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Original Article

The Role of *Thymus Vulgaris* Essential Oil on the Rate of Fecal Excretion of *Salmonella Typhimurium* in Broilers Infected with Bacteria

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Summary
Gastrointestinal tract infection with serotypes of *Salmonella* is common worldwide, and its treatment with antibiotics leads to problems such as drug resistance and drug side effects. With the spread of antibiotic resistance, the desire to use medicinal plants to control microorganisms has increased. The use of medicinal plants can be a solution to this problem. This experimental study aimed to investigate the antimicrobial activity of Thyme essential oil compared to two antibiotics, doxycycline, and oxytetracycline, in an animal model. So, 36 pieces of 14-day-old broilers of the Ross breed were used in 6 groups of 6. The broilers were infected with *Salmonella Typhimurium* (ATCC14028). After extracting the essential oil from thyme and analyzing by GC-MS, the minimum inhibitory concentration (MIC) of its growth and antibiotic was determined by broth microdilution method, and finally, the broilers were treated by gavage for seven days, twice every day with a time interval of 12 hours. To check the effectiveness of antibiotics and essential oils, broiler feces were cultured daily, and the number of *salmonella* colonies grown was counted. The findings were analyzed with SPSS 26 software and a two-way analysis of variance. The GC-MS analysis of T. vulgaris essential oil showed the presence of 12 chemical compounds among which thymol, m-Thymol, p-cymene, and carvacrol were major. The results showed that there is a significant difference between the groups in terms of reducing the number of bacteria, but there is no significant difference between the times. Both thyme essential oil and doxycycline and oxytetracycline antibiotics reduced the colonization and finally stopped the excretion of *Salmonella Typhimurium* in the feces of broiler chickens.
Keywords: Antibiotic Resistance, Broiler, *Salmonella Typhimurium*, Thyme essential oil, GC-MS.

Introduction

Acute gastrointestinal diseases are among the most important diseases worldwide. Gastrointestinal tract infection with *Salmonella* is prevalent in the world, especially in developing countries (Guerin et al., 2005). *Salmonella* has a wide geographical distribution and caused infection in a wide range of living organisms, including humans and birds. *Salmonella Typhimurium* and *Salmonella Enteritidis* are the most important and common serotypes isolated from poultry. *Salmonella* infection in humans occurs in the form of food

poisoning, gastroenteritis, typhoid fever, and sometimes septicemia. Most infections occur as a result of eating contaminated food products (Azizpour, 2018). In addition to causing significant losses in poultry farms, especially chickens less than two weeks old, avian salmonellosis causes great damage to the economy of our country, as a disease transmitted to humans through food. It is also very important from the public health point of view. However, adult birds can serve as lifelong hosts for this organism without showing signs of infection (Tariq et al., 2022). Currently, animals, specifically chicken and eggs, are considered the primary cause of salmonellosis and many other food-borne outbreaks. Previous studies have reported the isolation of *Salmonella* from foods of animal origin as well as human samples. Therefore, it is very important to control *salmonella* in poultry flocks for the success of the poultry industry (Azizpour, 2018; Elbediwi et al., 2020).

With the spread of commercial meat birds and the sensitivity of these birds, breeders have used antibiotics orally for many years for therapeutic purposes as well as non-therapeutic uses (subtherapeutic) as growth stimulants. This situation has gradually increased the resistance of birds to the treatment of their infectious diseases with antibiotics (Antibiotic Resistance; Alonso et al., 2017). The selective pressure caused by antibiotics on intestinal microbes led to the creation of resistant genes that are transmitted by horizontal gene transfer among pathogenic bacterial species. This leads to the overgrowth of resistant bacterial pathogens such as *Clostridium*, *Salmonella*, and *Campylobacter* in the host, resulting in harmful diseases (Allen et al., 2013). Furthermore, changes in the gut microbial population can make the host more vulnerable to infection by other environmental pathogens. Since bacteria already have antibiotic resistance mechanisms, it is necessary to explore different non-antibiotic alternatives. Thus, the issue of antibiotic resistance should be addressed through multimodal and interdisciplinary research to discover alternatives to antibiotics (Allen et al., 2013).

Medicinal plants are being explored to find new antimicrobial agents, especially against resistant drugs. Moreover, the antimicrobial activity of some of these plants against different strains has been reported (Allen et al., 2013). However, fewer data are available for testing these extracts in vivo. Previous studies have reported positive effects of thyme essential oils on growth performance and immune pathogens (Manandhar et al., 2019; Alsakhawy et al., 2022). The main compounds of the Thyme essential oil are terpenoids and phenolic derivates including thymol, carvacrol, α-terpineol, 1,8-cineole, borneol, geraniol, p-cymene, thujanol, γ-terpinene, and caryophyllene. Thymol (5-methyl-1-2-isopropyl phenol) and carvacrol (5-isopropyl-2-methyl phenol) are the main phenolic components in thymus vulgaris (Gavahian et al., 2012). Antibacterial activity of thyme extract, oil, and the major components against of Clostridium botulinum, Clostridium perfringens, Bacillus subtilis, S. sonnei, E. coli, H. pylori, S. typhimurium, S. sonnei, Bacillus cereus, L monocytogenes, C. jejuni and S. enteric were reported in previous literature (Thakare, 1999; Nevas et al., 2004). However, the composition of EOs is highly influenced by intrinsic, ecological, and technological factors. Thus, they can express different effects as bio-preservatives (Ed-Dra et al., 2021).

Considering the importance of salmonellosis disease, the present study was conducted to investigate the antimicrobial properties of thymus vulgaris essential oil on *Salmonella Typhimurium* in an animal model (broiler) in comparison with two antibiotics, doxycycline, and oxytetracycline.

Material and Methods

This study was performed in the form of a Preclinical Trial and Experimental type in the winter of 2022 at the Department of Pathobiology, Faculty of Veterinary Medicine, Tabriz Medical Sciences, Islamic Azad University, Tabriz, Iran.

Preparation of herbal essential oil

Dried garden thyme leaves were purchased from one of Tabriz's groceries. Before starting to prepare

the essential oil, the scientific identity of the thyme plant was confirmed by referring to the Herbarium of the Faculty of Agriculture of the Islamic Azad University, Tabriz branch. Extraction of essential oil was done by steam distillation using a Cloninger machine (Schott Duran, Germany) (Basti et al., 2007). In this way, 60 grams of drying each plant was transferred to a balloon and 300 mL of distilled water (ratio 1 to 5) was added to another balloon. The essential oil extraction operation was carried out for 3 hours. About 50 mL of pure essential oil was obtained, which was collected by the decanter funnel and poured directly into the bott,le and stored (Sepehry and Mortazavi, 2022).

Gas chromatography/mass spectrometry (GC/MS) analysis

The sample of thyme essential oil was analyzed by GC-MS (Gas chromatography (7890B, Agilent, USA), Mass spectrometer (5877A, Agilent, USA)) by following the protocol of Negahban and Saeedfar (2015). The instrument was equipped with a split/splitless injector. To analyze the desired compositions of the column HP5-MS 60 m and film thickness 0.25 μm with an inner diameter of 0.25 mm has been used. The injection site temperature, interface temperature, and ionization site temperature are 250, 270, and 230 °C, respectively. The temperature program of the column starts with an initial temperature of 50 °C, and it was kept at this temperature for 5 minutes, then the temperature of the column with a gradient of 10 minute reached the 150 °C and remained constant at this temperature for 2 minutes, and finally with a slope of 20 °C it reached a temperature of 280 °C per minutes, and remained at this temperature for 5 minutes, The split ratio was set as 1 to 5, and the injection volume was 0.5 microliter. The components of the oil were separated and the chromatogram obtained was identified by comparing the mass spectra to those from National Institute of Standards and Technology (NIST) libraries.

Preparation of antibiotics

Antibiotics doxycycline and oxytetracycline produced in Royan Daru were prepared. The amount of doxycycline antibiotic active ingredient is 100 mg/g and the amount of oxytetracycline antibiotic active ingredient is 200 mg/g.

Preparation of bacterial strain

The studied microorganism was *Salmonella Typhimurium* (ATCC 14028), which was obtained from the Scientific and Industrial Research Center of Iran. To revive the bacteria, first, a pellet dipped in the desired bacteria solution was removed from the cryobank and placed in 3 ccs of Brain Heart Infusion (BHI, Merk, Germany) broth medium. Then this culture medium was incubated for 24 hours at 37 °C for the bacteria to multiply again. After the revival study, to obtain pure colonies, from the BHI broth culture medium containing pathogenic bacteria, isolated culture was done on the BHI agar medium. Then, after 24 hours (in this case, it is in the logarithmic phase of growth), a suspension was prepared from the colonies obtained from the bacterial culture in the Hinton broth medium and standardized to 0.5 McFarland by visual observation in the light source. To prepare a McFarland dilution, 0.1% barium chloride and 9.9% sulfuric acid were added to the obtained solution (Serrano-Lobo et al., 2022). In the pilot test, we observed that the dilution of 0.5 McFarland did not cause experimental infection, therefore, the dilution of 1 McFarland was used.

Preparation of minimum inhibition concentration (MIC)

The minimum inhibitory concentration of essential oil and antibiotics was determined by the broth microdilution method using a 96-well microtiter plate (Manandhar et al., 2019). First, 100 microliters of Mueller Hinton Brat culture medium was added to each well, then 10 microliters of bacterial suspension with 0.5 McFarland dilution was added to all wells. Then, 100 microliters of serial two-fold dilutions of essential oil and antibiotics (equivalent to 100-0.195 mg/mL) were added to each of the wells. Well, 11 containing only culture medium plus bacterial suspension was considered as a negative control, and well 12 containing culture medium plus essential oil or antibiotic was considered a positive control. After mixing the samples with a vortex (Pars Azma, Iran), they were placed in an incubator (Pars Azma,

Iran) at 37 °C for 18 to 24 hours, and the wells were examined for the presence or absence of turbidity. The dilution of the well containing the lowest concentration that inhibited bacterial growth (no turbidity) was determined as the minimum inhibitory concentration (MIC).

Antimicrobial activity in vivo

In this study, we used broiler chickens 14 days after hatching because it has been previously shown that *Salmonella Typhimurium* infection in chickens may cause gastroenteritis in young birds. The study was carried out in such a way that 36, 14-day-old broilers of the Ross breed with an average weight of 150 grams were randomly divided into 6 groups of 6. Chickens were all kept in separate cages in a well-lit and ventilated room from the time of infection to the treatment periods, and this model offered many advantages as described by Bjerrum et al. (2003). This enables us to easily control infection by preventing the problem of re-infection, preventing the spread of bacteria between groups, and contamination of the environment. We housed all chickens in the same room but in separate cages to investigate the rate of airborne transmission of *Salmonella typhimurium* infection to healthy chickens. Food and water were freely available to the chickens. The ration contains 20.2% crude protein and 2900 Kcal/Kg ME was provided freely (Kyakma et al., 2022). Broilers were divided into six groups: group I: a healthy control group, group II: a gavage control group (to investigate the possible stress that gavage causes to chicks), group III: a sick control group, group IV: group treated with thyme, group V: group treated with doxycycline antibiotic, group VI: group treated with oxytetracycline antibiotic. Group I (healthy control) and group II (gavage control) did not receive any intervention. In subsequent groups, each animal was treated with a *Salmonella Typhimurium* suspension containing 3×10^8 cells/mL prepared using the McFarland standard (Oussalah et al., 2007), it was gavage orally by a syringe with a flexible tube attached. Stool samples were collected to investigate *salmonella* contamination. The samples were first dissolved in peptone buffer (Merk, Germany), then incubated in tetrathionate (Merk, Germany) for 18-24 hours at 37 °C. After the incubation period, centrifugation (Behdad, Iran) was performed with 360 rotations for 1 minute and after discarding the supernatant, a linear culture was made from the remaining sediment on the XLD culture medium and incubated for 24 hours at 37 °C (Singer et al., 2009). After confirming the infection through fecal culture, groups IV, V, and VI were subjected to MIC of thyme essential oil, doxycycline antibiotic and oxytetracycline antibiotic, in the amount of 1 ml, twice a day with 12 hours interval for 7 days were treated (Zaheer, 2015). Group II also received distilled water at the same time to investigate the effect of gavage and the resulting stress. 24, 48, 72, 96, 120, 144 and 168 hours after treatment, a stool culture was performed. In groups I to III that were not treated, stool cultures were also performed on the same days. To standardize the stool culture, one gram of each group's stool was cultured on XLD (Xylose Lysine Deoxycholate) agar culture medium after dissolving in 9 mL of physiological serum. After 24 hours of each culture, bacterial colonies were counted according to standard method of Iran Research and Industrial Institute and reported as CFU/mL (Mokhtarian et al., 2004). It should be noted that after the end of each test period, culture wastes were disinfected by autoclave. In the end, all the chickens were rendered unconscious by intramuscular injection of ketamine with a dose of 0.33 mL/kg and xylazine with a dose of 0.13 mL/kg, and with dislocation of the neck vertebrae without pain (Wang et al., 2008), and finally, after completing all the experimental and stool sample stages Removal, carcasses and microbial and laboratory waste were burned in a one meter deep pit and buried after liming (Hall et al., 2001).

Statistical analysis

The findings were analyzed with SPSS 26 software and two-way analysis of variance statistical test at a significant level of $p\text{-value} < 0.01$.

Results

In vitro findings

The GC–MS fingerprint profile of T. vulgaris was obtained and showed in Figure 1. By analyzing the results, hydro-distilled oil of T. vulgaris showed the presence of 12 chemical compounds. The compounds, its retention time, and area percentage are shown in Table 1. According to the broth microdilution test results, *Salmonella Typhimurium* was most sensitive to oxytetracycline antibiotic with the minimum growth inhibitory concentration of <0.00195 µg/mL. As can be seen in Table 2, the minimum growth inhibitory concentration of doxycycline antibiotic equal to 0.0078 µg/mL and the minimum growth inhibitory concentration of garden thyme blue essential oil equal to 0.25 µg/mL were obtained.

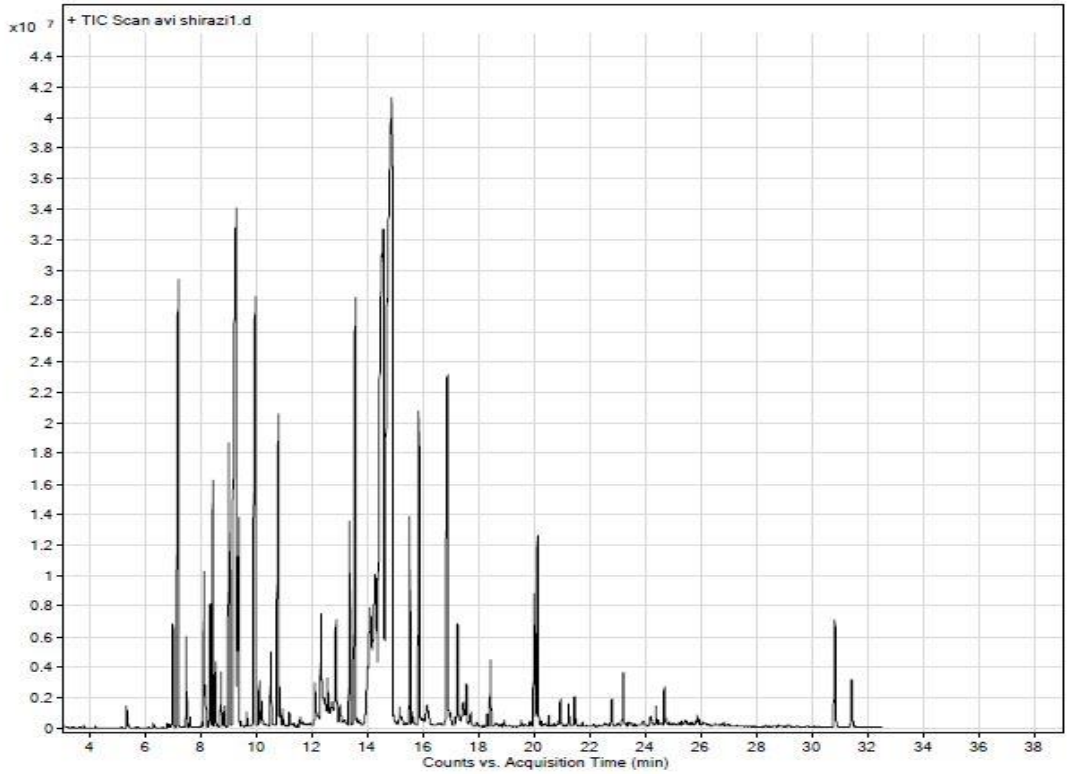

Fig. 1. GC–MS chromatogram of *T. vulgaris* analyzed on GC–MS (Agilent, USA) using a capillary column (5-MS) attached with mass detector. The chromatogram showed the presence of chemical components found in the *T. vulgaris.*

In vivo findings

In the group of animals that were infected and did not receive any drug (III), only 3 out of 6 chicks survived, while no deaths occurred in the other groups. No symptoms of lethargy and depression were observed in any of the groups receiving bacteria (III, IV, V, and VI). The bulk of food consumed in the groups receiving antibiotics (V, VI) increased compared to the control groups (p-<0.05). The amount of food consumed by the term infected groups after contamination was less than the control groups (p<0.05). Water consumption also increased in the groups receiving antibiotics compared to the control groups I and II. No reaction against gavage was observed in the second group, so it can be concluded that gavage does not cause any harmful stress to chickens.

Before the chickens were infected, the number of bacterial colonies in the feces of all animals was zero. In the treated groups, 24 hours after the start of treatment, the number of colonies in the group treated with thyme (IV) and oxytetracycline (VI) decreased (p<0.05), while in the group treated with doxycycline (V), first the number Bacteria increased and then decreased (p<0.05). In the screening of fecal cultures from the group treated

with thyme, there were no bacterial colonies in the cultures from the fourth day onwards, while in the groups treated with antibiotics, the fecal cultures became negative from the second day onwards. After that, no colonies were observed in the cultured samples from the feces of the groups. Of course, it should be noted that in the bacterial control group, *Salmonella* fecal culture was positive until the seventh day and did not reach zero until the end of the study (Table 3). The p-value between the compared groups in terms of the number of colonies grown on different days was 0.046. The results of the statistical analysis are shown in chart 1. It should be noted that groups I and II were excluded from the statistical analysis due to the lack of infection with *Salmonella Typhimurium*.

Table 1. List of the compounds present in the Thymus essential oil analyzed by GC–MS.

No.	Main Compounds	Area Sum[a] %	RI[b]	Molecular Formula	Identification Method[c]
1	Tymol	22.0	1299.0	C10H14O	1,2
2	carvacrol	9.0	1291.0	C10H14O	1,2
3	m-Thymol	7.65	1299.0	C10H14O	1,2
4	3-Carene	5.15	929.0	C10H16	1,2
5	p-Cymene	10.0	1104	C10H14	1,2
6	gamma.-Terpinene	5.0	1075	C10H16	1,2
7	Benzene, 2-methoxy-1-methyl-4-(1-methylethyl)-	4.15	1143	C11H16O	1,2
8	Caryophyllene	4.05	1579.0	C15H24	1,2
9	Eucalyptol	1.45	1032.0	C10H18O	1,2
10	Endo-borneol	0.6	1167	C10H18O	1,2
11	Camphene	0.6	952.0	C10H16	1,2
12	Caryophyllene oxide	3.67	1581	C15H24O	1,2

[a] Chemical composition (relative % peak area)
[b] Calculated based on the DB-5: 5% phenylmethylpolysiloxane.
[c] Identification Methods: 1-Retention indices; 2- NIST and Wiley library.

Table 2. The minimum inhibitory concentration of the aqueous essential oil of the thyme plant in comparison with the antibiotics Doxycycline and Oxytetracycline on *Salmonella Typhimurium* (in micrograms per milliliter)

The desired material	Parameters	Results
Thyme essential oil	MIC	0/25
Doxycycline	MIC	0/0078
Oxytetracycline	MIC	<0/00195

Discussion

After extracting the essential oil, its chemical composition was determined by GC-MS (Table 1). By using the broth microdilution method, the minimum inhibitory concentration (MIC) of doxycycline and oxytetracycline antibiotics and thyme essential oil was determined. Thyme essential oil's MIC was 0.25 µg/mL. The minimal inhibitory concentration for oxytetracycline was >0.00195 µg/mL and 0.0078 µg/mL for doxycycline (Table 2). The effectiveness of antibiotics and essential oils was evaluated about the number of germs found in stool samples (Table 3). Antimicrobial effects of thyme vulgaris plant on various pathogenic agents have been investigated

by researchers, and the results of their work have also led to different results. In 2021, Guilin et al. showed that thyme essential oil has an antibiofilm effect on *Salmonella typhimurium* bacteria with an MIC equal to 7 μg/mL and a growth inhibition percentage higher than 60% (Guillín et al., 2021), which is less than the amount obtained in this research. Also, in a research by Safari Samani et al. (2020), they tested the antimicrobial activity of thyme essential oil on a number of gram-positive and gram-negative pathogenic bacteria.

Table 3. The number of *Salmonella typhimurium* colonies grown in the fecal culture of broilers tasted in different groups after infection and receiving treatment in terms of CFU/g.

	Hours							
Groups	24	48	72	96	120	144	168	196
I	NS	NS	NS	NS	NS	NS	NS	NS
II	NS	NS	NS	NS	NS	NS	NS	NS
III	1.4×10^{8}	6.8×10^{8}	1.2×10^{5}	1.8×10	5.2×10^{8}	6×10^{8}	3×10^{7}	2.5×10^{6}
IV	1.9×10^{8}	5.1×10^{6}	9×10^{3}	10	NS	NS	NS	NS
V	5.2×10^{8}	1.3×10^{5}	NS	NS	NS	NS	NS	NS
VI	2.5×10^{5}	3×10^{5}	NS	NS	NS	NS	NS	NS

Group I: Negative control group, Group II: Gavage control group, Group III: Positive control group, Group IV: The group treated with thyme essential oil, Group V: The group treated with doxycycline, Group VI: The group treated with oxytetracycline. *NS means no excretion of *Salmonella* in the stool samples collected.

In the aforementioned study, the minimum inhibitory concentration (MIC) of thyme essential oil against *Salmonella typhimurium* was reported to be 0.25 μg/mL, which is consistent with the result of the present study (Saffari Samani et al., 2020). In 2018, Mustafa et al. investigated the antimicrobial activity of 5 plant extracts, including thyme plant extract, against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi* using the disk diffusion method in agar and at least the inhibitory concentration of thyme essential oil for all tested bacteria was reported as 10 mg/mL (Mostafa et al., 2018). It seems that the difference observed in the MIC of thyme essential oil in the above research can be caused by the difference in the chemical composition of the essential oils due to the difference in the place of growth of the plant and its harvesting season, the method of extracting the essential oil, as well as the type and strain of the microorganism, and the method of determining the antimicrobial effect is relevant (Ranjbarian et al., 2004).

In the current study, thyme essential oil treatment resulted in a reduction in the amount of *Salmonella Typhimurium* bacteria in infected chickens from the very first day of the treatment until the fourth day of treatment, when the number of bacteria reached zero (Table 2). In this case, Hamed et al. (2022) reported that essential oil of thyme reduced the number of *Salmonella* colonies after 5 days in infected chickens. In 2020, Elmi et al. reported that thyme essential oil reduced the growth of *Salmonella* bacteria in stool culture colonies on the eighth day of the experiment (Elmi et al., 2020). In 2017, during an experiment, Abousouh treated mice infected with *Salmonella typhimurium* bacteria with three doses of 250, 500 and 750 mg/kg. According to Abousouh reports, the number of bacterial colonies from stool culture in the doses of 500 and 750 mg/kg was zero from the beginning to the end, while the number of colonies in the group treated with the dose of 250 mg/kg initially decreased, and became zero on the third day and then it has increased (Abousouh, 2017). In a study by A Ahmed et al. in 2014, chickens infected with Salmonella bacteria were treated with thyme essential oil. They reported that thyme essential oil reduced the number of colonies in stool samples (Ahmed et al., 2014). All the reports given are consistent with the results of the current research on the treatment of salmonellosis. The

ability of thyme essential oil to treat salmonellosis in broilers can be related to its ability to directly kill *salmonella* and strengthen the immune system (Sokoudjou et al., 2019).

In a study by Salih in 2012, rabbits infected with *Salmonella* were treated with thyme essential oil. By the 7th day after treatment, no colony of bacteria grew on McConkey agar, but after 10 days, positive colony growth was reported in all groups treated with the sub-MIC dose of thyme (Salih, 2012). In 2010, Hoffman-Pensi and Wu also reported that thyme essential oil had no inhibitory effect on *Salmonella typhimurium* inoculated into broiler chickens (Hoffman-Pennesi and Wu, 2010), which is contrary to the results of this study (Table 2).

In 2022, Kalani et al. reported that the antibiotic oxytetracycline reduced the bacterial population of *Salmonella* in chickens challenged with this bacterium (Kalani et al., 2022). In 2019, Sokojo et al. reported that chickens infected with *Salmonella typhimurium* treated with oxytetracycline recovered on day seven after treatment. One day after the start of the treatment, a slight increase in the bacterial load was observed in the infected animals treated with oxytetracycline antibiotic, after this stage, a gradual decrease in the fecal load of these treated animals was observed until this load was removed on the seventh day (Sokoudjou et al., 2019). According to the report presented by Karim et al. in 2017, the antibiotic's oxytetracycline and neomycin did not affect the population of *Salmonella* bacteria in broilers (Kareem et al., 2017). In another study by Kodjio et al. in 2016, the positive effect of oxytetracycline on reducing the microbial load of *Salmonella Typhimurium* in mice infected with this bacterium was reported (Kodjio et al., 2016). Contrary to the studies done, in the present study, oxytetracycline antibiotic from the very beginning reduced the population of *Salmonella Typhimurium* in the cultured samples from the feces of the group treated with this antibiotic, and finally, after two days, it stopped the excretion of *Salmonella* from the feces of chickens (Table 2). The reason for the difference in the results can be due to the nature and severity of the disease in different animal models. Of course, it is important to mention that the infecting serotype, breed and genetic background, age, and immune status of the bird can also be involved in this matter (Gast and Porter Jr, 2020).

Unfortunately, reports on the effect of doxycycline antibiotic on *Salmonella Typhimurium* colonization in vivo are scarce. This study showed that the administration of 1 mL of the MIC dose of doxycycline twice a day caused a gradual decrease in colonization and finally stopped the excretion of *Salmonella Typhimurium* in chickens challenged with this bacterium (Table 2). Therefore, this dose of doxycycline can be suggested for the treatment of salmonellosis in poultry. Also, the consumption of 1 mL of thyme essential oil at a dose of 0.25 µg/mL twice a day with an hourly interval of 12 hours prevented the growth of *Salmonella Typhimurium*. This dose of thyme essential oil is also suggested for the treatment of salmonellosis in poultry.

Conclusion

According to the obtained results, it was observed that thyme essential oil inhibited the growth of bacteria and treated salmonellosis in broiler chickens with a small difference in time, like the antibiotics oxytetracycline and doxycycline. Therefore, according to the results obtained from this research and the increasing limitations of using antimicrobial chemicals, including side effects and the development of drug resistance, thyme essential oil can be suggested as an auxiliary and independent combination in the treatment of salmonellosis in broilers.

Ethical approval

Ethical clearance was obtained from research ethics committees' of Tabriz Medical Sciences, Islamic Azad University, Tabriz, Iran.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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