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In vitro antimicrobial activity and antagonistic effect of essential oils from plant species

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Abstract: *Kahramanmaras, is a developing city, located in the southern part of Turkey. Thymus eigii (M. Zohary and P.H. Davis) Jalas, Pinus nigra Arn. sub sp pallasiana and Cupressus sempervirens L. are the useful plants of the Kahramanmaras province and have been under study since 2004 for the traditional uses of plants empiric drug, spice, herbal tea industry, herbal gum and fuel. The study was designed to examine the antimicrobial activities of essential oils of these plants by the disc diffusion and minimum inhibitory concentration (MIC) methods. In addition, antimicrobial activity of Thymus eigii was researched by effects when it was used together with antibiotics and even when it was combined with other essential oils. When the results of this study were compared with vancomycin (30 mcg) and erytromycin (15 mcg) standards, it was found that Thymus eigii essential oil was particularly found to possess stronger antimicrobial activity, whereas other essential oils showed susceptible or moderate activity. However, antimicrobial activity changed also by in vitro interactions between antibiotics and Thymus eigii essential oil, also between essential oils of these plants and that of Thymus eigii causing synergic, additive, antagonist effect.*

Key words: *Antimicrobial activity, Essential oil, Thymus eigii, Pinus nigra, Cupressus sempervirens, Synergisms-additive-antagonism*
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Introduction

Turkey is one of the richest areas in the middle latitudes in terms of plant diversity. The main reasons for this are, climate varieties, geomorphological and soil diversities and the situation of the area at the junction of three flora region (Euro-Siberian, Mediterrenean and Irano-Turanian). When all these factors are combined, it provides many properties for the plants to grow up and discrepant. The flora of Turkey is relatively rich (about 12000 species) and still a great number of new species are being described. In Turkey, the rate of endemism is relatively high when compared with other European countries. The number of endemic species in Turkey is over 3000. Furthermore, there are endemic plants in genus' level. However, this diversity and endemic species are under considerable threat. The habitats in mountainous areas and coastal dunes are under the threat due to the tourism activities. That is the reason why it is really significant to protect the diversity of plants (Avci, 2005).

Turkish flora is rich in plants which are used in various forms as folk medicine and herbal tea (Sezik and Saracoglu, 1987). Essential oils of plants are good candidates for pharmaceutical and cosmetic ingredients, due to their low toxicity. The most attractive aspects of using essential oils that they are very low mammalian, fish, and environment toxicities compared with synthetic chemicals and their nonpersistence in fresh water and soil (Lai *et al*., 2006).

The genus *Thymus* (Lamiaceae) is represented by 38 species (64 taxa) in Turkey and 24 of which are endemic to Turkey (Azaz *et al*., 2004). The genus *Thymus* has numerous species and varieties, therefore their essential oil compositions and antimicrobial activities were studied earlier (Baser *et al*., 1992; Rasooli and Mirmostafa, 2003; Azaz *et al*., 2004). Herbal parts of this plant are used as tea and condiment. It is used as folk medicine in sore throat and gastrointestinal system disorders (Sezik and Saracoglu, 1987) and also used as expels intestinal worm, as a depressant, as an antiseptic and as a bloodstream stimulating (Baytop, 1984). It is a shrublet, up to 12-20 cm and narrowly distributed in E. Mediterranean area (Davis, 1982).

In Turkey, five pine species (*Pinus nigra*, *P. sylvestris*, *P. brutia*, *P. pinae* and *P. halepensis*) (*Pinaceae*) are indigenous. Different parts of these species are used for the same purpose regardless of the species. Due to their tannin contents, the dried barks were used as a tanning agent and as an infusion to produce constipation (Baytop, 1984). An essence obtained from the leaves of pines contain compounds such as pinene, cadinene, terpineol and bornyl acetate. The leaves can also be used as a mucus remover and as an antiseptic (Sezik *et al*., 1997). *Pinus nigra* is twigs blackish; leaves dark green, not twisted, up to 30 m (Davis, 1965).

The green *Cupressus sempervirens* L. (Cupressaceae) is of great interest for ornamental, reforestation and windbreak use in the whole Mediterranean basin (Cantini and Battisti, 2001). Since *Cupressus sempervirens* L. contains tanen, it is an antipyretic, constipating, diaphoretic, enhancing of urine, astringent. Externally it is used for hemoroid and anti-foot sweating. Internally, it is beneficial to cut urination in childreen in the night (Baytop, 1984) and is also used antibacterial and antifungal (Dagci *et al*., 2002). *Cupressus sempervirens* L. is branched quadrangular, horizontal, or erect and distributed in E. Mediterranean area (Davis, 1965).

According to World Health Organization (Santos *et al.*, 1995) medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Ellof, 1998; Nascimento *et al.*, 2000).

Many researches were studied on the antimicrobial activities of plants and in their application areas. However, the interactions of antibiotics with plant extracts can cause side effect and decrease effectiveness. Essential oil of plants, both endemic and nonendemic, is one of the very important subjects for public health and environmental quality. For this reason, in this study, the antimicrobial properties of essential oil from *Thymus eigii* that is endemic in Turkey, *Pinus nigra* and *Cupressus sempervirens* peculiar to Kahramanmaras region were investigated. In addition, antimicrobial activity of *Thymus eigii* was investigated for effects when it is used together with vancomycin, erytromycin antibiotics and it was studied for effects when it is used together with *Pinus nigra* and *Cupressus sempervirens*.

Materials and Methods

Study area: The Kahramanmaras City, falls within C_6 square of the Flora of Turkey adopted by Davis (1982). This area has different phytogeographic region, Irano-Turanian and Mediterranean and has rich biological resources (Davis, 1982). In this area, these plants are used as for empiric drug, spice, herbal tea industry, herbal gum and fuel (Fig. 1).

Materials: In this study, *Thymus eigii* (M. Zohary and P.H. Davis) Jalas, *Pinus nigra* Arn. sub sp *pallasiana*, *Cupressus sempervirens* L. were used. *T. eigii* was collected from Ahir mountain (Kahramanmaras), *P. nigra* and *C.sempervirens* were collected from Kahramanmaras region. A voucher specimen was deposited in the herbarium of the Department of Botany, University of Kahramanmaras Sutcu Imam *T. eigii* (M. Zohary and P.H. Davis) Jalas No: ILCIM 809 KSU H, *P. Nigra* Arn. sub-sp *pallasiana* No: ILCIM 899 KSU H, *C. sempervirens* L. No: ILCIM 727 KSU H). Plants were identified, dried and broken into small pieces under sterile conditions.

The tested microorganisms in this study were provided from the culture collections of Microbiology Laboratory of Sciences and Arts Faculty of the University of Kahramanmaras Sutcu Imam, in Kahramanmaras, in Turkey. *Micrococcus luteus* LA 2971, *Bacillus megaterium* NRS, *Bacillus brevis* FMC 3, *Enterococcus faecalis* ATCC 15753, *Pseudomonas pyocyaneus* DC 127, *Mycobacterium smegmatis* CCM 2067, *Escherichia coli* DM, *Aeromonas hydrophila* ATCC 7966, *Yersinia enterocolitica* AU 19, *Staphylococcus aureus* Cowan 1, *Streptococcus faecalis* DC 74 bacteria, and *Saccharomyces cerevisiae* WET 136, *Kluvyeromyces fragilis* DC

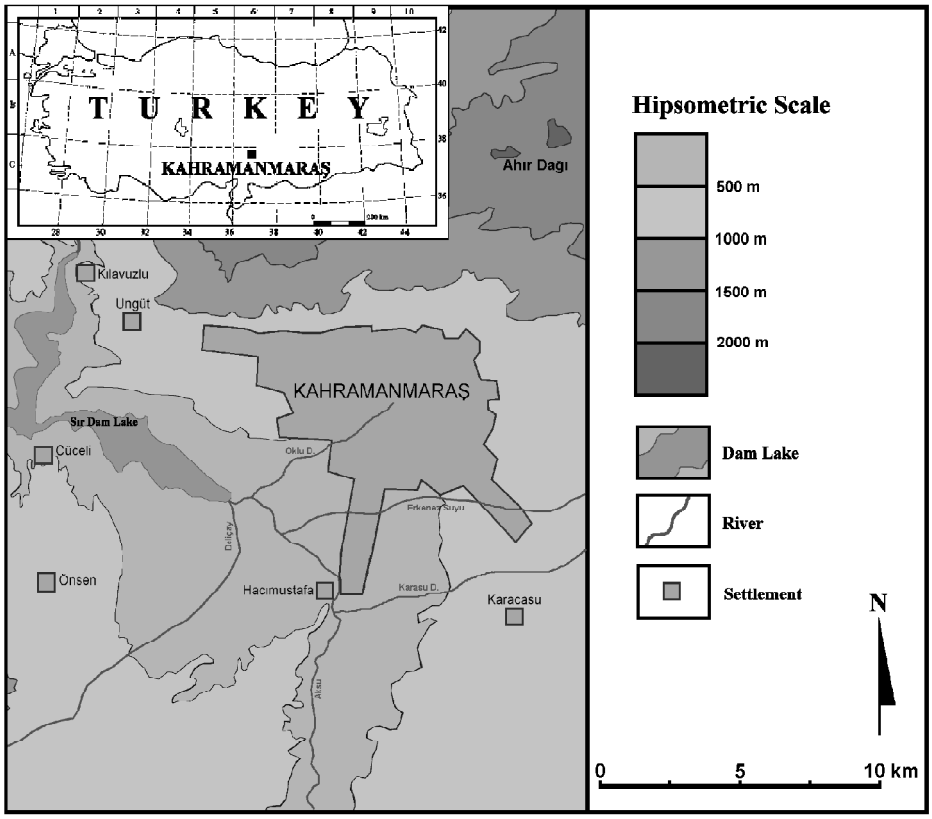

Fig. 1: Map showing the location of Kahramanmaras

fungi were used as microorganisms. Standard antibiotic discs such as vancomycin (30 mcg), erytromycin (15 mcg) (Bioanalyse) and Nystatin (100 U) (Oxoid) discs were used for comparision with the antimicrobial activities in essential oils. All discs were provided by the Microbiology Division of Faculty of the Medicine Sutcu Imam University in Kahramanmaras, Turkey.

The plants were distilled for 3 hr, using a clevenger-type apparatus according to the European Pharmacopoeia (1975). Disc diffusion method was employed for the determination of antimicrobial activities of single essential oil and when *T. eigii* was combined with antibiotics and with other plants. Bioactivities were determined by measuring diameter of inhibition zones.

Evaluation of antimicrobial effect of essential oils of *T. eigii*, *P. nigra*, and *C. sempervirens* on standard microbial strains: The essential oils thus obtained were injected into empty sterilized antibiotic discs having a diameter of 6 mm (Schleicher and Shull No: 2668, Germany), (Collins *et al*., 1989; Bradshaw, 1992). In addition, for reference antibiotic discs such as vancomycin, erytromycin and nystatin were used (Collins *et al*., 1989; Brooks *et al*., 1995).

Evaluation of the antagoni stic or synergistic effect of antibiotics and essential oil of *T. eigii* on standard microbial strains when essential oil of it was applied to standard antibiotic discs: 0.5 ml plant essential oil of *T. eigii*, an amount that starts inhibiting bacteria, was saturated to the standard antibiotic discs described above in order to determine the mutual influence of this essential oil and antibiotic combination (Collins *et al*., 1989; Brooks *et al*., 1995).

Evaluation of the antagonistic or synergistic effect of *T. eigii* and the other essential oils in combination on standard microbial strains when essential oil of it was mixed with the others: 0.5 ml plant essential oil of *T. eigii*, an amount that starts inhibiting bacteria, and separated for each other plant essential oil type (1 µl and 2 µl) was saturated to empty sterilized antibiotic discs described above in order to determine the mutual influence of plant essential oils combination (Collins *et al*., 1989; Brooks *et al*., 1995).

Determination of minimal inhibitory concentration (MIC):

A broth microdilution susceptibility assay was used, as recommended by NCCLS, for the determination of the MIC of essential oils of *T. eigii*, *P. nigra*, and *C. sempervirens* and some reference components (Anonymous, 1999). All tests were performed in Mueller Hinton Broth (MHB) supplemented with Tween 80 detergent (final concentration of 0.5% (v/v), with the exception of the yeasts (Sabouraud dextrose Broth (SDB) + Tween 80). Bacterial strains were cultured overnight at 37°C in MHB, and the yeasts were cultured overnight at 30°C in SDB. Geometric dilutions ranging from 0.01 to 5.0 µg/ml of the essential oils were prepared including one growth control (MHB + Tween 80) and one sterility control (MHB + Tween 80 + test oil). Test tubes were incubated under normal atmospheric conditions at 37°C for 24 hr for bacteria and at 30°C for 48 hr about the yeasts. The bacterial growth was indicated by the presence of a white 'pellet' on the well bottom.

Preparation of microbial cultures:

The bacteria were incubated in Nutrient Broth (NB) (Difco) at 37±0.1°C for 24 hr and the yeasts were incubated in Sabouraud Dextrose Broth (SDB) (Difco) at 25±0.1°C for 24 hr. The bacteria and yeasts (prepared as above) were injected into petri dishes (9 cm) in the amount of 0.01 ml (10^6/ml for the bacteria and 10^5/ml for the fungi)(Anonymous,1999), 15 ml of Mueller Hinton Agar (MHA, Oxoid) for bacteria and Sabouraud Dextrose Agar (SDA) for fungi (sterilized in a flask and cooled to 45-50°C) and homogenously distributed in the sterilized petri dishes (Collins *et al*., 1989).

Sterilized blank paper discs 6 mm in diameter were saturated with 0.5, 1, 2 and 4 ml of essential oil by micro- injector per disc. Standard antibiotic discs were unsaturated and saturated with 0.5 ml of essential oil of *T. eigii* and the combination of the other essential oils (*P. nigra* and *C. sempervirens*) (1 and 2 µl) with *T. eigii* per disc then placed onto the agar plates which had previously been inoculated with the above organisms. The petri dishes were kept at 4°C for 2 hr and then the plates inoculated with bacteria were incubated at 37±0.1°C for 24 hr, plates inoculated with fungi were incubated at 25±0.1°C for 48 hr (Collins *et al*., 1989; Bradshaw, 1992). At the end of the period, the diameter of inhibition zones were measured in millimeters (mm). These experiments were performed in triplicate.

Results and Discussion

As can clearly be seen in Table 1, antimicrobial activity of essential oil of *T. eigii* was the most efficacious in the whole essential oils. Both low (0.5 µl) and high (1 µl) concentration of *T. eigii* essential oil showed antimicrobial effects on all tested bacteria and fungi. However, the inhibition zones formed against the tested bacteria showed differences among other bacteria and fungi at low concentration, while the inhibition zones were higher in *S. aureus, Y. enterolitica, B. megaterium* at high concentration. The inhibition zones were the highest value in *A. hydrophila* in case of bacteria. Among fungi, *K. fragilis* was seen with the highest zone at both low and high concentration. The inhibition zones were lower in *E. coli* and *P. pyocyaneus* than the other bacteria at both high and low concentration.

Antimicrobial activities were highest value with *T. eigii* essential oil followed by *P. nigra* and *C. sempervirens* essential oils. A 1 µl concentration *P. nigra* and *C. sempervirens* essential oils had no antibacterial effects, but they had antifungal effects (except: *C. sempervirens* essential oil didn't form inhibition zone against *S. cerevisia*). Each of these essential oils concentrations of 2 µl and 4 µl was tested for bacteria and fungi in different rates. On the other hand, inhibition zones formed by each of these essential oils in 4 µl concentration were generally lower than inhibition zones formed by *T. eigii* essential oils in 0.5 µl concentration. *P. nigra* essential oils concentrations of 2 µl and 4 µl formed the biggest inhibition zone against *M. luteus* in whole tested bacteria, while inhibition zones against fungi formed equal. *C. sempervirens* essential oil concentration of 2 µl formed more inhibition zones against *M. luteus*

Table - 1: Antibacterial and antifungal activities of essential oils of *Thymus eigii*, *Pinus nigra* and *Cupressus sempervirens* (inhibition zones, mm)

Microorganisms	Inhibition zone (mm)													
	Thymus eigii			*Pinus nigra*				*Cupressus sempervirens*				V30	E15	N10
	0.5 µl/disc	1 µl/disc	MIC µg/ml	1 µl/disc	2 µl/disc	4 µl/disc	MIC µg/ml	1 µl/disc	2 µl/disc	4 µl/disc	MIC µg/ml	mcg/disc		
Escherichia coli	11	27	0.15	-	-	7	2.00	-	-	-	4.00	11	9	nt
Micrococcus luteus	17	36	0.05	-	14	16	0.50	-	10	13	0.25	21	34	nt
Staphylococcus aureus	19	28	0.15	-	7	8	0.75	-	7	8	0.75	20	26	nt
Mycobacterium simegmatis	15	31	0.10	-	8	9	0.25	-	10	11	0.75	22	27	nt
Pseudomonas pyocyaneus	11	16	0.15	-	7	12	0.50	-	9	11	0.75	17	36	nt
Yersinia enterolitica	19	25	0.15	-	8	10	0.75	-	8	9	0.25	17	28	nt
Aeoromonas hydrophila	17	38	0.05	-	7	10	0.50	-	7	10	0.75	12	26	nt
Enterococcus faecalis	15	30	0.15	-	7	10	0.50	-	7	9	0.25	27	28	nt
Bacillus megaterium	19	27	0.25	-	7	11	0.50	-	7	9	2.00	16	25	nt
Streptococcus faecalis	17	25	0.25	-	7	10	0.25	-	7	9	0.75	16	25	nt
Bacillus brevis	16	35	0.05	-	7	9	0.25	-	7	8	0.50	16	24	nt
Saccharomyces cerevisiae	16	24	0.15	10	15	20	0.50	-	9	10	0.25	nt	nt	18
Klyveromyces fragilis	30	50	0.25	6	15	20	1.00	7	15	17	0.75	nt	nt	18

MIC = Minimum inhibitory concentration of the oil, V30 = Vancomycin (30 mcg/disc), E15 = Erytromycin (15 mcg/disc), N10 = Nystatin 100 units (10 mcg/disc), nt = Not tested

and *M. simegmatis* in bacteria and against *K. fragilis* in fungi. Again, 4 µl concentration of *C. sempervirens* essential oil formed the biggest inhibition zone against *M. luteus* in bacteria and against *K. fragilis* in fungi.

Meanwhile, the MICs of the essential oils of *T. eigii*, *P. nigra* and *C. sempervirens* against the tested bacteria and fungi are presented in Table 1. As shown in this table, the essential oil of *T. eigii* has variable levels of inhibition, *M. luteus*, *A. hydrophila*, *B. Brevis* and *M. smegmatis* are the most sensitive microorganisms to the *T. eigii* essential oil because of their low MIC values, ranging from 0.05 to 1.0 µg/ml. Also, the highest MIC values of *T. eigii* essential oil (0.25 µg/ml) are determined against *B. megaterium*, *S. faecalis*, and *K. fragilis*. In addition to, the highest MIC values of both *P. nigra* essential oil (2.0 µg/ml) and *C. sempervirens* essential oil (4.0 µg/ml) are determined against *Escherichia coli*. Furthermore, the lowest MIC values of *P. nigra* essential oil (0.25 µg/ml) are observed against *M. simegmatis*, *S. faecalis*, and *B. brevis.* On the other hand, the lowest MIC values of *C. sempervirens* essential oil (0.25 µg/ml) are found against *M. luteus*, *Y. enterocolitica*, *E. faecalis* and *S. cerevisiae*.

Tepe *et al.* (2004) showed that the antimicrobial activity of undiluted *T. eigii* essential oil, the strongest activity was observed against *B. catarrhalis* and *C. perfringens*, followed by *B. cereus*, *S. aureus, S. pneumoniae* and *M. smegmatis* and the weakest activity was observed against *P. aeruginosa* in broth microdilution method. According to Bonjar (2004), the antimicrobial activity of methanolic extract of *T. kotschyanus*, was found against *Bordetella bronchiseptica*, *Micrococcus luteus* in disc diffusion method (Bonjar, 2004). Similarly, Nariman *et al.* (2004) indicated that *T. kotschyanus* showed anti-*Helicobacter pylori* activity by the disk sensitivity method. Additionally, Essawi and Srour (2000), reported that the most active antibacterial plants against both gram-positive and gram-negative bacteria were *T. vulgaris* and *T. origanium*.

Furthermore, Rasooli and Mirmostafa (2002), showed that *T. pubescens* and *T. serpyllum* essential oils were strongly bactericidal even at higher dilutions with the exception of *P. aeruginosa.* Similarly, *T. revolutus* Celak essential oils also showed high antimicrobial effects and *P. aeruginosa* was resistant at low concentrations of it (Karaman *et al.*, 2001). In contrast, Alzoreky and Nakahara (2003), found that acetone extract of *T. serpyllum* was inactive against *S. aureus* and *L. monocytogenes*.

Several studies were previously done on antifungal activity of *Thymus* species. The lowest minimum inhibitory concentration was 0.03% (v/v) thyme oil against *C. albicans* and *E. coli* (Hammer *et al.*, 1999), *T. vulgaris* essential oil has fungicidal effect against *Macrophomina phaseolina* and *Bipolaris spicifera* (Pourbaig *et al.*, 2004) and methanol extract of *T. vulgaris* showed anticandidal activities against Clotrimazole-resistant *Candida albicans* (Bonjar, 2004).

According to Digrak *et al.* (1999), the antimicrobial effects of several solvent (chloroform, acetone and methanol) based extracts of *P. nigra* were observed in case of several bacteria. The antimicrobial activity of chloroform extract of the cone of *P. nigra*, expressed as a diameter of inhibition zone in millimeters including the disc diameter of 6 mm, was 10 mm *E. coli* and the chloroform and acetone extracts of the cone inhibited the growth of *L. monocytogenes* and *P. aeruginosa*. The strongest antimicrobial activities of essential oils of *C. sempervirens*, were observed against *B. megaterium* followed by *M. luteus, P. fluorescens*, *M. smegmatis, E. aerogenes*, *Y. enterocolitica* (Dagci *et al.*, 2002).

Our study was similar to previous studies, despite the different extraction methods used. The latter depends on a number of parameters of essential oils such as environmental conditions, collection period, dehydration procedure, storage condition isolation method (Magiatis *et al.*, 2002).

Several components of essential oils were reported to possess biological activities. In previous studies, for instance, thymol, carvacrol, a-terpineol, and terpinen-4-ol were found to be active substance (Cosentino *et al.*, 1999; Dorman and Deans, 2000; Juliano *et al.*, 2000; Isman *et al.*, 2001; Friedman *et al.*, 2002; Tepe *et al.*, 2004). In studies, Camphene, α-Pinene, β-Pinene were found to be effective substances (Scortichini and Rossi, 1991) and large amounts of monoterpenes, hydrocarbons, and/or sesquiterpenes were found to be lower effective substances (Chalchat *et al.*, 1997). Plants contained microbial inhibitors (*i.e.*, flavonoids) are soluble in aqueous methanol, and the flavonoid aglycones are more active than their glycosidic forms naturally present in plants (Rauha *et al.*, 2000).

Values of the formed inhibition zones when *T. eigii* essential oil (0.5 µl) applied to two different antibiotic discs (vancomycin and erytromycin) against all tested bacteria *in vitro* can be seen in Table 2.

An antagonistic effect was seen against all tested bacteria while the combination of *T. eigii* essential oil (0.5 µl) and vancomycin and erytromycin antibiotic discs were applied. But, when *T. eigii* essential oil was applied to vancomycin antibiotic discs, the inhibition zone decreased the most in *E. faecalis* and similarly, when it was applied to erytromycin antibiotic discs, it decreased the most in *M. luteus* (antagonistic effect) (Table 2).

An antagonistic effect was seen against generally all tested bacteria while the combination of *T. eigii* essential oil (0.5 µl) and *P. nigra* (1 µl and 2 µl) were applied. A synergistic effect occured in *E. coli*, *P. pyocyaneus* and *E. faecalis* when the combination of *T. eigii* essential oil (0.5 µl) and *P. nigra* (1µl), but it occured in case of *E. coli*, *P. pyocyaneus*, *E. faecalis* and *M. smegmatis* when the combination of *T. eigii* essential oil (0.5 µl) and *P. nigra* (2 µl), additive effect occured in *B. brevis* (Table 3).

Also an antagonistic effect has been seen against generally all tested bacteria while the combination of *T. eigii* essential oil (0.5 µl) and *C. sempervirens* (1 µl and 2 µl) were applied. In combined application of *T. eigii* essential oil (0.5 µl) and *C.*

Table - 2: Antimicrobial activities of *Thymus eigii* essential oil and its antagonistic effect on antibiotics

Microorganisms	Inhibition zones (mm)								
	A	B		C		D		Effect	
	T	V30	E15	V30	E15	V30	E15	V30	E15
Escherichia coli	11	11	9	22	20	10	12	a	a
Micrococcus luteus	17	21	34	38	51	11	13	a	a
Staphylococcus aureus	19	20	26	39	45	28	29	a	a
Mycobacterium smegmatis	15	22	27	37	42	25	30	a	a
Pseudomonas pyocyaneus	11	17	36	28	47	26	35	a	a
Yersinia enterolitica	19	17	28	36	47	28	36	a	a
Aeoromonas hydrophila	17	12	26	29	43	23	27	a	a
Enterococcus faecalis	15	27	28	42	43	26	30	a	a
Bacillus megaterium	19	16	25	35	44	24	31	a	a
Streptococcus faecalis	17	16	25	33	42	23	35	a	a
Bacillus brevis	16	16	24	32	40	23	27	a	a
Saccharomyces cerevisiae	16	nt	nt	nt	nt	nt	nt	nt	nt
Klyveromyces fragilis	30	nt	nt	nt	nt	nt	nt	nt	nt

A = Inhibition zones that occured with plant essential oil (essential oil of *Thymus eigii* : 0.5 µl/disc),
B = Inhibition zones that occured with standard antibiotic disc, vancomycin (V30) and erytromycin (E15),
C = Inhibition zones that expected to occure when essential oil and standard antibiotic were used together, (essential oil of *Thymus eigii* : 0.5 µl/disc),
D = Inhibition zones that occured when essential oil and standard antibiotic were used together, (essential oil of *Thymus eigii* : 0.5 µl/disc),
(T = *Thymus eigii* , V30 = Vancomycin, E15 = Erytromycin, nt = Not tested, s = Synergism effect, e = Additive effect, a = Antagonism effect)

Table - 3: Antimicrobial activities of *Thymus eigii* essential oil and its combined effect with *Pinus nigra* essential oil

Microorganisms	Inhibition zones (mm)								
	A	B		C		D		Effect	
	T	P1	P2	P1	P2	P1	P2	P1	P2
Escherichia coli	11	-	-	11	11	14	15	s	s
Micrococcus luteus	17	-	14	17	31	13	16	a	a
Staphylococcus aureus	19	-	7	19	26	14	20	a	a
Mycobacterium smegmatis	15	-	8	15	23	14	25	a	s
Pseudomonas pyocyaneus	11	-	7	11	18	18	30	s	s
Yersinia enterolitica	19	-	8	19	27	14	23	a	a
Aeoromonas hydrophila	17	-	7	17	24	13	20	a	a
Enterococcus faecalis	15	-	7	15	22	17	27	s	s
Bacillus megaterium	19	-	7	19	26	16	21	a	a
Streptococcus faecalis	17	-	7	17	24	14	17	a	a
Bacillus brevis	16	-	7	16	23	13	23	a	e
Saccharomyces cerevisiae	16	10	15	26	31	20	25	a	a
Klyveromyces fragilis	30	6	15	36	45	16	26	a	a

A = Inhibition zones that occured with plant essential oil, (T = *Thymus* eigii: 0.5 µl/disc),
B = Inhibition zones that occured with plant essential oil, (P1 = *Pinus nigra*: 1 µl/disc, P2 = *Pinus nigra*: 2 µl/disc),
C = Inhibition zones that expected to occure when *Thymus* eigii (0.5 µl) and *Pinus nigra* (1 µl/disc and 2 µl/disc) were used together,
D = Inhibition zones that occured when *Thymus* eigii (0.5 µl/disc) and *Pinus nigra* (1 µl/disc and 2 µl/disc) were used together,
(nt =Not tested, s = Synergism effect, e = Additive effect, a = Antagonism effect)

sempervirens (1 µl) synergistic effect was seen in *E. coli*, *P. pyocyaneus* and *E. faecalis* and additive effect occured in *S. cerevisia*. When the combination of *T. eigii* essential oil (0.5 µl) and *C. sempervirens* (2 µl) was applied, synergistic effect was seen only in *P. pyocyaneus* (Table 4).

Synergistic, synergism and combined effect of two or more agents exceed the sum of their individual effects (Mac Faddin, 2000). Antimicrobial synergism can occur in several types of situations. Synergistic drug combinations must be selected by complex laboratory procedures. Two drugs may sequentially block a microbial metabolic pathway. A drug such as a cell wall inhibitor (a penicillin or cephalosporin) may enhance the entry of an aminoglycoside into bacteria and thus produce synergistic effects. One drug may affect the cell membrane and facilitate the entry of the

Table - 4: Antimicrobial activities of *Thymus eigii* essential oil and its combined effect with *Cupressus sempervirens* essential oil

Microorganisms	Inhibition zones (mm)								
	A	B		C		D		Effect	
	T	C1	C2	C1	C2	C1	C2	C1	C2
Escherichia coli	11	-	-	11	11	12	10	s	a
Micrococcus luteus	17	-	10	17	27	10	11	a	a
Staphylococcus aureus	19	-	7	19	26	13	18	a	a
Mycobacterium smegmatis	15	-	10	15	25	12	18	a	a
Pseudomonas pyocyaneus	11	-	9	11	20	15	28	s	s
Yersinia enterolitica	19	-	8	19	27	13	14	a	a
Aeoromonas hydrophila	17	-	7	17	24	14	9	a	a
Enterococcus faecalis	15	-	7	15	22	16	16	s	a
Bacillus megaterium	19	-	7	19	26	14	17	a	a
Streptococcus faecalis	17	-	7	17	24	13	13	a	a
Bacillus brevis	16	-	7	16	23	14	15	a	a
Saccharomyces cerevisiae	16	-	9	16	25	16	20	e	a
Klyveromyces fragilis	30	7	15	37	45	20	23	a	a

A = Inhibition zones that occured with plant essential oil, (T = *Thymus* eigii: 0.5 µl/disc),
B = Inhibition zones that occured with plant essential oil, (C1 = *Cupressus sempervirens*: 1 µl/disc, C2 = *Cupressus sempervirens*: 2 µl/disc),
C = Inhibition zones that expected to occure when *Thymus* eigii (0.5 µl/disc) and *Cupressus sempervirens* (1 µl/disc and 2 µl/disc) were used together,
D = Inhibition zones that occured when *Thymus* eigii (0.5 µl/disc) and *Cupressus sempervirens* (1µl/disc and 2 µl/disc) were" used together,
(nt =Not tested, s = Synergism effect, e = Additive effect, a = Antagonism effect)

second drug. The combined effect may then be greater than the sum of its parts. One drug may prevent the inactivation of a second drug by microbial enzymes. In such circumstances, a form of synergism takes place (Brooks *et al.*, 1995).

According to Settineri and Krassner's study, two plant-derived proprietary essential oil blends were tested for their antibacterial activity for five common strains of pathogenic bacteria using disk susceptibility test. This screening study in the laboratory indicates that essential oils may be considered to be used in combination with standard topical and antibiotic therapies (Settineri and Krassner, 2003).

According to Abascal and Yarnell (2002), tea (*Camellia sinensis*) has antimicrobial action against many pathogenic bacteria, including methicillin-resistant *S. aureus*; a synergistic effect when combined with beta-lactam antibiotics; reversal of resistance in MRSA and penicillin resistance in beta-lactamase-producing *S. aureus*. Epicatechin gallate from green tea lowered the minimum inhibitory concentration of oxacillin and other beta-lactams in MRSA and, when combined with oxacillin, exhibited bacteriocidal action. One of the liminoids, gedunin, had a synergistic effect when it combined with chloroquine. The focal point of Abascal and Yarnell (2002)'s study is on the combining of herbs with antibiotics to reduce drug resistance. Nascimento *et al.* (2000) determined that association of antibiotics and plant extracts showed synergistic antibacterial activity against antibiotic-resistant bacteria. According to the results of Adeleke and Olaitan's study suggest the potentials of using fixed non-mineral oils, especially King's vegetable oil, in the elimination of staphylococcal resistance to beta-lactamase sensitive antibiotics such as the parent penicillins and some semi-synthetic Beta-lactam antibiotics (Adeleke and Olaitan, 2003). However, the interactions of antibiotics with everyday foods or nutrition supplements can cause side effect-and decrease effectiveness (Anonymous, 1998; Anonymous, 2002).

In the previous study (Toroglu *et al.*, 2005) that, an antagonistic effect is seen against whole tested bacteria while the combination of *Thymbra spicata* L. var. *spicata* essential oil (0.5 µl) and Gentamicin, Cephalothin, Ceftriaxone antibiotic discs were applied. In this study was seen decreased antimicrobial activity when *Thymbra spicata* L. var. *spicata* essential oil was applied to above antibiotic discs *in vitro*. According to Tepe *et al.*, 2004, the activity, seen for the different extracts of *T. eigii,* is closely related to the content of phenolic compounds. It was seen that the amount of phenolic content was high in polar extracts. It seems clear that the presence of polar phenolics is another fact concerning the evaluation of the activity in free radical scavenging. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Komali *et al.*, 1999; Moller *et al.*, 1999). Moreover, radical scavenging activity is one of various mech-anisms to contribute overall activity, thereby creating a synergistic effect. Synergistic effects of thymol and carvacrol and an antagonistic effect of p-cymene are quite possible, and these facts would be considered when the antimicrobial activity of the oil from any particular plant was studied.

The biological activity of the oils can be compared with the activity of synthetically produced pharmacological preparations and should be investigated in the same way. Generally, their action is the result of the combined effect of both their active and inactive

compounds. These inactive compounds might influence resorption, rate of reactions and bioavailability of the active compounds. Several active components might have a synergistic effect (Svoboda and Hampson, 1999).

Antimicrobial properties of the essential oils and various extracts from many plants have recently been of great interest in both academia and food industry, because of their possible use as natural additives emerged from a growing tendency to replace synthetic antimicrobials with natural ones. Owing to strong antibacterial and excellent protective features exhibited in antimicrobial activity tests, the essential oil of *T. eigii* could be considered a natural source that can be freely used in the food industry as a culinary herb.

The focal point of this study on the combining of plants with antibiotics is to reduce drug resistance. The synergistic effects from the association of antibiotic with essential oils and combined essential oils against bacteria lead to new choices for the treatment of infectious diseases. This synergistic effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment. On the other hand, according to our results, *in vitro* testing demonstrated such a combination both to be synergistic and to be antagonistic. This study explained possible serious side effect on traditional uses and clinical applications of these plants for patients who are taking antibiotics. When consumed consciously and systematically, many wild plants are very important for human health because of such constituents. The effect of essential oils of plants on these microorganisms *in vivo* cannot be predicted from this study. Further *in vivo* studies are necessery.

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