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Antibacterial and antibiofilm activity of *Melaleuca alternifolia* (tea tree) essential oil against colistin resistant *Salmonella enterica* serotypes isolated from poultry environmental specimens

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ABSTRACT: The development of increasing resistance to antibiotics such as colistin, problems arise in the treatment of bacterial infections and make it necessary to search new alternative methods. For this purpose, plant-based approaches are among the important research topics depending on their traditional uses. The aim of the present study was to investigate the antibacterial and antibiofilm activity of tea tree oil purchased from a local market against a variety of 66 colistin resistant *Salmonella enterica* serotypes isolated from poultry farm environmental samples. Content analysis of TTO was determined by gas chromatography/mass spectroscopy. The antibacterial activity was determined by broth microdilution method, and antibiofilm activity was examined by crystal violet method. As a result, terpinen-4-ol was found as major component of TTO with 35.9% ratio. The MIC values of TTO were differed between 6250-12500 µg/mL. 27 of 66 isolates formed biofilm and 25 of 27 isolates belonged to *S.* Infantis. The biofilm reduction of TTO at sub-inhibitory concentration were found between 52-84.4%. Current study should be supported by future studies to determine the effectiveness of TTO to be among the agents that can be used together with antimicrobials in the attenuation of microorganisms.

KEYWORDS: antibacterial; biofilm; poultry; *Salmonella enterica*; tea tree oil; *Melaleuca alternifolia*; antibiotic resistance; colistin.

1. INTRODUCTION

Antimicrobial resistance is recognized by most countries around the world as one of the greatest global health threats [1]. This resistance, for example, occurs when bacteria change over time and are not affected by the therapeutic doses of antibiotics they were previously affected. This makes common infections more difficult to treat and increases the risk of serious illness and death [2]. Bacteria have the ability to cross-transmit antibiotic resistance, and this resistance can be indirectly transmitted between humans, animals, environments etc. spreads in a chain [3-5]. This causes a significant increase in the number of drug-resistant strains worldwide. Therefore, operating in only one of the health fields in the fight against resistance cannot eliminate an existing resistance problem. For this reason, all health sectors should act in coordination. This situation highlights the importance of the concept of "one health" once again [6, 7].

Alternative treatment approaches are being investigated including the activation of existing drugs with nanotechnologies, the search for new synthetic active substances, and additionally the development of methods based on aromatic or medicinal plants. Recently, an antimicrobial treatment approach to reduce the virulence of microorganisms has gained importance by developing methods based on aromatic and medicinal plants. In this context, the use of plants, spices and essential oils (EOs) as antimicrobial agents is among the most important researches [5, 8].

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The EOs are found highly valued for different type of industries like pharmaceutical, food, etc. worldwide due to their antimicrobial, antiinflammatory, antidepressant properties in vitro [9]. Tea tree oil (TTO), as an EO, is one of the examples, obtained from the plant *Melaleuca alternifolia* (*M. alternifolia*), which belongs to the *Myrtaceae* family. The genus *Melaleuca* contains about 250 species, and most *Melaleuca* species are restricted to Australia [10-12]. *M. alternifolia* is a mostly green plant that can reach up to 6 meters in height. It bears masses of fluffy, white flowers from spring to early summer and has narrow-to-wide leaves and flat spherical fruits. The EO is light yellow in color and smells of eucalyptus. Its active ingredient is cineole and terpinen-4-ol [13].

Non-typhoidal *Salmonella* species are facultative anaerobic, Gram-negative, bacilli belonging to the *Enterobacteriaceae* family [14]. *Salmonella* sp. can be found in the intestinal microbiota of especially animals, some serotypes are host specific and can only be found in one or a few animal species however among the pathogens of medical importance for them particulary in humans [15]. Asymptomatic chronic carrier humans are contagious if they cannot reach sanitation [16]. Since *Salmonella* sp. colonizes the gastrointestinal tract in general, it is excreted in the feces and transmission can occur between many organisms [17]. *Salmonella* sp. is one of the leading food-borne pathogenic bacteria in some countries, especially in the USA, and creates a high mortality and cost burden. The majority of *Salmonella* sp. outbreaks in recent years have been associated with poultry by the food chain, due to the consumption of contaminated chicken, turkey or eggs [18-20]. Some *Salmonella* serovars (e.g. s. typhi, s. typhimurium) that can potentially cause disease in human and/or animal hosts are capable of forming biofilms. It is thought that this feature provides an advantage to the bacteria in the development of the infection [21, 22] and also allows bacteria to live in adverse conditions and possibly contributes to their protection against various environmental stressors, including antibiotics [23]. Biofilm-forming bacteria account for 65% or more, mainly instrument-related infections, skin and soft tissue infections, and chronic infections, often referred to foreign body infections [24]. Foreign body infections are an important clinical problems due to the fact that they are not affected by standard treatment protocols with known efficacy [25]. Accordingly, the treatment of some infections becomes difficult and it is important to search for new and effective antimicrobial alternatives, especially against resistant pathogens.

Colistin is a cationic antibiotic in the class of polymyxins, that binds to negatively charged lipid A in the outer membranes of Gram-negative bacteria. Mechanims of action is by distrupting lipopolysaccharide, increasing the permeability of the bacterial membrane, and consequently causing cell death [26]. According to the World Health Organization, colistin is one of the critically important antibiotics [27] and although it was first used only for animals, it has been considered as an antibiotic of last resort in the treatment of human infections due to the decrease in the effectiveness of available drugs in human medicine and the increase in multidrug-resistant bacteria [28]. Food-producing animals, particularly poultry and pigs, appear to be the primary reservoir of colistin-resistant *Salmonella enterica* (*S. enterica*) strains [29, 30].

In order to find a solution to such problems, the present study aimed to investigate the antibacterial activity of TTO against a variety of colistin resistant *S. enterica* serotypes isolated from environmental samples of poultry farms and to determine the antibiofilm activity of TTO at sub-inhibitory concentration on biofilm producing serotypes.

2. RESULTS

In this study, TTO purchased from a commercial brand was subjected to GC and GC/MS analyses to identify their composition. Terpinen-4-ol was found as the major component of the commercial TTO with 35.9% ratio. γ-Terpinene 14.2% and p-Cymene 10.99% were components with a high % ratio after terpinen-4-ol. Other defined components in the TTO were listed in Table 1 and the related chromatogram was demonstrated in Figure 1.

Table 1. Compounds identified in TTO sample by GC/MS analyses.

No	Name	RT (min)	% Area	No	Name	RT (min)	% Area
1	α-Thujene	3.97	0.49	**13**	α-Terpineol	7.81	3.83
2	α-Pinene	4.08	1.64	**14**	α-Gurjunene	13.48	0.54
3	β-Pinene	4.63	0.43	**15**	Caryophyllene	13.83	0.44
4	β-Myrcene	4.71	0.42	**16**	Aromadendrene	14.49	1.55
5	α-Terpinene	5.11	9.65	**17**	Alloaromadendrene	15.27	0.63
6	p-Cymene	5.22	10.99	**18**	β-Cadinene	15.67	0.61
7	D-Limonene	5.28	1.35	**19**	(+)-Ledene	16.49	1.37
8	Eucalyptol	5.33	6.82	**20**	Bicyclogermacrene	16.54	1.21
9	γ-Terpinene	5.69	14.12	**21**	δ-Cadinene	17.33	2.32
10	Terpinolene	6.10	3.97	**22**	Epizonarene	17.40	0.39
11	Trans 2 Menthenol	6.61	0.41	**23**	(-)-Globulol	18.92	0.49
12	Terpinen-4-ol	7.63	35.90	**24**	Guaiol	19.12	0.44

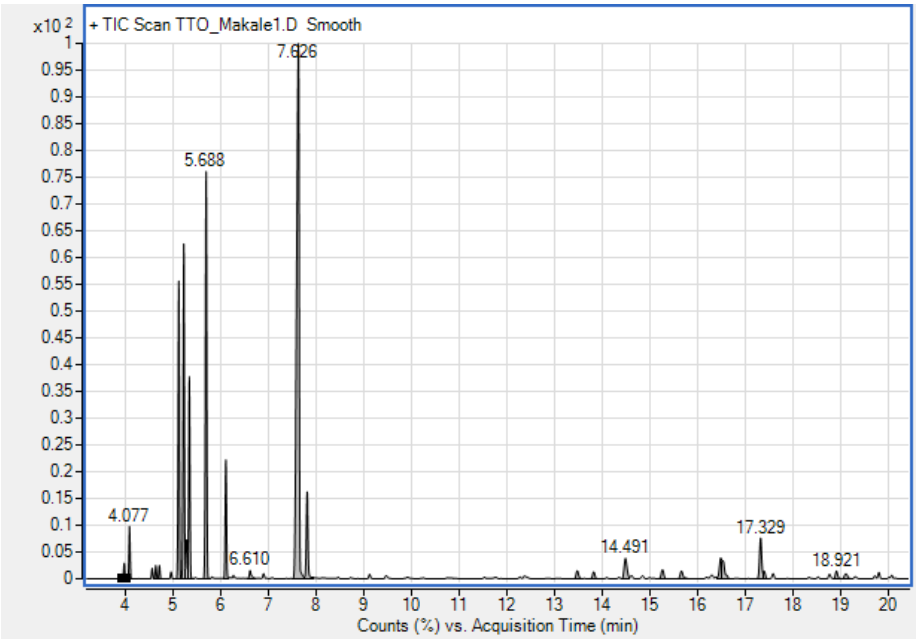

Figure 1. GC/MS chromatogram of the 50 mg/mL TTO sample.

Antibacterial activity results of TTO were shown as a MIC value and found between 6250- 12500 µg/mL (Table 2). Biofilm formation levels of the tested *S. enterica* serotypes were classified according to Pedonese, Longo [31]'s method as weak, moderate and strong. ODs were interpreted using following formulations: ODs ≤ ODc = no biofilm producers, ODc < ODs < 2ODc = weak biofilm producer, 2ODc < ODs < 3ODc = moderate biofilm producer, ODs ≥ 3ODc = strong biofilm producer. As a result, it was evaluated that 27 of 66 isolates were found as biofilm producer. Twenty five of 27 biofilm producer isolates were belonged to serotype Infantis (weak (n: 11), moderate (n: 10), strong (n: 4)), 1 of 27 was Enteritidis (weak) and the other one was Typhimurium (weak). Then the biofilm inhibition of TTO at MIC/2 concentrations was measured and determined as a reduction %. Biofilm reduction% calculation of isolates were evaluated according to Jadhav et al. [32]'s formulation: Inhibition % = 100 – [{OD620 experiment/OD620 control}X100]. Results were shown in Figure 2 and Table 3.

Table 2. Antibacterial activity results for *Salmonella enterica* serotypes as MIC (µg/mL).

Isolate Number (n)	Isolate code	*Salmonella* Serotype	TTO MIC (µg/mL)	Colistin MIC (µg/mL)
2	67, 85	*Salmonella* Abony	12500	4
1	62	*Salmonella* Abony	25000	0,5
1	78	*Salmonella* Anatum	12500	8
6	64, 66, 181, 189, 201, 297	*Salmonella* Enteritidis	12500	4
8	84, 90, 152, 153, 169, 170, 182, 194	*Salmonella* Enteritidis	12500	8
1	157	*Salmonella* Enteritidis	12500	16
2	188, 193	*Salmonella* Enteritidis	6250	4
1	128	*Salmonella* Hadar	12500	4
1	171	*Salmonella* Hadar	12500	16
1	75	*Salmonella* Havana	6250	16
2	79, 83	*Salmonella* Infantis	6250	16
2	76, 173	*Salmonella* Infantis	12500	16
16	59, 60, 65, 89, 132, 138, 143, 155, 187, 196, 210, 226, 251, 254, 267, 283	*Salmonella* Infantis	12500	4
7	73, 74, 77, 82, 154, 163, 211,	*Salmonella* Infantis	12500	8
1	133	*Salmonella* Kentucky	12500	4
1	166	*Salmonella* Kikoma	6250	8
1	160	*Salmonella* Kottbus	12500	8
1	161	*Salmonella* Kottbus	12500	16
1	159	*Salmonella* Lexington	12500	16
1	156	*Salmonella* Liverpool	6250	8
1	72	*Salmonella* Liverpool	6250	32
1	165	*Salmonella* Mbandaka	12500	16
1	80	*Salmonella* Newport	12500	16
1	175	*Salmonella* Paratyphi B	12500	8
1	68	*Salmonella* Thompson	12500	8
1	126	Salmonella typhimurium	6250	4
3	162, 164, 250	Salmonella typhimurium	12500	8
Control	*Escherichia coli* ATCC 25922		6250	1
Control	*Escherichia coli* NCTC 13846		12500	4

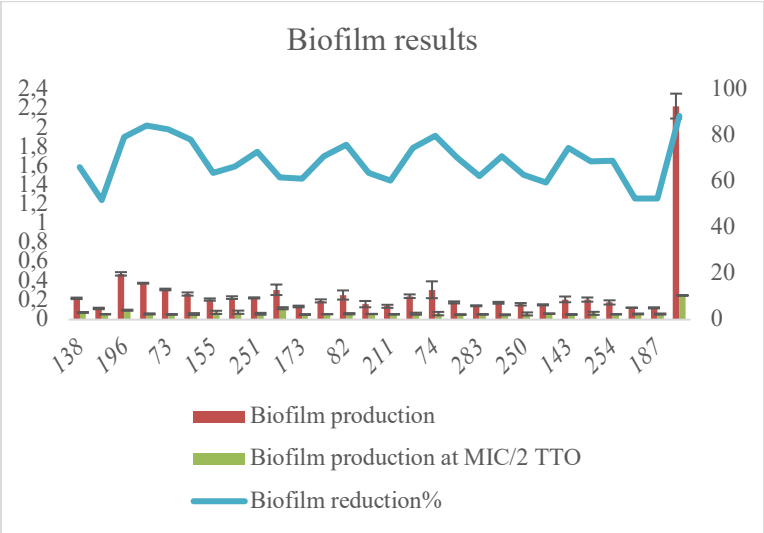

Figure 2. Biofilm production of isolates and biofilm inhibition at MIC/2 TTO.

Table 3. Biofilm results of *S. enterica* serotypes

	Isolate lab code	*Salmonella* serotype	Degree of biofilms formed	Biofilm production	Biofilm production at MIC/2 TTO	Reduction %
1	59	*S.* Infantis[a]	Strong	0,3115± 0,055	0,1187± 0,011	61,90884
2	60	*S.* Infantis[a]	Weak	0,1541± 0,006	0,0622± 0,003	59,6237
3	66	*S.* Enteritidis[b]	Weak	0,1617± 0,033	0,0583± 0,002	63,93814
4	73	*S.* Infantis[a]	Strong	0,3145± 0,009	0,0537± 0,003	82,92709
5	74	*S.* Infantis[a]	Strong	0,3119± 0,088	0,0625± 0,018	79,97221
6	76	*S.* Infantis[a]	Moderate	0,2673± 0,017	0,0577± 0,01	78,41646
7	77	*S.* Infantis[a]	Strong	0,3802± 0,005	0,0591± 0,006	84,44678
8	82	*S.* Infantis[a]	Moderate	0,2569± 0,048	0,0609± 0,007	76,2813
9	83	*S.* Infantis[a]	Weak	0,1242± 0,002	0,0587± 0,005	52,75168
10	89	*S.* Infantis[a]	Weak	0,1794± 0,011	0,0526± 0,001	70,68004
11	132	*S.* Infantis[a]	Moderate	0,2444± 0,019	0,0614± 0,011	74,89431
12	138	*S.* Infantis[a]	Moderate	0,2221± 0,008	0,0744± 0,004	66,48657
13	143	*S.* Infantis[a]	Moderate	0,2113± 0,03	0,0533± 0,001	74,75943
14	154	*S.* Infantis[a]	Moderate	0,2298± 0,014	0,0768± 0,017	66,58929
15	155	*S.* Infantis[a]	Moderate	0,2094± 0,012	0,0755± 0,017	63,92294
16	163	*S.* Infantis[a]	Weak	0,1756± 0,01	0,0506± 0,005	71,171
17	173	*S.* Infantis[a]	Weak	0,1369± 0,009	0,0529± 0,002	61,36806
18	187	*S.* Infantis[a]	Weak	0,1227± 0,006	0,0579± 0,006	52,8389
19	196	*S.* Infantis[a]	Strong	0,4780± 0,018	0,0979± 0,006	79,52723
20	210	*S.* Infantis[a]	Weak	0,1164± 0,008	0,0558± 0,002	52,04812
21	211	*S.* Infantis[a]	Weak	0,1389± 0,016	0,0549± 0,002	60,50864
22	226	*S.* Infantis[a]	Weak	0,1955± 0,016	0,0564± 0,001	71,17286
23	250	*S.* Typhimurium[b]	Weak	0,1601± 0,013	0,0592± 0,016	63,01541
24	251	*S.* Infantis[a]	Moderate	0,2265± 0,006	0,0608± 0,010	73,15673
25	254	*S.* Infantis[a]	Weak	0,1793± 0,022	0,0552± 0,001	69,22648
26	267	*S.* Infantis[a]	Moderate	0,2092± 0,022	0,0652± 0,016	68,81772
27	283	*S.* Infantis[a]	Weak	0,1444± 0,005	0,0542± 0,002	62,49712
Biofilm negative		(Abony, Anatum, Enteritidis, Hadar, Havana, Infantis,	-	-	-	-

Salmonella serotypes	Ketucky, Kikoma, Kottbus, Lexington, Liverpool, Mbandaka, Newport, Paratyphi B, Thompson, Typhimurium)[b]				
Biofilm control	*Staphylococcus epidermidis* ATCC 35984	Strong	2,2318± 0,13	0,2523± 0,004	88,69356
Biofilm control	*Escherichia coli* ATCC 25922	Weak	0,1008±0,009	0,0692±0,006	31,34921

[a, b]: Means for biofilm producer with unlike letter among *Salmonella* serotype differ significantly ($p < 0.05$).

3. DISCUSSION

Recently, the increase of multi-antibiotic-resistant and biofilm-forming bacteria poses a global threat as conventional antibiotic therapy is becoming ineffective [33]. Colistin resistance, especially in *Enterobacterales* members has hindered the clinical use of colistin. Treatment failures with colistin monotherapy and the emerging of drug resistance, have stimulated the search for another agent that can overcome resistance mechanisms against colistin-susceptible and colistin-resistant organisms [28]. As a search for an alternative solution, in present study, the inhibitory effect of EO obtained from *M. alternifolia* against colistin-resistant *S. enterica* serotypes was determined. In the last few years, many studies investigating the antimicrobial activity of plant EOs, which may have an important therapeutic potential due to the high chemical metabolite diversity they contain, as an alternative approach. EOs show antimicrobial activity, especially due to the secondary metabolites they contain (erpenes, phenols, alcohols, etc.) [34] and this makes EOs find use in many areas including food packaging, cosmetics and pharmaceutical industries. TTO is also one of these EOs and there are a limited number of studies that determined the antibacterial and antibiofilm activity against different *S. enterica* serotypes. The serotype diversity investigated in the present study will provide data for the first time to the literature. It is included in studies that TTO may affect the permeability barrier of cell membrane structures and also cause cellular potassium ions to leak, preventing the respiration of bacteria [35].

TTO has many beneficial properties such as antibacterial activity due to the constituents it contains. The commercially available TTO that used in present study was analyzed by GC and GC/MS and terpinen-4-ol was found as major component with 35.9%, other high content substances were γ-Terpinene 14.2% and p-Cymene 10.99%, respectively. Borotova et al. [36] was detected that TTO contains terpinen-4-ol as dominate content, γ-Terpinene, and p-cymene with 40.3%, 11.7%, and 6.2% respectively, similar to our results. Melo et al. [37] was found that contents of TTO were Terpinen-4-ol (39.8-40.4%), γ-Terpinene (17.8-19.5%) and p-Cymene (2.3-4.7%). Noumi et al. [38] was evaluated Terpinen-4-ol was the major compound of TTO (40.44%) and γ-Terpinene with 19.54% was found after. McMahon et al. [39] was underlined the major contents of TTO as Terpinen-4-ol (>35%). TTO contents of the present study are comparable to other analyzed TTOs, although the presence of some components varied with other studies. Many factors such as the genotype of *M. alternifolia,* the location from which plant was obtained, the growing conditions, the plant part (leaf or flower) and time from which the essential oil was obtained, affect the amount and composition of TTO [40-42].

In this study, the efficacy of TTO against *S. enterica* serotypes was determined as MIC and concentrations were found between 6250-12500 µg/mL. Borotova et al. [36] evaluated the antibacterial activity of TTO against *S.* enterica (MIC50: 11.82 µl/mL; MIC90: 16.36 µl/mL). Puvaca et al. [43] tested the antibacterial activity of TTO by disk difussion method and found effective with 15 mm zone of inhibition diameter against *S.* Typhi. Filimon et al. [44] concluded that S. enteridis was sensitive to different concentrations of TTO. Singh et al. [45] investigated the antibacterial activity of TTO against *S.* Gallinarum (n: 1), *S.* Abortusequi (n: 2), *S.* Adelaide (n: 1), *S.* Kentucky (n: 3), *S.* Typhimurium (n: 4) and found the MIC ranges between 160->5120 nL/mL. McMahon et al. [39] reported that TTO was an active antibacterial agent even at low concentrations (0.25-0.5%) for *S.* Enteritidis NCTC 12694, *S.* Typhimurium St11 and St17 strains. MIC values and *Salmonella* serotypes are variable in literature, but all researchers reported efficacy of TTO, which is consistent with our results. McMahon et al. [39] confirmed that TTO is an effective antibacterial agent for S. Typhimurium and Enteritidis. The present study provides data for the first time in the literature for antibacterial activity of TTO against *S. enterica* serotypes including Abony, Anatum, Hadar, Havana, Kikoma, Kottbus, Lexington, Liverpool, Mbandaka, Newport, Thompson.

It has been reported that public health pathogens with the ability to form biofilms, including *Salmonella*, may cause problems on surfaces in contact with food in addition to foreign body infections [46]. Foodborne

infections or poisonings may occur due to bacterial biofilms formed on foods (chicken, milk, etc.) or on production factory equipment [47]. Since *Salmonella* contamination in foods can occur in any part of the food chain, the "farm-to-table" approach is important. *Salmonella* species are considered to be a source of human infection that can contaminate food-producing or food-consumed animals and vegetables, especially poultry [48]. Biofilm formation can be seen as a physical barrier that allows bacterial pathogens to resist antibiotic treatment [49, 50]. In the present study, it was determined that 27 of 66 isolates formed different degrees of biofilm (weak, moderate and strong) and 25 of the biofilm positive isolates belonged to *S. enterica* serotype Infantis. Biofilm producer *Salmonella* serotypes differ significantly ($p<0.05$). In recent years, the most frequently isolated serotype from poultry environmental samples is Infantis [30]. Infantis serovar is reported worldwide as one of the most common causes of foodborne human infections. It was included in studies that Infantis serotype was isolated from food and animal sources, usually from broilers and associated with human salmonellosis that not easily removed by disinfection from farms or environments such as slaughterhouses [51-56]. For this reason, it is important to search for an effective alternative method especially for Infantis serotypes that form biofilms. In this study, it was determined that TTO reduced the biofilm formation of isolates by 52-84.4% at sub-inhibitory concentration. In the literature review, Borotova et al. [36] investigated the activity of 0.1% TTO against *S. enterica* biofilm by MALDI-TOF and stated that this concentration was not sufficient to break down the biofilm at the protein level. Since there are a limited number of studies investigating the antibacterial and antibiofilm activity of TTO against *Salmonella* serotypes, the results obtained in this study are important in terms of providing data to the literature.

4. CONCLUSION

Studies are limited about antibacterial and antibiofilm activity of TTO against *Salmonella* serotypes. The current work should be supported by future studies in order to determine the effectiveness of TTO against the virulence of pathogens and to be among the agents that can be used together with antimicrobials in the attenuation of microorganisms.

5. MATERIALS AND METHODS

5.1. Essential oil

Tea tree oil obtained by steam distillation from the leaf of *M. alternifolia* with serial number TR-34-K-183635 purchased from a local market in Turkey was included in the present study.

5.2. GC/MS analysis of tea tree oil

The components of the tea tree oil were analyzed by 6890/5973N model GC/MS (Agilent, Santa Clara, USA). A Restek 5MS gas chromatography column with the dimensions of 30 m × 0.25 mm i.d. × 0.25 µm was supplied by Ant Teknik, (Ankara, Turkey). As a carrier gas, helium (99.999%) was used with a flow rate of 1.5 mL/min. 50 mg of the tea tree oil sample was accurately weighed and dissolved in 1mL of analytical grade ethyl acetate (Merck, Darmstadt, Germany). The prepared solution was injected as 0.2 µL with a split ratio of 30:1. The temperature program of the column oven starting with the ascending from 60 °C to 120 °C stepped by 10 °C per minute, then it was increased to 140 °C at 2 °C/min. And lastly 250 °C at 10 °C/min. The injection port and transfer line (AUX) temperatures were set at 200 °C and the detector temperature was 300 °C. The mass spectrum was obtained by electron ionization (EI) at 70 eV in the mass range of m/z 33-350 atomic mass units, scanned by the detector. The signals were processed by Mass Hunter software (Qualitative Analysis B.07.00) and the integrated peaks were identified by NIST Mass Spectral Library (2014).

5.3. Bacteria

Different colistin resistant *S. enterica* serotypes including Abony (n: 3), Anatum (n: 1), Enteritidis (n: 17), Hadar (n: 2), Havana (n: 1), Infantis (n: 27), Ketucky (n: 1), Kikoma (n: 1), Kottbus (n: 2), Lexington (n: 1), Liverpool (n: 2), Mbandaka (n: 1), Newport (n: 1), Paratyphi B (n: 1), Thompson (n: 1) Typhimurium (n: 4) isolated from various environmental specimens taken from poultry farms in Turkey by the Aviagen Anatolian Poultry Diagnosis and Analysis Laboratory between 2014-2018 years were included in the present study. Colistin resistance (antibiotic testing range: 0,0625-128 µg/mL) of these isolates were confirmed by broth microdilution test according to European Committee of Antimicrobial Susceptibility Testing standards [57] by replicating the test.

5.4. Antibacterial activity

Antibacterial activity of TTO was determined by broth microdilution method as a minimum inhibitiory concentration (MIC) according to EUCAST [57] standards against a variety of colistin resistant *S. enterica* serotypes. *Escherichia coli* ATCC 25922 (*E. coli*, colistin suscetible) and *E. coli* NCTC 13846 (colistin resistant) were used as control strains.

For broth microdilution test, a cation-adjusted Mueller Hinton broth (MHB, Becton, Dickinson and Company, USA) was used. A total of 100 µl MHB was added to the all wells of 96 U-bottom microplate. The same volume of TTO was added (100 µl) to the first well and diluted. TTO concentrations were between 0,244-50 mg/mL. Then, the bacterial suspension prepared from fresh microorganism cultures at 0.5 McFarland turbidity in saline (0.85% NaCl) was diluted 1:100 to give a final bacterial concentration of $5x10^5$ cfu/mL and added to all wells. Then microplates were incubated at 35 ± 1 °C for 18 ± 2 h.

5.5. Antibiofilm activity

Antibiofilm activity of TTO at MIC/2 concentration was evaluated according to modified Christensen, et al.'s method [58] and results were achieved as a percentage reduction. *Staphylococcus epidermidis* ATCC 35984 was used as control. Luria bertani broth containing MIC/2 TTO was added to the microplate wells in a volume of 100 µl. The bacterial suspension was prepared at 0.5 McFarland turbidity in sterile saline (0.85% NaCl). This suspension was diluted 1:100 in Luria bertani broth (HiMedia, India) to give a final bacterial concentration of $5x10^5$ cfu/mL and added to the microplate wells in a volume of 100 µl. Microplates were incubated for 24 hours at 35±1 °C. At the end of the incubation, the wells were emptied and washed 3 times with sterile distilled water. After washing, 200 µl of 0.1% crystal violet was added to each well and stained for 15 minutes at room temperature. After staining, the wells were washed 3 times with sterile distilled water and dried at room temperature. 200 µl of 33% glacial acetic acid was added to the dried wells and incubated for 15 minutes at room temperature. The results were read in the spectrometer (Thermo Fisher Scientific, USA) at OD 620 nm. All experiments were performed in triplicate, also run with TTO-free controls, and the results were interpreted with comparing the control and MIC/2 TTO.

5.6. Statistical analysis

Comparisons of the differences for biofilm producer among *S. enterica* serotypes were made by the chi-square test. A probability value of less than 0.05 was considered significant.

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