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Antioxidant and antibacterial activities of essential oils and aromatic waters of some plants grown in the highlands

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Abstract

The essential oils from twenty-two plants grown in the highlands were isolated by hydrodistillation. The plants with an essential oil yield above one percent, including *Achillea millefolium, Asparagus plumosus*, *Matricaria chamomilla*, *Mentha piperita*, *Mentha pulegium*, and *Thymus vulgaris,* were tested for the antibacterial activity and total antioxidant capacity. Their antibacterial activities against the three most common foodborne pathogens and an opportunistic pathogen were evaluated. Results indicated that *T. vulgaris* essential oil had the highest total antioxidant capacity with 11.78 ± 0.01 mmol/L TEAC. The essential oils of plants inhibited the growth of pathogen bacteria tested, while their aromatic waters showed no inhibition. *T. vulgaris* oil was the most powerful antibacterial essential oil with the inhibition zones of 49.27±7.26 mm against *S. aureus,* 44.13±4.16 mm against *L. monocytogenes,* 39.55 ± 0.52 mm against *E. coli,* and 38.09 ± 4.15 mm against *M. luteus.* Furthermore, the volatile compounds of *T. vulgaris* essential oil were detected using GC-MS. Thymol, carvacrol, caryophyllene, 1,8-cineole, 2 acetyl-4,5-dimethylphenol, and γ-terpinene were determined as major compounds in *T. vulgaris* essential oil. The obtained results suggest that the essential oils of tested plants with high antimicrobial activity and antioxidant capacity might be used as natural antioxidants and antimicrobial agents.

Keywords: Antibacterial activity, Antioxidant capacity, Foodborne pathogen, *Thymus vulgaris,* Essential oil

Introduction

Antibiotic resistance, one of the largest hazards to global health and food security, is accelerated by the misuse and overuse of antibiotics. Development of new antibiotics and use of alternatives to antibiotics are the measures taken to control and prevent the spread of antibiotic resistance (WHO, 2018). The use of essential oils as antibiotics is safer for the people and the environment because of their natural origin and they play an important role in the fight against antibiotic resistance (Daferera et al., 2003). Essential oils containing natural compounds are used in folk medicine for their beneficial effects on the health. They have not only antimicrobial effects against several microbial pathogens, but also antioxidant activity. For example, the bactericidal (against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Enterococcus feacalis, Vibrio alginolyticus*), fungicidal (against *Verticillium fungicola* and *Trichoderma harzianum*), and antioxidant effects of thyme (*Thymus vulgaris)* essential oil have been reported (Miladi et al., 2013; Oussalah et al., 2007; Soković et al., 2010). Roby et al. (2013) evaluated the antimicrobial effect of chamomile (*Matricaria chamomilla* L.) essential oil on some pathogens (*E. coli, S. tyhpimurium, B. cereus, S. aureus, Candida albicans* and *Aspergillus flavus)* and found that chamomile oil inhibits the growth of tested pathogens. Essential oils of yarrow (*Achillea millefolium),* a plant belonging to the family *Asteraceae,* was tested for its antibacterial effect

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and it was found that its essential oil inhibited the growth of some pathogenic bacteria, including *B. cereus, Bacillus subtilus*, *S. aureus, S. typhimurium,* and *Salmonella agona* (El-Kalamouni, et al., 2017). Similarly, it has been shown that the peppermint oils (*Mentha* spp.; *M. piperita* and *M. pulegium)* exhibited antimicrobial effect against *E. coli*, *S. aureus*, *P. aeruginosa*, *Proteus* vulgaris, *Enterobacter aerogenes*, *S. typhimurium*, *C. albicans, Klebsiella pneumoniae, Yersinia enterocolitica*, *L. monocytogenes*, *B. cereus*, *S. epidermidis*, *Xanthomonas campestris* pv. *phaseoli, Pseudomonas syringae* pv. *phaseolicola*, *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas tolaasii* var. *tolaasii*, *Xanthomonas campestris* pv. *campestris, Verticillium fungicola var. fungicola,* and *T. harzianum* (Soković and van Griensven, 2006; İşçan et al. 2002). Moreover, Madikizela et al. (2014) reported the antibacterial effect of two *Asparagus* spp. (*A. africanus* and *A. falcatus*) on *Mycobacterium tuberculosis* and *Salmonella typhimurium.*

In the present study, the essential oils from twenty-two plants, which are grown in the highlands in Turkey and traditionally consumed due to their health benefits, were obtained. Among these, the plants containing over one percent essential oil (*Achillea millefolium, Asparagus plumosus*, *Matricaria chamomilla*, *Mentha piperita*, *Mentha pulegium*, and *Thymus vulgaris)* were tested for total antioxidant capacity and antibacterial effect on three widespread foodborne virulent pathogens, *S. aureus, E. coli*, and *L. monocytogenes,* and a low-virulence pathogen *M. luteus*.

Materials and methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), Plate Count Agar and Mueller Hinton Agar were purchased from Sigma-Aldrich (St. Louis, USA). Tryptic Soy Broth, n-butanol, and methanol were obtained from Merck (Darmstadt, Germany).

Plant material and isolation of essential oils

Cynara *syriaca, Sambucus ebulus, Psoralea bituminosa* L., *Sedum pallidum* M. Bieb., *Alchemilla mollis, Trifolium repens, Verbascum blattaria* L., *Phedimus stoloniferus, Echium vulgare, Hypericum perforatum, Prunella vulgaris* L., *Polygonum affine, Urtica dioica, Inula helenium, Rhododendron luteum, Vaccinium corymbosum* L., *Achillea millefolium, Asparagus plumosus*, *Mentha piperita*, *Mentha pulegium, Matricaria chamomilla*, and *Thymus vulgaris* were collected from highlands in Ordu and Giresun provinces, Turkey. The plant species were identified in the departments of biology and plant protection at Ordu University. The essential oils of plants were extracted by the hydrodistillation method according to Soković et al. (2010). After drying at room temperature, the plant samples were coarsely ground for extraction. 50 g of dried plants were taken into a 1 L flask and hydrodistilled using the Clevenger apparatus to extract the essential oils. The obtained essential oil and aromatic water, the residue after isolation of essential oil, were then stored at -18 °C until analyses.

Determination of total antioxidant activity

To determine the antioxidant activity of essential oils and aromatic waters, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was performed with a slightly modified method described by Shahidi and Liyana-Pathirana (2007). 40 µL of the samples were added to 1500 µL of DPPH solution (0.6 mM DPPH in n-butanol for essential oil samples and 0.1 mM DPPH in methanol for plant aromatic waters) in a microcuvette in which the reaction takes place. The mixture was allowed to stand at room temperature for 30 min. After the incubation, the absorbance of the mixture was measured at 515 nm. Trolox was chosen as the standard. Total antioxidant activity was calculated using standard curves. Results were expressed as millimoles per liter trolox equivalents.

Microbial strains and culture

Staphylococcus aureus NCTC 8530, *Escherichia coli* BL21, *Listeria monocytogenes* ATCC 7644, and *Micrococcus luteus* NCIMB 8166 were gifts of Dr. Ö. F. Çelik (Ordu University, Faculty of Agriculture) and were used as the indicator bacteria to determine antibacterial activity of the essential oils. All bacteria were inoculated from frozen stocks into the Tryptic Soy Broth and incubated at 37°C for cultivation purposes. Preceding the assay, the cultures were passaged in the same medium overnight.

Determination of antibacterial activity

The antibacterial activity of essential oils against *E. coli, L. monocytogenes, M. luteus,* and *S. aureus* was determined using a disc diffusion method according to Soković et al. (2010) with some modifications. Agars of Plate Count (PCA) and Mueller Hinton (MHA) were sterilized by autoclaving and used for the growth of *E. coli* and the remaining bacteria, respectively. Then, the agars were cooled to 50 ºC and 20 mL of each agar were poured into sterile petri dishes. After inoculation with 0.1 mL of the bacterial suspension adjusted to the turbidity of a 0.5 McFarland standard, the petri dishes were allowed to dry for 30 minutes. The blank sterile disc was placed on an inoculated agar plate and 15 µL of essential oil were added to the discs. After incubation at 37 ºC for 24 hours, the diameters of the inhibition zones were measured with a digital caliper and recorded in mm. The antibiotic discs including penicillin (10 µg/disc), streptomycin (300 µg/disc), ampicillin (10 µg/disc) and gentamycin (10 µg/disc) were used as positive controls.

Determination of volatile compounds

The volatile compounds of the essential oil of *Thymus vulgaris* were analyzed according to literature (Sahin et al., 2017) with some modifications by GC/MS (Thermo Fischer Scientific, USA) equipped with a TraceGOLD TG-5MS capillary column (30 m x 0.25 mm; film thickness 0.25 µm). Helium was used as the carrier gas and the flow rate was adjusted as 1.2 mL per minute. The oven temperature was programmed at 80°C for 2 min, then 2°C/min to 270°C. The MS conditions were as follow: ionization voltage, 70 eV; ion source temperature, 250°C; electron ionization mass spectra were acquired over the mass range of 50 to 550 m/z. The identification of the compounds in *Thymus vulgaris* essential oil was based on a comparison of their mass spectra with those obtained from the NIST, WinMain, mainlip, replib, and Wiley libraries spectra.

Statistical analysis

The results of the analysis were expressed as mean ± standard deviation (SD). Data were statistically analysed using repeated measures two-way ANOVA. The Tukey multiple comparison test was used as follow-up to ANOVA with a significance level of $p < 0.05$. Statistical analysis was performed using Minitab 18.1 (Minitab Software, Coventry, UK).

Results and Discussion

Yield of essential oils

The essential oil yields of *Cynara syriaca, Sambucus ebulus, Psoralea bituminosa* L., *Sedum pallidum* M. Bieb., *Alchemilla mollis, Trifolium repens, Verbascum blattaria* L., *Phedimus stoloniferus, Echium vulgare, Hypericum perforatum, Prunella vulgaris* L., *Polygonum affine, Urtica dioica, Inula helenium, Rhododendron luteum,* and *Vaccinium corymbosum* L. were less than one percent, while *Achillea millefolium, Asparagus plumosus*, *Matricaria chamomilla*, *Mentha piperita*, *Mentha pulegium*, and *Thymus vulgaris* were rich in essential oils. For further analysis, these six essential-oil rich plants were tested.

Antioxidant activity

The antioxidant capacities of plant essential oils and aromatic waters were detected using a DPPH radical scavenging assay and presented as mmol/L Trolox Equivalent Antioxidant Capacity (TEAC) values in Figure 1. The essential oil of *Thymus vulgaris* showed the highest antioxidant capacity, which was 3- to 11-fold higher than the other plant essential oils tested. The minimum TEAC value was found in *Asparagus plumosus* essential oil (1.07 ± 0.03 mmol/L TEAC). Many researchers reported that *Thymus vulgaris* essential oil exhibited high antioxidant capacity and there is a positive correlation between its antioxidant activity and amounts of its phenolic components like thymol and carvacrol (Chizzola et al., 2008; Kulisic et al., 2005; Miladi et al., 2013). El-Kalamouni et al. (2017) evaluated the antioxidant activity of *Achillea millefolium* essential oil using sunflower oxidation test and observed that it increased the stability of sunflower oil. The antioxidant activity of *Matricaria chamomilla* essential oil assessed by DPPH assay has been also reported (Abdoul-Latif et al., 2011; Stanojevic et al., 2016).

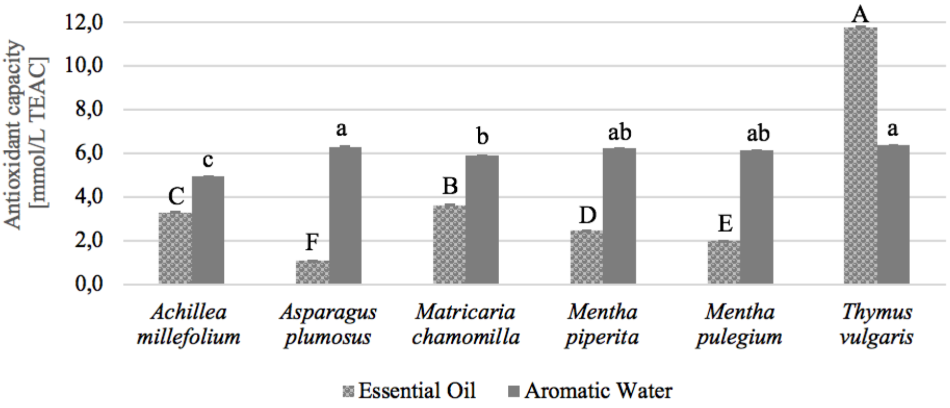

Figure 1. Total antioxidant capacity of plant essential oils and plant aromatic waters expressed as mmol/L TEAC values. The data are reported as mean ± standard deviation; n=6. The values indicated by different letters (a-c or A-F) on the columns in each group are different from each other at p <0.05 level.

When the aromatic waters of plants were tested for total antioxidant capacity, it was seen that *Thymus vulgaris* and *Asparagus plumosus* aromatic waters had higher antioxidant capacity, followed by *Mentha pulegium* and *Mentha piperita*, however, no difference between the means was found among these plant aromatic waters (p<0.05). The *Achillea millefolium* aromatic water had the least antioxidant capacity. The results also indicated that the aromatic waters of plants-except for *Thymus vulgaris*- showed higher antioxidant capacity when compared with their essential oils. This can be explained that the aromatic water is generally rich in water soluble compounds which may have higher antioxidant activity than the water-insoluble substances found in the essential oil of *Achillea millefolium, Asparagus plumosus*, *Mentha piperita*, *Mentha pulegium,* and *Matricaria chamomilla*. On the other hand, Candan et al. (2003) observed that the methanolic extracts (water-soluble) of *Achillea millefolium* had lower antioxidant capacity compared to *Achillea millefolium* essential oil. This suggests that methanol may not be a good choice to extract the strong, water soluble antioxidants.

Antibacterial activity

The plant essential oils and aromatic waters were tested for antibacterial effects against the three most common foodborne pathogens including *S. aureus, E. coli,* and *L. monocytogenes* (Bintsis, 2017) and an opportunistic pathogen *M. luteus* (Kao et al., 2014). *S. aureus,* an aerobic Gram-positive bacteria, can cause several infections in the skin such as cellulitis, and postoperative wound infections. Some serious infections, including bacteremia, pneumonia, osteomyelitis, cerebritis, etc., have been attributed to *S. aureus* infection (Archer, 1998; Bintsis, 2017). Pathogenic *E. coli*, a Gram-negative facultative anaerobe, is a fecal indicator bacteria and an important public health problem because of its low infectious concentration and its transmission via omnipresent media such as food and water (Bintsis, 2017). *L.monocytogenes,* a Gram-positive facultative anaerobic bacteria, is known as highly hazardous food-borne pathogen and causes some inflammation like meningitis, and gastroenteritis (Bintsis, 2017; Buchanan et al., 2017). *M. luteus,* Gram-positive aerobe, is an opportunistic pathogen in patients having a weakened immune system and found in soil, water, air, and the skin of humans and animals. *Micrococcus* species are associated with bacteremia, infection of the endocardium, inflammation of the ventricles in the brain, pneumonia, endophthalmitis, inflammation of the peritoneum, and infectious (Kao et al., 2014).

No inhibition zones were observed for the plant aromatic waters. The antibacterial activity (mean diameters of the inhibition zones of plant essential oils) on *S. aureus, E. coli, L. monocytogenes,* and *M. luteus* were given in Table 1. The highest antibacterial activity against the four pathogenic bacteria was observed in the essential oil of *Thymus vulgaris*. The essential oil of *Achillea millefolium* led to the least inhibitory effect on the growth of *S.aureus;* however, the difference between inhibition zones of *Mentha pulegium*, *Mentha piperita*, *Asparagus plumosus*, *Matricaria chamomilla*, and *Achillea millefolium* was not significant ($p > 0.05$). The essential oils of *Asparagus plumosus* and *Achillea millefolium* were the least effective against *E. coli*. The inhibition zones of *Mentha pulegium* and *Mentha piperita* on *E. coli* were similar. *Asparagus plumosus* essential oil had a high inhibition zone (17.14 ± 0.6 mm) on *L. monocytogenes*, whereas no significant difference in the mean diameters of inhibition zones of *Mentha pulegium*, *Mentha piperita, Matricaria chamomilla*, and *Achillea millefolium* was found. The essential oils of *Mentha pulegium* and *Mentha piperita* showed similar antibacterial effects against *M. luteus*. The lowest inhibition zones on *M. luteus* were determined with the essential oils of *Achillea millefolium*, *Asparagus plumosus*, and *Matricaria chamomilla*.

Table 1. Mean inhibition zones of plant essential oils [mm]. Data represent means ± SD of three replicates. Different superscript letters (a-d) in the same columns are significantly different from each other ($p < 0.05$).

Plant essential oils	**Pathogenic bacteria**			
	S. aureus	*E. coli*	*L. monocytogenes*	*M. luteus*
Achillea millefolium	9.4 ± 0.01^{b}	9.84 ± 0.49^{d}	11.96 ± 1.51^{c}	8.11 ± 0.10^{d}
Asparagus plumosus	10.72 ± 0.76^{b}	8.73 ± 1.68^{d}	17.14 ± 0.6^{b}	8.19 ± 0.08^{d}
Matricaria chamomilla	10.63 ± 1.01^{b}	14.63 ± 1.37^{cd}	11.04 ± 0.91^{c}	9.51 ± 2.1^{d}
Mentha piperita	15.93 ± 1.29^{b}	21.55 ± 7.36^{bc}	11.15 ± 0.42^{c}	11.69 ± 2.3^{bc}
Mentha pulegium	16.23 ± 0.91^{b}	24.3 ± 0.00^{b}	13.16 ± 2.31^{bc}	10.45 ± 0.58^{b}
Thymus vulgaris	49.27 ± 7.26^{a}	39.55 ± 0.52^{a}	44.13 ± 4.16^{a}	38.09 ± 4.15^{a}

El-Kalamouni et al. (2017) tested also the essential oil of *Achillea millefolium* grown in France for its antimicrobial activity against some pathogenic bacteria species (*B. subtilis*, *B. cereus, S. aureus, S. epidermidis, S. typhimurium, S. enteritidis, S. agona, E. coli)* and observed that the essential oil of *Achillea millefolium* had antibacterial activity against *S. aureus* but no activity against *E. coli*. Similarly, Candan et al. (2003) reported that the essential oil of *Achillea millefolium* subsp. *millefolium* grown in Sivas, in the Central Anatolian Region of Turkey, showed antimicrobial activity against *S. aureus,* while *E. coli* was resistant against *Achillea millefolium* essential oil. In our study, the essential oil of *Achillea millefolium,* which is grown in Ordu in the Black Sea Region of Turkey, exhibited antibacterial activity against both *S. aureus* and *E. coli*. Compared to other studies (El-Kalamouni et al., 2017; Candan et al., 2003), we used the PCA instead of the MHA for the growth of *E. coli* in this study, because the growth of *E. coli* on PCA was better monitored. It was also shown that the essential oils of some *Achillea* species (*A. clavennae* and *A. holosericea*) had antibacterial activity against *E. coli (*Bezić et al., 2003; Stojanović et al., 2005).

Some *Asparagus* spp. (*A. africanus* and *A. falcatus)* are grown in South Africa and known as South African medicinal plants. Madikizela et al., (2014) reported the antibacterial effect of two *Asparagus* spp. (*A. africanus* and *A. falcatus)* on *M. tuberculosis* and *S. typhimurium.* We tested here the essential oil of *Asparagus plumosus* for antibacterial activity and demonstrated that *Asparagus plumosus* essential oil inhibited the growth of *S.aureus, E. coli, L. monocytogenes,* and *M. luteus.* To our knowledge, the antibacterial effect of *Asparagus plumosus* essential oil has not been described before.

The essential oil of *Matricaria chamomilla* L. showed antimicrobial activity against all pathogenic bacteria tested (Table 1). Previously, the antibacterial effect of *Matricaria chamomilla* L. essential oil against *S. aureus* and *E. coli* was reported (Abdoul-Latif et al., 2011; Roby et al., 2013; Soković et al. 2010; Stanojevic et al., 2016). It was also shown that the essential oil of *Matricaria chamomilla* L. exhibited antibacterial effect on *L. monocytogenes* (Soković et al. 2010; Stanojevic et al., 2016).

As shown in Table 1, the essential oils obtained from *Mentha piperita* and *Mentha pulegium* inhibited the growth of pathogenic bacteria tested. Similarly, Soković et al. (2010) and İşçan et al. (2002) observed the antibacterial activity of *Mentha piperita* essential oil against *S. aureus, E. coli* and *L.*

monocytogenes. The antimicrobial effect of *Mentha piperita* essential oil on *M. luteus* has been also reported by Mattazi et al. (2015). Mahboubi and Haghi (2008) reported that the essential oil of *Mentha pulegium* showed antibacterial activity against *S. aureus* and *E. coli.*

Among the tested essential oils, *Thymus vulgaris* was the most active in terms of inhibition of bacterial growth and *S. aureus* was the most sensitive to the essential oil of *Thymus vulgaris.* Previous studies have also shown that *Thymus vulgaris* essential oils exhibited antimicrobial activity against *S. aureus, E. coli*, and *L. monocytogenes;* and *S. aureus* had the highest sensitivity (Soković et al., 2010; Oussalah et al., 2007). On the other hand, Miladi et al. (2013) reported that *S. aureus* was more resistant to the essential oil of *Thymus vulgaris* than *L. monocytogenes*, and *M. luteus* was the most sensitive among these four pathogenic bacteria.

Since *Thymus vulgaris* was the most potent antibacterial essential oil tested, its antibacterial effect was compared with the antibacterial effects of commonly used antibiotics (Table 2). Among tested antibacterials, penicillin and ampicillin were most effective against *S. aureus*, *L. monocytogenes,* and *M. luteus* and their effects were similar to the antibacterial effect of *Thymus vulgaris*. Furthermore, *Thymus vulgaris* had a higher antibacterial effect against *E. coli* than the tested antibiotics.

Table 2. Mean diameters of the inhibition zones [mm] of antibiotics. Data represent means ± SD of three replicates. Means with different superscript small letters in the same column are significantly different from each other ($p < 0.05$).

Antibiotics	**Pathogenic bacteria**			
	S. aureus	*E. coli*	*L. monocytogenes*	*M. luteus*
Ampicillin	36.40 ± 0.85^{ab}	23.85 ± 0.50^{b}	38.85 ± 0.50^{ab}	34.45 ± 0.07^{a}
Gentamycin	18.90 ± 0.00^{c}	14.10 ± 0.42^{c}	22.05 ± 0.64^{c}	24.10 ± 0.14^{b}
Penicilin	40.75 ± 1.91^{a}	14.25 ± 0.21^{c}	41.70 ± 2.69^{a}	38.35 ± 1.34^{a}
Streptomycin	24.85 ± 0.07^{bc}	$26.00 \pm 0.1b^{b}$	$31.45 \pm 0.64b^{bc}$	$26.10 \pm 0.85b^{b}$
Thymus vulgaris	49.27 ± 7.26^{a}	39.55 ± 0.52^{a}	44.13 ± 4.16^{a}	38.09 ± 4.15^{a}

Chemical composition of essential oil of Thymus vulgaris

Single volatile compounds of the essential oil of *Thymus vulgaris,* which showed the highest antioxidant and antibacterial effect, were determined by GC-MS. *Thymus vulgaris* essential oil was rich in thymol, carvacrol, 1,8-cineole, 2 acetyl-4,5-dimethylphenol, γ-terpinene, and caryophyllene (Figure 2). Soković et al. (2010) reported that the thymol, carvacrol, and 1,8-cineole isolated from *Thymus vulgaris* essential oil had antibacterial activity against *S. aureus*, *L. monocytogenes,* and *E. coli.* They found also a correlation between the levels of antibacterial activity and the percentage of the major components of essential oil (Soković et al., 2010). Thymol and carvacrol belong to the phenol-type oxygenated monoterpenes which show higher antimicrobial activity (Couladis et al., 2004; García-García et al.,2011; Soković and van Griensven, 2006; Soković et al., 2010; de Oliveira et al., 2015). Thymol and carvacrol also exhibit synergistic antibactericidal effects when used in combination (García-García et al., 2011). The synergic interaction between carvacrol and 1,8-cineole has been also reported by de Oliveira et al. (2015). Additionally, Ruberto and Baratta (2000) measured the antioxidative capacity of approximately 100 pure compounds of essential oils and observed that phenols like thymol and carvacrol have the highest antioxidant activity.

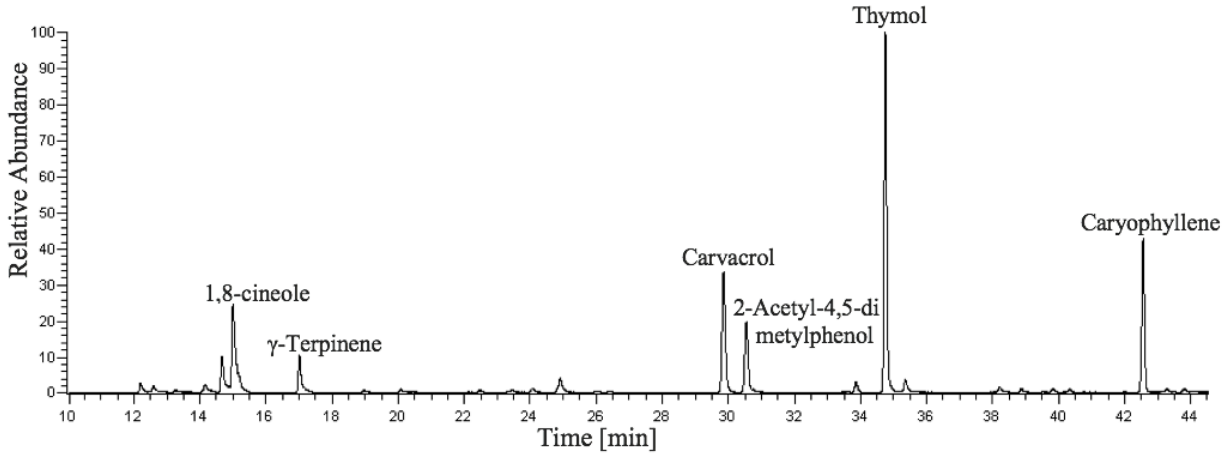

Figure 2 GC-MS chromatogram of a *Thymus vulgaris* essential oil

Conclusion

The use of natural products such as essential oils might be useful in the struggle against antibiotic resistance which is a serious problem for food security and public health. In this study, the essential oils and aromatic waters were isolated from *Achillea millefolium, Asparagus plumosus*, *Matricaria chamomilla*, *Mentha piperita*, *Mentha pulegium*, and *Thymus vulgaris.* Their antibacterial effects were detected against three foodborne pathogenic bacteria and an opportunistic pathogen. All essential oils tested indicated a high antioxidant capacity and antibacterial effect. *Thymus vulgaris* essential oil containing thymol, carvacrol, caryophyllene, 1,8-cineole, 2 acetyl-4,5-dimethylphenol, and γ-terpinene was the most effective against all pathogens tested. Further studies are now required to determine the antibacterial mechanism of the active compounds found in essential oil.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Not applicable.

Funding

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Data availability

Not applicable.

Consent for publication

Not applicable.

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