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ANTIBACTERIAL EFFECT OF ESSENTIAL OILS AGAINST BACTERIAL STRAINS ISOLATED FROM COWS WITH MASTITIS

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Abstract

Antibiotic resistance has become a global concern, with major implications in both human and veterinary medicine. In recent years, new therapies have been sought as alternatives to antibiotics. In this regard, essential oils extracted from medicinal plants have been shown to be effective in many cases. The purpose of our study was to test the efficacy of the essential oils of thyme, clove, peppermint, and cinnamon against ten bacterial strains isolated from cows with clinical mastitis. Sixty percent of the bacterial strains examined in the study were found to be resistant to five or more antibiotics. The effectiveness of essential oils was tested using the aromatogram method. The results varied depending on the bacterial strain and concentration of the tested essential oils. Cinnamon, thyme, and the mixture of the four oils were the most effective products. The highly resistant S. aureus isolate (7 from 12 antibiotic molecules), proved to be extremely sensitive to the essential oils of thyme and cinnamon and highly sensitive to the mixture of oils.

***Key words**: cow, mastitis, essential oils, antibacterial.*

INTRODUCTION

Since 1928, the year when penicillin was discovered, a new era of medicine has begun, saving millions of lives. In recent years, it has been found that infections considered common place have become a threat again, due to the development of antibiotic resistance. Alarm signals have been raised by most of the competent international institutions regarding the judicious use of antimicrobials and the rapid discovery of alternatives for them, since the research for the discovery of new molecules does not prove profitable for the pharmaceutical industry (Allen et al., 2014; Ventola, 2015). Tomanic et al. (2023) estimated that in veterinary medicine, the amount of antibiotics used is 73-100% higher than that used in human medicine. There is a worldwide concern about food safety and the effect of antibiotic use in animal husbandry, and a series of general recommendations have been issued for their administration, such as the selection of specific antimicrobials based on laboratory analysis, the use of appropriate therapeutic doses, optimal dosage, minimising the potential for transfer of genetic antimicrobial resistance, or administration of antimicrobials in order to reduce adverse effects for humans and animals (Guardabassi et al., 2008). Antibiotic resistance is estimated to cause 10 million deaths and $100 trillion in economic losses annually by 2050 (Tomanic et al., 2023).

Alternatives to which modern medicine is beginning to turn its face in recent years are phytotherapy, homeotherapy, apitherapy, acupuncture, phagitherapy, probiotics, immunotherapy, and vaccines. Compounds derived from plants could enter the current use in therapy due to their direct, antibacterial, but also indirect action, due to bioactive compounds that modify resistance to antibiotics and increase their effectiveness (Stephanovic, 2018).

The antibacterial activity of plants is mainly due to essential oils (Gîrd, 2014). The composition of all essential oils has a very high level of variability in terms of quality and quantity, which depends on intrinsic and extrinsic factors such as the area where the plant is grown (latitude and altitude), the amount of precipitation and the amount of UV radiation, the time of cultivation, the type of soil and its fertilisation, plant age, extraction conditions, drying and storage conditions. All these factors influence and interconnect, making them difficult to treat separately (Hassiotis et al., 2014; Dhifi et al., 2016; Kulyas & Batiyash, 2018; Haro-González et al., 2021).

MATERIALS AND METHODS

Milk samples were collected from 10 cows showing clinical signs of mastitis. Collected milk samples were plated on solid culture media: defibrinated sheep blood agar, MacConkey agar, and Sabouraud agar. The seeded plates were incubated for 24 hours at 37°C and 72 hours at 25°C for Sabouraud. The resulting cultures were examined macroscopically and microscopically. Based on cultural, morphological, and biochemical characteristics using Api 20 NE, Api 20 E, Api 20 Strep and ID 32 Staph galleries, the bacterial isolates from the milk samples were identified and preserved.

To determine antibiotic sensitivity, the disc diffusion method was used, which is based on the property of antimicrobials to diffuse in a solid culture medium (defibrinated sheep blood agar) on which the bacterial culture to be tested is seeded.

The essential oils used were purchased from a processing company in France, 100% pure oils, containing no other similar essential oils, 100% natural, from certified organic crops, not denatured by synthetic molecules, 100% integral, obtained by complete distillation with steam. Testing the antibacterial effect of the essential oils of *Thymus serpillum*, *Syzygium aromaticum*, *Mentha piperita* Franco-Mitcham, and *Cinnamomum zeylanicum* on the isolated bacterial strains was carried out by the aromatogram method.

Bacterial suspensions were prepared from cultures on solid medium in sterile saline solution, at a known concentration of 0.5 McFarland, a concentration equivalent to 1-2 x 10^8 CFU/ml. Blood agar plates were seeded with 100 µl of the previously prepared inocula, then left ajar for 10 min in the laminar flow hood. Of the test oils, dilutions of 1/2, 1/4, and 1/10 were made in a mixture of distilled water with 10% DMSO and 0.5% Tween 80. An equal-parts mixture was also made of the four essential oils to be tested, undiluted. In the same experiment, the antibacterial effect of coconut oil and grape seed oil was also tested. Discs were placed on the previously inoculated plate using sterile forceps at a minimum of 15 mm from the edge and not less than 24 mm apart, and a micro disc of norfloxacin was used as a positive control. The plates were incubated aerobically at 37°C for 24 hours. Each sample was run in duplicate.

After 24 hours of incubation, the diameter of the zones of inhibition around each disc was measured using a ruler.

The correlation between the diameters of the inhibition zones and the sensitivity of the bacteria was established according to the specialised literature using the Duraffourd scale (Vasquez & Guardia, 2021). The sensitivity of the bacteria was assessed, depending on the diameter of the zone of inhibition that appeared around the disc impregnated with volatile oil, and the sensitivity of the bacteria to each essential oil used was noted as follows: < 8 mm = resistant; between 8 and 14 mm = sensitive; between 14 and 20 mm = very sensitive; > 20 mm = extremely sensitive.

RESULTS AND DISCUSSIONS

The following bacterial strains were isolated and identified from breast milk: *Escherichia coli* (two strains), *Lactococcus lactis* ssp. *cremosis*, *Listeria* spp., *Pateurella multocida*, *Staphylococcus xylosus*, *Staphylococcus* spp., *Staphylococcus intermedius*, *Staphylococcus epidermidis,* and *Staphylococcus aureus*. Of the tested strains, 90% showed multiple resistance to the tested antibiotics, 60% proved to be resistant to at least 5 antibiotics, and 30% to 7 or more antibiotics, results that reflect the upward trend of the antibiotic resistance phenomenon (Table 1). Similar results were also obtained in the western part of Romania, where 39 bacterial strains, belonging to different species, isolated from cows with mastitis, were tested. All strains showed resistance to at least 3 antibiotics from those tested, but were resistant to 9 or more antibiotics, the most frequently recorded resistance to erythromycin, ampicillin, gentamicin, amoxicillin, and clavulanic acid (Pascu et al., 2022).

Table 1. Bacterial sensitivity to antibiotics

Strain / Atb.	1*	2	3	4	5	6	7	8	9	10
AK	S**	S	R	R	R	-	-	S	-	-
AML	S	-	-	-	-	S	R	S	R	R
AMP	-	R	R	I	S	S	R	-	-	R
C	-	-	-	-	-	S	-	-	-	R
CFM	R	S	S	R	S	S	-	R	R	R
CS	R	-	-	-	-	-	R	R	-	-
CD	I	R	I	R	R	S	-	I	R	I
DXT	S	S	S	R	S	S	R	S	-	S
E	-	-	-	-	-	-	R	-	-	-
ENR	-	S	I	I	S	-	R	-	R	S
GM	S	S	I	R	R	S	-	S	R	S
K	-	-	-	-	-	-	-	-	R	R
KF	S	R	S	R	S	-	-	S	R	R
N	-	-	-	-	-	-	-	-	R	R
NOR	S	S	S	S	S	S	S	S	R	S
OX	-	-	-	-	-	-	R	-	-	-
P	R	R	S	I	S	S	-	R	-	R
RA	S	R	S	S	S	S	-	S	-	-
S	S	I	R	R	R	S	S	I	-	R
SPE	I	-	-	-	-	S	R	I	-	-
SXT	S	S	I	I	S	S	R	S	-	-
VA	R	-	-	-	-	R	-	R	-	-
TE	-	I	S	I	I	-	-	-	R	R

*1,2 - *Escherichia coli,* 3 - *Lactococcus lactis* ssp. *cremoris,* 4 - *Listeria* spp., 5 - *Pasteurella multocida,* 6 - *Staphylococcus xylosus,* 7 - *Staphylococcus* spp., 8 -*Staphylococcus intermedius,* 9 - *Staphylococcus epidermitis,* 10 - *Staphylococcus aureus*
** S-sensitive, I-intermediar, R-resistant

The effectiveness of the essential oils varied depending on the strain and concentration tested, but cinnamon and thyme oils were the most effective, as was the mixture of the four oils (Figure 1).

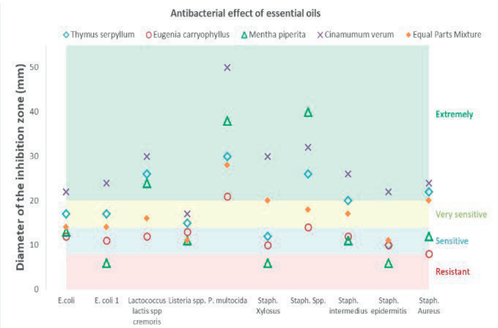

Figure 1. Antibacterial effect of essential oils

The strain of *S. aureus*, resistant to most of the antibiotics tested, was highly sensitive to the essential oils of thyme and cinnamon and very sensitive to the mixture of oils. Cinnamon oil had the strongest effect, with all bacteria being at least sensitive to the action of this oil. The smallest inhibition zone was 17 mm for *Listeria* spp., and the maximum inhibition zone was in the presence of the *Pasteurella multocida* strain, above 50 mm, a value that was preserved at all tested dilutions. Similar results were obtained by Casalina et al. (2023). For 117 strains of *E. coli* from birds, cinnamon oil proved effective against all strains tested. The same was reported by El Atki et al. (2019) for strains of *E. coli*, *S. aureus,* and *Pseudomonas aeruginosa* that were inhibited in the presence of cinnamon oil.

The sensitivity of the bacterial strains to the action of the essential oils, for the tested concentrations is shown in Table 2.

Table 2. Susceptibility of bacterial strains to essential oils

**	1*	2	3	4	5	6	7	8	9	10
Thymus serpillum										
A	17	17	26	15	30	2	26	22	10	20
B	15	12	24	12	28	10	24	18	8	18
C	10	10	14	10	26	7	10	12	6	12
D	6	6	8	6	6	6	7	6	6	6
Cinnamomum zeylanicum										
A	22	24	30	17	>50	30	32	24	22	26
B	22	22	28	16	>50	28	31	22	18	22
C	20	20	26	15	>50	26	30	20	17	20
D	14	16	22	12	>50	24	22	16	10	14
Syzygium aromaticum										
A	12	11	12	13	21	10	14	12	10	13
B	9	8	10	10	20	9	11	10	8	10
C	8	6	9	6	12	9	10	8	6	6
D	6	6	6	6	10	6	7	6	6	6
Mentha piperita										
A	13	6	22	11	38	6	40	11	6	12
B	8	6	16	8	30	6	24	9	6	10
C	6	6	12	6	12	6	16	6	6	6
D	6	6	16	6	6	6	6	6	6	6
Mixture E.O. 1:1:1:1										
	14	14	16	28	20	18	17	11	20	11

*1, 2 - *Escherichia coli,* 3 - *Lactococcus lactis* ssp. *cremoris,* 4 - *Listeria* spp., 5-*Pasteurella multocida,* 6 - *Staphylococcus xylosus,* 7 - *Staphylococcus* spp., 8 -*Staphylococcus intermedius,* 9 - *Staphylococcus epidermitis,* 10 - *Staphylococcus aureus.*
**A-integral E.O., B-1/2 dilution, C-1/4 dilution, and D-1/10 dilution

All tested bacterial strains were at least sensitive to the action of *Thymus serpyllum* essential oil. This is an oil rich in thymol and carvacrol, substances that induce changes in the integrity and permeability of the membrane where ATP and potassium ions are released, with changes in pH and the balance of organic ions; undergo oxidation at the level of the double bonds in the nucleus, releasing carbonyl, carboxyl, and hydrogen peroxide derivatives with an antiseptic effect at the cellular level. Both have phenolic structure; they are structural isomers; on the phenolic ring, only the position of the hydroxyl group differs. The activity of thymol and carvacrol is potentiated by p-cymene, without antimicrobial action but with hydrophobic action. Among the many uses of thyme oil, its anti-inflammatory and antimicrobial properties stand out (Gîrd, 2014; Mahboubi & Kazempour, 2011; Kovacevic et al., 2022).

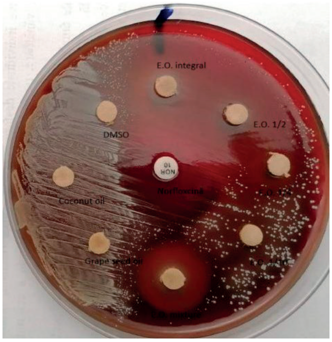

Figure 2. Efficacy of essential oils of thyme on the *Staphylococcus aureus* strain

Tested on bacteria isolated from cows with mastitis, the effect of *Thymus spp*. oil had remarkable results at concentrations of 2% and 3%, completely inhibiting the *in vitro* growth of bacteria *E. coli*, *S. aureus*, and *Str. agalactiae* (Mason et al., 2015). *Thymus* spp. oil used at a dose of 50 µl/ml against a strain of *S. aureus* and *Klebsiella pneumoniae* produced a zone of inhibition of 35 mm and 40 mm, respectively, and when the oil was combined with tetracycline, the zone of inhibition increased to 38 mm and, respectively, 41 mm (Pancu et al., 2022). The results are also confirmed by Sahin et al. (2003) concerning the extract of *Thymmus* spp., which he tested against 178 strains of microorganisms (55 species of bacteria, 4 species of fungi, and one fungus) *in vitro* using the disc diffusion method.
Thymus spp. extract inhibited the activity of 23 bacterial strains from 11 different species, as well as 6 strains of *Candida albicans*. Clove oil was most effective against the *Pasteurella multocida* strain (21 mm), retaining its effectiveness up to 1/10 dilution (10 mm). The same oil also proved effective against all other strains except *S. aureus*. The main component of *Syzygium aromaticum* oil is eugenol, at a percentage of 80.30%. All strains tested by Oyedemi et al. (2009) were the least sensitive to the action of clove oil. Eugenol caused a 7.9 log reduction when contacted with *E. coli* after 20 hours of incubation, followed by *Proteus vulgaris* with a 3-log reduction.

Yadav et al. (2015) tested the bactericidal effect of eugenol against some MRSA and MSSA strains. They were treated with serial dilutions of eugenol, carvacrol, or a combination thereof for 6 hours and incubated at 37ºC, after which the number of CFU was determined. The results suggested that eugenol had strong bactericidal activity during the first 3-6 hours of incubation.
Mint, known for its antispasmodic, antimicrobial, anti-inflammatory, carminative, antioxidant, analgesic, and more effects, showed a strong antibacterial effect against the 40 mm strain of *Staphylococcus* spp. The lowest activity of only 11 mm was observed for *S. epidermidis* and *Listeria* spp., against which only cinnamon oil showed high activity of 18 mm and 16 mm, respectively.
Peppermint essential oil was the only oil to which resistance was recorded from three bacterial strains (*Escherichia coli*, *Staphylococcus xylosus*, and *Staphylococcus epidermidis*). The results obtained by Muntean et al. (2019) on the efficacy of peppermint essential oil on multidrug-resistant Gram-positive and Gram-negative bacterial strains were superior to the present study, being effective against all strains tested, including *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.
The mixture in equal parts of the four integral essential oils showed an antibacterial effect on all 10 bacterial strains tested; the largest zone of inhibition was observed in the presence of the *Pasteurella multocida* strain (28 mm), with variations for the other strains between 11 and 20 mm, but did not show stronger activity than all the oils tested separately. In contrast, Kovacevic et al. (2022) for *Thymus vulgaris*, *Thymus serpyllum*, *Satureja montana*, and *Origanum vulgare* oils tested together had better results than when they were tested individually.
Grape seed oil and coconut oil did not inhibit the growth of any of the bacterial strains tested. A study conducted in 2019 to determine the effectiveness of coconut oil against strains of *S. aureus* isolated from goats with mastitis determined their inhibition, in vitro (Widianingrum et al., 2019).

Encouraging results were also obtained when coconut oil was used in concentrations of 25%, 50%, and 75% for the inhibition of *Streptococcus mutans* ATCC 25175, obtaining zones of inhibition of 17 mm, 21.75 mm, and 22 mm, respectively (Vásquez & Guardia, 2021). Coconut oil has been shown to be effective against some strains of *S. aureus*, *Str. pneumoniae*, and *E. coli*, but ineffective against the strain of *P. aeruginosa* tested. At the same time, two fungal strains were studied, thus, in contact with *Candida albicans*, coconut oil produced an inhibition zone of 18.5 mm, and in contact with *Aspergillus fumigatus* of 15.1 mm (Effiong et al., 2019).

The antibacterial activity of grape seed oil is primarily due to polyphenols, linolenic acid, but also to its higher acidity, compared to other carrier oils. Resveratrol also degrades the bacterial membrane without harming the host cells. Any change to the cell matrix will disrupt the normal functioning of the cell. The results in the scientific literature are varied, with greater efficacy against Gram-positive bacteria compared to Gram-negative ones. There are studies in which strains of *S. aureus* showed sensitivity to concentrations between 20 mg/mL and 1.25 mg/mL, with zones of inhibition ranging from 12.5 mm to 7 mm, but there are also studies in which strains of *S. aureus* were not sensitive to grape seed extract even at 20 mg/mL. Positive results have also been recorded against strains of *S. enteritidis*, *Bacillus subtilis*, *E. coli*, *L. monocytogenes*, and *Streptococcus pneumoniae*, but there are also strains of *E. coli*, *K. pneumoniae*, *C. parapsilosis*, *Listeria monocytogenes* and *Salmonella enteric*a that were not sensitive to any concentration tested (Belilli et al., 2018; Garaglavia et al., 2016; Memar et al., 2019; Bajpai et al., 2021).

CONCLUSIONS

According to the antibiogram, 90% of the strains showed multiple resistance to the tested antibiotics, 60% proved to be resistant to at least 5 antibiotics, and 30% to 7 or more antibiotics. All 10 bacterial isolates tested were found to be sensitive to cinnamon essential oil up to the 1/10 dilution, which had the strongest antibacterial effect among the oils tested. The mixture of equal parts of the four whole essential oils showed an antibacterial effect on all 10 bacterial strains tested. Both coconut oil and grape seed oil, through testing, demonstrated a lack of antibacterial activity against the strains studied.

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