Available online at https://journal.rescollacomm.com/index.php/ijqrm/index

International Journal of Quantitative Research and Modeling

e-ISSN 2721-477X
p-ISSN 2722-5046

Vol. 7, No. 2, pp. 205-220, 2026

Formulation and antibacterial activity testing of essential oil-based hand sanitizer spray citronella grass leaves (*cymbopogon nardus*)

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Abstract

Hand hygiene is an essential aspect of preventing healthcare-associated infections (HAIs). However, repeated use of alcohol-based hand sanitizers can cause skin dryness, irritation, dermatitis, and even allergic reactions, driving the demand for safer natural active ingredients. This study aims to formulate and evaluate the antibacterial activity of a spray hand sanitizer containing citronella (*Cymbopogon nardus*) essential oil. This research employed a quantitative–qualitative approach using a laboratory experimental design. The results showed that all HS-CO formulations were homogeneous, exhibited a color transition from clear to milky white as the concentration increased, possessed a characteristic leafy odour, exhibited good spreadability, and maintained pH values within the physiological range of human skin. The antibacterial activity test of citronella oil demonstrated zones of inhibition ranging from 8.93±2.09 mm to 17.02±4.13 mm against *Staphylococcus aureus* and from 9.28±1.44 mm to 18.19±4.06 mm against *Escherichia coli*. All formulations (P1, P2, P3, and P4) exhibited exceptional antibacterial efficacy, achieving a log reduction value of 8.18 within only 30 seconds of contact time. This value was equivalent to 100% bacterial reduction, indicating better antibacterial effectiveness than 70% alcohol.

Keywords: Antibacterial, citronella, formulation, hand sanitizer.

1. Introduction

Substandard healthcare services remain a critical global issue with profound implications for patient safety. According to WHO and UNICEF (2024), more than eight million deaths occur annually across 137 low and middle income countries due to poor quality of care, exacerbated by inadequate access to clean water, sanitation, and hygiene services. A major consequence of these deficiencies is the rising incidence of healthcare associated infections (HAIs). These infections occur when patients acquire pathogenic microorganisms during medical treatment. Based on a recent systematic review, the prevalence of HAIs in Southeast Asia has reached 22%, with Indonesia exhibiting a notably high rate of approximately 30,4% (Wah et al., 2023). This figure sits well above the global average of 7%–22% reported by the WHO (Khan et al., 2017).

Healthcare workers in medical facilities serve as the primary source of HAIs, transmitting pathogens to patients via hand contact (Wahyutomo, 2025). Consequently, hand hygiene represents the most effective preventive measure, capable of reducing the transmission of pathogenic microorganisms by 24% to 31% (Rundle et al., 2020). Alcohol based hand sanitizers are fast acting hand decontamination products recommended by the WHO. Their mechanism of protein denaturation is highly effective against various bacteria and viruses (Aodah et al., 2021 ; Hayati et al., 2022 ; Bayer et al., 2023 ; Ariani et al., 2024). However, repeated application of alcohol based sanitizers can dissolve skin lipids and disrupt the integrity of the skin cell barrier as a biological shield, thereby triggering skin dryness, dermatitis, irritation, and allergic reactions (Risti et al., 2019 ; Abo-Zeid et al., 2022 ; Lehtinen et al., 2022 ; Risma et al., 2023). Healthcare professionals are known to perform hand hygiene more than 10 times per day during clinical activities, which increases their risk of developing hand dermatitis by up to 55% compared to the general population (Dewantara, 2023). This painful condition can decrease hand hygiene compliance and subsequently increase the risk of HAI dissemination (de Kraker et al., 2022). Therefore, safer alternative active ingredients are urgently required to provide robust antibacterial activity without causing skin dryness, dermatitis, irritation, or allergies.

Citronella oil holds immense potential due to its high concentration of citronellal, which ranges from 42,28% to 55,78% (Kurniawan et al., 2020 ; Trindade et al., 2022 ; Itawani et al., 2024). In addition, other active compounds such as geraniol, geranial, neral, and citronellol provide synergistic antibacterial, antifungal, and antiviral activities

(Putri et al., 2017 ; Martihandini et al., 2023). Its strong antibacterial efficacy against *Staphylococcus aureus* and *Escherichia coli* has been demonstrated in numerous studies, yielding inhibition zone diameters ranging from 13 mm to 18,36 mm (Kamari et al., 2018 ; Rina et al., 2020 ; Dasmyta et al., 2024).

Furthermore, the hectic workflow of healthcare workers demands an innovative and practical dosage form. Spray type hand sanitizers serve as an ideal choice because they are easy to apply, quick drying, capable of covering hand surfaces evenly without rinsing, and highly portable (Risti et al., 2019 ; Ariani et al., 2024). Therefore, this study was conducted to formulate and evaluate the antibacterial activity of a citronella oil (CO) blend in a spray hand sanitizer format (HS-CO) as an effective, skin safe, and natural antiseptic alternative.

2. Literature Review

Essential oils (EOs) are natural secondary metabolites derived from plants, consisting of complex mixtures of volatile components characterized by a distinct odour and high volatility (Guandalini et al., 2020). Biosynthetically, these compounds are synthesized within the secretory cells of aromatic plants via the acetate-malonate pathway. This process involves isoprene precursors, such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which condense into geranyl pyrophosphate (GPP) for monoterpene formation and farnesyl pyrophosphate (FPP) for sesquiterpene synthesis (Bayala et al., 2020). Beyond this pathway, EOs can also be synthesized through the shikimate-chorismate pathway, which yields a phenylpropane skeleton (Mulyani et al., 2021).

Citronella (*Cymbopogon nardus*), a member of the Poaceae family, is widely recognized as an aromatic grass that yields highly economically valuable essential oil. It is an erect, perennial herb that can reach heights of 1–1,5 meters. Morphologically, citronella features green to reddish stems, long ribbon like green leaves resembling cogon grass, and dense clusters of adventitious roots. When crushed, the leaves emit a sharp, potent, and characteristic odour. Although citronella can adapt to various environmental conditions, optimal growth occurs in sandy soil with a neutral pH at altitudes of 350–600 meters above sea level, temperatures of 18–25°C, annual rainfall of 1,800–2,500 mm, and full sunlight exposure throughout the year (Kumoro et al., 2021). The oil from the citronella plant can be extracted using conventional methods, such as hydrodistillation, steam distillation, steam-water distillation, and cold pressing, as well as via innovative techniques, including supercritical carbon dioxide ($scCO_2$) fluid extraction and microwave-assisted extraction (Maes et al., 2019). Following extraction, Gas Chromatography-Mass Spectrometry (GC-MS) is commonly employed to separate and identify the volatile chemical constituents based on their boiling points.

GC-MS analysis reveals that citronella oil exhibits a golden brown (sherry) coloration and is predominantly composed of a complex mixture of monoterpenes and sesquiterpenes, including citronellal, citronellol, geraniol, nerol, and citral (geranial/neral) (Bayala et al., 2020 ; Guandalini et al., 2020 ; Prasad et al., 2022). Citronellal is a monoterpene containing an aldehyde group that undergoes oxidation, which contributes to its distinctive fragrant aroma (Risti et al., 2019). The profile of these components varies across the literature, for instance, Guandalini et al (2020) identified 16 compounds, with the major constituents being citronellal (27,53%), citronellol (25%), and nerol (2,89%). Conversely, Bayala et al (2020) successfully identified 43 compounds, highlighting five dominant components: citronellal (33,06%), geraniol (28,40%), nerol (10,94%), elemol (5,25%), and δ-elemene (4,09%). This variation in chemical composition and essential oil quality is influenced by genetic factors (chemotypes), plant age, harvest time, environmental conditions, and post-harvest treatments (drying and extraction techniques). While the application of fertilizers and the presence of rhizosphere fungi can enhance citral content, the optimal harvesting period is recommended at 5,5–6,5 months, as it yields the highest oil content with a geraniol increase of up to 45,95%.

The chemical composition of citronella oil directly governs its biological activities. Hydrocarbon monoterpenes generally exhibit weaker antibacterial activity compared to oxygenated monoterpenes, whereas phenolics and aldehydes frequently demonstrate the most potent efficacy (Mulyani et al., 2021). The components citronellol and citronellal operate by disrupting the structure of the cytoplasmic membrane and cell wall through alterations in physicochemical properties, such as hydrophobicity and surface charge (Guandalini et al., 2020). Meanwhile, the hydrophobic citral component partitions into the lipid bilayer of the bacterial cell membrane, increasing membrane fluidity and permeability. This disruption triggers the leakage of ions and essential intracellular components, ultimately causing cell lysis or metabolic failure (Martihandini et al., 2023). However, the efficacy of this antibacterial spectrum is also determined by the nature of the target organism. Gram-negative bacteria, such as *Escherichia coli*, tend to be more resistant than Gram-positive bacteria, such as *Staphylococcus aureus*. This resistance occurs because Gram-negative bacteria possess a more complex, three layered cell wall structure (composed of lipoproteins, lipopolysaccharides, and peptidoglycan) that restricts the penetration of hydrophobic compounds. In contrast, Gram-positive bacteria feature a thick yet simpler peptidoglycan cell wall, allowing active components to easily penetrate and damage intracellular structures (Ariani et al., 2024).

Despite its immense potential across various industries, utilizing essential oils in water-based formulations, such as hand sanitizers, faces a significant bottleneck due to their hydrophobic nature. This hydrophobicity makes the oil poorly soluble in water, leading to non-homogeneous preparations and diminished antibacterial efficacy. This physical stability issue can be mitigated by incorporating surfactants, such as polysorbate 80 (Tween 80), into gel or

spray formulations. Surfactants possess both polar (hydrophilic) and non-polar (hydrophobic) groups that lower interfacial tension, thereby enabling the oil and water phases to coalesce into a homogeneous and stable emulsion (Martihandini et al., 2023; Dasmyta et al., 2024). Furthermore, to minimize adverse dermatological side effects, hand sanitizer formulations are commonly combined with humectants, such as urea, glycerin, or propylene glycol, which attract water molecules to enhance hydration within the stratum corneum of the skin (Rundle et al., 2020).

3. Materials and Methods

3.1. Materials

The primary raw material used in this study was the aster cultivar of citronella (*Cymbopogon nardus*). The citronella plants were harvested from Dlingo, Bantul, Special Region of Yogyakarta. Plant materials aged 34 days were selected, and the leaves were utilized for the extraction process. The plants were cultivated at an altitude of approximately 137 meters above sea level.

3.2. Methods

This research employed a mixed quantitative–qualitative approach using a laboratory experimental design. Two independent variables were evaluated: the concentration of citronella oil consisting of five distinct treatments (Table 1) and two species of test bacteria, namely *Staphylococcus aureus* and *Escherichia coli*. Meanwhile, the dependent variables encompassed the physicochemical characteristics of the formulation and its antibacterial efficacy. The measured formulation characteristics included pH, color, appearance, odour, spreadability, and homogeneity. Concurrently, the antibacterial efficacy was assessed based on the zone of inhibition and the bacterial kill percentage.

Table 1: Citronella oil concentration treatments in HS-CO formulations.

HS-CO	Citronella Oil Consentration
P0	0×MIC
P1	0,5×MIC
P2	1× MIC
P3	1,5×MIC
P4	2×MIC

Note: MIC (Minimum Inhibitory Concentration): the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism.

3.2.1. Extraction of Citronella Oil (CO)

The extraction of CO was performed using the water-steam distillation method. The citronella leaves were initially dried under indirect sunlight for three to four hours. Subsequently, the plant materials were placed on a perforated grid inside the distillation still. Distilled water was added to the bottom of the vessel, reaching just below the grid level without making direct contact with the plant tissue. The system was heated and maintained at 100°C for three hours. The generated steam passed through the plant matrix, volatilizing the essential oil components, which were then routed into a condenser for cooling. The resulting distillate was collected through a valve at the bottom of the receiving vessel, separating into two distinct layers: an upper organic (oil) phase and a lower aqueous phase. Residual water in the oil phase was removed by adding anhydrous natrium sulfate (Na2SO4), followed by filtration. The yield percentage of the obtained essential oil was calculated based on Eq. (1) (Nur et al., 2019).

$$\textbf{Yield (\%)} = \frac{W_o}{W_b} \times 100\% \quad (1)$$

Note: W_o = the weight of the extracted essential oil (g), W_b = the initial weight of the plant material raw sample (g).

3.2.2. Chemical Composition Analysis

The chemical profile of the citronella oil sample was analyzed using a Gas Chromatography-Mass Spectrometry (GC–MS) instrument equipped with an AOC-20i autosampler. Chromatographic separation was achieved using an Rxi-5MS capillary column 30 m × 0,25 mm i.d., film thickness 0,25 μm. The operational parameters of the GC–MS system were configured as follows: helium was utilized as the carrier gas, the initial column temperature was set at 80°C and increased to 200°C at a heating rate of 4°C/ minutes, followed by a secondary ramp from 200°C to 240°C at 10°C/minutes, while the injector temperature was maintained at 250°C. The mass spectrometer was operated in electron-impact ionization (EI) mode at 70 eV with a scan time of 0,3 seconds and a mass acquisition range of 35–500 m/z. Chemical constituents were identified through chromatogram interpretation by comparing their retention times,

m/z values, and mass spectrum fragmentation patterns with data compiled in the National Institute of Standards and Technology (NIST) reference library (Afrizal et al., 2024).

3.2.3. MIC/MBC

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method (Silveira et al., 2014). Prior to the assay, all equipment and media were sterilized via autoclaving at 121°C for 15 minutes. Overnight cultures of *Staphylococcus aureus* and *Escherichia coli* were adjusted to match a 0,5 McFarland standard ($1–2 \times 10^8$ CFU/mL) and subsequently diluted at a 1:15 ratio to achieve a working bacterial density of approximately (5×10^5 CFU/mL). A stock solution of the citronella oil was prepared by dissolving the oil in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/mL. Into each well of a 96 well microplate, 100 µL of Mueller-Hinton Broth (MHB) was introduced, and 100 µL of the citronella oil sample was added to the first well, followed by a two-fold serial dilution across the rows. Each well was then inoculated with 10 µL of the bacterial suspension. To ensure the validity of the results, specific controls were included: a negative control containing only MHB and a positive control consisting of MHB inoculated with the bacterial suspension. The microplates were subsequently incubated at 37 °C for 24 hours. Post-incubation, bacterial growth was evaluated through visual inspection of turbidity in each well. The MIC value was defined as the lowest concentration of essential oil that demonstrated a complete absence of visible growth (clear medium).

The minimum bactericidal concentration (MBC) was determined from the microdilution plates used in the MIC assay. A 50 µL aliquot from the wells showing no visible growth (at and above the MIC) was subcultured onto solid agar plates and incubated. The MBC value was defined as the lowest sample concentration that completely eradicated bacterial growth following the incubation period.

3.2.4. Formulation and Characterization of the HS-CO

The HS-CO spray was formulated according to the compositions specified in Table 2, modifying the procedure described by Aodah et al (2021). The preparation was initiated by blending glycerin and polysorbate 80 (Tween 80). Concurrently, CO was incorporated into the mixture at the designated treatment concentrations. Aquadest was then added to bring the final volume to 50 mL. The pH of the resulting solution was adjusted dropwise using 0,4% natrium hydroxide (NaOH) until it reached a baseline pH of 6,3. The mixture was continuously agitated using a magnetic stirrer at a speed of 100 RPM for 45 minutes at room temperature to ensure complete homogeneity.

Following successful preparation, the physicochemical characteristics of the HS-CO spray were evaluated, encompassing color, appearance, odour, pH, spreadability, and homogeneity. Color, appearance, and odour were assessed via visual and olfactory inspection. The pH value was measured using a calibrated pH meter. The pH of the HS-CO formulation must align with the physiological pH range of human skin (4,5–6,5) to minimize the risk of dermatological irritation (Risti et al., 2019 ; Primasari et al., 2021). Spreadability was evaluated by spraying the HS-CO formulation onto a pre-weighed transparent plastic sheet from a fixed distance of 10 cm from the spray nozzle. After which the resulting deposition diameter was measured (Ariani et al., 2024). A formulation met the acceptance criteria if it was easily aerosolized, forming fine, uniformly distributed particles (Martihandini et al., 2023). The homogeneity test was performed by applying a few drops of the HS-CO formulation onto a glass slide to inspect for phase separation or coarse particulates (Ariani et al., 2024). A high quality formulation exhibits a uniform structure with no visible coarse grains (Risma et al., 2023).

Table 2: HS-CO formulation.

Materials	Concentration (%)				
	P0	**P1**	**P2**	**P3**	**P4**
CO	0×MIC	0,5×MIC	1×MIC	1,5×MIC	2×MIC
Glycerin	1,45%	1,45%	1,45%	1,45%	1,45%
Tween-80	1%	1%	1%	1%	1%
NaOH	0,4%	0,4%	0,4%	0,4%	0,4%
Aquadest	50 ml	50 ml	50 ml	50 ml	50 ml

3.2.5. Disk Diffusion Method

The antibacterial activity assay was performed to verify whether bacterial eradication was driven solely by the essential oil or enhanced by the synergistic effects of the formulation components (Table 2). This phase was executed using the Kirby-Bauer disk diffusion method against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. Sterile petri dishes were prepared by pouring molten MHA and allowing it

to solidify. Once solidified, a bacterial suspension adjusted to a 0,5 McFarland standard (1–2 × 10^8 CFU/mL) was inoculated onto the MHA medium and streaked uniformly using a sterile cotton swab to achieve a confluent lawn. Sterile filter paper disks were subsequently submerged in each respective treatment formulation for 30 seconds before being gently pressed onto the surface of the inoculated agar. For comparative analysis, a positive control using amoxicillin and a negative control using aquades were included. All petri dishes were incubated at 37°C for 24 hours. Post-incubation, the resulting zones of inhibition were measured precisely using a digital vernier caliper and calculated according to Eq. (2).

$$\textbf{Inhibition Zones} = \frac{(Dv-Dc)+(Dh-Dc)}{2} \quad (2)$$

Note: Dv = vertical diameter (mm), Dc = disk diameter (mm), Dh = horizontal diameter (mm).

3.2.6. Time-kill Kinetic Assay

The time-kill kinetic assay of the HS-CO formulation to evaluate the bacterial kill percentage was performed by modifying the procedure described by Yolanda et al (2017). A 0,5 mL aliquot of the bacterial suspension, adjusted to a 0,5 McFarland standard (1–2 × 10^8 CFU/mL), was mixed thoroughly with 4,5 mL of the HS-CO formulation. After an exposure time of 30 seconds, a 1 mL sample from the mixture was transferred into Tube A, which contained 9 mL of a neutralizer solution to halt the antimicrobial activity. Concurrently, after an exposure time of 60 seconds, another 1 mL sample from the original mixture was transferred into Tube B containing 9 mL of the neutralizer solution. Subsequently, a 1 mL aliquot from each neutralized tube was plated onto MHA plates using the pourplate technique. The plates were then incubated at 37°C for 24 hours. For the positive control group, a 0,5 mL bacterial suspension was mixed with 4,5 mL of 70% alcohol and subjected to the identical experimental procedure. Post-incubation, the number of surviving bacterial colonies was quantified using a colony counter, and the percentage kill was calculated according to Eq. (3).

$$\textbf{Percentage kill (\%)} = \frac{(C-X)}{C} \times 100\% \quad (3)$$

Note: C = initial colony count (CFU/mL), X = the surviving bacterial colony count in the treated sample after exposure (CFU/mL).

3.2.7. Statistical Analysis

Qualitative data were presented descriptively. Quantitative findings were obtained from five independent replicates and expressed as mean ± standard deviation (SD). Data normality was evaluated using the Shapiro-Wilk test. The inhibition zone data satisfied the assumption of normality (p > 0,05) but violated the homogeneity of variance assumption. Thus, a one-way Analysis of Variance (ANOVA) was still applicable at a 95% confidence level. For analyses demonstrating a significant treatment effect (p < 0,05), post hoc comparisons were performed using the Games–Howell test. Conversely, the time-kill kinetic data did not satisfy the assumptions of normality and homogeneity of variance, precluding the use of ANOVA. As an alternative, data were analyzed using the nonparametric Kruskal–Wallis test. If a statistically significant difference was detected (p < 0,05), post hoc analysis was subsequently conducted using Pairwise Comparisons.

4. Results and Discussion

4.1 Physicochemical Characteristics of the HS-CO Formulation

4.1.1 Extraction and Quality of Citronella Oil

The citronella oil (CO) utilized in this study was extracted via the water-steam distillation method. This method was selected to ensure that the plant material only interacted with saturated wet steam at moderate temperatures, thereby preventing thermal degradation typically caused by direct contact with boiling water (Sastrohamidjodjo, 2018). The quantitative yield of the extracted CO is presented in Table 3.

Table 3: Extraction yield of citronella oil.

Weight of citronella leaves (g)	Weight of extracted citronella oil (g)	Citronella oil ield (%)
85.000	600	0,71

Based on the data, 600 grams of CO were obtained, corresponding to an essential oil yield of 0,71%. This yield falls within the standard baseline range of 0,5–1,5% typically recovered from fresh citronella leaves (Kristiana et al., 2025). Furthermore, the yield observed in this study is comparable to the findings of Parman et al., (2023), who reported a yield of 0,78% using fresh leaves. This minor variance is postulated to stem from the pre-extraction wilting or drying treatment of the raw material. In the present study, the citronella leaves underwent a brief three hour drying

process prior to distillation, which served to mitigate the loss of volatile constituents and restrict the formation of undesirable artifact transformation products (Mohammed & Alfazi, 2026).

However, this yield profile contrasts significantly with the results reported by Dacosta et al., (2017), where citronella oil extracted at an altitude of 12 meters above sea level (asl) produced an oil content of 8,11 ± 0,4%, while cultivation at 1100 meters asl yielded 11,47 ± 0,4%. For comparison, the raw plant material in this study was harvested from a geographic location at an altitude of 137 meters asl. Theoretically, altitude closely dictates environmental temperature, higher elevations are characterized by lower ambient temperatures.

These microclimatic conditions exert a profound influence on plant development and secondary metabolite biosynthesis. Elevated ambient temperatures accelerate the transpiration rate in citronella plants. Conversely, the cultivation site in this study was characterized by canopy shading, which induced a restricted solar irradiance intensity. Such low-light stress conditions suppress the photosynthetic rate, thereby reducing the net assimilation yield (Danata et al., 2023). Under the physiological strain of light limitation, the scarce assimilatory products, chiefly total proteins, amino acids, and carbohydrates are heavily allocated toward chlorophyll biosynthesis (specifically chlorophyll b) to maximize light harvesting capacity (Thakur et al., 2019). This emergency energy reallocation triggers morphological adaptations, such as an expansion of leaf surface area and width, to optimize light capture.

Nevertheless, because the majority of these assimilatory products are consumed to sustain vegetative growth, cellular packing within the leaf tissue becomes less dense. Consequently, this leads to a reduction or a relatively lower accumulation of total plant biomass (Pratiksha et al., 2026). This energetic trade-off directly impairs the absolute quantity of the biosynthesized essential oil. The restricted energy pool derived from primary metabolism (utilized for leaf expansion) dominates the plant's metabolic budget. As a result, insufficient energy remains to feed secondary metabolic pathways, such as oil storage, which is reflected in a lower density of glandular trichomes (Silva & Rasp, 2021).

The restricted oil volume was further compounded by the harvesting age of the crops, which was only 34 days. This harvesting timeframe represents a relatively immature developmental stage compared to the standard commercial citronella harvesting cycle of approximately 3 to 4 months. Due to harvesting during this early vegetative phase, the anatomical structure of the glandular trichomes had not yet achieved full ontogenetic maturity in terms of both dimensions and storage capacity. Consequently, the extractable oil volume remained constrained, elucidating the lower essential oil yield observed in this research.

Despite the low quantitative yield, the vegetative morphology of the young leaves demonstrated robust elongation, driven by fertilization using the distillation by product liquid (hydrosol). Hydrosol is rich in organic carbon (C-organic), which activates rhizosphere microorganisms to decompose organic matter and enhance atmospheric nitrogen fixation, thereby providing essential macronutrients (Nitrogen, Phosphor, Kalium) to the plant (Bahar et al., 2020 ; Silva & Rasp, 2021). This adequate nutrient availability stimulates plastid organelles, specifically chloroplasts, which serve as the primary site for monoterpene biosynthesis. Thereby enhancing the concentration of essential oil constituents (Efendi et al., 2021).

This physiological phenomenon was validated in the present study. Although the interplay between early harvest maturity and environmental stressors resulted in a lower oil yield (0,71%), GC-MS analysis of the essential oil revealed an exceptional chemical profile characterized by high-quality major compounds: citronellal (37,78%), geraniol (18,89%), and citronellol (13,12%) (Table 4). This outcome strongly indicates that the application of hydrosol byproduct acts as an effective organic elicitor. It successfully stimulates secondary metabolic pathways, ensuring that the biosynthesis of major monoterpene compounds remains highly optimized within the leaf tissue despite the early developmental age of the crop.

This behavior stands in stark contrast to the study by Dacosta et al., (2017), which achieved a higher quantitative oil yield but recovered a chemical profile dominated predominantly by fatty acid groups with a negligible geraniol content. This demonstrates that a superior essential oil yield does not universally guarantee high chemical quality. While a elevated yield indicates a greater volume of oil extracted from the raw biomass, it does not ensure that the oil's chemical composition satisfies required standards.

Table 4: Chemical composition of citronella oil.

No	Compound Name	Retention Time	Area (%)
Monoterpenes			
Monoterpene hydrocarbons			
1	d-Limonene	4,88	3,9
Oxygenated monoterpenes			
Monoterpene alcohols			
2	Linalool	6,227	1,34
3	Isopulegol	7,73	0,82
4	Citronellol	9,492	13,12
5	Geraniol	10,279	18,89

Monoterpene aldehydes			
6	Citronellal	7,595	37,78
7	Geranial	10,673	0,39
Monoterpene esters			
8	Citronelyll acetate	13,025	2,76
9	(-)-Lavandulyl acetate	13,927	1,99
Monoterpene ethers			
10	Eucalyptol	4,952	0,46
Sesquiterpenes			
Sesquiterpene hydrocarbons			
11	(+)-Cyclosativene	13,533	0,46
12	(-)-Germacrene D	14,269	1,23
13	Caryophyllene	15,119	3,72
14	α-Bergamotene	15,523	0,58
15	Humulene	16,103	0,67
16	β-Gurjunene	16,901	0,97
17	(+)-epi-Bicyclosesquiphellandrene	17,222	0,41
18	α-Muurolene	17,43	0,46
19	γ-Muurulene	17,606	0,46
20	(+)-δ-Cadinene	18,103	2,79
Oxygenated sesquiterpenes			
Sesquiterpene alcohols			
21	Cubebol	17,844	0,77
22	β-Elemol	18,839	1,36
23	τ-Cadinol	21,451	0,66
24	τ-Muurolol	21,793	0,92
Others			
25	cis-Vaccenic acid	32,871	3,09
Total monoterpenes			3,9
Total oxygenated monoterpenes			77,55
Total sesquiterpenes			11,75
Total oxygenated sesquiterpenes			3,71
Total others			3,09
Grand total			100

Table 5: Quality profile of citronella oil based ISO standard benchmarks.

Parameter	Standard Range	Essential Oil Result	Testing Method
Citronellal	3–6 %	37,78 %	ISO 3849-2003
Citronellol	3–8,5 %	13,12 %	ISO 3849-2003
Geraniol	15–23 %	18,89 %	ISO 3849-2003

The therapeutic and commercial quality of citronella oil is strictly governed by its primary bio-active constituents, namely citronellal, citronellol, and geraniol. In this study, the concentrations of citronellal and citronellol exceeded the quality benchmarks set by the ISO 3849-2003 standard, while the geraniol content fell precisely within the designated standard range (Table 5). These findings confirm that the generated citronella oil possesses an excellent quality profile, aligning with Guandalini et al. (2020), who previously identified citronellal (27,53%) and citronellol (25%) as the primary dominant compounds. Variances in secondary metabolite concentrations are typically modulated by genetic characteristics and intrinsic physiological processes (Prasad et al., 2022). Furthermore, variables such as plant variety, soil fertility, the specific plant organs utilized, storage conditions, climate, altitude, harvesting season, geographic origin, chemotype, genotype, sample preprocessing, and extraction duration collectively shape the volatile profile of essential oils (Benoudjit et al., 2020 ; Permadi et al., 2024 ; Prasad et al., 2022). Nonetheless, specific chemical classes, including hydrocarbon terpenes, ketones, alcohols, esters, and aldehydes, are consistently identified within the essential oil matrix. The core major components universally found in citronella oil comprise citronellal, citronellol, geraniol, citral, and eugenol; these compounds serve as definitive marker compounds to verify the authenticity and quality of the essential oil (Kumoro et al., 2021)

4.1.1 Formulation and Characteristic of HS-CO

The successfully extracted CO was subjected to MIC evaluation to determine its baseline antimicrobial efficacy. Based on the MIC findings, a concentration of 22,73 mg/mL (Table 6) was designated as the baseline treatment

parameter for the formulation (Table 1). Subsequently, the citronella oil-based hand sanitizer (HS-CO) formulations were prepared according to the compositions detailed in Table 2. Visual observations revealed a distinct color transition in the formulations, shifting from completely clear (P0) to a milky white appearance (P1–P4). This color transformation demonstrated a concentration dependent trend, wherein higher concentrations of incorporated CO yielded an increasingly opaque appearance. This phenomenon serves as a macroscopic indicator of a stable emulsion or dispersion system formed between the volatile essential oil and polysorbate 80 (Tween 80) acting as the surfactant. Martihandini et al. (2023) previously reported that binary systems containing distinct oil and aqueous phases are inherently thermodynamically unstable and difficult to stabilize without the incorporation of an appropriate surfactant. The addition of 5% Tween 80 has been documented to yield highly stable formulations. Furthermore, surfactants induce a physical transition from transparency to opacity due to the formation of dispersed oil globules. The surfactant molecules lower the interfacial tension between the oil and water phases, allowing the essential oil to become uniformly dispersed within the continuous aqueous phase as microglobules (McClements, 2021). Despite their minute dimensions, these newly formed oil globules scatter incident light as it passes through the medium, a phenomenon recognized as the Tyndall effect. When the internal phase globule size is exceedingly small (< 50 nm), the system remains optically clear because light passes through without significant scattering. Conversely, larger globule dimensions (100–500 nm) drastically increase light scattering, thereby producing a characteristic milky white appearance (Gama et al., 2025).

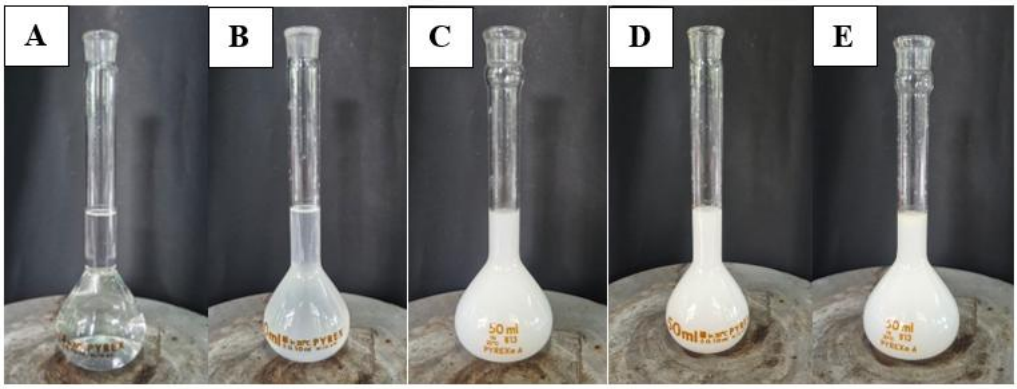


Figure 1: Physical appearance and color characteristics of the HS-CO formulation.

Notes: HS-CO formulation under treatment P0 (A), HS-CO formulation under treatment P1 (B), HS-CO formulation under treatment P2 (C), HS-CO formulation under treatment P3 (D),
HS-CO formulation under treatment P4 (E).

(Source: personal documentation)

In terms of appearance, all formulated preparations maintained a fluid, liquid consistency. This behavior is postulated to stem from the relatively low concentration of Tween 80 utilized in this study (1%), which was insufficient to induce a highly viscous gel network. This stands in contrast to the findings of Martihandini et al. (2023), who utilized 5% Tween 80, resulting in significantly more viscous preparations. Sanglyazzahra & Wikantyasning, (2020) observed that while surfactants successfully diminish interfacial tension, their incorporation in excessive quantities can markedly elevate system viscosity. Employing surfactants such as Tween 80 beyond a specific critical threshold triggers a structural transition toward more complex micellar architectures, including cylindrical micelles or liquid crystalline phases. These structured networks impose mechanical resistance against fluid deformation due to dense molecular entanglement, macroscopically manifesting as increased viscosity or gel like texturing (McClements, 2021). Maintaining a lower formulation viscosity is highly advantageous as it ensures an even spreadability across the human integumentary surface and facilitates the unhindered release of active volatile molecules, such as citronellal and geraniol, bypassing the mechanical entrapment of dense micellar structures to achieve optimal antimicrobial performance.

Regarding olfactory attributes, the formulations exhibited a significant sensory shift from completely odourless (P0) to a distinct, prominent leafy aroma typical of pure citronella oil (P1–P4). This olfactory profile is directly linked to the major chemical constituents of the CO resolved via GC-MS, particularly the oxygenated monoterpene fraction. Specifically, citronellal bears an aldehyde functional group that undergoes aromatization upon oxidation, imparting a characteristic crisp, citrus/lemon like fragrance note (Risti et al., 2019 ; Martihandini et al., 2023). Concurrently, the presence of monoterpene alcohols, such as geraniol, contributes a subtle, underlying rosy nuance to the overall scent profile (Martihandini et al., 2023).

Table 6: Characteristics of the HS-CO formulation.

Parameter	HS-CO Treatments				
	P0	**P1**	**P2**	**P3**	**P4**
Color	Clear	White	White	White	White
Appearance	Liquid	Liquid	Liquid	Liquid	Liquid
Odour	Odorless	Leafy	Leafy	Leafy	Leafy
Homogenity	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Spreadability (cm)	12,81 ± 1,13	12,29 ± 0,63	14,04 ± 1,53	14,68 ± 1,08	14,79 ± 1,06
pH	6,32 ± 0,02	6,57 ± 0,03	6,46 ± 0,03	6,33 ± 0,03	6,22 ± 0,07

Another critical physicochemical characteristic evaluated was homogeneity, which serves to confirm the uniform distribution of all formulation ingredients. A topical preparation is classified as possessing excellent homogeneity in compliance with the SNI 06-2588 standard when it exhibits a complete absence of localized coagulation, phase separation, or coarse particulates (Ariani et al., 2024). High homogeneity is imperative to guarantee that the bioactive components remain uniformly distributed throughout the vehicle matrix. Microscopic evaluation on glass slides (Figure 2) demonstrated that all HS-CO formulations (P0–P4) displayed excellent homogeneity with no detectable coarse particulates, thereby satisfying the standardized criteria. This structural stability is attributed to the amphiphilic Tween 80 molecules which effectively entrap the oil globules thereby preventing coalescence, the phenomenon where smaller droplets merge into larger aggregates. Upon forming micelles or coating the globule interfaces, the hydrophilic polyoxyethylene chains of Tween 80 extend into the continuous aqueous phase, creating a robust steric hindrance barrier that prevents the oil droplets from colliding or merging. This steric stabilization mechanism is highly critical. If coalescence occurs, the formulation visually develops a speckled or clumped appearance failing to meet the rigorous criteria for homogeneity (McClements, 2021).

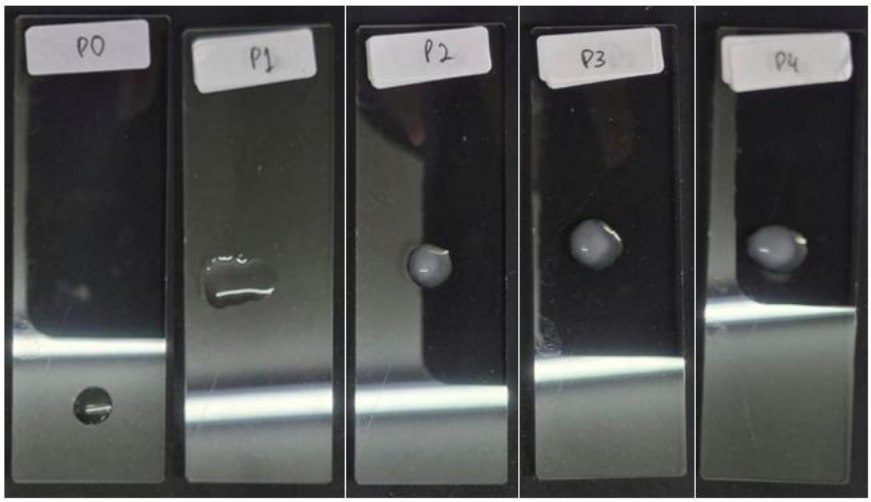


Figure 2: Homogenity characteristics of the HS-CO formulation.
(Source: personal documentation)

Furthermore, spreadability testing was conducted to assess the formulation's capacity to disperse efficiently across the human skin surface. An optimal hand sanitizer formulation must spread effortlessly and uniformly upon application (Ariani, 2024). The quantitative evaluation performed at a fixed distance of 10 cm demonstrated that the spreadability diameters ranged from 12,29 ± 0,63 cm to 14,79 ± 1,06 cm. The resulting data revealed a distinct upward trend in spreadability that correlated positively with increasing concentrations of CO within the vehicle matrix.

Oil, by its physical nature, functions as a lubricant. At the lowest essential oil concentration (P1), the volume of oil is insufficient to lubricate the continuous phase of the formulation. Instead, this limited fraction of oil is tightly bound by the surfactant molecules, forming densely packed globules that render the system structurally more rigid and coherent compared to the oil free control (P0). However, as the essential oil concentration is progressively increased, the intrinsic lubricating effect of the lipid phase becomes dominant. This breaks down and loosens the structural network of the vehicle base, significantly lowering resistance to flow and enabling the formulation to spread more readily than both P1 and P0.

Concurrently, pH evaluation revealed a systematic decline in pH values corresponding to increasing concentrations of the essential oil. The formulated preparations exhibited pH values within the range of 6,22 ± 0,07 to 6,57 ± 0,03.

This downward pH shift is hypothesized to occur due to a chemical interaction between the aldehyde functional group of citronellal and natrium hydroxide (NaOH). Under strongly basic conditions, aldehyde groups can undergo conversion into carboxylic acid, a reaction that consumes free hydroxyl ions (OH^-). The resulting depletion of unbound OH^- ions within the dispersion system directly accounts for the observed reduction in the formulation's pH (Chug et al., 2025). Nonetheless, these values remain well within the acceptable threshold defined by the SNI 06-2588 standard, which mandates a product pH range of 4,5–6,5 (Milenia & Ariani, 2023). Maintaining a slightly acidic pH is essential for preserving the structural integrity of the stratum corneum and sustaining the skin's natural acid mantle, which serves as a primary biological defense barrier (Martihandini et al., 2023). Moreover, a mildly acidic environment actively supports the viability of normal skin flora while concurrently suppressing the proliferation of transient pathogenic bacteria, which typically exhibit optimal growth kinetics at near-neutral pH values (7,0) (Booq et al., 2021). Consequently, the developed HS-CO preparations are categorized as dermatologically safe and are highly likely to minimize the risk of skin irritation because their pH values closely mirror the homeostatic physiological conditions of human skin (Selvan et al., 2022). These findings strongly align with Rahmasari et al. (2020), who previously reported that hand cleansing gels maintaining a pH profile between 6,5 and 6,7 induced no observable dermal irritation.

4.2 Antibacterial Activity of Citronella Oil

The experimental results demonstrated that the MIC and MBC values against *Escherichia coli* were 2,84 mg/mL and 5,68 mg/mL, respectively. Conversely, against *Staphylococcus aureus*, the MIC and MBC values were identical, established at 22,73 mg/mL. The equivalence of MIC and MBC values against *Staphylococcus aureus* indicates that at this specific concentration, the CO not only suppresses bacterial proliferation but also exerts a distinct bactericidal effect. This was visually confirmed by the complete absence of visible turbidity in the medium, signifying total growth inhibition. In contrast, at concentrations below this threshold, the medium exhibited prominent turbidity, indicating persistent bacterial proliferation. For *Escherichia coli*, although the MIC was achieved at 2,84 mg/mL, not all concentrations above this level presented a fully turbid medium. Due to these discrepancies in susceptibility profiles and growth kinetics between the two isolated test bacteria, establishing a single optimal concentration threshold capable of eradicating both species was imperative. Consequently, the concentration of 22,73 mg/mL was selected as the lowest baseline parameter that consistently prevented bacterial growth in both strains, serving as the fundamental treatment baseline for this study (Table 1).

Table 7: MIC and MBC values against the tested bacteria.

Bacteria	MIC (mg/mL)	MBC (mg/mL)
Staphylococcus aureus	22,73	22,73
Escherichia coli	2,84	5,68

The capacity of varying CO concentrations to inhibit bacterial growth was evaluated using the Kirby-Bauer disk diffusion method. The resulting data were expressed as inhibition zone diameters and classified according to the criteria established by Davis & Stout (1971) into four distinct categories: weak (≤5,0 mm), moderate (6,0–10,0 mm), strong (11,0–20,0 mm), and very strong (≥ 21,0 mm) (Ariani et al., 2024). Shapiro-Wilk normality testing indicated that the data were normally distributed (p>0,05) with a value of p=0,193, but violated the homogeneity of variance assumption (p< 0,05) with a value of p=0,010. A subsequent one-way Analysis of Variance (ANOVA) revealed a highly significant treatment effect (p=0,001, p<0,05) on the inhibition zone diameters across the different concentrations. To determine specific significant variances between groups, a Games-Howell post hoc test was conducted.

As summarized in Table 8, the oil free control formulation and contained only 2% DMSO as a solvent (P0), exhibited no inhibition against either *Staphylococcus aureus* or *Escherichia coli*. The P1 formulation demonstrated moderate inhibitory activity against both test strains. The P2 treatment yielded inhibition zones that were not statistically different from P1. Conversely, the P3 formulation produced strong zones of inhibition against *Staphylococcus aureus* or *Escherichia coli*, measuring 12,46 ± 3,81 mm and 16,48 ± 2,85 mm respectively. The highest antibacterial efficacy was observed in the P4 group, which generated an inhibition zone diameter of 18,19 mm against *Escherichia coli* and 17,02 mm against *Staphylococcus aureus.* According to the Davis & Stout classification, both values fall within the strong category. These findings confirm that increasing the concentration of CO effectively enhances the inhibition of bacterial growth (Tabel 8).

These observations align with the findings of Kowalska-Krochmal & Dudek-Wicher, (2021), who reported a direct, linear correlation between essential oil concentration and antibacterial efficacy, as evidenced by progressively larger inhibition zone diameters. However, this trend contrasts with the study by Risma Aprilia et al, (2023), which noted that increasing lemongrass extract concentrations from 5% to 10% enhanced the bacterial inhibition diameter, but further increases to higher concentrations (15% and 20%) led to a decline in antibacterial activity.

Tabel 8: Antibacterial efficacy of formulation components against zone of inhibition formation,

Sampel	Concentration	Inhibition Zones (mm)			
		Staphylococcus aureus	Categories	*Escherichia coli*	Categories
P0 (DMSO)	2%	$0,00 \pm 0,00^{a}$	Weak	$0,00 \pm 0,00^{a}$	Weak
P1	0,5×MIC	$8,93 \pm 2,09^{b}$	Moderate	$9,28 \pm 1,44^{b}$	Moderate
P2	1×MIC	$9,50 \pm 4,94^{bc}$	Moderate	$16,04 \pm 2,68^{bc}$	Strong
P3	1,5×MIC	$12,46 \pm 3,81^{c}$	Strong	$16,48 \pm 2,85^{c}$	Strong
P4	2×MIC	$17,02 \pm 4,13^{c}$	Strong	$18,19 \pm 4,06^{c}$	Strong
Positive Control (Amoxicillin)	20g/ml	$26,30 \pm 2,17^{d}$	Very Strong	$29,23 \pm 3,43^{d}$	Very Strong
Negative Control (Aquadest)	100%	0,00 ± 0,00	Weak	0,00 ± 0,00	Weak
Glycerin	1,45%	0,00 ± 0,00	Weak	0,00 ± 0,00	Weak
Tween-80	1%	0,00 ± 0,00	Weak	0,00 ± 0,00	Weak
NaOH	0,4%	0,00 ± 0,00	Weak	0,00 ± 0,00	Weak

Note: Different superscript letters within the same column indicate a statistically significant difference between the samples against the test bacteria based on the Games-Howell post hoc test ($\alpha = 0,05$).

The distinct inhibitory profiles observed between the two bacterial strains are primarily governed by their structural and morphological variations. Gram-negative bacteria, such as *Escherichia coli*, possess a cell wall characterized by a thin peptidoglycan layer, an inner phospholipid membrane, and a lipid-rich outer membrane composed predominantly of lipopolysaccharides, rendering the cell boundary highly nonpolar. This hydrophobic architectural layout facilitates the partitioning of lipophilic essential oil constituents into the cell membrane, inducing structural disruptions, ion leakage, and ultimate cellular lysis (Risti et al., 2019; Martihandini et al., 2023). Conversely, Gram-positive bacteria, such as *Staphylococcus aureus*, feature a thicker, more rigid cell wall dominated by peptidoglycan (50–80%) and teichoic acids, with minimal lipid content. Teichoic acids function as water-soluble polymers, imbuing the *Staphylococcus aureus* cell wall with a predominantly polar character. This polar barrier limits the ease with which hydrophobic compounds within the essential oil can penetrate the cell wall, thereby resulting in a lower inhibitory response in Gram-positive strains compared to Gram-negative counterparts (Risti et al., 2019; Risma et al., 2023).

The antibacterial potency documented in this study is attributed to the presence of key bioactive compounds, namely citronellal (37,78%), geraniol (18,89%), and citronellol (13,12%), which possess established cell membrane disrupting mechanisms of action. Guandalini et al. (2020) reported that citronellal and citronellol can destabilize the cytoplasmic membrane and cell wall structures of *Staphylococcus aureus* by altering vital physicochemical properties, including surface charge and hydrophobicity. Furthermore, compounds such as geraniol and citronellol trigger the intracellular leakage of potassium ions (K^{+}), driving an electrolyte imbalance, a severe decline in membrane potential, and subsequent cell lysis (Angane et al., 2022 ; Cebi & Erarslan, 2023).

Additionally, secondary active constituents within the essential oil matrix, such as limonene and linalool, are known to compromise microbial membrane integrity through direct molecular interactions with membrane proteins and phospholipids. The resulting structural damage destabilizes critical cellular homeostatic processes, including respiration, oxidative phosphorylation, and nucleic acid synthesis, ultimately leading to microbial cell death (Angane et al., 2022; Han et al., 2020 ; Liu et al., 2020).

Meanwhile, vehicle components such as glycerin and Tween 80 demonstrated no independent antibacterial activity (inhibition diameter of 0 mm), confirming that the observed antimicrobial efficacy stems purely from the citronella essential oil incorporated within the HS-CO formulation. Furthermore, distilled water utilized as a negative control group yielded no zones of inhibition, verifying that the solvent does not exert any confounding antibacterial effects.

4.2 Antibacterial Efficacy of the HS-CO Formulation

The time-kill kinetic assay was conducted to evaluate the bactericidal kinetics of the HS-CO formulation at designated exposure time intervals of 30 seconds} and 60 seconds. The antimicrobial efficacy was quantified in terms of logarithmic (log) reduction and bacterial kill percentage. As illustrated in Table 9 and Graph 1, all formulations incorporating citronella oil (P1, P2, P3, and P4) exhibited exceptional antibacterial potency, achieving a log reduction value of 8.18 within just 30 seconds of exposure. This reduction profile corresponds to a 100% bacterial mortality rate against both the Gram-positive strain (*Staphylococcus aureus*) and the Gram-negative strain (*Escherichia coli*). These results successfully comply with the EN 1276:2019 standard, which mandates a minimum of a ≥ 5-log reduction for effective hygienic handrubs preparations. Conversely, the oil-free control formulation (P0) only yielded log reduction values of 0,76 and 0,82 after 60 seconds, which translates to a limited mortality rate ranging between 82–84%. This

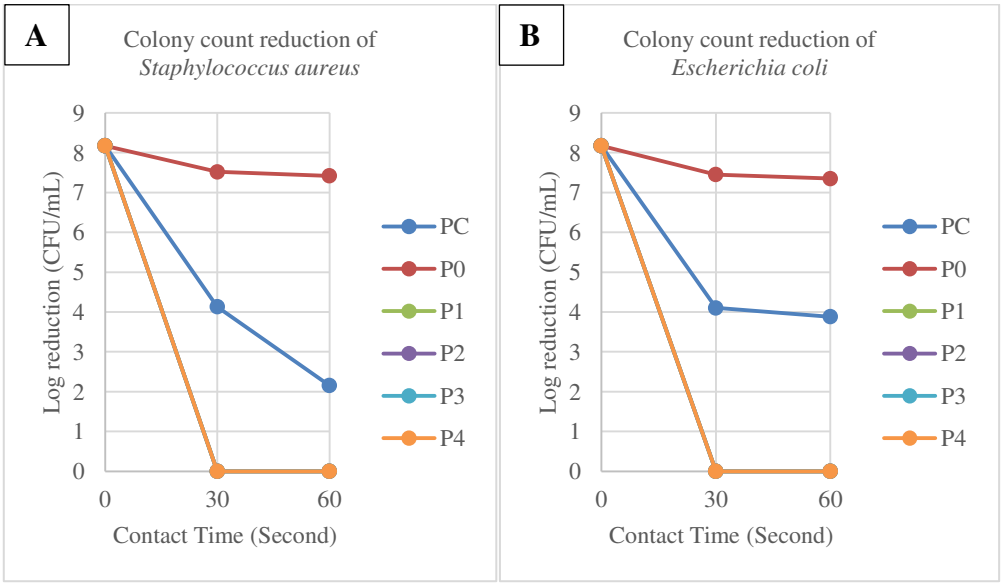
pronounced variance demonstrates that the incorporation of citronella oil plays a pivotal role in accelerating rapid bactericidal action. This high potency is closely linked to the major active constituents within the citronella oil, particularly citronellal, which possesses the capacity to disrupt the structural integrity of the bacterial cytoplasmic membrane, leading to the rapid leakage of intracellular components and subsequent cell death within a brief contact timeframe (Guandalini et al., 2020).

Tabel 9: Log reduction and bacteria kill percentages at various HS-CO formulation concentrations.

Bacteria	*Staphylococcus aureus* (Log 10 CFU/ml)				*Escherichia coli* (Log 10 CFU/ml)			
Contact time	30 seconds	% Kill	60 seconds	% Kill	30 seconds	% Kill	60 seconds	% Kill
HS-CO Treatment								
Positive Control (70% Alcohol)	4,04 ± 0,07[a]	99,99	6,02 ± 1,97[a]	99,99	4,07 ± 0,10[a]	99,99	4,29 ± 0,07[a]	99,99
P0	0,66 ± 0,03[a]	77,85	0,76 ± 0,08[a]	82,17	0,72 ± 0,05[a]	81,01	0,82 ± 0,05[a]	84,93
P1	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00
P2	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00
P3	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00
P4	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00	8,18 ± 0,00[b]	100,00

Note: Different superscript letters within the same column indicate a statistically significant difference between contact time against the samples against based on the Pairwise Comparison post hoc test (α = 0,05).

Theoretically, Gram-negative bacteria such as *Escherichia coli* frequently exhibit higher resistance to antimicrobial agents due to the presence of a lipid rich outer membrane composed of lipopolysaccharides. However, as shown in Graph 1, the P1–P4 formulations completely reduced the viable *Escherichia coli* colony count to zero at the 30 second mark. This finding indicates that the rate of bactericidal action against *Echerichia coli* and *Staphylococcus aureus* is relatively uniform. Such a pattern implies that even the lowest incorporated concentration of citronella oil successfully surpassed the minimum bactericidal concentration (MBC) threshold for both test pathogens. Furthermore, the excellent droplet dispersion within the emulsion vehicle facilitated an even distribution of the active hydrophobic compounds, enabling them to attack the bacterial cell membranes simultaneously. The lipophilic nature of essential oils is well documented to drive their antimicrobial activity, as it facilitates the partitioning and influx of active volatile fractions into the lipid bilayers of the bacterial cell membrane, thereby severely compromising cytoplasmic membrane integrity (Booq et al., 2021).



Graph 1: Time-kill kinetics of the HS-CO formulations against the tested bacteria.

Note: PC refers to the positive control (70% alcohol). Lines P1, P2, P3, and P4 overlap, indicating statistically equivalent efficacy with complete eradication of viable bacterial colonies (0 CFU/mL) beginning at the 30 second mark.

For comparative analysis, the positive control (PC) treated with 70% alcohol demonstrated a bacterial mortality rate of 99,99% within a 60 second exposure window. Nonetheless, the HS-CO formulations (P1–P4) displayed a more

rapid bactericidal onset, achieving total eradication within a 30 second contact timeframe. Alcohol typically exerts its antimicrobial action by denaturing structural proteins and dissolving bacterial lipid membranes (Rundle et al., 2020; Aodah et al., 2021). This mechanism is inherently more effective against Gram-positive bacteria like *Staphylococcus aureus*, which possess a less complex cell wall architecture compared to Gram-negative strains. This structural susceptibility is reflected in the higher log reduction values observed for *Staphylococcus aureus* compared to *Escherichia coli* in the PC group at 60 seconds (Graph 1).

Statistical analysis using the Kruskal–Wallis test on the bacterial log reduction data revealed a p-value of 0,813. Because this value exceeds the significance threshold (p>0,05), it can be concluded that the bacterial strain type did not exert a statistically significant effect on the log reduction outcomes. Conversely, statistical evaluations of the exposure time and concentration variance factors yielded p<0,05, indicating that both parameters significantly influenced bacterial log reduction. Based on the post hoc Pairwise Comparison test, all treatments containing citronella oil (P1–P4) demonstrated a significantly higher reduction in viable bacterial counts compared to both the oil free control (P0) and the positive control (PC). However, no statistically significant variances were detected among the P1, P2, P3, and P4. This suggests that the lowest concentration tested (P1) was already sufficient to elicit a maximum bactericidal response, meaning that subsequent concentration increments in P2, P3, and P4 did not yield further statistically observable benefits. Consequently, based on these statistical insights, the P1 formulation (0,5×MIC) was established as the optimal formulation parameter in this study and is recommended for scale-up production. Furthermore, the primary bactericidal event occurred predominantly within the 0–30 second interval, whereas the reduction rate stabilized between 30 and 60 seconds, yielding statistically equivalent survival outcomes.

5. Conclussion

Based on the experimental findings, it can be concluded that the developed HS-CO preparations maintain a fluid, liquid consistency, exhibiting a concentration dependent visual transition from transparent to an opaque milky white appearance as the citronella oil concentration increases. The formulation possesses a distinctive, leafy olfactory profile characteristic of pure citronella oil. All prepared formulas display excellent structural homogeneity, efficient spreadability diameters ranging from 12,29–14,79 cm and stable pH values within the range of 6,22–6,57, confirming that the preparations are dermatologically safe and biocompatible with human dermal physiology.

Furthermore, increasing the concentration of citronella oil as the primary bioactive agent significantly enhances the inhibition zone diameters. The resulting zones of inhibition ranged from 8,93 ± 2,09 mm to 17,02 ± 4,13 mm against *Staphylococcus aureus*, and from 9,28 ± 1,44 mm to 18,19 ± 4,06 mm against *Escherichia coli*.

The bactericidal efficacy of the HS-CO formulation was further validated by its superior performance compared to 70% alcohol in terms of rapid bacterial eradication. All formulations incorporating citronella oil (P1, P2, P3, and P4) demonstrated exceptional antibacterial kinetics, achieving a prominent logarithmic reduction of 8,18 within an exposure time of just 30 seconds, which corresponds to a 100% bacterial mortality rate.

Acknowledgments

The authors wish to express their sincere gratitude to the UMKM Shafaluna Atsiri for providing the citronella grass (*Cymbopogon nardus*) plant materials, as well as the necessary facilities, equipment, and access required for the extraction of the citronella oil used in this study.

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