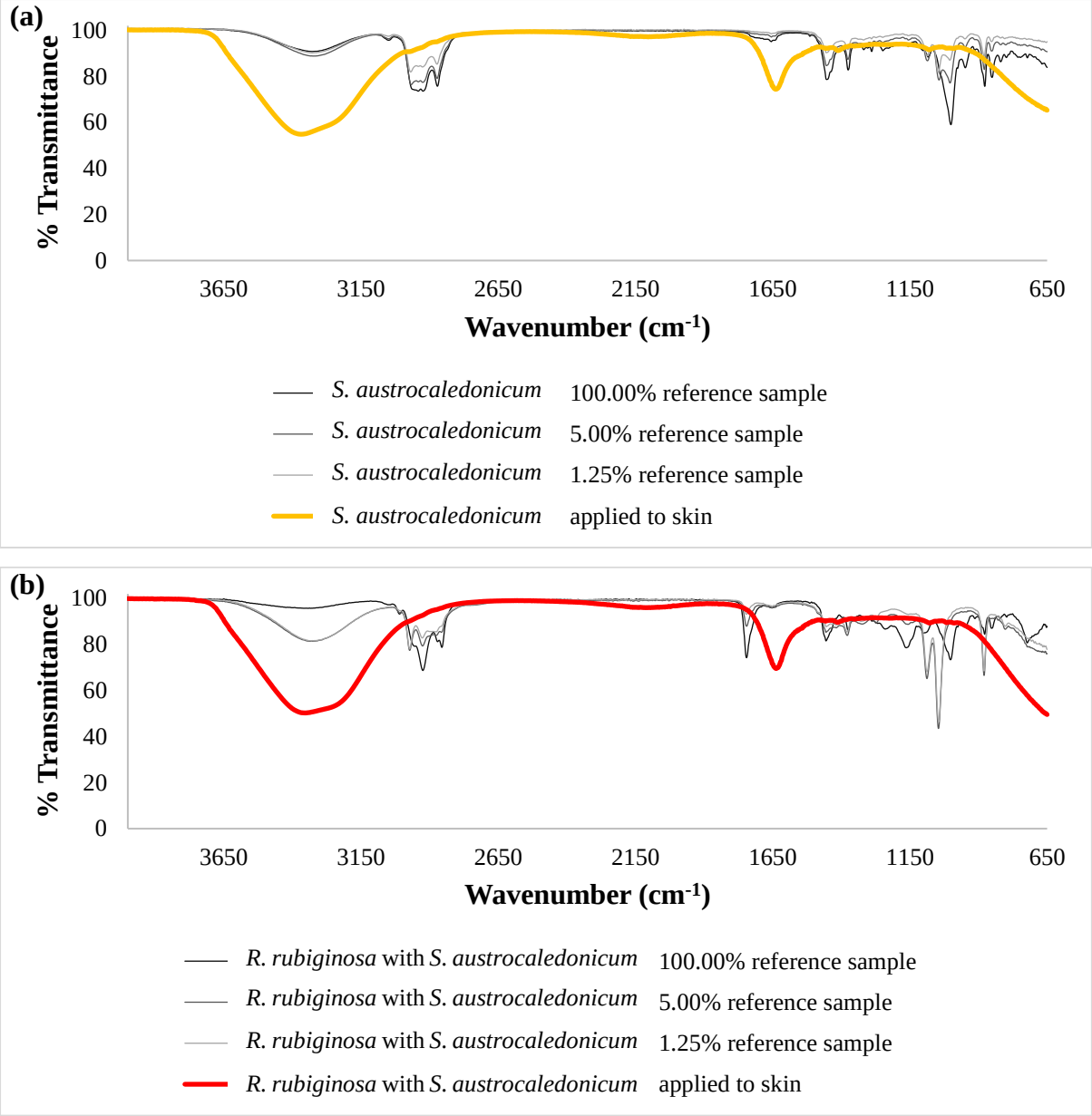

Figure 5.11. Mean FTIR spectrum (n ≥ 3) representing the infrared profile depicting the vibration transitions of **(a)** *S. austrocaledonicum* essential oil independently and **(b)** *R. rubiginosa* fixed oil and *S. austrocaledonicum* essential oil in 1:1 combination extracted from the skin, compared to the spectra of the respective sets of known concentration (v/v).

Similar to *M. officinalis* essential oil, the essential oil of *S. austrocaledonicum* was visually observed to be as viscous as *R. rubiginosa* fixed oil. Moreover, *S. austrocaledonicum* essential oil contains a mixture of multiple chemical constituents (z-α-santalol (42.80%), z-β-santalol (18.60%), z-lanceol (10.30%), epi-β-santalol (3.80%), z-α-bergamotol (3.20%), and farnesol (2.20%)) which may contribute to an overall high molecular weight (> 500 Dalton) that may hinder skin absorption. This suggests that the topical application of *S. austrocaledonicum*

essential oil diluted in *R. rubiginosa* fixed oil may rather offer an occlusive effect forming a protective skin barrier to retain moisture and lower TEWL to effectively treat the pathogenesis of acne vulgaris.

5.3.4. General trend

A general trend noted was that the observed viscosity of the undiluted essential oils (as compared to *R. rubiginosa* fixed oil) correlated to the skin absorption and retention properties. Essential oils which were just as viscous as *R. rubiginosa* fixed oil (including *M. officinalis* and *S. austrocaledonicum* essential oils) were not absorbed and retained within the skin. Essential oils which were less viscous than *R. rubiginosa* fixed oil (including *C. zeylanicum*, *E. caryophyllata*, and *S. benzoin* essential oils) were absorbed and retained within the skin. Essential oils which were the least viscous compared to *R. rubiginosa* fixed oil (including *K. ericoides*, *M. quinquenervia*, and *R. damascena* essential oils) may have been absorbed, however, were minimally or not at all retained within the skin.

5.4. Overview

- The infrared profile depicting the vibration transitions of *R. rubiginosa* fixed oil was not detected in the solvent from the skin samples, thereby indicating that *R. rubiginosa* fixed oil was not absorbed and retained within the skin.
- The infrared profiles depicting the vibration transitions of *C. zeylanicum*, *E. caryophyllata*, *S. benzoin*, and *R. damascena* essential oils were detected in the extraction solvent from the skin samples, when tested independently and in 1:1 combination with *R. rubiginosa* fixed oil, thereby indicating that the essential oils were absorbed and retained within the skin.
- *Rosa rubiginosa* fixed oil increased the skin retention of *C. zeylanicum*, *E. caryophyllata*, and *S. benzoin* essential oils suggesting that these oil combinations could be used to treat the pathogenesis of acne vulgaris when topically applied.
- *Rosa damascena* essential oil requires further dilution in *R. rubiginosa* fixed oil to increase its viscosity and improve skin retention when topically applied.
- The infrared profiles depicting the vibration transitions of *K. ericoides*, *M. quinquenervia*, *M. officinalis*, and *S. austrocaledonicum* essential oils were not detected

in the extraction solvent from the skin samples, when tested independently and in combination with *R. rubiginosa* fixed oil, thereby indicating that the essential oils were not absorbed and retained within the skin.

- *Kunzea ericoides* and *M. quinquenervia* essential oils require further dilution in *R. rubiginosa* fixed oil to improve skin retention when topically applied.

CHAPTER 6

Summary and conclusion

6.1. Summary

Essential oils are therapeutically combined with fixed oils prior to topical application as a means of dilution to subsequently reduce toxicity and skin irritation, however, the effect of fixed oils on essential oil bio-activity required further investigation. Fixed oils are also believed to reduce the evaporation and enhance the cutaneous absorption of essential oils (Orchard and van Vuuren, 2019), however, investigations concerning the effect of fixed and essential oil combination therapy on skin absorption required additional studies. This study investigated the *in vitro* antimicrobial, toxicity, anti-inflammatory and associated cytotoxicity, as well as the skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil independently and in combination with commercial essential oils to find promising oil combinations for the potential and efficient topical treatment of the pathophysiological mechanisms involved in acne vulgaris. The objectives and outcomes of the study are outlined as follows:

Objective 1: To determine the antimicrobial activity of *R. rubiginosa* fixed oil independently and in combination with a selection of essential oils against the reference strains of three acne-inducing pathogens (*C. acnes*, *S. epidermidis* and *S. aureus*) using the broth microdilution assay. The antimicrobial interactions were then evaluated by calculating the fractional inhibitory concentration index (ΣFIC), and interactions (synergy, additive interaction, or antagonism) were analysed using isobolograms.

Chapter 2: The minimum inhibitory concentration (MIC) values of *R. rubiginosa* fixed oil and 30 essential oils, were determined. *Rosa rubiginosa* fixed oil displayed poor antimicrobial activity (mean MIC = 3.33 mg/mL) against the three reference strains. When essential oils were combined with *R. rubiginosa* fixed oil in a 1:1 ratio, *C. zeylanicum* and *S. austrocaledonicum* essential oils maintained noteworthy antimicrobial activity (combined MIC = 0.42 mg/mL and MIC = 0.75 mg/mL, respectively) against the three reference strains. *Rosa rubiginosa* fixed oil combined with *C. zeylanicum* essential oil exhibited antimicrobial synergism (ΣFIC = 0.31)

against *S. epidermidis* and *S. aureus*. *Rosa rubiginosa* fixed oil combined with either of the six essential oils (*E. caryophyllata*, ΣFIC = 0.92; *K. ericoides*, ΣFIC = 0.92; *M. quinquenervia*, ΣFIC = 0.98; *M. officinalis*, ΣFIC = 0.88; *R. damascena*, ΣFIC = 0.81; and *S. benzoin*, ΣFIC = 0.99) displayed additive interactions against all three reference strains. *Rosa rubiginosa* fixed oil combined with *S. austrocaledonicum* essential oil exhibited antimicrobial antagonism (ΣFIC = 4.13) against *S. aureus*. The ΣFIC for the various ratios were expressed graphically on isobolograms, which revealed that the majority of the antimicrobial effects were attributed to the essential oils rather than *R. rubiginosa* fixed oil.

Objective 2: To screen the toxicity of *R. rubiginosa* fixed oil independently and in combination with the essential oils (whereby selection was based on those combinations displaying antimicrobial synergy, additive interactions, or antagonism) using the brine shrimp lethality assay (BSLA).

Chapter 3: *Rosa rubiginosa* fixed oil was found to be non-toxic, with a percentage mortality of 0.35-1.64% after 24 hrs and 0.74-2.00% after 48 hrs, across all concentrations in the range of 0.03-1.00 mg/mL. The toxicity of all eight essential oils (*C. zeylanicum*, *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, *S. austrocaledonicum*, and *S. benzoin*) in combination with *R. rubiginosa* fixed oil resulted in a reduction in toxicity. The greatest reduction in toxicity was observed for *R. rubiginosa* fixed oil combined with *K. ericoides* essential oil at 0.25 mg/mL, where the percentage mortality decreased significantly from 100% (*K. ericoides* essential oil independently) to less than 3% (1:1 combination) at 24 hrs (p-value = 0.04) and 48 hrs (p-value = 0.03). The toxicity of three (*K. ericoides*, *M. quinquenervia* and *S. benzoin*) of the eight essential oils were reduced to non-toxic levels through combination with *R. rubiginosa* fixed oil. At a 1:9 ratio (essential oil: fixed oil), *K. ericoides* essential oil displayed the greatest decrease in toxicity with a percentage mortality of 0.71% and 8.33% at 24 and 48 hrs, respectively.

Objective 3: To investigate the anti-inflammatory activity and cytotoxicity of *R. rubiginosa* fixed oil independently and in combination with the essential oils whereby selection was based on those combinations displaying antimicrobial synergy, additive interactions, or antagonism. The lipopolysaccharide (LPS) induced inflammatory pathway in a RAW 264.7 murine macrophage model was used, and cell viability was simultaneously evaluated using the MTT assay. The cytotoxicity was also assessed in A549 epithelial cells.

Chapter 4: *Rosa rubiginosa* fixed oil reduced LPS induced nitrite production by 18.75% compared to the LPS control. The best reduction in nitrite production was observed for the combination of *R. rubiginosa* fixed oil with *S. benzoin* essential oil, where nitrite production was only 1.51% higher than the aminoguanidine positive control. Simultaneous evaluation of cell viability (MTT assay) was used to confirm the absence of cytotoxicity of the test samples. *Rosa rubiginosa* fixed oil could be considered non-cytotoxic as a similar number of live cells remained after exposure compared to the cell culture medium. The combination of *R. rubiginosa* fixed oil with *M. quinquenervia* essential oil showed the highest percentage cell viability (non-cytotoxicity) and the potential to support A549 epithelial cell growth. *Rosa rubiginosa* fixed oil combined with essential oils substantially reduced the cytotoxic nature of most oil combinations in this study. This study found that since the BSLA toxicity results and the cytotoxicity results are comparable, the BSLA may be used as a simple, efficient, and economical preliminary screening tool for testing toxicity (Sarah *et al.*, 2017).

Objective 4: To detect porcine skin absorption and retention properties of topically applied *R. rubiginosa* fixed oil independently and in combination with the essential oils (whereby selection was based on those combinations displaying antimicrobial synergy, additive interactions, or antagonism) using qualitative Fourier Transform Infrared spectroscopy (FTIR) analysis.

Chapter 5: The infrared profile depicting the vibration transitions of *R. rubiginosa* fixed oil was not detected in the extraction solvent from the skin samples, thereby indicating that *R. rubiginosa* fixed oil was not absorbed and retained within the skin. The infrared profiles depicting the vibration transitions of *C. zeylanicum, E. caryophyllata, S. benzoin*, and *R. damascena* essential oils were detected in the extraction solvent from the skin samples, when tested in 1:1 combination with *R. rubiginosa* fixed oil, thereby indicating that these essential oils were absorbed and retained within the skin. The infrared profiles depicting the vibration transitions of *K. ericoides, M. quinquenervia, M. officinalis*, and *S. austrocaledonicum* essential oils were not detected in the extraction solvent from the skin samples, when tested in combination with *R. rubiginosa* fixed oil, thereby indicating that these essential oils were not absorbed and retained within the skin. This study suggested that FTIR could be a suitable method for detection and qualitative analysis of *R. rubiginosa* fixed oil and essential oils within the concentration range of 1.25-100.00% v/v. Furthermore, FTIR could be used as a possible

preliminary screening tool for *in vitro* and/ or *ex vivo* skin permeation studies associated with fixed and essential oils.

6.2. Limitations

6.2.1. Oil chemotype

In the current study, the chemical characterisation was determined, and the same batch of oils was used throughout the study thereby maintaining consistency. However, should other chemotypes be used, results may differ. Oil composition may differ between plants of the same species depending on the environment or location in which the plants were grown, harvest season, part of the plants harvested, the process of oil extraction, storage, and degradation (Gonçalves *et al.*, 2007, Kırmızıbekmez *et al.*, 2009, Riahi *et al.*, 2013). As the bio-activity of the oils depend on the chemical constituents, any variation in the chemotypes of the oils could cause differences in bio-activity.

6.2.2. Skin permeation studies

The present study suggested that FTIR could be a suitable method for the detection and qualitative analysis of *R. rubiginosa* fixed oil and essential oils within the concentration range of 1.25-100.00% v/v. As such, FTIR could be used as a possible preliminary screening tool for *in vitro* and/ or *ex vivo* skin permeation studies. Specialised static diffusion cells, such as the Franz diffusion cell systems, have evolved into one of the most important research methodologies for the assessment of *in vitro* skin permeation pertaining to conventional drugs and formulations (Ng *et al.*, 2010). However, owing to the volatile nature of essential oils (Orchard and van Vuuren, 2017), essential oil test samples may evaporate from the skin before detection and analysis, or even during analysis if not conducted in a well-controlled confined environment. Fixed and essential oils are often studied as permeation enhancers rather than as active ingredients. Additionally, because the oils are composed of mixtures of numerous chemical constituents, quantitative analysis of these constituents is challenging as the chemical constituents in each oil varies in concentration and diffusion profile. This was a limitation in the current study concerning the use of static diffusion cells for performing the skin absorption and retention experiments of the fixed and essential oils, and its quantification in terms of the

amount of oil permeated per square unit of skin. The skin absorption and retention experiment in the present study serves as an initial step in the complex process of investigating skin permeation. Hence, further research is required to develop an optimised *in vitro* and/ or *ex vivo* skin permeation model for studies involving fixed and essential oils as active ingredients, in conjunction with a sensitive detection instrument for quantifying permeation, absorption, and retention. Studies should focus on the various chemical compounds as each may have a different diffusion profile.

6.2.3. COVID-19 pandemic

The onset of the COVID-19 pandemic posed several constraints to the timeous completion of this research and was thus identified as another limitation. The nationwide lockdown restrictions prohibited university and laboratory access for approximately 12 weeks. Additionally, delays in the delivery of chemicals and laboratory consumables, as well as restrictions on the working hours and availability of working space in the laboratories disrupted the workflow and continuity of time-dependent experiments. To overcome the challenge of lost time, a rotating laboratory schedule was implemented with additional after-hours work policy during the weekdays (2-3 hours) and on the weekends (10-16 hours). Strict adherence to COVID-19 prevention protocols ensured a safe working environment and proficient operation of the laboratories during the pandemic.

6.3. Future recommendations

6.3.1. Anti-biofilm and anti-quorum sensing studies

A review by Brandwein *et al.* (2016) discussed that the persistent nature of acne vulgaris may be attributed to *C. acnes* biofilm formation on the *stratum corneum*, sebaceous gland and hair follicles of acne lesions. In addition, reports of the *C. acnes* genome containing genes responsible for producing quorum sensing molecules support the ability of *C. acnes* to elude eradication using conventional antibiotics. A study reported that *C. acnes* isolates from severe acne infections were more potent producers of biofilms *in vitro* compared to isolates from healthy skin. The reduced efficacy of antibiotic treatment seen in acne patients could be attributed to such biofilm formation (Brandwein *et al.*, 2016). Thus, warranting the

development of novel therapeutic agents with anti-biofilm and anti-quorum sensing properties. The anti-biofilm and anti-quorum sensing properties of topically applied *R. rubiginosa* fixed oil independently and in combination with essential oils could be further explored in future.

6.3.2. Advanced anti-inflammatory studies

Protein extracts, including peptidoglycan, lipoteichoic acid, cytosolic proteins, and membrane proteins (Jugeau *et al.*, 2005), of *C. acnes* stimulate macrophage expression of toll-like receptor 2 (TLR-2) *in vitro* (Kim *et al.*, 2002, Beylot *et al.*, 2014). *Staphylococcus epidermidis*, which produces virulent factors such as lipase and haemolysin delta, is also found in abundance in inflammatory acne vulgaris lesions (Kumar *et al.*, 2016, Jusuf *et al.*, 2020). Since inflammatory acne vulgaris is triggered by a cascade of immunoregulatory responses, the immunohistochemistry and immunophysiology of *R. rubiginosa* fixed oil independently and in combination with the essential oils could be further studied in additional cell lines such as THP-1 human monocytes and HaCaT human keratinocytes.

6.3.3. Wound healing studies

Enlarged, traumatised acne lesions develop fibrosis and depressed, hypertrophic or keloidal scarring (Sutaria *et al.*, 2020). Therefore, future research should investigate the ability of *R. rubiginosa* fixed oil independently and in combination with commercial essential oils to promote re-epithelisation and reduce acne scar formation (using human epidermal keratinocytes and dermal fibroblasts, respectively). Similarly, sebum production (using human sebocytes) and abnormal keratinisation (using human keratinocytes) could also be targeted to further support the use *R. rubiginosa* fixed oil independently and in combination with the essential oils for treating acne pathogenesis.

6.3.4. Varied ratio and triple oil combination studies

In the current study, *R. rubiginosa* fixed oil and the commercial essential oils were studied independently and in 1:1 ratio oil combination in terms of antimicrobial, toxicity, anti-inflammatory, and associated cytotoxicity, as well as skin absorption and retention properties. Varied ratio oil combinations were also investigated in terms of antimicrobial and toxicity properties. Further investigation of varied ratio oil combinations could extend to anti-

inflammatory and associated cytotoxicity as well as skin absorption and retention properties. Moreover, triple oil combinations involving the use of *R. rubiginosa* fixed oil and two commercial essential oils in 1:1:1 ratios presents an avenue for future research to improve and expand the favourable anti-acne profile of an oil combination. This may provide a strategy to further customise antimicrobial activity, anti-inflammatory properties, cytotoxicity, and permeation of an oil combination for the topical treatment of acne vulgaris.

6.3.5. Structure-activity relationships (SARs) and mechanisms of action

Rosa rubiginosa fixed oil and the essential oils contain a multitude of chemical constituents. Thus, the SARs between the chemical constituents of *R. rubiginosa* fixed oil and the essential oils requires further research. As such, future studies could elucidate the specific mechanisms of action of *R. rubiginosa* fixed oil and essential oil combination therapy for the targeted treatment of the pathophysiological mechanisms involved in acne vulgaris.

6.3.6. Formulation development for topical administration

The topical application of *R. rubiginosa* fixed oil offers an occlusive effect forming a protective skin barrier to retain moisture and lower transepidermal water loss (TEWL). This occlusive effect offered by *R. rubiginosa* fixed oil may enhance the permeation of essential oil into the skin when applied in the form of an oil blend, lotion, or cream. However, essential oils become unstable, and their composition may alter due to exposure of the oils to light, oxygen, extreme temperatures, and various storage or processing conditions over time (Gonçalves *et al.*, 2007, Kırmızıbekmez *et al.*, 2009, Riahi *et al.*, 2013). A useful strategy to overcome the issues of essential oil volatility, instability, and risk of toxicity, is encapsulation of the essential oil within a drug delivery systems such as micro- or nano-emulsions, liposomes, solid lipid nanoparticles, and nanostructured lipid carriers for the release of therapeutic, yet non-cytotoxic essential oil at the targeted acne site (Gunawardena *et al.*, 2015, Cimino *et al.*, 2021). The formulation of essential oils as advanced pharmaceutical systems is challenged by their highly volatile and hydrophobic nature, water insolubility, and chemical instability caused by hydrolysis and oxidation (Cimino *et al.*, 2021). Therefore, research regarding the different forms of essential oil encapsulation, suited to the unique properties of essential oils, is required to protect the delicate chemical stability and enhance the pharmacokinetic profile of topically administered oils.

6.4. Conclusion

Acne vulgaris persists as a globally prevalent skin disease caused by the overgrowth of *C. acnes* and subsequent inflammation (Zaenglein, 2018). Individuals afflicted with acne vulgaris often become predisposed to deep skin infection, tissue scarring, and declining psychosocial functioning associated with altered aesthetic appeal. The treatment of acne, with a combination of topical and systemic medicines (such as antibiotics and retinoids), generally has a positive outcome (Taub, 2007, Marson and Baldwin, 2019b). However, harsh side-effects, complicated medication regimens, and expensive remedies lower patient compliance and acceptability. Essential oils are one of the leading natural products used for dermatological applications and further research is necessitated to assess their potential use in the treatment of acne vulgaris.

This study demonstrated that *R. rubiginosa* fixed oil and commercial essential oil combinations had potential in managing bacterial proliferation and inflammation involved in acne vulgaris. Herein, *R. rubiginosa* fixed oil and 30 commercial essential oils were tested for antimicrobial activity. The poor antimicrobial activity demonstrated by *R. rubiginosa* fixed oil against the three reference strains (*C. acnes*, *S. epidermidis* and *S. aureus*) was expected as fixed oils typically do not exhibit antimicrobial activity, but rather anti-oxidant and anti-inflammatory properties owing to their composition of polyunsaturated fatty acids (Orchard *et al.*, 2019, Orchard and van Vuuren, 2019). When tested in different ratios, isobolograms showed that majority of the antimicrobial activity emanated from the essential oil rather than the *R. rubiginosa* fixed oil. This confirmed the inherent antimicrobial properties of the essential oils, and lack thereof in the fixed oil. Nonetheless, via its ability to synergistically quench the high intrinsic toxicity of the essential oils, *R. rubiginosa* fixed oil successfully demonstrated the ability to modulate the toxicity profiles of most of the essential oils in this study, to achieve non-toxicity.

A favourable anti-acne profile of a topically applied oil combination would be one that displayed antimicrobial synergy or additive interaction, low toxicity, effective suppression of LPS induced nitrite production, low cytotoxicity or improved cell viability, and absorption and retention within the skin. Table 6.1 summarises the antimicrobial interaction, toxicity, anti-inflammatory activity, cytotoxicity, and skin absorption and retention properties of *R. rubiginosa* fixed oil combined with the eight essential oils that were identified as having the

highest antimicrobial potential when tested in combination with *R. rubiginosa* fixed oil. From the oil combinations studied, *R. rubiginosa* fixed oil combined with *K. ericoides*, *M. quinquenervia*, *R. damascena*, and *S. benzoin* essential oils displayed either four or more of these favourable properties. *Rosa rubiginosa* fixed oil combined with *S. benzoin* essential oil demonstrated additive antimicrobial interaction in addition to effectively inhibiting LPS induced nitrite production. This combination showed non-cytotoxicity and the ability to be absorbed and retained within the skin (Table 6.1).

Table 6.1. Summary of the properties of *R. rubiginosa* fixed oil combined with the eight essential oils as determined by the relative assays performed in this study.

Essential oil	**Antimicrobial interaction**	**Toxicity**	**Anti-inflammatory activity**	**Cytotoxicity**	**Skin absorption and retention**
C. zeylanicum	Synergy	Toxic	No	Cytotoxic	Yes
E. caryophyllata	Additive	Toxic	No	Non-cytotoxic	Yes
K. ericoides	Additive	Non-toxic	Yes	Non-cytotoxic	No
M. quinquenervia	Additive	Non-toxic	Yes	Non-cytotoxic	No
M. officinalis	Additive	Toxic	No	Cytotoxic	No
R. damascena	Additive	Toxic	Yes	Non-cytotoxic	Yes
***S. benzoin*[a]**	**Additive**	**Non-toxic**	**Yes**	**Non-cytotoxic**	**Yes**
S. austrocaledonicum	Antagonism	Toxic	N/a	Cytotoxic	No

[a] Best combination of *R. rubiginosa* fixed oil with essential oil (bold).

This study identified that the combination of *R. rubiginosa* fixed oil (containing 41.93% of linoleic, 35.73% of linolenic, and 15.45% of oleic acids) with *S. benzoin* essential oil (containing 72.20% of dimethyl phthalate, 7.20% of benzyl acetate, and 5.70% of ethyl cinnamate) showed the best potential as a future treatment option for acne vulgaris. Not only was the antimicrobial interaction additive, but toxicity and cytotoxicity were decreased, and permeation studies showed that this combination was absorbed and retained within the skin – a vital prerequisite for the topical treatment of acne vulgaris. The use of fixed oils combined with essential oils (such as the *R. rubiginosa* fixed oil with *S. benzoin* essential oil combination) for improved bio-activity and skin absorption and retention, holds promise for easing the burden of conventional antibiotic prescribing and use for treating acne vulgaris amidst the global concerns of emerging antimicrobial resistance.

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APPENDIX A

Abstract for publication draft

In vitro bio-activity of *Rosa rubiginosa* (rosehip) oil combined with commercial essential oils for acne vulgaris

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Acne vulgaris is caused by *Cutibacterium acnes* overgrowth and inflammation. Essential oils are one of the leading natural products used for dermatological applications, including acne. Essential oils combined with fixed oils reduce irritation and toxicity, however, the effect of fixed oils on essential oil (EO) bio-activity requires further investigation. This study investigates the *in vitro* antimicrobial, toxicity, anti-inflammatory and associated cytotoxicity, as well as the skin absorption and retention properties of *Rosa rubiginosa* fixed oil (RRFO) as a carrier for EOs. The minimum inhibitory concentration (MIC) and the fractional inhibitory concentration index (ΣFIC) indicated that RRFO combined with *Cinnamomum zeylanicum* EO displayed antimicrobial synergy (MIC=0.42 mg/mL; ΣFIC=0.45) against three acne-inducing reference strains. The brine shrimp lethality assay revealed that RRFO combined with *Kunzea ericoides* EO significantly decreased the percentage mortality from 100% (EO independently) to less than 3% (oil combination) at 24 hrs and 48 hrs. Combined with *Melaleuca quinquenervia* EO, RRFO exhibited the lowest cytotoxicity in A549 cells. Combined with *Styrax benzoin* EO, RRFO supressed lipopolysaccharide (LPS) induced nitrite production to 66.20% in RAW 264.7 cells. Skin absorption and retention of topically applied RRFO and the EOs were further explored. The combinations of RRFO with *C. zeylanicum*, *E. caryophyllata*, and *S. benzoin* EOs were absorbed and retained within porcine skin after 24 hrs as qualitatively detected using Fourier Transform Infrared spectroscopy. This study revealed that RRFO combined with *S. benzoin* EO is a promising non-cytotoxic oil combination which may be used to treat the pathophysiological mechanisms in acne vulgaris.

APPENDIX B

Presentations

B1. FameLab 2021 WITS Preliminary Heat – University of the Witwatersrand, Johannesburg, South Africa, 6 May 2021

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No abstract was required for this presentation.

B2. FameLab 2021 WITS Final Heat – University of the Witwatersrand, Johannesburg, South Africa, 7 May 2021

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No abstract was required for this presentation.

B3. Indigenous Plant Use Forum (IPUF) 2021 Virtual Conference – Johannesburg, South Africa, 5-7 July 2021

In vitro antimicrobial, toxicity, cytotoxicity, and anti-inflammatory properties of *Rosa rubiginosa* L. (rosehip) fixed oil and essential oil combinations for acne treatment

Shivani Ramburrun[a], Ané Orchard[a] and Sandy van Vuuren[a]

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Acne vulgaris (comedones, papules, pustules, nodules, and cysts with consequent scarring) is the eighth most prevalent disease worldwide. *Cutibacterium acnes* overgrowth and inflammation are responsible for acne pathogenesis. Acne remedies are one of the leading dermatological applications of essential oils. Essential oils are combined with carrier oils to reduce irritation, however, the effect of carrier oils on essential oil (EO) bioactivities requires further investigation. This study investigates the *in vitro* antimicrobial, toxicity, cytotoxicity, and anti-inflammatory properties of Southern African harvested *Rosa rubiginosa* L. fixed oil (FO) as a carrier for essential oils. The minimum inhibitory concentration (MIC) and sum of the fractional inhibitory concentration index (ΣFIC) indicated *R. rubiginosa* FO combined with *Cinnamomum zeylanicum* Blume EO displayed antimicrobial synergy (MIC=0.42 mg/mL; ΣFIC=0.45) against three acne-inducing reference strains (*C. acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus*). The brine shrimp lethality assay revealed *R. rubiginosa* FO combined with *Kunzea ericoides* (A.Rich.) Joy Thomps. EO produced the greatest toxicity reduction. *Rosa rubiginosa* FO combined with *Melaleuca quinquenervia* (Cav.) S.T.Blake EO exhibited the lowest cytotoxicity (84.32% cell viability compared to the control) in the A549 cell line. *Rosa rubiginosa* FO combined with *Styrax benzoin* Dryand. EO supressed inflammation-induced nitrite production to 66.20% in the RAW 264.7 cell line. This study contributes to natural product science by exploring cosmetically-appealing and promising oil combinations for effective use in acne treatment. Research showing potential for adjusting cytotoxic and anti-inflammatory properties of essential oils provides credibility for commercially harvesting and marketing Southern African *R. rubiginosa* FO.

B4. Cross-Faculty Postgraduate Symposium 2021 – University of the Witwatersrand, Johannesburg, South Africa, 26-30 July 2021

In vitro investigation of *Rosa rubiginosa* (rosehip) oil and essential oil combinations for acne treatment

Shivani Ramburrun[a], Ané Orchard[a] and Sandy van Vuuren[a]

[a] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa.

Acne vulgaris, the eighth most prevalent disease worldwide, is caused by *Cutibacterium acnes* overgrowth and inflammation. Essential oils are one of the leading natural products used for dermatological applications, including acne. Essential oils combined with carrier oils reduce irritation and toxicity, however, the effect of carrier oils on essential oil (EO) bio-activity requires further investigation. This study investigates the *in vitro* antimicrobial, toxicity, and anti-inflammatory properties of *Rosa rubiginosa* L. fixed oil (RRFO) as a carrier for EOs. The minimum inhibitory concentration (MIC) and sum of the fractional inhibitory concentration index (ΣFIC) indicated that RRFO combined with *Cinnamomum zeylanicum* Blume EO displayed antimicrobial synergy (MIC=0.42 mg/mL; ΣFIC=0.45) against three acne-inducing reference strains. The brine shrimp lethality assay revealed that RRFO combined with *Kunzea ericoides* (A.Rich.) Joy Thomps. EO produced the greatest reduction in toxicity. Combined with *Melaleuca quinquenervia* (Cav.) S.T.Blake EO, RRFO exhibited the lowest cytotoxicity (84.32% cell viability) in A549 cells. Combined with *Styrax benzoin* Dryand. EO, RRFO supressed inflammation-induced nitrite production to 66.20% in RAW 264.7 cells. This study revealed that RRFO and *S. benzoin* EO is a promising non-toxic and effective anti-inflammatory combination for use in acne treatment, thus contributing to natural product science.

B5. FameLab 2021 South Africa Semi-Final – Johannesburg, South Africa, 28 September 2021

Shivani Ramburrun[a], Ané Orchard[a] and Sandy van Vuuren[a]

[a] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa.

No abstract was required for this presentation.

B6. FameLab 2021 South Africa Final – Johannesburg, South Africa, 6 October 2021

Shivani Ramburrun[a], Ané Orchard[a] and Sandy van Vuuren[a]

[a] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa.

No abstract was required for this presentation.

APPENDIX C

Human ethics waiver certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms S Ramburrun
School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University
E-mail: 1142109@students.wits.ac.za

CC: Supervisor: Professor S van Vuuren <Sandy.vanVuuren@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 26/03/2020

REF: *R14/49*

PROTOCOL NO: W-CBP-200326-02 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *In vitro investigation of Rosa rubiginosa (rosehip) oil and essential oil combinations for the treatment of acne*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

26/03/2020

Ref: W-CBP-200326-02

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Ms S Ramburrun
Student No. (if appropriate): 1142109
Staff No. (if appropriate):

Supervisor: Professor S van Vuuren

School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Medical School
University

Project title: *In vitro investigation of Rosa rubiginosa (rosehip) oil and essential oil combinations for the treatment of acne*

Reason: In vitro laboratory study.
No human participants will be involved in the study.

CB Penny

Dr CB Penny
Co-Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za.
Website:http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

APPENDIX D

Animal ethics waiver certificate

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 09/04/2020

Certificate reference: Waiver 09-04-2020-O
Category: O
Applicant: Shivani Ramburrun
Department: Department of Pharmacy and Pharmacology (University of the Witwatersrand)
Tel: 011 717 2157; **Email**: 1142109@students.wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Ms Shivani Ramburrun, MPharm student (#1142109) under the supervision of Prof. Sandy van Vuuren at the Department of Pharmacy and Pharmacology (University of the Witwatersrand), does not require full Animal Ethics Research Committee clearance to undertake the work titled '***In vitro* investigation of *Rosa rubiginosa* (rosehip) oil and essential oil combinations for the treatment of acne'.**

Reason for waiver
The project is using existing and commercially obtained porcine skin. The porcine skin will be obtained from the local abattoir, Mintko Meat Packers (Krugersdorp, Gauteng), which has been previously used by the Department of Pharmacy and Pharmacology.
The project is also using brine shrimp.

Details of the study
Permeation/ retention studies
Permeation studies will be performed using porcine skin in an FDC apparatus. After removing the hair and subcutaneous fat using scissors and a scalpel, the porcine skin will be cut into appropriately sized squares. The porcine skin will be clamped between the donor and receptor FDC compartments and will be conditioned by immersion in receptor fluid prior to use. The oil samples will be applied directly onto the porcine skin samples. After the experiment, the tape-stripping method will be used to remove the stratum corneum. The epidermis will be separated from the dermis with a scalpel and both will be weighed on an analytical scale. The remaining collected tissue (stratum corneum, epidermis and dermis) will be digested. Following solid phase extraction, the contents will be analysed by high-performance liquid chromatography.

Brine shrimp lethality assay (BSLA)

Artificial sea water will be prepared and dried *Artemia franciscana* (brine shrimp) eggs will be added to the saltwater and aerated with a rotary pump. A constant source of warmth and light will be provided with a lamp. After incubation, microtitre plates will be prepared for toxicity evaluation by adding saltwater containing 40 – 60 live brine shrimp to each of the 48 wells. The oil samples will then be added to each well. Potassium dichromate, a known toxic compound, will be used as a positive control. The plates will be viewed after 24 and 48 hrs under a light microscope and the dead brine shrimp counted. Thereafter, a lethal dose of acetic acid will be added to each well and a final count of dead brine shrimp will be taken to calculate the percentage mortality.

People involved

The individual covered by the waiver are Ms Shivani Ramburrun, Prof. Sandy van Vuuren, Dr Ané Orchard and Prof. Lisa du Toit (Department of Pharmacy and Pharmacology).

Please contact me should you require further information.

Yours sincerely

Prof. Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand

APPENDIX E

Rosa rubiginosa fixed oil certificate of analysis

Precision Oil Laboratories

T0802

Pty (Ltd) | Co. Reg No. 2013/091831/07 | VAT No. 4240267635
Consulting Room No 7, Ivory Tusk Lodge, 75 Douglas Street, Tzaneen | Po Box 4513 Tzaneen 0850
Tel: 015 307 7280 | Fax: 015 307 7290 | Cell: 072 491 6044
Technical: me.mostert@telkomsa.net | polaboratories@gmail.com | Accounts: drmostert1@gmail.com

Certificate of Analysis

Report no: R19/203

Suzette van der Schyff
Rosehip farm (Pty Ltd)
700 Cypress Road
Zonnehoewe
Ruimsig
Roodepoort
1742
Tel: 010 593 2752
Email: admin@rosehipfarm.co.za

Date: 13/6/2019

Certificate of Analysis

Sample Description: Rosehip oil

Sample: COA CH04020515 **Lab no: P19/203**

Analyses:

Received: 6/6/2019 Commenced: 10/6/2019 Completed: 13/6/2019

Methods used:

Method name	Method number	Method
Peroxide Value	POL 004	AOCS Method CD 8-53
Free Fatty Acids/Acid Value	POL 005	Based on AOCS Method Cd 3d-63
Unsaponifiable Material	POL 008	AOCS Method Ca 6a-40
Anisidine Value	POL 006	AOCS Method Cd 18-90
Saponification Value	POL 007	AOCS Method Cd 3-25
Iodine Value*	POL 009	AOCS Method Cd 1c-85
Fatty Acid profile	POL 015	Based on AOCS Ce2-66
Relative density*	POL 002	Guy Lussac Pyknometer
Refractive index*	POL 003	Atago refractometer

Results:

The results are applicable to sample condition at receipt. The results relate only to the items tested.
This certificate shall not be reproduced except in full, without the written approval of the Laboratory.

1 of 2

Sample: COA CH04021505 **Report no: P19/203**

The results are reported with the expanded relative uncertainty at a confidence level of 95% using a factor of k=2.

LOQ = limit of quantitation

Sample:	Result	Uncertainty	LOQ
Peroxide Value (meq/kg oil)	4.33	± 0.79	0.81
Acid Value (mg KOH/g sample)	0.85	± 0.13	0.112
Free Fatty Acids (g oleic acid/100g oil)	0.428	± 0.066	0.056
Unsaponifiable Material g/100g	0.79	± 0.36	0.24
Anisidine Value (mmol/kg oil)	4.26	± 0.70	0.52
Saponification Value (mg KOH to saponify 1g sample)	196.0	± 3.5	
Iodine Value*	179.61		
Refractive index* (20°C)	1.4792		
Relative Density* (20°C)	0.924		

*Not Sanas Accredited

Limit of detection (LOD) = 0.09 g Fatty acid/100g Fatty acids.
Limit of quantitation (LOQ) = 0.28 g Fatty acid/100g Fatty acids.
Values below the limit of quantitation cannot be accurately quantified.

Fatty Acid	g Fatty acids/100g Fatty Acids	Uncertainty
C14:0 Myristic acid	None detected	
C16:0 Palmitic acid	2.71	0.31
C16:1 Palmitoleic acid	None detected	
C17:0 Margaric acid	None detected	
C17:1 Glinkgolic acid	None detected	
C18:0 Stearic acid	1.62	0.08
C18:1 trans Oleic acid	None detected	
C18:1 cis Oleic acid	15.45	0.46
C18:2 trans Linoleic acid	None detected	
C18:2 cis Linoleic acid	41.93	1.2
C18:3 n6 Linolenic acid	0.34	
C18:3 n3 Linolenic acid	35.73	2.5
C20:0 Arachidic acid	1.10	0.23
C20:1 Eicosenoic acid	0.57	0.11
C20:2 Eicosadienoic acid	Lower than LOQ	
C21:0 Heneicosanoic acid	None detected	
C22:0 Behenic acid	Lower than LOQ	
C24:0 Lignoceric acid	None detected	
C24:1 Nervonic acid	None detected	

Do not hesitate to contact me should you need any additional information.

Dr. Mathilda Mostert, Pri.Sci.Nat
Technical Signatory and Laboratory manager

APPENDIX F

Chemical profiles and mean antimicrobial activities of the selected commercial essential oils

Table F1. Summary of the GC-MS data and mean MIC values (mg/mL) of the commercial essential oils selected for this study against acne-inducing micro-organisms, adapted from van Vuuren *et al.* (2014) and Orchard *et al.* (2017, 2018a, 2019).

	Supplier	**Previous studies**		
Sample	**Major chemical constituents**	**Major chemical constituents**	**MIC (mg/mL) average**	**Reference**
Cinnamomum zeylanicum Blume (true cinnamon)	Cinnamaldehyde (61.50%) β-Caryophyllene (6.80%) Cinnamyl acetate (6.50%) Eugenol (3.70%) β-Phellandrene (3.70%)	Cinnamaldehyde (61.50%) β-Caryophyllene (6.80%) Cinnamyl acetate (6.50%) Eugenol (3.70%) β-phellandrene (3.70%)	**1.00[a]**	(Orchard *et al.*, 2018a)
Citrus aurantium var. *amara* L. (bitter orange)	Linalyl acetate (41.80%) Limonene (15.30%) β-Pinene (10.10%)	Linalyl acetate (41.80%) Limonene (15.30%) β-Pinene (10.10%)	1.33	(Orchard *et al.*, 2017)
Commiphora molmol (Engl.) Engl. ex Tschirch (myrrh)	Furanoeudesma-1,3-diene (46.40%) Curzerene (22.00%) Lindestrene (5.00%)	Furanoeudesma-1,3-diene (52.90%) Lindestrene (15.80%)	**0.67**	(Orchard *et al.*, 2017)
Cymbopogon citratus (DC.) Stapf (lemon grass)	Geranial (42.70%) Neral (28.90%) Limonene (11.70%)	Geranial (42.70%) Neral (28.90%) Limonene (11.70%)	**0.83**	(Orchard *et al.*, 2017)
Cymbopogon martini (Roxb.) W.Watson (palmarosa)	Geraniol (77.80%) Geranyl acetate (9.60%) Linalol (3.02%)	Geraniol (80.70%)	**0.83**	(Orchard *et al.*, 2017)

	Supplier	Previous studies		
Sample	**Major chemical constituents**	**Major chemical constituents**	**MIC (mg/mL) average**	**Reference**
Cymbopogon nardus (L.) Rendle (citronella grass)	Citronellal (42.10%) Geraniol (20.60%) Citronellol (18.50%)	Citronellal (42.10%) Geraniol (20.60%) Citronellol (18.50%)	**1.00**	(Orchard *et al.*, 2018a)
Eugenia caryophyllata Thunb. (clove)	Eugenol (70.70%) Eugenyl acetate (19.20%) Caryophyllene (8.10%)	Eugenol (81.90%) Isoeugenol (13.10%)	**0.79**	(Orchard *et al.*, 2017)
Helichrysum italicum (Roth) G.Don (Italian strawflower)	α-Pinene (22.40%) γ-Curcumene + α-Terpineol (14.20%) β-Selinene (6.80%) Neryl acetate (6.40%)	1,8-Cineole (19.40%) α-Humulene (15.00%) β-Caryophyllene (12.40%) β-Pinene (7.60%)	1.33	(Orchard *et al.*, 2017)
Hyssopus officinalis L. (hyssop)	β-Pinocamphone (35.10%) β-Pinene (16.00%) α-Pinocamphone (8.80%) β-Phellandrene (5.60%)	Isopinocamphone (47.90%) Pinocamphone (15.10%) β-Pinene (10.10%)	1.25	(Orchard *et al.*, 2017)
Kunzea ericoides (A.Rich.) Joy Thomps. (kanuka)	α-Pinene (26.20-46.70%) ρ-Cymene (5.80-19.10%)	α-Pinene (26.20-46.70%) ρ-Cymene (5.80-19.10%)	3.33	(van Vuuren *et al.*, 2014)
Laurus nobilis L. (bay laurel)	1,8-Cineole (54.10%) Terpinyl acetate (7.50%) Sabinene (5.50%) α-pinene (5.30%)	Eugenol (54.40%) Myrcene (18.50%) Chavicol (11.50%)	**1.00**	(Orchard *et al.*, 2017)
Leptospermum petersonii F.M.Bailey (lemon-scented tea tree)	Geranial (30.40-40.70%) Neral (17.90-22.50%) Citronellol (6.50-26.80%) Citronellal (4.30-19.10%)	Geranial (30.40-40.70%) Neral (17.90-22.50%) Citronellol (6.50-26.80%) Citronellal (4.30-19.10%)	2.33	(van Vuuren *et al.*, 2014)

Sample	Supplier Major chemical constituents	Previous studies Major chemical constituents	MIC (mg/mL) average	Reference
Leptospermum scoparium J.R.Forst. & G.Forst. (manuka)	E-methyl cinnamate (9.20-19.50%) Eudesma-4(14),11-diene (6.20-14.50%) α-Selinene (5.90-13.50%)	E-Methyl cinnamate (9.20-19.50%) Eudesma-4(14),11-diene (6.20-14.50%) α-Selinene (5.90-13.50%)	**0.98**	(van Vuuren *et al.*, 2014)
Litsea cubeba (Lour.) Pers. (may chang)	Geranial (34.10%) Neral (32.80%) Limonene (14.30%)	Geranial (44.60%) Neral (28.80%)	**0.88**	(Orchard *et al.*, 2017)
Matricaria recutita L. (chamomile)	E(trans)-β-farnesene (45.73%) α-Bisabolol oxide B (9.56%) Bisabolol oxide A (5.80%) α-Bisabolol oxide A (5.22%)	Bisabolene oxide A (52.30%) β-Farnesene (19.40%) Chamazulene (3.00%)	**0.67**	(Orchard *et al.*, 2018a)
Melaleuca alternifolia (Maiden & Betche) Cheel (tea tree)	Terpinen-4-ol (44.60%) γ-Terpinene (16.60%) ρ-Cymene (9.60%)	Terpinen-4-ol (44.60%) γ-Terpinene (16.60%) ρ-Cymene (9.60%)	2.75	(Orchard *et al.*, 2017)
Melaleuca quinquenervia (Cav.) S.T.Blake (niaouli)	Linalyl acetate (49.80%) Linalool (30.10%)	Linalyl acetate (49.80%) Linalool (30.10%)	1.33	(Orchard *et al.*, 2017)
Melissa officinalis L. (lemon balm)	Citronellal (35.60%) Terpinyl acetate (14.20%) Geranial (8.40%) α-Terpineol (7.30%) cis-Geraniol (7.20%) Neral (7.10%)	Citronellal (35.60%) Terpinyl acetate (14.20%) Geranial (8.40%) α-Terpineol (7.30%) cis-Geraniol (7.20%) Neral (7.10%)	**1.00**	(Orchard *et al.*, 2017)
Nardostachys grandiflora DC. (spikenard)	β-Gurjunene (9.70%) Ledene (9.10%) Spirotetemol (5.60%) α-Panasinsen (3.40%)	β-Gurjunene (9.70%) Ledene (9.10%) α-Panasinsen (3.40%)	1.06	(Orchard *et al.*, 2018a)

	Supplier	Previous studies		
Sample	**Major chemical constituents**	**Major chemical constituents**	**MIC (mg/mL) average**	**Reference**
Ocimum sanctum L. (holy basil)	Eugenol (57.30%) β-Caryophyllene (30.90%) α-Humulene (3.60%)	Eugenol (57.30%) β-Caryophyllene (30.90%) α-Humulene (3.60%)	**0.67**	(Orchard *et al.*, 2018a)
Origanum majorana L. (marjoram)	1,8-Cineole (51.90%) Linalyl formate (22.40%)	1,8-Cineole (51.90%) Linalyl formate (22.40%)	1.33	(Orchard *et al.*, 2017)
Origanum vulgare L. (oregano)	Carvacrol (60.40%) γ-Terpinene (7.90%) ρ-Cymene (7.00%) Thymol (5.70%)	Carvacrol (51.70%) Thymol (22.50%) γ-Terpinene (10.20%) ρ-Cymene (7.20%)	**0.58**	(Orchard *et al.*, 2018a)
Pelargonium graveolens L'Hér. (sweet scented geranium)	Citronellol (27.80%) Geraniol (14.10%) Citronellyl formate (10.30%) Iso menthone (7.90%) Guiedene (7.40%)	Citronellol (26.70%) Eudesmol (12.50%) Citronellyl formate (11.50%) Menthone (7.80%) Linalool (7.20%) Neryl formate (5.40%)	**0.83**	(Orchard *et al.*, 2018a)
Pistacia lentiscus L. (mastic tree)	Myrcene (20.20%) α-Pinene (18.70%) Limonene (14.50%) α-Himachalene (13.10%)	Myrcene (20.20%) α-Pinene (18.70%) Limonene (14.50%) α-Himachalene (13.10%)	1.75	(Orchard *et al.*, 2017)
Pogostemon cablin (Blanco) Benth. (patchouli)	Patchouli alcohol (30.80%) δ-Guai'ene(α-Bulnesene) (18.00%) α-Guai'ene (15.00%) α-Patchoulene (7.40%)	β-Patchoulene (38.30%) α-Bulnesene (13.00%) α-Guaiene (11.90%)	**0.42**	(Orchard *et al.*, 2017)
Rosa damascena Herrm. (Damask rose)	Citronellol (29.90%) Phenelyl alcohol (22.40%) Geraniol (20.80%) Nerol (10.20%)	Citronellol (29.90%) Phenelyl alcohol (22.40%) Geraniol (20.80%) Nerol (10.20%)	**0.83**	(Orchard *et al.*, 2018a)

	Supplier	**Previous studies**		
Sample	**Major chemical constituents**	**Major chemical constituents**	**MIC (mg/mL) average**	**Reference**
Santalum austrocaledonicum Vieill. (New Caledonia sandalwood)	Z-α-santalol (42.80%) Z-β-santalol (18.60%) Z-lanceol (10.30%) Epi-β-santalol (3.80%) Z-α-bergamotol (3.20%) Farnesol (2.20%)	α-Santalol (52.70%) β-Santalol (15.70%) Bergamotol (7.10%) cis-Santalol (3.80%)	**0.33**	(Orchard *et al.*, 2018a)
Styrax benzoin Dryand. (gum benjamin tree)	Dimethyl phthalate (72.20%) Benzyl acetate (7.20%) Ethyl cinnamate (5.70%)	Dimethyl phthalate (72.20%) Benzyl acetate (7.20%) Ethyl cinnamate (5.70%)	1.67	(Orchard *et al.*, 2017)
Thymus vulgaris L. (thyme)	Thymol (43.30%) ρ-Cymene (19.20%) γ-Terpinene (11.20%)	ρ-Cymene (41.00%) Thymol (18.90%) γ-Terpinene (7.20%)	**0.92**	(Orchard *et al.*, 2017)
Vetiveria zizanioides (L.) Nash (vetiver grass)	Khusenic acid (15.90%) Khusimol (7.90%) Isovalencenol (7.40%) α-Vetivone (3.70%) Vetiselinenol (3.32%) β-Vetivone (2.70%)	Zizanol (12.80%) β-Vetirenene (8.80%)	**0.38**	(Orchard *et al.*, 2017)

[a] Noteworthy activity (bold)

APPENDIX G

Antimicrobial isobologram MIC values

Table G1. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *C. zeylanicum* essential oil against the three reference strains.

	Volume ratio	**Mean combination MIC values (mg/mL)**		
Isobologram plot number	***C. zeylanicum*: *R. rubiginosa***	***C. acnes***	***S. epidermidis***	***S. aureus***
1	9:1	0.13 ±0.00	1.00 ±0.00	1.00 ±0.00
2	8:2	0.13 ±0.00	1.00 ±0.00	1.00 ±0.00
3	7:3	0.13 ±0.00	1.00 ±0.00	1.00 ±0.00
4	6:4	0.22 ±0.05	1.00 ±0.00	1.00 ±0.00
5	5:5	0.25 ±0.00	0.50 ±0.00	0.50 ±0.00
6	4:6	0.31 ±0.11	1.00 ±0.00	1.00 ±0.00
7	3:7	0.38 ±0.13	1.75 ±0.43	2.00 ±0.00
8	2:8	0.75 ±0.25	1.75 ±0.43	2.00 ±0.00
9	1:9	1.50 ±0.50	2.00 ±0.00	3.00 ±1.00

Table G2. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *E. caryophyllata* essential oil against the three reference strains.

	Volume ratio	**Mean combination MIC values (mg/mL)**		
Isobologram plot number	***E. caryophyllata*: *R. rubiginosa***	***C. acnes***	***S. epidermidis***	***S. aureus***
1	9:1	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
2	8:2	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
3	7:3	0.75 ±0.25	2.00 ±0.00	2.00 ±0.00
4	6:4	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
5	5:5	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
6	4:6	1.00 ±0.00	2.50 ±0.87	3.50 ±0.87
7	3:7	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
8	2:8	1.50 ±0.50	4.00 ±0.00	4.00 ±0.00
9	1:9	2.00 ±0.00	4.00 ±0.00	4.00 ±0.00

Table G3. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *K. ericoides* essential oil against the three reference strains.

	Volume ratio	**Mean combination MIC values (mg/mL)**		
Isobologram plot number	***K. ericoides*: *R. rubiginosa***	***C. acnes***	***S. epidermidis***	***S. aureus***
1	9:1	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
2	8:2	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
3	7:3	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
4	6:4	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
5	5:5	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
6	4:6	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
7	3:7	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
8	2:8	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
9	1:9	1.50 ±0.50	4.00 ±0.00	4.00 ±0.00

Table G4. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *M. quinquenervia* essential oil against the three reference strains.

	Volume ratio	**Mean combination MIC values (mg/mL)**		
Isobologram plot number	***M. quinquenervia*: *R. rubiginosa***	***C. acnes***	***S. epidermidis***	***S. aureus***
1	9:1	0.75 ±0.25	2.00 ±0.00	2.00 ±0.00
2	8:2	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
3	7:3	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
4	6:4	0.75 ±0.25	2.50 ±0.87	2.00 ±0.00
5	5:5	0.75 ±0.25	4.00 ±0.00	2.00 ±0.00
6	4:6	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
7	3:7	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
8	2:8	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
9	1:9	1.25 ±0.43	4.00 ±0.00	4.00 ±0.00

Table G5. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *M. officinalis* essential oil against the three reference strains.

	Volume ratio	**Mean combination MIC values (mg/mL)**		
Isobologram plot number	***M. officinalis*: *R. rubiginosa***	***C. acnes***	***S. epidermidis***	***S. aureus***
1	9:1	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
2	8:2	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
3	7:3	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
4	6:4	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
5	5:5	1.00 ±0.00	3.00 ±1.00	2.00 ±0.00
6	4:6	1.00 ±0.00	4.00 ±0.00	3.00 ±1.00
7	3:7	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
8	2:8	1.50 ±0.50	4.00 ±0.00	4.00 ±0.00
9	1:9	2.00 ±0.00	4.00 ±0.00	4.00 ±0.00

Table G6. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *R. damascena* essential oil against the three reference strains.

	Volume ratio	**Mean combination MIC values (mg/mL)**		
Isobologram plot number	***R. damascena*: *R. rubiginosa***	***C. acnes***	***S. epidermidis***	***S. aureus***
1	9:1	0.75 ±0.25	2.00 ±0.00	2.00 ±0.00
2	8:2	0.75 ±0.25	2.00 ±0.00	2.00 ±0.00
3	7:3	0.75 ±0.25	2.00 ±0.00	2.00 ±0.00
4	6:4	0.75 ±0.25	2.00 ±0.00	2.00 ±0.00
5	5:5	1.00 ±0.00	2.00 ±0.00	2.00 ±0.00
6	4:6	1.00 ±0.00	4.00 ±0.00	3.00 ±1.00
7	3:7	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
8	2:8	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00
9	1:9	1.50 ±0.50	4.00 ±0.00	4.00 ±0.00

Table G7. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *S. benzoin* essential oil against the three reference strains.

Isobologram plot number	Volume ratio *S. benzoin*: *R. rubiginosa*	Mean combination MIC values (mg/mL)		
		C. acnes	*S. epidermidis*	*S. aureus*
1	9:1	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
2	8:2	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
3	7:3	0.50 ±0.00	2.00 ±0.00	2.00 ±0.00
4	6:4	0.50 ±0.00	3.00 ±1.00	3.00 ±1.00
5	5:5	0.75 ±0.25	4.00 ±0.00	3.00 ±1.00
6	4:6	1.50 ±0.50	4.00 ±0.00	4.00 ±0.00
7	3:7	2.00 ±0.00	4.00 ±0.00	4.00 ±0.00
8	2:8	2.00 ±0.00	4.00 ±0.00	4.00 ±0.00
9	1:9	2.00 ±0.00	4.00 ±0.00	4.00 ±0.00

Table G8. Mean combination MIC values of *R. rubiginosa* fixed oil in combination with *S. austrocaledonicum* essential oil against the three reference strains.

Isobologram plot number	Volume ratio *S. austrocaledonicum*: *R. rubiginosa*	Mean combination MIC values (mg/mL)		
		C. acnes	*S. epidermidis*	*S. aureus*
1	9:1	0.13 ±0.00	0.19 ±0.06	0.19 ±0.06
2	8:2	0.13 ±0.00	0.22 ±0.05	0.25 ±0.00
3	7:3	0.16 ±0.05	0.38 ±0.13	0.44 ±0.11
4	6:4	0.19 ±0.06	0.38 ±0.13	0.88 ±0.22
5	5:5	0.25 ±0.00	1.00 ±0.00	1.00 ±0.00
6	4:6	0.25 ±0.00	1.00 ±0.00	1.75 ±0.43
7	3:7	0.25 ±0.00	1.50 ±0.50	2.50 ±0.87
8	2:8	0.50 ±0.00	3.00 ±1.00	4.00 ±0.00
9	1:9	1.00 ±0.00	4.00 ±0.00	4.00 ±0.00

APPENDIX H

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Shivani Ramburrun - Dissertation Final - 21.04.2022 (report)

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