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Determination of the antimicrobial effectiveness of the essential oil of *Origanum vulgare* against strains of *Escherichia coli* isolated from bovine mastitis

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ABSTRACT
Milk production is mainly affected by pathologies such as mastitis, caused mainly by bacteria, where one of the most important is *Escherichia coli*. In this sense, the objective of the present investigation was to determine the antimicrobial effectiveness of the essential oil of *Origanum vulgare* against strains of *E. coli* isolated from bovine mastitis. Therefore, the presence of the bacterium was analyzed in the farm "La felicidad" located in the province of Chimborazo (Ecuador). The initial identification of the microorganism was made using the CMT diagnostic test. Subsequently, the isolation by means of plate culture and its characterization using the IMVIC tests were carried out. On the other hand, the essential oil of oregano was obtained by steam distillation. These oils were studied for their antimicrobial affectivity against the isolated strains of *E. coli*, and their MIC was also analyzed. As results, the total prevalence of mastitis was 26.25%. Of the 28 samples analyzed, 18 isolates (64.29%) corresponded to *Escherichia coli*. After the antimicrobial activity, oregano oil with a 60% concentration was the one that showed the best performance against the pathogen. In the CMI, from the concentration of 3% of essential oil there was an inhibitory effect. Finally, gas chromatography of oregano oil showed that formic acid and p-Cymeno were the most present. In conclusion, natural organ oil can be a phytopharmacological alternative against pathogens that cause bovine mastitis.

Keywords: Antimicrobial activity, Oregano essential oil, *Escherichia coli*, Bovine mastitis
Article type: Research Article.

INTRODUCTION
In dairy production, cows are the critical control point most evaluated and subjected to surveillance due to the multiple factors rooted in them that can affect production, where udder integrity is necessary to maximize milk production and quality, and therefore maintain profitability (Castillo *et al.* 2019). Milk production is mainly affected by pathologies such as mastitis, which causes inflammation of the secretory tissue and the mammary gland, caused by multiple factors, among which stand out: management, health, microorganisms, treatments and misdiagnoses applied, which affect the quality of milk. Currently, around 200 bacterial species with the ability to affect the mammary gland have been quantified. These pathogens have the ability to inhibit the defense mechanisms of the mammary gland and cause an inflammatory condition, giving rise to the transmission of contagious mastitis and environment (Pedroza 2018). Within the microbiological classification of mastitis, environmental mastitis is detailed, caused by pathogens found in the environment and in physical vectors such as; milking equipment and supplies. Among the main pathogens are: *Enterobacter, Klebsiella, Pseudomonas, Strepctococcus disgalactiae* and *Streptococcus uberis* (Calvinho 2017). Mastitis caused by *Esherichia coli*, are more predisposed at the beginning and end of lactation, since they do not live on the skin of the udder, but enter

the teat canal when the cow comes into contact with a contaminated environment. These bacteria are found in feces, bedding, and feed (Flores *et al*. 2021). *Escherichia coli* is generally a bacterium that inhabits the intestine of humans and animals. Currently, several pathotypes have been described that show variation in virulence. Ruminants, particularly cattle, are considered one of the main reservoirs of *E. coli* (Jiménez *et al*. 2017).

Diagnostic techniques of mastitis

The diagnosis of bovine mastitis is a great challenge, due to the fact that farms are environments with limited resources, and the high degree of complexity to determine a timely prevention of intramammary infection (Ashraf & Imran 2018). The most used method for diagnosis is the California Mastitis Test (CMT). This technique is useful for the somatic cell count and is a useful predictor of infection (Quevedo 2018; Kandeel *et al*. 2018). Enterobacteriaceae can mainly be identified by IMVIC profiles comprised of: Indole, which allows to identify if the bacterium possesses the tryptophanase enzyme); Methyl red, which manifests itself in the accumulation of acetic and formic acids, among others, and allows us to know if the medium is acidified by the growth of a bacterium; Voges-Proskauer, which allows determining the production of acetoin by the bacteria; and Citrate, which allows the identification of microorganisms that convert citrate to ammonium phosphate, ammonia and ammonium hydroxide (Locatelli *et al*. 2019; Younis *et al*. 2021; Khasanah *et al*. 2021).

Susceptibility testing

Susceptibility tests in recent years have been implemented based on the need to promote a correct therapeutic plan for bovine mastitis, where the most common method is the plate-disc diffusion method or Kirby-Bauer, which is promoted by the inhibition of the development of the bacteria, given mainly by the action of the antimicrobial agent (Kasai *et al*. 2022).

Essential oils

Essential oils are volatile compounds, structurally make up organs of plants and vegetables, including leaves, root stems, flowers, and fruits. They exhibit biological activity such as antibacterial, antifungal, and antioxidant. The use of these compounds dates back to the beginnings of human modernity where the Arabs were the pioneers in obtaining said oils by distilling water. These bioactives are used in different approaches, being able to be used in the cosmetic, chemical, food industry, etc. (Pavli *et al*. 2019; Chen *et al*. 2020). The diffusing capacity of essential oils is the main criterion used for the inhibition of the vital functions of a microorganism in question (Concha 2020; Manouchehri 2023). Essential oil can act at the level of the membrane or cytoplasmic organelles. Its main activity is defined by its hydrophobicity, which gives it a greater permeability capacity towards the interior of the cell (Mutlu *et al*. 2021; Seratnahaei *et al*. 2023). In this sense, the essential oil of oregano is considered as one of the bioactives exhibiting the highest concentration of phenolic compounds; carvacrol being the one found to a greater extent. This is the main responsible for depolarization, since it promotes greater permeability of the cytoplasmic membrane of the bacterium (Rodrigues *et al*. 2020). Also, some studies reported that oregano essential oil has activity to efficiently control *in vivo* the development of endogenous microorganisms in wheat, thanks to the presence of aromatic compounds and phenolic groups such as eugenol and carvacrol (AEMPS 2018). So, the objective of this work was to determine the antimicrobial effectiveness of the essential oil of *Origanum vulgare* against strains of *E. coli* isolated from bovine mastitis.

MATERIALS AND METHODS

The sampling was carried out at the "La felicidad" farm located in the Chambo canton, in Chimborazo Province. Subsequently, these were transferred and processed in the general laboratory of the Faculty of Agricultural Sciences, Natural Resources and the Environment of the State University of Bolivar.

Treatments

Table 1. Treatments considered for the investigation.

T_0: Enrofloxacin antibiotic disc 5µg (Control)
T_1: Oregano essential oil (*Origanum vulgare*) 20%
T_2: Oregano essential oil (*O. vulgare*) 40%
T_3: Oregano essential oil (*O. vulgare*) 60%

These treatments were analyzed against *E. coli* isolates.

Oregano essential oil extraction process

The steam distillation methodology was used, once the plant material was placed in the distillation equipment flask, assembled, it was boiled at an approximate temperature of 100 °C. So, 500 g oregano leaves was placed in the distillation equipment flask with 300 mL of distilled water. After extraction, the product obtained was bottled in 10 mL amber bottles to avoid the interaction of sunlight.

Isolation of *E. coli*

Exploration. Initially, the udder was subjected to a clinical examination in search of clinical findings revealing anatomical alterations coincident with an inflammatory picture. In addition, as a methodological part, the detection of mastitis was established by means of the California Mastitis test (CMT; NCM 2017).

Isolation of the microorganism. Culture media such as MacConkey agar and EMB (Eosin methylene blue) agar were prepared. These growth media are selective and differential, for the isolation and differentiation of enterobacteria, especially *E. coli* from samples positive for bovine mastitis. Once the media were prepared, the passage was proceeded through triple streaks on Mac Conkey agar from the sample. Given the morphological characteristics of the colony, a re-culture was carried out on EMB agar, to obtain a uniform and homogeneous bacterial growth without evidence of differentiation for further identification analysis.

Identification of *E. coli*

The *E. coli* colonies were 2 to 4 mm in diameter, with a dark center on EMB agar, and a characteristic bright metallic green in color. On MacConkey agar, they were red with a cloudy zone. Microscopic observation was performed using Gram stain and biochemical tests such as IMVIC (Indole, Methyl Red, Voges Proskauer and Citrate). For its application, nutrient media such as peptone water, MR-VP broth and Simmons citrate agar were used, which were packed in tubes in the following order: Tube with 5 mL of peptone water (Indole); Tube with 5 mL of MR-VP broth (Methyl Red); Tube with 5 mL of MR-VP broth (Voges-Proskauer); 5 mL tube with Simmons citrate agar (tilt until solid).

Case prevalence

In the investigation, the prevalence of cases with mastitis was considered, in addition to the prevalence of *E. coli* as the pathogen under study, since it was the cause of mastitis with the highest prevalence in dairy herds.

Antimicrobial activity

Initially, to establish susceptibility, Müeller-Hinton agar was prepared in Petri dishes, in which the bacteria were cultured. So that, the McFarland 0.5 scale was adjusted with the help of a sterile swab, the box was inoculated by culturing.

Application of discs impregnated with essential oil

After the inoculation of the Petri dishes with the bacterial strains under study, with the use of a sterile forceps, the cellulose filter paper discs impregnated with essential oil of oregano (*Origanum vulgare*) placed at a minimum approximate distance of 15 - 20 mm between the disc, and 1.5 cm from the edge of the Petri dish. Once this process was completed, the petri dishes were incubated at 37°C for 24-48 hours. The concentrations of oregano oil used were 60%, 40%, 20%. In addition, the MIC of oregano essential oil was analyzed at 1%, 2%, 2.5%, 3%, 5% and 10%. In order to obtain the desired concentrations, the essential oil was diluted with the help of a solvent (Dimethylsulfoxide). Finally, after 24 hours of incubation, the inhibition zones were measured.

Sensitivity. This variable allowed us to measure the ability of *E. coli* to be susceptible to the action of oregano essential oil. We can undoubtedly demonstrate its occurrence by measuring the inhibition zones (in mm); where the following parameters were considered for interpretation: Sensitive (+) > 8mm ≤ 14mm; Very Sensitive (++) >14 mm ≤ 19 mm; Extremely sensitive (+++) > 20 mm; Resistant (-) ≤ 8 mm.

Determination of volatile compounds present in oregano oil

The volatile compounds of oregano leaf oil obtained were characterized by gas chromatography, coupled with mass spectrometry (GC-MSD), the most abundant compounds, mainly those exhibiting antimicrobial activity against *E. coli*, the cause of mastitis.

RESULT AND DISCUSSION

Prevalence of mastitis

Table 2. Prevalence of bovine mastitis**.**

Property	Number of animals	Positive cases	Negative cases	Prevalence
La felicidad 1	40	6	34	**15%**
La felicidad 2	40	15	25	**37.5%**
Total	80	21	59	**26.25%**

The total prevalence of mastitis in the two properties is expressed as 26.25%, where the La Felicidad 2 farm was the one with the highest value. When comparing with Guzñay (2021) in his investigation of microbiological diagnosis of bovine mastitis in the canton Chambo, he obtained a prevalence of 58.33% of bovine mastitis applying CMT as a diagnostic test. Also Proaño & Vásconez (2013) in their investigation on the determination of bovine mastitis through CMT and other diagnostic tests reported a prevalence of 21.40% (n = 6) of a total of 28 cows analyzed, in such a way that the prevalence of bovine mastitis was dependent on the area of dairy activity and one of the factors was the sanitary management applied in the productions.

Identification of isolated microorganisms

Of the 21 animals positive for bovine mastitis using the California Mastitis Test (CMT), 28 samples were collected from the different mammary quarters of the positive cows for further analysis. So that, MacConkey agar and EMB were used as exclusive selective and differential media for enterobacteria, which were identified by biochemical tests.

Table 3. Result of IMViC profiles.

IMViC Profiles						Profiles TSI			
#	Code	I	RM	VP	C	Glu	Lac/sac	Gas	H_2SO_4
1	F1001	+	+	-	-	+	+	+	-
2	F1002					No growth			
3	F1003	+	+	-	-	+	+	+	-
4	F1004	+	+	-	-	+	+	+	-
5	F1005					No growth			
6	F1006	+	+	-	-	+	+	+	-
7	F1007	+	+	-	-	+	+	+	-
8	F1008					No growth			
9	F1009	+	+	-	-	+	+	+	-
10	F1010					No growth			
11	F1011	+	+	-	-	+	+	+	-
12	F1012	+	+	-	-	+	+	+	-
13	F2001	+	+	-	-	+	+	+	-
14	F2002	+	+	-	-	+	+	+	-
15	F2003	+	+	-	-	+	+	+	-
16	F2004	+	+	-	-	+	+	+	-
17	F2005					No growth			
18	F2006	+	+	-	-	+	+	+	-
19	F2007	+	+	-	-	+	+	+	-
20	F2008					No growth			
21	F2009	+	+	-	-	+	+	+	-
22	F2010					No growth			
23	F2011	+	+	-	-	+	+	+	-
24	F2012	+	+	-	-	+	+	+	-
25	F2013	+	+	-	-	+	+	+	-
26	F2014					No growth			
27	F2015	+	V	+	+	Not detected			
28	F2016	-	V	+	+	Not detected			

I: Indol, RM: Methyl Red, VP: Voges Proskauer, C: Citrate/ Glu: Glucose, Lac/sac: Lactose/Sucrose, H_2SO_4

After this analysis, a total of 18 isolates were identified as *Escherichia coli*.

Identification prevalence of isolated pathogens

Of the 28 samples, 18 *E. coli* strains were isolated and identified, corresponding to 64.29%, while 2 (7.14%) isolated strains belonged to another class of Enterobacteriaceae. Finally, 28.57% (n = 8) did not show growth, since the agars used allow inhibiting the growth of another genus not related to enterobacteria. Guzñay (2021) in his investigation of microbiological diagnosis of bovine mastitis in the Chambo canton, he isolated 30% Gram-negative bacillus bacteria. Bonifaz & Conlago (2016) isolated and identified *E. coli* in 13% at the first phase, while40% at the second phase. They suggested that these coliforms are the cause of environmental mastitis, interpreting that the etiological agent recovered occurs when there are hygiene deficits in the environment and udders, since it is the main entrance door to establish a mastitis picture. In agreement with the aforementioned report, hygienic limitations were evidenced at the time of milking.

Antimicrobial activity of the proposed treatments

After the comparison of means, the means of the treatments were statistically different, where T_3 (60%) obtained the highest average diameter of the inhibition zone with 62.40 mm, followed by T_2 (40%) with 46.15 mm, T_1 (20%) with a mean of 31.80 mm of inhibition zone and finally, the lowest average was obtained by T_0 (5 µg Enrofloxacin) with a mean of 21.60 mm (Fig. 1).

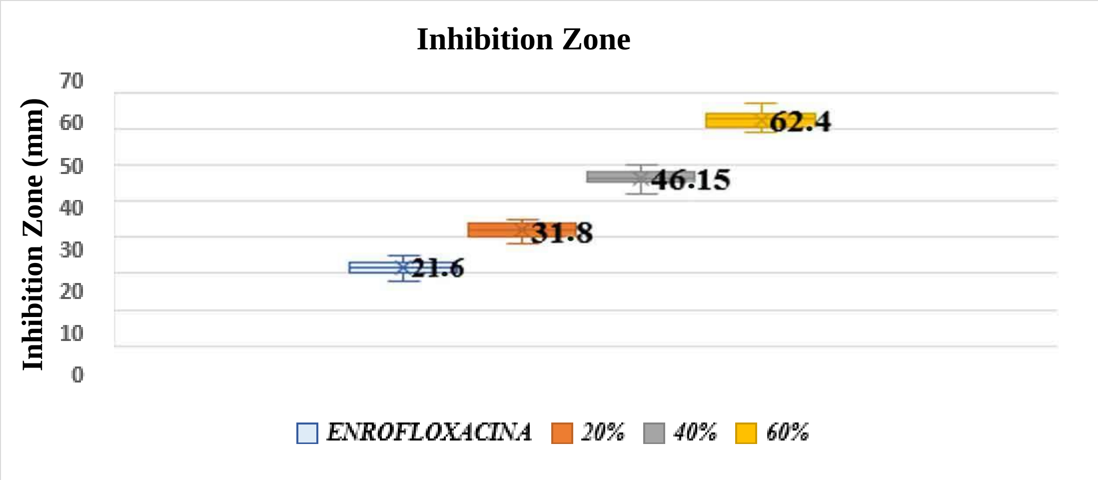

Fig. 1. Box and whisker plot of the inhibition zone diameter of the treatments.

Yerren & Salazar (2021) in their investigation of the antibacterial effect of oregano essential oil against *E. coli* ATCC 25922 strains, reported the concentration of 50 and 100% exhibiting averaged 11.68 mm and 15.28 mm inhibition zone. Likewise, Llanos (2022) in his investigation of the antimicrobial effect of oregano and rosemary against *E. coli* ATCC 25922 at the concentrations of 50, 75 and 100% obtained averaged inhibition zones of 11.1mm, 16.2 mm and 21.9 mm respectively. On the other hand, Medrano & Medrano (2020) in their research on the use of oregano essential oil as an antimicrobial against *E. coli* ATCC 25922 at concentrations of 100, 80, 40 and 20% obtained averaged inhibition zones of 12 mm, 15.7 mm, 9.3 mm, 8 mm respectively, resulting in all these data being lower than those obtained in the present study. These findings are presumably due to the distinction in the pathotypes of *E. coli* and the nature of the dilutions of the extracts in each investigation respectively.

Analysis of the antimicrobial susceptibility of enrofloxacin against *E. coli* isolated from bovine mastitis

Through the antimicrobial susceptibility analysis, only 45% (n = 9) were sensitive to 5 µg enrofloxacin and 55% (n = 11) exhibited intermediate resistance to that concentration. Sánchez (2018) examined antibiotic resistance of the causative agents of bovine mastitis, and found that *E. coli* against Enrofloxacin (5µg) exhibited 75% (n = 6) sensitivity and 25% (n = 2) intermediate resistance, which was not in agreement with our study. Likewise, Ayala (2019) isolated *E. coli* = 6 (24%) evaluating the antimicrobial sensitivity, which presented 100% sensitivity to 5 µg Enrofloxacin, different from those obtained in the present study. In the same way, Franco (2020) worked on the antimicrobial resistance profile of bacteria causing mastitis and isolated *Escherichia coli* = 11 (7.7%) where he reported that 100% were sensitive to 5 µg Enrofloxacin. These values are highly variable, since the susceptibility variable depends on the exposure to the antibiotics used to treat this pathology.

Analysis of the minimum inhibitory concentration (MIC) of oregano essential oil against *E. coli* isolated from bovine mastitis

The minimum inhibitory concentration (MIC) of oregano essential oil against *E. coli* isolated from bovine mastitis, exhibited that when using dilutions with a concentration of 3% of essential oil, average diameter of the inhibition zone was 9 mm, being considered as sensitive according to Duraffourd (1897), while by the concentration of 2.5%, the tests showed 8.10 mm in average. Concentrations higher than those mentioned above exhibited 16.45 mm and 23.20 mm in average at the 5% and 10% concentrations respectively, being very acceptable values to inhibit the growth of the bacterium examined, compared to 10% concentration of the essential oil of oregano with Erofloxacin. It was observed that the essential oil at the aforementioned concentration displayed a higher average in the inhibition zones against the antibiotic examined. When applying the concentrations of 2 % and 1 %, it was conclusively evidenced that they exhibited 4.45 mm and 1.95 mm in average respectively, being considered as insufficient to inhibit the growth of *E. coli* inducing bovine mastitis. Fig. 2 illustrates with a box and whisker diagram the results obtained in each of the applied treatments.

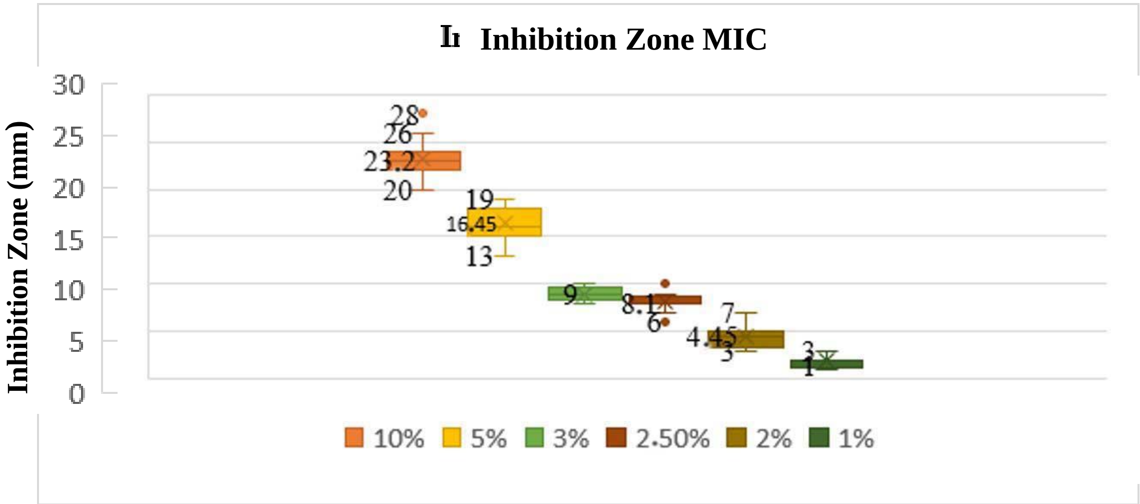

Fig. 2. Inhibition zones of the minimum inhibitory concentration (MIC) of oregano essential oil.

When comparing with Contreras (2017) in his investigation of the synergistic effect of oregano essential oil with ciprofloxacin against ampicillin-resistant E. coli, he obtained that at the concentration of 5% and 10% it exhibited average inhibition zones of 11.70 mm and 10.62 mm respectively, establishing that the MIC necessary to inhibit the growth of the aforementioned pathogen was 5%, data being lower than those found in the present investigation since the MIC of oregano essential oil was established at a concentration of 3%.

Analysis of the sensitivity scale of the concentrations used in the MIC of oregano essential oil against Escherichia coli.

The results obtained from the CMI were compared with the sensitivity scale described by Duraffourd (1897) for an essential oil, where the activity they presented is detailed through the concentrations analyzed.

Table 4. Analysis of the MIC sensitivity scale.

Number	Code	10 %		5 %		3 %		2.5 %		2 %		1 %	
1	F1001	23	+++	16	++	10	+	8	-	6	-	3	-
2	F1003	26	+++	16	++	8	-	8	-	5	-	2	-
3	F1004	24	+++	15	++	9	+	8	-	4	-	2	-
4	F1006	28	+++	19	++	10	+	8	-	3	-	1	-
5	F1007	23	+++	13	++	9	+	8	-	3	-	2	-
6	F1009	22	+++	15	++	8	-	7	-	3	-	1	-
7	F1011	21	+++	14	+	8	-	10	+	5	-	2	-

8	F1012	24	+++	17	++	9	+	9	+	5	-	2	-
9	F2001	24	+++	19	++	10	+	8	-	4	-	1	-
10	F2002	22	+++	15	++	9	+	6	-	3	-	2	-
11	F2003	22	+++	18	++	10	+	8	-	4	-	2	-
12	F2004	24	+++	17	++	9	+	10	+	5	-	2	-
13	F2006	23	+++	15	++	9	+	9	+	5	-	2	-
14	F2007	21	+++	16	++	9	+	7	-	5	-	1	-
15	F2009	25	+++	18	++	10	+	9	+	3	-	1	-
16	F2011	20	++	16	++	9	+	8	-	5	-	3	-
17	F2012	21	+++	15	++	8	-	7	-	4	-	2	-
18	F2012	22	+++	19	++	9	+	8	-	6	-	3	-
19	F2013	24	+++	17	++	8	-	8	-	7	-	3	-
20	F2013	25	+++	19	++	9	+	8	-	4	-	2	-

+++: Extremely sensitive, ++: Very sensitive, +: Sensitive, -: Null activity.

Table 5. Frequency and percentage of the sensitivity scale according to Duraffoud.

Concentration	+++		++		+		-		Total	
	Fr.	%	Fr.	%	Fr.	%	Fr.	%	Fr	%
10%	19	95	1	5	-	-	-	-	20	100
5%	-	-	19	95	1	5	-	-	20	100
3%	-	-	-	-	15	75	5	25	20	100
2.5%	-	-	-	-	5	25	15	75	20	100
2%	-	-	-	-	-	-	20	100	20	100
1%	-	-	-	-	-	-	20	100	20	100

+++: Extremely sensitive, ++: Very sensitive, +: Sensitive, -: Null activity.

Table 5 depicts the sensitivity scale according to Duraffourd (1897), where the 1% and 2% concentrations exhibited 100% null activity against the 20 *E. coli* isolates. Likewise, the 2.5% concentration displayed 25% sensitivity and 75% null activity, while the 3% concentration revealed 75% sensitivity and 25% null activity. Later the 5% concentration showed 5% sensitivity and 95% very sensitive. In the same way, 10% concentration exhibited 5% very sensitive and 95% extremely sensitive.

Results of volatile compounds by gas chromatography of the essential oil of oregano

In the present study, the identification of the volatile compounds of the essential oil in oregano is shown, the same one that stood out for having good antimicrobial activity against *Escherichia coli* isolated from bovine mastitis.

Table 6. Volatile compounds of oregano essential oil by steam stripping.

#	Compound	Time retention (minutes)	Area (%)
14	2-Pentanone, 4 - hydroxy - 4 – methyl (alcohol diacetona)	15.896	5.97
15	Alpha Pinene	19.080	1.83
17	Beta Myrcene	20.437	1.74
19	p-Cymene	21.579	11.54
22	Gamma -Terpinene	22.430	1.84
23	Linalool	23.307	2.17
29	Acid Formic	27.894	66.63
30	Caryophyllene	30.903	1.97

Forty-two peaks were recorded, where the highest intensity was demonstrated by peak 29 (acid formic) which obtained a retention of 27,894 minutes, followed by peak 19 (p-Cymene), and finally, the peak with the lowest intensity was obtained by peak 14 (2-pentatone, 4-hydroxy -4- methyl). Abalco (2020) worked on the phytochemical characterization of oregano essential oil by gas chromatography, reporting that the oregano essential oil obtained from the province of Pichincha exhibited 51 volatile compounds, where γ-Terpinene was found in a proportion of 21.28%, p-Cymene 20.88%, thymol 2.64%, β-Myrcene 1.75%, Caryphyllene 1.58 and linalool 0.043%. Among the compounds with the highest percentage, comparing with the results obtained in a higher percentage, formic acid and p-Cymene were obtained, exhibited the antimicrobial capacity against *E. coli* inducing bovine mastitis.

CONCLUSION

In conclusion, most of the bacteria that make up the battery of microorganisms that cause mastitis is *Escherichia coli*, which in fact in our study was 64.29%. It was also determined that the formic acid and p-Cymeno components are the metabolites most present in the essential oil and therefore presumably the ones that acted as *E. coli* growth inhibitors, ultimately being a 3% concentration of essential oil sufficient to inhibit pathogen growth.

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