Antimicrobial Formulation of \textit{Chrysopogon zizanioides} Essential Oil in an Emulsified Lotion for Acne

**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. \textit{Chrysopogon zizanioides} (vetiver) displayed noteworthy antimicrobial activity with a mean minimum inhibitory concentration of 0.14 mg/mL against \textit{Cutibacterium acnes}, \textit{Staphylococcus aureus}, \textit{Staphylococcus epidermidis}, and \textit{Streptococcus pyogenes}. Emulsified lotions containing \textit{C. zizanioides} were developed through the hydrophilic-lipophilic balance approach. At tested hydrophilic-lipophilic balance values of 8, 9, and 10, \textit{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 \textit{C. zizanioides} emulsified lotion at optimum hydrophilic-lipophilic balance 9 completely inhibited the growth of \textit{C. acnes} and killed \textit{S. aureus}, \textit{S. epidermidis}, and \textit{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 \textit{C. zizanioides} emulsified lotion demonstrated physical stability and antimicrobial efficiency, making it an ideal natural product anti-acne treatment.

**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 \textit{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-
Additionally, 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 antioxidiant and anti-inflammatory properties [9]. A total of 98 EOs have been recommended for dermatological use, of which 49 were specifically identified for the treatment of acne [10]. A previous study [11] 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.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (mg/mL)</th>
<th>Cutibacterium acnes (ATCC 11 827)</th>
<th>Staphylococcus epidermidis (ATCC 2223)</th>
<th>Staphylococcus aureus (ATCC 25923)</th>
<th>Streptococcus pyogenes (ATCC 12 344)</th>
<th>Mean MIC ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. zizanioides EO</td>
<td></td>
<td>0.25 ± 0.00</td>
<td>0.10 ± 0.02</td>
<td>0.17 ± 0.09</td>
<td>0.04 ± 0.00</td>
<td>0.14 ± 0.09</td>
</tr>
<tr>
<td>Positive control – Ciprofloxacin (µg/mL)</td>
<td>0.04 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>0.31 ± 0.00</td>
<td>0.11 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Negative control – Acetone</td>
<td>&gt; 8.00</td>
<td>&gt; 8.00</td>
<td>&gt; 8.00</td>
<td>&gt; 8.00</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Culture control</td>
<td>&gt; 8.00</td>
<td>&gt; 8.00</td>
<td>&gt; 8.00</td>
<td>&gt; 8.00</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

* N/A – not applicable as values cannot be calculated from current data set.

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

<table>
<thead>
<tr>
<th>Retention Time</th>
<th>Constituent*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>76.9</td>
<td>Vetisellinenol</td>
<td>3.32</td>
</tr>
<tr>
<td>80.3</td>
<td>β-Vetivone</td>
<td>2.67</td>
</tr>
<tr>
<td>80.5</td>
<td>Khusimol</td>
<td>7.93</td>
</tr>
<tr>
<td>81.9</td>
<td>Isovalencenol</td>
<td>7.39</td>
</tr>
<tr>
<td>82.1</td>
<td>α-Vetivone</td>
<td>3.73</td>
</tr>
<tr>
<td>101.2</td>
<td>Khusenic acid</td>
<td>15.87</td>
</tr>
<tr>
<td>N/A</td>
<td>Minor constituants</td>
<td>54.43</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>95.34</td>
</tr>
</tbody>
</table>

* 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 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.
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. 1a). 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. 1b). 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-
The susceptibility of the acne pathogen, S. aureus, S. epidermidis, and S. pyogenes to EO was assessed using a time-kill assay. The C. zizanioides emulsified lotion (HLB 9) was observed to completely inhibit S. aureus, S. epidermidis, and S. pyogenes within 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) remained turbid, 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.

**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.
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 25 923) and *S. epidermidis* (ATCC 22 223) were cultured in Tryptone Soya broth (Oxoid) while *S. pyogenes* (ATCC 12 344) 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 11 827) was cultured in Thioglycolate 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 hydrophilic-lipophilic 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 (Twee 80: GMS) was varied to obtain each HLB value, as in the following equations:

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

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

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 then 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

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

* quantity sufficient
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 × 10⁹ 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 anaerobically for 72 h. The absence or presence of broth turbidity was recorded for each sample. The inoculated aged sample was transferred into fresh 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). The ensuing results were plotted accordingly. Ciprofloxacin as the positive control, SCCO lotion as the negative, and a test culture of C. acnes 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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