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Research Article

Antimicrobial Effects of *Thymus vulgaris, Cinnamomum zeylanicum* and *Zingiber officinale* Essential Oils on *Salmonella enterica* serovar Enteritidis Infections

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Abstract

Salmonella enterica serovar Enteritidis infections are among the leading causes of human foodborne illness mainly due to the consumption of contaminated poultry meat and eggs. Therefore, the aim of this study was to investigate the antimicrobial effects of essential oils (EOs) derived from *Thymus vulgaris* (thyme), *Cinnamomum zeylanicum* (cinnamon) and *Zingiber officinale* (ginger) on these infections. These EOs were added to *Salmonella enterica* ser. Enteritidis cultures in 96-well microplates in a sealed pouch and incubated at 37°C for 24 h minimum inhibitory concentration was measured to determine their antimicrobial effects. Ciprofloxacin (1 mg/mL) added to the culture medium served as the positive control to test the antibacterial effect. In this in vitro cell culture study, the cytotoxic effects of all EOs on healthy fibroblasts were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide cell viability test. Cinnamon EO showed antibacterial effects at all concentrations, whereas ginger EO was effective only in combination with thyme EO at 50% concentration. Further, thyme EO was found to be bactericidal at 50% concentration and bacteriostatic at 25% concentration. None of the EOs were cytotoxic to fibroblasts. In conclusion, cinnamon EO is highly effective and safe for the treatment of *Salmonella enterica* ser. Enteritidis infections, followed by thyme EO.

Keywords: *Antibacterial effects, Thymus vulgaris, Thyme, Cinnamomum zeylanicum, Cinnamon, Zingiber officinale, Ginger, Essential oil, Salmonella enterica*

Thymus vulgaris, Cinnamomum zeylanicum ve *Zingiber officinale* Uçucu Yağlarının *Salmonella enterica* Serovar Enteritidis Üzerindeki Antimikrobiyal Etkileri

Öz

Salmonella enterica serovar Enteritidis enfeksiyonları, esas olarak kontamine kümes hayvanı eti ve yumurta tüketimi nedeniyle insan gıda kaynaklı hastalıkların önde gelen nedenleri arasındadır. Bu nedenle, bu çalışmanın amacı *Thymus vulgaris* (kekik), *Cinnamomum zeylanicum* (tarçın) ve *Zingiber officinale*'den (zencefil) elde edilen esansiyel uçucu yağların (EY) bu enfeksiyonlar üzerindeki antimikrobiyal etkilerini araştırmaktır. Bu EY'lar *S. enterica* serovarının bulunduğu 96 oyuklu mikroplakalarda enteritidis kültürlerinin bulunduğu ortamda 37°C'de 24 saat inkübe edildi. Antimikrobiyal etkileri belirlemek için minimal inhibitör konsantrasyon ölçüldü. Kültür ortamına eklenen siprofloksasin (1 mg/mL), antibakteriyel etkiyi test etmek için pozitif kontrol görevi gördü. Bu *in vitro* hücre kültürü çalışmasında, tüm EY'ların sağlıklı fibroblastlar üzerindeki sitotoksik etkileri 3-(4,5-dimetiltiyazol-2-il) -2,5-difeniltetrazolium-bromür hücre canlılığı testi kullanılarak araştırıldı. Tarçın EY'ı tüm konsantrasyonlarda antibakteriyel etkiler gösterirken, zencefil EY'ı sadece %50 konsantrasyonda kekik EY ile kombinasyon halinde etkiliydi. Ayrıca kekik EY'nın %50 konsantrasyonda bakterisidal ve %25 konsantrasyonda bakteriyostatik olduğu bulundu. Hiçbir EY fibroblast hücrelerine sitotoksik değildi. Çalışmamızın bulgularında tarçın EY'nın *S. enterica* serovarı tedavisinde oldukça etkili ve güvenli olduğunu göstermekte olup bunu kekik EY'ı takip etmektedir. Hiçbir EY fibroblast için sitotoksik değildi. Sonuç olarak, tarçın EY'ı *Salmonella enterica* ser. Enteritidis tedavisi için oldukça etkili ve güvenlidir. Enteritidis enfeksiyonlarına etkinlikte bunun ardından kekik EY'ı gelmektedir.

Anahtar sözcükler: *Antimikrobiyal etki, Thymus vulgaris, Kekik, Cinnamomum zeylanicum, Tarçın, Zingiber officinale, Zencefil, Esansiyel yağ, Salmonella enterica*

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INTRODUCTION

Salmonellosis caused by *Salmonella* spp. is a widely reported zoonotic disease, and it is transmitted to humans through the consumption of raw animal food products including poultry meat and eggs. *Salmonella* infections are highly prevalent in humans, animals and birds. Therefore, these infections are a major concern for public health, animals and the food industry worldwide [1]. *Salmonella enterica* serovar Enteritidis has been associated with a number of outbreaks of salmonellosis in humans.

In recent years, natural antimicrobials such as plant essential oils (EOs) and extracts have been considered popular alternatives to commercially used chemicals such as chlorine and hydrogen peroxide, which have harmful effects [2,3]. Plant EOs and extracts have been used in food preservation, pharmaceuticals, alternative medicine and natural therapies. Božik et al.[4] reported that there are potentially six plant EOs-antimicrobial volatile substances derived from plants with beneficial effects-which are suitable alternatives to synthetic pesticides and food preservatives, which can be used for the treatment of microbial infections [4]. These EOs are derived from cinnamon *(Cinnamomum zeylanicum* Nees.), thyme *(Thymus vulgaris* L.), oregano *(Origanum vulgare* L.), clove *(Syzygium aromaticum* L.), lemongrass [*Cymbopogon citratus* (DC) Stapf.] and ginger *(Zingiber officinale* Rosc.) [4]. For the study of the effects of the study EOs on *S. enteritidis*, the three plants were selected because scientific studies have commenced on this issue recently [5,6], although they are very rare.

The aim of this study was to investigate the effect of thyme, cinnamon and ginger EOs on *S. enterica* ser. Enteritidis cultures and to elucidate their cytotoxic effects on healthy fibroblasts. Elucidation of the antimicrobial effects of these EOs can facilitate the design of suitable therapies for the treatment of *S. enterica* ser. Enteritidis infections. Through this study, we hope to contribute to the literature in this field.

MATERIAL and METHODS

Plant Materials

The plant materials used in this study included *T. vulgaris* (thyme), *C. zeylanicum* (cinnamon) and *Z. officinale* (ginger). This study was conducted at the Kırıkkale University Scientific and Technological Research Application and Research Center.

EO Extraction: This *in vitro* study was approved by the Ministry of Agriculture, and three readymade test EO extracts from the same commercial centre, which are commercially available and sold under a specific barcode number, were used. Therefore, any powdered samples could not be extracted with methanol. Density (d) of EOs calculating with Mettler Toledo density meter was as follows: *C. zeylanicum* = 0.889 g/mL, *T. vulgaris* = 0.912 g/mL and *Z. officinale* = 0.868 g/mL.

Test Organisms: The *Salmonella enteritidis* bacterial strain used in this study was obtained from the Kırıkkale University Scientific and Technological Research Application and Research Center.

Cell Line

L929 (a healthy mouse adipose fibroblast cell line) cells that were frozen at -80°C in cryotubes in the cell library of the Kırıkkale University Scientific and Technological Research Application and Research Centre Biocompatibility Laboratory were used.

Minimum Inhibitory Concentration (Antimicrobial Effect)

In 96-well microplates, 90 µL of Mueller–Hinton broth medium was first introduced into all wells. Subsequently, 90 µL of the three EOs was added (cinnamon, ginger, thyme and their mixtures) to the first well in each of the 6 rows to achieve 1:1 concentration. Thereafter, EOs were added at different concentrations in the following ratios: 1:2 (50%), 1:4 (25%), 1:8 (12.5%), 1:16 (6.25%), 1:32 (3.13%), 1:64 (1.56%) and 1:128 (0.78%).

The concentration of 24-h fresh *Salmonella enterica* ser. Enteritidis culture was adjusted to 0.5 McFarland turbidity standard and diluted to a ratio of 1:100. Approximately 10 µL of this inoculum was added to each well to ensure that the final concentration was 10^5 microorganisms/well. For measuring the antibacterial effect, ciprofloxacin (1 mg/mL) was used as a positive control in a well containing only the medium. Thereafter, the 96-well microplates were placed in a sealed pouch and incubated (Binder CB150, USA) at 37°C for 24 h to observe the antimicrobial effects of the EOs.

Cytotoxicity Analysis

For cytotoxicity measurements, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) cytotoxicity test was performed. MTT was performed according to the ISO 10993-5:2009 standards. For adjusting cell density, 100 µL of Dulbecco's modified Eagle's medium (DMEM) was added to 10^5 L929 cells/well in a 96-well microtiter culture plate. L929 cells were incubated for 24 h at 37°C in 5% CO_2. Thereafter, EOs were added at the following different concentrations: 1:1 (100%), 1:2 (50%), 1:4 (25%), 1:8 (12.5%), 1:16 (6.25%), 1:32 (3.13%), 1:64 (1.56%) and 1:128 (0.78%).

Negative wells (containing cells only), positive wells (containing 20% DMSO plus EO with L929 cells), control wells and blind wells (DMEM only) were used in triplicates. Cells were incubated for 24 h at 37°C in 5% CO_2. At the end of the 24-h incubation, the medium was carefully removed from the 96-well microtiter culture plate and 10

µL of MTT solution was added to each well and incubated at 37°C for 1-4 h. After incubation, the incubation buffer was removed from the wells, cells were shaken by adding 100 µL of isopropanol to each well, and absorbance was measured on a microplate reader (Biotek PowerWave XS2) at a wavelength of 570 nm decrease in viability compared with that of cells grown in the blind wells is expressed as percentage. The result of the visually control and with sowing methods obtained four different results.

RESULTS

Antimicrobial Effect of the Three EOs and Their Mixtures

Cinnamon EO showed an antimicrobial effect on *S. enterica* ser. Enteritidis infections at all tested concentrations. Although thyme EO had bactericidal activity against *S. enterica* ser. Enteritidis infections at 50% concentration, it showed bacteriostatic activity at 25% concentration. Ginger EO did not show any antimicrobial effect. Further, cinnamon-thyme and cinnamon-ginger EO mixtures were found to have antimicrobial effects at all concentrations. However, the antimicrobial effect of thyme-ginger EO mixture was only found at 50% concentration. Regarding the *S. enteritidis* strain, antimicrobial effect and minimum bactericidal concentration *(Fig. 1)* of cinnamon EO (50%-0.78%), as well as its antimicrobial effect and MIC value (0.39%-0.006%, *Fig. 2)*, were detected.

Cytotoxic Effect of EOs

Fig. 3 shows the cytotoxic activity of all EOs. Our study showed that these EOs were not cytotoxic to fibroblasts at any concentration. No significant findings were obtained in cytotoxicity analysis.

DISCUSSION

Salmonellosis is a major zoonotic disease [7] which is prevalent worldwide. Marus et al.[7] analysed the data reported by the Centres for Disease Control and Prevention through the National Outbreak Reporting System from 2009 to 2014, with the primary mode of transmission listed as 'animal contact' or 'food contaminated'. They found 484 outbreaks through animal contact or foodborne transmission, of which 99 (20.5%) resulted from *Salmonella* transmission through animal contact and 385 (79.5%)

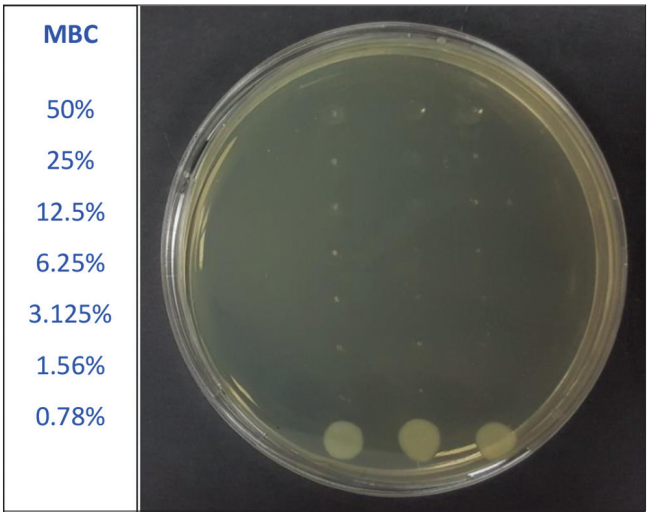
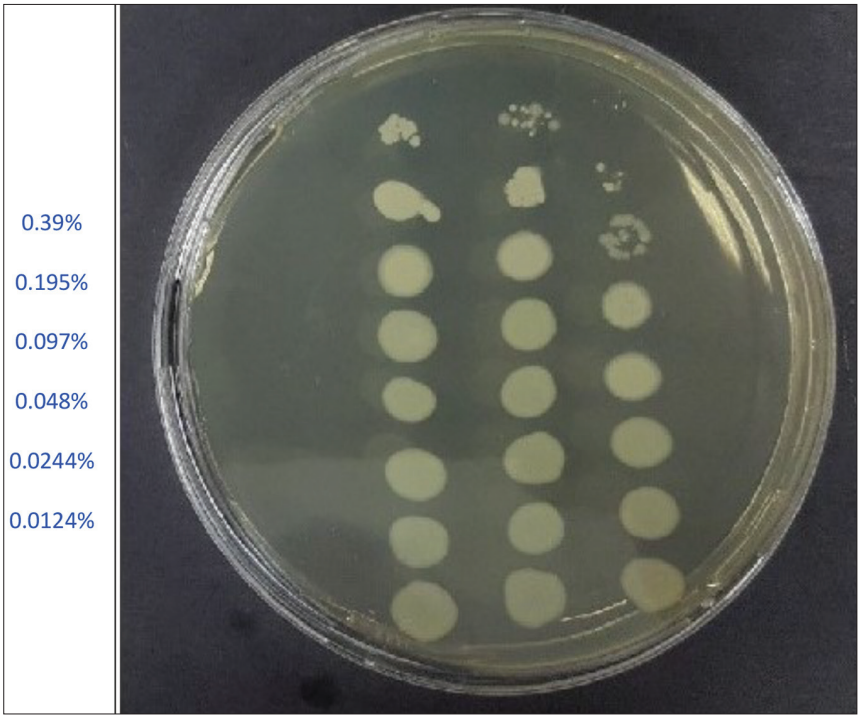


Fig 1. Antimicrobial effectand minimum bactericidal concentrationof cinnamon essential oil (50%-0.78%) inthe studied*Salmonella enteritidis* strain. Minimum inhibitory concentration: 0.39%-0.006%.

Fig 2. Antimicrobial effectand minimum inhibitory concentration of cinnamon essential oil (0.39%-0.006%) inthe studied *Salmonella enteritidis* strain



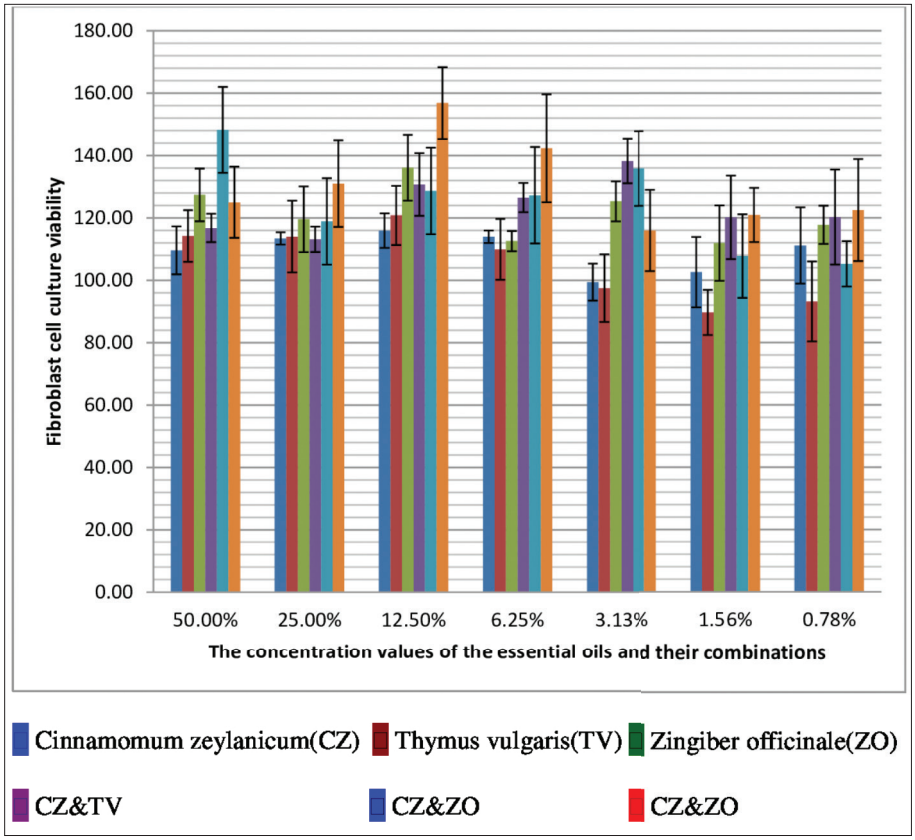


Fig 3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromidecell viability test results for cytotoxicity. The cytotoxic actiity of all studied EOs. Our study showed that these EOs were not cytotoxic to fibroblasts at any concentration. No significant findings were obtained in cytotoxicity analysis.

from the foodborne transmission; these resulted in 3.604 (19.8%) and 13.568 (80.2%) illnesses, respectively [7,8]. Giacometti et al.[9] reported highly suspected cases of salmonellosis in two cats fed a commercial raw meat-based diet [9]. Further, Tomaštíková et al.[10] reported a case wherein salmonellosis was transmitted from a reptile to an infant through indirect contact. Thus, salmonellosis can be transmitted through direct or indirect contact or through the consumption of contaminated poultry meat or eggs.

Some plant EOs have been reported to exhibit antimicrobial properties, and they are potential sources of novel antimicrobial compounds, particularly against bacterial pathogens. Warnke et al.[11] showed that plant EOs are a cheap and effective antiseptic treatment option, even for antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* and *S. enterica*. Zhu et al.[12] showed that carvacrol and cinnamaldehyde incorporated into apple-, carrot- and hibiscus-based edible films could inactivate *Salmonella* Newport in bagged organic leafy greens [12]. Al-Bayati [13] showed that EOs and methanol extracts derived from the aerial parts of *T. vulgaris* and *Pimpinella anisum* seeds had high inhibitory effects on most pathogenic bacteria such as *Bacillus cereus*, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Typhimurium and *Pseudomonas aeruginosa*, which are resistant to the standard antibiotic Maxipime [13]. Renata et al.[14] investigated the growth and survival of *Escherichia coli* 0157 and *S. enterica* ser. Enteritidis in the presence of garlic, ginger, mustard and cloves. Experiments performed in broth model systems supported with 0.25%-1% garlic and cloves showed bacteriostatic and bactericidal activities against both microorganisms.

Clove was the most effective antimicrobial agent, followed by garlic. However, mustard and ginger showed only little bacteriostatic activity [14]. Rosti and Gastaldi [15] reported a case in 2005 of the therapeutic effect of *C. zeylanicum* on an infant. The infant's mother had eaten raw seafood, and this exclusively breastfed 4-month-old infant became a carrier of *S. enterica* ser. Enteritidis. Stool samples of both mother and infant were positive for *S. enterica* ser. Enteritidis. However, they reported that chronic *Salmonella* carriage in the infant disappeared after cinnamon administration. Boskovic et al.[16] estimated the antibacterial effect of thyme EO (TEO) on four *Salmonella* serovars *(Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella montevideo* and *Salmonella infantis)* which were experimentally inoculated (10^6 CFU/g). The highest antibacterial effect was achieved by the combination of MAP and 0.9% TEO. They stated that all TEO concentrations in the studied pork had significant antibacterial effects ($P<0.05$). Travis et al.[17] reported that food poisoning outbreaks resulting from *Salmonella* growing on vegetables emphasise the need for the knowledge of pathogen evolution and adaptation in developing appropriate countermeasures for the prevention and policy development [17]. Therefore, the findings of this study are important in terms of both public health and prevention of zoonotic diseases.

Our study showed that cinnamon and thyme EOs had an effective antibacterial effect on *S. enterica* ser. Enteritidis. Ginger and thyme EOs were effective against *S. enterica* ser. Enteritidis at 50% concentration, but ginger EO alone did not have any antimicrobial effect, as shown in previous studies [11,13]. Pure *C. zeylanicum* and pure *T. vulgaris* or their mixtures were found to be effective against *S. enterica* ser. Enteritidis at ≤50% concentration, and these EOs and their mixtures can be considered in the development of therapeutic strategies for salmonellosis because they were not found to be cytotoxic to the fibroblast cell line.

Our *in vitro* study results confirm that the main EO which is effective and safe for the treatment of salmonellosis caused by *S. enterica* ser. Enteritidis is cinnamon EO, followed by TEO. It would be better if the extraction was performed in our laboratory, which is a limitation of this study. On the other hand, as the next steps, we aim to perform the same analysis with methanol extraction in our laboratory because this would allow the comparison of both extracts. However, as a preliminary study, we believe that this study is crucial in terms of foundation for future research.

Conflict of Interest

The authors declare no conflict of interes

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