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Research Article

Isolation, Characterization, and Application of Citronellal as an Antinociceptive Nanoemulgel

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Abstract
Citronellal is the major component of citronella essential oil and is widely used in perfumery, skincare, aromatherapy, and as a bio-additive, as well as a potential topical antinociceptive agent. However, its effectiveness is influenced by purity, volatility, and stability. This study first developed an efficient vacuum fractionation distillation method to isolate citronellal, evaluating variations in mass of fraction, reflux ratio, and temperature under –760 mmHg. Five methods (M1–M5) produced citronellal purities of 80.03%, 87.57%, 82.87%, 82.91%, and 79.94%, respectively, identifying 98ºC and a 12:6 reflux ratio (M2) as the most effective separation conditions. The isolated citronellal was then formulated into a nanoemulgel using high-pressure homogenization and evaluated for physicochemical properties, user acceptability, irritation, and antinociceptive activity. The antinociceptive activity was assessed using the tail immersion method, in which one-third of the animal tail was immersed in water at approximately 55 ˚C, the withdrawal latency was recorded and expressed as the percentage of maximum possible effect (%MPE). The optimized formula demonstrated small particle size (107.97 ± 0.84 nm), appropriate pH, good viscosity and spreadability, and stability under storage. The tail immersion test showed strong antinociceptive activity, with a maximum %MPE of 64.30%, which was higher than that of the commercial product (42.90%). Overall, optimized vacuum fractionation and NEGs formulation indicate promising potential for developing citronellal as a natural-based topical antinociceptive preparation.

Keywords: Antinociceptive, Citronellal, Nanoemulgel, Vacuum Fractional Distillation

1 Introduction

Pain is defined by the International Association for the Study of Pain (IASP) as a central nervous system response to tissue injury or emotional changes, which may be acute or chronic and significantly impacts quality of life and public health burden [1]. The process of pain perception, or nociception, therefore, serves as a crucial target in the development of antinociceptive therapies. Citronellal, the primary monoterpene in citronella essential oil, is known to exhibit diverse biological activities including antibacterial [2], antifungal [3], repellent [4], pesticide [5], cosmetics [6], bioadditive for fuel [7] and antinociceptive [8]. Using a chronic non-inflammatory muscle pain model, citronellal/β-cyclodextrin complex demonstrated superior duration of antihyperalgesic effects [9]. Citronellal has been reported to produce a dose-dependent antinociceptive effect in mice, comparable to morphine, with naloxone-sensitive responses indicating possible involvement of the opioid system [10].

Beyond the involvement of the opioid system, the antinociceptive effect of citronellal is also

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suggested to be mediated through complementary mechanisms involving transient receptor potential (TRP) channels. These channels, including TRPV1 and TRPV8, play a central role in nociceptive signal transduction, and their modulation has been recognized as an effective strategy for pain management. Evidence from monoterpenes structurally related to citronellal, such as citral, demonstrated significant antinociceptive effect in both acute and chronic pain models, which are attenuated by TRP channel antagonists, indicating direct involvement of these pathways. Furthermore, these effects were observed across multiple nociceptive models, suggesting the involvement of both peripheral and central pain pathways. These findings support the pharmacological potential of citronellal as a topical antinociceptive agent through TRP channel modulation [11].

Citronella oil from *Cymbopogon nardus* represents a readily available natural source of citronellal; however, its complex composition necessitates efficient separation methods to obtain citronellal with sufficient purity and reproducibility for pharmaceutical applications [12]. Previous studies have demonstrated that the selection of distillation technique plays an important role in determining the yield, purity, and chemical composition of essential oil [13]. A comparative study on patchouli oil reported that the water bubble distillation produced significantly higher patchoulol content than conventional water-steam distillation [14]. These findings indicate that steam distillation may still require further purification steps to obtain higher concentrations of specific target compounds. Therefore, in the present study, citronella oil obtained through steam distillation was subsequently subjected to vacuum fractional distillation to enrich citronellal content while minimizing thermal degradation and improving separation efficiency [15], [16].

Vacuum fractional distillation (VFD) is a separation technique based on differences in compound volatility, which are governed by their physicochemical properties as well as operating temperature and pressure [17]. The separation efficiency is further influenced by mass and heat transfer between the liquid and vapor phases within the distillation column. This approach is particularly suitable for essential oils, which are composed of thermolabile terpene compounds that are prone to degradation or oxidation at elevated temperatures. Accordingly, the application of vacuum conditions reduces the boiling point of the mixture, enabling distillation at lower temperatures and thereby minimizing thermal decomposition [18]. Previous studies have demonstrated that VFD applied to lemongrass essential oil successfully produces fractions with high citral content, reaching purity up to 95% with recovery of around 80% under optimal conditions [19]. These findings highlight the potential of VFD to improve both the quality and consistency of essential oil-derived compounds, which are critical for subsequent formulation and biological applications.

Despite these advantages, the application of VFD as a preparative step to obtain citronellal specifically tailored for nanoemulgels (NEGs) formulation and pharmacological evaluation remains underexplored [20], [21]. From a formulation and delivery perspective, NEGs systems have emerged as promising platforms for topical analgesic applications due to their ability to enhance solubility, stability, skin permeation, and controlled release of lipophilic bioactive compounds [22], [23]. Previous studies have reported the incorporation of citronellal into nanoemulsion systems, demonstrating improved stability and biological activity [24], [25]. However, to date, the development of citronellal-based NEGs that explicitly link isolation quality, physicochemical characterization, and antinociceptive performance has not been comprehensively reported [26], [27]. In particular, the influence of purified citronellal obtained via VFD on NEGs characteristics and subsequent antinociceptive efficacy has not been systematically investigated [28], [29].

Therefore, the novelty of this study lies in the integrated approach that combines (i) method optimization of citronella isolation from *Cymbopogon nardus* essential oil using VFD, (ii) detailed physicochemical characterization of the isolated compound, and (iii) its application in a NEGs delivery system for antinociceptive evaluation. By directly correlating isolation methodology, formulation properties, and biological activity, this work provides new insights into the rational development of citronellal-based NEGs as topical antinociceptive agents, advancing both applied pharmaceutical engineering and natural product utilization.

2 Materials and Methods

2.1 Materials

The materials used in this research included citronellal oil (*Cymbopogon nardus* L.), technical ethanol

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(Merck), technical acetone (Merck), 70% alcohol (Merck), Tween 80 (PT KAO Indonesia Chemicals), propylene glycol (Dow Chemical Pacific Singapore Private Limited), carbopol (Sigma-Aldrich), triethanolamine (TEA) (Merck), distilled water (aquadestillata), eugenol (PT Wingja Indonesia), peppermint oil (iSenso aroma Indesso), methyl salicylate (Merck), tissue and grease.

The instruments employed comprised standard laboratory glassware (Merck), a complete vacuum fractionation distillation apparatus, pycnometer (Pyrex), refractometer (ABBE), polarimeter (Atago Polax-2L), gas chromatography system (GC-2010-RS 23C COM3), gas chromatography mass spectrometry system (GC-MS Shimadzu QP 2010 SE), pH meter (ATC), Brookfield viscometer, a refrigerator, particle size analyzer (Horiba Scientific SZ-100), magnetic stirrer, ultrasonic processor and analytical balance (Mettler Toledo).

2.2 Methods

The overall experimental workflow of citronellal isolation, characterization, and its formulation into an antinociceptive NEGs, along with subsequent evaluations, is presented in Figure 1.

2.2.1 Distillation of citronella leaves

A total of 500 kg of citronella leaves were air-dried in ambient conditions for 24 hours, followed by steam distillation using a 500 kg-capacity distillation unit for 5 hours. This process was conducted over 20 cycles.

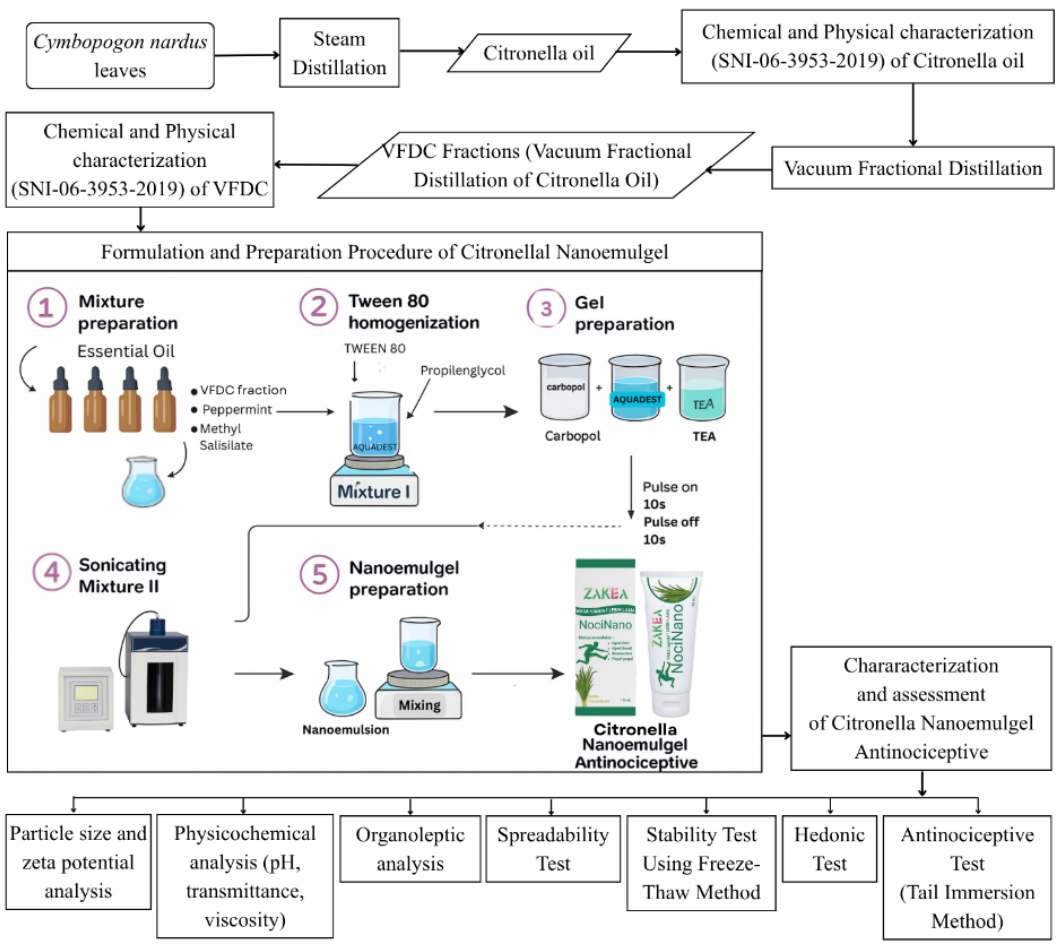

Figure 1. Experimental workflow for citronellal isolation, characterization, and antinociceptive NEGs formulation.

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2.2.2 Characterization of citronella oil

The characterization of citronella essential oil was performed in accordance with the Indonesian National Standard (SNI-06-3953-2019) for physicochemical parameters, including color, odor, specific gravity, refractive index, solubility in ethanol and optical rotation. The citronellal content was evaluated using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), with the ISO 3848 standard for component identification and quantification. GC analysis was conducted using a GC system (Shimadzu GC-2010, Japan), while GC-MS analysis was performed using a GC-MS system (Shimadzu QP 2010 SE, Japan). The instrumental conditions for GC and GC-MS analyses are presented in Tables 1 and 2, respectively.

Table 1: GC instrumental conditions for the analysis of citronella oil.

Parameters	GC Instrument
Carrier gas	N_2/Air
Maximum temperature	470 ˚C
Column thickness	1 µm
Column diameter	0.53 mm
Column lenght	30 m
Maximum column temperature	320 ˚C
Injection mode	Split
Injection temperature	300 ˚C
Pressure	11 kPa
Column flow	1.69 mL/min
Split ratio	58

Table 2: GC-MS instrumental conditions for the analysis of citronella oil.

Parameters	GC-MS Instrument
Oven temperature	80 ˚C
Injection temperature	300 ˚C
Injection mode	Split
Flow control mode	Pressure
Pressure	42.3 kPa
Total flow	117.5 mL/min
Column flow	0.74 mL/min
Split ratio	10
Temperature program	80-320 ˚C
Detector amplification mode	Relative
Gain detector	0.98 kV + 0.00 kV

2.2.3 Vacuum fractional distillation of citronella oil (VFDC)

Vacuum fractional distillation was performed using a total of 100 kg of citronella oil, divided into five experimental methods (M1-M5), as summarized in Table 3. Each method utilized 20 kg of citronella oil. The method differed in reflux ratio, temperature, and flow rate conditions. The reflux ratios investigated were 12:12, 12:6, 12:4, 12:2, 12:1 and 6:6, with temperatures ranging from 80- 140 °C and flow rates from 50 mL/h to 300 mL/h.

The selection of reflux ratios was based on their influence on separation efficiency and product purity. Higher reflux ratios promote stronger vapor–liquid equilibrium within the column, enhancing the separation of components with similar boiling points and increasing citronellal purity. Conversely, lower reflux ratios improve distillation throughput but may compromise separation efficiency. Therefore, a range of reflux ratios was evaluated to determine the optimal operating condition.

Preparation was performed by filling the trap tube with an ice-salt mixture to prevent vapor carryover into the vacuum pump, followed by connecting the distillation unit to a 1000–1500-watt power supply so that the system could be operated via control panel. Citronella oil was introduced into the feed flask, which was equipped with a sensor and a stirring rod to maintain homogeneity, a heating jacket to provide heat and maintain temperature stability, and a feed thermocouple to control the distillation temperature.

The citronella oil was heated for approximately 1-30 h under vacuum pressure of 30 mmHg. The condenser converted the vapor exiting the distillation column into liquid. The vacuum fractional distillation process was terminated, and the system was allowed to cool until the temperature reached 70 ºC. The schematic of a vacuum fractionation distillation apparatus is presented in Figure 2.

Table 3: Operational conditions of vacuum fractional distillation methods (M1-M5).

Method	Reflux Ratio Range	Temperature Range (ºC)
M1	12:1 – 6:6	84-127
M2	12:1 – 12:6	84-139
M3	12:1 – 12:2	85-133
M4	Mostly 12:2 and 6:12	89-140
M5	Mostly 12:2 and 6:12	93-140

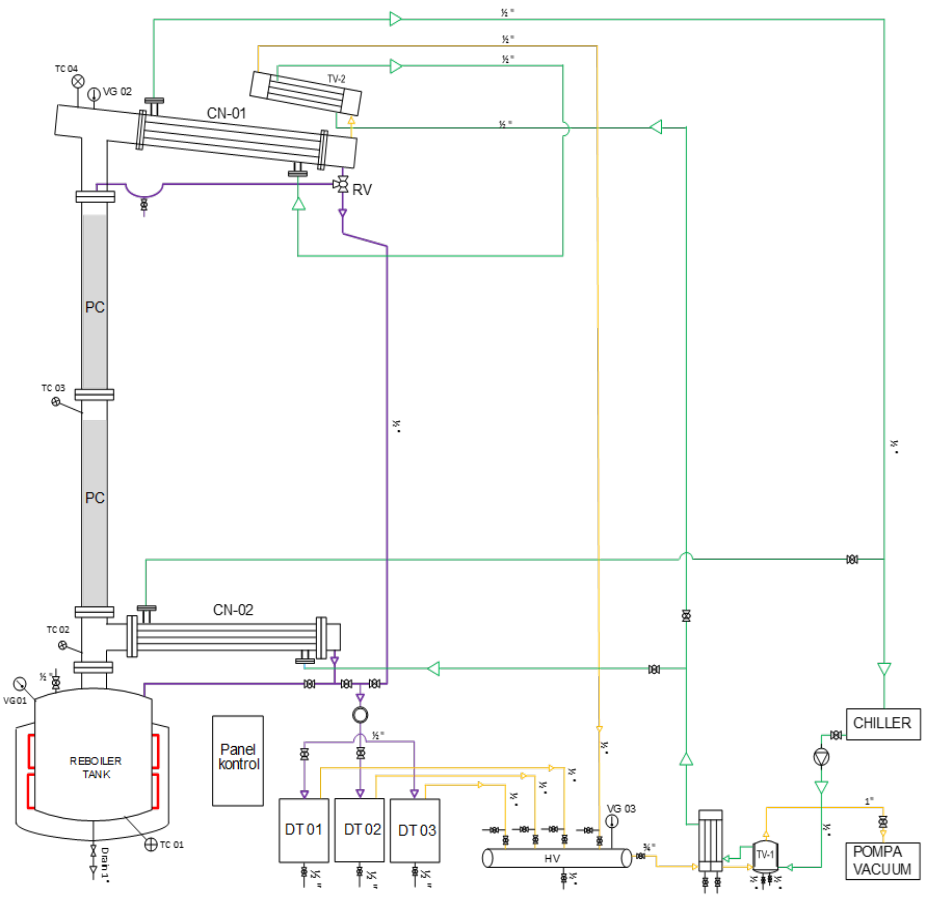

Figure 2: Schematic of a vacuum fractionation distillation apparatus (DT: Distillation Tank, CN: Condenser, PC: Packing Column, RV: Reflux Valve, HV: Header Vacuum, TV: Trap Vacuum).

2.2.4 Characterization of VFDC fractions

Characterization of the citronella essential oil fractions was performed in accordance with SNI-06-3953-2019 following the procedure described in point 2.2.2

2.2.5 Formulation and preparation procedure of citronellal NEGs

The citronellal NEGs were formulated into five formulations (FA-FE) based on a modified IDS000008797 patent, with varying concentrations of citronellal oil (0-6% w/w) and methyl salicylate (6-0 % w/w) [30]. The percentage composition of each formula is presented in Table 4.

Table 4 : Composition of citronellal NEGs formulation (% w/w).

Ingredient	FA	FB	FC	FD	FE
Methyl Salicylate	6.0	4.5	3.0	1.5	0.0
Citronellal	0.0	1.5	3.0	4.5	6.0
Tween 80	27.0	27.0	27.0	27.0	27.0
Propylene Glycol	27.0	27.0	27.0	27.0	27.0
Distilled Water	37.0	37.0	37.0	37.0	37.0
Peppermint Oil	2.0	2.0	2.0	2.0	2.0
Eugenol	0.5	0.5	0.5	0.5	0.5
Patchouli oil	0.5	0.5	0.5	0.5	0.5

Note: FA-FE represent formulations with varying concentrations of citronellal and methyl salicylate. All other components were kept constant.

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The oil phase was weighed and homogenized using a magnetic stirrer, followed by the addition of Tween 80. The mixture was processed using a probe ultrasonicator for 10 minutes (Mixture I). Subsequently, propylene glycol was added and further homogenized by ultrasonication for 10 minutes (Mixture II). This mixture was gradually diluted with distilled water while stirring at 700 rpm for 10 minutes and then subjected to an additional 10 minutes of ultrasonication. The resulting nanoemulsion was left to stand for 24 hours before being used as the base for the NEGs.

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2.2.6 Particle size and polydispersity index

Mean droplet size, polydispersity index (PDI), and zeta potential were measured by dynamic light scattering (DLS). This technique evaluates fluctuations in scattered light caused by Brownian motion, enabling particle size determination within the range of 0.3 nm to 10 µm. Zeta potential, reflecting the surface charge of nanoemulsion droplets, was determined using specialized cuvettes. The PDI, calculated as the ratio of standard deviation to mean droplet size, indicates the uniformity and size distribution of the dispersion [31].

2.2.7 pH measurement

The pH of the citronellal NEGs was carried out by immersing the pH meter electrode in the formulation and recording the displayed pH value. The measurement was performed in triplicate at room temperature.

2.2.8 Transmittance test

Measurement of the nanoemulsion transmittance was carried out using a UV-Vis spectrophotometer at a wavelength of 650 nm.

2.2.9 Viscosity

The viscosity of the NEGs was measured using a Brookfield viscometer at room temperature (25 °C) with a rotational speed of 5-10 rpm.

2.2.10 Organoleptic analysis

Organoleptic is a method used to assess the quality of material or product based on human sensory perception. The NEGs were visually examined for their color, odor, clarity, and consistency.

2.2.11 Spreadability test

A 0.5 g sample of the NEGs was placed at the center of a glass plate, and a second glass plate was positioned on top as an initial load for 1 minute. The diameter of the gel spread was then measured. The test was repeated with additional loads of 50 g, 100 g, and 200 g.

2.2.12 Stability using the freeze-thaw method

Stability testing was conducted using the freeze-thaw method for 6 cycles. Each cycle consisted of storing the formulation at 4°±2ºC for 24 hours, followed by storage at room temperature for the next 24 hours (one cycle).

2.2.13 Hedonic test

This evaluation was conducted through organoleptic testing of the color, odor and texture of the citronellal oil NEGs. The panelists selected were familiar with the sensory properties of the formulation and met the criteria to serve as panelists. The panelist requirements included: (a) the ability to detect, recognize, compare, distinguish, and provide hedonic responses; (b) attention to organoleptic attributes; (c) willingness to dedicate time; and (d) possessing the necessary sensitivity. Panelists were then asked to express their preferences for the citronellal oil NEGs formulations using a provided questionnaire. The testing procedure involved preparing five samples placed in ointment jars, each coded with a unique identifier. Each panelist evaluated the samples one by one and completed the organoleptic test form based on their responses. They rated the color, odor, and texture using numerical values on the form provided. In this study, the hedonic scale used was a five-point scale comprising: "like

very much," "like," "somewhat dislike," "dislike," and "strongly dislike" [32].

2.2.14 Experimental replication and statistical analysis

All physicochemical evaluations, including particle size, pH, transmittance, viscosity, and spreadability test, were performed in triplicate (n=3). Statistical analysis was conducted to determine the significant differences among formulations. Hedonic test data were analyzed using multivariate analysis of variance (MANOVA) to assess overall differences among formulation based on multiple sensory attributes simultaneously. To complement the multivariate analysis, one-way analysis of variance (ANOVA) was performed on each sensory attribute individually. Since sensory perception is inherently multidimensional, Canonical Discriminant Analysis (CDA) was subsequently applied to identify the principal dimensions responsible for discrimination among formulations and to visualize the multivariate sensory structure.

Canonical variables showing significant differences were evaluated using one-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post hoc test for pairwise comparisons among formulations [33]. This multistep approach anabled a comprehensive assessment of sensory acceptance and was used to identify the most preferred formulation for subsequent antinociceptive testing.

2.2.15 Antinociceptive test (tail immersion method)

The antinociceptive activity test was performed using the tail immersion method. The test animals had one-third of their tail immersed in hot water at approximately 55 °C, and the reaction time, indicated by the tail withdrawal reflex latency, was recorded. An increase in latency time compared to the control group was used as an indicator of antinociceptive activity, expressed as the percentage of maximum possible effect (%MPE).

3 Result and Discussion

3.1 Characterization of citronella oil

The physical characterization of citronella (*Cymbopogon nardus*) essential oil demonstrated a density of 0.886 ± 0.07 g/mL, which falls within the acceptable range for high-quality citronella oil, indicating proper molecular packing of its constituent compounds. A higher density indicates a greater mass fraction of the constituents contained in the oil [34]. The refractive index measured at 24 ºC was 1.4671 ± 0.0512, suggesting good optical clarity and minimal water contamination, as the refractive index is closely associated with the purity and compositional uniformity of essential oil. Furthermore, polarimetric analysis revealed a specific optical rotation of +0.45° at 26.3 ºC, confirming the presence of optically active constituents and indicating that the oil meets the purity and stereochemical requirements specified by the Indonesian National Standard (SNI). Solubility assessment was conducted using ethanol as a solvent to evaluate the polarity profile of the essential oil constituents. Oxygenated terpenes, which are key quality markers in citronella oil, exhibit higher solubility in alcohol compared to non-oxygenated terpenes due to their greater polarity. The citronella oil showed complete miscibility in 96% (v/v) ethanol at the tested ratio, fully complying with SNI specifications. This high degree of solubility reflects a substantial proportion of polar oxygenated compounds, such as citronellal, citronellol, and geraniol, thereby confirming the superior physicochemical quality and suitability of the oil for industrial and pharmaceutical applications [35].

The chemical composition of the essential oil, particularly citronellal content, was further analyzed using GC and GC-MS in accordance with ISO 3848. This analysis was conducted to confirm the presence and relative abundance of target compounds prior to fractionation. Table 5 presents the comparative chromatographic profile of the citronella oil, detailing the distribution of major volatile components relative to ISO 3848 standards and prior studies.

The GC-MS analysis that the unfractionated citronellal oil exhibits a citronellal-dominant profile (36.84% ± 2.95%), positioned near the upper limit of ISO 3848, with citronellol and geraniol present at moderate levels. Compared with Yuliani [36], the substantially higher citronellal content indicates superior intrinsic oil quality, while the close agreement with Kang [37] suggests chemical consistency with well-characterized Indonesian citronella oil. Importantly, the low variability across GC1-GC3 injections demonstrates high analytical reproducibility, establishing the compositional profile as a reliable representation of the raw oil rather than an artifact of instrumental variation. Beyond compositional reporting, this GC-MS dataset provides

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Table 5: Comparison of chemical component (% Area) of citronella oil with ISO 3848 and literature data.

No	Compound	%Area						
		GC ± SD	GC-MS	ISO 3848		Yuliani [36]		Kang [37]
				Min	Max	Riau	W	Java
1	Limonene	4.51 ± 0.99	3.46	2.0	5.0	0.883	3.228	3.7
2	Linalool	1.02 ± 0.14	1.21	0.5	1.5	1.301	0.849	0.86
3	Citronellal	36.84 ± 2.95	36.74	31.0	40.0	13.763	37.897	35.25
4	Isopulegol	0.21 ± 0.05	0.15	0.5	1.7	n.a.	n.a.	0.53
5	Citronellol	10.77 ± 3.04	14.2	8.5	14.0	6.226	10.219	12.44
6	Geraniol	18.55 ± 1.51	21.58	20.0	25.0	39.474	26.844	23.06
7	Citral	0.45 ± 0.06	0.36	0.3	1.0	n.a.	n.a.	0.64
8	Geranyl acetate	1.77 ± 0.89	1.13	2.5	5.5	2.851	1.365	3.14
9	Citronellyl acetate	2.79 ± 0.77	2.24	2.0	4.0	2.143	1.068	2.4
10	Beta elemene	1.83 ± 0.10	1.87	0.7	2.5	0.408	1.092	2.6
11	Germacrene	1.35 ± 0.12	1.23	1.5	3.0	2.293	1.815	1.56
12	Cadinene	1.36 ± 0.10	1.46	1.5	2.5	0.042	0.425	2.03
13	Elemol	2.06 ± 0.08	2.15	1.3	4.8	0.205	0.67	3.46

Note: Riau, WJ (West Java), and Java mean the locations where citronella samples were taken.

a critical chemical baseline for the process design of the vacuum fractional distillation method. The high citronellal content in citronella oil indicates that the essential oil is suitable for further fractionation to obtain a high-content citronellal fraction that can be used as an active ingredient in antinociceptive products. In this context, GC-MS characterization functions as a process-enabling tool, linking raw material chemistry to separation strategy. This approach advances essential oil research by shifting GC-MS analysis from a descriptive endpoint to a decision-making framework for engineering-oriented fractionation [38].

Beyond its role as a compositional marker, the citronellal-rich profile of the essential oil has important implications for formulation performance and pharmaceutical activity [12]. As the dominant oxygenated monoterpene, citronellal modulates the physicochemical behavior of the system by influencing intermolecular interactions, which may enhance the stability of the nanoemulsion through improved dispersion and reduced phase separation [1]. This is particularly relevant in nanoemulsion systems, where compositional uniformity and controlled droplet characteristics (e.g., size distribution and zeta potential) play a critical role in determining formulation stability and performance [24]. Therefore, by maintaining this structural integrity, the relatively high citronellal concentration in the raw material is expected to optimize drug delivery and enhance the overall formulation efficacy.

3.2 Method development of vacuum fractional distillation to isolate citronellal

The aim of method development is to identify the most effective and efficient separation conditions for citronellal isolation. In all methods, citronellal was concentrated mainly in the early-middle fraction (F1-F7), which corresponds to its intermediate volatility relative to lighter monoterpenes and heavier oxygenated compounds. The first fraction (F1) still contained moderate citronellal levels (30-41%), indicating co-distillation with more volatile constituents. Citronellal concentration increased markedly in F2-F4 and gradually decreased in later fractions (F7 onward), where heavier or less volatile compounds predominated.

From the distribution plot, M2 exhibits a pronounced and sharp peak at fraction F3 (87.57%), followed by a steep decline in subsequent fractions, indicating a highly concentrated enrichment. In contrast to other methods that exhibit broader distributions across multiple fractions, the M2 method confines citronellal predominantly within a narrow fraction window (F2–F4), reflecting a high degree of separation selectivity. Separation selectivity in vacuum fractional distillation is quantitatively reflected by the degree of concentration and confinement of the target compound within specific fractions [39], which can be evaluated through parameters such as peak purity, distribution width

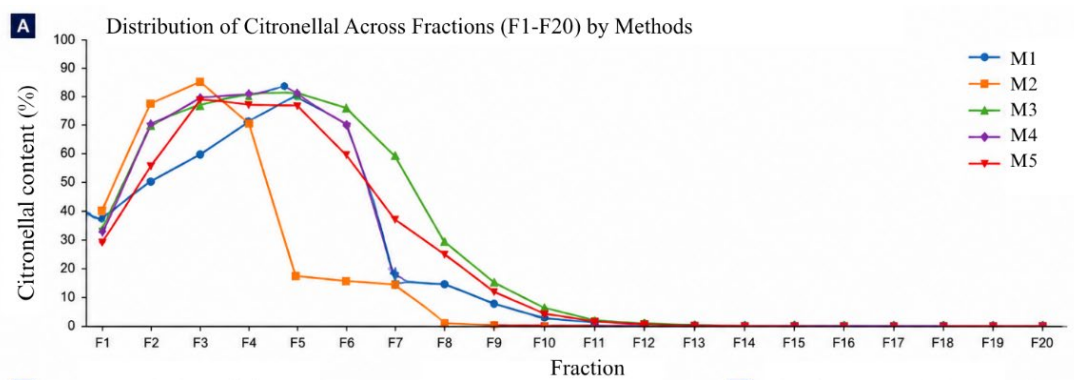
(e.g., FWHM), and the extent of fraction overlap with other components.

In this study, selectivity was evaluated based on sharpness and confinement of citronellal distribution, as assessed through three complementary metrics in Figure 3 : (a) peak concentration intensity, (b) fractional confinement at ≥70% of peak concentration and (c) distributin width expressed as full width at half maximum (FWHM).

The heatmap visualization further supports this observation, where M2 demonstrates a highly localized high-content region limited to F2-F4, with a rapid transition to low citronellal distribution across wider fraction ranges, indicating partial overlap between fractions and reduced separation precision, while M1 exhibits the most dispersed pattern, confirming its low selectivity. Quantitatively, M2 present the narrowest FWHM (three fraction), compared to broader distributions observed in the other methods. This trend is consistent under a stricter criterion (≥70% of peak content), where M2 remains confined to three fractions, indicating minimal tailing and high fractionation precision [40].

Collectively, these results demonstrate that M2 achieves superior selectivity by combining high citronellal enrichment with minimal distribution width. The narrow and well-defined distribution suggest improved vapor-liquid equilibrium control and reduced co-distillation with other components, allowing citronellal to be preferentially distilled and collected before the emergence of heavier oxygenated compounds. This selective enrichment simplifies downstream processing, reduces energy consumption, and enhances collection efficiency. Therefore, based on both qualitative visualization and quantitative metrics (peak intensity, high-content fraction range, and FWHM), M2 can be considered the most selective method for citronellal isolation, particularly requiring high-purity compounds such as pharmaceutical and topical formulations.

While selectivity and citronellal purity are critical indicators of separation performance, the practical effectiveness of a distillation process must also consider product recovery. Therefore, the mass of citronellal-rich fractions (≥70% of peak content) and the corresponding yield were evaluated for each method. M2 produced the highest mass recovery



Method	FWHM (≥ 50% peak)		Effective Range (≥ 70% peak)	
	Range (fraction)	Number of fraction	Range (fraction)	Number of fraction
M1	F2-F6	**5**	F4-F6	**3**
M2	F2-F4	**3**	F2-F4	**3**
M3	F2-F7	**6**	F3-F6	**4**
M4	F2-F6	**5**	F3-F6	**4**
M5	F2-F6	**5**	FE-F5	**3**

Note: FWHM = Full Width at Half Maximum

Figure 3: (A) Distribution of citronellal content, (B) Heatmap of citronellal content, (C) Distribution Width of citronellal content.

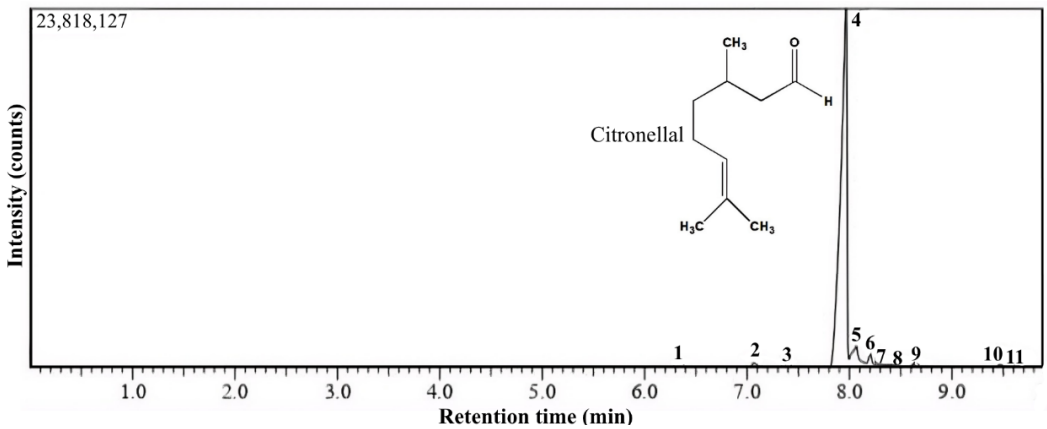


Figure 4: GC-MS chromatogram of the isolated citronellal.

(6675 g) and yield (33.38%), followed by M3 (5950 g; 29.75%) and M4 (5595 g; 27.98%), whereas M5 (4025 g; 20.13%) and M1 (3775 g; 18.88%) resulted in substantially lower recoveries. The superior yield obtained by M2 indicates that citronellal was not only concentrated into a narrow fraction range but was also recovered in larger quantities. This result suggests that the operating conditions applied in M2 provided a favorable balance between separation efficiency and product recovery, minimizing citronellal losses to adjacent fractions and reducing excessive retention in the vacuum fractional distillation residue.

3.3 Characterization of isolated citronellal

The characterization of citronellal was conducted in accordance with the Globally Harmonized System of Classification and Labelling of Chemicals. The results of physicochemical characterization of citronellal are presented in Table 6.

Table 6. Physicochemical characterization of isolated citronellal.

Test	Result
Appearance form	Liquid, yellowish
Odor	fresh citronellal
Relative Density	0.8168 g/mL (24.9 °C)
Optical rotation	(+) 0.45 (26.3°C)
Refractive index	1.4671 (24.9°C)
Vapor density	No data available
Citronellal content	87.57%

The chromatogram results of fraction 3 from method 2 (M2) are shown in Figure 4 with one dominant peak exhibiting the highest intensity at a retention time of 7.967 minutes (peak number 4). Mass spectral analysis identified this peak as citronellal (Similarity Index 97), with an area percentage of 87.57%. Based on the mass spectral analysis, a molecular ion peak at m/z 154 corresponding to a molecular weight of 1554 g/mol was clearly observed, indicating the presence of a monoterpenoid compound. This ion was assigned to citronellal ($C_{10}H_{18}O$), a cyclic oxygenated monoterpene, based on its fragmentation pattern and strong agreement with reference spectra from established databases such as the National Institute of Standards and Technology (NIST). The relatively stable molecular ion suggests a robust carbon framework, which is characteristic of monoterpenoid aldehydes under electron impact (EI) ionization conditions.

The fragmentation process is initiated by electron impact, generating a radical cation followed by hydrogen abstraction and rearrangement reactions. The presence of a fragment at m/z 136, corresponding to the loss of water ($-H_2O$), indicates intramolecular rearrangement involving the aldehyde functional group, which is commonly observed in oxygenated terpenoids. This behavior is consistent with classical fragmentation mechanisms such as McLafferty rearrangement, which involves hydrogen transfer and stabilization of the resulting ion [41].

Further fragmentation produces a peak at m/z 121 due to the loss of a methyl group ($-CH_3$), followed by a fragment at m/z 95 attributed to cleavage involving the loss of an ethyl group ($-C_2H_5$). The

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appearance of a fragment at m/z 69 suggests the formation of a stabilized carbocation through rearrangement processes, which is frequently reported as a diagnostic ion in monoterpene mass spectra. A dominant base peak at m/z 41 corresponds to a highly stable allylic or propenyl cation formed after the loss of an ethylene group ($-C_2H_4$), indicating a highly favorable fragmentation pathway.

Additionally, the molecular ion at m/z 154 undergoes fragmentation to yield a peak at m/z 111 via the loss of a propyl group ($–C_3H_7$), along with a fragment at m/z 55 derived from secondary fragmentation along the pathway leading to m/z 69. These fragmentation routes reflect a combination of bond cleavage and rearrangement processes that are characteristic of citronellal and related monoterpenoids. The presence of key diagnostic ions (m/z 136, 121, 95, 69, 41, and 55) strongly supports the structural assignment and is in good agreement with previously reported spectral data [42], [43].

Overall, the fragmentation pattern demonstrates a consistent and reproducible decomposition pathway involving stabilized carbocation intermediates, confirming the identity of citronellal. The high similarity index (SI = 97) obtained from spectral matching further strengthens this identification. These findings highlight the reliability of GC–MS analysis for confirming citronellal structure and provide detailed insight into its fragmentation behavior under EI conditions.

3.4 Analysis of particle size distribution

Particle size of the nanoemulsion was measured using a Particle Size Analyzer (PSA) based on the principle of Dynamic Light Scattering (DLS). The developed nanoemulsion exhibited a mean particle size of 107.97 ± 0.84 nm, a polydispersity index (PDI) of 0.29 ± 0.01 and zeta potential of -14.23 ± 0.32 mV (n=3). These parameters collectively indicate that the system was successfully formulated within the nanoscale range with a relatively uniform droplet distribution.

The particle size below 200 nm is critical for improving kinetic stability, as smaller droplets reduce the rate of gravitational separation phenomena such as creaming and sedimentation [44], [45]. In addition, the PDI value of 0.29 suggests a moderately narrow size distribution, reflecting a homogeneous system with minimal aggregation during the emulsification process. Comparable findings were reported in a nanoemulsion containing *Centella asiatica* leaf extract, which showed a particle size of 166.7 nm with a PDI of 0.413, demonstrating acceptable nanoscale characteristics and good homogeneity. The smaller droplet size and lower PDI obtained in the present study suggest a more uniform dispersion system, which may contribute to enhanced physical stability and more efficient topical delivery of citronellal [46].

The zeta potential value of −14.23 mV is lower than the conventional ±30 mV threshold typically associated with electrostatic stabilization [47]. However, this result is consistent with the use of Tween 80 as a non-ionic surfactant, where stability is primarily governed by steric rather than electrostatic mechanisms. Tween 80 forms a hydrated interfacial layer around the droplets, providing a steric barrier that prevents coalescence and ensures stability across a wide pH range and in electrolyte-rich environments [48], [49].

In combination, propylene glycol functions as a co-surfactant by reducing interfacial tension, facilitating droplet disruption during ultrasonication, and promoting the formation of smaller droplets. It also enhances interfacial flexibility and acts as a permeation enhancer, which is particularly advantageous for topical delivery. Together with the extended sonication time, these factors synergistically contribute to the observed nanoscale droplet size, improved homogeneity, and overall stability of the system [50].

A comparison with the formulation reported in Patent IDS000008797 further highlights the improvement achieved in the present study. The patented nanoemulsion system exhibited a particle size of approximately 136 nm, whereas the formulation developed in this study resulted in a smaller droplet size of 107.97 ± 0.84 nm.

This reduction in particle size can be attributed to key formulation and process modifications, including the substitution of Tween 20 and polyethylene glycol (PEG) with Tween 80 and propylene glycol, as well as the extension of ultrasonication time from 3 to 10 minutes. These changes enhance interfacial stabilization and droplet disruption efficiency, leading to a more homogeneous nanoscale system with potentially improved stability and topical delivery performance.

3.5 Physicochemical properties of citronellal NEGs

The physicochemical properties of the citronellal NEGs formulas (FA-FE), including pH, transmittance, and viscosity, are summarized in Table 7. The

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Table 7: Properties of citronellal NEGs formulation.

Formula	pH	Transmittance (%)	Viscosity (cP)
FA	6.18 ± 0.02	99.60 ± 0.00	23605.00 ± 5.22
FB	6.13 ± 0.03	99.64 ± 0.00	25164.67 ± 3.58
FC	6.13 ± 0.02	99.90 ± 0.00	21335.33 ± 1.03
FD	6.11 ± 0.03	100.46 ± 0.00	23605.00 ± 1.54
FE	5.81 ± 0.05	100.10 ± 0.00	24185.00 ± 2.65

Remark: FA= 0% citronellal : 6% methyl salicylate, FB= 1.5% citronellal : 4.5% methyl salicylate, FC= 3.0% citronellal : 3.0% methyl salicylate, FD= 4.5% citronellal : 1.5% methyl salicylate, FE= 6.0% citronellal : 0% methyl salicylate. n=3

evaluation began with pH assessment, which showed that all formulations remained within the physiological skin range (4.5–6.5), indicating their suitability for topical application without causing dryness or irritation. Compared to the initial nanoemulsion (pH 5.45–5.66), the nanoemulgel (NEG) formulations exhibited a slight increase in pH (5.81–6.18). In agreement with previous reports, topical gel formulations are considered acceptable if they have a pH comparable to the pH of human skin, thus not harming the skin [51]. This change is primarily attributed to the incorporation of Carbopol neutralized with triethanolamine (TEA), which induces ionization of the polymer's carboxyl groups. This process promotes polymer chain expansion, leading to the formation of a gel network that enhances viscosity and contributes to formulation stability. Therefore, the observed pH increase reflects the successful development of an optimal gel structure [52], [53].

The transmittance values above 99% indicate the formulation of a clear and stable nanoemulsion system. The highest transmittance value (100.46%) should not be interpreted as a physical transmittance exceeding the theoretical limit, but rather as a consequence of the combined effect of excellent optical clarity and inherent instrumental limitation in UV-Vis [54], [55]. In a highly transparent system with a very small droplet size, light scattering becomes negligible, allowing nearly complete transmission of incident light. However, slight deviations above 100% transmittance may arise from baseline drift, improper instrument zeroing, imperfect blank correction, photometric inaccuracies, and residual matrix effect associated with the reference measurement [56]. These factors are well recognized as sources of uncertainty in UV-Vis and may lead to a minor overestimation of the transmittance value.

The viscosity of the citronellal NEGs formulations was measured using a viscometer and ranged from 21,335.33 to 25,164.67 cP, with %RSD less than 2%, indicating excellent consistency and reproducibility of the measurements. These viscosity values fall within the optimal range for topical gel preparations (10,000–50,000 cP), indicating suitable rheological properties for dermal application [57].

From a rheological perspective, this viscosity range ensures a balance between ease of spreadability and sufficient structural integrity, allowing the formulation to form a stable film on the skin while prolonging residence time. The Carbopol-based gel system is also expected to exhibit pseudoplastic (shear-thinning) behavior, facilitating application under shear and recovery of viscosity afterward, which prevents runoff and supports uniform distribution [58].

Importantly, the rheological behavior is closely associated with the physicochemical characteristics of the dispersed system. The relatively small droplet size contributes to a more homogeneous dispersion within the gel matrix, enhancing physical stability and minimizing the risk of phase separation. In addition, an adequate zeta potential provides electrostatic or steric stabilization, reducing droplet aggregation and maintaining dispersion uniformity over time. The combination of appropriate viscosity, fine droplet size, and sufficient zeta potential indicates a well-structured nanoemulgel system with improved stability and performance for topical delivery.

From a formulation perspective, the observed viscosity can be attributed to an optimal balance between the oil phase, surfactant, and gelling agent within the NEGs system. Enhanced intermolecular interactions among these components contribute to increased resistance to flow, leading to higher viscosity. Overall, the viscosity characteristics of the citronellal NEGs are consistent with typical nanoemulsion-based gel systems, supporting its suitability for topical drug delivery applications.

3.6 Organoleptic Evaluation

Organoleptic evaluation was performed to assess the appearance, odor, and consistency of the citronellal NEG formulations (FA–FE) as preliminary quality parameters related to physical stability and user acceptability. Overall, all formulations exhibited a homogeneous semi-solid gel with a thick, light texture and rapid absorption upon application, indicating that the incorporation of citronellal and methyl salicylate did not negatively affect the gel matrix (Figure 5). This consistency is desirable for topical delivery because it supports ease of spreading, good skin adherence, and comfortable application without leaving excessive residue.

Visual observation showed that all formulations possessed a slightly clear white to translucent appearance with no significant macroscopic differences among formulations. The translucent characteristics observed in several formulations suggest the formation of a nanoemulsion system, where fine droplet dispersion reduces light scattering and enhances optical clarity [59]. This observation is consistent with the droplet size analysis, which demonstrated nanoscale dimensions with narrow PDI (0.29 ± 0.01), indicating a uniform droplet distribution.

The absence of phase separation and the visually homogeneous nature of all formulations further confirm the physical stability of the system, which is supported by the zeta potential result indicating sufficient stabilization to prevent droplet aggregation. This indicates that variations in the concentrations of citronellal and methyl salicylate in the formulations do not affect the visual appearance and physical properties of the citronellal NEGs formulations.

Odor evaluation revealed differences among formulations depending on the proportion of volatile components. Formula FA exhibited a dominant methyl salicylate odor, while formulas FB to FE progressively showed increasing citronellal intensity, indicating the presence and proportional variation of the active compounds within the formulations.

Overall, the organoleptic results indicate that all citronellal NEG formulations possessed acceptable physical characteristics, homogeneous appearance, and pleasant odor profiles, with no visible phase separation or instability. These findings are further supported by physicochemical parameters, including nano-sized droplets, narrow PDI, and zeta potential values, collectively confirming the physical stability of the system and its suitability for further evaluation as a topical nanoemulgel preparation [60].

3.7 Spreadability test

Spreadability is an important parameter in topical formulations as it determines the ease of application and uniform distribution of the drug on the skin surface [47]. The spreadability test results showed that all NEGs formulations (FA-FE) exhibited an increase in spreading diameter with increasing applied load, indicating good flow properties of the system (Table 8). Overall, all formulas fall within the ideal range for topical preparations (5–7 cm), indicating good gel spreadability on the skin surface [38]. The differences in spreadability values among formulations suggest that the viscosity influences the spreading ability of the preparation. Theoretically, an increase in viscosity leads to a decrease in spreadability due to greater resistance to flow. The result indicated an inverse relationship between viscosity and spreadability,

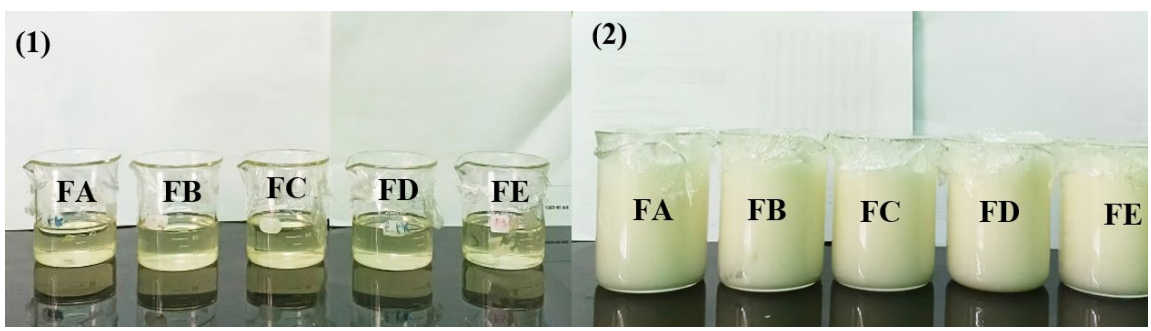

Figure 5: Visual appearance of (1) citronellal nanoemulsion and (2) citronellal NEGs formulas. All formulations possessed a slightly clear white appearance.

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Table 8: The spreadability test of citronellal NEGs.

Load Addition (g)	Spreadability (cm)				
	FA	FB	FC	FD	FE
50	4.83 ± 0.23	4.10 ± 0.17	4.00 ± 0.00	4.30 ± 0.00	3.87 ± 0.12
100	6.20 ± 0.10	5.17 ± 0.15	5.20 ± 0.17	5.00 ± 0.00	4.70 ± 0.17
200	6.67 ± 0.15	5.63 ± 0.21	5.83 ± 0.06	5.40 ± 0.17	5.73 ± 0.06

Remark: FA = 0% citronellal: 6% methyl salicylate, FB= 1.5% citronellal : 4.5% methyl salicylate, FC= 3.0% citronellal : 3.0% methyl salicylate, FD= 4.5% citronellal : 1.5% methyl salicylate, FE= 6.0% citronellal : 0% methyl salicylate. n=3

where formulations with lower viscosity, such as FC, tended to exhibit higher spreadability, while formulations with higher viscosity, such as FB, showed lower spreading ability. However, this relationship was not strictly linear. This can be attributed to the complex rheological behavior of NEGs systems, which are generally non-Newtonian in nature.

In addition to viscosity, spreadability is also influenced by other factors such as the gel network structure, interactions between nanoemulsion droplets and the gel matrix, as well as phase distribution within the system. The nanoscale droplet size (~107 nm) enables a more homogeneous distribution within the gel matrix, which may enhance spreading ability. On the other hand, the presence of a structured gel network contributes to the overall viscosity of the system, thereby affecting the resulting spreadability values.

3.8 Stability test

Stability testing using the cycle testing method was conducted by alternating storage at 4ºC, 30ºC, and 40ºC for three cycles. Observations showed that all formulations did not experience phase separation or aggregation, indicating good physical stability of NEG citronellal. This indicates that the nanoemulsion droplets are well dispersed within the gel matrix and maintain structural homogeneity despite thermal stress. Furthermore, the gel consistency remained stable without significant viscosity changes, reflecting the strong interaction between citronellal and the gel components [60], [61].

In addition to physical stability, the formulation strategy also contributes to chemical stability, especially considering that citronellal is an essential oil that generally exhibits high volatility, low stability, and susceptibility to degradation; however, encapsulation in a nanoemulsion system can mitigate these limitations by trapping the active compounds within the nanoscale droplets, thereby reducing evaporation and exposure to environmental factors such as oxygen and temperature [62], [63]. Nanoemulsion-based systems are also known to improve stability through better dispersion, reduced interfacial tension, and controlled release behavior, which collectively help maintain the integrity of the bioactive compounds during storage [63], [64]. Therefore, the observed stability of citronellal NEG is not only due to its gel structure but also to the protective effect of the nanoemulsion system in maintaining physical and chemical stability [64].

3.9 Hedonic test

The hedonic test results showed in Table 9 that all evaluated parameters of the citronellal NEGs scored an average above 4.00 for all sensory attributes. Among the tested formulations, F4 consistently exhibited the highest scores for odor, texture, overall liking, comfort, and post-application sensation, suggesting consumer acceptance.

Although F4 showed the highest average scores across most sensory attributes, univariate ANOVA revealed no significant differences among formulations for individual attributes (p-value > 0.05). Therefore, a multivariate approach was employed to evaluate overall sensory perception. The multivariate statistical results (Table 10) demonstrated significant differences among formulations when all sensory attributes were considered simultaneously (MANOVA, p-value > 0.05). Canonical discriminant analysis further revealed that the first two canonical variables explained 98.39% of the total variance, with F4 exhibiting the highest canonical score on the primary acceptance dimension. Subsequent Tukey HSD analysis confirmed that F4 differed significantly from several formulations, indicating that F4 possessed the most favorable overall sensory profile.

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Table 9: Hedonic evaluation for citronellal NEGs formulations.

Hedonic test	F1	F2	F3	F4	F5
Odor	4.32 ± 0.65	4.45 ± 0.60	4.50 ± 0.58	4.61 ± 0.63	4.40 ± 0.66
Texture (touched)	4.38 ± 0.70	4.50 ± 0.65	4.55 ± 0.64	4.69 ± 0.67	4.47 ± 0.68
Texture (smeared)	4.35 ± 0.68	4.48 ± 0.64	4.52 ± 0.60	4.61 ± 0.69	4.42 ± 0.67
Liked	4.30 ± 0.60	4.44 ± 0.58	4.48 ± 0.55	4.61 ± 0.57	4.39 ± 0.61

Note: Values are presented as mean ± standard deviation (SD), n=26.

Table 10: Multivariate statistical analysis of hedonic evaluation.

Analysis	Parameter	Value	F-value	*p*-value
MANOVA	Wilks' Lambda	0.678	1.751	0.011
	Pillai's Trace	0.352	1.683	0.017
	Hotelling–Lawley Trace	0.431	1.810	0.007
	Roy's Largest Root	0.277	4.829	< 0.001
CDA	Can1	64.22%	8.658	3.35×10^{-6}
	Can2	34.17%	4.606	0.0017
Tukey HSD	F4–F1 (Can1)	1.546	-	<0.001
	F4–F2 (Can1)	0.829	-	0.027
	F5–F4 (Can1)	-0.850	-	0.022
	F4–F3 (Can2)	0.953	-	0.007

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3.11 Hedonic test

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canonical score on the primary acceptance dimension. Subsequent Tukey HSD analysis confirmed that F4 differed significantly from several formulations, indicating that F4 possessed the most favorable overall sensory profile.

3.12 Antinociceptive activity (Tail Immersion Test)

The tail immersion method was conducted using several treatment groups for antinociceptive evaluation, which were divided into three min groups: baseline, negative control, and positive control. The baseline was used as a reference for the natural pain response. The negative control consisted of the gel base containing methyl salicylate to evaluate the effect of the matrix without the active compound citronellal. The positive control employed a commercial product as a comparator, as it is known to possess analgesic activity.

The tail immersion test results in Figure 6 show that the citronellal–methyl salicylate gel exhibited a relatively strong antinociceptive activity. The peak %MPE value reached 64.3% at the 15th minute, whereas the positive control only reached 42.9%. According to the literature, a %MPE ≥ 50% is generally considered indicative of a strong analgesic effect, while values between 20–40% are often categorized as moderate effects [65]. Therefore, the gel sample has potential for a good antinociceptive.

In preclinical studies, 50% MPE is often used as a reference for ED_{50}, which is the dose or treatment that produces half of the maximal possible effect [66]. Strong opioid drugs such as morphine typically achieve %MPE values above 50% at their peak effect phase, whereas some plant extracts or non-steroidal anti-inflammatory drugs (NSAIDs) only produce 20–40% MPE [67]. This aligns with this finding, where the citronellal–methyl salicylate gel not only surpassed the negative control but also provided higher %MPE than the positive control at all observation times.

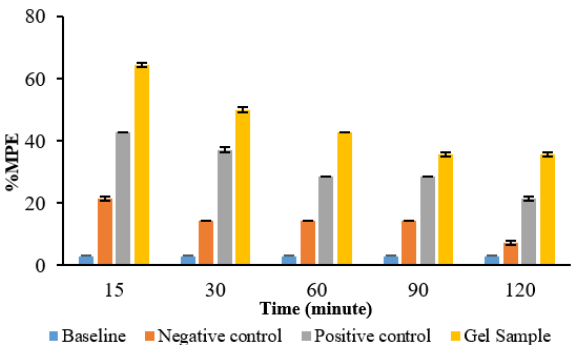

Figure 6: Percentage of maximum possible effect (%MPE) (n=2).

These findings indicate that the citronellal–methyl salicylate gel formulation can deliver a significant antinociceptive effect and potentially outperform the standard positive control. This 6fect was particularly evident during the early phase of observation, and although it decreased over time, the %MPE values of the sample remained above the positive control up to 120 minutes. Overall, these data strengthen the evidence that the gel sample has an effective and promising analgesic activity in the tail immersion test.

4 Conclusion

This study shows that vacuum fractional distillation is an effective method to isolate citronellal from citronella essential oil. The M2 method, using a reflux ratio of 12:6 at 98 °C and 760 mmHg, gave the best result. It increased citronellal purity from 36.74% to 87.57%. The isolated citronellal was formulated into a nanoemulgel. The formulation had a small particle size (107.97 ± 0.84 nm, a polydispersity index of 0.29 ± 0.01 and zeta potential of -14.23 ± 0.32 mV), which indicates good stability. It also showed a suitable pH, high transmittance, good spreadability, and appropriate viscosity. The product remained stable during cycling tests. Organoleptic evaluation showed high hedonic scores (>4.6), indicating good user acceptance. The tail immersion test demonstrated notable antinociceptive activity, with a maximum %MPE of 64.3%. This value was higher than the commercial product (42.9%) and remained stable for up to 120 minutes. However, further statistical analysis is required to confirm whether this difference is significant. Overall, these findings highlight the potential of the optimized isolation method and NEG formulation for developing citronellal as a natural topical antinociceptive agent, while future studies should focus on extended pharmacological evaluation, long-term stability studies, clinical validation, and scale-up for industrial application.

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Author Contributions

N.F. and W.F. : Conceptualization, Methodology, Resources, and Funding acquisition; N.F., I.H., O.M.I, N.A.F, and N.A.M: Formal Analysis, Investigation, Data Curation; N.F., W.F., and O.M.I: Data Validation; N.F., I.H., N.A.F, N.A.M: Writing original draft preparation; N.F., N.A.M., and W.F.: Writing review and editing; N.F., and N.A.M.: Visualization; O.M.I, and N.A.M: Project administration. All authors have reviewed and approved the final published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest**.**

Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized the ChatGPT tool to enhance the language and readability of the manuscript.

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