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Research Article

In vitro bio-activity of *Rosa rubiginosa* L. (sweet briar) rosehip fixed oil combined with essential oils for acne vulgaris

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Abstract
Cutibacterium acnes, *Staphylococcus epidermidis*, and *Staphylococcus aureus* proliferate within accumulated sebum and produce localised inflammation thereby inducing acne vulgaris. Essential oils are one of the leading natural products used for acne and are diluted in fixed oils to reduce irritation and toxicity. This study investigated the *in vitro* antimicrobial, anti-inflammatory, cytotoxicity, and skin retention properties of *Rosa rubiginosa* rosehip fixed oil in combination with essential oils having activity against acne vulgaris. The broth microdilution assay was used to determine the minimum inhibitory concentration (MIC), and the sum of the fractional inhibitory concentration index (ΣFIC) was calculated for the interactions. The lipopolysaccharide (LPS) induced inflammatory pathway in RAW 264.7 macrophages was used to assess anti-inflammatory activity. A dual-staining method in A549 epithelial cells was used to assess cytotoxicity. Qualitative Fourier Transform Infrared spectroscopy analysis was used for initial skin retention determination. *Rosa rubiginosa* oil combined with *Cinnamomum zeylanicum* essential oil displayed antimicrobial synergy (MIC = 0.42 mg/mL; ΣFIC = 0.45) against three acne-inducing strains. *Rosa rubiginosa* fixed oil combined with *Styrax benzoin* essential oil suppressed LPS-induced nitrite production by 33.80%. *Rosa rubiginosa* oil combined with *Melaleuca quinquenervia* essential oil exhibited the lowest cytotoxicity (84.32% cell viability). *Rosa rubiginosa* oil combined with either *C. zeylanicum*, *Eugenia caryophyllata*, *Rosa damascena* or *S. benzoin* essential oil was retained within the porcine skin. *Rosa rubiginosa* fixed oil serves as an ideal carrier oil that can potentiate selected essential oils for the treatment of acne vulgaris.

Keywords
Rosa rubiginosa, Essential oils, Acne, Antimicrobial, Anti-inflammatory, Cytotoxicity

INTRODUCTION

Acne is the eighth most prevalent disease worldwide, appearing throughout adolescence and frequently continuing into adulthood[1,2]. Acne vulgaris is a chronic inflammatory condition of the pilosebaceous unit[1]. The pilosebaceous unit, consisting of a hair follicle and its associated sebaceous gland, secretes sebum to maintain the *stratum corneum* and hair shafts[3]. Hyperkeratinisation and excessive holocrine sebum secretion lead to the obstruction of the follicular ducts. The obstruction of the follicular ducts creates an ideal growth environment for *Cutibacterium acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus*[4]. These pathogens proliferate within the accumulated sebum and produce localised inflammation[3].

Anti-acne treatments may consist of various topical and systemic preparations intended to reduce one or more of the following pathophysiological mechanisms of the disease; androgen-mediated sebum production, abnormal keratinisation, bacterial proliferation, and inflammation[5]. Although the general prognosis of acne is positive with a combination of topical and systemic

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treatments, the harsh adverse effects causing discomfort, complicated medication regimens, and high costs tend to lower compliance[6]. Most antibiotic acne treatments are used for both their antimicrobial and anti-inflammatory effects[5], however, 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, is emphasised. Essential oils have a promising directive for treating acne whereby approximately 49 different essential oils have been recommended for dermatological application[7-11].

Essential oils, however, are rarely applied undiluted, directly onto the skin due to potent skin irritancy[12]. They are combined within a base, such as a fixed or carrier oil, prior to application as a means of dilution to subsequently reduce toxicity and skin irritation. Fixed oils are believed to reduce the evaporation and enhance the cutaneous absorption of essential oils[13]. Fixed oils may also augment therapeutic efficacy of essential oils by moisturising, nourishing, and healing the skin[14]. Topical delivery systems provide localised delivery rather than systemic delivery, as opposed to transdermal delivery systems which permeate through the skin into the systemic circulation. Subsequently, topical skin treatments require high skin retention and minimal permeation into the systemic circulation to limit systemic side effects[15]. Since acne vulgaris is an inflammatory skin condition affecting the pilosebaceous unit, effective anti-acne treatment necessitates localised skin retention to avoid systemic permeation and side effects[16].

Literature reveals that 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 antimicrobial and anti-inflammatory activities[17,18]. *Rosa rubiginosa* L. (sweet briar), 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[19]. *Rosa rubiginosa* rosehip oil is a fixed oil comprising of high compositions of polyunsaturated fatty acids (encompassing linoleic, linolenic, and oleic acids), and as such, may offer relatively high protection against inflammation[20,21]. Clinical studies by van der Walt[22], found beneficial results including improved skin moisture levels, antiwrinkle and visco-elastic properties of *R. rubiginosa* fixed oil. The authors[22], concluded that *R. rubiginosa* fixed oil may be considered as a potential, valuable cosmetic constituent for the enhancement of healthy skin aesthetics.

With the global trend towards the use of naturally-occurring products[23], fixed and essential oil combinations offer an alternative and viable approach to discovering modern agents for anti-acne therapy. Noting that fixed oils contribute to a crucial aspect in dermatology, further research concerning their interaction with essential oils are necessary[24]. Despite literature profiling various *Rosa* species inciting notable antimicrobial and anti-inflammatory activities[21,25], there are no recent studies, other than that of the authors, concerning *R. rubiginosa* fixed oil as a carrier oil for essential oils[26]. Neither is there any acne research of this fixed oil in combination with essential oils. Hence, this study aimed to investigate the *in vitro* antimicrobial, anti-inflammatory and associated cytotoxicity, as well as the skin retention properties of *R. rubiginosa* fixed oil independently and in combination with essential oils to find promising oil combinations to topically treat the pathophysiological mechanisms of acne vulgaris.

Materials and Methods

Oil selection and procurement

Cold-pressed *Rosa rubiginosa* was donated by Wynand Gericke (Rosehip Farm, Gauteng, South Africa) and the certificate of analysis {fixed oil 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} supplied. Thirty steam distilled essential oils were selected for this study based on previous antimicrobial dermatological research within our research group[27-29]. 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[27,28,30]. Table S1 provides a list of the selected essential oils including a summary of the chemical profiles as previously reported.

Antimicrobial analysis

Minimum inhibitory concentration (MIC) assay

The American Type Culture Collection (ATCC) reference strains of *Cutibacterium acnes* (ATCC® 11827™), *Staphylococcus epidermidis* (ATCC® 12228™) and *Staphylococcus aureus* (ATCC® 25923™) were obtained from Davies Diagnostics (Pty) Ltd. *Cutibacterium acnes* was inoculated into Thioglycolate broth (TGB) (Oxoid) and incubated under 5% CO_2 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. 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.

Cold-pressed *Rosa rubiginosa* oil and the essential oils were tested independently, and then in 1:1 combinations (fixed oil: essential oil) using the broth microdilution assay[28]. The test samples (100 µL of cold-pressed *R. rubiginosa* oil or essential oil, or 50 µL of *R. rubiginosa* with 50 µL of essential oil for combinations) were serially diluted in acetone to achieve concentrations ranging from 8.00-0.063 mg/mL. The respective broths (100 µL) were included for the 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®), the standard antibiotics used for acne treatment, were included as positive controls (100 µL). Sterile water in acetone was included as a negative control. An approximate inoculum concentration of 1×10^6 colony forming units (CFU)/mL was prepared for each micro-organism and 100 µL was added to each well. After incubation, 40 µL of 0.04% w/v *ρ*-iodonitrotetrazolium violet solution (INT) (Sigma-Aldrich®) was added to each well, where the lowest concentration displaying no colour change was taken as the MIC value. An MIC value of ≤ 1.00 mg/mL was considered noteworthy activity and an MIC value of > 1.00 - ≤ 2.00 mg/mL was considered moderate activity[29].

Fractional inhibitory concentration index (ΣFIC)

The interactive profiles of the combinations were assessed by calculating the fractional inhibitory concentration index (ΣFIC)[31] as follows:

$$\text{FIC(i)} = \frac{\text{MIC value of } \textit{R. rubiginosa} \text{ in combination with essential oil}}{\text{MIC value of } \textit{R. rubiginosa} \text{ oil independently}}$$

$$\text{FIC(ii)} = \frac{\text{MIC value of essential oil in combination with } \textit{R. rubiginosa}}{\text{MIC value of essential oil independently}}$$

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

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[31]. Cold-pressed *Rosa rubiginosa* oil with essential oil combinations displaying antimicrobial synergy or an overall antimicrobial additive interaction were selected for further investigation (varied ratio studies, anti-inflammatory and associated cytotoxicity, as well as skin retention properties).

Varied ratio combinations

The 1:1 combinations that exhibited synergy against the reference strains were further investigated to determine their interactive profiles at various ratios. The concentration of the varied ratios were 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 where the ΣFIC calculated was presented graphically on an isobologram. Data points falling beneath and inclusive of the 0.5:0.5-line represent synergy. Data points between the 0.5:0.5 and 1:1-lines displayed additive interactions. 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[31].

Anti-inflammatory study and associated cytotoxicity

Materials and procurement

RAW 264.7 murine macrophages and A549

human epithelial cells were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), 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).

Anti-inflammatory and MTT assays

Nitric oxide (NO) inhibition was investigated using the LPS-induced inflammatory pathway in a RAW 264.7 murine macrophage model[32]. Changes in NO production were determined by measuring the concentration of nitrite (NO_2^-) in the culture medium. The RAW 264.7 macrophages were cultured in RPMI 1640 supplemented with 10% v/v FBS in a humidified, 5% CO_2 incubator at 37°C. The 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. To assess nitrite production as a parameter of macrophage activation and anti-inflammatory activity, 50 µL of 1 µg/mL LPS in RPMI complete medium was added to all wells except for the untreated cells (culture control), which received 50 µL RPMI complete medium. Samples were prepared in absolute dimethyl sulfoxide (DMSO) to a concentration of 0.02% v/v. 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 ambient 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 untreated cells, LPS, and aminoguanidine. Increased nitrite production (compared to the untreated cells and LPS control) was generally related to lower anti-inflammatory activity of the test samples, whereas decreased nitrite production (compared to the untreated cells 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, a 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 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 as a contributory factor[32]. 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.

Cytotoxicity assay

The cytotoxicity of cold-pressed *R. rubiginosa* oil and the selected essential oils, independently and in 1:1 combinations, was assessed in A549 epithelial cells. A549 epithelial cells were cultured in DMEM supplemented with 10% v/v FBS in a humidified, 5% 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 and then incubated for 24 hrs. Samples were prepared to a concentration of 0.02% v/v. The attached A549 epithelial cells were treated with 0.01% v/v of test sample diluted in culture medium. The untreated cells

were 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. After a further 48 hrs incubation, 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) was added. Thereafter, A549 epithelial cells were stained with PI (100 µg/mL). Epithelial cells (A549) 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[33]. Results were expressed as a percentage of the untreated cells.

Initial skin retention determination via qualitative Fourier Transform Infrared spectroscopy (FTIR) analysis

Skin retention experiment

Porcine ears, 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[34]. The skin was cut into 2 cm × 2 cm sized squares[35]. An ethics waiver certificate for the use of porcine skin was obtained from the University of the Witwatersrand Animal Research Ethics Committee, reference number: 09-04-2020-O.

The skin retention of cold-pressed *R. rubiginosa* oil and the selected essential oils, independently and in 1:1 combinations, in porcine ear skin was determined via qualitative FTIR analysis. The skin retention experiment was adapted from the skin penetration and accumulation studies[35]. Prior to the experiment, porcine ear skin samples were washed and conditioned in phosphate-buffered saline (PBS) solution, pH 7.4, at ambient temperature. To remove excess moisture, the skin samples were blotted dry, and were then placed in 25 mL glass ointment jars. The test samples (100 µL of undiluted cold-pressed *R. rubiginosa* oil or undiluted essential oil, or 50 µL of undiluted *R. rubiginosa* oil with 50 µL of undiluted essential oil for 1:1 combinations) were applied to the epidermis and tested in triplicate (n = 3). The essential oil constituents, cinnamaldehyde and eugenol, have previously been shown to be absorbed by the skin[36,37], thus were included as positive controls (100 µL, undiluted). Untreated skin samples were included as a blank reference (negative control). After 24 hrs of incubation, the skin samples were then placed in 400 µL of absolute methanol (ACE Chemicals) to extract the test samples that were retained within the skin. The skin samples were macerated for extraction at ambient temperature for a further 24 hrs.

Qualitative FTIR analysis

The qualitative FTIR analysis, used to detect the skin retention of the test samples, was adapted from the skin penetration and accumulation studies[35]. After 24 hrs of extraction in methanol, the solvent was collected and centrifuged at 3500 rpm for 10 min to separate the extracted test samples from the skin particles. The supernatant (containing the extracted test samples) was collected, vortexed and then scanned within the 4000-650 cm^{-1} wavelength range using a Spectrum™ 100 FTIR Spectrometer (PerkinElmer®). Fourier Transform Infrared spectra averaging 20 scans were obtained for the extracted test samples, controls, and undiluted reference samples. The peak positions (wavenumbers) and intensities (% transmittance) of the scanned samples were determined using the PerkinElmer® Spectrum (Version 10.4.1) software. The background spectrum of air was subtracted from the spectra of the scanned samples.

The mean FTIR spectrum (n = 3) of the test samples was calculated and compared to two references: the untreated skin (blank) and the undiluted oil sample[35]. Specifically, the five most intense % transmittance peaks (identified by their corresponding wavenumbers) in the FTIR spectrum of the undiluted oil reference samples were used to determine the presence of the test samples in the extraction solvent from the skin. This served as a qualitative indicator of skin retention of the test samples.

Statistical analysis

For the MIC, anti-inflammatory and cytotoxicity analysis, each sample and combination were tested in quadruplicate. Data including the MIC values, percentage nitrite produced, and percentage cell viabilities were expressed as mean values with ±standard deviation. Data was entered into Microsoft Excel Office 365 (Version 2202) software. The isobolograms were constructed using GraphPad Prism® software version 5.0 (GraphPad Prism Software, CA, USA)

Results and Discussion

Antimicrobial analysis

The antimicrobial efficacy of cold-pressed *R. rubiginosa* oil and 30 essential oils, independently and in 1:1 combinations, against the reference strains of three acne-inducing pathogens was investigated (Table 1 and Table S2). Attention was given to the 1:1 oil combinations that displayed either synergy or additive interactions against the three reference strains (mean ΣFIC ≤ 1.0) (Table 1). *Rosa rubiginosa* oil independently displayed moderate antimicrobial activity against *C. acnes* and poor antimicrobial activity against *S. epidermidis* and *S. aureus*. This was expected as cold-pressed oils typically do not exhibit noteworthy antimicrobial activity[24,29].

Like the present study, the essential oil of *Cinnamomum zeylanicum* Blume (true cinnamon) has previously demonstrated antimicrobial activity against *C. acnes*, *S. epidermidis*, and *S. aureus*[38-40]. The antimicrobial activity of *Eugenia caryophyllata* Thunb. (clove) essential oil was reported against multiple strains of *S. epidermidis* and *S. aureus*[41]. When tested at higher concentrations (75.00-100.00% v/v) 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 treating skin and soft tissue infection[42]. Similar to the present study, another study[30] reported overall poor antimicrobial activity of *Kunzea ericoides* (A. Rich.) Joy Thomps. (kanuka) essential oil against *C. acnes*, *S. epidermidis*, and *S. aureus*. *Melaleuca quinquenervia* (Cav.) S.T. Blake (niaouli) essential oil showed promising antimicrobial activity against *C. acnes* and *S. epidermidis* when formulated in a topical anti-acne treatment[43]. In addition, promising antimicrobial activity of *M. quinquenervia* essential oil against *S. aureus* has been reported in previous studies[44,45]. In contrast to the present study, *Melissa officinalis* L. (lemon balm) essential oil exhibited noteworthy antimicrobial activity against *S. epidermidis* (MIC = 1.00 mg/mL)[46] and *S. aureus* (MIC = 0.12 mg/mL)[47]. The antimicrobial activity of *Rosa damascena* Herrm. (Damask rose) 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*[48,49]. The topical application of *Styrax benzoin* Dryand. (Gum Benjamin) essential oil to treat acne has been described in several reviews[50,51]. Compared to the present study, *S. benzoin* essential oil displayed noteworthy antimicrobial activity against *C. acnes* (MIC = 1.00 mg/mL) and moderate antimicrobial activity against *S. aureus* (MIC = 2.00 mg/mL)[28,52]. Extensive literature exists elucidating the antimicrobial efficacy of essential oils; however, there is a paucity of research specifically investigating the antimicrobial properties of cold-pressed *R. rubiginosa* oil in combination with essential oils.

Of the synergistic or additive 1:1 oil combinations, the combination of *R. rubiginosa* oil with *C. zeylanicum* essential oil lowered the MIC from 1.00 mg/mL (*C. zeylanicum* essential oil independently) to 0.50 mg/mL (1:1 combination) against *S. epidermidis* and *S. aureus* (Table 1). This combination displayed an additive interaction (ΣFIC = 0.73) against *C. acnes*; and synergy (ΣFIC = 0.31) against *S. epidermidis* and *S. aureus*, with a mean synergistic interaction (ΣFIC = 0.45) across all three reference strains (Table 1). Thus, the favourable antimicrobial synergy of cold-pressed *R. rubiginosa* oil in 1:1 combination with *C. zeylanicum* essential oil was considered ideal for inhibiting the bacterial pathogens responsible for acne vulgaris.

Cold-pressed *Rosa rubiginosa* oil combined with *C. zeylanicum* essential oil, displaying

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

Sample	Mean MIC values (mg/mL) and ΣFIC									Mean ΣFIC[3]
	C. acnes (ATCC® 11827™)			*S. epidermidis* (ATCC® 12228™)			*S. aureus* (ATCC® 25923™)			
	Independent MIC[1]	Combination MIC[2]	ΣFIC	Independent MIC	Combination MIC	ΣFIC	Independent MIC	Combination MIC	ΣFIC	
Rosa rubiginosa L. (sweet briar)	2.00 ±0.00	-[4]	-	4.00 ±0.00	-	-	4.00 ±0.00	-	-	-
Cinnamomum zeylanicum Blume (true cinnamon)	**0.19 ±0.06**[5]	**0.25 ±0.00**	*0.73*[6]	**1.00 ±0.00**	**0.50 ±0.00**	***0.31***[7]	**1.00 ±0.00**	**0.50 ±0.00**	***0.31***	***0.45***
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*
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*
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*
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*
Styrax benzoin Dryand. (Gum Benjamin)	**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*
Culture control	> 8.00 ±0.00	-	-	> 8.00 ±0.00	-	-	> 8.00 ±0.00	-	-	-
Benzoyl peroxide (positive control)	0.78 ±0.00	-	-	1.56 ±0.00	-	-	3.13 ±0.00	-	-	-
Ciprofloxacin (positive control)	6.25×10^{-4} ±0.00	-	-	6.25×10^{-4} ±0.00	-	-	6.25×10^{-4} ±0.00	-	-	-
Erythromycin (positive control)	0.39×10^{-4} ±0.00	-	-	1.25×10^{-3} ±0.00	-	-	1.25×10^{-3} ±0.00	-	-	-
Sterile water in acetone (negative control)	2.00 ±0.00	-	-	4.00 ±0.00	-	-	4.00 ±0.00	-	-	-

[1] Values for MIC of samples independently; [2] values for MIC of *R. rubiginosa* fixed oil combined with essential oils; [3] overall mean ΣFIC of the three reference strains; [4] not applicable (-); [5] noteworthy activity (MIC ≤ 1.00 mg/mL; bold); [6] additive interaction (ΣFIC > 0.5 - ≤ 1.0; italic); and [7] synergy (ΣFIC ≤ 0.5; bold, italic)

antimicrobial synergy, was further investigated in various ratios. Fig. 1 depicts the contributory effect of each of the varied ratios and Table S3 provides the exact oil concentrations for each combination represented as data points labelled 1-9 on the isobologram. The various ratio combinations of *R. rubiginosa* oil with *C. zeylanicum* essential oil indicated mostly additive interactions against *C. acnes* (Fig. 1a). When there was an increased proportion of the cold-pressed oil, this interaction shifted toward indifference. Of interest, was that a synergistic effect was observed only with the 1:1 combination against *S. epidermidis* (Fig. 1b) and *S. aureus* (Fig. 1c), therefore suggesting this to be the optimal ratio for the *R. rubiginosa* oil with *C. zeylanicum* essential oil combination. The main contribution to antimicrobial activity was provided by *C. zeylanicum* essential oil.

Six combinations (cold-pressed *R. rubiginosa* oil with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *M. officinalis*, *R. damascena*, or *S. benzoin* essential oil) displayed mean additive interactions (ΣFIC = 0.81-0.99) against all three acne-inducing reference strains (Table 1). Furthermore, *R. rubiginosa* oil combined

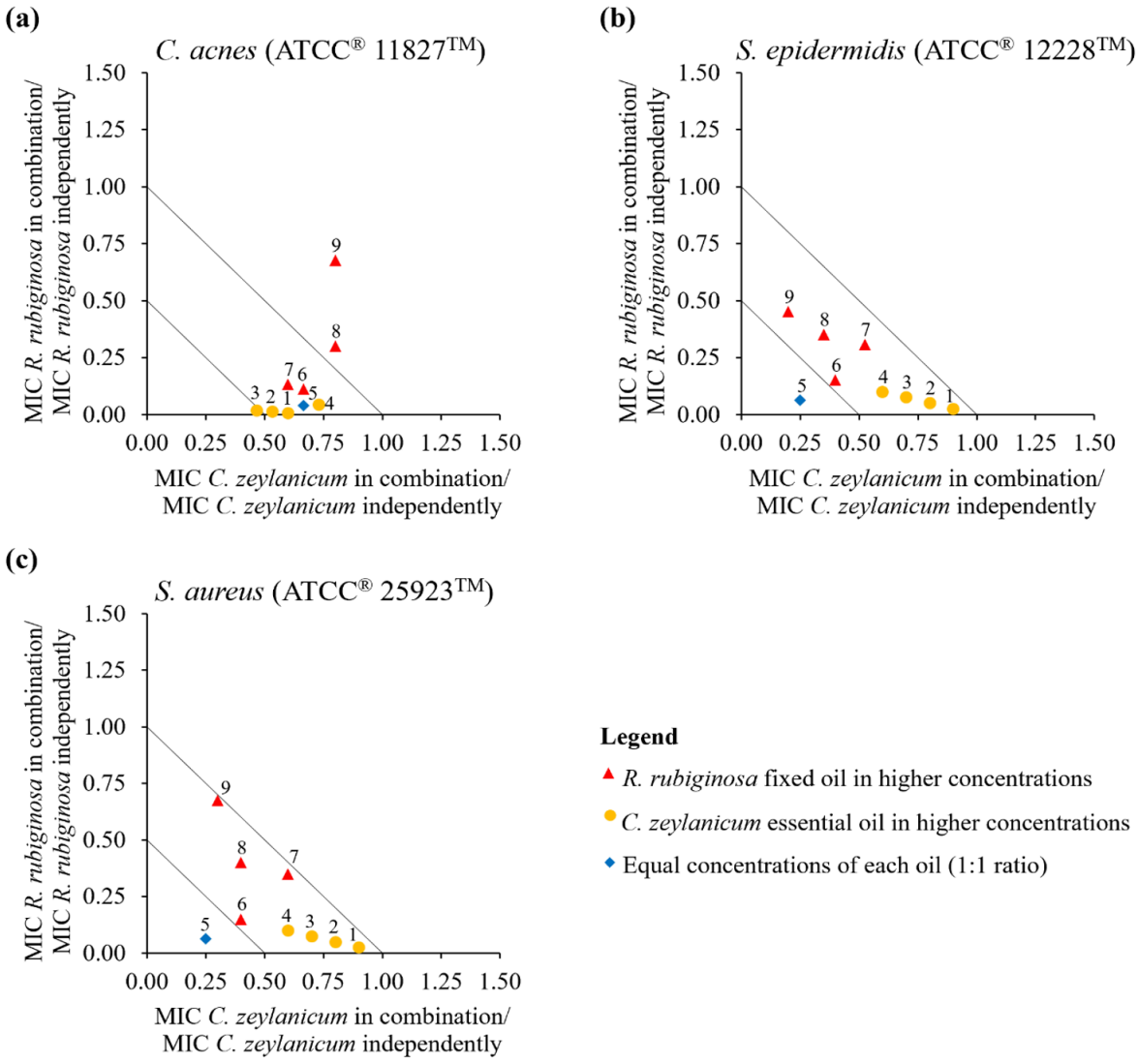


Figure 1. Isobologram representation of *R. rubiginosa* fixed oil in combination with *C. zeylanicum* essential oil against the reference strains of (a) *C. acnes*, (b) *S. epidermidis*, and (c) *S. aureus*

with *R. damascena* essential oil in a 1:1 ratio showed an additive interaction against each of the three acne-inducing reference strains. Cold-pressed *Rosa rubiginosa* oil in a 1:1 ratio with *C. zeylanicum* essential oil was considered the best antimicrobial combination as this combination exhibited antimicrobial synergy across all three reference strains. These combinations (six additive and one synergistic) were studied further in the anti-inflammatory, cytotoxicity, and skin permeation assays.

Anti-inflammatory effects and associated cytotoxicity

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 cold-pressed *R. rubiginosa* oil and the seven essential oils, independently and in 1:1 combinations (Fig. 2). To determine whether the cytotoxicity of the test samples affected nitrite production via the LPS-induced inflammatory pathway, cell viability was simultaneously evaluated using the MTT assay (Fig. 3). Therefore, the anti-inflammatory response (Fig. 2) should be considered together with the relative cell viability (Fig. 3).

Cold-pressed *Rosa rubiginosa* oil reduced LPS-induced nitrite production by 18.75% compared to the LPS control (Fig. 2). This anti-inflammatory effect may have been due to the high cell viability of *R. rubiginosa* oil (Fig. 3), which was similar to macrophages treated with aminoguanidine – a potent inhibitor of LPS stimulated inducible nitric oxide synthase (iNOS)[53]. The high content of polyunsaturated fatty acids (linoleic, linolenic, and oleic acids) present in the cold-pressed oil of various *Rosa* species is known to protect against inflammation[20,21]. Several *in vivo* studies have demonstrated the anti-inflammatory effect of *R. rubiginosa* oil[54,55]. Furthermore, an *in vivo* murine model revealed that cold-pressed *R. rubiginosa* oil reduced inflammation and induced macrophage proliferation when applied as a topical treatment[18]. A study, investigating a close relative of *R. rubiginosa*, showed that *Rosa canina* L. (dog rose) inhibited inflammatory NO in RAW 264.7 macrophages without impairing

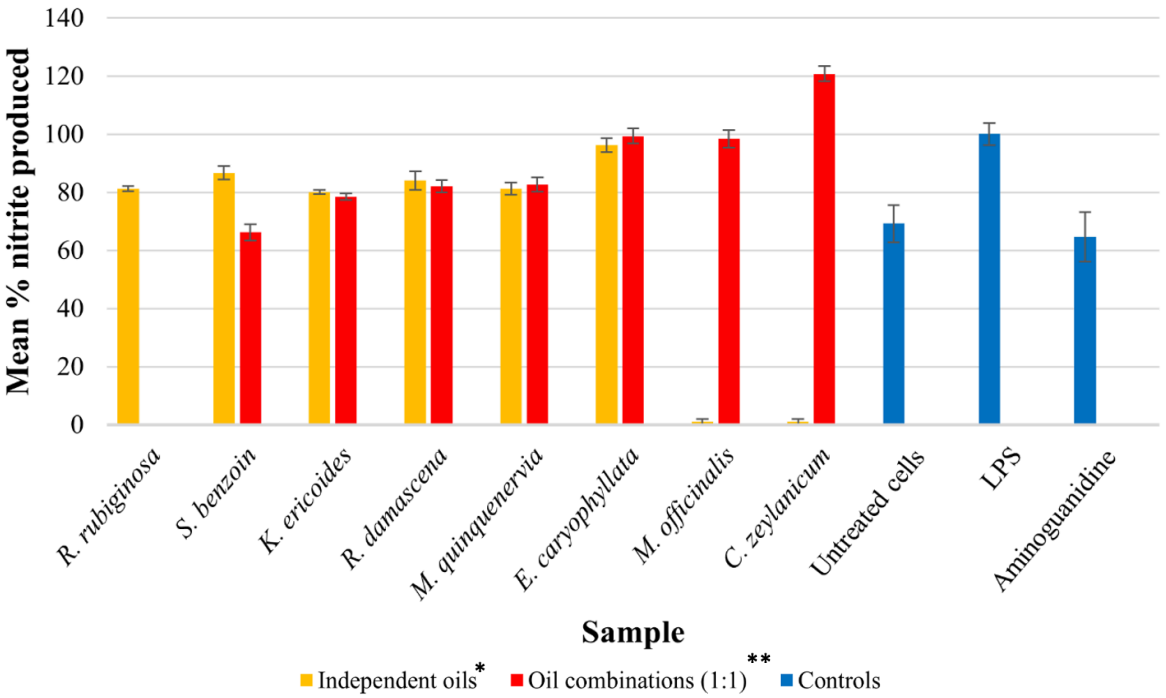


Figure 2. Mean % nitrite produced (n = 4) as a parameter of macrophage activation and anti-inflammatory response for test samples in RAW 264.7 macrophages after 24 hrs.
*Variance = 1342.34, ** Variance = 274.03

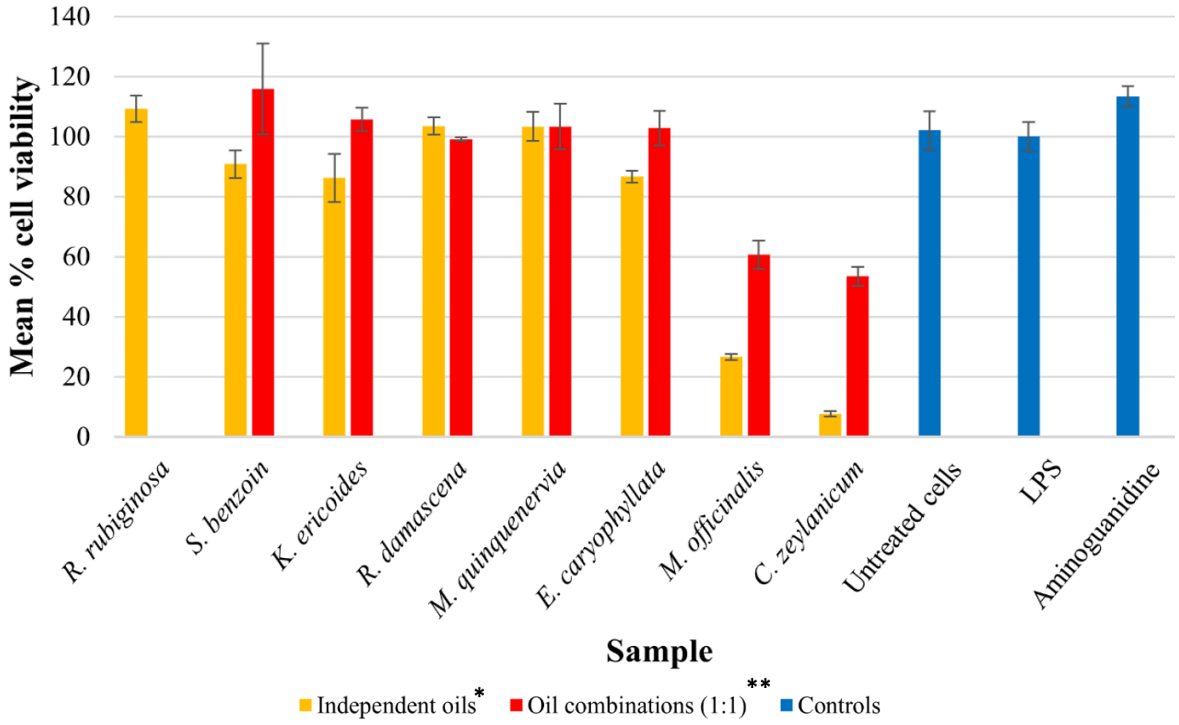


Figure 3. Mean % cell viability (n = 4) measuring cytotoxicity (as per the MTT assay) for test samples in RAW 264.7 macrophages after 24 hrs. *Variance = 1269.30, ** Variance = 503.79

cell viability[56]. A similar mechanism may explain the observation noted in the current study where *R. rubiginosa* oil inhibited nitrite production in RAW 264.7 macrophages, thereby encouraging macrophages to proliferate in the presence of reduced inflammation. *Rosa rubiginosa* oil is most commonly utilised in skin cosmetics for its regenerative and anti-inflammatory properties due to the high concentrations of polyunsaturated fatty acids[19,20].

Styrax benzoin essential oil was able to decrease nitrite production by 13.34% compared to the LPS control (Fig. 2). Although *S. benzoin* resins are commonly used in products to treat skin diseases such as irritated, dry, or inflamed skin[57], scientific evidence supporting anti-inflammatory activity, especially of *S. benzoin* essential oil, is lacking. The combination of cold-pressed *R. rubiginosa* oil with *S. benzoin* essential oil reduced nitrite production by 20.46% compared to the independent essential oil (Fig. 2), representing the best improvement in nitrite inhibition as a parameter of anti-inflammatory activity of the oil combination compared to the oils tested independently. Furthermore, cold-pressed *R. rubiginosa* oil in combination with *S. benzoin* essential oil produced the best improvement in cell viability, increasing RAW 264.7 macrophage proliferation by 25.06% compared to the independent essential oil (Fig. 3). This combination greatly reduced nitrite production and was non-cytotoxic.

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 (Fig. 2). Research[58] 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 application due to its anti-inflammatory activity and absence of adverse allergic reaction resulting from cytokine release[58]. *In vivo* studies have reported potent anti-inflammatory activity of *R. damascena*[59,60]

and *M. quinquenervia*[61,62] essential oils, yet cell studies using the LPS-induced inflammatory pathway in RAW 264.7 macrophages are limited. Cold-pressed *Rosa rubiginosa* oil combined in 1:1 ratio with either *K. ericoides* or *R. damascena* essential oil slightly increased the ability of the oil combinations to suppress LPS-induced nitrite production compared to the independent essential oils (Fig. 2). The combination of *R. rubiginosa* oil and *K. ericoides* essential oil improved cell viability by 19.45%, while that of the *R. rubiginosa* oil and *R. damascena* essential oil combination slightly decreased by 4.39%, compared to the essential oils tested independently (Fig. 3). *Rosa rubiginosa* oil combined with *M. quinquenervia* essential oil slightly decreased the ability of the oil combination to supress LPS-induced nitrite production compared to the independent essential oil (Fig. 2).

Eugenia caryophyllata essential oil slightly reduced LPS-induced nitrite production by 3.80% compared to the LPS control (Fig. 2). In contrast, a study using *E. caryophyllata* essential oil, described a significant increase in nitrite production, both in the absence or presence of LPS-induction in RAW 264.7 macrophages. Lang *et al.*[63] suggested that incubation time may influence iNOS regulation, however, both the current study and the study by Lang *et al.*[63] pre-treated RAW 264.7 macrophages for 24 hrs. The bio-activity of essential oils depends on their chemical constituents. As such, the variations in the chemotypes of *E. caryophyllata* essential oil could have caused differences in its bio-activity. The anti-inflammatory activity of *E. caryophyllata* essential oil has been observed in human dermal fibroblasts (HDF3CGF)[64], and in several *in vivo* models[65,66]. Cold-pressed *Rosa rubiginosa* oil combined with *E. caryophyllata* essential oil slightly decreased the ability of the oil combination to suppress LPS-induced nitrite production compared to the independent essential oil (Fig. 2).

Decreased nitrite production of the studied test samples 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. This was specifically noted with the anti-inflammatory response (Fig. 2) and cell viability (Fig. 3) of the independent essential oils: *M. officinalis* and *C. zeylanicum*. These two essential oils lacked nitrite production as the cell viability of the respective samples were very low at 26.59% and 7.62%, respectively (Fig. 2 and 3). The combinations of cold-pressed *R. rubiginosa* fixed oil with either *M. officinalis* or *C. zeylanicum* essential oil in a 1:1 ratio, improved cell viability by 34.09% and 45.75% respectively, compared to the independent essential oils (Fig. 3). As a result of improved cell viability, an LPS-induced inflammatory response became apparent, however, nitrite production of these oil combinations increased. 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 cold-pressed *R. rubiginosa* oil with the essential oil, *C. zeylanicum*. *Rosa rubiginosa* oil combined with *C. zeylanicum* essential oil increased nitrite production by 20.75% compared to the LPS control (Fig. 2), hence eliciting an inflammatory response within RAW 264.7 macrophages. *Rosa rubiginosa* oil combined with *M. officinalis* essential oil produced an inflammatory response like that of the LPS control (Fig. 2).

Cold-pressed *Rosa rubiginosa* oil combined with *S. benzoin* essential oil was considered the best anti-inflammatory combination as this combination inhibited nitrite production the most and exhibited the highest cell viability.

Epithelial cell (A549) cytotoxicity

The cytotoxicity results in A549 epithelial cells are represented in Fig. 4. The cell viability (as a percentage of the untreated cells) was determined after 48 hrs exposure to cold-pressed *R. rubiginosa* oil and the seven essential oils, independently and in 1:1 combinations. *Rosa rubiginosa* oil could be considered non-cytotoxic as cell viability (98.56%) was similar

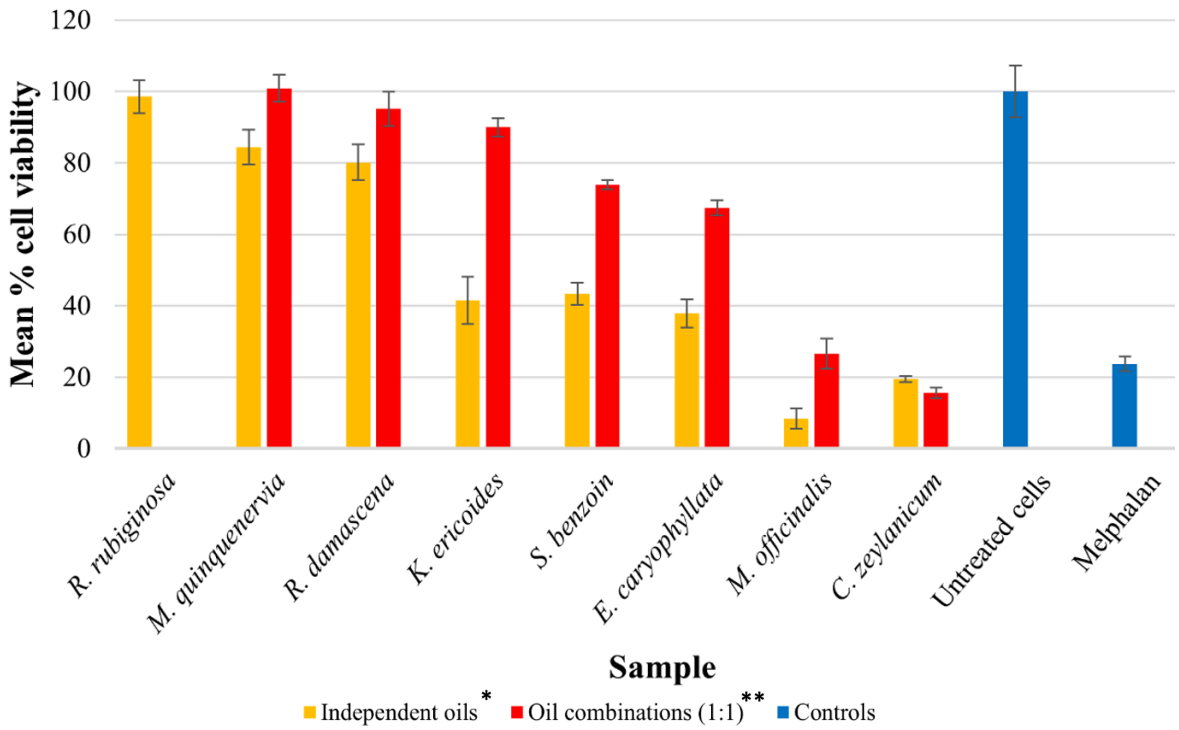


Figure 4. Mean % cell viability (n = 4) measuring cytotoxicity (as per the cytotoxicity assay) relative to the untreated cells (culture control) for test samples in A549 epithelial cells after 48 hrs. *Variance = 918.06, ** Variance = 969.70

to that of the untreated cells. The least cytotoxic essential oil was *M. quinquenervia* (84.32% cell viability) followed by *R. damascena* (80.13% cell viability) (Fig. 4).

Previous cytotoxicity studies investigating *M. quinquenervia* essential oil are lacking. In contrast, *R. damascena* essential oil demonstrated strong cytotoxicity in A549 epithelial cells using the MTT assay[37]. *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 for comparison. Research[67] 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 *M. officinalis* (8.42% cell viability) and *C. zeylanicum* (19.49% cell viability) (Fig. 4).

Compared to the independent essential oils, the 1:1 combinations of cold-pressed *R. rubiginosa* oil with either *M. quinquenervia* or *R. damascena* essential oil decreased cytotoxicity by 16.54% and 14.98%, respectively (Fig. 4). This resulted in a non-cytotoxic profile similar to that of the untreated cells. In this case, *R. rubiginosa* oil combined with either *M. quinquenervia* or *R. damascena* essential oil was non-cytotoxic and could support A549 epithelial cell growth. Compared to the independent essential oils, the 1:1 combinations of *R. rubiginosa* oil with either *K. ericoides*, *S. benzoin*, or *E. caryophyllata* essential oil reduced cytotoxicity by 48.41%, 30.52%, and 29.57%, respectively (Fig. 4). Although *M. officinalis* was the most cytotoxic essential oil tested in this study, the 1:1 combination of *R. rubiginosa* oil with *M. officinalis* essential oil lowered cytotoxicity by three-fold compared to the independent essential oil (Fig. 4).

Except for cold-pressed *R. rubiginosa* oil combined with *C. zeylanicum* essential oil which slightly increased the cytotoxicity, this study showed that *R. rubiginosa* oil combined with essential oils could substantially reduce the cytotoxic nature of most of the oil combinations. This effect may be mediated by the ability of *R. rubiginosa* oil, due to its high content of polyunsaturated fatty acids, to inhibit essential oil induced inflammation and cell death[20,68]. *Rosa rubiginosa* oil combined with *M. quinquenervia* essential oil was considered the best non-cytotoxic combination as this combination exhibited the highest cell viability.

Initial skin retention determination via qualitative FTIR analysis

Qualitative FTIR analysis was used to detect the skin retention of topically applied cold-pressed *R. rubiginosa* oil independently and in 1:1 combinations with each of the seven essential oils after 24 hrs, within porcine skin (Fig. 5 and 6, and Fig. S1-S7). *Rosa rubiginosa* oil (Fig. 5) and *C. zeylanicum* essential oil (Fig. 6a) independently, and in 1:1 combination (Fig. 6b), served as representative FTIR spectra and infrared profiles depicting the vibration transitions. The remainder of the samples are discussed here but for the sake of brevity, the figurative profiles are presented in Fig. S1-S7.

Fig. 5 displays the mean FTIR spectrum (n = 3) of *R. rubiginosa* oil extracted from the skin, compared to the infrared profile depicting the vibration transitions of the undiluted reference sample of *R. rubiginosa* oil. The infrared profile depicting the vibration transitions of the undiluted *R. rubiginosa* oil reference sample was then used as a baseline to identify whether the extraction solvent from the skin contained *R. rubiginosa* fixed oil. The five most intense transmittance peaks (identified by their corresponding wavenumbers) in the FTIR spectrum of the undiluted *R. rubiginosa* fixed oil reference sample were used to determine the presence of *R. rubiginosa* oil in the extraction solvent from the skin (Fig. 5). The bolded wavenumbers in the FTIR spectrum of the undiluted oil reference sample correspond to the % transmittance peaks present in the FTIR spectrum of the test sample applied to the skin. This served as a qualitative indicator of skin retention of the test sample. Conversely, non-bolded wavenumbers in the FTIR spectrum of the undiluted oil reference sample indicated the absence of the corresponding % transmittance peaks in the FTIR spectrum of the test sample applied to the skin. This, in turn, suggested a lack of skin retention of the test sample. Fig. S7a displays the mean FTIR spectrum (n = 3) of the untreated skin sample in methanol. This infrared

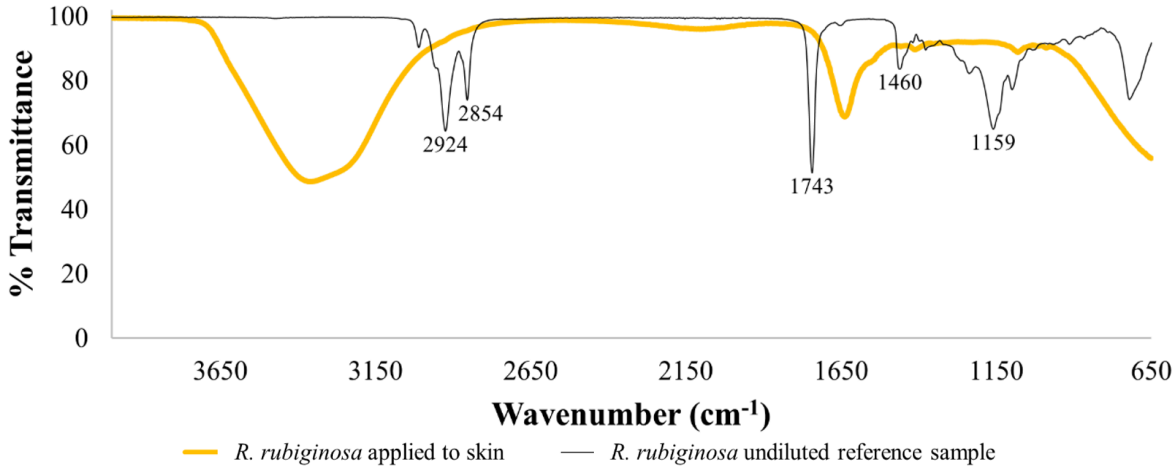


Figure 5. Mean FTIR spectrum (n = 3) representing the infrared profile depicting the vibration transitions of *R. rubiginosa* fixed oil extracted from the skin, compared to the spectrum of the undiluted oil reference sample

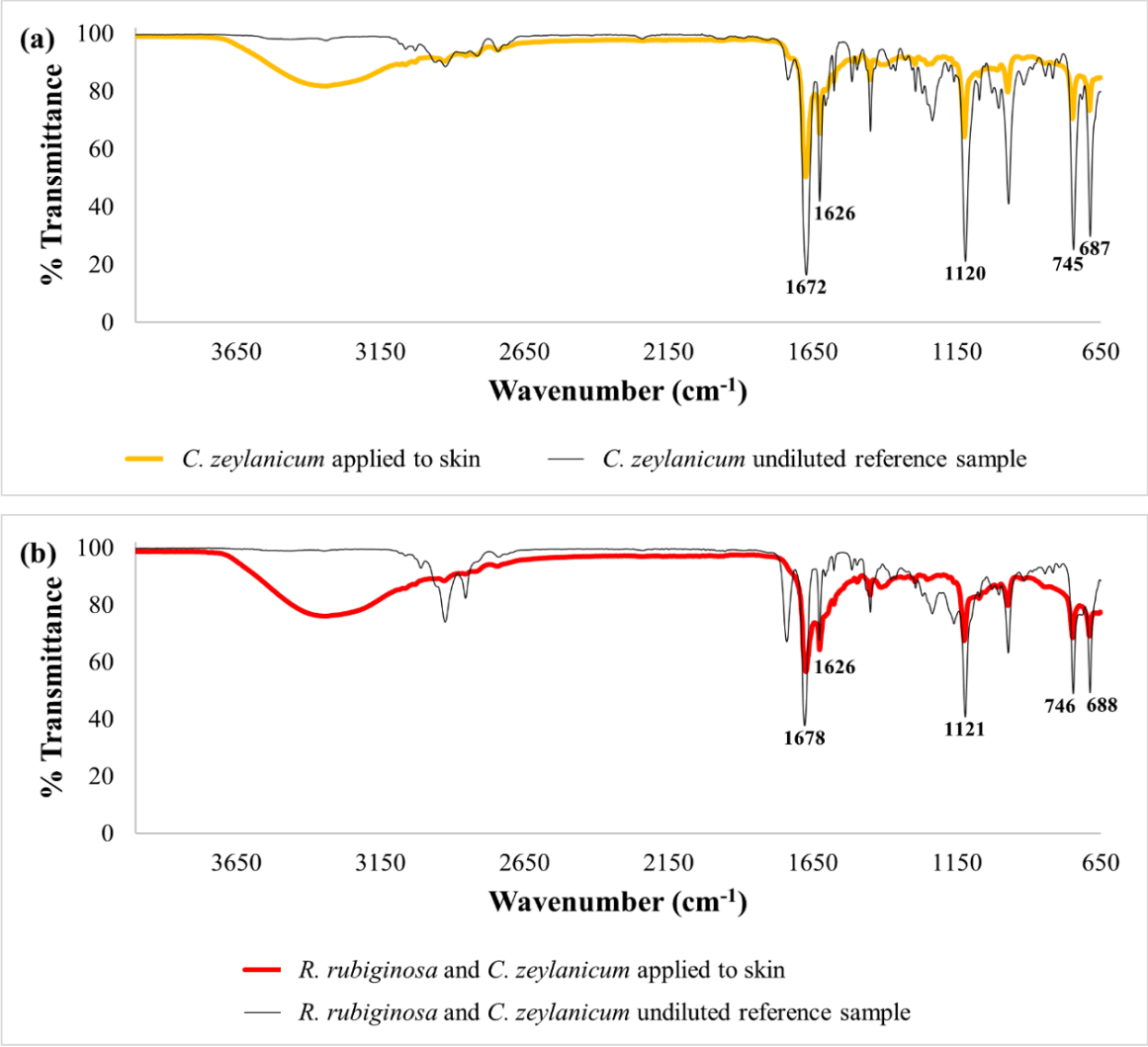


Figure 6. Mean FTIR spectrum (n = 3) representing the infrared profile depicting the vibration transitions of (a) *C. zeylanicum* essential oil independently and (b) *R. rubiginosa* fixed oil and *C. zeylanicum* essential oil in 1:1 combination extracted from the skin, compared to the spectrum of the respective undiluted oil reference samples

profile depicting the vibration transitions served as a reference since a similar spectrum was observed for the test samples which were not detected in the extraction solvent from the skin. The same process was applied for all the test samples in Fig. 6 and Fig. S1-S7.

The infrared profile depicting the vibration transitions of the cold-pressed *R. rubiginosa* oil was not detected in the extraction solvent from the skin samples (Fig. 5). The five most intense % transmittance peaks in the FTIR spectrum of the undiluted oil reference sample were absent in the FTIR spectrum of the test sample applied to the skin (Fig. 5), thereby qualitatively indicating that *R. rubiginosa* oil was not retained within the skin. In comparison, an *in vivo* study demonstrated that various fixed oils mostly remain at the surface of the skin, without deep penetration into the *stratum corneum*, when topically applied[67]. Moreover, Bos and Meinardi[69] argued that the molecular weight of a compound must be < 500 Dalton to penetrate the *stratum corneum* and allow skin absorption ("500 Dalton Rule"). As such, the skin penetration of high molecular

weight molecules remains a challenge[70,71]. *Rosa rubiginosa* fixed oil contains high concentrations of polyunsaturated fatty acids which may contribute to an overall high molecular weight (> 500 Dalton) that may hinder skin penetration. Dryness and dehydration of the skin stimulate sebum production, consequently exacerbating the condition of acne-prone skin[72]. The topical application of cold-pressed *R. rubiginosa* oil may rather offer an occlusive effect forming a protective skin barrier to retain moisture and lower transepidermal water loss, as do various other fixed oils[20,73]. By preventing dryness and dehydration of the skin, the occlusive effect of fixed oils (such as *R. rubiginosa* oil) may be beneficial for acne-prone skin[72].

The infrared profiles depicting the vibration transitions of *C. zeylanicum* (Fig. 6), *E. caryophyllata* (Fig. S1), *S. benzoin* (Fig. S2), and *R. damascena* (Fig. S3) essential oils were detected in the extraction solvent from the skin samples, when tested independently and in combination with cold-pressed *R. rubiginosa* oil. Two or more of the five most intense % transmittance peaks in the FTIR spectra of the undiluted oil reference samples were present in the FTIR spectra of the test samples applied to the skin, thereby qualitatively indicating that these essential oils and oil combinations were retained within the skin. *Cinnamomum zeylanicum*, *E. caryophyllata*, *S. benzoin*, and *R. damascena* essential oils contain a mixture of low molecular weight (< 500 Dalton) chemical constituents which may more readily penetrate and be retained within the skin. Fixed or cold pressed oils are the commonly used base in aromatherapy, reduce evaporation and enhance the cutaneous absorption of essential oils[13]. Thus, *R. rubiginosa* oil may reduce the evaporation of the essential oils when in combination. This in turn increases skin retention of these essential oils to potentially treat the pathogenesis of acne vulgaris when topically applied. The cold-pressed *R. rubiginosa* oil and *R. damascena* essential oil combination may be retained within the skin (Fig. S3b), albeit to a lesser extent than *R. rubiginosa* oil combined with either *C. zeylanicum* (Fig. 6b), *E. caryophyllata* (Fig. S1b), or *S. benzoin* (Fig. S2b) essential oil tested in this study. This suggests that *R. damascena* essential oil may require further dilution in *R. rubiginosa* oil to improve skin retention and effectively treat the pathogenesis of acne vulgaris when topically applied.

The infrared profiles depicting the vibration transitions of *K. ericoides* (Fig. S4), *M. quinquenervia* (Fig. S5), and *M. officinalis* (Fig. S6) essential oils were not detected in the extraction solvent from the skin samples, when tested independently and in combination with *R. rubiginosa* oil. The five most intense % transmittance peaks in the FTIR spectra of the undiluted oil reference samples were absent in the FTIR spectra of the test samples applied to the skin, thereby qualitatively indicating that the essential oils and oil combinations were not retained within the skin. *Kunzea ericoides* and *M. quinquenervia* essential oils contain a mixture of low molecular weight (< 500 Dalton) chemical constituents which may penetrate the skin. This suggests that similar to *R. damascena* essential oil, these essential oils may require further dilution in *R. rubiginosa* oil to improve skin retention. *Melissa officinalis* essential oil contains a mixture of multiple (albeit low molecular weight) chemical constituents which may contribute to an overall high molecular weight (> 500 Dalton) that may hinder skin penetration. This suggests that the topical application of *M. officinalis* essential oil diluted in *R. rubiginosa* oil may rather offer an occlusive effect to effectively treat the pathogenesis of acne vulgaris.

Cold-pressed *Rosa rubiginosa* oil combined with either *C. zeylanicum* or *E. caryophyllata* essential oil were considered the best skin retention combinations as the five most intense % transmittance peaks in the FTIR spectra of the undiluted oil reference samples were present in the FTIR spectra of the test samples applied to the skin, serving as a qualitative indicator of skin retention.

In summary, Table 2 provides an overview of the antimicrobial interactions, anti-inflammatory activity, cytotoxicity, and skin retention properties of cold-pressed *R. rubiginosa* oil combined

Table 2. Summary of the properties of *R. rubiginosa* fixed oil combined with the selected essential oils as determined by the relative assays performed in this study

Essential oil	Antimicrobial interaction (ΣFIC analysis)	Anti-inflammatory activity (RAW 264.7 macrophages)	Cytotoxicity (RAW 264.7 macrophages)	Cytotoxicity (A549 epithelial cells)	Skin retention (FTIR analysis)
C. zeylanicum	Synergy	No	Cytotoxic	Cytotoxic	Yes
E. caryophyllata	Additive	No	Non-cytotoxic	Non-cytotoxic	Yes
K. ericoides	Additive	Yes	Non-cytotoxic	Non-cytotoxic	No
M. quinquenervia	Additive	Yes	Non-cytotoxic	Non-cytotoxic	No
M. officinalis	Additive	No	Cytotoxic	Cytotoxic	No
R. damascena	Additive	Yes	Non-cytotoxic	Non-cytotoxic	Yes
S. benzoin	Additive	Yes	Non-cytotoxic	Non-cytotoxic	Yes

with the seven essential oils that were identified as having the most promising antimicrobial interactions when tested in combination with *R. rubiginosa* oil. A favourable anti-acne profile of a topically applied oil combination would be one that displays antimicrobial synergy or additive interaction, effective suppression of LPS-induced nitrite production, non-cytotoxicity or improved cell viability, and retention within the skin. From the oil combinations studied, *R. rubiginosa* oil combined with either *E. caryophyllata*, *K. ericoides*, *M. quinquenervia*, *R. damascena*, or *S. benzoin* essential oil displayed three or more of these favourable properties (Table 2).

Conclusions

This study demonstrated that cold-pressed *R. rubiginosa* oil in combination with a selection of essential oils has potential in decreasing bacterial proliferation and inflammation involved in acne vulgaris. This study identified that the combination of *R. rubiginosa* 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 this combination represented the best improvement in nitrite inhibition as a parameter of anti-inflammatory activity, and decreased cytotoxicity. Furthermore, this combination was retained within the skin— a vital prerequisite for the topical treatment of acne vulgaris. *Rosa rubiginosa* oil combined with *R. damascena* essential oil (containing 29.90% of citronellol, 22.40% of phenelyl alcohol, 20.80% of geraniol, and 10.20% of nerol) followed closely, as this combination displayed antimicrobial additive interaction, reduced nitrite production, was non-cytotoxic, and was retained within the skin. The use of fixed or cold-pressed oils combined with essential oils (such as *R. rubiginosa* oil combined with either *S. benzoin* or *R. damascena* essential oil) for improved bio-activity and skin 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.

The skin retention 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 quantifying permeation, absorption, and retention involving fixed, cold-pressed or essential oils as active ingredients. Future research directions may include investigating the ability of cold-pressed *R. rubiginosa* oil independently and in combination with essential oils to promote re-epithelisation, reduce acne scar formation,

and for formulation development into a topical preparation for administration in *in vivo* studies. Another aspect worthy of further study is the role of phytoconstituents independently and in combination to further understand the synergistic and additive principles at play in these favourable combinations.

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Conflict of interest statement

Authors declare no conflict of interest.

Supplementary data

Figures S1 to S7 and Tables S1 to S3 are provided as supplementary data available online.

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