Research Article

The Combined Effect of *Mentha spicata*, *Perilla frutescens*, and *Ocimum basilicum* Essential Oils with Norfloxacin Against Antibiotic-Resistant *Escherichia coli* Responsible for Pyometra in Dogs

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Abstract | Pyometra is a serious reproductive condition in female dogs, often caused by antibiotic-resistant *Escherichia coli*, which complicates treatment due to its resistance to common antibiotics. This study explores the potential of essential oils from *Mentha spicata*, *Perilla frutescens*, and *Ocimum basilicum* to combat *Escherichia coli* strains isolated from pyometra cases, both alone and in combination with the antibiotic norfloxacin. Using the disk diffusion method, we assessed the antibiotic resistance profiles of the bacteria, revealing significant resistance to amoxicillin, ampicillin, ceftazidime, streptomycin, colistin, and doxycycline. The bactericidal activity of the essential oils was determined through minimum bactericidal concentration (MBC) tests, with *Mentha spicata* exhibiting the highest MBC, significantly outperformed by norfloxacin. Notably, *Ocimum basilicum* and *Mentha spicata* demonstrated synergistic and additive effects with norfloxacin, while *Perilla frutescens* had no significant interaction. This study highlights the promising role of essential oils as adjuncts to antibiotics in treating antibiotic-resistant *Escherichia coli* infections in dogs, providing new insights into potential therapeutic strategies for pyometra.

Keywords | Essential oils, Antibiotic-Resistance, *Escherichia coli*, Pyometra, Synergistic, Antibacterial

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INTRODUCTION

As society advances, the growing interest in pet ownership has resulted in a notable increase in the population of pet dogs. However, this growing dog population also heightens the risk of disease outbreaks. Among the various affecting dogs, pyometra in female dogs is one of the most prevalent (Hagman, 2023). The uterus plays a vital role in reproduction, serving as the site for fetal development. This condition is becoming more widespread, significantly affecting the health, fertility, and reproductive capacity of dogs. Without timely treatment, it can pose a severe threat, potentially leading to high mortality rates (Rafael *et al*., 2024).

Multiple studies have identified *Escherichia coli* as the most frequently isolated bacterium from endometritis fluid (Hagman and Kuhn, 2002; Harada *et al*., 2024; Ylhäinen *et al*., 2025). This pathogen enters through the vaginal tract and causes uterine damage. The infection primarily occurs during estrus, mating, childbirth, or abortion. Antibiotics are widely used for both the prevention and

treatment of infections caused by *Escherichia coli*. However, this bacterium develops antibiotic resistance at a rapid rate, highlighting the urgent need for effective treatment options. *Escherichia coli* exhibits resistance to several commonly used antibiotics, including β-lactams (ampicillin, piperacillin, amoxicillin, cefazolin, ceftazidime, cefepime, meropenem), aminoglycosides (streptomycin, amikacin), tetracyclines (tetracycline), and folate pathway inhibitors (trimethoprim, sulfamethoxazole) (Carvalhoa *et al*., 2016; Harada *et al*., 2024). Long-term use of synthetic antibiotics can contribute to increased resistance, making bacterial infections more difficult to manage. Moreover, they can facilitate the transfer of antibiotic resistance from bacteria in animals to those in humans (Nair *et al*., 2018). In recent years, antibiotic resistance has emerged as a critical global health challenge. Globally, approximately 50-80% of all antibiotics are used for livestock rather than for humans. Moreover, approximately 75% of antibiotics used remain in the environment because they are not fully metabolized by animals (Xu *et al*., 2022). This also contributes to the development of antibiotic-resistance genes in environmental bacteria (Bava *et al*., 2024).

To address these challenges and promote sustainable approaches to disease prevention and treatment in pets, many scientific studies have investigated the potential of antibacterial essential oils derived from herbs as substitutes for synthetic antibiotics (Galgano *et al*., 2022; Aouadhi *et al*., 2024). In Vietnam, aromatic herbs such as *Mentha spicata*, *Perilla frutescens*, and *Ocimum basilicum* are commonly used as food ingredients. These herbs are not only popular as culinary spices but are also widely utilized for extracting essential oils with medicinal properties. The essential oils derived from these three herbs contain valuable bioactive compounds. *Mentha spicata* essential oil is rich in 1,8-cineole (17.0%), limonene (20.8%), and carvone (40.8%) (Mahboubi, 2021). *Perilla frutescens* essential oil primarily consists of β-caryophyllene (4.89%), limonene (5.61%), and perillaldehyde (77.49%) (Ahmed and Al-Zubaidy, 2020). *Ocimum basilicum* essential oil contains 1,8-cineole (7.4%), linalool (16%), and estragole (52.2%) (Pandey *et al*., 2014). Numerous studies have highlighted the antibacterial effects of these bioactive compounds. Kamatou *et al*. (2008) reported that these compounds exhibited strong antibacterial activity against both Gram-negative and Gram-positive bacteria. Their effectiveness was evaluated against three Gram-positive bacteria (*Staphylococcus aureus, Micrococcus luteus*, and *Bacillus subtilis*) and two Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) (Sharmaa *et al*., 2020), as well as against *Escherichia coli* and *Listeria monocytogenes* (Angane *et al*., 2023).

As a result, these essential oils hold significant potential for eliminating *Escherichia coli*, a bacterium responsible for pyometra in dogs. Additionally, research suggests that combining herbal essential oils with antibiotics can produce synergistic effects (Moussaoui and Alaoui, 2016; Boonyanugomol *et al*., 2017; Romo-Castillo *et al*., 2023; Drioiche *et al*., 2024). Previous studies have suggested that combining essential oils with antibiotics may reduce the required dosage and enhance efficacy against resistant strains. This study aims to evaluate the bactericidal effects of essential oils from *Mentha spicata*, *Perilla frutescens*, and *Ocimum basilicum*, both individually and in combination with an antibiotic, against antibiotic-resistant *Escherichia coli* isolated from pyometra in dogs.

MATERIALS AND METHODS

Extraction and Analysis of Bioactive Compounds in Essential Oils

Mentha spicata, *Perilla frutescens*, and *Ocimum basilicum* were harvested from an herb garden in Tra Vinh Province, Vietnam, in June 2024. These herbs were grown without the use of pesticides or growth stimulants. Prior to use, all herbs were authenticated by the Department of Crop Science, Tra Vinh University, Vietnam, based on the morphological characteristics of the stem, branches, and leaves. Essential oils were obtained through steam distillation as described by Elhafez (2022). Following harvest, the herbal leaves were dried at 40°C. Subsequently, 150 g of dried leaves and 500 ml of water were added to a 1000 ml round-bottom flask in the Clevenger essential oil distillation system (Witeg, Germany). The mixture was heated to 100°C for 5 hours. The extracted essential oils were then separated from water using sodium sulfate and stored at a temperature of 4°C until use. The composition and relative content of volatile compounds in the essential oils were analyzed using gas chromatography-mass spectrometry (GC-MS) (Agilent 6890N, Agilent Technologies, USA) (Adams, 2007).

Culturing and Isolating

Escherichia coli was isolated from five clinical samples (uterine inflammatory fluid). The samples were collected from dogs with pyometra at veterinary clinics in Tra Vinh Province, Vietnam. These dogs were diagnosed with pyometra and treated with ovariohysterectomy, and had not been treated with antibiotics prior to collection. The culturing and isolation process was carried out following the guidelines of Carroll and Pfaller (2023). After collecting samples at the veterinary clinic, the uterine inflammatory fluid was placed into Buffered Peptone Water (BPW) and incubated at 37°C for 24 hours. A swab was streaked onto Mac Conkey (MC) and Eosin Methylene Blue (EMB) media, then incubated at 37°C for 24 hours. Subcultures were made until uniform colonies appeared. On MC agar, the bacteria formed large, round, pale pink colonies with slightly raised, non-mucoid surfaces, and distinct edges,

while the surrounding medium turned pink. On EMB agar, the *E. coli* colonies were round, shiny, dark purple, and had a metallic green sheen. Once uniform colonies were obtained, a pure culture was transferred to Nutrient Agar (NA) medium and incubated at 37°C for 24 hours, resulting in round, moist, pale white colonies with slightly darker centre and a diameter of 2-3 mm. These colonies on NA medium were used for biochemical testing and antibiotic susceptibility testing.

Bacterial Identification Following Isolation

After isolation, the bacteria were identified biochemically using the API 20E kit (Biomerieux, France), and the positive samples were further analyzed through gene sequencing via polymerase chain reaction (PCR). PCR amplification targeted the *E. coli* 16S rRNA gene, utilizing specific primers: ECO-1 (Forward Primer): 5'-GACCTCGGTTTAGTTCACAGA-3' and ECO-2 (Reverse Primer): 5'-CACACGCTGACGCTGACCA-3'. The expected amplicon size for this gene target was 585 base pairs (bp). Genomic DNA was extracted using a bacterial DNA extraction kit and used as a PCR template. DNA from a known *E. coli* strain served as a positive control, while nuclease-free water was the negative control. PCR was performed following Mamun *et al.* (2016), and the products were analyzed via 1.5% agarose gel electrophoresis. A distinct 585 bp band under UV light confirmed the presence of *E. coli*.

Antibiotic Resistance Testing of Isolated *Escherichia coli*

Antibiotic susceptibility of the isolated *Escherichia coli* was assessed using the disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2024). Twelve antibiotics were tested, including Ampicillin 10µg (Am), Amoxicillin 10µg (Ax), Ceftazidime 30µg (Cz), Cefuroxime 30µg (Cu), Colistin 10µg (Co), Doxycycline 30µg (Dx), Gentamicin 10µg (Ge), Kanamycin 30µg (Kn), Norfloxacin 10µg (Nr), Streptomycin 10µg (Sm), Tetracycline 24µg (Te), and Tobramycin 10µg (Tb). The standard inhibition zone diameter and resistance classification for the 12 antibiotics tested are presented in Table 1. The experiment was repeated three times.

The Minimum Bactericidal Concentration (MBC) of Essential Oils and Norfloxacin Against Isolated *Escherichia coli*

The minimum bactericidal concentration (MBC) was determined using a broth microdilution susceptibility assay, following the guidelines of the Clinical and Laboratory Standards Institute for dilution antimicrobial susceptibility tests (CLSI, 2024). The bacterial suspension was adjusted to a turbidity of 0.5 McFarland, corresponding to a concentration of 10^6-10^8 CFU/ml. Essential oils were diluted in concentrations ranging from 1-15 mg/ml in 10% DMSO, while norfloxacin was diluted from 31.25 to 1,000 µg/ml in distilled water. These concentrations were selected based on preliminary tests to identify the range that could inhibit bacterial growth. The essential oils and antibiotics at the specified concentrations were added to the test tubes containing the bacterial suspension and Tryptic Soy Broth (TSB) medium, and incubated at 37°C for 24 hours. The positive control contained the bacterial suspension, TSB medium, and the antibiotic at the minimum bactericidal concentration for the isolated bacteria. The negative control contained the bacterial suspension and TSB. Additionally, the negative control was used to test the effect of the essential oil solvent, including the bacterial suspension, TSB medium, and DMSO (10%). After incubation, the contents from each test tube were plated on MC agar and incubated again at 37°C for 24 hours. The MBC was defined as the lowest concentration at which no bacterial colonies grew on the MC medium. Each experiment was repeated three times.

Table 1: The standard inhibition zone diameter and resistance classification for the 12 antibiotics tested (CLSI, 2024).

Antibiotics	Levels (µg)	Zone of growth inhibition (mm)		
		Sensitive (S) (≥)	Intermediate (I)	Resistant (R) (≤)
Ampicillin (Am)	10	17	14-16	13
Amoxicillin (Ax)	10	16	14-15	13
Ceftazidime (Cz)	30	21	18-20	17
Cefuroxime (Cu)	30	18	15-17	14
Colistin (Co)	10	15	13-14	12
Doxycycline (Dx)	30	17	15-16	14
Gentamycin (Ge)	10	15	13-14	12
Kanamycin (Kn)	30	15	13-14	12
Norfloxacin (Nr)	10	18	16-17	15
Streptomycin (Sm)	10	15	13-14	12
Tetracycline (Te)	24	17	15-16	14
Tobramycin (Tb)	10	15	13-14	12

The Minimum Bactericidal Concentration (MBC) of Essential Oils and Norfloxacin used in Combination Against Isolated *Escherichia coli*

The interaction between essential oils and antibiotics was assessed using the fractional bactericidal concentration (FBC) index, following the microdilution checkerboard method outlined by Drioiche *et al.* (2024). At the minimum bactericidal concentration (MBC), essential oils and antibiotics were serially diluted in broth to 1/64 of their initial concentrations. The positive control contained the bacterial suspension, TSB medium, and the antibiotic at the minimum bactericidal concentration for the isolated

bacteria. The negative control contained the bacterial suspension, TSB, and distilled water. The MBC was determined for each combination of essential oil and antibiotic.

The FBC indices were determined by adding FBC^A and FBC^B, where FBC^A and FBC^B represent the minimum concentrations needed to eliminate bacteria for drugs A and B, respectively. These were calculated as follows: FBC^A = MBC^A (in combination) / MBC^A (alone), and FBC^B = MBC^B (in combination) / MBC^B (alone). The mean FBC index was then derived using the formula: FBC index = $FBC^A + FBC^B$, with the following classifications: synergistic (≤0.5), additive (>0.5 but <1), indifferent (≥1 but <4), or antagonistic (≥4.0). The experiment was repeated three times.

Statistical Analysis

Statistical analysis was conducted using SPSS software (version 22). Variations in MBC values between essential oils and antibiotics were evaluated through ANOVA, followed by Tukey's post hoc test. A P-value below 0.05 was regarded as statistically significant. Results were expressed as mean ± standard deviation (SD).

Table 2: The primary biologically active compounds found in the essential oils of *Mentha spicata*, *Perilla frutescens*, and *Ocimum basilicum*.

Components	Retention time (min)	Relative percentage (%)		
		Mentha spicata	Perilla frutescens	Ocimum basilicum
3-Octenol	10.080		0.60	
3-Octanol	10.917		0.34	
β-Myrcene	11.960	0.42		
D-Limonene	12.635	17.60	6.54	0.42
Eucalyptol	12.726			0.65
Menthone	13.340	1.15		
1,8-Cineol	13.490	15.4		6.72
cis-β-Ocimene	13.898			0.59
Terpinen-4-ol	15.420	0.86		
α-Linalool	17.076		1.3	
β-Linalool	17.101	0.30		5.34
Fenchol	17.689			0.86
Camphor	19.346			0.96
Borneol	20.461			0.54
γ-Terpinene	21.230	0.36		
α-Terpineol	21.699	0.53	0.45	0.48
Estragole	22.240		1.17	52.65
Fenchyl acetate	22.972			0.68
Dihydrocarveol	23.034	0.32		
cis-Dihydrocarveol	23.326	1.24		
Dihydrocarvone	23.432	0.57		
trans-Carveol	23.540	0.40		
Carvone	23.650	48.9		
Dihydrocarvyl acetate	24.120	0.46		
L-Carveol	24.470	0.54		
β-Bourbonene	24.870	7.56		
Perilla aldehyde	25.177		65.23	
γ-Amorphene	25.234	0.21		
α-Amorphene	25.357	0.12		
Bornyl acetate	25.467		0.45	0.26
Perillyl alcohol	25.901		1.33	
Eugenol	27.852		0.50	
α-Copaene	28.437		0.15	
β-Elemene	28.949		0.11	1.21
Methyl eugenol	29.324			12.72
Isocaryophyllene	29.398		1.25	
β-Caryophyllene	29.765	1.52	8.32	0.25
trans-α-Bergamotene	30.240			3.49
α-Guaiene	30.326			0.47
Humulene	30.761		0.68	0.20
cis-Muurola-4(15),5-diene	31.029			0.56
Germacrene D	31.521		0.55	0.64
(3Z,6E)-α-Farnesene	31.841		7.75	
Bicyclogermacrene	31.916		0.12	0.80
δ-Guaiene	32.134			0.45
α-Farnesene	32.142		0.75	
γ-Cadinene	32.326			1.85
δ-Cadinene	32.522		0.26	0.62
Nerolidol	33.333		0.25	0.15
Spathulenol	33.685			0.23
Caryophyllene oxide	33.804	0.30	0.46	
Epicubenol	34.384			0.45
tau-Cadinol	34.830			3.74
β-Eudesmol	35.012			0.25
α-Cadinol	35.057			0.18
Phytol	39.634		0.09	
Total		98.76	98.65	98.41

RESULTS

Essential Oil Yield and Composition

Through steam distillation, essential oils were extracted from 300 g of dried leaves of each herb, yielding 1.70 ml (0.57%) of *Mentha spicata* essential oil, 1.10 ml (0.37%) of *Perilla frutescens* essential oil, and 2.2 ml (0.74%) of *Ocimum basilicum* essential oil. The chemical compositions of these essential oils are summarized in Table 2. A total of 20 compounds were identified in *Mentha spicata*, 23 in

Perilla frutescens, and 30 in *Ocimum basilicum*, collectively accounting for over 98% of the total constituents in these oils. The major components of *Mentha spicata* were cis-dihydrocarveol (1.24%), β-bourbonene (7.56%), 1,8-cineole (15.40%), D-limonene (17.60%), and carvone (48.90%). In *Perilla frutescens*, the predominant compounds included α-linalool (1.30%), perillyl alcohol (1.33%), estragole (1.17%), D-limonene (6.54%), (3Z,6E)-α-farnesene (7.77%), β-caryophyllene (8.32%), and perilla aldehyde (65.23%). Meanwhile, the key constituents of *Ocimum basilicum* essential oil were trans-α-bergamotene (3.49%), tau-cadinol (3.74%), β-linalool (5.34%), 1,8-cineole (6.72%), methyl eugenol (12.72%), and estragole (52.65%).

Table 3: Results of culture and isolation E. coli from intrauterine inflammatory fluid samples.

Bacterial culture media	Positive samples	Ratio (%)	Bacterial culture characteristics
BPW	5/5	100	Bacteria grow in a uniformly turbid medium
MC	5/5	100	The colonies are large, round, uniform, light pink in color, with a slightly convex surface, non-mucoid, well-defined edges, and the surrounding medium also turns pink
EMB	5/5	100	The colonies are round, shiny, dark purple in color, with an iridescent metallic green sheen

BPW: Buffered Peptone Water; **MC:** MacConkey Agar; **EMB:** Eosin Methyl Blue Agar.

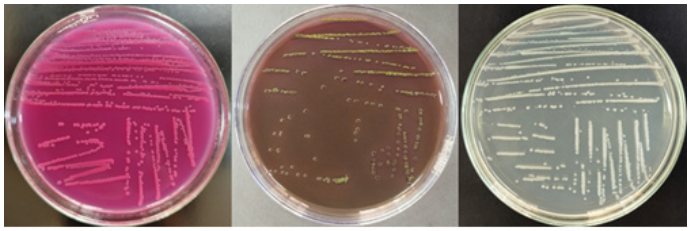

Figure 1: Suspected *Escherichia coli* colonies on (A) MacConkey agar, (B) Eosin Methyl Blue agar, and (C) Nutrient agar.

Bacterial Cultivation and Isolation

The samples were first enriched in BPW medium, where all showed turbidity, confirming a 100% positivity rate. Bacteria from the BPW medium were then transferred to MC and EMB media for further culturing. On MC medium, positive colonies were large, round, pale pink, slightly convex, non-mucoid, with distinct edges, and caused the surrounding medium to turn pink. On EMB medium, the colonies were round, glossy, dark purple-black, and exhibited a metallic sheen, also indicating a 100% positivity rate. The bacteria were subsequently purified and sub-cultured on NA medium to generate biomass for biochemical identification and gene sequencing. The findings of bacterial culture and isolation are shown in Table 3 and Figure 1.

Bacterial Identification Through the API 20E Biochemical Test Kit

The bacterial identification results using the API 20E biochemical kit are displayed in Table 4. The results reveal that three of the five test samples tested positive for *Escherichia coli*, which were isolated from uterine inflammatory fluid.

Table 4: Biochemical identification of bacteria isolated from intrauterine inflammatory fluid using the API 20E kit.

Identification code determined	Identified bacterial species
5144572	*Escherichia coli*
5144552	*Escherichia coli*
5144573	*Escherichia coli*
5205773	*Raoultella ornithinolytica*
7214773	*Klebsiella pneumoniae*

Table 5: Identification of bacteria isolated from intrauterine inflammatory fluid using the PCR method.

Number of samples	Positive samples	Identified bacterial species
3	2	*Escherichia coli*

PCR-Based Identification of Isolated Bacteria

Table 5 presents the results of bacterial identification by PCR. The findings show that two out of five test samples were positive for *Escherichia coli*. A comparison of the sequenced genes with the NCBI database revealed 99.86% similarity and 100% coverage with *Escherichia coli (S1),* 100% similarity and 100% coverage with *Escherichia coli (S2)*.

Antibiotic Resistance Testing Results for Isolated *Escherichia coli*

The results of the antibiotic susceptibility testing of the isolated *Escherichia coli* are shown in Table 6. The antibiotic susceptibility profiles of the two isolated bacteria, *Escherichia coli (S1)* and *Escherichia coli (S2)*, were identical in terms of sensitivity and resistance to the tested antibiotics. The results show that among the 12 antibiotics tested, the isolated *Escherichia coli* was sensitive to norfloxacin, cefuroxime, tetracycline, kanamycin, gentamycin, and tobramycin. Among these, the bacteria showed the highest sensitivity to norfloxacin. Therefore, norfloxacin was selected in combination with essential oils to determine the bactericidal role of the interaction between the essential oils and antibiotics in the next experiment. However, the isolated *Escherichia coli* exhibited high insensitivity to amoxicillin, ampicillin, ceftazidime, streptomycin, colistin, doxycycline.

Table 6: Antibiotic susceptibility results of the isolated *Escherichia coli*.

Antibiotics	Levels (µg)	Zone of growth inhibition (mm)					
		Escherichia coli - S1			Escherichia coli - S2		
		S	I	R	S	I	R
Ampicillin (Am)	10			6.33±0.58			7.33±1.00
Amoxicillin (Ax)	10			10.00±1.00			7.00±1.15
Ceftazidime (Cz)	30			16.67±0.58			15.33±0.58
Cefuroxime (Cu)	30	18.00±1.00			20.00±1.15		
Colistin (Co)	10			10.67±1.15			8.67±1.53
Doxycycline (Dx)	30		16.33±0.58			15.67±1.15	
Gentamycin (Ge)	10	16.67±1.15			16.00±1.00		
Kanamycin (Kn)	30	16.33±1.15			15.33±0.58		
Norfloxacin (Nr)	10	30.00±1.00			31.00±1.15		
Streptomycin (Sm)	10			11.67±0.58			11.33±0.58
Tetracycline (Te)	30	19.67±1.53			18.67±0.58		
Tobramycin (Tb)	10	16.33±1.15			15.33±0.58		

S: sensitive; **I:** intermediate; **R:** resistant. The values are expressed as the mean ± standard deviation.

Table 7: The minimum bactericidal concentration (MBC) and fractional bactericidal concentration index (FBC) of essential oils (*Mentha spicata, Perilla frutescens*, and *Ocimum basilicum*) and norfloxacin used alone and in combination against isolated Escherichia coli

Compounds		Escherichia coli (S1)			Escherichia coli (S2)		
		MBC (µg/ml)	FBC (index)	Type of interaction	MBC (µg/ml)	FBC (index)	Type of interaction
Alone	MS	13,000±150[a]			14,000±150[a]		
	PF	12,000±100[b]			12,000±100[b]		
	OB	12,000±200[b]			12,500±100[b]		
	NR	125±5[c]			130±5[c]		
Combination	OB + NR	4,500/15.62	0.50	Synergistic	4,000/16.25	0.45	Synergistic
	MS + NR	7,500/31.25	0.83	Additive	8,500/32.5	0.86	Additive
	PF + NR	12,000/62.5	1.50	Indifferent	10,500/65	1.38	Indifferent

MS: *Mentha spicata*, **PF:** *Perilla frutescens*, **OB:** *Ocimum basilicum*, **Nr:** Norfloxacin. Superscript letters (a, b, and c) within the same column indicate statistically significant differences (P<0.05). The values represent mean ± SD.

Antibacterial Activity of Essential Oils and Norfloxacin used Alone and in Combination Against Isolated *Escherichia coli*

Table 7 outlines the bactericidal effects of the essential oils and the antibiotic, along with their interactions when combined with norfloxacin against isolated *Escherichia coli*. The findings showed that the essential oils and the antibiotic exhibited similar effects on the two bacterial strains, *Escherichia coli* (S1) and *Escherichia coli* (S2). Among the tested essential oils, *Mentha spicata* had the highest MBC value, which was significantly different from those of the other essential oils and norfloxacin for both bacterial strains. Meanwhile, the MBC values of *Ocimum basilicum* and *Perilla frutescens* were comparable to each other and significantly higher than that of norfloxacin ($P<0.05$). This indicates that *Mentha spicata* exhibited lower bactericidal activity against *Escherichia coli* compared to *Ocimum basilicum* and *Perilla frutescens*. Norfloxacin displayed strong bactericidal activity, with an MBC value of 125–130 µg/ml. Although the MBC values of the essential oils were nearly 100 times greater than that of norfloxacin, all essential oils demonstrated antibacterial effects against the isolated *Escherichia coli*. Analysis of their interactions with norfloxacin revealed that *Ocimum basilicum* essential oil exhibited a synergistic effect when combined with norfloxacin, whereas *Mentha spicata* and *Perilla frutescens* essential oils displayed additive and indifferent effects, respectively, in eradicating *Escherichia coli*. The MBC of norfloxacin against the isolated *Escherichia coli* was reduced by 8-fold when combined with *Ocimum basilicum* essential oil, 4-fold with *Mentha spicata*, and 2-fold with *Perilla frutescens*. Additionally, the MBC values of the essential oils signifi-

cantly decreased in the presence of norfloxacin, reaching 4,000–4,500 µg/ml for *Ocimum basilicum*, 7,500–8,500 µg/ml for *Mentha spicata*, and 10,500–12,000 µg/ml for *Perilla frutescens*.

DISCUSSION

The culture isolation results confirmed the presence of *Escherichia coli* bacteria in the uterine exudate of dogs affected by pyometra. This suggests that *Escherichia coli* plays a role as a causative agent of pyometra in dogs. These results align with findings from previous research (Hagman and Kuhn, 2002; Harada *et al*., 2024; Ylhäinen *et al*., 2025). The results of bacterial identification using the API 20E biochemical kit and gene sequencing were consistent, confirming the high reliability of the API 20E kit. This suggests that it is a useful tool for preliminary screening before applying gene sequencing for bacterial identification. Antibiotic susceptibility testing revealed that the two isolated *Escherichia coli* strains exhibited complete resistance to amoxicillin, ampicillin, ceftazidime, streptomycin, colistin, and doxycycline - antibiotics widely used in both veterinary and human medicine. These findings align with previous studies on antibiotic resistance in *E. coli* strains isolated from pyometra in dogs. The increasing resistance of microorganisms to antibiotics presents a major challenge, highlighting the urgent need for further research to develop alternative antimicrobial agents, including plant-derived essential oils. In addition, the two isolated *Escherichia coli* strains showed identical resistance and susceptibility profiles to the tested antibiotics. This may be attributed to the fact that the samples were collected from the same region, where animals were treated within the same veterinary network. The prolonged and frequent use of specific antibiotics in feed, water, and disease management by veterinarians and livestock farmers has likely contributed to the emergence of similar resistance patterns in certain microorganisms, including *Escherichia coli*.

This study assessed the bactericidal effects of essential oils extracted from *Mentha spicata*, *Perilla frutescens*, and *Ocimum basilicum* against isolated *Escherichia coli*. The findings revealed that all three essential oils effectively eliminated the bacteria. Their bactericidal activity may be attributed to the presence of specific chemical constituents with antimicrobial properties or the synergistic interaction of bioactive compounds, which enhance their effectiveness.

Numerous studies have highlighted the strong bactericidal potential of bioactive compounds present in these three essential oils. *Perilla frutescens* and *Ocimum basilicum* essential oils contain estragole, linalool, and thymol, which have been shown to inhibit *Shigella flexneri* (Ngome *et al*., 2018). Additionally, 1,8-cineole, a key component in the essential oils of *Mentha spicata* and *Ocimum basilicum*, exhibits bactericidal activity against *Klebsiella pneumoniae* (Moo *et al*., 2021) and *Escherichia coli* (Wang *et al*., 2022). Furthermore, carvone and D-limonene, found in both *Mentha spicata* and *Perilla frutescens* essential oils, have been reported to eliminate common bacterial pathogens affecting humans and livestock (Jacob *et al*., 2017; Agougui *et al*., 2022). Moreover, eugenol, a major component of *Ocimum basilicum* essential oil, has demonstrated bactericidal activity against *Staphylococcus aureus* and *Escherichia coli* (Jeyakumar and Lawrenceb, 2021; Bai *et al*., 2023).

The results indicated that the bactericidal effectiveness of the three essential oils varied, with *Ocimum basilicum* and *Perilla frutescens* showing higher bactericidal activity than *Mentha spicata*. The differences in bactericidal effectiveness among these essential oils can be explained by the diversity of bioactive compounds present in each oil, which leads to different mechanisms of action against bacteria. Additionally, the synergistic or inhibitory effects of bioactive compounds within essential oils also influence their bactericidal effectiveness. Previous studies have identified the synergistic interactions or inhibitory effects of bioactive compounds on the bactericidal activity of herbal essential oils (Kamatou *et al*., 2008; Sharmaa *et al*., 2020; Angane *et al*., 2023). The bioactive compounds in essential oils are hydrophobic, which disrupts the lipid components of bacterial cell membranes and mitochondria, increasing their permeability. This disruption leads to the breakdown of the cell wall and cytoplasmic membrane, causing lysis and leakage of intracellular compounds, adversely affecting metabolism and resulting in cell death (Nazzaro *et al*., 2013). In addition, the active components of essential oils affect bacterial intracellular proteins, impacting cell division (Domadia *et al*., 2007). Although gram-negative bacteria have higher resistance to essential oils than gram-positive bacteria cause of the unique structure of their cell walls, which include an additional outer membrane linked to peptidoglycan by lipoproteins, this double membrane limits the effects of essential oils (Swamy *et al*., 2016). Carvone, estragole, and perilla aldehyde, found in *Mentha spicata*, *Ocimum basilicum*, and *Perilla frutescens*, exhibit stronger antibacterial activity against Gram-negative bacteria than Gram-positive bacteria. This is due to their ability to disrupt bacterial membranes, interfere with ion and ATP transport, and alter fatty acid composition. Carvone, a hydrophobic compound, integrates into bacterial cell membranes, disturbing their fluidity and permeability. This disruption leads to ion leakage (e.g., K^+, Na^+), ATP depletion, and ultimately, cell lysis. Moreover, carvone inhibits bacterial enzymes crucial for ATP synthesis, reducing energy production. It also interferes with DNA replication and protein synthesis, slowing bacterial growth. Since biofilms help bacteria evade antibiotics and immune responses, carvone plays a vital role in disrupting quorum sensing, thereby preventing *Escherichia coli* and other bacteria from forming biofilms (Bouyahya *et*

al., 2021). Estragole, due to its lipophilic nature, embeds itself in bacterial membranes, compromising their integrity and increasing permeability. This causes intracellular leakage, leading to bacterial cell death. Moreover, estragole inhibits essential bacterial metabolic enzymes, particularly those involved in energy production and cell wall synthesis, thereby impairing bacterial growth and replication (Song *et al*., 2016). As an aldehyde, perilla aldehyde interacts with membrane lipids and proteins, altering their structure and function. This results in membrane disruption and intracellular leakage. Similar to carvone, perilla aldehyde also induces the production of reactive oxygen species (ROS), which damage bacterial cellular components. The oxidative stress ultimately triggers apoptosis, leading to bacterial cell death (Li *et al*., 2022).

The combination of essential oils with norfloxacin lowered the MBC values of both the essential oils and the antibiotic against the isolated *Escherichia coli*. The combination of *Ocimum basilicum* essential oil with the antibiotic demonstrated a synergistic interaction, whereas the combinations of *Mentha spicata* and *Perilla frutescens* essential oils with the antibiotic resulted in additive and indifferent effects, respectively.

This finding is in agreement with previous studies on the synergistic interactions between essential oils and antibiotics (Moussaoui and Alaoui, 2016; Boonyanugomol *et al*., 2017; Romo-Castillo *et al*., 2023; Drioiche *et al*., 2024). The minimum bactericidal concentration of norfloxacin against *Escherichia coli* was significantly lowered when combined with essential oils. Both experimental findings and existing research indicate that integrating essential oils with antibiotics can reduce the required antibiotic dosage and help mitigate bacterial resistance. In other words, this combination enhances bacterial sensitivity to antibiotics, presenting a promising strategy for developing novel treatments against multidrug-resistant pathogens. The synergistic and additive effects of *Ocimum basilicum* and *Mentha spicata* essential oils in combination with norfloxacin result from their ability to enhance the elimination of *Escherichia coli*. Norfloxacin, a second-generation quinolone antibiotic, enters bacterial cells and inhibits DNA gyrase, a crucial enzyme for DNA replication in *Escherichia coli*. This penetration becomes more efficient with the assistance of antibacterial compounds found in *Ocimum basilicum* and *Mentha spicata* essential oils. These compounds disrupt the bacterial cell membrane, facilitating the transport of norfloxacin across the membrane and enabling it to reach an effective concentration more rapidly. At the same time, the enhanced inhibition of *Escherichia coli* by norfloxacin decreases the required amount of *Ocimum basilicum* and *Mentha spicata* essential oils to achieve bactericidal effects. Conversely, the combination of *Perilla frutescens* essential oil with norfloxacin exhibited an indifferent interaction in eliminating *Escherichia coli*, as both acted independently without enhancing each other's bactericidal effects. Perillaldehyde, a key component of *Perilla frutescens* essential oil, can penetrate deeper and more complex regions of the cell, depleting intracellular ATP. However, it does not disrupt the outer membrane of *Escherichia coli* (Dorman and Deans, 2000), meaning it does not facilitate norfloxacin's permeability, nor does norfloxacin enhance its activity. As a result, while the combination requires a lower concentration than when used separately, the interaction remains indifferent.

CONCLUSIONS AND RECOMMENDATIONS

The study concluded that essential oils from *Ocimum basilicum*, *Mentha spicata*, and *Perilla frutescens* were effective in killing antibiotic-resistant *Escherichia coli* isolated from dogs with pyometra. The combination of *Ocimum basilicum* and *Mentha spicata* essential oils with norfloxacin demonstrated synergistic and additive effects in eliminating *Escherichia coli*, whereas *Perilla frutescens* essential oil showed an indifferent interaction when paired with the antibiotic. These essential oils, when used alongside antibiotics, could be integrated into treatment strategies for canine diseases, potentially reducing antibiotic dosage, minimizing side effects, and helping to combat antibiotic resistance in the future.

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NOVELTY STATEMENT

This study presents an innovative method that combines essential oils from *Ocimum basilicum*, *Mentha spicata*, and *Perilla frutescens* with the antibiotic norfloxacin to combat antibiotic-resistant *Escherichia coli* isolated from dogs with pyometra. The findings indicate that *Ocimum basilicum* and *Mentha spicata* essential oils, when used alongside norfloxacin, produced synergistic and additive effects in eradicating *Escherichia coli*, whereas *Perilla frutescens* essential oil exhibited an indifferent interaction with the antibiotic.

AUTHOR'S CONTRIBUTIONS

Nguyen Van Vui conceptualized and designed the experiments. Nguyen Van Tung Lam, Nguyen Thuy Linh and Le My Duyen carried out the experimental work. Nguyen Van Vui and Le My Duyen analyzed the data and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

REFERENCES

Adams RD (2007). Identification of essential oil components by gas chromatography/ mass spectrometry. (Allured: IL, USA).

Agougui C, Cecilia JA, Saad H, Franco-Duro F, Essid R, Khabbouchi M, Frini-Srasra N (2022). Adsorption of Carvone and Limonene from Caraway essential oil onto Tunisian montmorillonite clay for pharmaceutical application. Sci. Rep., 12: 19814. https://doi.org/10.1038/s41598-022-24268-5

Ahmed HM, Al-Zubaidy AMA (2020). Exploring natural essential oil components and antibacterial activity of solvent extracts from twelve Perilla frutescens L. Genotypes. Arab. J. Chem., 13: 7390–7402. https://doi.org/10.1016/j.arabjc.2020.08.016

Angane M, Swift S, Huang K, Perera J, Butts, Chen X, AC, Quek, Young S (2023). Synergistic antimicrobial interaction of plant essential oils and extracts against foodborne pathogens. Food Sci. Nutr., 12: 1189–1206. https://doi.org/10.1002/fsn3.3834

Aouadhi C, Jouini A, Maaroufi K, Maaroufi A (2024). Antibacterial Effect of Eight Essential Oils against Bacteria Implicated in Bovine Mastitis and Characterization of Primary Action Mode of Thymus capitatus Essential Oil Chedia. Antibiotics, 13: 1–12. https://doi.org/10.3390/antibiotics13030237

Bai J, Li J, Chen Z, Bai X, Yang Z, Wang Z, Yang Y (2023). Antibacterial activity and mechanism of clove essential oil against foodborne pathogens. LWT 173: 114249. https://doi.org/10.1016/j.lwt.2022.114249

Bava R, Castagna F, Lupia C, Poerio G, Liguori G, Lombardi R, Naturale MD, Mercuri C, Bulotta RM, Britti D, Palma E (2024). Antimicrobial Resistance in Livestock: A Serious Threat to Public Health. Antibiotics, 13: 1–17. https://doi.org/10.3390/antibiotics13060551

Boonyanugomol W, Kraisriwattana, Kairin, Rukseree K, Boonsam K, Narachai P (2017). In vitro synergistic antibacterial activity of the essential oil from Zingiber cassumunar Roxb against extensively drug-resistant Acinetobacter baumannii strains. J. Infect. Public Health, 10: 586–592. https://doi.org/10.1016/j.jiph.2017.01.008

Bouyahya A, Mechchate H, Benali T, Ghchime R, Charfi S, Balahbib A, Burkov P, Shariati MA, Lorenzo JM, Omari N El (2021). Health Benefits and Pharmacological Properties of Carvone. Biomolecules, 11: 1–26. https://doi.org/10.3390/biom11121803

Carroll KC, Pfaller MA (2023). Manual of Clinical Microbiology. (ASM Press).

Carvalhoa AC, Barbosab AV, Araisb LR, Ribeirob PF, Carneirob VC, Cerqueirab AMF (2016). Resistance patterns, ESBL genes, and genetic relatedness of *Escherichia coli* from dogs and owners. Brazilian J. Microbiol., 47: 150–158. https://doi.org/10.1016/j.bjm.2015.11.005

CLSI (2024). Performance Standards for Antimicrobial Susceptibility Testing. (Wayne, PA: Clinical and Laboratory Standards Institute).

Domadia P, Swarup S, Bhunia A, Sivaraman, Dasgupta D (2007). Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde. Biochem. Pharmacol., 74: 831–840. https://doi.org/10.1016/j.bcp.2007.06.029

Dorman HJD, Deans SG (2000). Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J. Appl. Microbiol., 88: 308–316. https://doi.org/10.1046/j.1365-2672.2000.00969.x

Drioiche A, Baammi S, Zibouh K, Kamaly O Al, Alnakhli AM, Remok F, Saidi S, Amaiach R, Makhoukhi F El, Elomri A, Zair T (2024). A Study of the Synergistic Effects of Essential Oils from *Origanum compactum* and *Origanum elongatum* with Commercial Antibiotics against Highly Prioritized Multidrug-Resistant Bacteria for the World Health Organization. Metabolites, 14: 1–34. https://doi.org/10.3390/metabo14040210

Elhafez ZAA (2022). The Effect of Different Extraction Methods (Steam Distillation and Supercritical Fluid Extraction) on Essential Oil Production of Aromatic Tagetes and Sweet Basil. European J. Med. Plants, 33: 1–6. https://doi.org/10.9734/ejmp/2022/v33i630468

Galgano M, Capozza P, Pellegrini F, Cordisco M, Sposato A, Sblano S, Camero M, Lanave G, Fracchiolla G, Corrente M, Cirone F, Trotta A, Tempesta M, Buonavoglia D, Pratelli A (2022). Antimicrobial Activity of Essential Oils Evaluated In Vitro against *Escherichia coli* and *Staphylococcus aureus*. Antibiotics, 11: 1–13. https://doi.org/10.3390/antibiotics11070979

Hagman R (2023). Pyometra in Small Animals 3.0. Vet. Clin. North Am. Small Anim. Pract., 53: 1223–1254. https://doi.org/10.1016/j.cvsm.2023.04.009

Hagman R, Kuhn I (2002). Escherichia coli strains isolated from the uterus and urinary bladder of bitches suffering from pyometra: comparison by restriction enzyme digestion and pulsed-field gel electrophoresis. Vet. Microbiol., 84: 143–153. https://doi.org/10.1016/S0378-1135(01)00449-7

Harada K, Miyamoto T, Sugiyama M, Asai T (2024). First report of a blaNDM-5-carrying *Escherichia coli* sequence type 12 isolated from a dog with pyometra in Japan. J. Infect. Chemother., 30: 938–941. https://doi.org/10.1016/j.jiac.2024.02.013

Jacob MI, Gondo GDGA, Pierri EG, Pietro RCLR, Soares CP, De DP, Sousa, Santos AG dos (2017). Evaluation of antimicrobial, cytotoxic and chemopreventive activities of carvone and its derivatives. Brazilian J. Pharm. Sci., 54: 1–8.

Jeyakumar E, Lawrenceb R (2021). Mechanisms of bactericidal action of Eugenol against Escherichia coli. J. Herbal Med., 26: 100406. https://doi.org/10.1016/j.hermed.2020.100406

Kamatou GPP, Zyl RL van, Davids H, Vuuren SF van, Viljoen AM (2008). Synergistic and Antagonistic Interactions of Essential Oils on the Biological Activities of the Solvent Extracts from Three Salvia species Guy. Nat. Prod. Commun., 3: 1111–1115. https://doi.org/10.1177/1934578X0800300714

Li X, Chen F, Xiong Y, Guo L, Xu J, Lin Y, Ni K, Yang F (2022). Perilla frutescens as potential antimicrobial modifier to against forage oat silage spoilage. Front. Microbiol., 13: 1053933. https://doi.org/10.3389/

fmicb.2022.1053933

Mahboubi M (2021). *Mentha spicata* L. essential oil, phytochemistry and its effectiveness in flatulence. J. Tradit. Complement. Med., 11: 75–81. https://doi.org/10.1016/j.jtcme.2017.08.011

Mamun MM, Parvej MS, Ahamed S, Hassan J, Nazir KHMNH, Nishikawa Y, Rahman MT (2016). Prevalence and characterization of shigatoxigenic *Escherichia coli* in broiler birds in Mymensingh. Bangladesh J. Vet. Med., 14: 5–8. https://doi.org/10.3329/bjvm.v14i1.28809

Moo CL, Osman MA, Yang SK, Yap WS, Ismail S, Lim SHE, Chong CM, Lai KS (2021). Antimicrobial activity and mode of action of 1,8-cineol against carbapenemase-producing Klebsiella pneumoniae. Sci. Rep., 11: 20824. https://doi.org/10.1038/s41598-021-00249-y

Moussaoui F, Alaoui T (2016). Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. Asian Pac. J. Trop. Biomed., 6: 32–37. https://doi.org/10.1016/j.apjtb.2015.09.024

Nair DVT, Venkitanarayanan K, Johny AK (2018). Antibiotic-Resistant Salmonella in the Food Supply and the Potential Role of Antibiotic Alternatives for Control. Foods, 7: 1–24. https://doi.org/10.3390/foods7100167

Nazzaro F, Fratianni F, Martino L De (2013). Effect of Essential Oils on Pathogenic Bacteria. Pharmaceuticals, 6: 1451–1474. https://doi.org/10.3390/ph6121451

Ngome MT, Guilherme J, Ferreira L, Cristina A, Oliveira F De, Machado S, Lucía O, Bernal M, Piccoli RH (2018). Linalool, citral, eugenol and thymol: control of planktonic and sessile cells of Shigella flexneri. AMB Express, 8: 1–10. https://doi.org/10.1186/s13568-018-0634-z

Pandey AK, Singh P, Tripathi NN (2014). Chemistry and bioactivities of essential oils of some Ocimum species: An overview. Asian Pac. J. Trop. Biomed., 4: 682–694. https://doi.org/10.12980/APJTB.4.2014C77

Rafael Gariglio Clark Xavier, Santana CH, Silva PHS da, Paraguassú AO, Rafael Romero Nicolino PMCF, Santos R de L, Silva ROS (2024). Association between bacterial pathogenicity, endometrial histological changes and clinical prognosis in canine pyometra. Theriogenology, 214: 118–123. https://doi.org/10.1016/j.theriogenology.2023.10.007

Romo-Castillo M, Flores-Bautista VA, Guzmán-Gutiérrez SL, Reyes-Chilpa R, León-Santiago M, Luna-Pineda VM (2023). Synergy of Plant Essential Oils in Antibiotic Therapy to Combat Klebsiella pneumoniae Infections. Pharmaceuticals, 16: 1–17. https://doi.org/10.3390/ph16060839

Sharmaa K, Guleriaa S, Razdanb VK, Babu V (2020). Synergistic antioxidant and antimicrobial activities of essential oils of some selected medicinal plants in combination and with synthetic compounds. Ind. Crops Prod., 154: 112569. https://doi.org/10.1016/j.indcrop.2020.112569

Song YR, Choi MS, Choi GW, Park IK, Oh CS (2016). Antibacterial activity of cinnamaldehyde and estragole extracted from plant essential oils against Pseudomonas syringae pv. Actinidiae causing bacterial canker disease in kiwifruit. Plant Pathol. J., 32: 363–370. https://doi.org/10.5423/PPJ.NT.01.2016.0006

Swamy MK, Akhtar MS, Sinniah UR (2016). Antimicrobial Properties of Plant Essential Oils against Human Pathogens and Their Mode of Action: An Updated Review. Evidence-Based Complement. Altern. Med., 3012462. https://doi.org/10.1155/2016/3012462

Wang Y, Zhang Y, Song X, Fang C, Xing R, Liu L, Zhao X, Zou Y, Li L, Jia R, Ye G, Shi F, Zhou X, Zhang Y, Wan H, Wei Q, Yin Z (2022). 1,8-Cineole inhibits biofilm formation and bacterial pathogenicity by suppressing luxS gene expression in *Escherichia coli*. Front. Pharmacol., 13: 988245. https://doi.org/10.3389/fphar.2022.988245

Xu C, Kong L, Gao H, Cheng X, Wang X (2022). A Review of Current Bacterial Resistance to Antibiotics in Food Animals. Front. Microbiol., 13: 1–16. https://doi.org/10.3389/fmicb.2022.822689

Ylhäinen A, Mölsä S, Thomson K, Laitinen-Vapaavuori O, Rantala M, Grönthal T (2025). Bacteria associated with canine pyometra and concurrent bacteriuria: A prospective study. Vet. Microbiol., 301: 110362. https://doi.org/10.1016/j.vetmic.2024.110362