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Designing an ideal essential oil combination nanoemulsion formulation for the management of respiratory tract infections

Essential oils are well studied for their antimicrobial effects; however, blends extending to formulations are rarely scientifically explored. In this study, we aimed to quantify and optimise the synergy of an essential oil blend by means of computational interpretation in order to create a nanoemulsion formulation ideal for use against respiratory tract pathogens. The nanoemulsion blend consisted of essential oils from *Hyssopus officinalis* var. *angustifolius* in combination with *Salvia rosmarinus* var. *angustifolius*. The prediction tool SynergyFinder (Version 2.0) was implemented to determine optimal synergy blends. According to the synergy maps derived, an optimal blend of these two essential oils is composed of 49.57% of *H. officinalis* and 50.43% of *S. rosmarinus*. This optimised blend was then formulated into a nanoemulsion, using the two-component, self-emulsification technique. The essential oil nanoemulsion showed strong in vitro antimicrobial activities against pathogens of the respiratory tract including *Streptococcus pneumoniae* (ATCC 49619), *Haemophilus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246), with an average six-fold improvement in antimicrobial effect when compared to the neat essential oils. The blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion therefore holds potential to be developed as a natural antimicrobial agent for the management of respiratory tract infections.

Significance:

This study holds significance as it goes beyond the use of single essential oils to explore the effect of essential oil blends for synergistic potential. By leveraging computational tools, we have optimised the combination of *Hyssopus officinalis* and *Salvia rosmarinus* oils for a potent antimicrobial effect. The subsequent creation of a nanoemulsion formulation enhances the stability and bioavailability of these oils and remarkably causes them to exhibit six-fold greater antimicrobial efficacy against respiratory pathogens when in formulation than as individual oils. As antibiotic resistance looms, this natural remedy offers a promising alternative for managing respiratory infections. In bridging the gap between theory and practice, this research contributes to essential oil science, potentially shaping future antimicrobial strategies.

Introduction

Essential oils were used for their antimicrobial properties as early as 4500 BCE, when the Ancient Egyptians made use of these oils in cosmetics and in ointments for medicine.[1] As a result of the empirical knowledge of this antimicrobial potential, which has been preserved for thousands of years, research performed in laboratories globally has substantiated these effects, thus promoting the application of pharmaceutical products containing essential oils.[2]

Despite the antimicrobial and other pharmacological properties associated with essential oils, their pharmaceutical use is often limited because of challenges in formulation development. Limitations in the development of essential oils into pharmaceutical products include physicochemical properties (e.g. poor water solubility), toxicity, and environmental instabilities as a result of heat, oxygen and moisture.[3] Essential oils are hydrophobic (which is a limitation for inclusion in some delivery systems); however, novel pharmaceutical technology, such as nanoemulsions, can be employed to improve the inclusion capabilities of essential oils in formulations.[4,5] Emulsions are formulated from two immiscible fluids, with one fluid dispersed as droplets, created by a barrier provided by a surfactant within the other fluid.[6] When an emulsion contains droplets with a mean diameter smaller than 200 nm, it is referred to as a nanoemulsion.[6] According to the literature, oil-in-water (O/W) nanoemulsions are especially suitable for the delivery of essential oils because of their oil-based core, high-loading capability, and the large variety of available emulsifiers and stabilisers available for use.[6,7] Previous studies incorporating essential oils into nanoemulsion systems for antimicrobial purposes have provided promising results.[8,9] In an earlier study, Sugumar et al.[10] aimed to determine the antimicrobial effect of the essential oil of *Eucalyptus globulus* Labill. (eucalyptus) when encapsulated into a nanoemulsion system. The nanoemulsion demonstrated a greater antimicrobial effect against *Staphylococcus aureus* than when compared to the essential oil alone.[10] Further studies have demonstrated similar improved antimicrobial effects.[2,11]

Much of the work produced concerning essential oils incorporated into nanoemulsion formulations has considered the effect of the essential oils when used alone.[12-18] Despite the positive findings that support the potential use of these individual essential oils in nanoformulations for antimicrobial purposes, the practice of aromatic medicine predominantly makes use of essential oils in combination with other essential oils to achieve an enhanced therapeutic effect.[19,20] Few studies have explored the effects of essential oils in combination when formulated as nanoemulsions for antimicrobial purposes.[21-25] Much of the published findings are limited to essential oils used against pathogens associated with food spoilage. The rationale for combination use for synergistic antimicrobial purposes within a pharmaceutical formulation is to encourage the use of lower doses of each component while maintaining optimised

antimicrobial effects.[21,22] In order to optimise the antimicrobial activity of an essential oil combination for formulation development, it is advisable to use an experimental design to define the most suitable proportions of each ingredient (oil) in an effective mixture.[22] The use of computer-aided software to quantify synergy for drug design and development has been used within the pharmaceutical industry for a number of years, with great success.[26] In pre-clinical formulation studies involving a combination of drugs, functional screening assays that probe the effects of the drug combination as per a dose-response matrix assay are commonly employed.[27] The use of this technology aims to develop innovative formulations based on optimised interactions and is considered the favoured method for interactive assessment in formulation design.[21]

In our previous studies[28,29], we provided results of the antimicrobial potential of essential oils alone and in equal combinations against nine pathogens of the respiratory tract. Based on the findings of these studies, one unique combination composed of the essential oils *Hyssopus officinalis* var. *angustifolius* (M.Bieb.) Benth. (hyssop) and *Salvia rosmarinus* (previously classified as *Rosemarinus officinalis*) var. *angustifolius* (Mill.) DC. (rosemary) was identified as the most promising for use, having broad-spectrum antimicrobial activity, and anti-inflammatory and non-toxic effects when used in equal ratio combinations.[28,29] In this study, we therefore aimed to explore this unique combination of essential oils to quantify the optimum essential oil dose ratio that would provide antimicrobial synergy, and to further explore this combination as an antimicrobial nanoformulation.

Methods

One essential oil combination was selected for this study: *Hyssopus officinalis* var. *angustifolius* and *Salvia rosmarinus* var. *angustifolius*. These essential oils were procured from a flavour and fragrance industry provider, Prana Monde (Hainaut, Belgium). The two essential oils used in this study have been previously chemically characterised using gas chromatography coupled with a mass spectrometer (GC-MS) and reported on in our earlier studies.[28,29] The major chemical constituents of these oils are provided in Supplementary table 1.

As a result of the lipid nature of essential oils, caprylocaproyl macrogol-8-glyceride (Labrasol) was selected as the surfactant for the formulation. Labrasol is a non-ionic water-dispersible surfactant specifically used in lipid-based formulations to solubilise poorly water-soluble ingredients. Labrasol was obtained as a donation from Gattefossé (Saint-Priest, France). Propylene glycol, procured from Sigma-Aldrich (St. Louis, Missouri, USA) was selected as a co-surfactant to the formulation to provide further stability to the essential oil nanoemulsion.

Preparation of microbial cultures

Microbial cultures were selected based on their relevance to respiratory infections and included the Gram-positive strains *S. aureus* (ATCC 25924), *Streptococcus agalactiae* (ATCC 55618), *Streptococcus pneumoniae* (ATCC 49619) and *Streptococcus pyogenes* (ATCC 12344). The Gram-negative strains included *Haemophilus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246). The non-pathogenic *Mycobacterium* strain *Mycobacterium smegmatis* (ATCC 19420) was investigated to determine the antimicrobial effect of these essential oils against tuberculosis strain classes, as well as against the yeast strain *Cryptococcus neoformans* (ATCC 14116) because of its pathogenic effect in the respiratory tracts of immunocompromised patients. All cultures were prepared as per our earlier studies.[28,29] A waiver for the use of these microorganisms was granted by the University of the Witwatersrand Human Research Ethics Committee (reference W-CJ-160720-2).

Antimicrobial combination optimisation using SynergyFinder

SynergyFinder (Version 2.0) (Helsinki Institute for Information Technology) facilitates the analysis of drug combination experiments and provides an interface for visualising the drug combination landscapes in an interactive manner.[27] The essential oil combination selected (Supplementary table 2) was investigated at varying ratios as a means to complete a dose-response matrix for evaluation by the SynergyFinder software, using the broth microdilution assay as previously reported.[30]

The 96-well microtitre plates were prepared by aseptically adding 100 μL of sterile broth into each of the wells. (Tryptone Soya broth was used for all bacterial cultures, with the exception of the *Streptococcus* species, *H. influenzae* and *M. smegmatis* for which *Haemophilus* test medium base with *Haemophilus* test medium supplement (ThermoFisher) was used.) A stock concentration of each essential oil (32 mg/mL in acetone) was added to the first row at varying ratios (as indicated in Supplementary table 2). The essential oil combinations were then serially diluted to concentrations ranging from 8 mg/mL to 0.06 mg/mL. A 100 μL of the positive, negative and culture controls were included for each microorganism. The positive controls (Sigma-Aldrich) were 0.01 mg/mL ciprofloxacin (for bacteria) or 0.10 mg/mL amphotericin B (for the yeast), and were included to ensure microbial susceptibility. The negative control of 32 mg/mL water in acetone was included to exclude the attribution of antimicrobial effects as a result of the solvent. The culture controls consisted of growth medium with the relevant pathogen to ensure that the appropriate broth supported microbial growth.

Streptococcus agalactiae, S. pneumoniae, S. pyogenes and *H. influenzae* were grown in *Haemophilus* test medium base and supplement, while *Mycobacterium smegmatis* required Middlebrook broth supplemented with glycerol and ADC enrichment. All other tested bacterial pathogens and *Cryptococcus neoformans* were cultured in Tryptone Soya broth (TSB). An inoculum concentration of approximately 1×10^6 colony-forming units per mL (CFU/mL) was prepared, as per the McFarland standard for each microorganism studied, and 100 μL of the inoculum was added to each well. Before incubation, each microtitre plate was sealed with sterile adhesive sealing film. Incubation conditions varied by bacterial species. The pathogens *M. smegmatis* and *C. neoformans* were incubated aerobically for 48 h at 37 °C, while *S. pneumoniae, S. pyogenes* and *H. influenzae* were maintained in anaerobic environments with 5% CO_2 at 37 °C for 24 h, and *S. agalactiae* and other test organisms were incubated aerobically at 37 °C for 24 h. Following liquid culture, all bacterial and fungal samples were streaked onto appropriate selective media to verify culture purity and ensure experimental reliability.

Following incubation, 40 μL of 0.04% w/v *p*-iodonitrotetrazolium violet solution (Sigma-Aldrich) was added into each well of the microtitre plate to determine microorganism viability. A change of colour in the well from clear to a purple-pink indicates microbial growth. Therefore, the lowest concentration displaying no colour change was recorded as the minimum inhibitory concentration (MIC). Each experiment was performed in triplicate and the mean value was recorded.

The data generated from the varied ratio combination MIC assay were entered into SynergyFinder (Version 2.0). A log-logistic model was applied to the varied ratio MIC findings in order to generate dose-response curves for the essential oils in each combination. Essential oil combination antimicrobial responses were then plotted as heat maps to identify the concentration ratios at which the essential oils used in combination had the maximum inhibitory effect on microbial growth. The degree of synergy per essential oil combination was analysed using the response surface model based on the Bliss reference synergy model. The synergy score determined for the essential oil combination investigated was averaged over all the dose combination measurements, and a 3D synergy map was generated that highlights synergistic and antagonistic dose regions in red and green, respectively.[27] On interpretation, the summary synergy scores of less than −10 were considered antagonistic, a range from −10 to +10 as additive, and greater than +10 as synergistic.[31]

Preparation of the nanoemulsion

The development of an essential oil nanoemulsion formulation was undertaken because of their oil-based core, high-loading capability and widely adopted application as the drug vehicle of choice in pulmonary delivery.[32] The nanoemulsion was prepared using the two-component, self-emulsification method.[33] Labrasol was first mixed with propylene glycol at a ratio of 2:1 and stirred magnetically (IKA Plate, RCT digital, Deutschland, Germany) for 5 min at a speed of 500 rpm. The essential

oils were subsequently added at optimal ratios to produce a formulation of a final concentration of 2.5% (v/v) to the Labrasol and propylene glycol mixture, which was stirred for a further 15 min. Sterile distilled water was then added dropwise and stirred magnetically until a homogeneous translucent appearance was achieved. The completed formulation was then stored at ambient temperature in sealed amber bottles to prevent any loss of the essential oil by evaporation.

Characterisation of the nanoemulsion

The physical attributes of a nanoemulsion formulation determine the formulation stability and efficacy, as well as its in vitro behaviour.[34] Important physical attributes include the average particle size distribution and the polydispersity index (PDI). The particle size distribution and PDI of the samples were determined using dynamic light scattering at 25 °C using a zeta-potential and particle size analyser (ELSZ-2000, Otsuka Electronics Co. Ltd. Japan). In the case of pulmonary drug delivery for systemic absorption, aerosols with a small particle size are required to ensure peripheral penetration.[35,36] Particles smaller than 3 μm have an approximately 80% probability of reaching the lower airways, while around 50–60% of these particles will be deposited in the alveoli, and hence this was a parameter for formulation.

The essential oil nanoemulsion was centrifuged at 1956 × *g* for 30 min to evaluate the stability. Stability was investigated by storing 5 mL of the nanoemulsion at 4±1 °C, 25±1 °C, 37±1 °C and 60±1 °C in duplicate for one month.[34,35] The samples were observed for visual indications of instability, including creaming or flocculation.

Antimicrobial activity of the nanoemulsion

The broth microdilution method[37] was used to quantify the antimicrobial activity of the formulated essential oil nanoemulsion against pathogens selected for their relevance to respiratory infections. The microtitre plates were prepared aseptically, as per the antimicrobial studies undertaken for the combination optimisation using SynergyFinder (Version 2.0). Variations to the method included the addition of 100 μL of the essential oil nanoemulsion, added to the first row. The positive controls remained 0.01 mg/mL ciprofloxacin (for bacteria) or 0.10 mg/mL amphotericin B (for the yeast) to ensure microbial susceptibility. The negative control of 15% (v/v) Labrasol and 7% (v/v) propylene glycol in distilled water was included to exclude the attribution of antimicrobial effects from the formulation's solution base. The culture controls consisted of growth medium with the relevant pathogen to ensure that the appropriate broth supported microbial growth. Following incubation, 40 μL of 0.04% w/v *p*-iodonitrotetrazolium violet solution (Sigma-Aldrich) was added into each well of the microtitre plate in order to determine the MIC. Each experiment was performed in triplicate and the mean value was recorded.

Results and discussion

Antimicrobial optimisation

The antimicrobial interaction of *H. officinalis* essential oil in combination with *S. rosmarinus* essential oil against a selection of nine respiratory pathogens was investigated using SynergyFinder (Version 2.0). According to the synergy maps (Figure 1), an average synergy score of 9.76 was determined against all nine pathogens studied. The highest synergy was noted against *H. influenzae,* for which a synergy score of 27.621 was determined, with greater incidence of synergistic interactions assigned to ratios higher in *H. officinalis* than *S. rosmarinus*. Synergy was also noted against *S. pyogenes, M. smegmatis* and *C. neoformans* with synergy scores of 10.470, 11.864 and 13.263, respectively. Marginal synergy was determined for the combination against the pathogen *S. aureus* (synergy score = 9.505), while additive scores were noted against all other pathogens, with scores of −0.029 (*S. agalactiae*), 7.602 (*S. pneumoniae*), 3.763 (*K. pneumoniae*) and 3.780 (*M. catarrhalis*). The results of SynergyFinder (Version 2.0) established that the optimal blend of each essential oil consists of 49.57% of *H. officinalis* and 50.43% of *S. rosmarinus* to achieve an average synergy score ranging from 14.25 to 57.14 against the pathogens studied.

Characterisation of the nanoemulsion

The blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion was stable to coalescence. The average particle size of the blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion was determined as 47.89 nm using a zeta-potential and particle size analyser (Figure 2). Oil in water emulsions are classified based on the size of the droplet, with nanoemulsions classified by droplet sizes of less than 100 nm.[38] Therefore, these findings indicate that the emulsion was within nano-range. Other studies incorporating the use of essential oils in nanoemulsions have defined a range of nanodroplet sizes between 23.4 nm and 100 nm with droplet sizes varying depending on the constituents selected, the operating conditions and preparation methods used.[39-42] The PDI value provided by the nanosizer informs if the nanoemulsion droplets are well dispersed, with a measure of 0.20 to 0.50 expected for pharmaceutical preparations.[43] The PDI value for this study was determined to be 0.202, which is desirably low and suggestive of a well-dispersed nanoemulsion. The obtained value of 0.202 falls within the narrow size distribution range (0.08 to 0.30), indicating high uniformity of droplet size. Monodispersed samples have PDI values lower than 0.08, while PDI values between 0.08 and 0.30 prove a narrow size distribution, and PDI values greater than 0.30 indicate a broad size distribution.[44] Therefore, higher PDI values suggest lower uniformity of droplet size in nanoemulsions.

Stability of the nanoemulsion

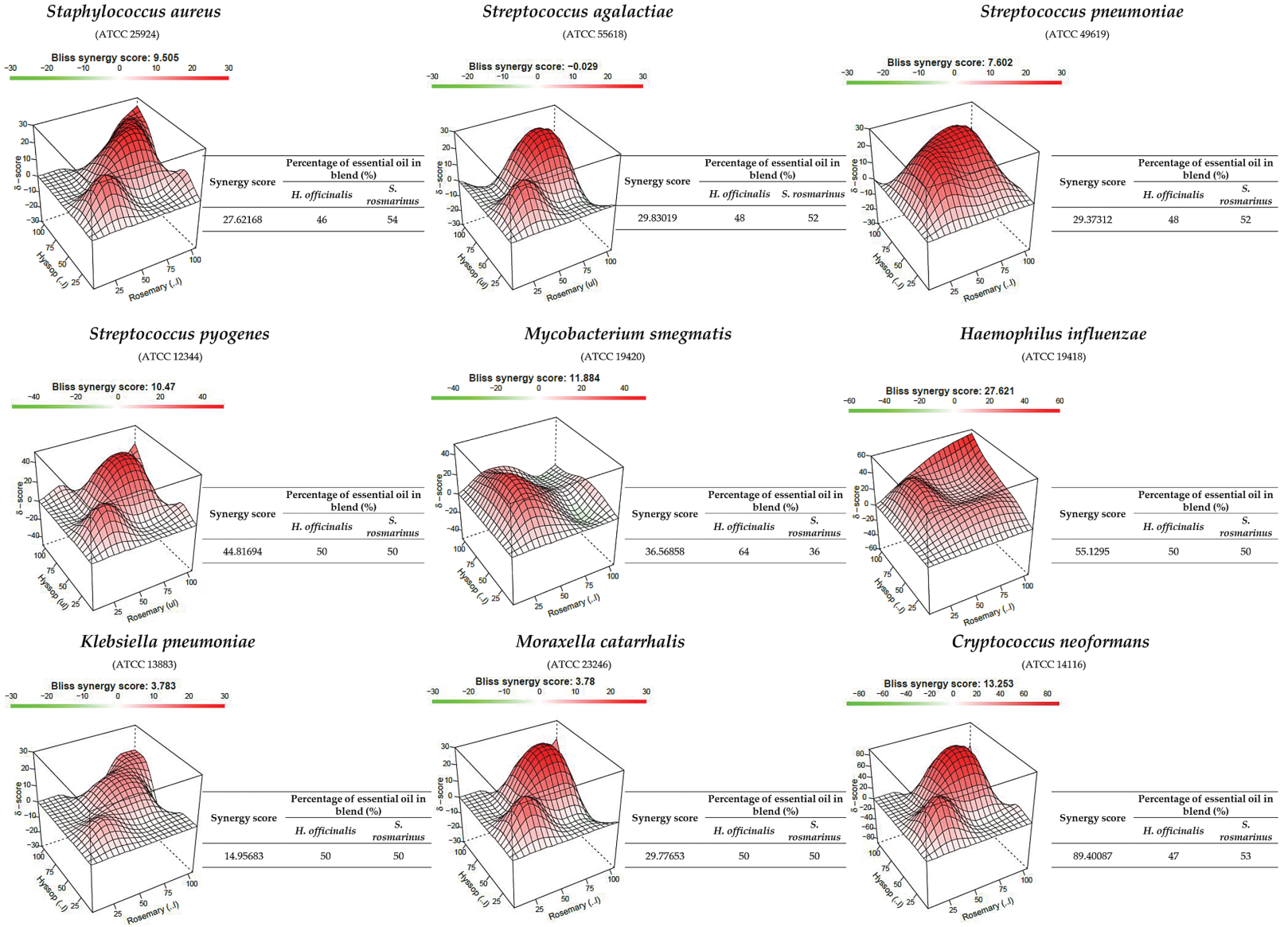
The appearance of the blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion following formulation was of a clear and transparent liquid, without observed turbidity or precipitate (Figure 3A). After centrifugation, the formulation maintained the same clear and transparent appearance (Figure 3B). The particle size of the nanoemulsion droplets was again measured using a zeta-potential and particle size analyser. The droplet sizes were maintained after centrifugation, with droplet sizes slightly reduced to 37.55 nm, with a PDI of 0.189. The reduction in PDI value after centrifugation is not statistically or practically significant. The slight drop is expected and, because of the negligible variance, is indicative of a stable formulation. This stability suggests that the nanoemulsion maintains its structural integrity under stress conditions, which is crucial for pharmaceutical applications.

The nanoemulsion, stored at 4±1 °C, 25±1 °C, 37±1 °C and 60±1 °C for one month, showed no phase separation or creaming properties at all temperatures, indicating stability of the formulation (Figure 4).

Antimicrobial validation of the formulation

The blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion was investigated against nine pathogens of the respiratory tract by means of MIC analysis. A noteworthy antimicrobial effect was determined for the blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion against all nine respiratory pathogens, with MIC values ranging between 0.07 mg/mL to 1.17 mg/mL (Table 1). The average MIC of the combined essential oils prior to formulation against the nine pathogens studied was 2.17 mg/mL.[30] The nanoemulsion exhibited better inhibitory effects across all pathogens studied, with a six-fold increase in antimicrobial activity compared with when the neat essential oils were blended.

The findings of this study are congruent with those of previous studies that showed that the conversion of essential oils into nanoemulsions produces an improved antimicrobial effect. A previous study[45] aimed to create an essential oil nanoemulsion containing *Cymbopogon flexuosus* (Nees ex Steud.) Will. Watson for antimicrobial use. The neat oil of *C. flexuosus* showed activity against *Candida albicans* at a concentration of 1.22 mg/mL while the nanoemulsion of the essential oil demonstrated a MIC of 0.28 mg/mL. This improved antimicrobial effect was also noted against the microorganisms *Cryptococcus grubii* (MIC of neat oil was 0.58 mg/mL and the nanoemulsion was 0.28 mg/mL) and *Pseudomonas aeruginosa* (no antimicrobial effect by neat oil, and the nanoemulsion showed potential bactericidal activity at concentrations above 11.33 mg/mL). These findings are further supported by previous essential oil studies, e.g. *Origanum vulgare* L.[46] and *Thymus daenensis* Čelak[47]. It has been noted that this enhanced antimicrobial effect demonstrated by essential oils when encapsulated into a nanoemulsion is a result of the nanometric size and resultant improved diffusion of nanoemulsions.[48] These enhanced characteristics offered by nanoformulations provide opportunity for further exploration of varying essential oil blends.



Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
27.62168	46	54

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
29.83019	48	52

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
29.37312	48	52

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
44.81694	50	50

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
36.56858	64	36

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
55.1295	50	50

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
14.95683	50	50

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
29.77653	50	50

Synergy score	Percentage of essential oil in blend (%)	
	H. officinalis	*S. rosmarinus*
89.40087	47	53

Figure 1: Visual representations of the antimicrobial interaction of the combination of *Hyssopus officinalis* and *Salvia rosmarinus* essential oils against nine microorganisms responsible for respiratory tract infections. Positive (red regions) of the response surface plot indicate synergistic effects, while negative (green regions) indicate antagonistic effects. Optimal ratios of *H. officinalis* and *S. rosmarinus* essential oils are indicated in adjacent tables, per microorganism.

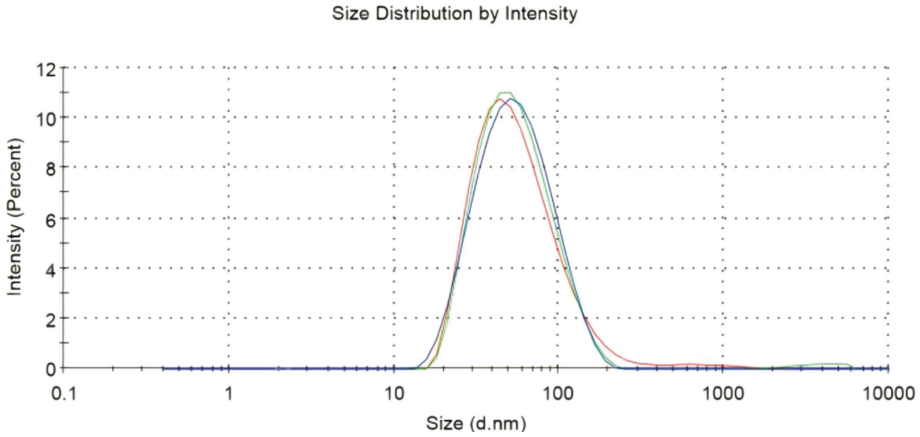


Figure 2: Particle size of the essential oil nanoemulsion as determined by zeta-potential and particle size analyser. The assorted colour bands represent repeat measurements of the same sample.

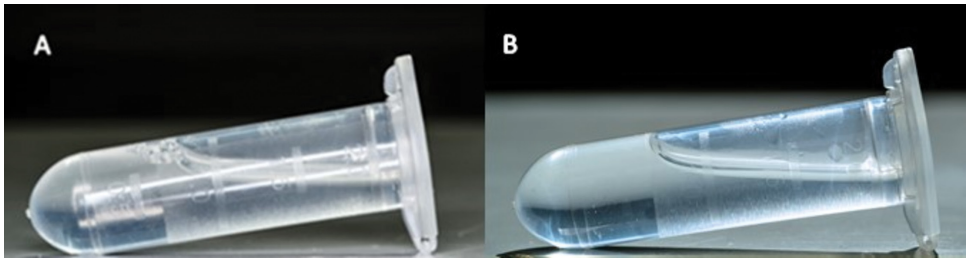


Figure 3: (A) The appearance of the blended *Hyssopus officinalis* and *Salvia rosmarinus* essential oil nanoemulsion following formulation as a clear and transparent liquid, without observed turbidity or precipitate. (B) Following centrifugation, the formulation maintained the same clear and transparent appearance.

Figure 4: The appearance of the blended *Hyssopus officinalis* and *Salvia rosmarinus* essential oil nanoemulsion (in duplicate), following storage at 4±1 °C, 25±1 °C, 37±1 °C and 60±1 °C for one month, showed no phase separation or creaming properties at all temperatures.

Conclusion

In conclusion, to our knowledge, this study is the first to apply SynergyFinder software for the visual interpretation of antimicrobial synergy between the essential oil combination *H. officinalis* blended with *S. rosmarinus*. The essential oil combination of *H. officinalis* and *S. rosmarinus* demonstrated broad-spectrum antimicrobial potential with a Bliss model synergy score of 9.76. As a result of this potential, a nanoemulsion formulation was created for a blend of *H. officinalis* and *S. rosmarinus* essential oils at optimised ratios as per the generated synergy maps for nine respiratory-associated pathogens. At the optimal concentration (49.57% of *H. officinalis* and 50.43% of *S. rosmarinus*) of the blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion, the average particle size was 47.89 nm and PDI was 0.202. The nanoemulsion maintained good stability and dispersion after centrifuging and at various storage conditions. Furthermore, at optimum concentrations, the blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion showed noteworthy antimicrobial activity against all nine pathogens studied, with MIC values ranging from 0.07 mg/mL to 1.17 mg/mL. The optimised nanoemulsion formulation exhibited better inhibitory effects across all pathogens studied, with a six-fold increase in antimicrobial activity than when the neat essential oils were blended.

Aerosolised nanoemulsions are promising alternatives for non-invasive drug delivery to the respiratory tract because of their improved and targeted antimicrobial approach. These characteristics of a nanoemulsion system and the proven enhanced interactions of the blended *H. officinalis* and *S. rosmarinus* essential oil nanoemulsion against pathogens of the respiratory tract further provide a rationale for the potential therapeutic use of this blend. This research indicates the potential for optimised essential oil combinations to be identified using a computational

Table 1: The mean minimum inhibitory concentration in mg/mL ($n = 3$) of the blended *Hyssopus officinalis* and *Salvia rosmarinus* essential oil nanoemulsion investigated against test pathogens

Pathogen	Neat essential oils in combination[29]	Essential oil nanoemulsion	Ciprofloxacin (positive control)	Amphotericin B (positive control)	Formulation solution base (negative control)
Staphyloccocus aureus (ATCC 25924)	2.00	0.07	0.50×10^{-3}	NA	> 4.93
Streptococcus agalactiae (ATCC 55618)	2.00	0.29	0.50×10^{-3}	NA	> 4.93
Streptococcus pneumoniae (ATCC 49619)	3.00	1.17	0.50×10^{-3}	NA	> 4.93
Streptococcus pyogenes (ATCC 12344)	1.00	0.29	0.50×10^{-3}	NA	> 4.93
Mycobacterium smegmatis (ATCC 19420)	1.50	0.29	0.50×10^{-3}	NA	> 4.93
Haemophilus influenzae (ATCC 19418)	4.00	0.29	0.25×10^{-3}	NA	> 4.93
Klebsiella pneumoniae (ATCC 13883)	4.00	0.59	1.00×10^{-3}	NA	> 4.93
Moraxella catarrhalis (ATCC 23246)	2.00	0.15	0.50×10^{-3}	NA	> 4.93
Cryptococcus neoformans (ATCC 14116)	0.09	0.07	NA	0.50×10^{-3}	> 4.93

software workflow and further formulated into nanosystems for targeted antimicrobial effects. As of now, research on the application of essential oils into nanoemulsions as antimicrobial agents is in a fast-growing phase; however, there is a paucity in the literature on the use of essential oils in combination. Advancements in this field of research require further exploration for commercial consideration and potential product development.

Limitations and future directions

This study has demonstrated the potential of a nanoemulsion formulation using a synergistic blend of *Hyssopus officinalis* and *Salvia rosmarinus* essential oils for managing respiratory tract infections. However, several limitations should be considered. The findings are based on in vitro studies against selected pathogens; therefore, the lack of in vivo validation means the formulation's efficacy and safety in biological systems remain unknown. The toxicity and safety profiling of this formulation was not comprehensively evaluated. Additionally, limited stability testing was performed, leaving questions about long-term stability. To address these limitations, future research may consider expanding the pathogen scope to include emerging resistant strains. In vivo studies may be considered to validate therapeutic efficacy, bioavailability, and safety in physiological conditions following detailed toxicity profiling. By addressing these limitations and advancing research in these directions, this study provides a foundation for the development of innovative, natural antimicrobial solutions for respiratory health.

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Data availability

The data supporting the results of this study are available upon request to the corresponding author.

Declarations

We have no competing interests to declare. We have no AI or LLM use to declare. A waiver for the use of these microorganisms was granted by the University of the Witwatersrand Human Research Ethics Committee (reference W-CJ-160720-2).

Authors' contributions

S.L.: Acquisition, analysis, or interpretation of data; drafting the work or revising. A.V.: Conception or design; drafting the work or revising. P.K.: Conception or design; drafting the work or revising. S.v.V.: Conception or design; drafting the work or revising. All authors read and approved the final manuscript.

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