OPEN ACCESS Advances in Animal and Veterinary Sciences

Research Article

The Impact of *Melaleuca leucadendron* and *Eucalyptus camaldulensis* Essential Oils in Combination with Norfloxacin on Antibiotic-Resistant *Salmonella* spp. Isolated from Chickens

Nguyen Van Vui[1*], Ly Thi Thu Lan[2], Nguyen Van Tung Lam[3], Huynh Thanh Tan[1]

[1]Department of Animal Science and Veterinary Medicine, Faculty of Agriculture and Aquaculture, Tra Vinh University, Vietnam; [2]Faculty of Agriculture and Aquaculture, Tra Vinh University, Vietnam; [3]Department of Assessment, Tra Vinh University, Vietnam.

Abstract | The overuse of antibiotics in livestock production has contributed to the rise of antibiotic-resistant bacteria, posing a significant risk to both animal and human health. Essential oils from *Melaleuca leucadendron* and *Eucalyptus camaldulensis* contain bioactive compounds with bactericidal properties. This study aimed to assess their effectiveness, both individually and in combination with norfloxacin, against antibiotic-resistant *Salmonella* spp. isolated from diseased chickens. Antibiotic susceptibility of the bacterial isolates was evaluated using the disk diffusion method, while their bactericidal activity was determined by measuring the minimum bactericidal concentration (MBC). The interaction between essential oils and antibiotics was assessed using the fractional bactericidal concentration index through a microdilution checkerboard assay. *Salmonella typhimurium* and *Salmonella enteritidis* were isolated from infected chickens, showing notable resistance to ampicillin, streptomycin, and colistin. The MBC of *Melaleuca leucadendron* essential oil was 13,000 µg/ml, whereas *Eucalyptus camaldulensis* exhibited a higher MBC of 20,000 µg/ml. When combined with norfloxacin, both essential oils demonstrated a synergistic effect, significantly enhancing bacterial eradication. These findings highlight the potential of using *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils alongside norfloxacin to combat antibiotic-resistant *Salmonella* spp. in poultry. The study suggests that integrating essential oils into treatment strategies could improve antibiotic efficacy and help mitigate resistance in livestock.

Keywords | *Melaleuca leucadendron*, *Eucalyptus camaldulensis,* Antibiotic-resistance, *Salmonella* spp., Synergistic, Antibacterial

Received | March 19, 2025; **Accepted** | March 31, 2025; **Published** | May 03, 2025
***Correspondence** | Nguyen Van Vui, Department of Animal Science and Veterinary Medicine, Faculty of Agriculture and Aquaculture, Tra Vinh University, Vietnam; **Email:** nvvuity@tvu.edu.vn
Citation | Vui NV, Lan LTT, Lam NVT, Tan HT (2025). The impact of *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils in combination with norfloxacin on antibiotic-resistant *Salmonella* spp. isolated from chickens. Adv. Anim. Vet. Sci., 13(6): 1149-1159.
DOI | https://dx.doi.org/10.17582/journal.aavs/2025/13.6.1149.1159
ISSN (Online) | 2307-8316

INTRODUCTION

Currently, the poultry farming industry is expanding at a rapid pace, with increasing poultry populations to meet the rising demand for poultry products. However, this rapid growth has also heightened the risk of disease outbreaks, which pose a significant threat to poultry health and farm productivity. Among the various diseases affecting poultry, diarrhea is one of the most frequent and persistent health challenges. There are multiple causes of diarrhea in poultry, including bacterial, viral, and parasitic infections, as well as poor management practices related to nutrition and hygiene. Among these, *Salmonella* spp. is one of the most prevalent and dangerous bacterial

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pathogens responsible for diarrhea in poultry. *Salmonella* spp. is widely distributed in poultry environments and can persist in the digestive system, faeces, feed, drinking water, and bedding materials of chickens (Lutful, 2010). Under normal conditions, the immune system of healthy chickens can control the bacterial load, preventing infection. However, when poultry experience stress due to poor nutrition, overcrowding, environmental fluctuations, or co-infections with other pathogens, their immune defences weaken, allowing *Salmonella* spp. to invade, multiply, and cause disease.

To manage bacterial infections and prevent disease outbreaks, poultry farmers have increasingly relied on the routine use of antibiotics in feed and drinking water as a preventive measure. Additionally, when disease occurs, antibiotics are often administered in high doses to treat infections. However, the excessive and indiscriminate use of antibiotics in poultry farming has led to the emergence of antibiotic-resistant *Salmonella* strains. Numerous studies have shown that *Salmonella* spp. isolated from diseased poultry exhibit a high level of resistance to commonly used antibiotics (Castro-Vargas *et al*., 2020; Tan *et al*., 2022). Alarmingly, some strains of *Salmonella* spp. have developed multidrug resistance, meaning they can withstand the effects of multiple antibiotic classes, making treatment increasingly difficult (Punchihewage-Don *et al*., 2023). Furthermore, *Salmonella* spp. has demonstrated resistance to several broad-spectrum antibiotics, including fluoroquinolones and cephalosporins, which are critical for treating bacterial infections in both animals and humans. The ability of *Salmonella* spp. to acquire and transfer antibiotic resistance genes poses a significant risk, as these resistant bacteria can spread from poultry to humans through direct contact, contaminated food, or environmental exposure. Studies have confirmed that *Salmonella* spp. can transfer antibiotic resistance to human-associated bacteria, further exacerbating the global issue of antibiotic resistance (Nair *et al*., 2018).

To control pathogenic bacteria in poultry and combat antibiotic resistance, researchers are exploring essential oils as natural alternatives to synthetic antibiotics. Several scientific studies have explored and confirmed the efficacy of essential oils as natural antibacterial agents, offering a promising alternative to conventional antibiotics in livestock production (Galgano *et al*., 2022; Aouadhi *et al*., 2024). In our country, a wide variety of medicinal plants have been traditionally used for their therapeutic properties. Among them, *Melaleuca leucadendron* and *Eucalyptus camaldulensis* stand out due to their high essential oil content and potent bioactive properties. These plants are particularly notable for their ability to produce substantial amounts of essential oils, with yields ranging from 1-2% (Pujiarti *et al*., 2011; Abrahim *et al*., 2024). Their essential oils contain a rich and diverse composition of bioactive compounds, including β-Eudesmol, Eucalyptol (1,8-Cineole), α-Terpineol, D-Limonene, β-Caryophyllene, β-Pinene, p-Cymene, and β-Cymene (Pujiarti *et al*., 2012; Nidal *et al*., 2023; Arisandi *et al*., 2023). These bioactive compounds have been scientifically validated for their strong antimicrobial activities, particularly against a range of bacterial pathogens (Swamy *et al*., 2016; Gayuh *et al*., 2022; Constantia *et al*., 2023; Zaki *et al*., 2024). Studies have demonstrated that these compounds can disrupt bacterial cell membranes, interfere with cellular metabolism, and ultimately inhibit or kill bacteria. Given their potent antibacterial properties, the essential oils derived from *Melaleuca leucadendron* and *Eucalyptus camaldulensis* hold great potential in combating *Salmonella* spp., a major causative agent of diarrhea in poultry.

In addition to their direct bactericidal effects, essential oils have also been studied for their potential synergistic interactions with antibiotics. Several studies have reported that the combination of herbal essential oils with antibiotics can enhance the overall antibacterial efficacy, leading to a synergistic effect (Moussaoui and Alaoui, 2016; Boonyanugomol *et al*., 2017; Romo-Castillo *et al*., 2023; Drioiche *et al*., 2024). Combining these essential oils with antibiotics could help combat antibiotic resistance in *Salmonella* spp. while lowering the necessary antibiotic dosage in poultry farming. This study investigated the bactericidal effects of *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils, both alone and in combination with an antibiotic, against antibiotic-resistant *Salmonella* spp. isolated from infected chickens. This approach supports the urgent search for alternative solutions to improve livestock health, reduce production costs, eliminate antibiotic residues in animal products, and enhance the overall efficiency and sustainability of poultry farming.

MATERIALS AND METHODS

Analysis and extraction of volatile compounds from essential oils

Melaleuca leucadendron and *Eucalyptus camaldulensis* leaves were collected from the natural in Tra Vinh Province, Vietnam. The Department of Crop Science, Tra Vinh University, identified the plants based on stem, branch, and leaf morphology. Essential oils were extracted via steam distillation. Leaves were dried at 40°C, then 150 g of dried leaves and 500 ml of water were distilled in a 1000 ml Clevenger flask at 100°C for 5 hours. The oils were separated using sodium sulfate and stored at 0-4°C until use.

Gas chromatography-mass spectrometry (GC-MS) was used to analyse the composition and relative abundance of volatile compounds in the essential oils (Adams, 2007).

Bacterial cultivation and isolation

Salmonella spp. was isolated from 10 clinical samples (cloacal secretion and internal organs) collected from diseased chickens in Tra Vinh Province, Vietnam. Infected chickens showed typical symptoms, including lethargy, emaciation, white diarrhea, and liver necrosis. Samples were enriched in Buffered Peptone Water (BPW) at 37°C for 24 hours, then selectively enriched in Rappaport Vassiliadis Soya Broth (RVS) under the same conditions. The bacterial solution was cultured on Xylose-Lysine Deoxycholate Agar (XLD) and MacConkey Agar (MC) to identify *Salmonella* spp. colonies.

Bacterial identification after isolation

After isolation, the bacterial strains were biochemically identified using the API 20E kit (Biomerieux, France), a standardized system designed for the identification of Enterobacteriaceae and other Gram-negative bacteria. The kit consists of 20 miniaturized biochemical tests, allowing for the metabolic profiling of the isolates. The bacterial suspension was prepared and inoculated into the wells of the API 20E strip, followed by incubation at 37°C for 18-24 hours. The test results were interpreted based on colour changes and compared with the API database for bacterial identification.

Samples that tested positive for *Salmonella* spp. were further analysed through polymerase chain reaction (PCR) for molecular confirmation. Genomic DNA was extracted from the isolates and used as a template for PCR amplification. Specific primers targeting *Salmonella* spp.-specific genes were employed to confirm the presence of the bacteria. PCR products were then subjected to gel electrophoresis to verify amplification, and positive samples were further processed for gene sequencing to determine their genetic profiles.

Testing the antibiotic susceptibility of isolated bacteria

The antibiotic susceptibility of the isolated *Salmonella* spp. was evaluated using the disk diffusion method as described by Bauer *et al.* (1966). This method involves placing antibiotic-impregnated disks onto the surface of Mueller-Hinton agar plates that have been evenly inoculated with the bacterial suspension. After incubation at 37°C for 18-24 hours, the diameter of the inhibition zones surrounding each antibiotic disk was measured to determine bacterial susceptibility.

A total of 12 antibiotics were tested, including ampicillin (10 µg), amoxicillin (10 µg), ceftazidime (30 µg), cefuroxime (30 µg), colistin (10 µg), doxycycline (30 µg), gentamicin (10 µg), kanamycin (30 µg), norfloxacin (10 µg), streptomycin (10 µg), tetracycline (24 µg), and tobramycin (10 µg). The interpretation of susceptibility or resistance was based on the Clinical and Laboratory Standards Institute (CLSI, 2024) guidelines.

To ensure the reliability of the results, the experiment was conducted four times, and the average inhibition zone diameters were recorded.

The minimum bactericidal concentration (MBC) of essential oils and antibiotic against isolated bacteria

MBC was determined using a broth microdilution assay following CLSI (2024) guidelines. The bacterial suspension was adjusted to 0.5 McFarland (10^6-10^8 CFU/ml). Essential oils (10-50 mg/ml) and norfloxacin (13.25-1,000 µg/ml) were tested based on preliminary results. These concentrations, along with the bacterial suspension, were incubated at 37°C for 24 hours. After incubation, samples were spread on XLD agar and incubated for another 24 hours. MBC was recorded as the lowest concentration where no bacterial growth was observed. The experiment was repeated four times.

The minimum bactericidal concentration (MBC) of essential oils and antibiotic used in combination against isolated bacteria

The interaction between essential oils and antibiotics was assessed using the fractional bactericidal concentration (FBC) index via the microdilution checkerboard method (Moody, 2003). To perform this assessment, both the essential oils and antibiotics were subjected to serial dilution, reducing their concentrations stepwise to 1/64 of their original minimum bactericidal concentration (MBC). The FBC index was calculated as $FBC^A + FBC^B$, where $FBC^A = MBC^A$ (in combination)/ MBC^A (alone) and $FBC^B = MBC^B$ (in combination)/ MBC^B (alone).

The FBC index classified interactions as synergistic (≤ 0.5), additive (>0.5 to <1), indifferent (≥1 to <4), or antagonistic (≥4.0). The experiment was repeated four times.

Statistical analysis

Statistical analysis was conducted using SPSS software, version 22. To compare the differences in minimum bactericidal concentration (MBC) values between the tested essential oils and the antibiotic Norfloxacin, a one-way analysis of variance (ANOVA) was performed. A post hoc analysis was conducted using Tukey's multiple comparison test to determine which specific groups differed from each other. A P-value of less than 0.05 was considered indicative of statistical significance. The results of the analysis were expressed as the mean value along with the corresponding standard deviation (mean ± SD).

RESULTS

Essential oil composition and yield

Following steam distillation extraction, essential oils were obtained from 150 g of dried leaves of each plant species. Specifically, 0.48 g of *Melaleuca leucadendron* essential oil was collected, corresponding to a yield of 0.32%, while *Eucalyptus camaldulensis* essential oil was obtained in a quantity of 3.3 g, yielding 2.2%. These results indicate a significantly higher extraction efficiency for *Eucalyptus camaldulensis* compared to *Melaleuca leucadendron*. The chemical composition of the extracted essential oils was analyzed using gas chromatography-mass spectrometry (GC-MS), and the results are summarized in Table 1. The predominant constituents detected in *Melaleuca leucadendron* essential oil included β-Eudesmol (11.61%), β-Caryophyllene (10.08%), α-Pinene (8.58%), Caryophyllene oxide (6.53%), o-Cymene (6.29%), α-Humulene (5.72%), Guaiol (5.30%), γ-Eudesmol (5.03%), Methyl Eugenol (4.55%), Humulene epoxide II (3.12%), γ-Terpinene (3.07%), D-Limonene (2.68%), Calamenene (2.47%), α-Terpinene (2.27%), α-Terpinolene (2.18%), β-Selinene (1.98%), α-Selinene (1.60%), 4-Terpineol (1.29%), β-Linalool (1.25%), and Bulnesol (1.23%). Among these components, β-Eudesmol was the most abundant, followed closely by β-Caryophyllene and α-Pinene, suggesting that these compounds may contribute significantly to the biological properties of *Melaleuca leucadendron* essential oil. In contrast, the essential oil of *Eucalyptus camaldulensis* exhibited a different chemical profile, with the major constituents being β-Pinene (21.84%), α-Pinene (19.80%), Eucalyptol (18.17%), D-Limonene (6.11%), α-Terpineol (5.37%), o-Cymene (3.42%), Globulol (3.04%), γ-Terpinene (2.51%), β-Eudesmol (2.33%), 4-Terpineol (1.91%), cis-Caryophyllene (1.82%), Caryophyllene oxide (1.50%), Spathulenol (1.23%), Borneol (1.08%), and Fenchol (1.04%). Notably, β-Pinene, α-Pinene, and Eucalyptol were the dominant components, collectively accounting for nearly 60% of the total composition. The high concentration of monoterpenes, particularly α-Pinene and β-Pinene, suggests that *Eucalyptus camaldulensis* essential oil may exhibit strong antimicrobial properties.

Bacterial culture and isolation

The collected samples were initially subjected to an enrichment process in BPW medium to facilitate bacterial growth. Following this primary enrichment step, an aliquot of the culture was transferred to RVS selective enrichment medium to further enhance the proliferation of target bacteria. The presence of bacterial growth in both BPW and

Table 1: The composition profile of *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils.

Constituents	Retention time (min)	Relative percentage (%)	
		Melaleuca leucadendron	*Eucalyptus camaldulensis*
Isovaleric acid	4.219	0.08	
α-Thujene	7.332	0.48	0.08
α-Pinene	7.629	8.58	19.80
α-Fenchene	8.319		0.21
Camphene	8.378		0.60
β-Pinene	9.732	0.31	21.84
Sulcatone	10.311	0.18	
β-Myrcene	10.568	0.04	0.53
α-Phellandrene	11.368	0.16	
3-Carene	11.503	0.11	
α-Terpinene	12.006	2.27	
o-Cymene	12.460	6.29	3.42
D-Limonene	12.736	2.68	6.11
Eucalyptol	12.915		18.17
β-cis-Ocimene	13.395		0.25
β-trans-Ocimene	14.029		0.08
γ-Terpinene	14.583	3.07	2.51
cis-Linalool oxide	15.410	0.08	
α-Terpinolene	16.317	2.18	0.17
p-Cymenene	16.604	0.11	
β-Linalool	17.455	1.25	
Fenchol	18.480		1.04
Nopinone	19.597		0.15
trans-Pinocarveol	19.800		0.79
Camphene hydrate	20.609		0.19
Borneol	21.615		1.08
4-Terpineol	22.082	1.29	1.91
p-Cymen-8-ol	22.467	0.62	
α-Terpineol	22.837	0.76	5.37
Fenchyl acetate	23.933		0.10
m-Thymol	26.939	0.07	
α-Terpineol acetate	28.793		0.60
o-Thymol	27.198	0.10	
α-Cubebene	28.776	0.24	
Eugenol	28.904	0.16	
α-Ylangene	29.475	0.23	
α-Copaene	29.678	0.21	
β-Elemene	30.132	0.17	0.06
Methyl Eugenol	30.485	4.55	
cis-Caryophyllene	30.564	0.13	1.82
α-Gurjunene	30.657	0.15	
β-Caryophyllene	31.024	10.08	
γ-Elemene	31.340	0.09	

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Constituents	Retention time (min)	Relative percentage (%)	
		Melaleuca leucadendron	*Eucalyptus camaldulensis*
α-Guaiene	31.515	0.08	
Aromadendrene	31.570	0.16	0.91
Guaia-6,9-diene	31.680	0.66	
α-Humulene	32.066	5.72	0.23
Alloaromadendrene	32.194	0.09	0.30
4,5-Di-epi-aristolochene	32.489	0.06	
γ-Selinene	32.596	0.38	
α-Amorphene	32.761	0.35	
β-Selinene	33.015	1.98	
β-Eudesmene	33.038	0.15	
Ledene	33.122	0.07	
α-Selinene	33.218	1.60	
Bicyclogermacrene	33.235		0.25
α-Muurolene	33.299	0.09	
trans-α-Farnesene	33.464	0.93	
Hedycaryol	34.492		0.13
γ-Cadinene	33.657	0.09	
Epiglobulol	34.795		0.36
δ-Cadinene	33.800	0.30	
Calamenene	33.859	2.47	
Cubenene	34.119	0.14	
α-Calacorene	34.305	0.25	
Palustrol	34.940	0.16	
Spathulenol	35.071	0.32	1.23
Caryophyllene oxide	35.184	6.53	1.50
Globulol	35.256	0.18	3.04
Guaiol	35.476	5.30	
Humulene epoxide II	35.720	3.12	
Rosifoliol	35.772	0.17	
Selina-6en-4-ol	35.955	0.09	
γ-Eudesmol	36.129	5.03	0.95
Caryophylladienol II	36.215	0.97	
Agarospirol	36.262	0.70	
δ-Cadinol	36.315	0.28	
β-Eudesmol	36.538	11.61	2.33
Bulnesol	36.691	1.23	
Juniper camphor	37.284	0.04	
cis-Farnesol	37.443	0.11	
Farnesyl acetate	38.939	0.03	
Total		98.16	98.11

The essential oil of *Melaleuca leucadendron* is primarily composed of β-Eudesmol, β-Caryophyllene, α-Pinene, o-Cymene, and Linalool, whereas *Eucalyptus camaldulensis* essential oil contains β-Pinene, α-Pinene, Eucalyptol, and D-Limonene as its major constituents. These compounds possess potent antibacterial activity.

RVS media was indicated by visible turbidity, confirming a 100% positivity rate for bacterial presence in the samples.

Subsequently, cultures from the RVS medium were streaked onto two types of selective and differential agar media, namely XLD and MC agar, to allow for the isolation and preliminary identification of bacterial colonies. The results obtained from these culture plates revealed bacterial growth, with colonies exhibiting distinct morphological characteristics. On XLD agar, the colonies displayed a faint red halo surrounding a black-centered colony, which is indicative of hydrogen sulfide (H_2S)-producing bacteria. Meanwhile, on MC agar, the observed colonies appeared in shades ranging from pink to red or beige, characteristic of lactose-fermenting and non-lactose-fermenting bacterial strains, respectively. The overall positive rate of bacterial isolation from both media was determined to be 40%.

Following the initial isolation, the bacterial colonies were further purified through subculturing on Tryptic Soy Agar (TSA) medium to obtain a sufficient biomass for subsequent biochemical identification and gene sequencing analyses. This step ensured the generation of pure bacterial cultures necessary for precise characterization. The detailed results of bacterial culture and isolation are presented in Table 2 and visually depicted in Figure 1.

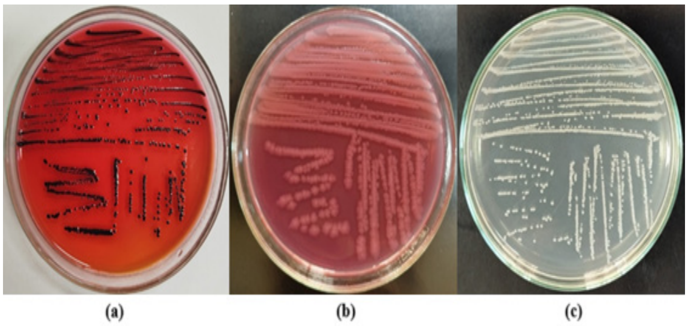

Figure 1: *Salmonella* spp. colonies cultivated on (a) Xylose-Lysin Deoxycholate (XLD) agar, (b) MacConkey (MC) agar, and (c) Tryptic Soy Agar (TSA).

Identification of bacteria using the API 20E biochemical kit

The identification of bacterial isolates was performed using the API 20E biochemical kit. The results obtained from the biochemical assays are summarized in Table 3. According to the biochemical identification results, out of the four tested bacterial isolates, two were confirmed to be *Salmonella* spp. These positive isolates were specifically recovered from the internal organs of diseased chickens, suggesting a possible correlation between the presence of *Salmonella* spp. and the observed disease symptoms in the affected birds. The identification of these pathogenic bacteria underscores the significance of proper microbiological surveillance and diagnostic testing in poultry health management.

Table 2: Results of *Salmonella* spp. isolation from diseased chicken specimens.

Culture media	Positive specimens	Percentage (%)	Characteristics of bacterial culture
Buffered Peptone Water	10/10	100	Bacterial growth leads to a uniformly turbid medium
Rappaport Vassiliadis Soya Broth	10/10	100	Bacterial growth leads to a uniformly turbid medium
Xylose-Lysin Deoxycholate Agar	04/10	40	Colonies feature a black center surrounded by a pale halo
MacConkey Agar	04/10	40	Colonies are light pink in colour

Table 3: The results of the biochemical examination of the isolated bacterial strains were obtained through the use of the API 20E identification kit.

Sample categories	Assigned identification code	Bacterial species identified
Cloacal secretion	2577000	*Proteus mirabilis*
Cloacal secretion	2536000	*Proteus mirabilis*
Internal organs	6727556	*Salmonella enterica*
Internal organs	6717552	*Salmonella enterica*

Biochemical identification of bacterial strains isolated from cloacal secretions and internal organs. *Proteus mirabilis* was found in cloacal samples, whereas *Salmonella enterica* was detected in the internal organs of chickens.

Table 4: The outcomes of the bacterial identification process were determined through the use of the PCR technique for detecting isolated strains.

Specimen categories	Number of specimens	Positive specimens	Bacterial species identified
Cloacal secretion	2	00	-
Internal organs	2	02	*Salmonella typhimurium* and *Salmonella enteritidis*

The identification of *Salmonella typhimurium* and *Salmonella enteritidis* is crucial as they are major poultry pathogens and zoonotic risks. They cause salmonellosis, leading to economic losses and potential human infections. Their detection underscores the need for strong biosecurity and antimicrobial measures in poultry farming.

PCR-based identification of isolated bacteria

The bacterial identification results obtained through polymerase chain reaction (PCR) analysis are documented in Table 4. PCR-based identification was conducted to achieve a more precise and reliable classification of the bacterial species, complementing the biochemical identification results. The findings from the PCR analysis revealed that out of the four test samples, two were confirmed as *Salmonella enterica* subsp. *Enterica* with high confidence levels. One isolate showed 99.20% identity with *Salmonella enterica* subsp. *Enterica*, while the second isolate displayed a 99.37% match with the same bacterial subspecies. Further comparison of the sequenced genes with the NCBI database demonstrated a 99.06% similarity and 100% coverage with *Salmonella enterica* subsp. *Enterica* serovar Typhimurium. Similarly, another sequence comparison revealed a 99.66% similarity and 100% coverage with *Salmonella enterica* subsp. *Enterica* serovar Enteritidis.

Results of the antibiotic susceptibility test for isolated bacteria

The results of the antibiotic susceptibility testing conducted on the isolated *Salmonella* spp. are systematically presented in Table 5. The two bacterial isolates identified as *Salmonella typhimurium* and *Salmonella enteritidis* exhibited identical antibiotic susceptibility profiles, indicating a consistent pattern of sensitivity and resistance to the tested antimicrobial agents. A total of 12 different antibiotics were assessed in this study to determine the efficacy of these drugs against the bacterial isolates. The results demonstrated that the isolated *Salmonella* spp. displayed notable sensitivity to three specific antibiotics: norfloxacin, ceftazidime, and gentamycin. Among these, norfloxacin exhibited the highest level of bacterial inhibition, making it the most effective antibiotic against the tested isolates. Due to its superior antibacterial activity, norfloxacin was selected for combination with essential oils in subsequent experiments to investigate the potential synergistic or bactericidal effects of the interaction between essential oils and antibiotics. Conversely, the tested *Salmonella* isolates demonstrated a high degree of insensitivity to several other antibiotics, including amoxicillin, kanamycin, tobramycin, colistin, ampicillin, streptomycin, cefuroxime, doxycycline, and tetracycline. The results further indicated that the isolates exhibited complete resistance to ampicillin, streptomycin and colistin, suggesting that these antibiotics would be ineffective in treating infections caused by these bacterial strains. The observed antibiotic resistance profile highlights the potential challenges in treating *Salmonella* infections and underscores the importance of continued monitoring of antimicrobial resistance patterns.

Table 5: Susceptibility of the isolated *Salmonella* spp. to antibiotics.

Antibiotics	Concen-trations (µg)	Growth inhibition zone (mm)					
		Salmonella typhimurium			*Salmonella enteritidis*		
		S (≥21)	I (18-20)	R (≤17)	S (≥21)	I (18-20)	R (≤17)
Ampicillin (Am)	10			00			00
Amoxicillin (Ax)	10		20.00±1.00			18.00±1.00	
Ceftazidime (Cz)	30	21.67±0.53			21.00±1.15		
Cefuroxime (Cu)	30			9.67±0.58			8.67±0.58
Colistin (Co)	10			00			00
Doxycycline (Dx)	30			11.67±0.58			11.33±1.00
Gentamycin (Ge)	10	21.33±1.15			21.00±0.58		
Kanamycin (Kn)	30		18.33±1.53			18.00±0.58	
Norfloxacin (Nr)	10	39.67±1.00			34.67±1.15		
Streptomycin (Sm)	10			00			00
Tetracycline (Te)	30			14.00±1.00			9.67±1.00
Tobramycin (Tb)	10		19.00±1.53			18.33±1.15	

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

Table 6: The minimum bactericidal concentration (MBC) and fractional bactericidal concentration index (FBC) of essential oils and norfloxacin, whether used individually or in combination, against isolated *Salmonella* spp.

Substances		*Salmonella typhimurium*			*Salmonella enteritidis*		
		MBC (µg/ml)	FBC (index)	Interaction type	MBC (µg/ml)	FBC (index)	Interaction type
Alone	ML	13,000±163.30^{b}			13,000±163.30^{b}		
	EC	20,000±244.95^{c}			20,000±408.25^{c}		
	NR	15.625±0.16^{a}			62.50±0.41^{a}		
Combination	ML + NR	3,250/0.98	0.31	Synergistic	3,250/3.91	0.31	Synergistic
	EC + NR	5,000/1.95	0.37	Synergistic	5,000/7.81	0.37	Synergistic

ML: *Melaleuca leucadendron*, EC: *Eucalyptus camaldulensis*, Nr: norfloxacin. Superscript letters (a, b, and c) within the same column denote statistically significant differences between essential oil and antibiotic treatments used individually ($P < 0.05$). The values are expressed as mean ± SD. The FBC index categorizes interactions as synergistic (≤0.5), additive (>0.5 to <1), indifferent (≥1 to <4), or antagonistic (≥4).

The antibacterial effects of essential oils and norfloxacin, individually and in combination, against isolated bacteria

Following the assessment of antibiotic resistance in the isolated bacteria, norfloxacin was determined to be the most effective and was subsequently chosen for further experiments. Table 6 outlines the bactericidal activity of the essential oils and norfloxacin, along with their combined effects against the isolated *Salmonella* spp. The findings of this study demonstrated that the antibacterial effects of the tested essential oils on the two strains, *Salmonella typhimurium* and *Salmonella enteritidis*, were comparable. Specifically, the essential oil extracted from *Melaleuca leucadendron* exhibited MBC of 13,000 µg/ml. In contrast, the essential oil derived from *Eucalyptus camaldulensis* had a significantly higher MBC value of 20,000 µg/ml (P<0.05). This observed difference in MBC values suggests that the ability of *Eucalyptus camaldulensis* essential oil to exert a bactericidal effect against *Salmonella* spp. was lower than that of *Melaleuca leucadendron* essential oil. On the other hand, norfloxacin showed varying effects on the two isolated *Salmonella* spp. strains. Its MBC values were 15.625 µg/ml for *Salmonella typhi* and 62.5 µg/ml for *Salmonella enteritidis*. While the MBC values of the essential oils were considerably higher than that of norfloxacin, they still demonstrated antibacterial activity against the isolated *Salmonella* spp. The interaction between essential oils and norfloxacin demonstrated a synergistic effect, as both *Melaleuca leucadendron* and *Eucalyptus camaldulensis* enhanced the antibiotic's bactericidal activity against *Salmonella* spp. When combined with *Melaleuca leucadendron*, the MBC of norfloxacin decreased 16-fold, while with *Eucalyptus camaldulensis*, it was reduced 8-fold. Moreover, the MBC values of the essential oils also dropped significantly in combination with norfloxacin, reaching 3,250 µg/ml for *Melaleuca leucadendron* and 5,000 µg/ml for *Eucalyptus camaldulensis*.

DISCUSSION

The findings of this study revealed that the internal organ specimens collected from the field tested positive for the presence of *Salmonella* spp. Upon further analysis, it was determined that the specific strains detected were *Salmonella typhimurium* and *Salmonella enteritidis*. Conversely, the specimens collected from cloacal fluid tested negative for *Salmonella* spp. A possible explanation for this discrepancy is that cloacal fluid contains a diverse population of intestinal bacteria, many of which are highly competitive and exhibit rapid growth. This microbial competition may hinder the successful isolation of *Salmonella* spp. from cloacal fluid samples compared to internal organ specimens, where bacterial populations may be more stable and favourable for detection. These findings are consistent with the results of Shen *et al.* (2023) who similarly isolated *Salmonella typhimurium* and *Salmonella enteritidis* from the internal organs of diseased chickens, reinforcing the validity of the present study's observations. To confirm bacterial identification, two different methodologies were employed: The API 20E biochemical kit and gene sequencing. The results from both methods were in agreement, demonstrating that the API 20E biochemical kit is a reliable tool for the identification of Salmonella spp. Due to its high level of accuracy, this kit is recommended for preliminary screening before implementing gene sequencing for more comprehensive bacterial identification.

The antibiotic susceptibility tests conducted in this study further revealed that the two isolated *Salmonella* spp. strains exhibited complete resistance to three widely used antibiotics: Ampicillin, streptomycin, and colistin. These antibiotics are widely available on the market and are frequently utilized in both veterinary and human medicine. The results obtained in this study align with findings from previous research on antibiotic resistance in *Salmonella* spp. strains isolated from diseased poultry (Castro-Vargas *et al.*, 2020; Tan *et al.*, 2022; Punchihewage-Don *et al.*, 2023). The multidrug resistance (MDR) observed in the isolated *Salmonella* spp. has significant implications for both animal and human health, particularly given the zoonotic nature of *Salmonella*. Antibiotic-resistant *Salmonella* strains can be transmitted from poultry to humans through direct contact, consumption of contaminated poultry products, or environmental exposure. In human infections, MDR *Salmonella* poses a major public health concern as it can lead to more severe illness, prolonged disease duration, and limited treatment options due to reduced antibiotic effectiveness. This can result in higher medical costs, increased hospitalization rates, and a greater risk of complications, especially in vulnerable populations such as children, the elderly, and immunocompromised individuals. Furthermore, the overuse and misuse of antibiotics in poultry farming contribute to the selection and spread of resistant bacterial strains, which may further exacerbate the antimicrobial resistance crisis. Addressing this issue requires the implementation of responsible antibiotic use in agriculture, improved biosecurity measures, and the exploration of alternative antimicrobial agents, such as essential oils, to help mitigate the spread of resistant *Salmonella* strains. Future research should focus on monitoring antibiotic resistance trends, evaluating the efficacy of alternative treatments, and investigating strategies to reduce the transmission of MDR *Salmonella* from poultry to humans. Another noteworthy observation in this study was that the two isolated *Salmonella* spp. strains exhibited identical resistance and sensitivity profiles to the tested antibiotics. This uniformity in antibiotic susceptibility patterns may be attributed to the fact that the samples were collected from the same geographical area, where the animals were subjected to similar veterinary treatments and management practices. The prolonged and frequent use of specific antibiotics in livestock farming whether administered through feed, drinking water, or disease treatment protocols may have contributed to the emergence of similar antibiotic resistance mechanisms among bacterial populations, including *Salmonella* spp.

In the present study, essential oils extracted from *Melaleuca leucadendron* and *Eucalyptus camaldulensis* were evaluated for their bactericidal activities against *Salmonella* spp. isolated from various sources. The experimental results demonstrated that both *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils exhibited significant bactericidal effects against the isolated *Salmonella* spp., indicating their potential as natural antibacterial agents. The bactericidal activity of these essential oils can likely be attributed to the presence of bioactive chemical constituents with known antimicrobial properties. These compounds may act individually or in combination, enhancing their overall effectiveness through a synergistic effect. The interaction between multiple bioactive compounds within the essential oils may lead to increased bacterial membrane permeability, disruption of cellular integrity, inhibition of key metabolic pathways, or interference with bacterial biofilm formation. Such mechanisms contribute to the overall antibacterial potency of these essential oils. Previous studies have extensively reported the antimicrobial potential of the major constituents found in *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils. Specifically, the essential oil of *Melaleuca leucadendron* contains β-Eudesmol and β-Selinene, which have been demonstrated to inhibit the growth of five Gram-negative bacterial species (Hoa *et al.*, 2009). In addition, β-Caryophyllene, another key component of *Melaleuca leucadendron* essential oil, has been shown to alter membrane permeability and compromise the structural integrity of *Bacillus cereus*, leading to bacterial cell death (Moo *et al*., 2020). Furthermore, several monoterpenes, including α-Pinene, α-Terpineol, and 1,8-Cineole (Eucalyptol), are present in both *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils. These compounds have been reported to exhibit potent bactericidal activity against *Bacillus cereus* (Akacha *et al*., 2023), highlighting their broad-spectrum antimicrobial potential. Additionally, *Melaleuca leucadendron* essential oil contains Linalool, a well-known antimicrobial compound that has been demonstrated to effectively inhibit the growth of *Shigella flexneri* (Ngome *et al*., 2018), a pathogenic bacterium responsible for severe gastrointestinal infections. Similarly, *Eucalyptus camaldulensis* essential oil is rich in 1,8-Cineole (Eucalyptol), an active compound known for its strong bactericidal properties. Studies have demonstrated that 1,8-Cineole exhibits antimicrobial effects against *Klebsiella pneumoniae* (Moo *et al*., 2021) and *Escherichia coli* (Wang *et al*., 2022), two major pathogens associated with respiratory and gastrointestinal infections. Moreover, D-Limonene, another significant component found in both *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils, has been widely reported to exert antibacterial activity against various pathogenic bacteria affecting both humans and livestock (Jacob *et al*., 2017; Agougui *et al*., 2022).

The findings revealed that the bactericidal effectiveness of the two essential oils differed, with *Melaleuca leucadendron* exhibiting stronger antibacterial activity than *Eucalyptus camaldulensis*. This variation can be attributed to the distinct compositions of bioactive compounds in each oil, leading to different mechanisms of action against bacteria. In addition, the synergistic and inhibitory interactions among bioactive compounds within essential oils also play a role in determining their overall bactericidal potency. Previous research has highlighted the influence of these interactions on the antimicrobial effects of herbal essential oils (Sharmaa *et al*., 2020; Angane *et al*., 2023). Since bioactive compounds in essential oils are hydrophobic, they disrupt the lipid structure of bacterial cell membranes and mitochondria, increasing membrane permeability. This disruption weakens the cell wall and cytoplasmic membrane, ultimately leading to cell lysis, leakage of intracellular components, metabolic disruption, and bacterial death (Nazzaro *et al*., 2013). Furthermore, certain essential oil components target bacterial intracellular proteins, interfering with cell division (Domadia *et al*., 2007). Specifically, in *Melaleuca leucadendron*, the bioactive compounds are believed to act synergistically, amplifying their overall antibacterial effect. For example, these compounds can destabilize bacterial membranes, making it easier for other compounds to penetrate and disrupt metabolic processes. In contrast, the bioactive compounds in *Eucalyptus camaldulensis* do not exhibit the same level of synergism, reducing their collective bactericidal efficiency. This explains why the MBC value of *Melaleuca leucadendron* was lower than that of *Eucalyptus camaldulensis*.

The combination of essential oils with norfloxacin resulted in a notable reduction in the MBC values for both the essential oils and the antibiotic when tested against the isolated *Salmonella* spp. This finding highlights the potential of essential oils in enhancing antibiotic efficacy. Specifically, the combination of *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils with norfloxacin demonstrated a synergistic interaction, meaning that their combined antibacterial effect was greater than the sum of their individual effects. Such synergy has been previously reported in several studies investigating the interactions between essential oils and antibiotics (Moussaoui and Alaoui, 2016; Boonyanugomol *et al*., 2017; Romo-Castillo *et al*., 2023; Drioiche *et al*., 2024). One of the most significant implications of this synergy is the substantial reduction in the required concentration of norfloxacin needed to effectively eliminate *Salmonella* spp. This reduction is particularly important as lowering antibiotic dosage can help mitigate the risk of antibiotic resistance, a growing global health concern. Published research and experimental results suggest that combining essential oils with antibiotics can both enhance bacterial susceptibility to antibiotics and minimize the selective pressure that drives the emergence of multidrug-resistant bacterial strains. In other words, the synergistic effect between essential oils and antibiotics presents a promising strategy for developing new therapeutic approaches against resistant pathogens.

The enhanced antibacterial effect observed with the combination of *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils with norfloxacin can be attributed to their complementary modes of action in eliminating *Salmonella* spp. Norfloxacin, a second-generation quinolone antibiotic, acts by penetrating bacterial cells and inhibiting DNA gyrase, a critical enzyme required for bacterial DNA replication. However, the efficiency of norfloxacin's penetration into bacterial cells was significantly improved when combined with the essential oils. This improvement is primarily due to the membrane-disrupting activity of the bioactive compounds found in *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils. These bioactive compounds integrate into the bacterial lipid bilayer, increasing membrane permeability and weakening the structural integrity of the cell. As a result, norfloxacin molecules were more efficiently transported across the bacterial membrane, reaching effective intracellular concentrations more quickly. At the same time, the enhanced bactericidal action of norfloxacin reduced the amounts of essential oils required to achieve antibacterial activity. By weakening *Salmonella* cells through DNA replication inhibition, norfloxacin made the bacteria more susceptible to the antibacterial compounds present in *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils. This reciprocal enhancement in effectiveness explains the strong synergistic interaction observed between norfloxacin and these essential oils. The ability of essential oils to enhance antibiotic penetration while simultaneously exerting their own bactericidal effects highlights their potential as adjuvants in antimicrobial therapies aimed at combating antibiotic-resistant bacteria.

CONCLUSIONS

The study found that *Melaleuca leucadendron* (13 mg/ml) and *Eucalyptus camaldulensis* (20 mg/ml) essential oils exhibited significant bactericidal activity against antibiotic-resistant *Salmonella* spp. isolated from infected chickens in vitro. Their combination with norfloxacin showed a synergistic effect, significantly enhancing bacterial elimination. This suggests that incorporating essential oils into treatment regimens could boost antibiotic efficacy while reducing required dosages. Given these promising results, further *in vivo* studies are needed to confirm their effectiveness and safety in real poultry production settings.

ACKNOWLEDGEMENTS

We sincerely thank Tra Vinh University for providing financial support for this research under grant contract number 18/2025/HĐ. HĐKH&ĐT-ĐHTV.

NOVELTY STATEMENT

This study presents an innovative method that combines essential oils from *Melaleuca leucadendron* and *Eucalyptus camaldulensis* with the antibiotic norfloxacin to combat antibiotic-resistant *Salmonella* spp. isolated from chicken. The findings indicate that *Melaleuca leucadendron* and *Eucalyptus camaldulensis* essential oils, when used alongside norfloxacin, produced synergistic effects in eradicating *Salmonella* spp.

AUTHOR'S CONTRIBUTION

Nguyen Van Vui was responsible for conceptualizing and designing the experiments. Ly Thi Thu Lan, Nguyen Van Tung Lam and Huynh Thanh Tan conducted the experimental work. Nguyen Van Vui performed the data analysis and prepared the initial manuscript draft. All authors reviewed and approved the final version of the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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