

Growth Inhibition of Pathogenic Bacteria and Some Yeasts by Selected Essential Oils and Survival of *L. monocytogenes* and *C. albicans* in Apple–Carrot Juice

Reyhan Irkin¹ and Mihriban Korukluoglu²

Abstract

Food safety is a fundamental concern of both consumers and the food industry. The increasing incidence of foodborne diseases increases the demand of using antimicrobials in foods. Spices and plants are rich in essential oils and show inhibition activity against microorganisms, which are composed of many compounds. In this research, effects of garlic, bay, black pepper, origanum, orange, thyme, tea tree, mint, clove, and cumin essential oils on *Listeria monocytogenes* AUFE 39237, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Proteus mirabilis* AUFE 43566, *Bacillus cereus* AUFE 81154, *Saccharomyces uvarum* UUFE 16732, *Kloeckera apiculata* UUFE 10628, *Candida albicans* ATCC 10231, *Candida oleophila* UUPP 94365, and *Metschnikowia fructicola* UUPP 23067 and effects of thyme oil at a concentration of 0.5% on *L. monocytogenes* and *C. albicans* in apple–carrot juice during +4°C storage (first to fifth day) were investigated. Strong antibacterial and antifungal activities of some essential oils were found. Thyme, origanum, clove, and orange essential oils were the most inhibitory against bacteria and yeasts. Cumin, tea tree, and mint oils inhibited the yeasts actively. It is concluded that some essential oils could be used as potential bio-preservatives capable of controlling foodborne pathogens and food spoilage yeasts.

Introduction

AROMATIC PLANTS HAD BEEN USED since ancient times for their preservative and medicinal properties, and to impart aroma and flavor to food. Essential (volatile) oils from aromatic and medicinal plants have been known since antiquity to possess biological activity, notably antibacterial, antifungal, and antioxidant properties. Essential oils are natural, complex, multicomponent systems composed mainly of terpenes in addition to some other nonterpene components (Edris, 2007; Fu *et al.*, 2007).

Bacterial and fungal infections pose a greater threat to health, most notably in immunocom-

promised subjects; hence, cheap and effective antimicrobial agents are needed. The essential oils can be used as growth inhibitors of foodborne and food spoilage microorganisms (Fisher and Phillips, 2008; Sharef *et al.*, 2008). Researchers are interested in biologically active compounds isolated from plant species for inhibiting pathogenic microorganisms because they have built up resistance to antibiotics. The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds such as carvacrol, thymol, citral, eugenol, 1–8 cineole, limonene, pinene, linalool, and their precursors. Differences in the antimicrobial activity should be attributed to their

¹Susurluk College, Balikesir University, Balikesir, Turkey.

²Department of Food Engineering, Uludag University, Bursa, Turkey.

chemical composition and relative proportions of the individual constituents in the essential oils (Viuda-Martos *et al.*, 2008). In general, Gram-negative bacteria have been found to be more resistant to essential oils than Gram-positive bacteria because of their lipopolysaccharide cell wall (Mangena and Muyima, 1999). The essential oils are hydrophobic, and their primary site of activity is the membrane. They lead to disruption of the membrane structure and function by accumulation in the lipid bilayer (Liolios *et al.*, 2009).

Most of the outbreaks are caused by pathogenic microorganisms after consumption of fresh products, and *Listeria monocytogenes* is an important microorganism that caused the high number of outbreaks in the last years, and it can be found in unpasteurized fruit juices (Raybaudi-Massilia *et al.*, 2009). *Candida albicans* is a major spoilage microorganism for fresh cut apples and soft drinks (Rupasinghe *et al.*, 2006; Stratford *et al.*, 2007).

The aims of this study were to evaluate the antibacterial and antifungal properties of commercial essential oils obtained from 10 plants—garlic (*Allium sativum* L.), bay (*Pimenta racemosa*), black pepper (*Piper nigrum*), oregano (*Origanum vulgare*), orange (*Citrus sinensis*), thyme (*Thymus vulgaris*), tea tree (*Melaleuca alternifolia*), mint (*Mentha longifolia*), clove (*Syzygium aromaticum*), and cumin (*Cuminum cyminum*)—and to determine 0.5% (v/v) thyme oil inhibition activity against inoculated *L. monocytogenes* and *C. albicans* in apple–carrot juice during storage period at +4°C. Penicillin G (10 IU) was used as a standard antimicrobial agent.

Materials and Methods

Essential oils

Essential oil of tea tree (*M. alternifolia*) was kindly supplied by Dr. Valerie Edwards-Jones, Research Development and Innovations Unit of Manchester Metropolitan University, Manchester, UK. Other essential oils were purchased from Sefer Yasemin Spice Food Botanic Company (Manisa, Turkey). Their quality parameters (appearance, color, purity, odor, density at –20°C, and refraction index at –20°C) were described in an accompanying technical report.

Bacteria

Stock cultures of *L. monocytogenes* AUF 39237, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Proteus mirabilis* AUF 43566, and *Bacillus cereus* AUF 81154 were obtained from Ankara University's Department of Food Engineering. Stock cultures were maintained on nutrient agar (NA) slants at 4°C. All bacteria were incubated at 35°C for 24 h by inoculation into nutrient broth. After incubation, bacteria cultures (~10⁶ cfu/mL) were inoculated (1.5 mL bacteria culture/150 mL medium) into sterilized and cooled (45–50°C) NA and distributed in Petri dishes (Moreira *et al.*, 2007).

Yeasts

Saccharomyces uvarum UUF 16732 and *Kloeckera apiculata* UUF 10628 were isolated from foods in Food Engineering Department and *Metschnikowia fructicola* UUP 23067 and *Candida oleophila* UUP 94365 were obtained from Plant Production Department of Uludag University in Bursa, Turkey, and identified according to the standard fungi determination procedures described by Samson *et al.* (1995) and Korukluoglu *et al.* (2005). *C. albicans* ATCC 10231, a medically important yeast, was obtained from Uludag University Medicine Faculty in Bursa, Turkey. Stock cultures were maintained on malt extract agar (MA) slants at 4°C. Yeasts were grown in 50 mL sterile malt extract broth (MB) for 18 h on a shaker incubator (Bottmingen model; Infors AGCH-4103, Switzerland) at 50 rpm/30°C, producing yeast suspensions of approximately 10⁶ cfu/mL. All yeasts in MB were serially diluted and enumerated on MA agar at 22–25°C for 4–5 days (Tournas *et al.*, 1998).

Disc diffusion method

Disc diffusion procedure using filter paper discs was used for the screening of antimicrobial activity of essential oils (Souza *et al.*, 2005). For this, 0.2 mL of the yeast suspension was uniformly spread on the sterile MA Petri dishes. Sterile filter paper discs (6 mm diameter; Schleicher & Schüll 2668, Germany) were soaked with 50 µL of each essential oil and placed at the center of MA Petri dishes inoculated with yeast suspension. The incubation time was

48 h at 30°C. For bacteria sterile filter discs on inoculated NA (1.5 mL bacteria culture/150 mL medium), Petri dishes were soaked with 50 µL essential oil, and they were incubated at 35°C for 48 h. Sterile filter discs on inoculated NA Petri dishes were soaked with 50 µL penicillin G (10 IU) (Fluka Biochemika 13752, Austria) tested against for bacteria. At the end of the incubation period, the inhibition halo diameters were measured using calipers and expressed in millimeters. Controls included in the assay were essential oil replaced by sterile water.

Apple–carrot juice preparation

Apples and carrots at commercial ripeness were purchased in a supermarket of Susurluk, Balıkesir, for preparing fruit juice. Each fruit was washed, peeled, and cut into pieces and blended using a blender (Braun MP80, Germany). Juice was filtered using a filter paper and mixed (50% [v/v] apple juice + 50% [v/v] carrot juice), and 100 mL of the mixed (apple–carrot) juice was bottled in glass containers, and autoclaved (Hirayama Hiclave HV-50, Japan) at 121°C for 15 min (Raybaudi-Massilia *et al.*, 2009). The pH and brix of apple–carrot juice were 4.8 and 10, as determined using pH meter (HI221 Microprocessor; Hanna Instruments, US) and a hand refractometer (Kernco, 400L, US), respectively.

L. monocytogenes and *C. albicans* cultures and inoculations

L. monocytogenes AUF 39237 was grown in brain heart infusion broth (Oxoid, CM 225 B, UK) at 35°C for 24 h, and *C. albicans* ATCC 10231 was grown in sterile MB for 18 h on a shaker incubator (Bottmingen model; Infors AGCH-4103) at 50 rpm/30°C. Concentrations were adjusted to 10⁷ cfu/mL using saline peptone water (0.1% [w/v] peptone + 0.85% [w/v] NaCl). An aliquot of 1 mL of *L. monocytogenes* AUF 39237 and *C. albicans* ATCC 10231 at approximately 10⁷ cfu/mL was inoculated to fruit juice samples containing 0.5% (v/v) thyme oil. A control of fruit juice without thyme oil was inoculated with microorganism cultures. All experiments were performed in triplicate for each replicate ($n = 3 \times 2$).

Statistical analysis

The data about the numbers of *L. monocytogenes* and *C. albicans* counts in apple–carrot juice were statistically analyzed by analysis of univariate variance (General Linear Model) using SPSS 10.0. Tukey test was used at a significance level of 0.05 (Ozdamar, 2004).

Results and Discussion

The data presented in Table 1 show the inhibition zones of plant essential oils against the some bacteria and yeasts.

Clove oil

In our study, *L. monocytogenes* AUF 39237 was the most resistant microorganism to the clove oil, but *K. apiculata* UUF 10628 and *C. albicans* ATCC 10231 were the most sensitive microorganisms to this oil. In Fu *et al.*'s (2007) study, clove oil was found to be inhibitory against *C. albicans* with 32 mm and *E. coli* with 16.3 mm. In another similar study, clove oil showed the highest antibacterial activity against five strains of *Staphylococcus epidermidis*, and inhibition activity was explained with eugenol, which is the major active component in the clove oil (Chaieb *et al.*, 2007).

Bay oil

Smith-Palmer *et al.* (1998) found zones of inhibition for bay, 10.1 and 11.1 mm; clove, 9.7 and 11.1 mm; garlic and orange, 4 and 4 mm; peppermint, 6.8 and 6.3 mm; and thyme, 8.3 and 11.1 mm against *E. coli* and *S. enteritidis*, respectively. In our study, bay oil did not show great effect on tested bacteria, but it was the most effective on *C. albicans* ATCC10231 and *K. apiculata* UUF 10628 among the microorganisms.

Thyme and origanum oils

Origanum oil was one of the most effective oil, and only *C. oleophila* UUP 94365 was not sensitive to this oil. Thyme oil had inhibitory effects on all the microorganisms and especially to the tested yeasts. Origanum, orange, and thyme oils showed higher inhibitory effect than penicillin G against *E. coli* ATCC 25922 and *L. monocytogenes* AUF 39237. Origanum and

TABLE 1. AVERAGE INHIBITION ZONES (MM ± SD) OF ESSENTIAL OILS AGAINST THE TESTED MICROORGANISMS

Essential oil	Inhibition zone (mm)									
	Bacteria					Yeast				
	1	2	3	4	5	1	2	3	4	5
Garlic	0 ^a	10 ± 0.3	13 ± 1.1	24 ± 1.2	25 ± 0.4	0	0	0	0	0
Bay	15 ± 0.6	11 ± 0.2	12 ± 2.6	10 ± 1.0	14 ± 0.8	12 ± 0.5	18 ± 1.2	17 ± 1.2	0	18 ± 1.1
Black pepper	0	10 ± 1.3	14 ± 0.5	24 ± 3.3	12 ± 0.4	12 ± 1.2	23 ± 0.4	0	0	0
Origanum	55 ± 0.8	45 ± 1.0	42 ± 0.1	48 ± 1.5	24 ± 1.2	≥	≥	0	≥	≥
Orange	20 ± 1.2	35 ± 1.5	19 ± 0.2	17 ± 1.8	26 ± 3.6	64 ± 3.6	53 ± 4.1	50 ± 3.4	63 ± 3.8	58 ± 1.2
Thyme	62 ± 0.6	50 ± 0.6	40 ± 1.6	45 ± 1.2	38 ± 2.1	≥	≥	≥	≥	≥
Tea tree	0	16 ± 2.4	19 ± 1.0	14 ± 3.6	10 ± 1.0	60 ± 2.8	27 ± 2.2	30 ± 1.2	12 ± 1.2	32 ± 1.1
Mint	18 ± 1.3	17 ± 3.2	18 ± 1.2	13 ± 2.3	12 ± 0.6	28 ± 2.2	35 ± 3.6	20 ± 1.4	25 ± 1.9	17 ± 1.8
Clove	17 ± 2.2	25 ± 1.1	33 ± 1.5	35 ± 1.7	32 ± 1.8	63 ± 5.4	≥	36 ± 2.3	58 ± 1.0	≥
Cumin	0	13 ± 0.4	10 ± 1.0	10 ± 0.8	10 ± 0.3	22 ± 3.5	40 ± 2.8	47 ± 3.6	34 ± 0.5	28 ± 2.3
PEN G	38 ± 0.4	33 ± 2.3	47 ± 0.7	39 ± 1.3	40 ± 2.8	ND	ND	ND	ND	ND

Bacteria: 1, *Listeria monocytogenes* AUF 39237; 2, *Escherichia coli* ATCC 25922; 3, *Salmonella enteritidis* ATCC 13076; 4, *Proteus mirabilis* AUF 43566; 5, *Bacillus cereus* AUF 81154.

Yeast: 1, *Saccharomyces uvarum* UUF 16732; 2, *Kloeckera apiculata* UUF 10628; 3, *Candida oleophila* UUP 94365; 4, *Metschnikowia fructicola* UUP 23067; 5, *Candida albicans* ATCC 10231.

^aGrowth was observed, and there was no any halo diameters around the 6 mm filter paper discs.

The symbol "≥" indicates values equal to or greater than the diameter of Petri dishes. ND, not determined; Not, zones of inhibition were 0 for all controls.

thyme essential oils were also more inhibitory to *P. mirabilis* AUF 43566 than penicillin G. Burt and Reinders (2003) found 18.7, 15.7, 24.3, and 25.7 mm zones of inhibition for bay, clove, oregano, and thyme essential oils against *E. coli* O157:H7, respectively. It was indicated that essential oils with high concentrations of thymol and carvacrol, for example, oregano, savory, and thyme, usually inhibit Gram-positive more than Gram-negative pathogenic bacteria (Chaieb *et al.*, 2007; Edris, 2007). Liolios *et al.* (2009) determined 17, 9.1, and 10.2 mm zones of inhibition for *Origanum dictamnus* essential oil against *S. epidermidis*, *E. coli*, and *C. albicans*, respectively. *O. vulgare* oil contains mostly active phenolic compounds that are responsible of its antimicrobial activity. In our study, inhibitory effects were determined for origanum oil against the tested yeasts and bacteria. Thyme and oregano essential oils could inhibit some pathogenic bacterial strains such as *E. coli*, *S. enteritidis*, *Salmonella choleraesuis*, and *Salmonella typhimurium* in some studies (Krist *et al.*, 2007). Gutierrez *et al.* (2008) found that oregano and thyme were the most inhibitory essential oils against *B. cereus*, *E. coli* ATCC 25922, *L. monocytogenes* IL323, and *P. aeruginosa* ATCC 27853 in their study. Carvacrol is the major component in the essential oil fraction of oregano (60–74%) and thyme (45%); it inhibits the growth of many

microorganisms. Ultee *et al.* (1998, 1999, 2002) determined its strong inhibitory effects against *B. cereus* even at low concentrations. Another study has shown that carvacrol can be used to inhibit *Saccharomyces cerevisiae* and *Salmonella enterica* sv. Typhimurium adhered to stainless steel (Roller and Seedhar, 2002). Busatta *et al.* (2008) found 0.069 mg/mL as a minimum inhibitory concentration against *Bacillus subtilis* with *Origanum majorana* essential oil.

Cumin oil

Among the yeasts, *C. oleophila* UUP 94365 and *K. apiculata* UUF 10628 were the most sensitive to cumin oil. But cumin oil has no strong inhibitory activities for tested bacteria, especially to *L. monocytogenes* AUF 39237. Cuminal, cuminic alcohol, terpinene, safranal, *p*-cymene, and pinene were determined as the major antimicrobial components in cumin oil by Li and Jiang (2004). Viuda-Martos *et al.* (2008) found high inhibitory effects of cumin oil against *Staphylococcus xylosus* and *Staphylococcus carnosus*.

Black pepper oil

P. mirabilis AUF 43566 and *K. apiculata* UUF 10628 were sensitive to black pepper oil, and tested bacteria were found resistant to black

pepper oil. In their study, Singh *et al.* (2005) determined that black pepper oil had no effect on *S. typhi* and *E. coli*.

Mint oil

Menthol, menthone, and menthyl acetate were found to be the main components of mint oil, and mint oil gave 16 mm, the greatest inhibition zones against *B. cereus* in Sutour *et al.* (2008) research. Mint oil strongly inhibited *K. apiculata* UUFE 10628 in our study. Tassou *et al.* (1995) determined that inhibitory effect of mint oil against *S. enteritidis* is depended mainly on concentrations of oil and pH of food.

Garlic oil

In this study, garlic oil had no effect on tested yeasts, but *B. cereus* AUFE 81154 was the most sensitive to the garlic oil with 25 mm of inhibition zone. Ahsan *et al.* (1996) found that garlic extract and allicin, which is the main compound of garlic, have inhibitory effects against drug-resistant strain enterotoxigenic *E. coli*. Al-lisin and ajoene were the major components of garlic oil that are very effective to inhibit the growth of microorganisms (Benkeblia, 2004).

Tea tree oil

In our research, tea tree oil was not found to be effective on bacteria generally, but *S. uvarum* UUFE 16732 and *C. albicans* ATCC 10231 were inhibited effectively with tea tree oil. Moreira *et al.* (2005) found 27, 61, 12, and 17 mm zones of inhibition against *E. coli* ATCC 25158 for tea tree,

clove, organum, and mint oils, respectively. It was expressed that the Australian tea tree oil from *M. alternifolia* and other *Melaleuca* species has strong antimicrobial potential. The problem with many *Myrtaceae* is that of their genetic variation or the production of spontaneous chemotypes, giving many different essential oil compositions with differing bioactivities (Friedman, 2007).

Orange oil

Fisher and Phillips (2008) expressed that citrus oils are the largest sector of essential oils produced in the world. In our study, orange oil was more inhibitory to the yeasts than the tested bacteria. *S. uvarum* UUFE 16732 was very sensitive to orange oil with 64 mm of inhibition zone. In this study, orange oil gave 19 and 35 mm of inhibition zones against *S. enteritidis* ATCC 13076 and *E. coli* ATCC 25922, respectively. In the study of O'Bryan *et al.* (2008), orange oil affected *S. enteritidis* with 12.7–30 mm zones of inhibition. Fisher and Phillips (2008) recognized inhibitory effect of *Citrus limonum* against *E. coli*.

Inhibition activity of thyme oil in apple–carrot juice

The growth of *L. monocytogenes* AUFE 39237 and *C. albicans* ATCC 10231 was monitored over a 5-day period, and results were compared with the control groups in Figs. 1 and 2. Apple–carrot juice stored at 4°C did not show growth of *L. monocytogenes* AUFE 39237 after the third day. Control groups reached 7.94 log cfu/mL

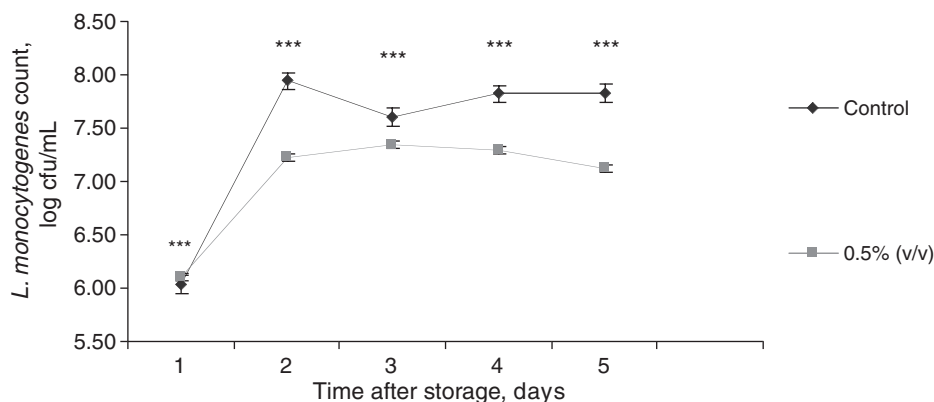


FIG. 1. Effect of thyme essential oil on the *Listeria monocytogenes* viable cell number in apple–carrot juice at 4°C storage. Data represent mean values of triplicate measurements, and error bars are indicated. Significant difference from control is shown as *** $p < 0.001$.

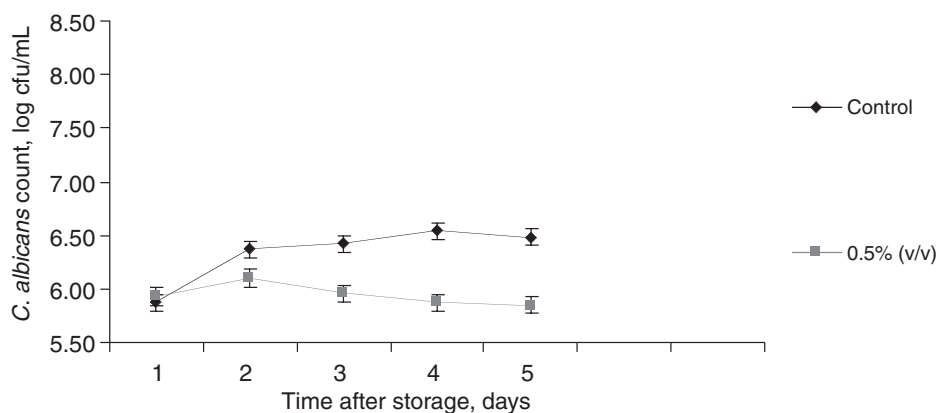


FIG. 2. Effect of thyme essential oil on the *Candida albicans* viable cell number in apple-carrot juice at 4°C storage. Data represent mean values of triplicate measurements, and error bars are indicated.

after the second day, and the oil-treated group count was 7.23 log cfu/mL. *Listeria* count of thyme oil-treated juice was 0.71 log units, which is lower than that of the control groups after the fifth day. According to the results, number of microorganisms in control and 0.5% oil-treated groups were different significantly ($p < 0.001$) during each day of storage. Gutierrez *et al.* (2008) concluded that great inhibitory activity against *L. monocytogenes* was obtained with thyme oil. Liolios *et al.* (2009) showed inhibitory activity of thymol, which is the main component of thyme oil, against the *L. monocytogenes*.

Beletti *et al.* (2008) found that *L. monocytogenes* Scott A and yeasts were inhibited with different amounts of citron essential oil in fruit-based salads. Smith-Palmer *et al.* (2001) showed important inhibition effects of 1% thyme oil against *L. monocytogenes* in soft-cheeses, and Tassou *et al.* (1995) determined inhibitory effects of mint oil against *L. monocytogenes* in some Greek foods, tzatziki (pH 4.5), taramosalata (pH 5.0), and pate (pH 6.8), at 0.5%, 1%, 1.5%, and 2% (v/w) concentrations at 4°C. Inhibition activity is expressed as a major disruption of the cell wall, together with increased roughness and lack of cytoplasm in *L. monocytogenes* on treatment with thyme essential oil (Fisher and Phillips, 2008).

C. albicans ATCC 10231 counts increased sharply and reached 6.37 log cfu/mL on the second day for control groups. *Candida* counts decreased after the second day in thyme oil-treated juice; their counts were 5.85 log cfu/mL, and 0.63 log unit lower than the control counts at the fifth day. *Candida* counts were significantly

different ($p < 0.001$) between the control and oil-treated groups, but counts for each day were not different significantly ($p > 0.05$).

Braga *et al.* (2007a, 2007b) reported that thymol was the most inhibitory compound for the *C. albicans* in their studies. Marti *et al.* (2007) and Giordani *et al.* (2004) determined strong growth inhibition of *C. albicans* with *Thymus piperella* and *T. vulgaris* essential oils, respectively.

Conclusions

Our data confirm the antimicrobial activity of some essential oils. Among the oils, origanum, orange, clove, and thyme oils have high inhibitory effects against the bacteria, and thyme, orange, clove, tea tree, and cumin were effective inhibiting the yeasts. Garlic, black pepper, tea tree, mint, and cumin have low effects on tested bacteria. The data reported here show the thyme oil as a potent inhibitor for *L. monocytogenes* AUFE 39237 and *C. albicans* ATCC 10231 in apple-carrot juice. Using low concentrations of oils or low doses of their active compounds in foods is possible during production; for example, carvacrol is added to flavoring foods such as baked goods (15.75 ppm), nonalcoholic beverages (28.54 ppm/0.18 mM), and chewing-gums (8.42 ppm). Their minimum inhibitory concentration doses can be determined and also antimicrobial activity can be obtained besides their organoleptic properties in foods. Further investigations are necessary to identify the most active molecules of the essential oils and their interaction with microbial growth.

Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Reyhan Irkin, Ph.D.
Susurluk College
Balikesir University
Balikesir TR10600
Turkey
E-mail: rirkin@hotmail.com

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