Scientific Papers. Series D. Animal Science. Vol. LXIX, No. 1, 2026
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF THYME (*Thymus vulgaris*) AND TEA TREE (*Melaleuca alternifolia*) ESSENTIAL OILS

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Abstract

Essential oils are explored as natural alternatives to synthetic additives because of their diverse biological activities and potential use in poultry production. This study evaluated the chemical composition and in vitro biological properties of thyme (Thymus vulgaris) essential oil (TEO) and tea tree (Melaleuca alternifolia) essential oil (TTEO) as potential bioactive candidates for broiler nutrition. Gas chromatography–mass spectrometry (GC–MS) analysis showed that TEO was dominated by thymol (52.84%), whereas TTEO was characterized mainly by terpinen-4-ol (37.14%). Antimicrobial activity against Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, and Clostridium perfringens assessed by broth microdilution and disc diffusion assays, showed stronger inhibition by TEO, MICs of 7.5 µg/mL against S. typhimurium and C. perfringens, while TTEO exhibited MICs of 7.5 µg/mL against L. monocytogenes and C. perfringens, 15 µg/mL against S. typhimurium, and 62.5 µg/mL against E. coli. Antioxidant activity, determined by the DPPH assay, was higher for TEO (65.48% at 0.09 mg/mL), whereas TTEO reached 56.02% at 17 mg/mL Both oils showed modest anti-inflammatory effects and relevant in vitro bioactivity, with thyme oil showing superior antimicrobial and antioxidant performance.

Key words*: antimicrobial activity, DPPH radical scavenging, GC–MS profiling, Melaleuca alternifolia essential oil, phytogenic feed additives, Thymus vulgaris essential oil.*

INTRODUCTION

Essential oils (EOs) are complex mixtures of volatile compounds derived from aromatic plants and have been extensively investigated because of their diverse biological properties (Bakkali et al., 2008). In recent years, interest in the use of natural compounds as alternatives to synthetic products has increased substantially, largely driven by the demand for safer solutions for both human health and the environment (Burt, 2004). In poultry production, the interest in EOs has expanded beyond their conventional use as flavouring agents, as they are increasingly regarded as multifunctional phytogenic candidates capable of simultaneously modulating microbial pressure, oxidative stability, and inflammatory status (Movahedi et al., 2024; Toader et al., 2025). Such a multifunctional profile is particularly relevant in modern production systems, where nutritional strategies are expected not only to support performance, but also to contribute to gut health, product quality, and reduction of reliance on conventional antimicrobial inputs (Toader et al., 2025; Toader et al., 2024). For this reason, the integration of chemical characterization with biological testing is essential for establishing meaningful structure-activity relationships and for identifying which oils are most suitable for future application as functional additives in broiler nutrition.

Thyme (*Thymus vulgaris*) essential oil (TEO) is particularly valued for its high content of phenolic constituents, especially thymol and carvacrol, which are largely responsible for its antimicrobial and antioxidant activities (Marchese et al., 2016). Likewise, tea tree (*Melaleuca alternifolia*) essential oil (TTEO) is characterized by the presence of terpinen-4-ol and other terpenes that contribute to its antimicrobial and anti-inflammatory effects (Carson, Hammer, & Riley, 2006). Importantly, the biological efficacy of an essential oil cannot be predicted solely from its botanical origin, because its activity depends on chemotype, the relative abundance of dominant constituents, and potential synergistic or antagonistic interactions among minor compounds (Benomari et al., 2023). The antioxidant potential of these oils is

important for limiting oxidative processes, while their antimicrobial and anti-inflammatory properties also support their considerable potential in animal production, in addition to applications in the food, pharmaceutical, and cosmetic industries (Miguel, 2010).

Therefore, this study aimed to investigate the chemical composition and *in vitro* biological activities of TEO and TTEO, with particular emphasis on their antimicrobial, antioxidant, and anti-inflammatory properties, to determine their suitability as natural functional additives with prospective use in broiler production.

MATERIALS AND METHODS

In vitro evaluation of antimicrobial activity

The microdilution broth method was used to evaluate the antimicrobial susceptibility, which involved testing serial 2-fold dilutions of the EO in liquid growth medium distributed into 96-well microtiter plates (Balouiri, Sadiki, & Ibnsouda, 2016). Each well was inoculated with a standardized microbial suspension containing EO of interest, adjusted to a turbidity equivalent to the 0.5 McFarland scale, and further diluted in the same growth medium. Following inoculation, the 96-well microtiter plates are incubated under conditions suitable for the microorganism being tested. The minimum inhibitory concentration (MIC) was selected as the lowest concentration of antimicrobial agent that completely inhibited microbial growth in the wells.

In vitro antimicrobial testing was performed against the bacterial strains *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Listeria monocytogenes* (ATCC 19114), and *Clostridium perfringens* (ATCC 13124). A 10^{-2} dilution of fresh bacterial culture, standardized to 0.5 McFarland (1.5×10^8 CFU/ml), was used for the assay (Balouiri et al., 2016). The reference strains (ATCC) were initially revived through 24h incubation in Brain Heart Infusion (BHI) broth (Oxoid, CM1135) at 37°C, followed by subsequent transfer to BHI agar for 24 hours at 37°C. Each bacterial strain was then adjusted to an optical density (OD) equivalent to a 0.5 McFarland standard using BHI broth. Subsequently, bacterial suspensions were dispensed into flat-bottomed 96-well microtiter plates, with a total working volume of 200 µl per well. Using an electronic Brand Transferpette pipette (15–300 µl), 100 µl of bacterial suspension were added to each well. Test samples, diluted from a stock solution, were applied to the wells at concentrations ranging from 7.5 µg/ml to 4000 µg/ml Agar plates were covered and incubated overnight at 37°C. Post-incubation, the optical OD was quantified at 540 nm via an ELISA Microplate Absorbance reader (BIORAD PR 1100). All tests were conducted in triplicate, with bacterial suspensions in BHI serving as positive controls. To complement MIC results, the disc diffusion assay was performed according to a previously reported study and readapted for essential oils (Balta, Marcu et al., 2021). Briefly, 100µl of *L. monocytogenes* (ATCC 19114), *S. enterica typhimurium* (ATCC 14028), *E. coli* (ATCC 25922) bacterial cultures were accurately spread onto Tryptone Soya Agar yeast extract (TSAYE Oxoid, UK), and *Campylobacter jejuni* (NCTC 11168) was plated on Blood Agar Base Nr. 2 (BAB, Oxoid Ltd., UK) supplemented with 5% (vol/vol) horse blood (Aquilant Scientific N.I.). Next, each EO was diluted in tenfold increments from 100% to 0.50% (v/v) in DMSO, and 20µl of corresponding antimicrobial concentration was pipetted on 6 mm paper discs (Oxoid, UK). The antimicrobial activity of each essential oil was evaluated with a calliper by measuring the diameter of the inhibition zone around the disc for each concentration.

Determination of volatile compounds from EOs

The analysis of the EOs was performed by Gas Chromatography-Mass Spectrometer (GC-MS) with a Thermo Electron system consisting of a Focus GC chromatograph equipped with DB-5MS capillary column (25 m × 0.25 mm; 0.25 µm film thickness) and TriPlus auto sampler, connected to a Polaris Q ion trap mass detector. Helium (noble gas) was used as carrier gas at a flow rate of 1 mL/min. The injector and temperature detectors were set up at 250°C. The oven temperature was programmed from 60ºC (3 min) to 200ºC at a rate of 10ºC/min, then from 200ºC to 240ºC at a rate of 12ºC/min. The mass spectra were recorded at 70 eV (EI) and were scanned in the range 35-450 *m*/*z*. The injection volume was 1 µL from 1:100 hexane dilution. Identification of the volatile components was carried out using Xcalibur® software and NIST

11 Mass Spectral Library, an alkane standard solution for GC (C8-C20 in hexane, Sigma Aldrich Co., St. Louis, USA) being used for retention indexes (RI/tR) determination (Marinas et al., 2023). The relative percentage (%) content of each component was calculated based on the GC-peak areas without any correction factors. - Mass Spectrometry (GC-MS).

Anti-inflammatory activity determination

The erythrocytic suspension was prepared through the method previously described, with minor modifications, where the blood samples were collected in heparinized vacutainers and centrifuged at 3000 rpm for 10 minutes (Gunathilake, Ranaweera, & Rupasinghe, 2018). Following the centrifugation, the supernatant was discarded, and the erythrocyte pellet was washed with an equal volume of isotonic sodium chloride (0.9%) solution. This centrifugation and washing process was repeated three times. Subsequently, the erythrocyte pellet was reconstituted as a 40% suspension in isotonic Phosphate-Buffered Saline (PBS, pH 7.4). The protocol was approved and licenced by the Bioethics Committee from the University of Life Sciences "King Mihai I" from Timisoara (No.56/7.07.2021).

The heat-induced hemolysis test was performed according to the method earlier described by Okoli et al. (2008), incorporating modifications (Gunathilake et al., 2018; Okoli et al., 2008). Different concentrations (i.e. 10 µl/ml, 20 µl/ml, 40 µl/ml, 80 µl/ml, and 160 µl/ml) of essential oil (extract) were dissolved in 5 ml isotonic PBS (pH 7.4) (RemedLab, Bucharest, Romania), to which 100 µl erythrocyte suspension was added. After gentle mixing, samples were incubated at 54°C for 20 minutes in a water bath. Following incubation, samples were centrifuged at 2500 rpm for 3 minutes, and the absorbance of the supernatant was detected at 540 nm using a UV/Vis spectrophotometer (Specord 205-Analytik Jena AG, Germany). A control sample consisted of PBS and 100 µl erythrocyte suspension. The percentage inhibition of hemolysis was calculated using the following formula:

(%) Inhibition of Hemolysis = 100 - A1/A2 × 100

where: A1 = absorbance of the test sample, and A2 = absorbance of the control sample.

Protein denaturation evaluation was performed as previously described with small modifications [13]. Different concentrations (i.e. 10 µl/ml, 20 µl/ml, 40 µl/ml, 80 µl/ml, and 160 µl/ml) of essential oils were added to 1 ml of 1% egg albumin (Oxford Lab Fine Chem LPP, Maharashtra, India) including 4 ml of PBS (pH 6.4). The mixture was gently mixed and incubated in a water bath at 37°C for 15 minutes. Subsequently, it was heated at 70°C for 5 minutes. After cooling, turbidity was measured at 660 nm optical density using a UV/VIS spectrophotometer (Optima SP-3000, Tokyo, Japan). The control sample consisted of PBS without extract.

(%) Inhibition of denaturation = 100 - A1/A2 × 100

where: A1 = absorbance of the control sample, and A2 = absorbance of the test sample.

Antioxidant capacity using the DPPH [1,1-diphenyl-2-picrylhydrazyl] radical scavenging activity assay

The DPPH assay was also performed as previously described with small modifications (Ciulca et al., 2021). In order to describe the antioxidant capacity of TTEO and TEO, a stock methanolic solution was prepared by dissolving 1 ml of essential oil in 10 ml of methanol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). From this stock solution, multiple dilutions were prepared, and from each dilution, 3 ml were taken and combined with 1 ml of 0.3 mM DPPH solution, followed by incubation in the dark for 30 minutes. Absorbance was quantified at 517 nm using a UV/VIS spectrophotometer (Specord 205; Analytik Jena AG, Jena, Germany). Each sample was analyzed in triplicate, and the mean values were recorded. The control sample was also prepared, where the extract was replaced by methanol, keeping the same concentrations and volume of DPPH solution. Ascorbic acid was used as a positive control at a concentration of 0.016 mg/ml in methanol, provided by Lach-Ner (Neratovice, Czech Republic).

The radical scavenging activity (RSA) was calculated according the following formula:

$$(\%)\ \text{RSA} = (\text{A control} - \text{A samples}) / (\text{A control}) \times 100$$

where: A control = absorbance value of the control sample and A sample = absorbance value of the tested extracts.

Statistical analysis

All assays were conducted in triplicate, and data are presented as mean ± standard deviation (SD); comparative evaluation of biological activities of the tested EOs was performed based on the recorded experimental responses.

RESULTS AND DISCUSSIONS

Chemical profile of EOs

Chemical profiling of TEO was evaluated by GC-MS which detected 29 volatile compounds as presented in Table 1. Thymol emerged as the most abundant constituent, representing 52% of its chemical content. This was followed by p-Cymene, which accounted for 18.54%, β-(E)-Caryophyllene at 7.33%, and γ-Terpinene at 5.71%. linalool and carvacrol were identified in lesser quantities, each comprising less than 3% of the oil. We also detected 23 volatile compounds present in trace amounts, ranging from 0.05% to 1.26%, respectively.

A chromatographic analysis characterized TEO volatile compounds by depicting their distribution patterns through prominent peaks that appeared in the chromatogram (Figure 1). Thymol served as the principal volatile component of Thyme EO as it produced a major peak at retention time (tR) 11.89 minutes which represented 99% of the total relative abundance. The next most prevalent compound was β-(E)-Caryophyllene that appeared at 13.62 tR and reached a 35% relative abundance level. Carvacrol and γ-Terpinene, displayed tR of 11.97 and 8.16 minutes, and relative abundances of 14%, while p-Cymene was observed at a 7.61 tR with a 29% relative abundance. The remaining volatile compounds within the Thyme EO exhibited lowered detection thresholds because their concentrations were detected under 5% relative abundance.

Similarly, GC-MS analysis of TTEO revealed a different composition, identifying 34 volatile compounds (Table 2). Predominantly, Terpinen-4-ol was identified as the principal constituent, comprising 37.14% of the oil's composition. This was closely followed by g-Terpinene, constituting 20.11%, and α-Terpinene at 11.23%. Among the detected volatile compounds in the oil analysis were α-Terpinolene, p-Cymene, Eucalyptol, α-Muurolene, γ-Cadinene, α-Terpineol, Aromadendrene, and α-Pinene, totaling 4% to 1% of the overall chemical composition. The remaining 29 compounds were discerned at concentrations below 1%. The subsequent chromatographic examination confirmed the quantity distribution of volatile substances in TTEO.

The chromatogram (Figure 2) indicated that Terpinen-4-ol produced the strongest peak at a tR of 10.24 minutes because it represented the overwhelming distribution. The g-Terpinene peak appeared at an 8.18 tR position with a relative abundance level amounting to 63%. γ-Cadinene (14.84 tR) was one of several distinctive compounds together with α-Muurolene (14.54 tR), Alloaromadendrene (13.84 tR), α-Terpineol (10.36 tR), α-Terpinolene (8.65 tR), and α-Terpinene (7.45 tR) that made up a total 45% to 5% of the constituents. The chromatogram also revealed other compounds within the oil, albeit at lower relative abundances.

Recent meta-analytical research corroborates that EOs are potential growth promoters in poultry farming, indicating that their inclusion in feed could lead to improved productivity (Irawan, Hidayat, Jayanegara, & Ratriyanto, 2021). The main effects of EOs on poultry production and growth promotion from the recent findings is depicted in Figure 1.

Table 1. Volatile compounds identified in TEO

tR (min)	RI calculated	Compound	CAS	%
5.93	926	a-Thujene	2867-05-2	0.54
6.02	931	a-Pinene	80-56-8	0.31
6.30	946	Camphene	79-92-5	1.26
6.97	983	b-Pinene	127-91-3	0.77
7.61	1019	p-Cymene	99-87-6	18.54
7.73	1026	Eucalyptol	470-82-6	0.98
8.16	1051	g-terpinene	99-85-4	5.71
8.34	1061	cis-Sabinene hydrate	15537-55-0	0.09
8.65	1079	α-Terpinolene	586-62-9	0.14
8.83	1090	Linalool	78-70-6	2.33
9.63	1139	Camphor	76-22-2	0.77
9.98	1161	Borneol	507-70-0	1.10
10.11	1170	Terpinen-4-ol	562-74-3	0.80
10.31	1182	α-Terpineol	98-55-5	0.11
10.89	1221	Thymol methyl ether	1076-56-8	1.06
11.03	1230	Carvacrol methyl ether	6379-73-3	0.90
11.89	1289	Thymol	89-83-8	52.84
11.97	1295	Carvacrol	499-75-2	2.41
12.58	1339	α-Cubebene	17699-14-8	0.10
12.96	1367	α-Copaene	3856-25-5	0.17
13.62	1417	β-(E)-Caryophyllene	87-44-5	7.33
13.69	1422	β-Copaene	18252-44-3	0.05
14.02	1448	n.i.		0.10
14.12	1456	Alloaromadendrene	25246-27-9	0.05
14.23	1465	n.i.		0.05
14.26	1467	γ-Muurolene	30021-74-0	0.12
14.53	1488	Germacrene D	23986-74-5	0.22
14.75	1506	n.i.		0.11
14.83	1513	γ-Cadinene	39029-41-9	0.59
14.96	1524	n.i.		0.05
15.67	1583	Caryophyllene oxide	1139-30-6	0.31
16.28	1636	τ-Cadinol	5937-11-1	0.10
				100.00
		Total identified		**99.69**

[1] n.i.- not identified

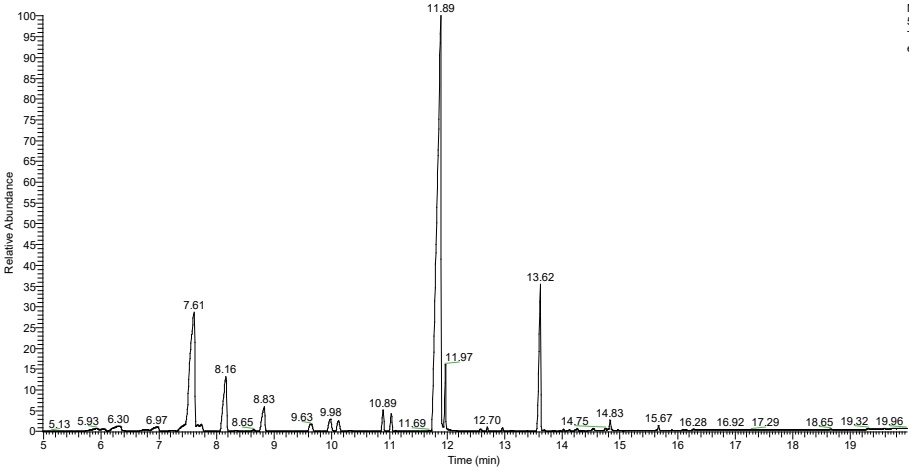

Figure 1. GC chromatogram of TEO

Table 2. Volatile compounds identified in TTEO

tR (min)	RI calculated	Compound	CAS	%
5.91	925	α-Thujene	2867-05-2	0.70
6.03	932	a-Pinene	80-56-8	1.08
6.72	969	Sabinene (β-Thujene)	3387-41-5	0.56
6.96	982	b-Pinene	127-91-3	0.43
7.23	997	α-Phellandrene	99-83-2	0.41
7.45	1009	α-Terpinene	99-86-5	11.23
7.59	1017	p-Cymene	99-87-6	3.20
7.73	1026	Eucalyptol	470-82-6	3.18
8.18	1052	g-Terpinene	99-85-4	20.11
8.65	1079	a-Terpinolene	586-62-9	3.70
8.82	1089	trans-Sabinene hydrate	17699-16-0	0.08
9.23	1114	cis-2-p-Menthen-1-ol	29803-82-5	0.15
9.53	1133	trans-2-p-Menthen-1-ol	29803-81-4	0.18
10.24	1178	Terpinen-4-ol	562-74-3	37.14
10.36	1185	α-Terpineol	98-55-5	2.49
10.57	1199	cis-Piperitol	16721-38-3	0.09
12.56	1336	α-Cubebene	17699-14-8	0.09
12.91	1361	α-Ylangene	14912-44-8	0.14
12.96	1365	α-Copaene	3856-25-5	0.32
13.25	1386	n.i.		0.09
13.43	1399	α-Gurjunene	489-40-7	0.82
13.58	1411	β-Caryophyllene	87-44-5	0.50
13.68	1419	n.i.		0.12
13.84	1432	Aromadendrene	489-39-4	2.21
13.94	1440	n.i.		0.21
14.12	1454	Alloaromadendrene	25246-27-9	0.77
14.23	1463	γ-Muurolene	30021-74-0	0.88
14.45	1481	Germacrene D	23986-74-5	0.54
14.54	1489	α-Muurolene	10208-80-7	3.07
14.84	1513	γ-Cadinene	39029-41-9	2.63
14.89	1518	δ-Cadinene	483-76-1	0.71
14.97	1524	n.i.		0.34
15.37	1558	n.i.		0.17
15.48	1567	n.i.		0.13
15.59	1576	Spathulenol	6750-60-3	0.16
15.68	1584	Globulol	51371-47-2	0.35
15.78	1592	Viridiflorol	552-02-3	0.31
16.11	1621	Ledol	557-27-5	0.14
16.15	1625	Cubenol (1-epi)	19912-67-5	0.28
16.31	1639	τ-Cadinol	5937-11-1	0.33
				100.00
		Total identified		**98.94**

[1]n.i. - not identified

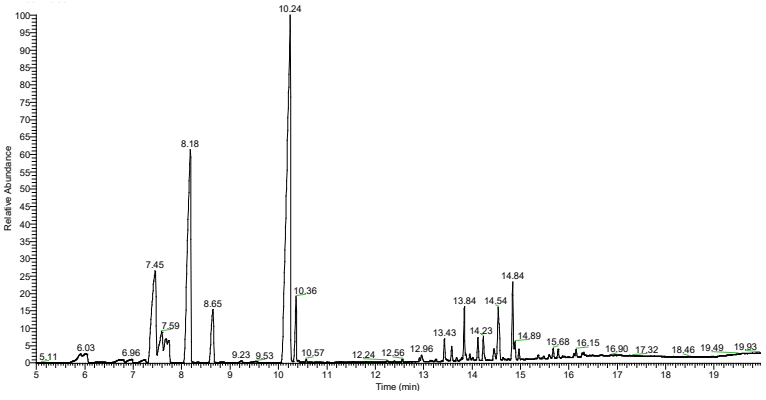

Figure 2. GC chromatogram of TTEO

Antimicrobial activity of EOs

First, we have determined the overall MIC values of all EOs are presented in Table 3. TEO had a more efficient inhibitory effect against *S. typhimurium* and *C. perfringens* at sub-inhibitory concentrations at an MIC of 7.5 µg/ml. Our results demonstrated that the TTEO had the same MIC value against *L. monocytogenes* and *C. perfringens* at 7.5 µg/ml. In addition, the MIC of TEO on *E. coli* and *L. monocytogenes*, was higher (015 µg/ml). The MIC of TTEO on *S. typhimurium* MIC was at 15 µg/ml, however, when used against *E. coli* an MIC of 62.5 µg/ml was recorded. These results clearly indicate and efficient antimicrobial activity of EOs against all bacterial pathogens investigated. These MIC findings were further supported by the disc diffusion method, which revealed clear inhibitory zones (Figure 3 and Figure 4) against all four examined pathogens.

First, *C. jejuni* NCTC 11168, as shown by the differential inhibitory zones measured in millimetres (mm), had a pronounced response across the various concentrations. Specifically, the zones of inhibition spanned from 58.75 mm at the highest concentration (100%) to 7.85 mm at the lowest concentration (1%) (Figure 3A). In comparison, TTEO exhibited a lesser antimicrobial effect, with the largest zone of inhibition observed at 24.11 mm for the highest concentration and 8.10 mm at 1 % (Figure 4A). When we assessed the antimicrobial effect against *L. monocytogenes* NCTC 4885, (Figure 4B) the TEO had a higher inhibitory zone effect ranging from 28.40 mm at 100% concentration, to 11.20 mm at 25% concentration. However, the TTEO had a reduced efficacy, producing inhibitory zones of 13.45 mm, 7.5 mm, and 6.8 mm for concentrations ranging from 100% to 50% (Figure 3B).

Moreover, the inhibitory action of both EOs against *E. coli* was comparable, yielding the largest inhibition zones of 21.43 mm for TEO and 17.41 mm for TTEO, with the smallest of 11.93 mm and 8.36 mm, respectively (Figure 3C and Figure 4C.). Smaller inhibitory zones were observed when testing both EOs against *Salmonella enterica* serovar *Typhimurium* (ATCC 14028), of 9.63 mm for TEO and 7.68 mm for TTEO at 100% concentration (Figure 3D and Figure 4D). Conclusively, these results confirm the MIC efficacy of EOs and underlines their potential use as antimicrobial agents against all pathogens investigated.

Table 3. Antimicrobial activity of TTEO and TEO, MIC method

EO	Concentration µg/mL	*E. coli*	*S. typhimurium*	*L. monocytogenes*	*C. perfringens*
TTEO					
	4000	0.107	0.177	0.109	0.113
	2000	0.174	0.277	0.130	0.117
	1000	0.291	0.385	0.163	0.149
	500	0.383	0.477	0.197	0.184
	250	0.587	0.502	0.222	0.256
	125	1.039	0.817	0.241	0.307
	62.5	**1.062***	0.973	0.314	0.314
	31	1.096	1.025	0.352	0.374
	15	1.220	**1.061***	0.438	0.396
	7.5	1.271	1.123	**0.472***	**0.418***
TEO					
	4000	0.233	0.155	0.122	0.175
	2000	0.252	0.166	0.195	0.191
	1000	0.298	0.198	0.212	0.210
	500	0.397	0.210	0.271	0.291
	250	0.414	0.258	0.285	0.338
	125	0.497	0.391	0.361	0.371
	62.5	0.594	0.410	0.393	0.397
	31	0.616	0.486	0.395	0.420
	15	**0.717***	0.506	**0.413***	0.422
	7.5	1.082	**0.654***	0.479	**0.474***
Control		1.072	1.101	0.477	0.485

*Results expressed as optical density, MIC values are highlighted with asterisk

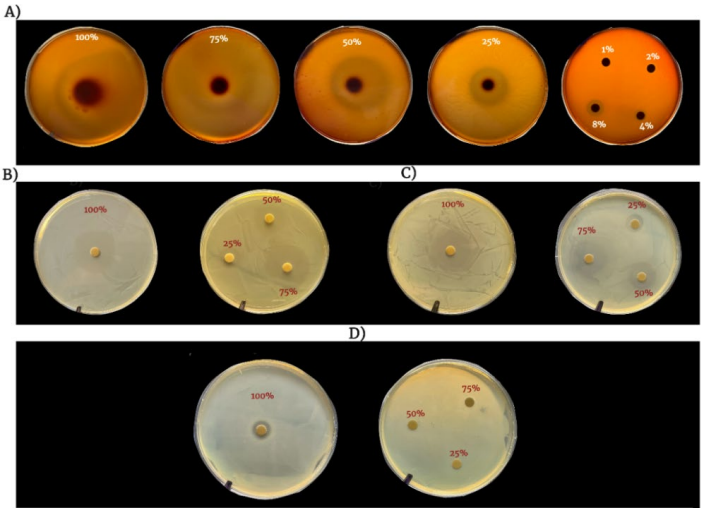

Figure 3. Antimicrobial activity of TEO visualized through disc diffusion assay (original source). This figure presents representative images of Petri dishes illustrating the zones of inhibition at varying concentrations of TEO against four pathogens: A) *C. jejuni*; B) *L. monocytogenes*; C) *E. coli*; D) *S. typhimurium*. Each panel (A-D) demonstrates the differential susceptibility of the pathogens to TEO, highlighting its concentration-dependent antimicrobial efficacy

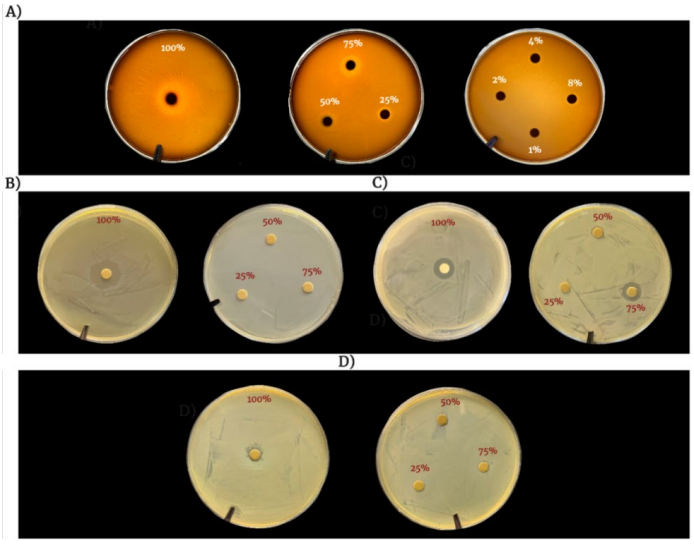

Figure 4. Inhibitory zones elicited by TTEO across varied concentrations, as observed in Disc diffusion assays (original source). Displayed are representative Petri dish images that delineate the antimicrobial effect of TTEO against: A) *C. jejuni*; B) *L. monocytogenes*; C) *E. coli*; D) *S. typhimurium*. Each subsection (A-D) details the pathogen-specific response to different concentrations of TTEO

Membrane lysis evaluation

Next, we have investigated the ability of EOs to prevent hemolysis upon exposure to heat. We have observed that TEO and TTEO, at their maximum tested concentration of 160 µl/ml. Specifically, the protective effect of dexamethasone was 47.49%, while TTEO provided a protection level of 5.04% and TEO oil offered a much lower protection level of just 0.05%. The results of membrane lysis assessment contributed by both EOs are depicted in Table 4. Conclusively, these results show that EOs can inhibit haemolysis by protecting the red blood cells membranes. The protective mechanism might require further investigation.

Table 4. Anti-inflammatory activity of TTEO and TEO

Concentration of EO	OD values	Hemolysis (%)	Hemolysis inhibition (%)
TTEO			
10 µl/ml	2.341	111.16	-
20 µl/ml	2.297	109.07	-
40 µl/ml	2.252	106.90	-
80 µl/ml	2.198	104.37	-
160 µl/ml	2.000	94.96	5.04
TEO			
10 µl/ml	2.491	118.28	-
20 µl/ml	2.394	113.68	-
40 µl/ml	2.310	109.69	-
80 µl/ml	2.200	104.47	-
160 µl/ml	2.105	99.95	0.05
Negative Control	2.106	100	-
Positive Control	1.003	52.50	47.49

[1]Negative Control was represented by PBS; Control Positive with dexamethasone.

Antioxidant capacity

To further improve our knowledge on the efficacy of these EOs we have studied their ability to reduce oxidative stress. The antioxidant activity according to DPPH assay of TTEO and TEO are represented in Table 5. The antioxidant capacity of Tea Tree and Thyme essential oils, quantified by their IC50 values (the concentration required to inhibit 50% of the target radical), varies depending on the assay used and the specific chemical profile of the oil. Lower IC50 values represent higher antioxidant potency.

Tea Tree Essential Oil (*Melaleuca alternifolia*) (TTEO) demonstrates significant potency, particularly in scavenging primary free radicals. Thyme oil (TEO) performance is strongly linked to its high phenolic content (thymol and carvacrol). Its IC50 values vary significantly by plant region and species, in this case show a higher antioxidant activity than TTEO.

Table 5. Antioxidant activity of activity of TTEO and TEO

Sample	IC50 DPPH assay
Tea tree (*Melaleuca alternifolia*) - TTEO	147 µg/ml
Thyme (*Thymus vulgaris*) - TEO	125 µg/ml

DPPH free radical neutralization activity related to ascorbic acid

DISCUSSIONS

Recent *in vitro* molecular data indicated that TTEO incorporated in nanoliposomes (TTONL), enhanced their antibacterial activity against different *E. coli* strains (Bo et al., 2023). The MIC and MBC of TTONL for *E. coli* were about 4-fold lower than those of diluted EO. The *in vivo* application of TTONL nanoliposomes in broiler chickens challenged with *E. coli* demonstrated promising results and alleviated clinical symptoms and intestinal lesions while significantly reducing mRNA expression of pro-inflammatory markers like NLRP3 and NF-kB in the duodenum and cecum (Bo et al., 2023). Similar observations regarding the inclusion of Thyme (Almremdhy & Al-Khafaji, 2020; Belali et al., 2024; Hassan et al., 2024; Hassanin et al., 2024; Naderiboroojerdi et al., 2022; Noruzi et al., 2022; Olfati & Hosseini, 2022; Puvača et al., 2022; Vlaicu et al., 2023) and Tea tree (Lv et al., 2022; Qu et al., 2019; Wang et al., 2025; Wang et al., 2024; Yang et al., 2022; Yu et al., 2025; Yuan et al., 2023) EO were previously reported which also suggested their role as growth promoters, as antimicrobials, and as inducers of antioxidant and anti-inflammatory effects with a effective impact on poultry health and meat quality. The effects of TTEO are primarily attributed to its main bioactive compound, terpinen-4-ol, alongside other significant constituents such as γ-terpinene and α-terpinene (≈ 40%, 23%, and 10%), respectively) (Lv et al., 2022; Qu et al., 2019; Y. Wang et al., 2025;

Yachao Wang et al., 2024; Yang et al., 2022; Yu et al., 2025; Yuan et al., 2023). The antimicrobial efficiency of both EOs is linked to its membrane-interacting mechanism which modifies the membrane permeability and membrane fluidity until the microbial cells become leaky and eventually die (Hassanzadeh et al., 2024; Lv et al., 2022; Salinas-Chavira & Barrios-García, 2024). TTEO has a remarkable antibacterial effect, as previously described against antibiotic resistant strains of *E. coli*, *Enterococcus* species and *Staphylococcus aureus* (Iseppi, Mariani, Benvenuti, Truzzi, & Messi, 2023). Moreover, our results show that TTEO has an anti-inflammatory through reduced inflammatory markers. These anti-inflammatory effects occurs through various mechanisms starting with lowering IL-2, IL-6, IL-17 and TNF-α while decreasing NLRP3 mRNA expression, but inhibiting Caspase-3 and Bax and boosting Bcl-2 anti-apoptotic gene activity (Bo et al., 2023; Lv et al., 2022; Wang et al., 2025; Wang et al., 2024). The treatment shaped the immune state of broilers through immune modulation that included $CD^{3+}CD^{8+}$ T-cell subset growth together with reduced expression of inflammatory cytokines (IFN-γ, TNF-α, IL-1β), which collectively reduced systemic inflammation (Wang et al., 2025).

In our study, the TEO also has robust antimicrobial properties similar to observations from other studies, emphasizing its beneficial impact on many poultry pathogens including *E. coli*, *S. typhimurium*, *C. perfringens*, *L. monocytogenes* and *C. jejuni* (Balta et al., 2021; Sakkas & Papadopoulou, 2017; Stratakos et al., 2018). For example, thymol and carvacrol act as the main active components which disrupt microbial membranes to induce membrane permeability leaks and cause impairment of vital metabolic functions (Kachur & Suntres, 2020; Sakkas & Papadopoulou, 2017). *In vitro* tests using subinhibitory MIC concentrations demonstrated that TEO affects the growth rate of *Salmonella enterica*, serovar Enteritidis, confirming its ability to suppress bacterial growth at low concentrations (Hassanzadeh et al., 2024). Other results exposed its ability to inhibit the activity of virulence genes (*hilA*, *spv*, *sefA*, *invA*) and other virulence factors which are vital for its pathogenicity (Hassanzadeh et al., 2024). *In vivo*, TEO encapsulated particles improved performance in broiler chicks after the finisher period and prevented *Salmonella* infection (Olfati & Hosseini, 2022). In a pilot study, the antibacterial properties of TEO, at 5% concentration, reduced *Enterobacteriaceae* and *Salmonella* Derby infection by 88.9% concomitantly with a reduction in *Mammaliicoccus lentus* by 77.7% (Galgano et al., 2023). All these data suggests that TEO is practical replacement alternative for standard chemical disinfectants which the poultry industry can be used to address poultry litter management. TEO, at 200 mg/kg, was reported to reduce the harmful effects of aflatoxin B1 in broiler diets and significantly enhance BW/BWG and FCR compared to other treatments (Fawaz et al., 2022).

The antioxidant effect of TTEO identified in our study was also confirmed in earlier studies which described its role in enhancing SOD and CAT enzyme activity and simultaneously reducing MDA productions (Qu et al., 2019; Wang et al., 2025; Wang et al., 2024). The primary active compounds of terpinen-4-ol along with other monoterpenes act as antioxidants to efficiently reduce oxidative stress along with inflammatory responses which was documented in previous studies involving broiler chickens and quails (Deminicis et al., 2024; Khan et al., 2023; Qu et al., 2019; Wang et al., 2025; Wang et al., 2024). Our research data matches previous observations showing antioxidative effects because it revealed better meat quality together with decreased drip loss. The antioxidant capacity of TEO, evidenced by neutralization of free radicals (DPPH assay), closely mirrors previous findings. Similarly to TTEO, the antioxidant activities of thymol and carvacrol from TEO function by directly reacting with free radicals and simultaneously strengthening endogenous antioxidant defenses through SOD, CAT and GPx (Saim et al., 2024) enhancement (Deeb et al., 2024; Ghalandari et al., 2022; Gümüş et al. 2023).

The antimicrobial properties of TTEO extended its beneficial impact on preventing common spoilage bacteria *S. aureus* and *E. coli* thereby increasing poultry meat shelf life. In broilers, experiments with other essential oils support this trend. Jimoh et al. (2024) gave broilers various spice EOs in drinking water and found that drip loss ranged from 4.5% in control birds down to

1.5% in the cinnamon EO group (Jimoh et al., 2024).

CONCLUSIONS

Both EOs effectively inhibited the growth of bacterial pathogens (*S. typhimurium, E. coli*, *C. perfringens*, *L. monocytogenes*, including *C. jejuni*), confirming their potential use as natural antimicrobials in broiler feed. Additionally, TEO demonstrated a more efficient antioxidant capacity over TTEO, suggesting an enhanced potential in combating oxidative stress in poultry. Our results indicate a protective effect of both EOs against protein denaturation and erythrocyte membrane lysis suggesting anti-inflammatory benefits.

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