

Antimicrobial Formulation of *Chrysopogon zizanioides* Essential Oil in an Emulsified Lotion for Acne

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ABSTRACT

Acne is a skin condition arising from excess sebum production and microbial overgrowth within the pilosebaceous unit. Several commercial essential oils have shown promising activity against acne-related pathogens. Due to their volatility and thermal instability, the formulation of essential oils into commercial products remains a pharmaceutical challenge. Thus, this study aimed to develop a viable anti-acne topical treatment as an oil-in-water emulsified lotion to overcome these challenges. *Chrysopogon zizanioides* (vetiver) displayed noteworthy antimicrobial activity with a mean minimum inhibitory concentration of 0.14 mg/mL against *Cutibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*. Emulsified lotions containing *C. zizanioides* were developed through the hydrophilic-lipophilic balance approach. At tested hydrophilic-lipophilic balance values of 8, 9, and 10, *C. zizanioides* emulsified lotions displayed maximum stability at hydrophilic-lipophilic balance 9 with a minimum change in mean droplet size and polydispersity index of 20.61 and 33.33%, respectively, over 84 days. The *C. zizanioides* emulsified lotion at optimum hydrophilic-lipophilic balance 9 completely inhibited the growth of *C. acnes* and killed *S. aureus*, *S. epidermidis*, and *S. pyogenes* within 24 h. Additionally, the lotion retained antimicrobial activity against these test micro-organisms over the 84-day stability test period. Thus, the *C. zizanioides* emulsified lotion demonstrated physical stability and antimicrobial efficiency, making it an ideal natural product anti-acne treatment.

ABBREVIATIONS

EO	essential oil
GMS	glycerol monostearate
HLB	hydrophile-lipophile balance
INT	<i>p</i> -iodonitrotetrazolium violet solution
log CFU/mL	log colony-forming units per mL
MDD	mean droplet diameter
MIC	minimum inhibitory concentration
PDI	polydispersity index
PIT	phase inversion temperature
SCCO	<i>Simmondsia chinensis</i> (jojoba) carrier oil

Introduction

Acne is a disease of the skin arising from the pilosebaceous unit [1]. It is the 8th most prevalent disease worldwide and affects approximately 80% of all adults at some point during their lifetime [2]. Although not a fatal condition, it can lead to permanent scarring and in severe cases, facial disfigurement [1]. Current anti-acne treatments on the market often consist of lengthy antibiotic regimens, which contribute to the antibiotic resistance crisis experienced worldwide. Evidence of emerging resistance is seen by studies that have identified 50% of *Cutibacterium acnes* strains being resistant to topical macrolides [3], as well as resistance towards doxycycline, minocycline, and trimethoprim-sulphamethoxazole, all commonly used in the treatment of acne [4, 5]. Ad-

► **Table 1** MIC values (mg/mL) of *C. zizanioides* EO against acne pathogen reference strains (n = 3).

Sample	MIC (mg/mL)				Mean MIC ± SD
	<i>Cutibacterium acnes</i> (ATCC 11 827)	<i>Staphylococcus epidermidis</i> (ATCC 2223)	<i>Staphylococcus aureus</i> (ATCC 25 923)	<i>Streptococcus pyogenes</i> (ATCC 12 344)	
<i>C. zizanioides</i> EO	0.25 ± 0.00	0.10 ± 0.02	0.17 ± 0.09	0.04 ± 0.00	0.14 ± 0.09
Positive control – Ciprofloxacin (µg/mL)	0.04 ± 0.00	0.02 ± 0.00	0.08 ± 0.00	0.31 ± 0.00	0.11 ± 0.13
Negative control – Acetone	> 8.00	> 8.00	> 8.00	> 8.00	N/A ^a
Culture control	> 8.00	> 8.00	> 8.00	> 8.00	N/A

^a N/A ~ not applicable as values cannot be calculated from current data set

ditionally, the current treatments are not without side effects, which may cause poor patient compliance and contribute towards the development of antibiotic resistance [6]. Moreover, a large percentage of people attempt to self-diagnose and self-treat acne as they cannot afford expensive clinician fees [7]. This highlights the need for readily available alternative herbal therapies that do not contribute to resistance [8].

EOs provide a feasible alternative to antibiotics with a range of bioactivities, extending from antimicrobial to antioxidant and anti-inflammatory properties [9]. A total of 98 EOs have been recommended for dermatological use, of which 49 were specifically indicated for the treatment of acne [10]. A previous study [11] identified *Chrysopogon zizanioides* (L.) Roberty (vetiver) as the most antimicrobially effective EO out of 19 commercial EOs tested against the causative pathogens of acne.

Due to the volatility, water insolubility, and thermal instability of EOs, their development into stable formulations remains a pharmaceutical challenge. Preparations containing EOs require precise formulation methods to produce a system that allows them to be delivered to the cutaneous tissue without compromising their stability or efficacy [12, 13]. The emulsification of an EO presents an avenue of opportunity in order to overcome these challenges, and improve on the stability of formulations [14]. Previous studies have investigated the formulation of EO emulsions for various applications, from food preservation to cosmetic use [15–17]. However, these studies often investigate either the physical stability or the antimicrobial effectiveness of such formulations and the link between the two aspects are often overlooked. Both aspects are critical for the overall performance of the formulation, as the physiochemical properties have been found to play a decisive role in the efficacy of a formulation [18]. It is thus critical to investigate the physical stability and antimicrobial efficacy of a formulation in tandem to ensure optimum performance of both. Hence, this study set out to validate the antimicrobial activity of *C. zizanioides* EO, prepare a stable emulsified lotion thereof, and concurrently investigate the antimicrobial efficacy of the resulting formulation.

► **Table 2** GC-MS analysis (major compounds) of *C. zizanioides* essential oil.

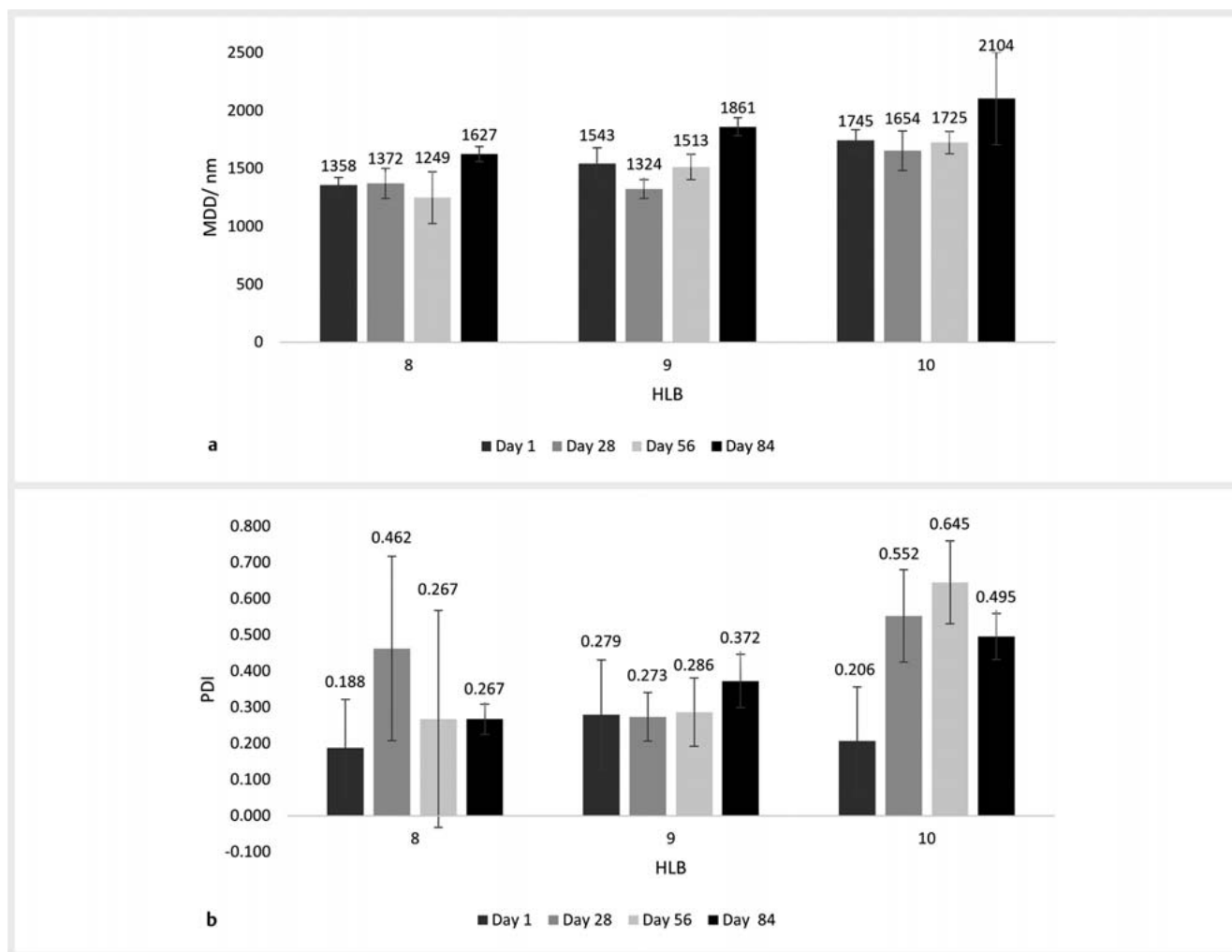
Retention Time	Constituent ^a	%
76.9	Vetiselinenol	3.32
80.3	β-Vetivone	2.67
80.5	Khusimol	7.93
81.9	Isovalencenol	7.39
82.1	α-Vetivone	3.73
101.2	Khusenic acid	15.87
N/A	Minor constituents	54.43
Total		95.34

^a Only major constituents (> 2.5%) are listed in detail for brevity. Complete list of chemical constituents are available on request.

Results and Discussion

The first component of the study was to validate the antimicrobial activity of *C. zizanioides* EO against the four selected pathogen reference strains, and the MIC values (0.04–0.25 mg/mL) are shown in ► **Table 1**.

The MIC values of *C. zizanioides* EO obtained in this study were slightly more effective than previous reports of MIC values (both 0.50 mg/mL against *C. acnes* and *Staphylococcus aureus*) [19, 20]. Analysis of the GC-MS data of the *C. zizanioides* EO (► **Table 2**) revealed that a different chemotype from the previous studies was investigated, as there were no major chemical constituents in common, therefore discrepancies in antimicrobial activity were expected. Currently, data on the bioactivities of *C. zizanioides* EO remains largely unexplored. It is thus likely that *C. zizanioides* EO has not been intensively studied due to its complex structure with over 170 chemical constituents [21]. Additionally, other studies often employ unreliable antimicrobial methods such as disc diffusion [22–24], which makes comparison a challenge. Therefore, the antimicrobial investigation and formulation development investigated in this study sheds light and reports on a novel application of *C. zizanioides* EO.



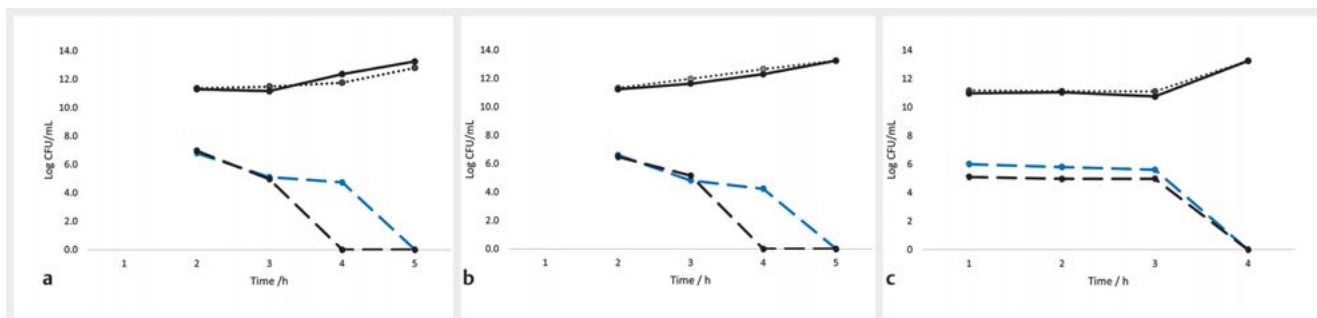
► **Fig. 1** The percentage change in (a) mean droplet diameter and (b) polydispersity index of *C. zizanioides* EO emulsified lotions as a measure of stability with time. Day 1, day 28, day 56, and day 84.

Once the efficacy of *C. zizanioides* EO was validated, the ensuing step was the incorporation of the EO into a formulation. In order to produce stable emulsified lotion of *C. zizanioides* EO, the HLB was employed. According to Orafidiya and Oladimeji [18], optimal stability is achieved when an emulsion is formulated at an HLB value that yields the smallest MDD. In addition, the PDI is a measure of the standard deviation of the MDD and may also be employed as a useful parameter. Fluctuations in droplet diameter may occur, especially in the initial days after preparation as the two phases are in dynamic equilibrium and the oil droplets are continuously being disrupted and reformed [25]. Therefore, a large change in the MDD or PDI over time can be viewed as a precursor of physical instability mechanisms (such as creaming and cracking). Hence, *C. zizanioides* emulsified lotions were prepared at HLB 8, 9, and 10 [11] and were all monitored over 84 days to find the HLB at which the emulsion displayed the minimum change in MDD and PDI. This HLB would reveal the emulsified lotion of peak physical stability.

Initial measurement of the MDD of the *C. zizanioides* EO emulsified lotions showed a trend of increasing MDD with an increase

in the HLB (► **Fig. 1 a**). The lowest MDD was obtained for the HLB 8 emulsified lotion (1358 nm) while the highest MDD was obtained at HLB 10 (1745 nm). After 84 days, similar increases in the MDD were seen with all HLB values (19.81, 20.61, and 20.57% for HLB 8, 9, and 10, respectively) for the emulsified lotions. As similar rates of destabilisation were obtained for each emulsion regardless of HLB, the MDD could not be used as an indicator of stability.

The initial PDI for all three HLB values of the emulsified lotions were acceptable (<0.5) and indicated uniformly sized droplets had formed for each HLB value on day 1 (► **Fig. 1 b**). However, a sharp increase of 140.23% over 84 days was observed for the PDI of the HLB 10 emulsified lotion, followed by an increase of 42.03% for the HLB 8 emulsified lotion. This indicates that *C. zizanioides* EO lotion at these HLBs might present with a limited shelf life. A clear minimal percentage change of 33.33% in the PDI (from 0.279 to 0.372) was noted for emulsified lotions formulated at an HLB of 9, signifying it to be the most resistant to fluctuations and internal system changes. The *C. zizanioides* lotion emulsified at an HLB of 9 could therefore be concluded to display optimum physical sta-



► **Fig. 2** Time-kill dynamics of stable *C. zizanioides* emulsified lotion (at HLB 9) against acne-inducing pathogens (a) *S. aureus* (b) *S. epidermidis*, and (c) *S. pyogenes*. Blue-grey disrupted line: *C. zizanioides* emulsified lotion (HLB 9), black disrupted line: positive control (0.01 mg/mL ciprofloxacin), black solid line: negative control (SCCO lotion), and grey dotted line: culture control.

bility and was selected for further antimicrobial assessment. In order to assess whether the stable emulsified *C. zizanioides* lotion at HLB 9 displayed antimicrobial activity, time-kill assays were undertaken.

Total bactericidal activity was observed against *S. aureus*, *S. epidermidis*, and *S. pyogenes* within 24 h, measured with log CFU/mL (► **Fig. 2**). As a negative control, a SCCO lotion was prepared and assayed. Carrier oils, in general, lack the potent chemical constituents present in EOs to have notable antimicrobial effects. The SCCO lotion showed no antimicrobial activity, as expected, and displayed comparable growth to the culture controls. This shows that none of the formulation excipients possessed or contributed towards antimicrobial activity, and therefore the efficacy of the *C. zizanioides* emulsified lotion (HLB 9) can be solely attributed to the *C. zizanioides* EO. The positive control ciprofloxacin eliminated *S. aureus*, *S. epidermidis*, and *S. pyogenes* within 6–24 h, demonstrating susceptibility of the acne pathogen reference strains.

While *S. aureus*, *S. epidermidis*, and *S. pyogenes* were all killed within 24 h, the *C. zizanioides* emulsified lotion (HLB 9) displayed a slightly faster kill dynamic against *S. pyogenes*, as can be seen with a lower starting log CFU/mL. This is in accordance with the antimicrobial activity of the neat *C. zizanioides* EO, where *S. pyogenes* proved to be the most susceptible pathogen, with the lowest MIC of 0.04 mg/mL. A previous study [26] found three strains of *Streptococcus*, including *S. pyogenes*, to be more susceptible to EO when compared to *S. aureus*. As a Gram-positive microorganism, *S. pyogenes* consists of a thick peptidoglycan cell wall that protects it from the invasion of antimicrobial agents. However, certain chemical constituents have been shown to not only penetrate this cell wall, but enhance the permeability and thereby facilitate cell death, as observed in the case of *S. pyogenes* [27]. Therefore, the antimicrobial efficacy results of both the neat *C. zizanioides* EO and *C. zizanioides* emulsified lotion (HLB 9) are in accordance with each other.

Due to irreproducible results and inconsistencies observed in the growth of *C. acnes* on aerobic agar plates, the more reproducible time-kill method was employed as a measure of inhibition. The *C. zizanioides* emulsified lotion (HLB 9) was observed to completely inhibit *C. acnes* as no signs of turbidity were seen after 72 h of incubation, signifying total elimination of the bacterial load.

The positive control, ciprofloxacin, also presented with clear broth, while the negative control (SCCO lotion) and the culture control (*C. acnes*) presented with turbid broth, indicating microbial growth. Thus, the *C. zizanioides* emulsified lotion (HLB 9) was comparable in activity to the commercial antibiotic ciprofloxacin against *C. acnes*. As *C. acnes* is the most important pathogen involved in acne [28], the prevention of its growth provides an excellent marker of the potential of the *C. zizanioides* emulsified lotion (HLB 9) as a suitable treatment regimen.

As changes in physical stability were noted in the emulsion system with time, antimicrobial assays were repeated at the end of the storage period to ensure maintenance of antimicrobial activity. The aged (84 days) *C. zizanioides* emulsified lotion (HLB 9) displayed total elimination of *S. aureus*, *S. epidermidis*, and *S. pyogenes*. The positive control, ciprofloxacin, remained at 100% inhibition, while the negative control, SCCO lotion, and the culture controls for *S. aureus*, *S. epidermidis* and *S. pyogenes* showed growth on their respective agar plates. The *C. zizanioides* emulsified lotion (HLB 9) also displayed complete inhibition of *C. acnes* 84 days after preparation. The positive control, ciprofloxacin, negative control (SCCO lotion), and the culture control (*C. acnes*) responded as expected. Therefore, regardless of fluctuations in MDD and PDI over time and changes in emulsion stability, the *C. zizanioides* emulsified lotion (HLB 9) remained antimicrobially effective against *C. acnes*, *S. aureus*, *S. epidermidis*, and *S. pyogenes* over an extended shelf life of 84 days. As EO products are often prone to degradation due to the volatility of the EOs themselves, the retention of antimicrobial activity over an extended period is a worthy point for consideration. While previous studies investigated the physical stability of EO emulsions over a long-term period, concurrent antimicrobial studies were conducted, as once-off and long-term data is lacking [25,29–36]. Only two studies could be found with extended antimicrobial activity studies [37,38], however, these were conducted over a significantly shorter time period (42 days and 20 days, respectively) compared to the current study (84 days). As the maintenance of antimicrobial activity is a priority required for the application of EO emulsions, this critical step seems to have been overlooked in the past. This study thus adds value demonstrating a full antimicrobial assessment carried out to ensure efficacy after three months of storage.

► **Table 3** Base formulation to prepare *C. zizanioides* emulsified lotion (40 g).

Required HLB	Mass (g)						
	<i>C. zizanioides</i> EO	Cetyl alcohol	Stearic acid	Glycerine	Tween 80	GMS	Water
8	4.00	1.20	0.60	2.00	1.50	2.50	q. s. ^a
9	4.00	1.20	0.60	2.00	1.86	2.14	q. s.
10	4.00	1.20	0.60	2.00	2.21	1.79	q. s.

^a quantity sufficient

This study successfully prepared an anti-acne *C. zizanioides* emulsified lotion that demonstrated optimum physical stability at HLB 9 and optimum antimicrobial activity (total bactericidal activity against *S. aureus*, *S. epidermidis*, and *S. pyogenes* within 24 h and total inhibition of *C. acnes*). Despite the volatility and instability of EOs and their products, the *C. zizanioides* emulsified lotion formulated in this study maintained its bioactivity for a minimum of three months. This demonstrates the long-term viability of the formulation and provides proof of the practicality of this concept. Thus, *C. zizanioides* emulsified lotion qualifies as a strong candidate for the topical treatment of acne.

Materials and Methods

Essential oil procurement

The *C. zizanioides* EO was procured from Prana Monde and a certificate of analysis containing the GC-MS data was provided by the supplier (► **Table 2**).

Preparation of cultures

Test organisms were selected based on their pathogenesis in acne and were procured from Davies Diagnostics. The facultative anaerobes *S. aureus* reference strain, (ATCC 25923) and *S. epidermidis* (ATCC 2223) were cultured in Tryptone Soya broth (Oxoid) while *S. pyogenes* (ATCC 12344) was cultured in Haemophilus test medium supplemented with nicotinamide adenine dinucleotide (Oxoid). Incubation was carried out at 37 °C for 24 h. The aerotolerant anaerobe *C. acnes* (ATCC 11827) was cultured in Thioglycollate broth (Oxoid) and incubated at 37 °C under anaerobic conditions in a CO₂ incubator (5% CO₂) for 72 h.

Validation of antimicrobial activity of *Chrysopogon zizanioides* essential oil

The broth microdilution method [19] was used to determine the MIC values of the EO. A 100 µL *C. zizanioides* EO, diluted in acetone to obtain a stock concentration of 32.00 mg/mL, and 100 µL of respective broth was introduced into the first well of each column of a microtitre plate (Thermo Fisher Scientific). Doubling serial dilutions were then performed on each sample and test culture (100 µL) containing approximately 1 × 10⁶ CFU/mL was transferred into each well. After incubation (as per respective pathogen conditions), 40 µL of indicator INT (Sigma-Aldrich) at 0.04 mg/mL were added to each well and the MIC was interpreted as the first

clear well, i.e., lowest concentration of sample required to inhibit microbial growth. All tests were performed in triplicate (n = 3) on consecutive days. Ciprofloxacin (purity ≥ 98.0%), purchased from Sigma-Aldrich (100 µL of a concentration of 0.01 mg/mL), was used as a positive control to ensure microbial susceptibility. A 100 µL 32.00 mg/mL water in acetone preparation was employed as the negative control to ensure that antimicrobial activity was not as a result of the solvent. A 100 µL culture control was included to ensure viable growth of the pathogen.

Emulsified lotion design through hydrophile-lipophile balance

The aqueous phase of the emulsified lotion contained 5% w/w glycerine and 72% w/w autoclaved distilled water, while the oil phase consisted of 10% w/w *C. zizanioides* EO (HLB≈7) [11], 2% w/w cetyl alcohol (HLB ≈ 15), and 1% w/w stearic acid (HLB≈15). The total emulsifier was set at 10% w/w and consisted of the hydrophilic emulsifier Tween 80 (HLB≈15) and lipophilic emulsifier GMS (HLB≈3.8). In order to achieve the required HLBs of 8, 9, and 10 for physical stability testing, the ratio of the hydrophilic to lipophilic emulsifier (Tween 80: GMS) was varied to obtain each HLB value, as in the following equations:

$$\% \text{ of Tween 80} = \frac{100 (\text{Desired HLB} - \text{HLB of GMS})}{\text{HLB of Tween 80} - \text{HLB of GMS}}$$

$$\% \text{ of GMS} = 100 - \% \text{ of Tween 80}$$

Total quantities of all ingredients are shown in ► **Table 3**.

Emulsified lotion preparation

The lotions were prepared using a combination of the PIT method and ultrasonification [39,40]. The lipophilic emulsifier, GMS, was weighed and added to the oil phase ingredients (cetyl alcohol and stearic acid), while Tween 80 was dissolved in the aqueous phase (autoclaved distilled water and glycerine). The two phases were placed separately in a water bath and heated to 55 °C. Once all the lipophilic components of the oil phase were melted, the beaker was removed from heat and allowed to cool to 40 °C, whereupon the *C. zizanioides* EO was added and mixed thoroughly at 750 rpm on a magnetic stirrer. The aqueous phase, maintained at 55 °C, was then added dropwise into the oil phase under constant stirring over a period of 30 s. The formulation was subjected to further stirring at 1500 rpm for 30 min until emulsification was achieved. To further reduce the mean oil droplet size and remove

trapped air bubbles, the formulation was placed in a sonicator bath and ultrasonicated for 120 s. The freshly formed emulsion (n = 3) was transferred and stored in appropriate sterile containers for physical stability testing through MDD and PDI.

Physical stability testing (mean droplet size and polydispersity index) of *Chrysopogon zizanioides* emulsified lotion

The MDD of emulsion at each HLB (8, 9, and 10) was measured using a Malvern Zetasizer Nanoseries at 25 °C equipped with a 173 °C backscatter detector. To avoid multiple scattering effects, each sample was diluted 1:100 using Milli-Q water and mixed thoroughly. The MDD was expressed as a Z-average size and the PDI was also recorded as a measure of homogeneity of the emulsion [41]. Data was collected as an average of 13 runs and each measurement was performed in triplicate. Runs were repeated on day 1, 28, 56, and 84 after preparation.

Antimicrobial validation of *Chrysopogon zizanioides* emulsified lotion

For the facultative anaerobes, the adapted broth microdilution method [11] was followed. Under sterile conditions, 100 µL of the optimum culture medium, 100 µL of freshly prepared *C. zizanioides* emulsified lotion at optimum HLB, and 100 µL of test culture (*S. aureus*, *S. epidermidis*, or *S. pyogenes* containing approximately 1×10^6 CFU/mL) were added into each well of the first row of a 48-well microtitre plate. Thereafter, the contents of each well (containing broth, formulation, and inoculum) were thoroughly mixed and 100 µL were transferred to the subsequent well containing 900 µL of sterile 0.90% sodium chloride (NaCl). Successive serial dilutions were carried out on each sample and the microtitre plates were then incubated as per the respective pathogen's optimal incubation requirements. Aliquots of 50 µL samples were periodically withdrawn from the wells at 0, 3, 6, and 24 h, spread onto previously prepared agar plates (Tryptone Soya agar for *S. aureus* and *S. epidermidis* and Haemophilus agar for *S. pyogenes*), and incubated as per respective conditions. The number of colonies with time for each sample was quantified and the logarithm of CFU/mL present in the original well was calculated as follows:

$$\text{Log (CFU/mL)} = \log_{10} \text{ colony count} \times 1 \times 10^9$$

The ensuing results were plotted accordingly. Ciprofloxacin as the positive control, SCCO lotion as the negative, and a test culture of the relevant pathogen strain (all at 100 µL) were also assayed as a reference point. All assays were conducted in triplicate.

For the aerotolerant anaerobe *C. acnes*, the broth turbidity principle [11] was employed. Under sterile conditions, 100 µL of the required test medium, freshly prepared *C. zizanioides* emulsified lotion at optimum HLB, and *C. acnes* test culture (containing approximately 1×10^6 CFU/mL) were added into each well of the first row of a 48-well microtitre plate. The plates were sealed and incubated anaerobically for 72 h. Thereafter, a 100-µL aliquot of each well was removed and transferred to 9.9 mL of Thioglycollate broth in a sterile test tube. The resulting dilution was incubated for 72 h anaerobically, upon which the test tubes were visually in-

spected. Visual inspection was the preferred measure of assessment as incubation over the 72 h test period resulted in colour changes of the formulation, making UV measurements (standard microbial methods) redundant. The absence of turbidity was an indicator of complete inhibition of *C. acnes* while turbid broth signified growth. Comparable controls as stated previously (i.e., ciprofloxacin, SCCO, and test culture) were employed. All measurements were conducted in triplicate.

Preservation of antimicrobial efficacy of *Chrysopogon zizanioides* emulsified lotion

The *C. zizanioides* emulsified lotion at optimum HLB was stored at room temperature (25 °C) for 84 days, upon which the retention of its antimicrobial efficacy over time was assessed. In a 48-well microtitre plate, 100 µL of broth, 100 µL of aged formulation (84 days), and 100 µL of test culture (*S. aureus*, *S. epidermidis*, *S. pyogenes* or *C. acnes*) were mixed in a well. The plate was sealed with a sterile seal and incubated as per the respective pathogen reference strain's requirements. Thereafter, a 50-µL sample was removed from each well. For the facultative anaerobes, the sample was spread onto Tryptone Soya agar for *S. aureus* and *S. epidermidis* and Haemophilus agar for *S. pyogenes* and incubated accordingly. A clear plate would indicate total inhibition and therefore the preservation of antimicrobial activity, while the presence of colonies would signify loss of efficacy. For the aerotolerant anaerobe *C. acnes*, the inoculated aged sample was transferred into 9.9 mL of Thioglycollate broth and incubated for a further 72 h. The absence or presence of broth turbidity was recorded for each *C. zizanioides* emulsified lotion sample in an identical manner to the initial assay, and comparisons between the two were drawn.

Contributors' Statement

FK: experimental, data analysis, drafting of manuscript; AO, PD and SvV: supervision, conceptual design, editorial input to the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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