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Research Article

The antimicrobial potential of essential oils and their nanoparticulate formulations as effective antifungals

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Abstract
Fungal infections present a challenge when it comes to topical treatment failure thereby necessitating the need for more effective topical delivery systems. Essential oils have demonstrated noteworthy antifungal activity; however, their volatility limits therapeutic use for skin conditions. This study aimed to investigate the antifungal activity of essential oils alone and in combination. Thereafter, antifungal nanoparticulate formulations were designed using three essential oil combinations against *Microsporum canis*, *Trichophyton mentagrophytes* and *Candida albicans*. The antifungal activity was investigated using the broth microdilution assay to determine the minimum inhibitory concentration (MIC). The formulations were prepared by forming nanoparticles (polymer-based and composite lipid-polymer-based systems), with the preferred formulation encapsulating the essential oil combinations. Characterisation of the formulations was performed with a Zeta-sizer and using attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR). The *in vitro* antifungal activity of the three nanoparticulate formulations was then determined, where a significant ($p = 0.002$) increase in the average antidermatophyte activity could be observed for all three formulations. The combination of *A. sativum* and *C. martinii* displayed the lowest MIC value across all three reference strains (MIC values 0.06-0.38 mg mL^{-1}) and the formulation maintained the antifungal activity with MIC values ranging 0.08-0.36 mg mL^{-1}. A successful combination of essential oils with antifungal activity was achieved thereby emphasising an effective nanoparticulate formulation.

Keywords
Dermatophytes, Nanoparticulate formulations, Combinations, Essential oils, Synergy

INTRODUCTION

Some of the most frequently encountered infections are affiliated to superficial topical sites. Fungi are amongst the various pathogens that cause topical infections, and it has been estimated that approximately 20-25% of the global population is suffering from fungal skin infections[1]. Skin and nail fungal infections can be caused by two classes of fungi: namely yeasts and dermatophytes. *Candida albicans* is a skin commensal that can invade the skin and cause infection, particularly in immunocompromised individuals. It commonly causes infections within skin folds such as at the axilla (intertrigo), the groin (balanitis) and corners of the mouth (angular cheilitis) as well as nappy rash in infants[2]. In addition, it is the most common yeast to cause infections of the nail folds (onychomycosis)[3].

Dermatophytes, such as *Microsporum canis* and *Trichophyton mentagrophytes*, are found in keratinised tissues such as the skin, nails, and hair due to their ability to digest the keratin, which is essential for growth[2]. They are filamentous and cause infections such as *tinea pedis* (athlete's foot), *tinea capitis* (infection of the scalp), *tinea cruris* (infection of the upper thigh and groin),

and *tinea corporis* (infection of the body)[2,4]. The infection site is commonly erythematous, pruritic, and scaling[5]. Infections of the nail bed and plate, caused by *tinea inguinium* is also very common, as the nail has a decreased cell-mediated immunity, making it more susceptible to fungi[3].

Superficial infections can be treated with topical or oral agents. Topical agents are advantageous as they can limit side effects and allow for direct access to the site of infection. Topical agents include miconazole, clotrimazole, nystatin, and terbinafine[2]. *Tinea inguinium* and onychomycosis can specifically be treated topically with 5% amorolfine. However, due to factors such as slow nail growth and topical agents lacking effective nail penetrating ability, oral treatment is usually recommended in addition to topical treatment[3]. Oral treatments with terbinafine, griseofulvin, or itraconazole are used in severe cases[3]. A challenge arises for treatments lasting six weeks to three months as there is emerging resistance to the antifungal agents[2]. This is of particular concern for *Candida* spp., with developing resistance to both first- and second-line agents[4].

The emerging resistance, inadequate penetration of topical agents to nail infections, and the long treatment regimens have highlighted the need to identify alternatives to use in conjunction with and/or instead of current treatments. Essential oils have been identified as displaying antifungal activity[6]. They are predominantly used in combination and the antimicrobial and anti-candidal activity of these combinations are well reported[6]. Studies against *M. canis* and *T. mentagrophytes*, however, are lacking, especially when considering essential oil combinations. A limiting factor for the use of essential oils, however, is the volatile nature and poor distribution to target sites. Thus ideally, the delivery method of the essential oils needs to reduce evaporation of these volatile oils, while increasing stability of the formulation.

Nanoparticles are a means of overcoming these practical challenges by significantly improving the delivery of topical treatments, together with minimizing the dose and side effects[7]. Nanoparticles facilitate the delivery of volatile compounds, such as those found within essential oils. The nanosized particles between the ranges of 10-1000 nm provide a greater surface area which allows for improved permeability, solubility, absorption, and bio-availability of the drug molecule[7]. The most commonly employed nanoparticles for enhancing the stability, permeation and retention of drugs for topical and/or transdermal drug delivery include polymeric nanoparticles (10-1000 nm), nanoemulsions (20-200 nm) and lipid-based nanoparticles (<100 nm) (liposomes and solid-lipid nanoparticles)[8-10]. Furthermore, the volatile components of the essential oil are retained for a longer time, with slow release of compounds that can improve the availability of the essential oil over time, providing long-lasting antimicrobial activity[11]. The inclusion of a lipid component within the nanoparticles for enhancing the encapsulation of essential oils within a nanoparticulate system is a promising approach for further enhancing the antimicrobial activity and stability of essential oils[12]. Formulating essential oils into liposomal nanoemulsions assists in overcoming the issues of volatility and poor distribution to target sites. Encapsulation of the oil in a nanosystem reduces evaporation of these volatile oils, increases skin and nail penetration as well as increases stability and renders the oils less reactive to light, oxygen, and water, thereby enhancing efficacy[13]. In developing a nanoparticulate system, however, cognisance of their limitations and challenges is important, including scale-up considerations and assessment of the clinical impact of the preferred nanosystem[8].

Thus, this study aimed to investigate the antifungal activity of essential oils and their combinations, and thereafter formulate, and characterize three particle systems as topical antifungal formulations. The effect of inclusion of lipids within the system to form a composite lipid-polymer nanoparticulate system for enhancing the entrapment and permeation of the essential oil was also investigated.

Material and Methods

Essential oil selection

The selection of essential oils and combinations

was based on recommendations in the aromatherapeutic literature for fungal infections, and frequently mentioned combinations[14-23]. In total, 51 combinations were studied against the three skin fungal pathogens, totaling 153 combinations.

The essential oils were procured from international flavour and fragrance industries Givaudan© (Dübendorf, Switzerland), Robertet© (Grasse, France), Burgess and Finch, PranaMonde, Essentia, and Scatters Oils© (Gauteng, South Africa).

The chemical analysis of the essential oils was previously undertaken using Gas chromatography-mass spectrometry (GC-MS)[24].

Preparation of cultures

The micro-organisms were obtained from Davies Diagnostics (South Africa) and are from the American Type Culture Collection (ATCC). The dermatophytes, *T. mentagrophytes* (ATCC 9533) and *M. canis* (ATCC 36299) were grown and maintained in Sabourauds Dextrose agar and broth (Oxoid) or in Malt Extract broth (MEB) and incubated at 35°C for seven days at 100% relative humidity. *Candida albicans* (ATCC 10231) was grown in Tryptone Soya broth (TSB) (Oxoid) and incubated at 37°C for 48 hr. Each fungal reference strain was streaked onto their respective agar plates and incubated accordingly, to confirm purity. A waiver for the use of these micro-organisms was granted by the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-131026-3).

The minimum inhibitory concentration (MIC)

The broth microdilution method[13] was used to determine the minimum inhibitory concentration (MIC) of the essential oils, alone and in combination. First, 100 µL of appropriate media was aseptically added into all 96 wells of a micro-titre plate, followed by 100 µL starting concentration of 32.00 mg mL^{-1} of the essential oil diluted in acetone (or 50 µL of one essential oil and 50 µL of another essential oil for the combinations), into the first well. This was serially diluted. Antifungal susceptibility was confirmed using the positive control amphotericin B (starting concentration 0.10 mg mL^{-1}) (Sigma-Aldrich). A water in acetone negative control was included to determine whether the antimicrobial activity was due to the solvent. The respective growth media per fungal reference strain was also included to ensure culture viability. After the preparation of an approximate inoculum concentration of $1x10^6$ colony forming units per ml (CFU mL^{-1}) for each micro-organism, 100 µL was added to each well. Sterile adhesive sealing film was used to seal each micro-titre plate and the plates were incubated accordingly. After incubation, 40 µL of 0.04% (w/v) *p*-iodonitrotetrazolium violet solution (INT) (Sigma Aldrich®) was added to each well for *C. albicans*. Culture growth was indicated by a colour change of the indicator to pink, and the lowest concentration displaying no growth was taken as the MIC value. For the dermatophytes, macroscopic assessment of growth matching that of the culture control was used as the measure of growth or inhibition. Each sample was tested in triplicate and the mean MIC value calculated. An MIC value less than or equal to 1.00 mg mL^{-1} was considered as noteworthy inhibition[24].

The fractional inhibitory concentration index (ΣFIC) was calculated for the combinations[25] as follows:

$$\text{FIC (i)} = \frac{\text{Value of (a *) combined with value of (b *)}}{\text{Value of (a) independently}}$$

$$\text{FIC (ii)} = \frac{\text{Value of (b) combined with value of (a)}}{\text{Value of (b) independently}}$$

*Where (a) is the MIC value of the first essential oil sample in the combination and (b) is the MIC value of the second essential oil sample.

The FIC index was then calculated as ΣFIC = FIC (i) + FIC (ii). An ΣFIC of ≤ 0.5 was interpreted as synergy, an ΣFIC of between > 0.5 and ≤ 1.0 was indicative of an additive interaction, > 1.0 - ≤ 4.0 indicated indifference and > 4.0 indicated antagonism[25].

Three combinations with the lowest average MIC values across all fungal reference strains were selected for formulation.

Pre-formulation material

The phospholipids i.e., cholesterol and distearoyl phosphatidyl choline (DSCP); organic solvents i.e., chloroform and methanol; deionized water

and polymers were used for formulation. All materials were obtained from Sigma-Aldrich.

Pre-formulation of essential oil liposomes

Pre-formulation studies were conducted with and without phospholipids using different polymers to determine the most suitable formulation. Polymer options include 5% solutions of Polyacrylic acid (PAA), Polyvinyl pyrrolidone (PVP), Polyvinyl alcohol (PVA), pectin and carrageenan[26].

Liposomes were prepared using the method adapted from van Vuuren, du Toit[13]. Prior to liposome formation, the phospholipids, i.e., distearoyl phosphatidyl choline (DSPC) (100 mg) and cholesterol (50 mg), were stored at 2-8°C and -20°C, respectively. The two phospholipids were then dissolved into an organic solvent phase of a 9:1 mL chloroform to methanol. Subsequently, 175 mg of the essential oil combination was also added to the organic solvent phase. Thereafter the aqueous phase, i.e., 5% polymer in deionized water, was added. The formulations were then placed onto a magnetic hotplate stirrer, at ambient temperature, for 1 hr (MKLab, South Africa) to remove the solvent. Quantitative estimation of the amount of EO incorporated within the preferred nanoparticulate system was undertaken employing FTIR spectroscopy following quantitative comparison between the spectra of the pure EO and the common peaks representing the EO in the final formulation[27].

The EO incorporation efficiency and the EO loading was determined via the following equations:

$$\text{EO Incorporation Efficiency (EOIE\%)} = \frac{\text{Actual quantity of EO in nanoparticle}}{\text{Total quantity of EO employed}} \times 100$$

$$\text{EO Loading (\%)} = \frac{\text{Actual mass of EO in formulation}}{\text{Total mass of formulation}} \times 100$$

Characterisation of the size and stability of the essential oil liposomes with identification of the final formulation

The size distribution, polydispersity index (PDI) and average diameter of the liposomes comprising the pre-formulation, a Zetasizer Nano ZS (Malvern Instruments Ltd. U.K.) was applied to characterise the formulations and to determine the surface charge and physical stability[13]. The final composition was identified based on optimal particle size, stability, and visual appearance and used for further studies. Zeta-potential indicates surface charge and physical stability of nanoparticles. A zeta-potential of ≥ +30 mV or ≤ -30 mV denotes acceptable stability and bio-availability. A value closer to 0 mV represents an unstable system where nanoparticles are more likely to agglomerate[28]. The size variability of the formulations was determined by the PDI[13]. A size variability less than 0.5 is indicative of a uniform nanoparticulate system.

Chemical characterisation of the final formulations

Three nanoparticulate systems were prepared, based on the preferred composition. Each contained essential oil combinations selected as described in the pre-formulation studies. It should be noted that the formulation of the nanoparticulate systems contained 175 mg each of the essential oil combinations with a ratio of 1:1 of each oil. In addition, a control formulation, containing no essential oil was also prepared. The formulation with the chosen polymer and all raw materials was characterised using ATR-FTIR spectroscopy with a single reflection diamond MIRTGS detector (PerkinElmer® Spectrum 100 Series FTIR Spectrometer, PerkinElmer Ltd., Beaconsfield, UK). This was undertaken to determine vibrational energy transitions and chemical bonds formed within the chosen formulation[29].

Antimicrobial analysis of formulations

The incorporation of essential oils into formulation has previously been shown to affect the release of the oil, depending on the formulation[30]. Thus, to determine whether the antifungal activity of the essential oils was maintained once in formulation, the antifungal activity was investigated for the formulations. The MIC of the nanoparticulate systems against *C. albicans*, *M. canis* and *T. mentagrophytes* was determined using the broth microdilution assay using the respective broths and incubation periods. The commercial product, Covarex®

(2% miconazole nitrate) was used as a positive control.

Statistical analysis

All antimicrobial studies were replicated and performed in triplicate on consecutive occasions. The average and standard deviation for the MIC values were calculated using Microsoft Excel Office 365. To statistically analyse the shifts in antifungal activity, the student T-Test was used to determine whether the shift was significant ($p \leq 0.05$), or not ($p > 0.05$).

Results and Discussion

Essential oil chemistry frequently varies according to the geographical location, species, harvest time and storage[31]. The chemical profiles for each essential oil has been previously reported[24] and is available in the supplementary data (Table S1).

The antimicrobial data of the 25 selected essential oils alone and in combination and the interactive profiles of the combinations that were investigated are presented in Table 1. *Microsporum canis* was the most susceptible of the fungal reference strains, with each essential oil inhibiting the micro-organism at noteworthy MIC values ranging between 0.13-0.69 mg mL^{-1}. *Candida albicans* was the more resilient of the fungal reference strains with MIC values ranging from 0.25-4.00 mg mL^{-1}. *Allium sativum* L. (garlic), *Cymbopogon martinii* Stapf (palmarosa) and *Santalum album* L. (sandalwood) were identified as the essential oils with the most noteworthy MIC values against the three reference strains (values ranging from 0.06-1.00 mg mL^{-1}). *Santalum album* has been highlighted previously for its noteworthy antimicrobial activity against a range of micro-organisms[24]. *Vetiveria zizanioides* Stapf (vetiver), *Commiphora myrrha* Engl. (myrrh), *Lavandula angustifolia* Bubani (lavender), *Citrus limon* (L.) Burm.f. (lemon), and *Pogostemon patchouli* Benth. (patchouli) also displayed strong antifungal activity against at least two tested strains (MIC values ranging from 0.06-0.13 mg mL^{-1}).

A total of 51 combinations were investigated against each of the fungal reference strains. Noteworthy activity ($\leq$ 1.00 mg mL^{-1}) across all three reference strains could be reported for 59% of the combinations. The dermatophytes *T. mentagrophytes* and *M. canis* were found to be more susceptible to the inhibition of the essential oil combinations than *C. albicans*. Two combinations, *A. sativum* with *C. limon* and *Cedrus atlantica* (Endl.) G.Manetti ex Carrière (cedarwood) with *Rosmarinus officinalis* (rosemary), displayed synergy against both dermatophytes.

Two combinations that have the lowest MIC values across the three fungal reference strains are *A. sativum* with *C. martinii* (MIC values 0.06-0.0.38 mg mL^{-1}), and *A. sativum* with *C. limon* (MIC values 0.11-0.44 mg mL^{-1}). The combination *Melaleuca alternifolia* Cheel (tea tree) with *S. album* is also highlighted. It is one of the combinations to display noteworthy antifungal activity against all three fungal reference strains, and has on average, the lowest MIC values against the two dermatophytes (MIC values 0.06 mg mL^{-1} against *M. canis* and 0.13 mg mL^{-1} against *T. mentagrophytes*). The essential oil combination of *M. alternifolia* and *S. album* has also previously been reported to display noteworthy MIC values against bacteria associated with wounds (*Pseudomonas aeruginosa*, *Staphylococcus aureus* including two methicillin-resistant *S. aureus* reference strains, and *Escherichia coli*) with MIC values ranging from 0.38-2.00 mg mL^{-1} [25], thereby adding to the potential benefit of preventing secondary bacterial infections. Based on these MIC results, the combinations *A. sativum* with *C. limon, A. sativum* with *C. martinii,* and *M. alternifolia* with *S. album* were selected for formulation development.

Various nanoparticulate systems incorporating preferred biopolymers as emulsifying agents were formulated (Table 2). Characterisation studies were conducted on the formulations with and without a lipid phase to identify their function in the formulation. Nanoparticulate system size and zeta potential analysis was undertaken initially to identify which biopolymer combination was best suited in terms of size and stability. The liposomes within the nanoparticulate system

Table 1. Antimicrobial activity (MIC in mg/mL) of essential oils and combinations against the three fungal reference strains (n=3)

Essential oil combinations		Mean MIC (mg/mL) (n=3) and ΣFIC											
		M. canis (ATCC 36299)				*T. mentagrophytes* (ATCC 9533)				*C. albicans* (ATCC 10231)			
Essential oil 1	**Essential oil 2**	**MIC1[a]**	**MIC2[a]**	**MIC[b]**	**ΣFIC[c]**	**MIC1[a]**	**MIC2[a]**	**MIC[b]**	**ΣFIC[c]**	**MIC1[a]**	**MIC2[a]**	**MIC[b]**	**ΣFIC[c]**
Allium sativum L. (garlic)	*Citrus limon* (L.) Burm.f. (lemon)[d]	**0.25**	**0.53**	**0.11**	***0.39***	**0.38**	**0.38**	**0.13**	***0.33***	**0.25**	4.00	**0.44**	0.93
	Cymbopogon martinii Stapf (palmarosa)	**0.25**	**0.25**	**0.16**	0.63	**0.38**	**0.19**	**0.06**	***0.25***	**0.25**	**0.75**	**0.38**	1.00
	Pogostemon patchouli Benth. (patchouli)	**0.25**	**0.38**	**0.06**	***0.21***	**0.38**	**0.25**	**0.50**	1.66	**0.25**	**1.00**	**0.38**	0.94
Boswellia carteri Birdw. (frankincense)	*Cedrus atlantica* (Endl.) G.Manetti ex Carrière (cedarwood)	**0.50**	**0.38**	**0.31**	0.73	**0.25**	3.00	**0.50**	1.08	2.00	**1.00**	2.00	1.50
	Citrus limon (L.) Burm.f. (lemon)	**0.50**	**0.53**	**0.50**	1.30	**0.25**	**0.38**	**0.50**	1.66	2.00	4.00	1.67	0.63
Cedrus atlantica (Endl.) G.Manetti ex Carrière (cedarwood)	*Citrus bergamia Risso* (bergamot)	**0.38**	**0.50**	**0.50**	1.17	3.00	8.00	**1.00**	***0.23***	**1.00**	**1.00**	**1.00**	1.00
	Juniperus virginiana L. (juniper)	**0.38**	**0.63**	**0.19**	***0.40***	3.00	**0.50**	**0.50**	0.58	**1.00**	**1.00**	**1.00**	1.00
	Rosmarinus officinalis L. (rosemary)	**0.38**	**0.50**	**0.19**	***0.44***	3.00	**0.75**	**0.50**	***0.42***	**1.00**	**1.00**	**1.00**	1.00
	Vetiveria zizanioides Stapf (vetiver)	**0.38**	**0.09**	**0.16**	1.04	3.00	**0.19**	**0.50**	1.40	**1.00**	**1.00**	**1.00**	1.00
Citrus aurantium var. amara flower L. (neroli)	*Citrus bergamia* Risso (bergamot)	**0.38**	**0.50**	**0.50**	1.00	**0.38**	8.00	**0.50**	0.69	**1.00**	**1.00**	2.00	2.00
	Commiphora myrrha Engl. (myrrh)	**0.38**	**0.50**	**0.31**	0.94	**0.38**	**0.25**	**0.50**	1.66	**1.00**	**1.00**	**1.00**	1.00
Citrus bergamia Risso (bergamot)	*Cupressus sempervirens* L. (cypress)	**0.50**	**0.19**	**0.31**	1.15	8.00	**0.75**	**0.50**	***0.36***	**1.00**	**1.00**	1.50	1.50
	Lavandula angustifolia Bubani (lavender)	**0.50**	**0.69**	**0.38**	0.88	8.00	**1.00**	**0.50**	***0.28***	**1.00**	**1.00**	3.00	3.00
Citrus limon (L.) Burm.f. (lemon)	*Cananga odorata* (Lam.) Hook.f. & Thomson (ylang ylang)	**0.53**	**0.31**	**0.75**	2.40	**0.38**	**0.50**	**0.50**	1.16	4.00	**1.00**	3.00	1.88
	Eucalyptus globulus Labill. (eucalyptus)	**0.53**	**0.38**	**0.38**	1.10	**0.38**	**0.25**	**0.50**	1.66	4.00	**1.00**	**1.00**	0.63
	Juniperus virginiana L. (juniper)	**0.53**	**0.63**	**0.50**	1.20	**0.38**	**0.50**	**0.50**	1.16	4.00	**1.00**	**1.00**	0.63
	Lavandula angustifolia Bubani (lavender)	**0.53**	**0.69**	**0.50**	1.47	**0.38**	**1.00**	**0.50**	0.91	4.00	**1.00**	**1.00**	0.63
	Styrax benzoin Dryand. (benzoin)	**0.53**	**0.25**	8.00	**5.83**	**0.38**	**0.50**	**0.25**	0.58	4.00	2.00	1.67	0.63
Commiphora myrrha Engl. (myrrh)	*Boswellia carteri* Birdw. (frankincense)	**0.50**	**0.50**	**0.25**	0.75	**0.25**	**0.25**	**0.75**	3.00	**1.00**	2.00	1.50	1.13
	Lavandula angustifolia Bubani (lavender)	**0.50**	**0.69**	**0.25**	0.83	**0.25**	**1.00**	**0.50**	1.25	**1.00**	**1.00**	2.00	2.00
	Melaleuca alternifolia Cheel (tea tree)	**0.50**	**0.63**	**0.19**	0.53	**0.25**	**0.25**	**0.50**	2.00	**1.00**	1.50	**0.50**	***0.42***
	Pelargonium odoratissimum [Soland.] / graveolens L'Hér. (geranium)	**0.50**	**0.38**	**0.25**	0.75	**0.25**	**0.25**	**0.50**	2.00	**1.00**	**1.00**	**1.00**	1.00
	Pogostemon patchouli Benth. (patchouli)	**0.50**	**0.38**	**0.59**	1.98	**0.25**	**0.25**	**0.50**	2.00	**1.00**	**1.00**	**1.00**	1.00

Table 1 ***cont.***

Essential oil combinations		Mean MIC (mg/mL) (n=3) and ΣFIC											
		M. canis (ATCC 36299)				*T. mentagrophytes* (ATCC 9533)				*C. albicans* (ATCC 10231)			
Essential oil 1	**Essential oil 2**	**MIC1[a]**	**MIC2[a]**	**MIC[b]**	**ΣFIC[c]**	**MIC1[a]**	**MIC2[a]**	**MIC[b]**	**ΣFIC[c]**	**MIC1[a]**	**MIC2[a]**	**MIC[b]**	**ΣFIC[c]**
Cymbopogon martinii Stapf (palmarosa)	*Boswellia carteri* Birdw. (frankincense)	**0.25**	**0.50**	**0.19**	0.56	**0.19**	**0.25**	**0.25**	1.16	**0.75**	2.00	**0.75**	0.69
	Cananga odorata (Lam.) Hook.f. & Thomson (ylang ylang)	**0.25**	**0.31**	**0.50**	1.80	**0.19**	**0.50**	**0.25**	0.91	**0.75**	**1.00**	1.50	1.75
	Cedrus atlantica (Endl.) G.Manetti ex Carrière (cedarwood)	**0.25**	**0.38**	**0.09**	***0.31***	**0.19**	3.00	**0.25**	0.70	**0.75**	**1.00**	**0.75**	0.88
	Pelargonium odoratissimum [Soland.] / graveolens L'Hér. (geranium)	**0.25**	**0.38**	**0.16**	***0.47***	**0.19**	**0.25**	**0.25**	1.16	**0.75**	**1.00**	**1.00**	1.17
Eucalyptus globulus Labill. (eucalyptus)	*Lavandula angustifolia* Bubani (lavender)	**0.38**	**0.69**	**0.75**	2.00	**0.25**	**1.00**	1.25	3.13	**1.00**	**1.00**	3.00	3.00
Helichrysum italicum (Roth) G.Don (immortelle)	*Anthemis nobilis* L. (Roman chamomile)	**0.53**	**0.22**	**0.50**	1.13	**0.50**	**0.38**	**0.50**	1.16	2.00	**1.00**	**1.00**	0.75
Lavandula angustifolia Bubani (lavender)	*Anthemis nobilis* L. (Roman chamomile)	**0.69**	**0.22**	**0.63**	2.08	**1.00**	**0.38**	**0.75**	1.36	**1.00**	**1.00**	1.50	1.50
	Melaleuca alternifolia Cheel (tea tree)	**0.69**	**0.63**	**0.50**	1.07	**1.00**	**0.25**	**0.50**	1.25	**1.00**	1.50	**1.00**	0.83
Leptospermum scoparium J.R.Forst. and G.Forst (manuka)	*Commiphora myrrha* Engl. (myrrh)	**0.13**	**0.50**	**0.25**	1.17	**0.13**	**0.25**	**0.13**	0.73	**1.00**	**1.00**	**0.75**	0.75
	Lavandula angustifolia Bubani (lavender)	**0.13**	**0.69**	**0.19**	0.84	**0.13**	**1.00**	**0.19**	0.81	**1.00**	**1.00**	**1.00**	1.00
Melaleuca alternifolia Cheel (tea tree)	*Citrus limon* (L.) Burm.f. (lemon)	**0.63**	**0.53**	**0.50**	1.20	**0.25**	**0.38**	**0.50**	1.66	1.50	4.00	1.25	0.57
	Rosmarinus officinalis L. (rosemary)	**0.63**	**0.50**	**0.50**	0.90	**0.25**	**0.75**	**0.50**	1.33	1.50	**1.00**	4.00	3.33
	Santalum album L. (sandalwood)	**0.63**	**0.13**	**0.06**	***0.30***	**0.06**	**0.25**	**0.13**	1.29	**1.00**	1.50	**1.00**	0.83
Pelargonium odoratissimum [Soland.] / graveolens L'Hér. (geranium)	*Citrus aurantium var. amara* L. flower (neroli)	**0.38**	**0.38**	**0.50**	1.00	**0.25**	**0.38**	**0.38**	1.24	**1.00**	**1.00**	2.00	2.00
	Citrus bergamia Risso (bergamot)	**0.38**	**0.50**	**0.50**	1.00	**0.25**	8.00	**0.50**	1.03	**1.00**	**1.00**	2.00	2.00
	Citrus limon (L.) Burm.f. (lemon)	**0.38**	**0.53**	**0.31**	0.81	**0.25**	**0.38**	**0.50**	1.66	**1.00**	4.00	**1.00**	0.63
	Citrus sinensis Pers. (orange)	**0.38**	**0.25**	**0.13**	***0.38***	**0.25**	**0.50**	**0.50**	1.50	**1.00**	2.00	2.00	1.50
	Lavandula angustifolia Bubani (lavender)	**0.38**	**0.69**	**0.50**	1.17	**0.25**	**1.00**	**0.50**	1.25	**1.00**	**1.00**	**1.00**	1.00
	Santalum album L. (sandalwood)	**0.38**	**0.13**	**0.13**	0.63	**0.25**	**0.06**	**0.25**	2.58	**1.00**	**1.00**	2.00	2.00
Pogostemon patchouli Benth. (patchouli)	*Cedrus atlantica* (Endl.) G.Manetti ex Carrière (cedarwood)	**0.38**	**0.38**	**0.19**	***0.50***	**0.25**	3.00	**0.50**	1.08	**1.00**	**1.00**	2.00	2.00
	Citrus bergamia Risso (bergamot)	**0.38**	**0.50**	**0.38**	0.88	**0.25**	8.00	**1.00**	2.06	**1.00**	**1.00**	2.00	2.00
	Pelargonium odoratissimum [Soland.] / graveolens L'Hér. (geranium)	**0.38**	**0.38**	**0.16**	***0.36***	**0.25**	**0.25**	**0.25**	1.00	**1.00**	**1.00**	**1.00**	1.00

Table 1 ***cont.***

Essential oil combinations		Mean MIC (mg/mL) (n=3) and ΣFIC											
		M. canis (ATCC 36299)				*T. mentagrophytes* (ATCC 9533)				*C. albicans* (ATCC 10231)			
Essential oil 1	**Essential oil 2**	**MIC1**[a]	**MIC2**[a]	**MIC**[b]	**ΣFIC**[c]	**MIC1**[a]	**MIC2**[a]	**MIC**[b]	**ΣFIC**[c]	**MIC1**[a]	**MIC2**[a]	**MIC**[b]	**ΣFIC**[c]
Santalum album L. (sandalwood)	*Citrus limon (L.)* Burm.f. (lemon)	**0.13**	**0.53**	**0.25**	1.40	**0.06**	**0.38**	**0.50**	4.82	**1.00**	4.00	**1.00**	0.63
	Commiphora myrrha Engl. (myrrh)	**0.13**	**0.50**	**0.13**	0.75	**0.06**	**0.25**	**0.50**	5.17	**1.00**	**1.00**	2.00	2.00
	Cymbopogon martinii Stapf (palmarosa)	**0.13**	**0.25**	**0.38**	2.25	**0.06**	**0.19**	**0.13**	1.37	**1.00**	**0.75**	**1.00**	1.17
	Pogostemon patchouli Benth. (patchouli)	**0.13**	**0.38**	**0.25**	1.33	**0.06**	**0.25**	**0.38**	3.88	**1.00**	**1.00**	**1.00**	1.00
Thymus vulgaris Willk. (thyme)	*Citrus limon* (L.) Burm.f. (lemon)	**0.50**	**0.53**	**0.38**	0.98	**0.50**	**0.38**	**0.50**	1.16	**1.00**	4.00	**0.75**	***0.47***
	Rosmarinus officinalis L. (rosemary)	**0.50**	**0.50**	**0.75**	1.50	**0.50**	**0.75**	**1.00**	1.67	**1.00**	**1.00**	**1.00**	1.00
Positive control (Amphotericin)		12.50 µg/mL				25.00 µg/mL				6.25×10^3 µg/mL			

[a]The MIC value of essential oil alone, investigated by this study or reported in Orchard *et al.*[24]. MIC1 represents the first oil and MIC2 represents the second oil. Noteworthy activity is indicated in bold; [b]The MIC value of combination. Noteworthy activity is shown in bold; [c] Interactive profile of the combinations expressed as ΣFIC. Synergistic interactions is shown in italics bold, antagonistic interactions is shown with underline; [d] Shaded areas indicated combinations with noteworthy antifungal against each of the fungal reference strains

were expected to have a negative charge due to the use of phospholipids[13]. The zeta-potential and size distribution of the liposomes within the pre-formulation nanoparticulate system were determined and is provided in Table 2.

The only formulation identified as having an acceptable stable charge was the nanoparticulate system that contained pectin with phospholipids (-31.6 mV). All formulations had an acceptable size distribution, polydispersity index (PDI) and were analogous with previous studies[32,33]. From the characterisation studies, it was evident that the nanoparticulate system containing pectin with the phospholipids was the most suitable composition for final formulation, as it possesses the most favourable stable surface charge, which resulted in evident physical stability on visualisation, and an acceptable particle size.

Particle size plays an important role in a drug delivery system. Nanoparticles have allowed for improved intracellular uptake of topical formulations since a decreased particle size results in better permeability[28]. A larger liposome also indicates greater instability[33]. For effective penetration of nanoliposomes into superficial body surfaces, an average diameter of 50-300 nm is preferred[8]. The average diameter of all the formulations characterised in this study fell within the preferred limit, with sizes ranging from 106.0-286.4 nm. These results were superior to a previous study[13], where a comparable method of essential oil encapsulation to this study was employed, however, considerably larger liposomes were produced; ranging from 885 nm-9.28 µm. Other studies of essential oil-encapsulated nanoemulsions indicated liposomal sizes ranging from 70-220 nm; comparable with four of the pre-formulation nanoemulsions and only marginally smaller than the pectin with and without phospholipid formulations investigated in this study[32,33]. Visually it was also noted that the formulations with phospholipids physically formed a semi-solid cohesive nanoparticulate system, indicating the importance of its role in the composition of the formulation.

The ATR-FTIR spectra was used to identify intermolecular interactions and chemical bonds that formed through changes of the functional

Table 2. Zeta-potential and size distribution of pre-formulation liposomes

Pre-formulation nanolipoemulsions	Zeta-potential (mV)	Size distribution		Quality report result	
		Z-Average (d.nm)	PdI	Zeta Potential	Size distribution
Pectin with phospholipids[a]	-31.6	268.3	0.232	Pass	Pass
Pectin without phospholipids	-20.7	286.4	0.195	Pass	Pass
PVP with phospholipids	-17.1	190	0.234	Pass	Pass
PVP without phospholipids	-18.6	229.1	0.237	Pass	Pass
PAA with phospholipid	-9.02	119.8	0.408	Poor	Poor
PAA without phospholipid	-22.2	106	0.35	Pass	Pass

[a]Shaded area highlights the formulation with most suitable characteristics

groups in the final formulation to the native polymer and essential oils[29]. Using ATR-FTIR spectroscopy, all raw materials (phospholipids, EO's and polymer) and the chosen pre-formulation liposomes were characterised to determine vibrational energy transitions, an indication of the formation of the nanoliposomes. *Cymbopogon martinii* was the essential oil used for pre-formulation studies. *Cymbopogon martinii*, one of the essential oils with the lowest overall MIC, was used to identify whether bonds were being formed between the essential oil, phospholipids, and the pectin polymer i.e., that the essential oil was being encapsulated by the polymer. The resulting spectra is given in Fig. 1 and the peaks assigned are listed in Table 3.

The results showed common peaks between pectin polymer and the final formulation at ± 3450 and 3272 cm^{-1}, ± 1635 and 1639 cm^{-1}, and 1100-1040 cm^{-1} and 1015cm^{-1}, indicating -OH and $-COO^-$ stretching and C-O-C stretching vibrations, respectively. The peaks at 1745 and 1250 cm^{-1} in the pectin polymer, assigned to the C=O and C-O components of an ester bond, are not visualised in the final formulation. Common peaks were also observed between the phospholipids (DSCP and cholesterol) and *C. martinii* at ± 2931.14 cm^{-1} and ± 1055.14 cm^{-1}, indicating -CH and C-O-C stretching, respectively[33]. Multiple absorption peaks were identified for *C. martinii* revealing the intricacy and complexity of the essential oil makeup. The -CH stretch was not present in the final formulation and the C-O-C stretch decreased to 1015.92 cm^{-1}, possibly signifying that hydrogen bonding occurred leading to incorporation of the essential oil within the phospholipids.

There was no chemical reaction initiated, thus chemical bonds were not formed. The similarity between the pectin polymer and final formulation spectra suggests that the outer coating of the nanoliposome is in fact pectin. This explains the mechanism of formation of the system where the hydrophobic phospholipids were found within the hydrophilic outer coating (pectin) facilitating incorporation and transport of the lipophilic essential oil within the nanosystem. These results signify successful encapsulation of the essential oils within the composite nanoliposome structure (Fig. 2).

The Incorporation Efficiency (%) of *Cymbopogon martini* was also estimated via quantitative comparison between the spectra of the pure EO and the common peaks representing *Cymbopogon martinii* in the final formulation. As described, 175 mg of the EO was added to the organic solvent phase. The EOIC was ~60.5% and the total loading of EO within the formulation was ~16.8%.

The three formulations, employing the preferred nanoparticulate system formulation, and containing the three essential oil combinations, were then tested for antifungal activity using the broth microdilution assay. The average MIC values (n=3) for the formulations and controls against *C. albicans, T. mentagrophytes* and *M. canis* are given in Table 4.

As expected, the positive control inhibited all

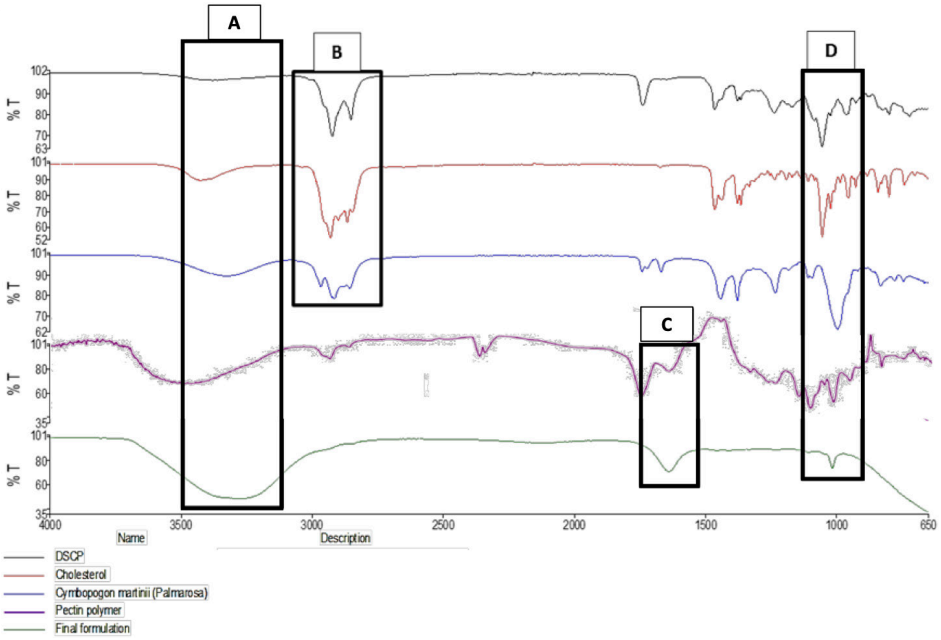

Figure 1. ATR-FTIR spectroscopy result of all raw materials and pre-formulation nanolipoemulsion. The blue line indicates C. martinii; A) -OH Stretch, B) -CH Stretch, C) -COO- Stretch and D) C-O-C Stretch

Table 3. FTIR peak assignments of raw materials and the preferred pre-formulation nanolipoemulsion

Sample Identity	A	B	B	C	D
	-OH Stretch (cm^{-1})	$-CH_3$ Asymmetric Stretch (cm^{-1})	$-CH_2$ Asymmetric Stretch (cm^{-1})	$-COO^-$ Stretch (cm^{-1})	C-O-C Stretch (cm^{-1})
DSCP	–		2924	–	1055
Cholesterol	–		2931	–	1022
C. martinii	-	2967		–	1093
Pectin polymer	3450	2930	2850	1635	1100 and 1040
Final formulation	3271		–	1639	1015

three fungal reference strains, and the negative control displayed poor antifungal activity. The formulation containing *A. sativum* and *C. martinii* displayed the most prominent antifungal activity against all three fungal reference strains, followed by the formulation containing *A. sativum* and *C. limon*. The essential oil combinations in these two formulations were also the two combinations with the lowest mean MIC values when the essential oils were investigated prior to formulation via incorporation in the nanoparticulate systems (Table 1). A significant (p = 0.002) increase in the average antidermatophyte activity could be observed for all three formulations, which may be due to the increased surface area from the nanosized encapsulation, which contributes towards increased bio-availability, permeability, and efficacy of the essential oils, as was previously observed[7,13].

Conclusion

The formulation studies allowed for identification of a successful composition that effectively encapsulated the essential oil

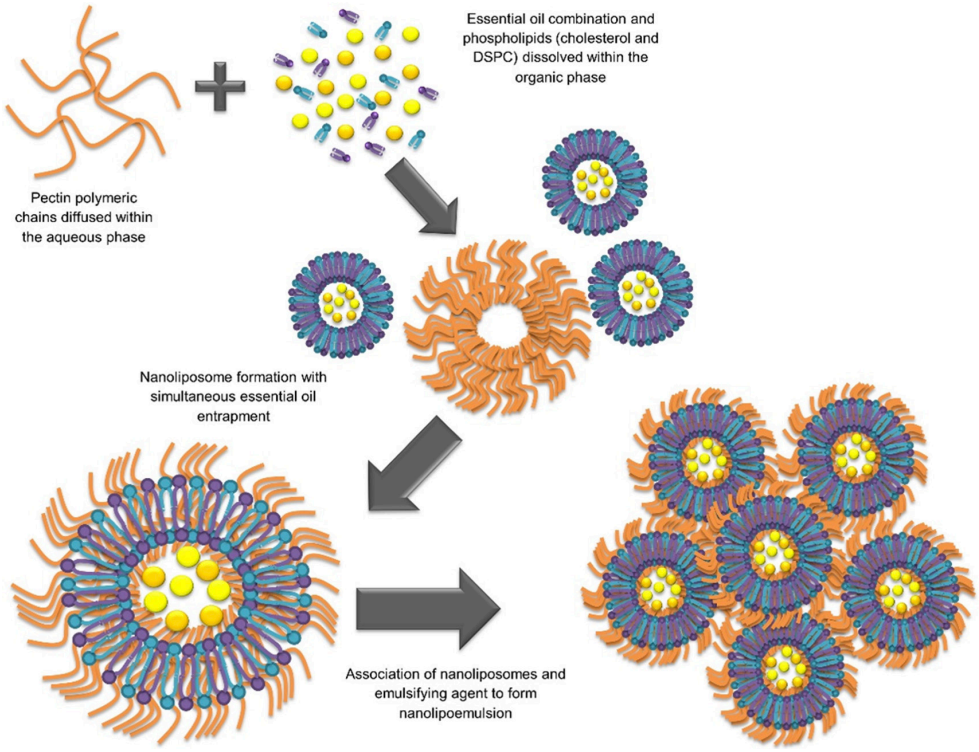

Figure 2. Formation and composition of nanoparticulate systems

Table 4. Average MIC values for formulations

Test samples	Average MIC (mg/mL) (SD)		
	M. canis **(ATCC 36299)**	***T. mentagrophytes*** **(ATCC 9533)**	***C. albicans*** **(ATCC 10231)**
A. sativum and *C. martinii*	**0.08**[a] (± 0.030)	**0.09** (± 0.049)	**0.36**[b] (± 0.140)
A. sativum and *C. limon*	**0.09** (± 0.035)	**0.08** (± 0.058)	**0.73** (± 0.279)
S. album and *M. alternifolia*	**0.09** (± 0.034)	**0.20** (± 0.069)	3.44 (± 1.171)
Covarex® 2% miconazole nitrate (mg/mg) positive control	0.00006	0.00006	>0.005
Negative control	8.75	8.75	> 8.75

[a] Noteworthy values are given in bold

combinations, with two formulations displaying noteworthy antifungal activity against all three fungal reference strains. Although the two most noteworthy formulations each contain *A. sativum*, which has unpleasant organoleptic properties, it needs to be considered that the potential benefit could provide effective treatment of difficult to treat fungi, which would outweigh the odour. Added to that, there was a decrease in the pungent *A. sativum* odour when formulated. The successful formulation of the reported nanoparticulate system could pave the way for finding potential alternatives to current commercial antifungals.

This is the first study reporting the antimicrobial activity of essential oil combinations against *M. canis*, and although previous studies have incorporated essential oils into various formulations, investigations on incorporating essential oil combinations in nano-formulations

are still limited. Considering that the preferred use of essential oils is as blends, more attention should be given to combinations in formulation. It should be noted that this study focused on two essential oils in combination. Three or more essential oils in combination could also be considered. Alternative essential oil combinations could also be considered for formulations, with selection being around oils that present with more pleasant organoleptic properties. This nanosystem developed herein possesses a novel composite polymeric-lipid composition for potentially enhancing the retention of essential oils in order to prolong the antifungal activity. Ideally these formulations highlighted in this study would further benefit from *in vivo* studies where the formulations can be compared to that of conventional antifungal treatments. Considering that antifungal management is usually a long-term treatment, identifying alternatives that are able to inhibit the fungal growth, within a shorter period of time, would be a great clinical benefit.

In further developing this system for clinical applications, consideration of the challenges of nanoparticulate systems is pertinent. With reference to the small number of advanced clinical studies, the clinical impact of nanoparticulate systems as topical or transdermal drug delivery systems has been limited, as the translation of these systems into clinically used products has been slow. Further quantitative studies are required relating the dose, nanoparticle penetration and therapeutic efficacy, as well as nanoparticle toxicity. Comparisons between healthy skin versus diseased skin related to nanoparticle penetration efficiency are also recommended[8]. Further stability analysis, cell toxicity, and animal studies, with comparison to a commercially relevant control, are thus recommended as the next step to determine the suitability of the formulations for further clinical studies.

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Data availability statement

Data is available on request.

Conflict of interest

No conflict of interest declared.

Supplementary data

Table S1 is provided as supplementary data.

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