Antimicrobial Properties of Some Essential Oils against Some Pathogenic Microorganisms

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Abstract

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Investigations were carried out to assess the efficiency of five plant essential oils: thyme, myrtle, laurel, sage, and orange oils as natural food preservatives. The effect of the plant essential oils against *Escherichia coli, Listeria monocytogenes*, *Staphylococcus aureus* and *Candida albicans* at concentrations of 5–20 µl/disk (diameter 6 mm) and 0.5–3% (v/v) was studied in agar diffusion test medium and milk medium. The essential oils of these extracts exhibited markedly antibacterial and bacteriostatic activity, with thyme showing the highest inhibition and orange the lowest. However, with thyme extract, high inhibitory activity was observed for all tested concentrations, *L. monocytogenes* showed less sensitivity towards essential oil extracts.

Keywords: essential oils; antimicrobial activity; pathogens; milk

In spite of the modern improvements in food hygiene and food production techniques, food safety is an increasingly important public health issue (WHO 2002a, b). It has been estimated that as many as 30% people in the industrialised countries suffer from foodborne diseases each year, and in 2000 at least two million people died of diarrhoeal diseases word wide (Burt 2004). Therefore, are still needed new methods for the reducing or inhibiting foodborne pathogens, possibly in combination with the existing methods (the hurdle principle, packaging under modified atmosphere, heating, refrigeration, the addition of antimicrobial compounds) (Burt 2004; Holley & Patel 2005; Valero & Francés 2006). Especially the industrialised societies appear to experience the trend towards green consumption, desiring fewer synthetic food additives and products with a low impact on the environment (Burt 2004; Sacchetti *et al.* 2005). For this reason there, is scope for new methods of producing safe foods that have a natural or green image. Another problem is the use of animal wastes as organic fertilisers, whether in organic or non-organic agriculture, that gives rise to concerns about the possible contamination of agricultural produce with pathogens and the possible contamination of ground and surface water (Moreira *et al.* 2005). One of such possibilities is the use of essential oils for different reasons (Halander *et al.* 1998; Menon & Garg 2001; Burt 2004; Valero & Francés 2006).

Essential (volatile) oils from aromatic and medicinal plants have been known since antiquity to possess biological activity, notably antibacterial, antifungal, and antioxidant properties (Baratta *et al.* 1998; Cosentino *et al.* 1999; Bounatirou *et al.* 2007).

Biological activity of essential oils depends on their chemical composition, which is determined

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by the plant genotype and is greatly influenced by several factors such as geographical origin and environmental and agronomic conditions (Rota *et al*. 2004; Yesil Celiktas *et al.* 2007).

Plant volatile oils are variable mixtures of essential terpenoids, specially monoterpenes (C_{10}) and sesquiterpenes (C_{15}), although diterpenes (C_{20}) may also be present, and of a variety a low-molecular-weight aliphatic hydrocarbons, acids, alcohol, aldehydes, phenolic compounds, acyclic esters, or lactones (Rota *et al.* 2004).

Many species and herbs exert antimicrobial activity due to their essential oil fractions. Some scientists reported the antimicrobial activity of essential oils from oregano, thyme, sage, rosemary, clove, coriander, garlic, and onion against both bacteria and molds. The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity (Omidbeygi *et al*. 2007; Yesil Celiktas *et al.* 2007).

The compounds containing phenolic groups are usually most effective (Dorman & Deans 2000; Holley & Patel 2005). The components present in essential oils like these have been known to possess antimicrobial activity and some are classified as Generally Recognised as Safe (GRAS) substances and therefore can be used to prevent post-harvest growth of native and contaminant bacteria. The essential oil fractions sensitise the cell membrane, causing an increase in permeability and leakage of vital intracellular constituents, as well as the impairment of bacterial enzyme system and cell respiration (Singh *et al.* 2002; Moreira *et al.* 2005).

The aim of the present work was to evaluate the antimicrobial effect of some essential oils, however, little quantitative data (MIC) are available on the antimicrobial activity against food borne pathogens of essential oils isolated from the plants cultured in different geographical areas of Izmir (Turkey).

MATERIAL AND METHODS

Material

Strain and preparation of inoculum. Three collection strains, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538 and *Candida albicans* ATCC 10239 obtained from American type culture collection and *Listeria monocytogenes (L. monocytogenes*) CECT 4031 obtained from Spanish Standard cultures collection were used in the study. Stock cultures of all strains were maintained on Nutrient Broth (Oxoid CM1, England) at 4°C. Microorganisms inocula were prepared by growing the cells in Nutrient Broth for 24 h at 37°C. These cell suspensions were diluted with Peptone water (Oxoid CM9, England) to provide initial cell counts of about 10^7–10^{10} CFU/ml. Approximately 0.1 ml of culture was transferred to 9.0 ml of Nutrient Broth at 2 consecutive 24 h intervals immediately before each experiment.

Essential oils. The essential oils investigated in this work were purchased from flora Izmir (Turkey) from Turer Tarim which supplies food grade oils. Depending on the amount of the plant material available, essential oil was obtained by hydrodestillation for 3 h using a Clevenger-type apparatus (Bounatirou *et al.* 2007). The oils used were those of thyme, sage, myrtle, laurel, and orange, and the active components carvacrol, thymol, estragol, linalool, and *p*-cymene were all obtained from Sigma-Aldrich (Steinheim, Germany).

Methods

Analysis of essential oils and volatile compounds by GC. GC analyses were carried out on a Shimadzu GC-9A gas chromatograph equipped with Thermon-600 T (30 m × 0.25 mm × 0.25 µm film thickness). The oven temperature was programmed at 15–200°C/min for the total of 15 minutes. Other operating conditions were: carrier gas, nitrogen with a flow rate of 10.0 ml/min; injector and detector temperatures were 250°C and 300°C, respectively; split ratio 1:20; column pressure 56.8 hPa (Bagamboula *et al*. 2004).

Determination of antibacterial activity of thyme, myrtle, laurel, sage, and orange essential oils

The sensivity of *E. coli, S. aureus, L. monocytogenes*, and *C. albicans* were determined by the agar diffusion method. Sterile paper discs (Oxoid antibacterial susceptibility blank test disc – England) were soaked with pure essential oils and placed on the surface of the inoculated Baird Parker Agar (Oxoid CM 275), VRB agar (Oxoid CM 107) , Palcam Listeria Selective agar (Base) (Merck 1.11755), PDA (Oxoid CM 139) agar plates. The sensivity to the individual oils was classified

by the diameter of the inhibition zones as follows (Ponce *et al.* 2003; Moreira *et al.* 2005):

- Not sensitive (–) for total diameter smaller than 8 mm
- Sensitive (+) for total diameter 9–14 mm
- Very sensitive (++) for total diameter 15–19 mm
- Extremely sensitive (+++) for total diameter larger than 20 mm

Each assay was performed in duplicates on two separate experimental runs.

Effect of essential oils on some pathogens in milk medium. The survival and growth of *E. coli, L. monocytogenes, S. aureus,* and *C. albicans* was monitored in sterilised milk (sterilised at 115°C for 15 min) medium at 37°C and 25°C (for fungi) for 24 h and 72 h (for fungi). The sterilised milk was supplemented with thyme, myrtle, laurel, sage, and orange essential oils at the concentrations of 0.5; 1; 2; 3% (v/v).

RESULTS AND DISCUSSION

Composition of plant essential oils

The main volatile components of crude essential oils recovered by steam distillation of thyme,

Table 1. Major components (in %) of thyme, sage, myrtle, laurel and orange essential oils separated by gas chromatography

Compound	Thyme	Sage	Myrtle	Laurel	Orange
1,8-Cineole	–	38.6	24.8	56.7	–
α-Pinene	1.3	7.0	23.5	4.9	–
Limonene	–	1.4	14.2	–	84.0
Linalool	15.4	–	7.5	–	1.1
Mirtenyl acetate	–	–	6.7	–	–
α-Terpineol	1.7	3.3	4.5	7.0	–
Nerol	–	–	2.9	–	–
Linoleic acetate	–	–	2.8	–	–
Geranil acetate	–	–	1.9	–	–
Isobutanoate	–	–	1.4	–	–
Carvacrol	58.1	–	1.1	–	–
Geraniol	–	–	1.1	–	–
Sabinene	–	–	–	6.8	4.2
β-Pinene	–	5.0	–	3.6	–
Terpinen-4-ol	–	–	–	2.5	–
p-Cymene	3.9	–	–	1.6	1.3
Trans pinocarveol	–	–	–	1.4	–
1-Terpinene	5.2	–	–	1.0	–
Thymol	2.1	–	–	–	–
β-Caryophyllene	1.6	5.6	–	–	–
Myrcene	1.3	1.8	–	–	1.3
α-Terpinene	1.0	–	–	–	–
Caphure	–	9.6	–	–	–
α-Thujone	–	3.7	–	–	–
Camphene	–	3.3	–	–	–
α-Humulene	–	2.5	–	–	–
β-Thujone	–	2.3	–	–	–

For essential oil, compounds ≥ 1% are presented

myrtle, laurel, sage and orange are given in Table 1 showing typical monoterpene hydrocarbon patterns of the individual oils. Monoterpene hydrocarbons such as thymol, eugenol, or carvacrol may inactivate thee essential enzymes, react with the cell membrane activity, or disturb the genetic material functionality, energy production, and structural components synthesis (Davidson 2001).

As shown by their proportions given in Table 1, the most abundant components in thyme essential oil are carvacrol 58.1%, linalool 15.4% and γ-terpinene 5.2%, while the minor components are *p*-cymene, thymol, caryophyllene, α-terpineol, mycene, and α-terpinene. The most abundant components in sage, myrtle, and laurel essential oils are 1–8 cineole (38.6; 24.8; 56.7%), α-pinene (7.0; 23.5; 4.9%), limonene (0; 0; 14.2%). Smaller amounts were found of α-terpineol, β-pinene, β-caryophyllene, myrcene, caphure, α-thujone, camphene, α-humulene, and β-thujone in sage essential oil; linalool, mirtenyl acetate, α-terpineol, nerol, linoleic acetate, geranil acetate, isobutanoate, carvacrol, and geraniol in that of myrtle; α-terpineol, sabinene, β-pinene, terpinen-4-ol, *p*-cymene, trans pinocarveol, and γ-terpinene in that of laurel. The principle component detected in orange essential oil is limonene 84%. This oil also contains sabinene 4.2% and smaller levels of *p*-cymene and mycene.

It was reported that the bactericidal properties and antibiotic activity of thyme essential oil are supposed to be associated with high levels of carvacrol and linalool (Sivropoulou *et al.* 1996; Rota *et al.* 2004). Moreover the genus *Thymus* comprises numerous species and varieties whose essential oil compositions have been studied. This species also contains linalool, β-pinene, α-terpineol, camphor, borneol, and thymol (Guillén & Manzanos 1998; Mangena & Muyima 1999; Bounatirou *et al.* 2007). Farag *et al.* (1989) reported that thyme essential oil contains 43% thymol and 36% *p*-cymene. Antibacterial, antifungal, and antioxidant properties and chemical compositions of essential oils obtained from cinnamon, y lang-y lang , basil, lemon, lemon grass, frankincense, marjoram, and rosemary were reported by Baratta *et al.* (1998). Thus, it can be said in the present study that the percentages of these compounds varied slightly across different collection periods, numerous species, varieties, and geographical regions (Sacchetti *et al.* 2005; Bounatirou *et al.* 2007).

Antimicrobial effects of essential oils on some pathogens

Table 2 summarises the antimicrobial properties of the five essential oils (thyme, sage, myrtle, laurel, and orange). Bacteria susceptibility to the essential oils, as determined by the agar diffusion method, showed that oils with the highest inhibitory effects produced inhibition zones of 20–25 mm diameter. In the dose response study, the inhibition zone increased with the increasing concentration of essential oil. Low concentrations (10 µl) of thyme, sage, myrtle, laurel, and orange essential oils inhibited weakly the development of bacteria. However, *E. coli*, and *S. aureus* were more sensitive than *L. monocytogenes* in the medium containing thymol essential oil. At a high concentration (20 µl/ml), the essential oil extracts

Table 2. Antimicrobial activity of thyme, sage, myrtle, laurel, and orange essential oils (in µl) against some pathogens (inhibition zones in mm)

Microorganisms*	Thyme			Sage			Myrtle			Orange			Laurel		
	5	10	20	5	10	20	5	10	20	5	10	20	30	60	100
E. coli (3.5×10^8 CFU/ml)	7	14	28	–	7	19	–	9	15	–	5	14	13	nt	nt
L. monocytogenes (1.5×10^8 CFU/ml)	4	12	23	–	6	18	–	10	16	–	4	17	12	16	nt
S. aureus (4.2×10^7 CFU/ml)	8	14	30	–	8	20	–	6	13	–	6	18	19	nt	nt
C. albicans (8.2×10^7 CFU/ml)	7	15	35	3	13	29	–	10	18	–	7	24	–	7	12

*initial colony count (CFU/ml); nt – not tested (antimicrobial effect observed by smaller essential oil levels)

exhibited a marked inhibition activity against bacteria, and the inhibition of the essential oil extract of thyme was stronger than that of the others, showing inhibition zones ranging from 23–30 mm. Comparatively, *E. coli* and *S. aureus* were less sensitive to the inhibitory activity of the orange and myrtle essential oils than *L. monocytogenes* which was more inhibited at the same concentrations of the same essential oils extracts. On the other hand, all bacteria showed low susceptibility to laurel essential oil, with 12–19 mm diameter inhibition zones compared to the same levels of thyme, sage, myrtle, and orangen essential oils.

Previous research into the essential oils has remarkably increased, mainly with regard to the antimicrobials used to control food pathogens and native microflora (Moreira *et al*. 2005; Cox *et al.* 2000; Menon & Garg 2001; Ponce *et al.* 2003; Sacchetti *et al.* 2005), and to the knowledge of the possible mechanism of the action of plant essential oils (Singh *et al.* 2002). The results revealed the potential of some oils, such as those of clove, thyme, sage, laurel, sweet, onions, garlic, etc., as natural preservatives in food technology. But the results found in this study are not in agreement with those reported by Elgayyar *et al.* (2001), Sacchetti *et al.* (2005), and Leuschner and Ielsch (2003).

The antilisteric activity of clove oil was examined in meat and cheese at both 30°C and 7°C by Menon and Garg (2001). At the concentrations of 0.5% and 1%, clove oil restricted the growth of *L. monocytogenes* in food items at both temperatures. The inhibitory activity of clove oil was more pronounced at the concentration of 1%. The results obtained revealed the potential of clove oil as a natural preservative for meat and cheese.

The differences we have found between our and other authors' results can be attributed to the fact that essential oils are a heterogeneous group of complete mixtures of organic substances, the quality and quantity of which vary with the growth stages, ecological conditions, and other factors based on the way in which the essential oil is extracted (Kim *et al.* 1995; Özcan & Erkmen 2001; Moreira *et al.* 2005).

The results of the antimicrobial disc-diffusion assay are summarised in Table 2; most of essential oils (thyme, sage, myrtle, and orange) showed a moderate inhibitory activity against the yeast *C. albicans* tested. In particular, the oils of thyme and sage showed very good effectiveness and the widest activity spectrum. On the other hand, *C. albicans* showed a strong resistance against laurel oil. Although the antifungal activity of essential oils has been reported several times, its activity against food-spoilage and pathogen yeast was scarcely investigated. At the same time, the different performances offered by essential oils can be also related to essentially different chemical compositions and other factors such as biological properties, geographical regions, etc. As previously reported, yeasts and fungi are markedly inhibited by oils rich in phenolics, aldehydes, and alcohols (Sacchetti *et al.* 2005; Bruni *et al.* 2003).

Antimicrobial effects of some essential oils on *E. coli*, *S. aureus*, *L. monocytogenes*, and *C. albicans* in milk medium

The growth of *E. coli, L. monocytogenes, S. aureus* and *C. albicans* was monitored at 37°C for 24 h in sterilised milk medium; initial inoculums of 2×10^8, 1×10^8, 3.5×10^7, and 8×10^7 CFU/ml, respectively, were used. SM systems were supplemented with thyme, sage, myrtle, laurel, and orange essential oils at different concentrations, i.e. 0.5, 1, 2, 3% (v/v), and the growth was monitored in comparison with the control that contained no essential oils. In the dose response study, the inhibition zone increased with increasing concentrations of essential oils. That of myrtle displayed bacteriostatic properties and that of thyme was bacteriocidal. Thyme essential oil effectively limited the cell numbers of all pathogenic microorganisms at the concentration of 0.5 v/v in the sterilised milk systems.

The colony forming units of *E. coli, S. aureus, L. monocytogenes*, and *C. albicans* in sterilised milk supplemented with 0.5; 1; 2; 3% thyme, sage, myrtle, laurel, and orange essential oils, respectively, at the incubation temperature of 37°C are presented in Table 3 in comparison with the control batch.

Thyme essential oil in sterilised milk supplemented to 0.5% displayed a bactericidal effect that resulted in only slightly lower counts compared to myrtle essential oil. Sage and myrtle essential oils had a bactericidal effect that was less pronounced at higher concentrations. Within the first 24 h, the viable cell counts of *L. monocytogenes*, *S. aureus*, and *C. albicans*, not *E. coli*, were significantly reduced from the initial counts in the presence of 1% sage essential oil, whereas those of laurel and orange displayed a bacteriostatic effect that resulted in only 50% of counts and less than the control.

Table 3. Effects of thyme, sage, myrtle, laurel, and orange essential oils (in ml) at concentrations of 0.5; 1; 2; 3% (v/v) in sterilised milk at 37°C on the cell concentration of 10^7–10^8 CFU/ml

Microorganisms*	Thyme				Sage				Myrtle				Orange				Laurel			
	0.5	1	2	3	0.5	1	2	3	0.5	1	2	3	0.5	1	2	3	0.5	1	2	3
E. coli (2×10^8 CFU/ml)	+	nt	nt	nt	–	–	+	nt	–	–	±	+	–	–	–	–	–	–	±	±*
L. monocytogenes (1×10^8 CFU/ml)	+	nt	nt	nt	–	+	nt	nt	–	±	+	nt	–	±	+	nt	–	–	±	+
S. aureus (3.5×10^7 CFU/ml)	+	nt	nt	nt	±	+	nt	nt	±	±	+	nt	–	–	+	nt	–	±	+	nt
C. albicans (8×10^7 CFU/ml)	+	nt	nt	nt	–	+	nt	nt	±	±	+	nt	–	–	+	nt	–	–	–	–

*initial colony count (CFU/ml); nt – not tested (antimicrobial effect observed by smaller essential oil levels); + antimicrobial effect observed; – no antimicrobial effect observed; ± comparatively less inhibitory activity

The antibacterial activities of essential oils of garlic, clove, thyme, basil, orange, vervan, and savage carrots in culture media were reported by several researchers (Chaiba *et al.* 1997; Lis-Balchin & Deans 1997; Bagamboula *et al.* 2004; Benkeblia 2004; Holley & Patel 2005; Moreira *et al.* 2005). Plant oils revealed only a limited action in food substrates. Menon and Garg (2001) stated that microorganisms were neither eliminated nor completely inhibited, while clove oil was able to restrict the proliferation of *L. monocytogenes* in both meat and cheese. The effect was more pronounced with 1% concentration of clove oil as compared to low concentrations and temperatures. On the other hand, Leuschner and Ielsch (2003) did not observe any antimicrobial effects on *L. monocytogenes* when the species was added to cheese as compared with the control batches.

Hao *et al.* (1998) reported only a slight inhibitory effect of clove extract on *L. monocytogenes* in refrigerated beef. I was also described in literature that foods with high protein and fat and low water contents required increased concentrations of essential oils to control the growth of microorganisms (Shelef *et al.* 1984; Smith-Palmer *et al.* 1998; Leuschner & Ielsch 2003). Aureli *et al.* (1992) observed the effects of thyme oil on the control of *L. monocytogenes* in minced pork meat. Cutter (2000) also explained a similar reduction of the viable counts of *L. monocytogenes* on the surface of beef when using a commercial herb extract (Leuschner & Ielsch 2003). These results pointed out the potential application of plant species and essential oils of them as antimicrobials, and also the need to use extended concentrations of them in food systems such as milk or cheeses with high fat contents.

Essential oils containing active antimicrobial compounds derived from many plants are known to possess antifungal and antibacterial activities (Ponce *et al.* 2003). In sterilised milk medium without essential oils, *C. albicans* grew to approximately 8×10^7 CFU 7 ml within 5 days. With all essential oils tested, the antifungal activity was found to be concentration dependent. The addition of 2% (v/v) sage, myrtle, and orange essential oils produced in immediate reduction in the viability of *C. albicans* in sterilised milk. The addition of 1% thyme and 3% of sage, myrtle, and orange essential oils to the medium also completely inhibited the growth of *C. albicans*. The different performances of essential oils in this research can be linked to their chemical compositions such as the contents of phenolics, aldehydes, and alcohols (Bruni *et al.* 2003; Sacchetti *et al.* 2005). On the other hand, the use of plant essential oils at the different concentrations required to be effective in milk and milk products such as cheese, whey cheese, and yoghurt could raise concerns regarding the changes in the sensory properties of the product, as in our study. A number of options can be considered to overcome this problem. One of them is to view the essential oil not only as a preservative but also as a flavour component, especially as many herb and spice flavoured products already exist and, alternatively, they could be also incorporated into the products to mask the presence of the plant essential oil. An other alternative to try is the use of some of the most active components

rather than the whole oil. This may, hopefully, reduce changes of the sensory properties whilst retaining the antimicrobial activity.

CONCLUSION

Thyme, sage, myrtle, laurel, and orange essential oils have a potential to inhibit and inactivate four microorganisms in agar and milk medium at different concentrations. The inhibitory effects of essential oils increased with increasing concentration.

It is suggested to investigate higher essential oils concentrations than were those used in research, and to study the effects over a longer time period in milk and other available milk products to access the potential of plant species essential oils as preservatives.

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