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FULL PAPER

Evaluation of the effectiveness of essential oil tea tree (*Melaleuca alternifolia*) against three bacterial genera isolated from bovine mastitis

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Bovine mastitis, a disease with a global negative impact on the dairy industry, is responsible for the spread of multidrug-resistant bacteria. This study evaluated the antimicrobial effectiveness of tea tree essential oil (*Melaleuca alternifolia*) against *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus*, pathogens previously isolated from bovine mastitis. The essential oil was obtained through steam distillation, revealing a yield of 2.12% of oil in relation to the volume of plant material. The evaluated concentrations (75%, 50%, 30%, 20%, and 10%) showed highly significant statistical differences ($p < 0.05$) in the inhibition zones. Volatile compounds were identified through gas chromatography and mass spectrometry, with L-terpinen-4-ol standing out as predominant (34.02% area). It was concluded that a minimum concentration of 50% of the essential oil is needed to completely inhibit the bacteria causing bovine mastitis, emphasizing the relevance of L-terpinen-4-ol in the antimicrobial activity. These findings highlight the potential of tea tree essential oil as an antimicrobial agent in the fight against bovine mastitis, with significant implications for health and productivity in the global dairy industry.

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KEYWORDS

Mastitis; distillation; antibiogram; volatile compounds.

Introduction

Bovine mastitis, with a prevalence ranging from 20% to 65% in herds, represents one of the most significant challenges in global milk production [1].

This disease, caused by pathogens such as *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus*, not only leads to culling and a loss of milk quality but also imposes considerable economic burdens [1].

The mentioned pathogens have specific characteristics, such as the high pathogenicity of *Escherichia coli* due to the production of endotoxins, the clinical severity of *Klebsiella* spp., and the antimicrobial versatility of *Staphylococcus aureus*, further complicating the sanitary landscape [2,3]. Antibiotic resistance has emerged as a critical problem, affecting the effectiveness of conventional treatments and posing threats to public health and the agricultural industry [4].

The search for sustainable alternatives has led to explore the potential of phytopharmaceutical products, including essential oils, as viable strategies to address infections without contributing to the issue of bacterial resistance [5].

In this context, Tea Tree essential oil, derived from *Melaleuca alternifolia*, emerges as a promising alternative. Originating from Queensland, Australia, this tree belonging to the Myrtaceae family is distinguished by its essential oil with anti-inflammatory, antifungal, and antimicrobial properties. Its versatility has positioned it in various industries, from pharmaceuticals to cosmetology [6].

Tea Tree essential oil contains key components such as 1,8-cineol, terpinen-4-ol, and methyl eugenol, which have been the subject of numerous studies for their biological activity [7,8]. The chemical variability of these compounds presents phytopharmacological benefits that can be harnessed to address health issues in livestock. Previous research supports its potential as a therapeutic and preventive agent, emphasizing the richness of its composition and its applications in traditional medicine and cosmetics [9]. The mechanisms of action of Tea Tree essential oil are diverse and complex. Studies have demonstrated its ability to interfere with the MDR P-gp drug transport in resistant cells, destroy periodontopathic bacterial strains, and suppress the production of inflammatory factors. These mechanisms provide a solid foundation for evaluating its effectiveness as an antimicrobial agent [10,11].

The importance of this study lies in the impact on dairy cattle production due to diseases such as bovine mastitis, where bacterial resistance to antimicrobials poses a growing threat. The lack of natural alternatives has led to an increase in the use of chemical substances, generating negative impacts on both production and public health. The assessment of Tea Tree essential oil as a natural antimicrobial presents an opportunity to address these challenges [12-14].

In addition, considering that bovine mastitis affects up to 65% of herds and antimicrobial resistance is on the rise, the assessment of the antimicrobial effectiveness of Tea Tree essential oil against the agents causing this disease becomes a valuable contribution. The World Health Organization (WHO) recognizes the importance of addressing antimicrobial resistance, and in this context, the research of natural solutions such as Tea Tree essential oil gains special significance [15]. With all the background described, the present study aimed to evaluate the effectiveness of Tea Tree essential oil against bacterial strains isolated from bovine mastitis. Its relevance lies in the potential to provide a natural and effective alternative in controlling infectious diseases in dairy cattle, mitigating risks associated with bacterial resistance and the overuse of antimicrobials. The chemical richness and mechanisms of action of Tea Tree offer an exciting perspective for improving health and productivity in the dairy industry.

Experimental

Materials and methods

In the present investigation, we worked with 30 bacterial isolates, 10 of *Escherichia coli.* 10 Staphylococcus aureus and 10 *Klebsiella* spp. Its antimicrobial effect was tested with tea tree essential oil (*Melaleuca alternifolia*). In this context, the following factors are taken into consideration:

Factor A: A0 (Cefoxitin antibiotic disk "Control"), A1 (10% Tea Tree essential oil); A2 (20% Tea Tree essential oil), A3 (30% Tea Tree essential oil), A4 (50% Tea Tree essential oil), and A5 (75% Tea Tree essential oil).

Factor B: B (Three bacterial genera "*Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella*"). The combination of factors is indicated in Table 1.

TABLE 1 Treatments

Tratamiento	Code	Description
T0	A0B	Cefoxitin Antibiotic Disc + Strain of *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella*
T1	A1B	10% essential oil + Strains of *Escherichia coli, Staphylococcus aureus*, and *Klebsiella*
T2	A2B	20% essential oil + Strains of *Escherichia coli, Staphylococcus aureus*, and *Klebsiella*
T3	A3B	30% essential oil + Strains of *Escherichia coli, Staphylococcus aureus*, and *Klebsiella*
T4	A4B	50% essential oil + Strains of *Escherichia coli, Staphylococcus aureus*, and *Klebsiella*
T5	A5B	75% essential oil + Strains of *Escherichia coli, Staphylococcus aureus*, and *Klebsiella*

A completely randomized block design (DBCA) was applied, with the following mathematical model; Yij = μ+ YBLOCKS +ti+ tj+ Ɛij; also the 5% Tukey Test, as a comparison between the means of the treatments.

Steam distillation of tea essential oil

The essential oil of *Melaleuca alternifolia* was obtained through steam distillation. Leaves from the tea tree were collected and carefully selected, then washed and dried. Due to the small size of the leaves, filling the distillation flask (DR Instruments, MS-E104, USA) was facilitated. Five distillations were conducted using an average of 154 grams of plant material. The equipment was maintained at 100 °C for 3 hours in each distillation. After a 24-hour rest period, the essential oil was decanted and packaged.

Calculation of the yield of plant extract

The estimation of the yield of the essential oil extracted from the tea tree leaves was based on its weighing at the beginning of the extraction, in the same way the total volume of essential oil obtained during the 3 hours of distillation was weighed, these values allowed us to obtain the yield, for this the net value of the essential oil was divided by the net value of the initial weighing of the plant material, multiplying by the factor (100) to obtain said result as a percentage and ended with the calculation of the percentage established in the formal Melo *et al.* [16]:

$$P = (M1/M2) * 100$$

Where,

M1 = Final mass of the essential oil; *M2* = Initial mass of the foliage.

Reactivation of the microorganisms under study

The cryoprecervated microorganisms used in the present study were provided from the microbial conservation bank of the Faculty of Agricultural Sciences of the State University of Bolivar. These microorganisms were initially isolated from cases of bovine mastitis in Ecuador.

Buffered peptone water was prepared as a nutrient medium to revive cryopreserved strains. The medium was inoculated with 100 µl of the bacterial suspension from the cryovial and incubated at 37 °C for 24 hours. The previously isolated and identified strains were subjected to thermal shock by exposing the cryovial to room temperature for 1 hour. Subsequently, they were incubated at 37 °C for 1 hour to reactivate bacterial metabolism. The reactivated strains were plated on solid agar and incubated for 24 hours at 37 °C to identify their morphological characteristics.

Establishing sensitivity and resistance

Mueller-Hinton Agar was prepared in petri plates to measure the susceptibility of the pathogens. Twenty milliliters were placed in each plate, solidifying within 10 minutes. Colonies suspended in sterile distilled water, adjusted to 0.5% on the McFarland turbidity scale, were evenly spread on the agar with a sterile swab. Subsequently, the antibacterial agent Cefoxitin Disk (Control) was applied, and

it was incubated for 24 hours at 37 °C to assess susceptibility.

Dilution of tea tree essential oil (Melaleuca alternifolia)

Concentrations of Tea Tree essential oil (*Melaleuca alternifolia*) at 10%, 20%, 30%, 50%, and 75% were evaluated, diluted in 99% Dimethyl sulfoxide (DMSO), (The percentage levels were in relation volume/volume). A micropipette was used to prepare the dilutions, ensuring precision in the proportions. Aliquots were mixed with DMSO to achieve the desired concentrations. These antimicrobial agents were impregnated onto cellulose filter paper discs and subjected to susceptibility tests against *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus.*

The antibiogram involved placing the cellulose discs impregnated with the essential oil on the culture medium inoculated with the bacterial strain. This process was repeated for each bacterial genus, maintaining a separation of 20 to 30 mm between discs. This method allowed the evaluation of the inhibitory capacity of the essential oil at each concentration against the studied pathogens.

Measurement of the inhibition halos

The antimicrobial effect of the concentrations was measured through inhibition zones using a Vernier caliper. Dimensions were recorded from the edges of the halo, subtracting 6 mm (the diameter of the discs) to obtain the net value of the inhibition zone (Table 2). The sensitivity scale of Duraffourd *et al.* (1897) was used as a reference to interpret the values, indicating the sensitivity or resistance of bacteria to Tea Tree essential oil.

TABLE 2 Sensitivity scale of a phytopharmaceutical according Duraffourd *et al.* (1897)

Inhibition	Inhibition zone diameter
Null Activity (-)	≤ 8 mm
Sensitive (+)	> 8 mm ≤ 14 mm
Very sensitive (++)	> 14 mm ≤ 20 mm
Extremely sensitive (+++)	> 20 mm

Gas Chromatography-Mass Spectrometry (GC-MS) study of the volatile compounds in Tea Tree essential oil The identification of volatile compounds was carried out through Gas Chromatography-Mass Spectrometry (GC-MS), using a chromatograph (Agilent Technologies 7890ª, CN12181050, USA) and a mass spectrometer (Agilent Technologies 5977A MSD, US1439M1416, USA) with an HP-5MS capillary column (Agilent Technologies, USE556037H, USA). One microliter of derivatized sample was injected in Split mode (ratio 50:1) at an injection chamber temperature of 220 °C. The thermal program involved a gradual increase in the oven temperature from 60 °C to 240 °C at a rate of 3 °C/min, with a total run time of 60 minutes. Compound identification was performed by comparing mass spectra with the NIST14 library.

Statistical analysis

The data obtained as a result of the analyses were processed using the assembly of the Completely Randomized Block Design (CRBD), with the assistance of the statistical packages SAS 9.4 and SPSS version 25.

Results and discussion

Extraction of the essential oil

TABLE 3 Results obtained from weighing the plant material to be distilled and the essential oil obtained through steam distillation

Mat.	W. g	X̄	Sol. H2O	T. °C	Ext. v	X̄
	180		500	100	3.5 mL	
	150		500	100	3.3 mL	
Leaves	120	**154**	400	100	3.0 mL	**3.28**
	150		500	100	3.1 mL	
	170		500	100	3.5 mL	

Note. Mat.: material, W.w.: weight in grams, X̄: mean, Sol.: solvent, and Ext. v: extracted volume in mL

According to the results obtained (Table 3) through the processing of tea tree plant material (leaves) using the steam distillation technique, an average of 154 grams of plant material was obtained, which was processed for a duration of 3 hours, yielding an average quantity of essential oil of 3.28 mL.

Yield of tea tree essential oil

From the processing of samples of plant material, specifically the leaves of the tea tree, essential oil was extracted, and a yield of 2.12% was obtained over an extraction period of 3 hours. This means that 3.28 mL of essential oil is obtained for every 154 g of sample. These results are comparable to those obtained by Carson *et al.* [16] in their study on the medicinal properties of tea tree essential oil, where they achieved a yield of 2%. In addition, they note that such yield can vary depending on the hydration conditions of the plant.

Analysis of antimicrobial susceptibility of the essential oil against Escherichia coli isolated from bovine mastitis

According to the Tukey test at 5%, the means of the treatments differ statistically. However, there is an equality between T0 (control) and T5 (75% essential oil) with an average of 18.40 mm. Next is T4 (50% essential oil) with 11.50 mm, followed by T3 (30% essential oil) with an average of 8.90 mm. In contrast, T2 (20% essential oil) and T1 (10% essential oil) showed the lowest averages, 5.70 mm and 2.25 mm, respectively, indicating no activity against *Escherichia coli*, responsible for mastitis (Figure 1).

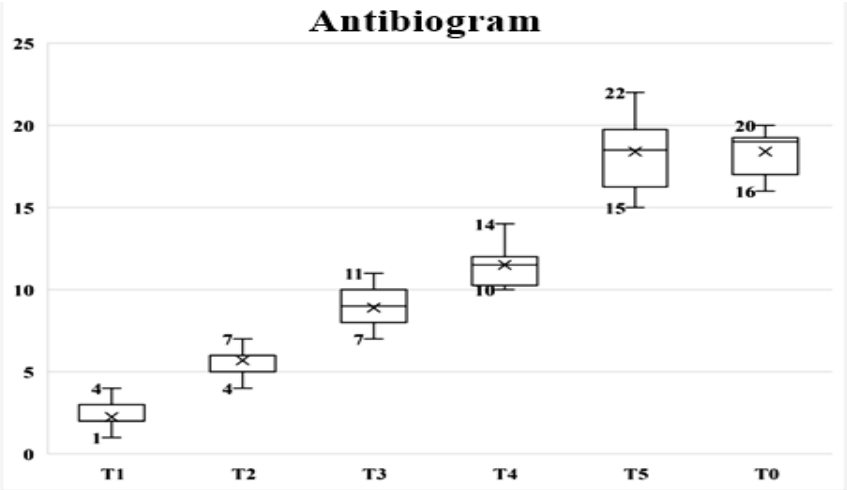

FIGURE 1 Box and whisker diagram for the diameters of the inhibition zones of the essential oil against *Escherichia coli*

Comparing with Zhang *et al.* [17], who evaluated the antimicrobial activity of *Melaleuca alternifolia* essential oil by disk diffusion, impregnating 0.1 mL of pure oil, a diameter of 12 mm was observed against *Escherichia coli*. In contrast, this study presents superior results. Carson *et al.* [16] explain that the inhibition of *E. coli* by tea tree essential oil is attributed to disruptions in potassium homeostasis, cellular respiration, and membrane synthesis, a result of volatile terpenes. These differences highlight the variability in antimicrobial activity depending on the conditions and concentrations used.

Sensitivity analysis of Escherichia coli against different concentrations of the essential oil

The sensitivity analysis of Escherichia coli against the essential oil is shown in Figure 2.

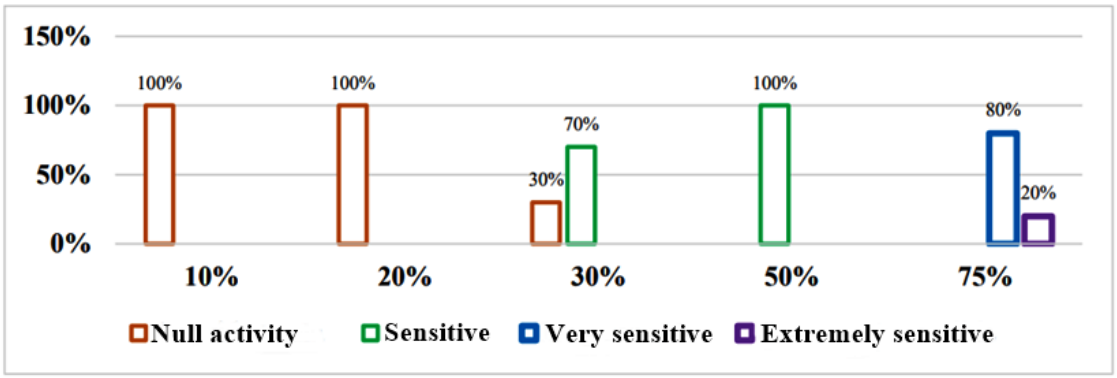

FIGURE 2 Percentage of sensitivity of *Escherichia coli* to different concentrations of tea tree essential oil

Analysis of antimicrobial susceptibility of the essential oil against Klebsiella spp., isolated from bovine mastitis

The Tukey test at 5% revealed significant differences between the treatments. T5 (75% essential oil) showed the highest average, with an inhibition zone of 23.35 mm. However, there was equality between T0 (control/FOX-30) and T4 (50% essential oil), both with an average of 17.70 mm. Next, T3 (30% essential oil) had an average of 14.20 mm, followed by T2 (20% essential oil) with 10.50 mm, and T1 (10% essential oil) recorded the lowest average, with a 7.05 mm diameter of the inhibition zone (Figure 3). These results differ from the study by Kulkarni *et al.* [19], which evaluated *Melaleuca alternifolia* essential oil against antibiotic-resistant *Klebsiella pneumoniae*, obtaining an average of 19 mm. However, the comparison is limited to variations in the strains and concentrations used.

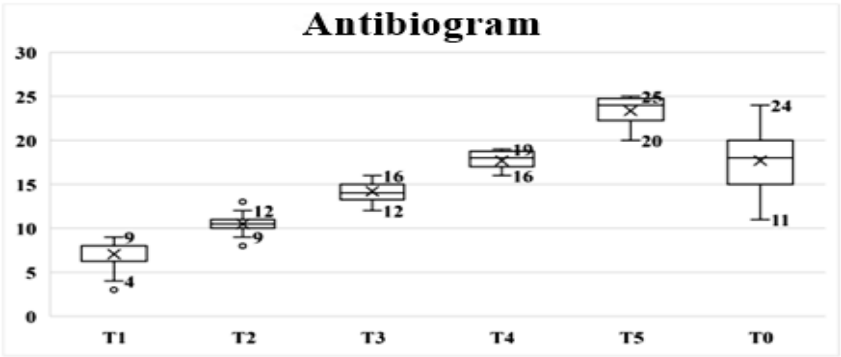

FIGURE 3 Box and whisker diagram for the diameters of the inhibition zones of the essential oil against *Klebsiella* spp.

Comparatively with the research by Thomsen *et al.* [18], which evaluated the antimicrobial effect of Tea Tree essential oil at concentrations of 5%, 10%, and 15%, the inhibition zone diameters obtained were 22 mm, 27 mm, and 28 mm, respectively. These values surpass those found in the present study, with the disparity attributed to differences in the origin of the Escherichia coli isolate and its variations in resistance to bioactive components based on the clinical source.

Kulkarni *et al.* [19] observed that 100% *Melaleuca alternifolia* essential oil exhibited antimicrobial activity with values ranging from 22 mm (maximum) to 17 mm (minimum) in the inhibition zone diameter. In comparison, this study showed superior results at 75% concentrations (T5), reaching a maximum of 25 mm and a minimum of 20 mm. In another study, Oliva *et al.* [20] demonstrated high antimicrobial potency of 100% essential oil, obtaining inhibition zones of 15 mm through the disk diffusion technique. These results are similar to those found in this study when evaluating 30% concentrations (T3). The variability may be attributed to differences in strains and methodologies used.

Sensitivity analysis of Klebsiella against different concentrations of the essential oil

The sensitivity analysis of *Klebsiella* against the essential oil is seen in Figure 4. In the research by Corona *et al.* [21] on the antimicrobial effect of tea tree essential oil, thymol, and carvacrol, 20 µL (2%) of essential oil and thymol were used against *Klebsiella*, resulting in an average inhibition zone diameter of 29 mm (+++). They attribute this finding to the antimicrobial effect of thymol, which acts on bacterial cell wall synthesis. Comparatively, the values exceed those found in our study because thymol enhances the antimicrobial effect against Gram-negative bacteria. In another study, Ramadan *et al.* [22] used 25µL (2.5%) of *Melaleuca alternifolia* essential oil against *Klebsiella pneumoniae*, obtaining an inhibition zone of 18 mm, surpassing the results of our study.

Analysis of antimicrobial susceptibility of the essential oil against Staphylococcus aureus isolated from bovine mastitis

According to the Tukey test at 5%, the averages of the treatments differ statistically. Treatment T5 (75% essential oil) shows the highest average diameter of the inhibition zone, with 25.55 mm. It is followed by T4 (50% essential oil) with an average of 19.85 mm, followed by T3 (30% essential oil) with 15.10 mm. T2 (20% essential oil) reaches an average of 12.70 mm, and T1 (10% essential oil) has the lowest average, with 9.80 mm in the diameter of the inhibition zone (Figure 5).

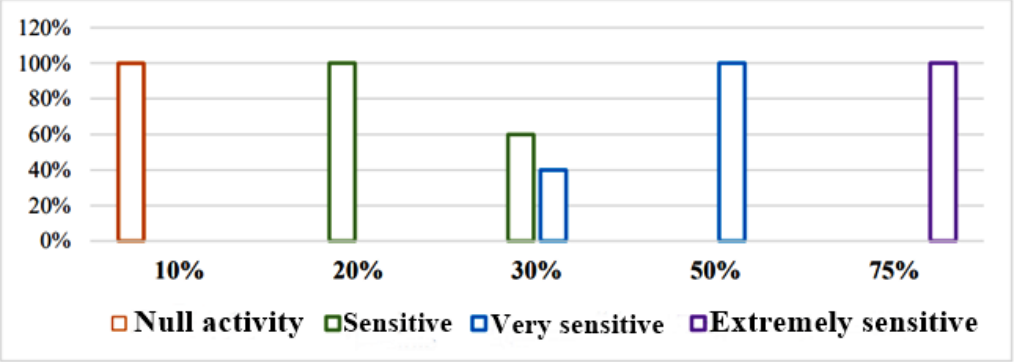

FIGURE 4 Percentage of sensitivity of *Klebsiella* to different concentrations of tea tree essential oil

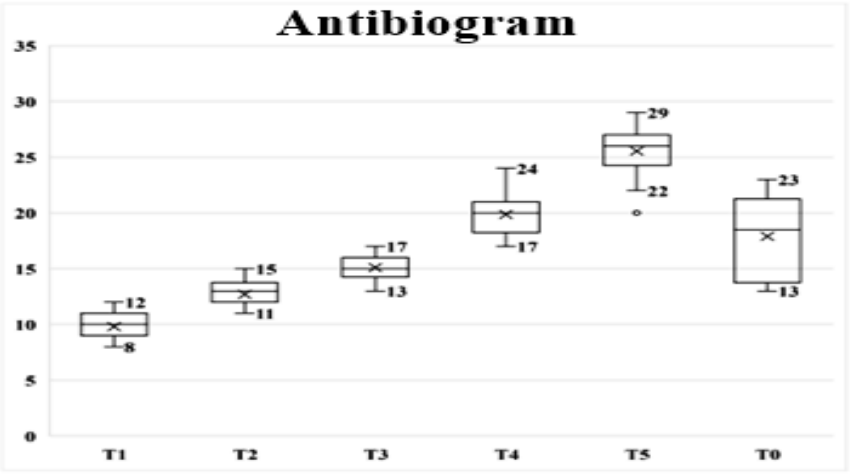

FIGURE 5 Box and whisker diagram for the diameters of the inhibition zones of the essential oil against Staphylococcus aureus

Alves Battisti *et al.* [23], in their study on the antimicrobial activity of tea tree essential oil against antibiotic-resistant pathogens, used a concentration of 100%, achieving an average inhibition zone of 16 mm against 30 isolates of *Staphylococcus aureus*. With a maximum of 22 mm and a minimum of 10 mm, the authors highlighted the antimicrobial efficacy of tea tree essential oil. In our study, at a concentration of 30% (T3), we obtained comparable results, with an average of 15.10 mm in the diameter of the inhibition zone against 10 isolates of *Staphylococcus aureus* responsible for bovine mastitis.

Sensitivity analysis of Staphylococcus aureus against different concentrations of the essential oil

The sensitivity analysis of *Staphylococcus aureus* against the essential oil is seen in Figure 6.

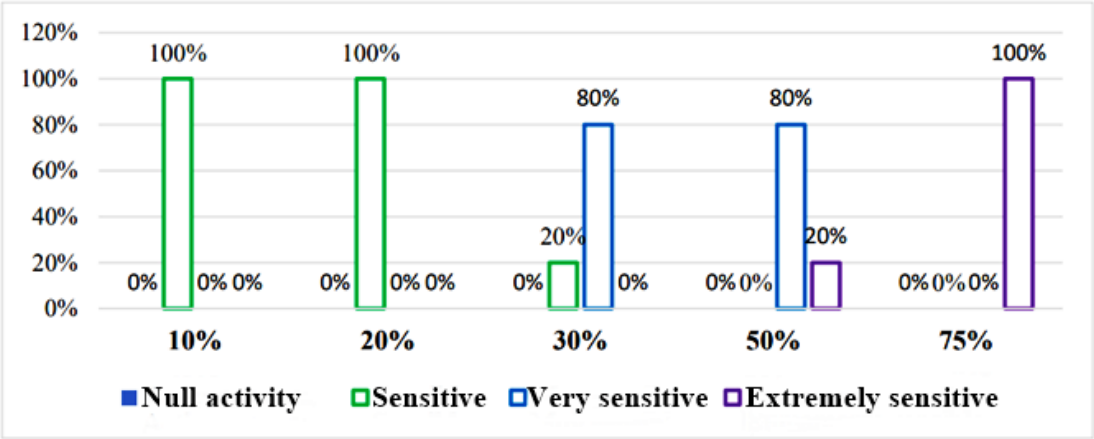

FIGURE 6 Percentage of the sensitivity scale of *Staphylococcus aureus* against different concentrations of tea tree essential oil

In the study of Puvača *et al.* [24], the in vitro antimicrobial activity of tea tree essential oil at 50% was evaluated, achieving an inhibition zone of 18 mm, results that coincide with those found in our research. In contrast, Sichieri et al. [25] used 100% *Melaleuca alternifolia* essential oil, obtaining an inhibition zone of 24 mm against *Staphylococcus aureus*. In our research, at a concentration of 75%, we achieved an average of 25.55 mm in the inhibition zone, showing significant differences. Furthermore, Esmael *et al*. [26] tested tea tree essential oil at 20%, achieving inhibition zones of 15.5 mm, surpassing the results of our research. It is crucial to highlight that, although both studies used the same concentration of essential oil, they differ in the methodology employed.

Gas chromatography-mass spectrometry (GC-MS) analysis of the volatile compounds present in the essential oil of the tea tree (*Melaluca alternifolia*) obtained by steam stripping.

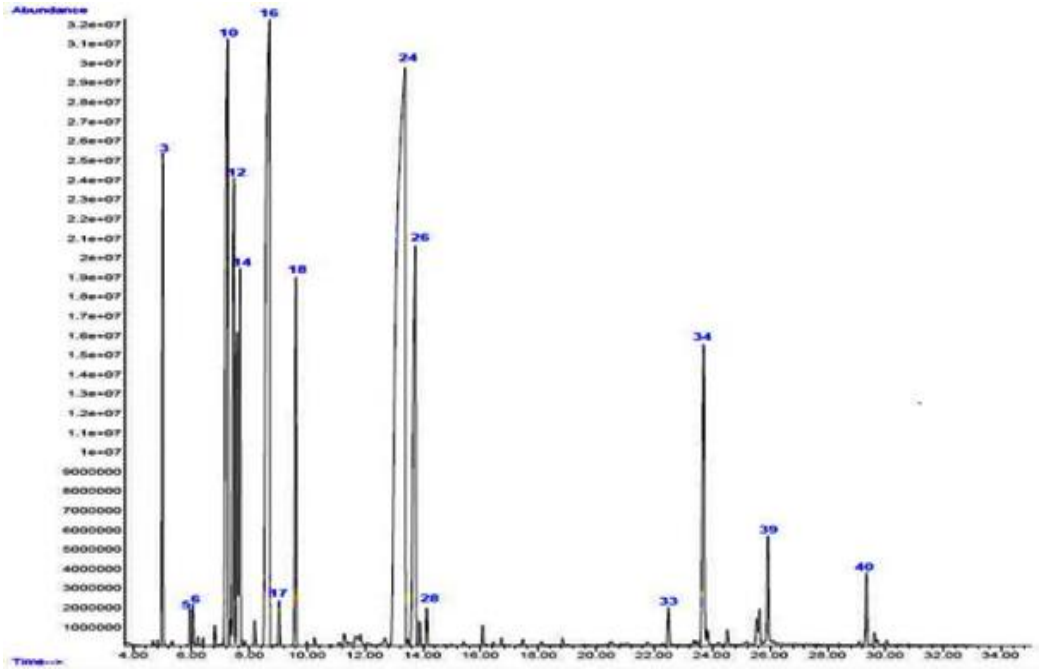

FIGURE 7 Chromatogram of the volatile compounds obtained through gas chromatography-mass spectrometry (GC-MS) of the essential oil from the tea tree *Melaleuca alternifolia* obtained by steam distillation

Using gas chromatography with mass spectrum (GC-MS), the essential oil of the tea tree was processed, where 41 volatile compounds were identified, observing in Figure 7 the peaks of the volatile compounds, highlighting the compounds of longer retention time at the time of analysis.

Library

According to the results obtained in Table 4, and Figure 8 the volatile compound with the highest percentage is L-terpinen-4-ol, with an area of 34.02% in relation to the other volatile compounds, followed by γ-Terpinene with 16.71%. Subsequently, α-Terpinene represented 9.61%, followed by α-Terpineol with 7.78%. Similarly, β–Cymene accounted for 5.18%, followed by Aromandendrene with 4.38%, compounds attributed to antimicrobial activity against bacteria causing mastitis in the study. Finally, with 26.69%, other compounds with smaller areas are represented in the chromatogram.

TABLE 4 Gas chromatography with mass spectrometry (GC-MS) analysis of the volatile compounds of the essential oil of tea tree (Melaleuca alternifolia) obtained by steam stripping

Nº	Compound	Holding time (min)	Area (%)
10	α - Terpinene	7.234	9.61
12	β - Cymene	7.457	5.18
13	D-Limonene	7.585	2.99
14	Eucalyptol	7.670	3.04
16	γ-Terpinene	8.684	16.71
18	α - Terpinolene	9.606	3.82
24	L-terpinen-4-ol	13.368	34.02
26	α-Terpineol	13.737	7.78
34	Aromandendrene	23.711	4.38
39	Viridflorene	25.937	1.27
40	(-)-Globulol	29.339	0.85

Note. The volatile compounds obtained from tea tree essential oil were identified using the NIST 14.L

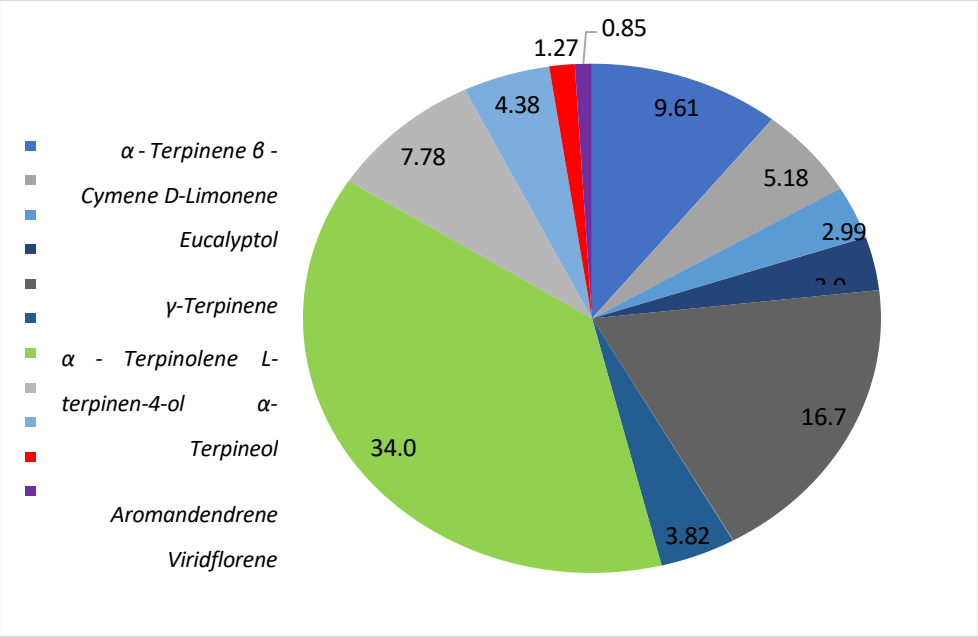

FIGURE 8 Percentage of volatile compounds in greater presence of tea tree essential oil

In the study by *Ramadan et al.* [22] on the antimicrobial activity of tea tree essential oil, GC-MS analysis identified 40 volatile compounds, with Terpinen-4-ol standing out at 44.41% area and γ-Terpinene at 21.88%. Although the results are slightly higher than those obtained in our research, other compounds like α-Terpinene were expressed at 6.83%, showing lower values. In the study by Ossa-Tabares *et al.* [27], Terpinen-4-ol was the dominant compound at 39%, slightly higher than in our research, while γ-Terpinene was expressed at 10%, lower than our results. These variations are attributed to agroecological characteristics, extraction time, and equipment used, factors that quantitatively affect the concentration of volatile compounds.

Conclusion

In the distillation of 154 grams of plant material, 3.28 mL of Tea Tree essential oil (*Melaleuca*

alternifolia) was obtained in 3 hours, with a yield of 2.12%. Statistically significant differences ($P<0.05$) were found in the antimicrobial spectra of the oil concentrations against *Escherichia coli*, *Klebsiella* spp, and *Staphylococcus aureus*, agents of bovine mastitis. It was determined that to inhibit 100% of *Escherichia coli, Klebsiella* spp, and *Staphylococcus aureus*, concentrations of 50%, 20%, and 10%, respectively, were needed, with no halos ≤ 8 mm. Chromatographically, L-terpinen-4-ol (34.02%), γ-Terpinene (16.71%), α-Terpinene (9.61%), and α-Terpineol (7.78%) stood out for their antimicrobial activity, with other compounds representing 36.25% of the chromatogram. These results support the antimicrobial potential of Tea Tree essential oil, crucial in the fight against bacteria causing mastitis.

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Authors' Contributions

The contribution of the authors was: Sahara Belén Veloz Carrasco collaborated with obtaining data and the experimental part; Riveliño Ramón Curay collaborated with the folding of the material; Dagnny Mazon-Velez performed the statistical and antimicrobial activity analyses; Mauricio Chávez Morales collected the data and interpreted it; Jenny Martínez Moreira collaborated with the methodological standardization; Favian Bayas-Morejón collaborated with the writing and interpretation of the research results.

Conflict of Interest

The authors declare that they have no conflict of interest.

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