Research Article [Araştırma Makalesi]

# *In vitro* antioxidant and antibacterial properties and total phenolic contents of essential oils from *Thymus vulgaris*, *T. kotschyanus*, *Ziziphora tenuior* and *Z. clinopodioides*

[*Thymus vulgaris, T. kotschyanus, Ziziphora tenuior* ve *Z. clinopodioides*'den elde edilen esansiyel yağların bütün fenolik içeriklerinin, antioksidan ve antibakteriyel özelliklerinin *in vitro* olarak incelenmesi]*

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**ABSTRACT**

**Objective:** The objective of present study was to evaluate the antibacterial and antioxidant activities of essential oils from *Thymus vulgaris*, *Thymus kotschyanus*, *Ziziphora tenuior* and *Ziziphora clinopodioides*.

**Methods:** The antioxidant potency of essential oils was determined by 2,2-diphenyl-1-picrylhydrazyl and reducing power assays. Total phenolic contents of essential oils were determined using the Folin-Ciocalteu reagent assay. The antibacterial activity of essential oils was evaluated using agar disc diffusion and minimum inhibitory concentration (MIC) methods.

**Results:** The essential oils of *Ziziphora clinopodioides* and *Thymus vulgaris* showed the highest antioxidant activity. The essential oils of *Thymus vulgaris* showed the strongest antibacterial activity with the widest inhibition zone and the lowest MIC value (2.5 µl/ml). The essential oils of *Thymus vulgaris* had the highest concentration of total phenolics (116.5 mg GAE/g).

**Conclusion:** In conclusion, the essential oils of *Thymus vulgaris* and *Ziziphora clinopodioides* can be used as potent antibacterial and antioxidant for food preservation.

**Key words:** Essential oils, *Thymus vulgaris*, *Thymus kotschyanus*, *Ziziphora clinopodioides*, *Ziziphora tenuior,* antibacterial, antioxidant.

**Conflict of Interest:** The authors declare no conflict of interest.

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**ÖZET**

**Amaç:** *Thymus vulgaris*, *Thymus kotschyanus*, *Ziziphora tenuior* ve *Ziziphora clinopodioides*'den elde edilen esansiyel yağların antibakteriyel ve antioksidan özelliklerinin incelenmesi amaçlanmıştır.

**Yöntem:** Esansiyel yağlardaki antioksidan potansiyel 2,2-difenil-1-pikril hidrazil ve indirgeyici güç yöntemi ile; total fenolik içerik Folin-Ciocalteu reaktifi ile; antibakteriyel aktivite disk difüzyon agar ve minimum inhibisyon derişimi (MIC) yöntemleri kullanılarak saptanmıştır.

**Bulgular:** *Ziziphora clinopodioides* ve *Thymus vulgaris*'den elde edilen esansiyel yağların en yüksek antioksidan aktiviteye sahip olduğu bulunmuştur. *Thymus vulgaris*'den elde edilen esansiyel yağlar ise en geniş inhibisyon alanı ve en düşük MIC değeri (2.5 µl/ml) ile birlikte en güçlü antibakteriyel aktiviteye sahiptir. En yüksek total fenolik derişimi (116.5 mg GAE/g) *Thymus vulgaris*'den elde edilen esansiyel yağlarda saptanmıştır.

**Sonuç:** *Thymus vulgaris* ve *Ziziphora clinopodioides*'in içerdiği esansiyel yağlar yiyeceklerin korunmasında etkili antibakteriyel ve antioksidan olarak kullanılabilir.

**Anahtar Kelimeler:** Esansiyel yağlar, *Thymus vulgaris*, *Thymus kotschyanus*, *Ziziphora clinopodioides*, *Ziziphora tenuior,* antibakteriyel, antioksidan.

**Çıkar Çatışması:** Yazarların çıkar çatışması bulunmamaktadır.

## Introduction

Although various methods are available to delay spoilage and extend the shelf-life of raw and processed foods, one simple method is the addition of preservatives to food systems [1]. Food preservatives have an important role in controlling lipid oxidation and microbial growth [2]. The researchers focused on finding and using natural preservatives in foods, due to toxic and carcinogenic effects of chemical preservatives [3]. A considerable amount of literature has been published on antioxidant and antimicrobial activities of Essential oils (EOs) from various aromatic plants [4-6]. The EOs and extracts of many aromatic plants and spices can be used as natural food preservative.

The genus *Thymus* (Persian name: Avishan) belongs to Lamiaceae family and comprises 14 species in Iran, four of which are endemic [7]. *Thymus* species are commonly used as herbal teas, flavoring agents (condiment and spice) and medicinal plants [8]. *Thymus vulgaris* is one of the well-known species in this genus. The antioxidant and antimicrobial activity of *Thymus* species, particularly *T. Vulgaris*, has been extensively investigated [9,10]. In spite of *T. vulgaris*, limited information is exists on biological activities of *T. Kotschyanus* essential oil.

The genus *Ziziphora* L. (Persian name: Kakuti), a member of Labiatae family, consists of four species in Iran, including *Z. clinopodioides, Z. tenuior, Z. capitata* and *Z. persica* [7]. In the Iranian and Turkish traditional medicine, the infusions of *Ziziphora* species have been used as sedative, antiseptic and carminative [11]. In the Iranian folk medicine, these plants have been used as culinary herb and for the treatment of common cold and cough [12].

Chemical composition, antibacterial and antioxidant activities of *Z. clinopodioides* have been reported [13]. Several studies have been reported the antibacterial activity of *Z. clinopodioides* [13-16]. However, there is only one report on antioxidant activity of this plant [13]. Many studies showed that pulegone is the main component of *Ziziphora* species essential oil [17,13].

To our knowledge, there is no report on antioxidant and antibacterial properties of *Z. tenuior* essential oil in literature.

Thus, the aim of present work was to evaluate and compare the antibacterial and antioxidant activities of essential oils of *Thymus vulgaris, T. kotschyanus, Ziziphora tenuior* and *Z. clinopodioides*.

## Materials and methods

### Plant materials

The aerial parts of *Thymus vulgaris, T. kotschyanus, Ziziphora tenuior* and *Z. clinopodioides* were collected during summer 2011 from northwest of Iran (Urmia, Khoy and Sanandaj) and identified in Agricultural Research Center of West Azarbaijan province, Iran.

### Chemicals

Analytical grade ethanol, methanol, Folin-Ciocalteu's phenol reagent, disodium hydrogen phosphate ($Na_2HPO_4$), sodium dihydrogen phosphate ($NaH_2PO_4$), sodium carbonate, anhydrous sodium sulfate, trichloroacetic acid, all culture media and standard antibiotic discs were purchased from Merck (Darmstadt, Germany). Ferric chloride, potassium ferricyanide ($K_3Fe(CN)_6$), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,6,-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

### Essential oil isolation

The aerial parts of plants were ground and subjected to hydro distillation for 3 h using Clevenger type apparatus. The isolated essential oils were dried over anhydrous sodium sulfate and stored in dark at 4 °C until analyzed.

### Antioxidant potential assays

### Free radical scavenging assay

The capacity of the essential oils to donate a hydrogen atom or electron and scavenge DPPH radical was evaluated by the method of Blois [18]. Briefly, 50 μl of the different concentrations (2.5, 5 and 10 μl/ml) of EOs in methanol was mixed with 2 ml of methanol solution of DPPH (24 μg/ml). The mixture was incubated at room temperature for 60 min in the dark. Then, the absorbance was measured against a blank at 517 nm with a UV/Vis spectrophotometer. Radical scavenging activity (RSA) was calculated according to the following formula: RSA $(\%) = (A_{DPPH} - A_{EO} / A_{DPPH}) \times 100$

Where $A_{DPPH}$ was the absorbance value of DPPH solution, and $A_{EO}$ was the absorbance value of the test solution. Butylated hydroxytoluene (BHT) was used as a positive control. All experiments were carried out in triplicate and results were reported as means ± SD of triplicates.

### Reducing power

The reducing power of three different concentrations (2.5, 5 and 10 μl/ml) of EOs was determined according to the method of Oyaizu [19]. Different concentrations (1 ml) of EOs were mixed with 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1 %). After incubation at 50 °C for 20 min, 2.5 ml of trichloroacetic acid (10 %) was added to the mixture and then centrifuged at 4000 rpm for 10 min. Finally, 2.5 ml of upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1 %). The absorbance at 700 nm was measured after 10 min. A higher absorbance indicates a higher reducing power. BHT was used as a standard. All experiments were repeated tree times.

### Total phenolics determination

Total phenolic contents of EOs were determined using the Folin-Ciocalteu reagent assay [20], with gallic acid

as standard. Briefly, 500 µl of the EOs in methanol (2.5 mg/ml) was mixed with 2.25 ml distilled water and then 250 µl of Folin-Ciocalteu reagent was added. The mixture was vortexed for 1 min and was allowed to react for 5 min. Then, 2 ml of $Na_2CO_3$ solution (7.5%) were added. After incubation at room temperature for 120 min, the absorbance of each mixture was measured at 760 nm. The same procedure was also used to the standard solution of gallic acid, and a standard curve was obtained. Total phenolic contents were expressed as mg of gallic acid equivalent per g of the EO. All tests carried out in triplicate.

## Antibacterial assays

### Bacterial strains

The antibacterial activity of each EO was tested against two gram positive bacteria, *Bacillus cereus* (ATCC 11778) and *Listeria monocytogenes* (PTCC 1163), and two gram negative bacteria, *Salmonella* Typhimurium (ATCC 1730) and *Escherichia coli* O157: H7 (ATCC 25222).

### Agar disc diffusion assay

The antibacterial activity of EOs against four pathogenic bacteria was evaluated by agar disc diffusion assay [21]. A bacterial suspension containing $10^6$ cfu/ml bacteria (100 µl) was spread on brain heart infusion (BHI) agar. The sterile paper discs (diameter 6 mm) were impregnated with different doses (2.5, 5 and 10 µl) of each EO and placed on the inoculated agar. After incubation at 37 °C for 24 h, inhibition zones were measured and recorded. Erytromycine (15 µg/disc) was used as a positive reference. Each assay was repeated tree times.

### Micro-well dilution assay

Minimum inhibitory concentration (MIC) values of essential oils against bacterial strains were determined based on a micro-well dilution method as previously described [22]. The essential oils, dissolved in 10% dimethyl sulfoxide (DMSO), were first diluted to the highest concentration (40 µl/ml) to be tested, and then serial twofold dilutions were made in a concentration range from 0.078 to 40 µl/ml in test tubes.

The 96-well plates were prepared by dispensing 95 µl of the cultures media and 5 µl of the inoculum into each well. A 100 µl aliquot from the stock solutions of the essential oils initially prepared at the concentration of 40 µl/ml was added into the first well. Then, 100 µl from their serial dilutions were transferred into seven consecutive wells. The last well containing 195 µl of BHI broth without the test materials and 5 µl of the inoculum on each strip was used as negative control. Erythromycine was used as positive control. The plates were sealed with parafilm and shaked at 300 rpm for 20 s. Finally, the plates incubated at 37 °C for 24 h. Bacterial growth was determined by the presence of a white pellet on the well bottom and confirmed by plating 5 µl samples from clear wells on BHI agar. The MIC value

was defined as the lowest concentration of the essential oil required for inhibiting the growth of each bacterium. The experiment was repeated tree times.

## Statistical analysis

Statistical analysis of data was performed using SAS software package (Version 9.1) and the Tukey's test was used to the compare differences among the mean values. A correlation between total phenolic content and antioxidant activity was done using the function CORREL from Microsoft Excel software.

## Results and Discussion

### Antioxidant activity

### DPPH radical scavenging activity

DPPH assay is considered as a simple and rapid method for determining radical scavenging activity. In this assay, purple color of DPPH solution changed to yellow color upon acceptance of hydrogen atoms or electrons from an antioxidant [23]. Free radical scavenging capacity of essential oils (EOs) from *Thymus vulgaris*, *T. kotschyanus*, *Ziziphora tenuior* and *Z. clinopodioides* are given in Table 1. The EOs of *T. vulgaris* and *Z. clinopodioides* showed the highest radical scavenging activities ($P > 0.05$) with no statistical differences between them while the EO of *Z. tenuior* had the lowest activity ($P < 0.05$).

Viuda-Martose et al. reported radical scavenging activity of 36.71% and 64.29 % for *T. vulgaris* EO at concentrations of 5 and 10 mg/ml, respectively [6]. In the case of *T. Kotschyanus* EO, Amiri reported higher radical scavenging activity [24]. This investigator found the $IC_{50}$ of 278 µg/ml for *T. Kotschyanus* EO.

### The reducing power of EOs

In reducing power assay, the yellow color of the test solution changes to various shades of green and blue color depending upon the reducing capacity of each sample. Reducing capacities are generally associated with the presence of reductants in the antioxidant samples [25].

The reducing power of EOs of *T. vulgaris*, *T. kotschyanus*, *Z. tenuior* and *Z. clinopodioides* are shown in Table 2. The EO of *Z. clinopodioides* had the strongest reductive potential ($P < 0.05$). This potential was followed by EOs of *T. vulgaris* and *T. kotschyanus*. The EO of *Z. tenuior* showed the weakest reducing power among EOs tested ($P < 0.05$).

The antioxidant and antibacterial activities of EOs obtained from some Egyptian aromatic plants were determined [6]. After black cumin, *T. vulgaris* had the the highest reducing capacity. Reductive potential of *Thymus capitatus* EOs isolated during vegetative, flowering and post-flowering stages was investigated [26]. The EOs isolated during the post-flowering stage showed a reducing power similar to that of BHT and BHA.

**Table 1.** DPPH radical scavenging activity (%) of essential oils of *Thymus vulgaris, T. kotschyanus, Ziziphora tenuior* and *Z. clinopodioides*

| Sample | Concentration (µl/ml) | | |
|---|---|---|---|
| | 2.5 | 5 | 10 |
| *T. vulgaris* | 22.88 ± 2.0 [b] | 33.15 ± 3.1 [a] | 44.94 ± 1.7 [a] |
| *T. kotschyanus* | 14.35 ± 1.1 [c] | 23.11 ± 1.8 [b] | 32.19 ± 1.6 [b] |
| *Z. tenuior* | 6.14 ± 1.25 [d] | 9.42 ± 1.5 [c] | 12.61 ± 0.8 [c] |
| *Z. clinopodioides* | 21.51 ± 1.4 [b] | 33.88 ± 1.4 [a] | 45.53 ± 1.3 [a] |
| BHT | 96.32 ± 0.9 [a] | – | – |

The values are mean ± SD. Values with the same letters in each column are not significantly different.

BHT, butylated hydroxytoluene

**Table 2.** Reducing power of *T. vulgaris, T. kotschyanus, Z. tenuior* and *Z. clinopodioides* essential oils

| Sample | Concentration (µl/ml) | | |
|---|---|---|---|
| | 2.5 | 5 | 10 |
| *T. vulgaris* | 0.719 ± 0.15 [bc] | 1.003 ± 0.09 [b] | 1.73 ± 0.09 [b] |
| *T. kotschyanus* | 0.449 ± 0.04 [c] | 0.703 ± 0.05 [c] | 0.9 ± 0.002 [c] |
| *Z. tenuior* | 0.056 ± 0.02 [c] | 0.121 ± 0.02 [d] | 0.346 ± 0.14 [d] |
| *Z. clinopodioides* | 0.791 ± 0.09 [b] | 1.372 ± 0.02 [a] | 2.232 ± 0.05 [a] |
| BHT | 2.553 ± 0.11 [a] | – | – |

The values are mean ± SD. Values with the same letters in each column are not significantly different.
BHT, butylated hydroxytoluene

## Total phenolic contents

The total phenolic contents of EOs are presented in Figure 1. The highest concentration of total phenolics was found in EOs of *T. vulgaris* (116.47 mg GAE/g) and *Z. clinopodioides* (114.83 mg GAE/g). The EO of *Z. tenuior* had the lowest total phenolics content ($P < 0.05$). Antiradical and antioxidant activities of EOs can be attributed to their phenolic contents. Therefore, the EOs of *T. vulgaris* and *Z. clinopodioides* which had higher antiradical and antioxidant activity also had higher total phenolics content.

In present study, total phenolic contents of EOs was positively correlated with DPPH ($R^2 = 0.95$) and reducing power ($R^2 = 0.90$) assays. Similar results were obtained by other researchers [27].

Viuda-Martos et al. reported a high content of total phenols (913.17 mg GAE/L) for *T. vulgaris* EO [6].

Total phenolic content of some *Thymus* extracts was determined. Safaei-Ghomi et al. reported that the amount of total phenolics in methanol extract of *Thymus caramanicus* was 124 µg/mg [10]. Similar results were reported for methanol extract of *Thymus spathulifolius* (141 µg/mg) [28]. Gursoy et al. found that total phenolic content of methanol extract of *Z. clinopodioides* was 129.55 µg/mg [29].

Antioxidant activity of herbs and spices depends on content of phenolic compounds [30]. Meanwhile, it was claimed that phenolic compounds present in plants might also play an important role in their antimicrobial activities [31].
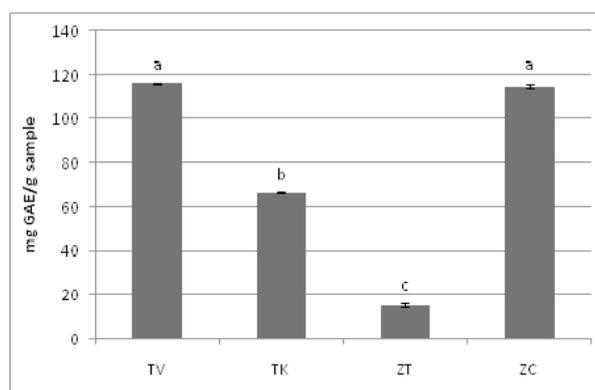


**Figure 1.** Total phenolics content of essential oils from *T. vulgaris* (TV), *T. kotschyanus* (TK), *Z. tenuior* (ZT) and *Z. clinopodioides* (ZC).

## Antibacterial activity

## Agar disc diffusion assay

The antibacterial activity of EOs against two Gram-positive and two Gram-negative bacteria was evaluated for presence or absence of inhibition zone using agar

disc diffusion assay. As can be seen from Table 3, the EO of *T. vulgaris* showed the strongest antibacterial activity and this activity was followed by the EOs of *T. kotschyanus, Z. tenuior* and *Z. clinopodioides*. The antibacterial activity of *Thymus* species was greater than that of *Ziziphora* species. *Bacillus cereus* was the most sensitive bacterium tested. In general, Gram-positive bacteria were more sensitive than Gram-negative ones.

The antibacterial activity of essential oils from three *Thymus* species (*T. hyemalis, T. vulgaris* and *T. zygis*) was studied against 10 pathogenic bacteria [32]. The EO of *T. vulgaris* showed the widest inhibition zones (19.6–45.0 mm) against tested bacteria.

Sokmen et al. investigated the antimicrobial activity of essential oil of *Thymus spathulifolius*. According to the results, EO had inhibitory effect against all 25 bacteria tested and inhibition zones were in the range of 7–32 mm [28].

The antibacterial activity of EOs of the aerial parts of *Thymus longicaulis* and *T. pulegioides* was investigated [33]. Both EOs showed relatively good antibacterial activity against tested bacteria such as *Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, B. cereus, Proteus mirabilis, Enterobacter coli, Salmonella typhi* and *Pseudomonas aeruginosa*, with inhibition zones that ranged between 9 and 20 mm.

The major constituents of essential oil of *T. kotschyanus* were thymol (38.6%) and carvacrol (33.9%) [34]. The antimicrobial activity of EOs from *Thymus* genus was associated with phenolic compounds such as thymol and carvacrol [32]. The presence of a phenolic hydroxyl group, in carvacrol particularly, is credited with its activity against pathogens such as *B. cereus* [35].

Antibacterial activity of *Z. clinopodioides* EO against bacteria such as *E. coli, B. subtilis* and *S. aureus* was evaluated and inhibition zones were in the range of 11–18 mm. This oil was not active against *P. aeruginosa* [13].

## MIC determination

The results of MIC values are presented in Table 4. The EO of *T. vulgaris* had the lowest MIC value against the four bacteria tested (0.312–1.25 µl /ml). The next most effective EO in this respect was *Z. clinopodioides*, which showed MIC values of 0.625–2.5 µl /ml.

Several authors have reported that the major constituent of thyme EO is thymol [36], and antibacterial activity of this compound has been confirmed on *E.coli, Shigella flexneri* and *Bacillus cereus* [37-39].

Rota et al. investigated the antimicrobial activity and chemical composition of *Thymus vulgaris, Thymus zygis* and *Thymus hyemalis* essential oils [32]. The essential oils with the most bactericidal and bacteriostatic properties were: *T. hyemalis* (thymol and carvacrol chemotypes), *T. zygis* (thymol ch.) and *T. vulgaris* (thymol ch.) with MIC $\leq 0.2$ µl/ml against strains tested.

Salehi et al. indicated that *B. subtilis* was the most sensitive bacteria tested to essential oil of *Z. clinopodioides*, with the lowest MIC value (3.8 mg/ml) [13]. These investigators reported moderate inhibitory activity for EO against *E.coli* with MIC value of 15 mg/ml.

**Table 3.** Inhibition zones (mm) of EOs against four food-borne bacteria using agar disc diffusion assay

| EOs | Dose (µl) | Bacteria | | | |
|---|---|---|---|---|---|
| | | B. cereus | L. monocytogenes | E.coli | S. typhimurium |
| T. vulgaris | 2.5 | 37.1 ± 1 | 32.6 ± 1.4 | 23.1 ± 2.1 | 19.4 ± 1.2 |
| | 5 | 38.2 ± 1.2 | 34.2 ± 1.5 | 24.3 ± 2.3 | 23.2 ± 1.8 |
| | 10 | 41.5 ± 14 | 37.5 ± 1.3 | 26.2 ± 1.9 | 27.0 ± 2.1 |
| T. kotschyanus | 2.5 | 23.1 ± 0.9 | 14.5 ± 1.5 | 13.5 ± 1.6 | 12.7 ± 1.3 |
| | 5 | 31.0 ± 1.2 | 21.2 ± 1.7 | 17.7 ± 1.3 | 15.5 ± 1.3 |
| | 10 | 35.0 ± 1.5 | 28.5 ± 2.1 | 20.4 ± 1.9 | 16.8 ± 1.4 |
| Z. tenuior | 2.5 | 13.4 ± 1.1 | 9.2 ± 1.2 | 8.7 ± 0.9 | 8.6 ± 0.7 |
| | 5 | 17.2 ± 1.3 | 10.3 ± 1.5 | 10.1 ± 1 | 10.1 ± 0.5 |
| | 10 | 23.1±1.4 | 14.6 ± 1 | 13.5 ± 1.2 | 11.8 ± 0.6 |
| Z. clinopodioides | 2.5 | 10.4 ± 0.8 | 8.9 ± 1.1 | 9.1 ± 0.4 | 8.1 ± 0.7 |
| | 5 | 17.3 ± 0.9 | 10.1 ± 1.3 | 10.2 ± 0.6 | 9.5 ± 0.3 |
| | 10 | 19.4 ± 1.1 | 14.2 ± 1.5 | 13.1 ± 0.2 | 12.3 ± 0.5 |
| **Antibiotic** | | | | | |
| Erytromycine | | 13.7 ± 0.3 | 41.2 ± 0.2 | 12.4 ± 0.1 | 14.1 ± 0.2 |

The values are mean ± SD.

**Table 4.** The minimum inhibitory concentration (MIC) values (μl/ml) of EOs against four food-borne bacteria

| EOs | Bacteria | | | |
|---|---|---|---|---|
| | *B. cereus* | *L. monocytogenes* | *E.coli* | *S.* typhimurium |
| *T. vulgaris* | 0.312 | 0.625 | 0.625 | 1.25 |
| *T. kotschyanus* | 5 | 5 | 5 | 5 |
| *Z. tenuior* | 2.5 | 5 | 5 | 5 |
| *Z. clinopodioides* | 1.25 | 2.5 | 1.25 | 2.5 |

In another study, the MIC value of 3.75 mg/ml was reported for EO of *Z. clinopodioides* against *Staphylococcus aureus, S. epidermidis, B. Subtilis* and *E.coli* [14].

The antioxidant and antibacterial activity of EOs are associated with their phenolics content [40] and phenolics content can be influenced by many factors such as geographic location, environmental and climate conditions, season, soil type, and the method of drying and extraction of the oil [38].

## Conclusion

This study indicated that the EOs of ZC and TV had the highest antioxidant activity. The order of antioxidant activity was: ZC > TV >TK > ZT. The EO of TV showed the strongest antibacterial activity against four food borne pathogenic bacteria. Then, the EOs of ZC and TV can be used as effective antibacterial and antioxidant to preserve of foods. A positive linear correlation was found between antioxidant activity and total phenolic content of essential oils tested. Meanwhile, this is the first study to provide data on antioxidant and antibacterial activities of essential oil of *Ziziphora tenuior*.

## Acknowledgments

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