Citral Chemotype of *Pectis elongata* Essential Oil: Nanoemulsification and Enhanced Antimicrobial Properties for Food Safety

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ABSTRACT

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| **Aims:** Essential oils of *Pectis elongata* from the Brazilian Amazon were analyzed to estimate the citral (neral and geranial stereoisomers) content in the vegetative and reproductive phases and their circadian cycle. Also, a nano emulsification process investigated whether it interferes with the oil's composition and antimicrobial potential against food-contaminating agents.**Methodology:** Gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC-MS) analyzed the oil composition, nanoemulsion, and the citral standard. Nanoemulsion was prepared using low-energy input methodology with phase inversion, and its mean diameter, polydispersity index, and volatile composition were evaluated on the 1st, 7th, and 15th-day post-processing.**Results: T**he citral content of *P. elongata* oil ranged from 70.0% to 92.5% in the performed studies. The disk diffusion and minimum inhibitory concentration bioassays evaluated the antimicrobial activity.Nanoemulsion showed an average diameter between 140.7 ± 6.79 and 177.07 ± 9.24 nm, a polydispersity index between 0.36 and 0.47 ± 0.09, and a zeta potential between -2.54 ± 2.6 and -7.25 ± 2.37.**Conclusion:** Citral was maintained as the main constituent, preserving its biological properties and significantly enhancing its antimicrobial activity up to two times against various pathogenic agents contaminating and spoiling food. This finding highlights the potential use of a citral nanoemulsion in ensuring food safety. |

*Keywords: Essential oil composition; citral (neral and geranial stereoisomers); seasonal and circadian evaluation; oil nanoemulsification; antimicrobial activity.*

***INTRODUCTION*** *:*

In a previous study, we analyzed the composition and variability of *Pectis elongata* oil in the Brazilian Amazon [1,2]. This study aims to estimate the citral (a mixture of the neral and geranial stereoisomers) content in the growing vegetative and reproductive phases of *P. elongata* and test its oil's antimicrobial properties.

The food industry's interest in using essential oils and their isolated components has grown in recent years, especially regarding their application as a food additive and natural preservative to replace synthetic compounds [3]. Essential oils are natural products that undergo variations in the total content and relative proportions of their secondary metabolites at different levels, such as seasonal, circadian, and inter/intraspecific variations. Even under control, gene expression can promote changes resulting from the interaction of biochemical, physiological, ecological, and evolutionary processes [4]. Their hydrophobicity and volatility control essential oils' incorporation into foods and the biological activity of essential oils is mediated by components present in the food matrix, such as lipids, carbohydrates, proteins, antioxidants, preservatives, and other additives [5,6].

The use of essential oils in foods can be optimized with the identification, planning, and production of plants with their distinct chemotypes and active principles of interest in association with technological applications, such as the development of nanoemulsions, which can disperse and increase the bioavailabiliy of these compounds by encapsulating them within structured nanometric droplets [5,7,8]. Studies have shown that the reduced size of nanoemulsion droplets can improve the appearance, physical stability, and biological activity of essential oils in food and beverages [5,9].

*Pectis elongata* Kunth [syn. *Pectis elongata* var. *ﬂoribunda* (A. Rich.) D. J. Keil, *P. ﬂoribunda* A. Rich., *P. elongata* var. *oerstediana*(Rydb.) D. J. Keil, *P. oerstediana* Rydb., *P. elongata* var. *fasciculiﬂora*(DC.) D. J. Keil, among others] 10], belongs to Asteraceae and has a wide geographical distribution, spreading from Central America to Brazil, Colombia, Venezuela, and Guianas. It is an annual herb (15-50 cm), erect, with green leaves and slightly yellow flowers, pointed and full of glandular oil, commonly known as “cuminho-bravo” (wild cumin) and “limãozinho” (little lemon) in Pará and Amapá states, North Brazil. These trivial names arise from the scents of the two varieties of *P. elongata*, which are reminiscent of cumin (*Cuminum cyminum* L.) and citronella [*Cymbopogon citratus* (DC.) Stapf].

*Pectis* species generally have pleasant citrus, cumin, and oregano-like scents due to the presence of monoterpene constituents in their volatile compositions, such as citral (neral plus geranial), α- and β-pinene, limonene, perillaldehyde, cumin aldehyde, carvone*, p*-cymene, and thymol. The oils of other *P. elongata* samples collected in Belém and Bujaru, Pará state, and Ferreira Gomes, Amapá state, Brazil, showed perillaldehyde and limonene as their major compounds [1]. Neral and geranial were previously identified as primary constituents in the essential oils of *Pectis elongata*, which is rich in citral and occurs in Martinique, West Indies, and Santarém, Brazil [2,11]. In the oil of *Pectis brevipedunculata* (Gardner) Sch. Bip., with occurrence in Rio de Janeiro, Brazil, also has predominated the isomeric oxygenated monoterpenes, neral, and geranial [12]. The discovery of these new chemical varieties opens perspectives for several commercial applications with the plant since citral has been used as a flavoring ingredient in food, cosmetics, perfumes, soaps, and detergents [13]. Citral also has significant biological properties, such as antifungal, anti-inflammatory, anti-carcinogenic, insecticide, antispasmodic, analgesic, antipyretic, diuretic, sedative, and antibacterial [14-20].

The present study examined the variations in the composition of essential oil from *Pectis elongata* during different phases of the plant’s vegetative and reproductive development, and throughout its circadian cycle. The essential oil nanoemulsification process was also evaluated using a low-energy input technique capable of producing physically stable nanoemulsions. Furthermore, the nanoemulsification process's efficiency in maintaining the oil's main constituents, its antimicrobial potential against bacteria frequently associated with food contamination, and the physical-chemical and biological stability of the nanoemulsion for 15 days were investigated.

2. material and methods

**2.1. Chemicals**

Dichloromethane and *n*-hexane were supplied by Merck (Rio de Janeiro, Brazil). The culture media were obtained from Himedia (Mumbai, India). Citral, tween 80, resazurin, ampicillin, and anhydrous sodium sulfate were purchased from Sigma-Aldrich and Sigma-Vetec (St. Louis, USA, and Rio de Janeiro, Brazil). The bacterial strains in lyophilized form were supplied by Cefar (São Paulo, Brazil). Polysorbate 80 was purchased from Dinamica, Química Contemporânea (Indaiatuba, Brazil).

**2.2. Plant material**

Samples of *Pectis elongata* (whole herbs, excepting the roots), cultivated in plant beds and under open field conditions at Universidade Federal do Oeste do Pará (UFOPA) (coordinates: 02°25'03.2" S / 54°44'25.1" W), Santarém, Pará, Brazil, were collected to obtain the essential oil and the analysis of its composition, from different stages of planting development: (1) end of the fourth month (vegetative stage, May) and (2) end of the eighth month (reproductive stage, August). For the evaluation of the oil in the plants during the circadian cycle, samples were collected at different times of the day (6 am, 9 am, 12 pm, 3 pm, 6 pm, and 9 pm), with the following environmental parameters: average precipitation of 972 mm (media/day: 6.35 mm), the temperature of 25.7 ºC and relative humidity of 84%. For plant cultivation, a mixture of black earth was used as a substrate in a 2:1 ratio, with a space of 30 cm between plants and rows. Plants were identified by Prof. Chieno Suemitsu from the Botany Laboratory of UFOPA and deposited under the number HSTM003432 (UFOPA Herbarium). The project was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimentos Tradicionais Associados (SisGen) of the Brazilian Government under the code A192EBE.

**2.3. Oil processing**

The essential oils of vegetative and reproductive phases were obtained by hydrodistillation, using a Clevenger-type apparatus (3 h), after the plants dried at room temperature for two days. The oils of the circadian cycle were obtained from fresh plants, collected, and hydrodistilled on the same day. The oil percentage was the same for the three replicates in the hydrodistillation process. Then, the oils were centrifuged with anhydrous sodium sulfate to remove residual water, stored in a labeled amber glass bottle, and refrigerated at 10°C. The oil yield was calculated by the plant biomass free from moisture, utilizing the relationship between the volume of oil and the dry biomass used in the extraction (v/w%).

**2.4. Oil composition analysis**

The essential oil analysis was performed on a GCMS-QP2010 Ultra system (Shimadzu Corporation, Tokyo, Japan), equipped with an AOC-20i auto-injector and the GCMS-Solution software containing mass spectra libraries for standard constituents of essential oils [21,22]. For the analysis, a silica capillary column (Rxi-5ms, 30m x 0.25mm; 0.25μm film thickness, Restek Corporation, Bellefonte, PA, USA) was utilized under the following conditions: injector temperature of 250 °C; oven temperature programming of 60-240 °C (3 °C/min); Helium was used as the carrier gas, adjusted at a linear velocity of 36.5 cm/s (rate of 1.0 ml/min); injection of 1 μL of the sample in the split mode (5 μL of the essential oil to 500 μL of *n*-hexane); split ratio 1:20; ionization by electronic impact at 70 eV; and temperatures of ionization source and transfer line at 200 and 250 °C, respectively. The mass spectra were obtained by scanning every 0.3 s, with mass fragments in the 35-400 m/z range. The retention index was calculated for all volatile components using a homologous series of C8-C20 *n*-alkanes (Sigma-Aldrich, USA), according to the linear equation of van den Dool and Kratz [23]. The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a Shimadzu GC 2010 ultra-system, coupled to the FID Detector, operated under similar conditions to the GC-MS system. The constituents were identified by comparing their retention indices and mass spectra (molecular mass and fragmentation pattern) with those existing in the GCMS-Solution system libraries in duplicate [21,22]. Expressing standard deviation values was unnecessary because the two ion chromatograms generated were identical, presenting the same peak-area values.

**2.5. Nanoemulsions preparation and characterization**

According to Ortiz-Zamora and co-workers [24], the nanoemulsions were prepared using a low-energy input method at 25 ºC. Briefly, the nonionic surfactant polysorbate 80 was vigorously mixed with the essential oil for 1.0 min on a vortex mixer. Then, water was added slowly (drop by drop) to obtain the final mass of 3.05 g, 0.8% essential oil, and 0.8% surfactant. All nanoemulsions were stored in clear glass vials at room temperature (25 ± 2 ºC) and protected from light. The nanoemulsions were defined according to macroscopic parameters: color, visual aspect, phase separation, creaming, and sedimentation during zero, 7, and 15 days. The particle size and polydispersion index (PDI) of nanoemulsions were evaluated by dynamic light scattering using a NanoZS Zetasizer (Malvern, UK), equipped with a 10mW red laser, at a wavelength of 632.8 nm and angle incidence of 173°. The dilution of the nanoemulsion in water (10 times) was performed before the analysis to avoid the multiple scattering effects. The readings were taken in triplicate using a UV quartz cuvette, and the results were expressed as mean ± standard deviation. The stability of the nanoemulsion was evaluated for 15 days according to the methodology described by Yazgan and colleagues [25]. Extractions with *n*-hexane (1500 µL) were performed for each formulated nanoemulsion (500 µL) to evaluate the oil composition incorporated into these nanoemulsions during the first, seventh, and fifteenth days. GC and GC-MS analyzed the *n*-hexane fractions under the same conditions. These procedures were performed in duplicate.

**2.6. Microorganisms and growing conditions**

The strains used in the antimicrobial assays were *Escherichia coli* (E003), *Staphylococcus aureus* (ATCC25923), *Salmonella typhimurium* (S004), *Pseudomonas aeruginosa* (P003), *Bacillus subutilis* (B005), *Enterococcus faecalis* (E002), and *Shigella flexneri* (S006), commercially purchased in lyophilized form (Cefar Diagnóstica, Brazil). The microorganisms were hydrated in Mueller Hinton broth at 37 ± 2 °C for 24 h. Inocula were prepared by directly inoculating colonies into sterile saline solution (1.0 mL) and adjusted to a 0.5 McFarland scale, corresponding to 1.5x108 CFU/mL [26].

**2.7. Antimicrobial activity – Disc-diffusion bioasssay**

The antimicrobial activity of the essential oil was evaluated using the agar disk diffusion method [26]. Mueller Hinton Agar (MHA) served as the growth medium for the microorganisms. Filter paper discs with a diameter of 6 mm, each containing 10 µL of essential oil from *P. elongata*, were gently placed on the surface of the culture medium previously inoculated with the test microorganism. After 30 minutes, the cultures were placed in a bacteriological incubator at 37 ± 2 °C for 24 hours. The diameters of the inhibition zones formed in the culture medium were then measured in millimeters. Ampicillin (10 μg/disk) was used as a positive standard to validate the experiment. Additionally, the citral standard (comprising neral and geranial) was employed to confirm whether the antimicrobial activity of the essential oil was associated with this primary compound.

**2.8. Determination of minimum inhibitory concentration (MIC)**

The initial concentration of bioassay was prepared by dissolving the oil or citral (35 mg) in Tween 80 (960 mL, 0.5%). Then, concentrations from 0.14 to 17.5 mg/mL were similarly prepared by serial dilution (dilution factor 1:1), using Mueller Hinton Broth (MHB) as the diluent. Nanoemulsion concentrations ranged from 0.032 to 4.16 mg/mL. The inoculum was initially adjusted to the standard 0.5 MacFarland scale, with further dilution (dilution factor of 1:10, sterile saline solution) to obtain a final concentration of 1.5×104 CFU/mL [27]. The tests were performed in 96-well plates, where each well received 90 μL of a specific concentration of oil plus 90 μL of sterile MHB and 20 μL of the inoculum, resulting in a final volume of 200 μL. The tests were performed simultaneously to control microbial growth, medium sterility, and solvent activity. Bacterial growth was inhibited after adding aqueous resazurin solution (20 µl, 0.02%) and re-incubation for 3 h. The permanence of blue staining in the wells determined the minimum inhibitory concentration (MIC). The change from blue to pink color (dye reduction) indicated the growth of viable cells, according to the National Committee for Clinical Laboratory Standards guidelines, with modifications [27]. Following the same described procedures, an MIC test was performed against *Staphylococcus aureus* (gram-positive) and *Escherichia coli*, gram-positive and gram-negative bacteria, after the nanoemulsification process to assess the biological stability of the nanoemulsions on the first, seventh, and fifteenth day.

**2.9. Statistical analysis**

Principal Component Analysis (PCA) was applied to verify the interrelationship in the composition of the oils (>0.5%) produced in the plant vegetative and reproductive phases and the different nanoemulsion times. The data matrix was standardized for the multi-variate analysis by subtracting and dividing the mean by the standard deviation. The Hierarchical Cluster Analysis (HCA), considering the Euclidean distance and complete linkage, was used to verify the similarity of the samples based on the distribution of the selected constituents in the PCA analysis using Minitab 18 software (free version, Minitab Inc., State College, PA, USA).

3. results and discussion

**3.1. Essential oil analysis**

In the vegetative and reproductive phases of *Pectis elongata*, the yields of essential oils were 1.0% and 1.2%, respectively. GC-MS identified thirty constituents, totaling more than 99.0% (see Table 1). A slight variation in the main constituent citral (neral + geranial) content was observed, which increased from 90.0% in the vegetative phase to 92.5% in the reproductive phase. The vegetative and reproductive phases also noted an inversion in neral (39.9% to 37.2%) and geranial content (50.1% to 55.3%). It is important to emphasize that the collection of *P. elongata* in the vegetative phase was carried out in the rainy period (May), while the collection of the plant in the reproductive phase was in the dry period (August). As it is an annual plant, the effects of seasonality may be similar to those of metabolic changes related to the plant's development process. Thus, plant cultivation must consider possible changes and incorporate field experiments with diverse plant origins to generalize results. It is recommended that *P. elongata* be collected during the reproductive phase and in the dry period to obtain greater oil yield and citral content. A similar study with *P. elongata* in Martinique, West Indies, showed different results, with higher content of neral (27.5%) and geranial (40.2%) in young plants when compared to plants close to senescence, which exhibited a slight variation for neral (15.7% to 16.7%) and geranial (24.6% to 28.5%) [11].

**Table 1. *Pectis elongata* oil composition in the vegetative and reproductive stages and in the nanoemulsification postprocessing.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Constituents (%)** | **RIC** | **RIL** | **Phases** | **Nanoemulsion** |
| **Vegetative** | **Reproductive** | **Day 0** | **Day 7** | **Day 15** |
| 5-Methyl-3-Heptanone | 940 | 936a | 0.1 | 0.1 | 0.3 | 0.4 | 0.7 |
| Camphene | 949 | 946a | 0.1 | 0.1 | 0.3 | 0.5 | 0.8 |
| (2*E*)-Hepten-1-ol | 964 | 958a |  |  |  | 0.1 | 0.3 |
| 6-methyl-5-Hepten-2-one | 984 | 981a | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| *n*-Decane | 997 | 1000a |  |  |  |  | 0.2 |
| Limonene | 1027 | 1024a | 0.2 | 0.2 |  | 0.1 |  |
| (*E*)-β-Ocimene | 1045 | 1044a |  | 0.1 |  |  |  |
| *trans*-Linalool oxide (furanoid) | 1087 | 1084a | 0.1 | 0.1 |  |  |  |
| Linalool | 1098 | 1095a | 0.3 | 0.2 | 0.1 | 0.1 |  |
| *exo*-Isoctral | 1142 | 1140a | 0.2 | 0.1 |  |  | 0.3 |
| Citronellal | 1151 | 1148a | 0.1 | 0.1 |  |  |  |
| (*Z*)-Isocitral | 1162 | 1160a | 1.9 | 1.0 |  | 1.0 |  |
| (*E*)-Isocitral | 1180 | 1177a | 2.8 | 1,4 | 0.5 | 1.7 |  |
| α-Terpineol | 1189 | 1186a |  | 0.1 |  |  |  |
| Nerol | 1227 | 1227a | 0.4 | 0.9 |  |  |  |
| **Neral** | 1240 | 1235a | **39.9** | **37.2** | **40.4** | **40.3** | **30.9** |
| Geraniol | 1253 | 1249a | 0.7 | 1.6 | 0.3 | 0.3 |  |
| **Geranial** | 1271 | 1264a | **50.1** | **55.3** | **56.4** | **54.5** | **51.1** |
| Neryl formate | 1278 | 1280a | 0.1 | 0.1 | 0.4 |  | 5.0 |
| Methyl nerolate | 1282 | 1280a |  |  | 0.5 |  | 6.9 |
| 1-Tridecene | 1290 | 1290a | 1.2 | 0.4 |  |  |  |
| Geranic acid | 1358 | 1354b |  |  |  |  | 2.0 |
| β-Elemene | 1392 | 1389a | 0.3 | 0.1 |  |  |  |
| Cyperene | 1399 | 1398a | 0.1 |  |  |  |  |
| (*E*)-Caryophyllene | 1417 | 1417a | 0.1 | 0.1 |  |  |  |
| α-Humulene | 1453 | 1452a | 0.7 | 0.4 |  | 0.3 |  |
| γ-Muurolene | 1480 | 1478a | 0.1 |  |  |  |  |
| 2-Tridecanone | 1499 | 1495a | 0.1 |  |  |  |  |
| (*E*,*E*)-α-Farnesene | 1507 | 1505a | 0.1 |  |  |  |  |
| Humulene epoxide II | 1609 | 1608a | 0.1 | 0.1 | 0.1 |  |  |
| Total (%) | 99.8 | 99.5 | 98.4 | 99.4 | 98.3 |

RIC = Calculated Retention Index (Rxi-5ms column); RIL = Literature Retention Index, aAdams, 2007 [21] and bMondello, 2011 [22]; Bold = Major constituents

The CP1 component of the Principal Component Analysis (PCA, Figure 1) plot showed a positive correlation with geranial, geraniol, neral, nerol, *E*-isocitral, α-humulene, *Z*-isocitral, and 1-tridecene. Moreover, the CP2 component displayed a positive correlation with neral and geranial. The PCA plot analysis allows the identification of the samples into three groups. Group I was formed by the vegetative and reproductive samples with 50,46% similarity. Group II was ordered by nanoemulsion formulation at 0 and 7 days with 70.53% similarity. Group III was composed of the nanoemulsion formulation on the 15th day.

PCA plot was confirmed by Hierarchical Cluster Analysis plot (HCA, Figure 2), with the dendrogram showing the formation of three groups. However, HCA can only be considered in this arrangement if the percentage of minor constituents is relevant since the contents of neral (57.7%) and geraniol (53.4%) are quite significant. In group I (vegetative and reproductive phases), in addition to the primary constituents neral (57.7%) and geranial (53.4%), the minor components nerol (0.4 and 0.9%) and 1-tridecene (1.2 and 0.4%) were also detected. Regarding the nanoemulsion preparation, nerol and 1-tridecene are absent in groups II and III. In group III, the nanoemulsion sampled on the 15th day showed considerable contents of neryl formate (5.0%), methyl nerolate (6.9%), and geranic acid (2.0%), which are probably products of oxidation of neral and geranial, generated after the seventh day of preparation and during the nanoemulsion storage.



**Fig. 1.** **Principal Component Analysis (PCA) plot of Pectis elongata essential oils, resulting**

**from the composition of vegetative and reproductive phases and nanoemulsions.**



**Fig. 2. HCA dendrogram representing the similar relationship of P. elongata oils in the**

**vegetative and reproductive phases and the nanoemulsions composition.**

The composition of the essential oils of *Pectis elongata* in the evaluation of the circadian cycle during the vegetative phase is presented in Table 2, which shows fifty-two constituents comprising about 97% of the total oils. The percentage of the components changed according to the time of collection. As expected, the main constituents were neral (24.5 to 35.4%) and geranial (45.5 to 55.2%), confirming the essential oil as a citral chemical type. The citral (neral + geranial) production was influenced by solar radiation because of its increased content between 12 pm and 6 pm. Citral increase can be attributed to the higher radiation level at the collection time since biosynthetic reactions are stimulated by photosynthesis [28,29]. The plant's metabolism was slower during the night and early morning, resulting in a lower percentage of citral. Previously, these same variations in citral content were observed in the circadian cycle of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae), occurring in the Western region of Pará state, Brazil [7].

**Table 2. Essential oil composition of *Pectis elongata* in the evaluation of its circadian cycle.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Constituents (%)** | **RIC** | **RIL** | **Circadian cycle** |
| **6am** | **9am** | **12pm** | **3pm** | **6pm** | **9pm** |
| 5-Methyl-3-Heptanone | 940 | 936a | 0.1 | 0.1 | 0.1 | 0.1 |  | 0.1 |
| Camphene | 949 | 946a | 0.1 |  | 0.1 | 0.1 |  |  |
| β-Pinene | 976 | 974a |  |  |  | 0.1 |  |  |
| 6-methyl-5-Hepten-2-one | 984 | 981a | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 |
| Octanal | 1002 | 998a |  |  |  | 0.1 |  | 0.1 |
| Limonene | 1027 | 1024a | 0.4 | 0.2 |  | 1.1 | 0.3 | 0.1 |
| (*E*)-β-Ocimene | 1045 | 1044a |  |  |  | 0.1 |  |  |
| *n*-Octanol | 1067 | 1063a |  |  |  |  |  | 0.1 |
| *cis*-Linalool oxide (furanoid | 1070 | 1067a |  | 0.1 |  |  |  |  |
| *trans*-Linalool oxide (furanoid) | 1087 | 1084a |  | 0.1 |  | 0.1 | 0.1 |  |
| Linalool | 1098 | 1095a | 0.6 | 0.7 | 0.4 | 0.6 | 0.5 | 0.6 |
| *exo*-Isocitral | 1142 | 1140a | 0.1 | 0.1 |  | 0.1 | 0.1 | 0.1 |
| Citronellal | 1151 | 1148a | 0.1 |  |  | 0.1 | 0.1 |  |
| (*Z*)-Isocitral | 1162 | 1160a | 0.2 | 0.1 | 0.3 | 0.5 | 0.4 | 0.4 |
| (*E*)-Isocitral | 1180 | 1177a | 0.4 | 0.2 | 0.4 | 0.8 | 0.7 | 0.7 |
| α-Terpineol | 1189 | 1186a | 0.1 | 0.1 |  | 0.1 | 0.1 | 0.2 |
| Prenyl angelate | 1193 | 1189a |  | 0.1 | 0.1 |  | 0.1 | 0.1 |
| dihydro-Myrcenol acetate | 1219 | 1214a | 0.1 | 0.2 | 0.1 |  |  | 0.1 |
| Citronellol | 1222 | 1223a | 0.1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 |
| Nerol | 1227 | 1227a | 0.7 | 1.5 | 0.4 | 0.4 | 0.5 | 0.6 |
| *cis*-*p*-Mentha-1(7),8-dien-2-ol | 1232 | 1230b | 0.2 |  |  | 0.2 | 0.1 | 0.1 |
| **Neral** | 1240 | 1235a | **28.6** | **24.5** | **30.9** | **35.4** | **32.9** | **32.2** |
| Geraniol | 1253 | 1249a | 2.8 | 4.5 | 0.9 | 1.8 | 2.3 | 2.6 |
| **Geranial** | 1271 | 1264a | **50.9** | **45.5** | **55.2** | **52.8** | **53.9** | **50.8** |
| Neryl formate | 1278 | 1280a | 0.4 | 1.0 | 0.9 | 0.2 | 0.3 | 0.3 |
| Methyl nerolate | 1282 | 1280a |  | 0.6 | 0.5 |  |  |  |
| 1-Tridecene | 1290 | 1290a | 0.6 | 0.6 | 0.2 | 0.4 | 0.8 | 0.3 |
| Geranyl formate | 1301 | 1298a | 0.1 | 0.1 |  |  |  |  |
| Methyl geranate | 1322 | 1322a | 0.1 | 0.3 | 0.1 |  |  |  |
| cis-(*E*)-Jasmonol | 1334 | 1328a |  | 3.6 | 1.8 | 0.8 | 1.5 | 2.5 |
| Verbenol acetate | 1342 | 1340a |  | 0.1 |  |  |  |  |
| Geranic acid | 1358 | 1354b | 0.8 | 1.4 | 0.7 | 0.1 | 0.2 | 0.5 |
| 10-Undecenol | 1361 | 1361a |  | 1.0 | 0.3 | 0.1 | 0.2 | 0.3 |
| (2*E*)-Undecenol | 1373 | 1370b | 4.4 | 5.4 | 3.5 | 1.4 | 2.8 | 4.3 |
| Geranyl acetate | 1384 | 1379a | 0.1 | 0.1 |  |  | 0.1 | 0.1 |
| β-Elemene | 1392 | 1389a |  |  |  | 0.1 |  |  |
| Decyl acetate | 1409 | 1407a | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| 4,8-β-epoxy-Caryophyllane | 1424 | 1423a | 0.1 | 0.1 |  | 0.1 | 0.1 | 0.1 |
| α-Humulene | 1453 | 1452a |  |  | 0.1 | 0.2 | 0.2 | 0.1 |
| (*Z*)-Jasmolactone | 1491 | 1487a |  | 0.1 |  |  |  |  |
| 2-Tridecanone | 1499 | 1495a | 0.1 | 0.1 |  |  |  | 0.1 |
| Geranyl isobutanoate | 1512 | 1514a | 0.1 | 0.1 |  |  |  |  |
| (*Z*)-Nerolidol | 1538 | 1531a | 0.1 | 0.1 | 0.1 |  |  |  |
| (*E)*-Nerolidol | 1563 | 1561a | 0.1 | 0.1 | 0.1 | 0.1 |  |  |
| (2*E*)-Tridecenol | 1578 | 1568a | 0.1 | 0.1 |  |  |  |  |
| 1-Hexadecene | 1593 | 1591a |  |  | 0.1 | 0.1 |  |  |
| Humulene epoxide II | 1609 | 1608a | 0.4 | 0.8 | 0.3 | 0.2 | 0.3 | 0.3 |
| α-Cadinol | 1654 | 1652a |  | 0.1 | 0.1 | 0.1 | 0.1 |  |
| β-Davanone-2-ol | 1724 | 1718a |  | 0.2 |  |  |  |  |
| Linoleic acid | 2137 | 2132a |  | 0.7 | 0.4 | 0.1 | 0.3 | 0.4 |
| Oleic acid | 2145 | 2141a |  | 0.3 | 0.2 | 0.1 | 0.1 | 0.2 |
| Total (%) | 93.3 | 95.7 | 98.6 | 99.0 | 99.4 | 98.8 |

RIC = Calculated Retention Index (Rxi-5ms column); RIL = Literature Retention Index, aAdams, 2007 [21] and bMondello,

2011 [22]; Bold = Major constituents.

The principal component analysis (PCA, Figure 3) was conducted to examine the chemical variability among the plant oils from the circadian cycle. It explained 88.1% of the variance between samples, with the first component (PC1) explaining 75.3% and the second component (PC2) explaining 12.8% of the data variation. PC1 showed a positive correlation with neryl formate, geranic acid, cis-(*E*)-jasmonol, (2*E*)-undecenol, nerol, and geraniol, while PC2 displayed a positive correlation with neral and geranial. In the PCA plot, the noon collection was characterized by an effective geranial content, while the 3 pm, 6 pm, 9 pm, and 6 am collections were evidenced by a high level of neral. In contrast, the 9 am collection revealed a more significant influence of nerol and geraniol, the citral precursors. Additionally, in the PCA plot, neral and geranial were found to be opposite to the other oil constituents, indicating an inverse relationship.



**Fig. 3. Principal Component Analysis (PCA) plot of the *Pectis elongata* essential oils,**

**resulting from the circadian cycle.**



**Fig. 4. The dendrogram from the HCA plot shows the similarity relationship of *P. elongata* oils,**

**based on the circadian cycle analysis.**

The HCA plot (Figure 4) confirmed data from the PCA plot (Figure 3), with a dendrogram showing the formation of two groups. Group I, with 50.98% similarity, was formed by the 6 am, 12 pm, 3 pm, 6 pm, and 9 pm collections, which showed oils with higher citral production, between 79.5% and 88.2%. Group II was formed by the 9 am collection, which was plotted separately due to the lower content of citral (70.0%) in association with the increased contents of neryl formate, geranic acid, *cis*-(*E*)-jasmonol, (2*E*)-undecenol, nerol, and geraniol, recognizing the inverse relationship previously observed in the PC1 component analysis.

In addition to *P. elongata* from Martinique, West Indies [11], and Santarém, Brazil [2], which were already mentioned, essential oils from other citral-rich *Pectis* species have been previously reported. For instance, two *P. apodocephala* Baker oil samples from Ceará, Brazil, showed significant percentages of citral, followed by a-pinene and limonene [30,31]. Additionally, the oil of *P. odorata* Griseb. from Córdoba, Argentina, exhibited citral and limonene as its primary constituents [32].

**3.2. Oil nanoemulsification process**

In the process of *P. elongata* oil nanoemulsification from the reproductive phase, despite the use of a low-energy method, the main constituents, neral, and geranial, showed a slight change over two weeks, as was expected, ranging from 94.8% (first day) to 82.0% (fifteenth day) (see Table 1), whose reduction can be attributed to citral volatility or oxidation. Few studies on nanoemulsions have evaluated the content of volatile constituents of essential oils regarding their final formulation. However, this analysis is critical since the process can change the composition of nanoemulsified oils, interfering with their biological potential [33]. As seen in Table 1, the nanoemulsion produced with *P. elongata* oil (reproductive phase) presented a reduction in the proportion of its constituents. However, it kept citral as the primary compound and its biological potential.

In many cases, the techniques for preparing nanostructured systems contain heating, solvent evaporation, and high-pressure homogenization steps, in which the essential oil components are subject to conditions that cause degradation and loss by volatilization [24]. For example, the nanoemulsification process of Cinnamon oil by phase inversion temperature (PIT) method reduced the concentration of cinnamaldehyde after its exposure to higher temperatures [5]. On the other hand, the nanoemulsification process using a low-energy method, with the solvent displacement technique, preserved the main components of Lavandin's oil and demonstrated the method's efficiency [34]. In this sense, the low-energy nanoemulsion method by phase inversion, used in the present work, in addition to the benefit of low cost and easy application, caused a reduced impact on the oil composition, preserving the neral and geranial as its main constituents.

**3.3. Nanoemulsions preparation and characterization**

The nanoemulsion containing the *P. elongata* oil at 0.8% showed a milky, translucent color, bluish reflections, and no creaming. The mean diameter and polydispersion index (PDI), measured on the first, seventh, and fifteenth day, are displayed in Table 3.

**Table 3*.* Particle size of the nanoemulsion prepared with P. elongata oil.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Storage time (days)** | **Mean Diameter (nm)** | **PDI** | **Zeta Potential** |
| 1 | 140.73 ± 6.79 | 0.37 ± 0.03 | -7.25 ± 2.37 |
| 7 | 141.90 ± 10.0 | 0.39 ± 0.02 | -2.95 ± 4.34 |
| 15 | 177.07 ± 9.24 | 0.47 ± 0.09 | -2.54 ± 2.60 |

Values expressed as mean ± standard deviation.

Nanoemulsions are kinetically stable colloidal systems with droplets ranging from 50 to 500 nm. The nanoemulsions are transparent from 50 to 200 nm and milky from 200 to 500 nm [35-37]. The polydispersion index describes the nanoemulsion’s particle size variation. If less than 0.2 indicates homogeneous size distribution. If greater than 0.7, it is related to a heterogeneous and unstable particle size distribution [38,39]. Zeta Potential (ZP) evaluates the colloidal stability, where the ZP values ±0–10 mV, ±10–20 mV, ±20–30 mV, and ˃ ±30 mV are classified as highly unstable, relatively stable, moderately stable, and highly stable, respectively. However, some water-in-oil emulsions are highly stable despite having low ZP values [40]. Previously, low ZP values were related to the low polarizability of the constituents of *P. elongata* essential oil and using non-ionic surfactants [41]. The results obtained in this study ensured the development of a stable nanoemulsion during the evaluated period.

Sesame oil and Tween 80 nanoemulsions, obtained by high energy method, containing citral as a bioactive compound at concentrations of 0.1%, 0.5%, and 1.0%, showed particle diameters of 131.08 nm, 66.67 nm, and 79.69 nm, and the PDI values of 0.35, 0.37, and 0.47, respectively [42]. Nanoemulsions obtained by the high energy method, containing citral standard at a concentration of 10%, exhibited diameters between 28 and 410 nm [43]. This droplet size variation was related to the difference in the hydrophilic-lipophilic balance (HLB) values from the mixture of the surfactants sorbitan trioleate (Span-85) and polyoxyethylene alkyl ethers (Brij-97). A nanoemulsion containing essential oil of *Cymbopogon citratus* L. (1%) and Tween 20 (2%), produced by the low energy method, resulted in droplets of 275 nm diameter and PDI of 0.36 [44]. Nanometric drops significantly affect the encapsulation efficiency of bioactive compounds because the reduced particle size results in more excellent retention of encapsulated components [43]. In addition, they ensure better stability and directly affect the physicochemical properties, enabling better applicability in food and beverages, as they cause a minor change in their optical properties [8,44].

Citral (neral + geranial), as it is widely used as an additive in the food, beverage, cosmetics, and pharmaceutical industries, has been the subject of several studies to prevent or delay its degradation, including the development of nanoemulsions that, as seen in the present study, can reduce its degradation, protecting it from reactive molecules present in the aqueous phase.

**3.4. Antimicrobial activity**

The essential oil of *P. elongata* and a citral standard exhibited antimicrobial activity against some pathogenic microorganisms that most commonly affect food. The results are shown in Table 4 and support the hypothesis that citral is the main active component in the oil.

**Table 4. Antimicrobial activity of *P. elongata* oil and citral standard by disk-diffusion method against**

**food-contaminating bacteria.**

|  |  |  |  |
| --- | --- | --- | --- |
| Bacteria | *P. elongata* oil (10 µL) | Citral (10 µL) | Ampicillin (10 µg/disco) |
| *Escherichia coli* | 22.68 ± 0.46a | 29.60 ± 1.73 | 20.56 ± 0.54 |
| *Staphylococcus aureus* | 35.9 ± 0.34a | >40.0 ± 0.0 | 37.00 ± 1.88 |
| *Bacillus subtilis* | >40.0 ± 0.0b | >40.0 ± 0.0 | 21.00 ± 0.25 |
| *Enterococcus faecalis* | 31.42 ± 0.15a,b | 37.49 ± 2.16 | 16.50 ± 0.26 |
| *Salmonella typhimurium*  | 11.36 ± 0.89b | 12.25 ± 0.39 | 19.10 ± 0.20 |
| *Shigella flexneri* | 28.48 ± 1.74a,b | >40.0 ± 0.0 | 25.10 ± 0.21 |
| *Pseudomonas aeruginosa* | 11.99 ± 0.27a,b | 9.37 ± 0.10 | 0 |

**a**Statistical significance related to citral; **b**Statistical significance related to ampicillin; (p < 0,05); Values expressed as

mean **±** standard deviation.

Formerly, the essential oil of *P. elongata* (citral, 40.20-67.62%) exhibited antimicrobial activity against the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis*, and *Enterococcus faecalis* [11]. Isolated studies with citral showed antimicrobial activity against *Bacillus brevis*, *B. circulans*, *Klebsiella* spp, *Salmonella typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Staphylococcus aureus*, *Cronobacter sakazakii*, and *Escherichia coli* [45-48].

While the antimicrobial activity of citral is a known subject, the precise mechanisms of growth inhibition, cell damage, and inactivation are not fully understood [46].A compelling study with Cronobacter sakazakii suggests that citral could bind to the microbial cell, causing the surface to collapse without cell disintegration, leading to a reduction of intracellular adenosine triphosphate (ATP) by an increase in the rate of hydrolysis within the cells, and an increase in membrane permeability which can cause leakage of intracellular ATP or membrane hyperpolarization due to pH reduction [47]. A striking example is the inactivation of Escherichia coli by citral, which is linked to damage to cytoplasmic and outer membranes [48].Despite essential oils and isolated constituents being recognized as antimicrobial agents for decades, their use in foods is significantly limited due to their hydrophobicity and interaction with the food matrix, which causes a drastic reduction in biological activity. This limitation underscores the need for alternative solutions, such as the nanoemulsification of the essential oil, to enhance their effectiveness [49].

This is the first work about the nanoemulsification of *P. elongata* essential oil with an antimicrobial focus on foods. The minimal inhibitory concentration (MIC) results support the hypothesis that this process intensifies the antimicrobial action of the essential oil. The essential oil samples inhibited the growth of all bacteria, tested after 24 hours of incubation. The nanoemulsion potentiated the antimicrobial activity of *P. elongata* oil up to two times against *S. typhimurium* and *P. aeruginosa*, and four times against *E. faecalis*, and *S. flexineri*, and remained active against *S. aureus*, *B. subtilis*, and *E. coli* (see Table 5).

**Table 5. Minimum inhibitory concentration (MIC, mg/mL) from *P. elongata* oil, its nanoemulsion, and the citral standard against food-contaminating bacteria.**

|  |  |  |  |
| --- | --- | --- | --- |
| Bacteria | *P. elongata* oil | *P. elongata* oil emulsion  | Citral standard |
| *Escherichia coli* | 0.54 | 0.54 | 0.54 |
| *Staphylococcus aureus* | 1.09 | 1.04 | 1.09 |
| *Bacillus subtilis*  | 4.38 | 4.16 | 4.38 |
| *Enterococcus faecalis*  | 4.38 | 1.04 | 1.09 |
| *Salmonella typhimurium* | 1.09 | 0.52 | 2.19 |
| *Shigella flexneri*  | 1.09 | 0.26 | 0.54 |
| *Pseudomonas aeruginosa* | 2.19 | 1.04 | 4.38 |

Encapsulating essential oils in nanoemulsions enhances their dispersibility in food matrices and improves their physicochemical stability, significantly impacting their interaction with microbial cells. The exposure of hydrophilic groups within the nanoemulsion facilitates the transport of essential oils through the porin proteins in the outer membrane. As a result, there is no significant difference in the antimicrobial activity of nanoemulsions against Gram-positive and Gram-negative bacteria [50]. A recent study indicates that the nanoemulsified citral enhances its antimicrobial activity compared to the isolated compound and that the mechanism of action for the nanoemulsion involves the disintegration of bacterial membranes in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, leading to the loss of cytoplasmic contents and allowing the antimicrobial agent to take effect [51]. These findings support the current work.

A minimum inhibitory concentration (MIC) test was performed on the first, seventh, and fifteenth-day post-emulsification to assess the biological stability of the *P. elongata* oil nanoemulsion against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria. The MIC values were about 1.09 mg/mL against *S. aureus* and 0.54 mg/mL against *E. coli* in the three periods analyzed (Figure 5). As seen in Table 1, although there was a reduction in citral content on the fifteenth day of the nanoemulsification process, its biological activity was not compromised. Thus, the nanoemulsion of *Pectis elongata* oil, formulated under these conditions, exhibited stability and maintained its antimicrobial activity throughout the evaluation period. Long-term stability studies on emulsions and evaluation of their effectiveness on a larger scale will be the next step toward their validation.



**Fig. 5. MIC values of P. elongata oil nanoemulsion against S. aureus and E. coli**

**bacteria on the first, seventh, and fifteenth-days of nanoemulsion postprocessing.**

4. Conclusion

The essential oil of *Pectis elongata* showed a yield of 1.2% and presented citral (mixture of neral and geranial stereoisomers) as the primary constituent, ranging from 70.0% to 92.5% in the studies of the vegetative and reproductive phases and during the circadian cycle of plant development. For greater yield and citral content in the essential oil of *P. elongata*, the material of plants can be collected both in the reproductive and the vegetative phase, but preferably in the afternoon of the less rainy season. When nanoemulsified by the low-energy method, the oil maintained citral as the main constituent, enhancing the antimicrobial activity against the tested bacteria contaminating food. It cannot be ruled out that minor oil constituents also have contributed to the observed nanoemulsion antimicrobial action, which showed equivalent antimicrobial activity for the essential oil and isolated citral against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subutilis* and a higher antimicrobial activity against the bacteria *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Shigella flexneri*. These results confirm that *Pectis elongata* is an aromatic species with the potential use of its essential oil in the food industry, particularly when associated with nanotechnology.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The author(s) declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of the manuscript.

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