Original Article

In Vitro Evaluation Of The Antimicrobial Activity Of Thyme (Thymus Zygis) Essential Oil On Staphylococcus Aureus Isolated From Bovine Mastitis

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Abstract

The aim of this research was to evaluate in vitro the antimicrobial activity of essential oil of thyme (Thymus zygis) using the Kirby-Bauer technique on Staphylococcus aureus isolated from bovine mastitis. For which, the California Mastitis Test (CMT) technique was used for selective culture media and antimicrobial activity. After CMT, it was determined that the prevalence rate was 48.33%, out of a total of 60 cows tested, resulting in 29 positive animals at diagnosis, of which 20 cows exhibited mastitis caused by Staphylococcus aureus, said pathogen represented 68.96% of the total number of the agents that were able to identify, in addition it was possible to stratify the presentation of said disease where it was exhibited that those animals of the Holstein breed. In the analysis of the antimicrobial activity of the essential oil of thyme, it was possible to observe that the best average was presented by T3 (75%) with a mean of 64.45 mm, followed by T2 (50%) with a mean of 54.95 mm, these versus at T4: Erythromycin 15µg that presented a mean of 19.90 mm. In the analysis of the antimicrobial susceptibility of Erythromycin against Staphylococcus aureus, according to the cut-off points established by CLSI, 95% of the isolates exhibited intermediate resistance and only 5% proved to be sensitive. The minimum inhibitory concentration (MIC) of Essential oil for Staphylococcus aureus, which causes bovine mastitis, was found in a concentration of 7% of thyme essential oil.

Keywords: bovine mastitis, Staphylococcus aureus, thyme essential oil, Erythromycin.

INTRODUCTION

Bovine mastitis is one of the main pathologies with the greatest productive and economic repercussion in dairy herds around the world. **O'Neill (2016) and World Bank Group** (**2017**), mentioned that; By the year 2050, antimicrobial resistance will be a significant challenge for global public health, since it will cause 300 million human losses, as well as financial losses of millions of dollars, affecting developing countries to a greater extent. In studies carried out in Ecuador by **Requelme & Bonifaz,** (**2011**) they showed that there is a high prevalence and incidence of Mastitis in dairy herds, this is mainly due to the inadequate application of good milking practices, lack of hygiene in the milking routine, as well as the lack of an adequate technique of identification of the causal pathogen.

Mastitis is one of the main diseases and the one that most affects dairy cows, causing important consequences in production and reproduction (**Rojas, 2018**). It should be noted that the production of quality milk is the primary mission of a dairy farm, where the integrity of the udder is necessary to maximize production in quantity and quality in addition to maintaining the profitability of the farm. Mastitis can be classified under many criteria. Thus, in general terms it is classified into: "Clinical Mastitis" and "Subclinical Mastitis" (**Quevedo, 2018**). Today the most widely used technique for the detection of this disease is the CMT (California Mastitis Test), the basis of this technique is to show changes in the characteristics of milk such as viscosity once the CMT reagent containing detergent and Bromocresol purple, which

acts by breaking the cell membrane of somatic cells and the nucleic acid forms a gel-like matrix with cellular debris, giving rise to a gelation that is proportional to the size of the total somatic cell count (SCC). with alterations (**Swinkels et al., 2020**).

Among all the infectious pathogens that cause mastitis, bacteria are the ones that cause the greatest number of intramammary infections, and the most common are: Staphyloccocus aureus, Streptoccocus agalactiae, Streptoccocus dysgalactiae, Estreptococcus uberis, Escherichia colia, Streptococcus pyogenes, Corynebacterium pyogenes, Pseudomonas aureoginosa, Mycoplasma bovis, etc. (**Jingao et al., 2020**).

In this context, the Sthaphylococcus aureus bacterium is characterized by having multiple virulence factors, some of which are closely related to intramammary infection, and often generating resistance to drugs such as β-lactams, widely used by the way in this field. type of infections (**Buldain et al., 2020**). There are several mechanisms that mediate resistance to β-lactams, the production of β-lactamase inactivates certain antimicrobials of this family through hydrolysis of the β-lactam ring. The hydrolysis product lacks antibacterial activity. A part of the enzyme that is produced is excreted into the external environment and part remains attached to the cell membrane (**Seija, 2018**). The global rise of multi-resistance to antibiotics and the increasing cases of bovine mastitis in dairy herds has exceeded the possibilities of developing new pharmacological agents, putting them at a disadvantage when it comes to controlling this pathology. However, plant species still offer an alternative capable of counteracting this problem. (**Ramírez Vásquez et al., 2018; Bayas-Chacha et al., 2022; Poaquiza-Caiza et al., 2022**).

At present, phytotherapeutics has been able to demonstrate properties of pharmacological interest of many plants, among this extensive list Thymus zygis has been considered for its multiple benefits, where generally the Kirby-Bauer disk diffusion test is the most used to determine a diameter of inhibition of the phytopharmaceutical against some pathogen (**Singh et al., 2018**).

Phytotherapy is based on the use of plant species capable of providing favorable curative benefits. Due to this, for many years an attempt has been made to deepen the knowledge of the medicinal properties of essential oils and extracts provided by these plants in order to create alternative products that counteract the resistance of many microorganisms to the drugs commonly supplied in different pathologies. **(Delgado, 2020**).

Plants and herbs comprise the main natural antimicrobials. Its effectiveness against pathogenic microorganisms is generally attributed to its phenolic compounds that are present in the various extracts or essential oils, among which are thyme, among others. (**Montero et al., 2018; Bayas-Morejón et al., 2021).** Thyme is classified as a shrubby plant with aromatic properties, it has countless properties, among which are antibacterial, antirheumatic, calming, expectorant, as well as an antihistamine that helps relieve itching caused by insect bites, as well as healing (**Agexport, 2020).**

Due to the aforementioned in the present research work, the antimicrobial activity of the essential oil of Thymus zygis was evaluated using the Kirby-Bauer technique against Staphylococcus aureus isolated from bovine mastitis, in order to be able to propose new natural therapeutic alternatives that allow to counteract the multiresistance to antibiotics and therefore decrease the rates of bovine mastitis.

MATERIALS AND METHODS

This research was carried out on the "Edith" farm, located in the cantón Penipe, province of Chimborazo (Ecuador). Twenty strains of Staphylococcus aureus from the Bolivar State University microorganism bank were used, on the other hand, essential oil of thyme was used (Thymus zygis).

Study Factors

We worked with different concentrations of vegetable oil (Table 1).

Treatment	Code	Description
1	a1b1	Essential oil at 25% + strain of Staphylococcus aureus.
2	a1b2	Essential oil at 50% + strain of Staphylococcus aureus.
3	a1b3	Essential oil at 75% + strain of Staphylococcus aureus.
4	a1b4	Erythromycin disc (control) + strain of Staphylococcus aureus.

Mastitis prevalence

The prevalence of mastitis on the "Edith" farm was determined with the following formula;

$$\text{Prevalence} = \frac{\text{Positive cases}}{\text{Total poblation}} * 100$$

The prevalence in the affected mammary quarter was also considered, as well as the isolates that were determined from the samples obtained.

Obtaining Thyme Essential Oil

The essential oil of thyme (Thymus zygis), was obtained from the company (Aromalab®) that is dedicated to the production and sale of essential oil obtained by steam drag, where through the technical sheet it provides us with the necessary data that demonstrates purity and quality.

Isolation and Identification of Staphylococcus Aureus

For isolation and identification, firstly the cows were sampled on the "Edith" farm. Then we proceeded to the identification by means of the clinical examination of the udder to obtain characteristic signs of inflammation, in addition, the application of the diagnostic methods of the California Mastitis Test (CMT) was used, in cows that were identified with alteration in the milk, For its analysis, sanitization was carried out according to the protocols established in Ecuador, to guarantee the safety of the sample, later, 5 to 10 mL of milk was collected from the positive samples for mastitis after CMT. Finally, the sample was stored at a temperature of 4°C - 10°C for later analysis in the laboratory.

Enrichment and Culture of the Sample.

The milk sample was enriched with sterile peptone water in a 1:10 ratio (milk: peptone water), then it was allowed to incubate at 37°C for 24 hours. After this period, Sal Mannitol agar was used as a selective medium, where 100 µL of enriched culture was poured homogeneously onto the agar plate and left to incubate at 37ºC for 24 hours.

Identification of Staphylococcus Aureus Isolates from Bovine Mastitis

For the identification of the bacterial strains, morphological characteristics, Gram staining, and biochemical tests were used, among the biochemical tests used are: Catalase test (The enzyme catalase breaks down hydrogen peroxide into water and oxygen); Coagulase test (Staphylococcus aureus has two types of coagulase, one that is Endo-coagulase or bound coagulase "Clumping factor", it also has an exocoagulase or free coagulase that acts by activating a CRF complex that will produce a fibrin clot); The fermentation of mannitol.

Antimicrobial Activity of Thyme Essential Oil

The antimicrobial activity was carried out according to the following steps.

Suspension of the microorganism of interest: For this analysis, a suspension of microorganism on the 0.5 McFarland scale was used, corresponding to 1.5 CFU/mL.

Antimicrobial activity by disk-plate diffusion (Kirby Bauer): At this stage, starting from the microbial suspension (0.5 McFarland) and with the help of a sterile swab, we proceeded to sow on Muller Hinton agar plates, then left to rest during 15 minutes.

Dilution of the essential oil of thyme: The dilution of the essential oil of Thyme was made at 25%, 50% and 75%, these dilutions were made with dimethyl sulfoxide as a diluent.

For the analysis of the MIC (Minimum Inhibitory Concentration) of the essential oil, the concentrations of 5, 7 and 10% were considered, in the same way dimethyl sulfoxide was used as the dilution unit.

Application of the inhibition discs, with the help of a sterile forceps, the discs were placed on the surface of the agar, all the cellulose filter paper discs were previously submerged in each of the essential oil dilutions, in a ratio of 20 discs for each concentration of essential oil.

In parallel, 15µg Erythromycin discs were tested as the control to comply with the proposed experimental design. Once the antibiotic discs and the essential oil were placed, they were incubated for 24 hours at 37°C.

Measurement of the inhibition halos: Finally, after incubation, the diameter of the inhibition halo was measured, with the help of a vernier caliper, the interpretation was made of the general scale of sensitivity to phytopharmaceuticals (table 2).

Table 2. General scale of sensitivity of a phytopharmaceutical

Inhibition	Inhibition halo diameter (mm)
Null (-)	(-) ≤ 8 mm
Sensitive (+)	(+) > 8 mm ≤ 14 mm
Very Sensitive (++)	(++) >14mm ≤ 20 mm
highly sensitive (+++)	> 20 mm

Source: Duraffourd et al, (1897).

The antimicrobial susceptibility of Erythromycin is established by the cut-off points established by the Clinical & Laboratory Standards Institute (CLSI, 2020) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022), where for 15µg Erythromycin it establishes, according to the CLSI; a diameter in mm of ≥ 23 (Sensitive), 14 – 22 (Intermediate Resistance), ≤ 13 (Resistant); and for the EUCAST; a diameter in mm of ≥ 21 (Sensitive) and ≤ 20 (Resistant).

RESULTS AND DISCUSSION

Occurrence of Mastitis by Breed.

Of a total number of 60 animals sampled, 29 were positive for mastitis by CMT, the frequency was calculated, where the Holstein breed expressed the highest number of positive cases with 65.6% (n=19), followed by the Jersey breed. with 24.12% (n=7) and finally, the Brown Swiss breed expressed the lowest casuistry with 10.78% (n=3). According to **Vidales et al, (2017)** in their research, they obtained that the Holstein breed presented the highest prevalence of clinical mastitis with 26%, versus the other breeds and their crosses, which obtained a low prevalence.

Isolation of the Bacterial Strain.

A prevalence rate of 48.33% of bovine mastitis was evidenced, that is, 29 animals were diagnosed as positive for mastitis in different degrees of chronicity by CMT.

In the work of **Suarez, (2007),** developed in the province of Chimborazo (Ecuador), the author obtained a prevalence of 39.21%, in the same way, **Cuzco, (2015)** obtained a 40% prevalence in the same province. In the same way, **Agrocalidad, (2015)** collects mastitis data obtaining a prevalence of 42.60%, data that agrees with those obtained in our work.

Identification Of Staphylococcus Aureus Isolates from Bovine Mastitis.

After phenotypic and biochemical identification tests, within 29 samples collected, 68.96% (n=20) presented specific characteristics for Staphylococcus aureus and 31.04% (n=9) presented other non-diagnostic microbiological characteristics. According to the work of **Guzñay, (2016)** where he analyzed samples from 16 cows with bovine mastitis, of which through microbiological and microscopic tests he registered 48 causative agents where 60.42% of the isolates were confirmed as Staphylococcus., also **Maldonado et al, (2022)** reported that of 14 cows with bovine mastitis, 51.4 % of the isolates correspond to Staphylococcus aureus, likewise, **Bhakat et al, (2020)** point out that the spread of bovine mastitis produced by contagious agents is due to milking line protocols. poorly established.

Analysis of the antimicrobial effect of thyme essential oil at 75%, 50% and 25% against Staphylococcus aureus isolates.

After the comparison of the Tukey means of the behavior of the treatments proposed against the 20 isolates of Staphylococcus aureus causing bovine mastitis, it was evidenced that the highest mean value of diameter in size of the inhibition halo was presented in treatment 3 (75% oil) with 64.45 mm (Figure 1), followed by T2 (50% oil) with 54.95 mm, T1 (25% oil) with 37.05 mm, and finally the witness with 19.90 mm. In a work carried out by **Montero et al, (2018),** he tested the antimicrobial efficacy of thyme essential oil on Staphylococcus aureus strains where he concluded that 30, 50, 70 and 90% could not be decisive in sensitivity since growth bacterial plate was totally inhibited, agreeing with the results obtained in the present investigation. **Ramos et al, (2019)** for their part reported that the antimicrobial activity of thyme essential oil at 25%, 50% and 100% against Staphylococcus aureus (ATCC 25923) the results were 11.6, 17.6 and 22 mm in diameter. inhibition halo respectively. In the same way, Ramos et al, (2019) and **Zeghad & Merghem (2013)** in their research worked with thyme oil at a concentration of 30% on Staphylococcus aureus, obtaining a diameter of inhibition halos of between 10 and 15 mm, being These results are lower than those obtained in our research.

Figure 1: Antimicrobial activity of thyme essential oil against Staphylococcus aureus

Analysis of the antimicrobial susceptibility of Erythromycin against Staphylococcus aureus

Table 3 shows the analysis of susceptibility to 15µg erythromycin against Staphylococcus aureus, where according to the cut-off points established by the **CLSI (2020),** it can be seen that 95% of the isolates have intermediate resistance, Likewise, the cut-off points established by the **EUCAST, (2022),** it was evidenced that 70% were resistant to said drug.

Table 3. Frequency of antimicrobial susceptibility of Erythromycin against Staphylococcus aureus

Bacteria	**S**		**I**		**R**	
	Fr	**%**	**Fr**	**%**	**Fr**	**%**
Staphylococcus aureus "CLSI"	1	5%	19	95 %	0	0 %
Staphylococcus aureus "EUCAST"	6	30%	0	0%	14	70 %

S: sensitive, I: intermediate, R: resistant.

According to **Calvinho et al. (2002),** in their investigation of the susceptibility of coagulase-positive Staphylococcus spp of a total of 101 isolated strains, the inhibitory activity of Erythromycin at 15µg presented 38.6% (n=39) sensitivity, 17.8% (n=18) of intermediate resistance and 2% (n=2) of resistances, according to the criteria established by the CLSI in the present investigation our results differ from the aforementioned.

Analysis of minimum inhibitory concentration (MIC) of the essential oil of Thyme, against Staphylococcus aureus.

The minimum inhibitory concentration (MIC) was carried out with the plate disc diffusion method at concentrations (5%, 7% and 10%) of thyme essential oil.

After Tukey's comparison of means, the highest average was exhibited by the 10 % concentration with 19.60 mm of inhibition halo diameter, followed by 7 % with a mean of 9.85 mm of halo diameter. Finally, 5% exhibited an average of 3.40 mm, thus determining that 7 % of the thyme essential oil was sufficient to inhibit the growth of the pathogen, since halos with measurements greater than 8 mm in diameter were obtained in the 20 isolates. from Staphylococcus aureus. However, in the work carried out by **Montero et al, (2018)**, a study of thyme essential oil and after CMI, showed that at 1% concentration it was sufficient to inhibit the strain of Staphylococcus aureus ATCC ® BAA-976 ™*, In this sense, our research shows that the isolated wild strain of Staphylococcus aureus requires a higher concentration of oil to inhibit it.

Figure 2. MIC inhibition halos of thyme essential oil against Staphylococcus aureus strains

Definitively, in our work we have determined that thyme oil inhibits the development of Staphylococcus aureus, and according to **Zhengkal et al, (2014)** in their work they found that thyme oil produces damage to the cytoplasmic membrane

of St. aureus that they can be caused by the synergy of the mechanisms of action of the compounds that potentiate the antimicrobial activity.

It is important to note that **Luengo (2006)** mentions that in thyme extracts, within their chemical composition, flavonoids, thymol and carvacol, among others, stand out. Which are volatile compounds that give it innumerable pharmacological properties, as well as antimicrobial properties.

CONCLUSIONS

Through the analysis, the Holstein breed expressed more than 60% prevalence of Staphylococcus aureus.

In the antimicrobial activity of the essential oil of thyme, 75% concentration was effective with an average of 64.45 mm of inhibition halo, and against Erythromycin 15µg that presented an average of 19.90 mm.

At the minimum inhibitory concentration, 7% thyme essential oil was sufficient to inhibit the pathogen studied.

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