Decontamination effects of lemon peel and mint leaf extracts on salad vegetables

REYHAN IRKIN – SEMA CARIKCI – SUMEYRA AKALIN – ZEHRA BATU

Summary
Salad vegetables are rich in vitamins, minerals and fibre. However, some food-borne diseases are associated with the consumption of this type of raw food. Effective disinfection processes are therefore important to obtain it microbiologically safe for consumption. This study aimed to evaluate the antimicrobial properties of the essential oils and extracts of fresh lemon (*Citrus lemon* L.) peel as well as fresh and dried mint (*Mentha spicata* L.) leaves on certain bacteria associated with salad vegetables. Lemon peel and mint extracts used for the disinfection process were obtained by the Soxhlet extraction method while their essential oil was obtained by hydrodistillation using a Clevenger-type apparatus. Study results showed that the most effective disinfectant regarding total microbial counts was mint essential oil with a reduction of 7.56 log CFU·g^{-1}. For *Escherichia coli* ATCC 25933, lemon peel Clevenger extract was determined to be the most effective with a reduction of 7.37 log CFU·g^{-1}. The disinfectant applications statistically significantly reduced total microbial counts on salad vegetables ($p < 0.05$). The results of the study suggested that natural extracts and/or essential oils can be used as effective disinfectants.

Keywords
Escherichia coli; disinfectant; antimicrobial; essential oil

Nutrition is defined as the process of consuming food to take in the necessary elements for growth, development and a healthy life. Good nutrition is the regular intake of nutrients of sufficient variety and quality according to one's gender, age and physiological needs [1]. In a balanced diet, raw vegetables and fruits are important foods and their regular consumption is known to reduce the risk of various diseases because they contain substances such as polyphenolic compounds, vitamins, minerals and fibre [2]. However, consuming vegetables and fruits that have not been effectively decontaminated may cause various illnesses due to pathogenic microorganisms. Worldwide, the consumption of raw vegetables ranks second among food-borne risks [3]. In terms of salad vegetables, the microorganisms most commonly associated with contamination are *Listeria monocytogenes, Bacillus cereus, Escherichia. coli, Staphylococcus aureus, Salmonella typhimurium, Pseudomonas aeruginosa, Shigella sonnei* and *Clostridium perfringens* [4].

Disinfection processes play an important role in preventing food-borne diseases. According to good hygiene practices, disinfection of foods in restaurants and cafeterias involves the removal of microorganisms by physical and/or chemical means without affecting the properties of foods and materials in contact with food [5]. Chlorine and chlorinated compounds are the preferred chemical disinfectants. However, in recent years, it has been found that chlorine reacts with organic substances in food and forms harmful by-products such as trihalomethanes and halo acetic acids. King et al. reported that trihalomethanes cause tumour formation in in vivo studies and may cause cancer in humans [6]. So, studies focused on safe disinfectants that can be used as an alternative to chlorine [7].

One such alternative is essential oils. Obtained

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from the root, stem, leaf or fruit parts of the plants by various methods such as distillation, pressing or extraction, essential oils are important secondary metabolites that exhibit many biological effects due to components such as monoterpenes, sesquiterpenes or phenolics [8]. Extracts and essential oils demonstrated antimicrobial effects on various microorganisms. These antimicrobial effects are associated with their chemical composition [9].

Salad vegetables are grown close to the ground and can therefore be contaminated by microorganisms, especially *E. coli*, from irrigation water and other conditions. Another important aspect from the food safety point of view is that they are consumed raw and can cause food-borne illnesses. Therefore, we aimed to evaluate the disinfection effect of washing solutions (fresh mint, dry mint and lemon peel essential oils and their hydrosols) on salad vegetables (lettuce, parsley and dill were selected as salad material). The test bacterium chosen was *E. coli* ATCC 25933 standard strain, since it is the most common contaminant of vegetables. Total microbial counts of salad vegetables before and after the washing treatments were determined and compared to the control groups. The chemical components of the essential oils were screened by gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials

Commonly used salad vegetables such as lettuce, parsley and dill were selected as disinfection experiment materials. Samples were obtained from a local market in Izmir (Turkey). Test materials were prepared using equal amounts of salad vegetables. In this way, three application samples were prepared. The experiments were repeated three times using the same methodology.

Fresh leaves of mint (*Mentha spicata* L.) and fruits of lemon (*Citrus lemon* L.*)* were used for preparing the washing solution. Lemon samples were obtained from a local market in Izmir. Mint samples were firstly collected from a grower, identified by a botanist, and then dried in a shaded place in laboratory conditions. After that, fresh mint samples were taken from the same grower and same places as where the samples were collected before, thereby ensuring that mint samples were taken from the same sources.

Preparation of samples for analysis

Lettuce, parsley, and dill samples were washed with tap water after purchase. Then, they were drained and placed on filter paper. The samples were kept under the UV light in a laminar flow cabin to decrease microbial load on the salad vegetables. The samples were treated by UV for a total of 50 min. After the UV application, the samples were mixed together and inoculated with *E. coli* ATCC 25933. Microbiological analyses were performed on these inoculated samples.

Production of lemon peel and mint extracts

The extraction process was carried out using the Soxhlet extraction method. Immediately after peeling, 50 g of the grated lemon peel was weighed and placed in the Soxhlet extractor. A volume of 300 ml of distilled water was added to the Soxhlet flask. Soxhlet extraction was performed for 4 h after the water in the flask started to boil. The same was done for 50 g of fresh mint leaves. After the extraction, extracts were concentrated with a rotary evaporator and stored in amber bottles at 4 °C for 24 h until analysis.

Extraction of essential oils

Fresh lemon peel and dried mint leaves were subjected to hydrodistillation using a Clevenger-type apparatus to produce essential oils. Seventy grams of grated fresh lemon peel was weighed into a 500 ml glass flask and 150 ml of distilled water was added. Similarly, 50 g dried mint leaves was weighted into a 500 ml glass flask and 300 ml of distilled water was added. The distillation process was performed for 3 h and repeated three times. The obtained essential oils were dried over anhydrous sodium sulphate and stored in amber vials at 4 °C for 24 h until analysis. The mean amounts of oil obtained from the weighed dry plant material were 1.10 g (1.6 %, w/w) for lemon peel oil and 0.86 g (1.2 %, w/w) for mint essential oil.

Gas chromatography-mass spectrometry

GC analysis of the essential oils was carried out using Agilent 7000 Series Triple Quad Mass Spectrometer (MS) system (GC-MS/MS; Agilent Technologies, Santa Clara, California, USA). Oil samples were diluted 1 : 100 with dichloromethane. Diluted oil samples were injected into the capillary column (DB-WAX, length 60 m, inner diameter 250 μm, film thickness 0.25 μm; Agilent Technologies) of the GC-MS device. The temperature programme started at 40 °C and increased to 240 °C with an increase of 3 °C·min^{-1}. Helium (1 ml·min^{-1}) was used as the carrier gas. Split ratio was 1 : 20. The mass range determined was 50–600 (m/z). NIST mass spectral library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA) was used to identify volatile com-

pounds. Furthermore, after the hydrodistillation process, the water in the distillation flask in which the lemon and dried mint leaf samples were boiled, and the hydrosol part accumulated under the essential oils, were also collected for use as a disinfectant. They were named Clevenger extract (C) and hydrosol (H), respectively.

Preparation of *Escherichia coli*

The *E. coli* ATCC 25933 standard strain was used to determine the efficacy of the washing solutions. Microorganism from a stock culture was transferred to 100 ml Nutrient broth (Merck, Darmstadt, Germany) and incubated for 24 h at 37 °C without shaking [10]. After the incubation, the active culture contained 3.16×10^{10} CFU·ml^{-1} live cells. A volume of 0.5 ml of this culture was inoculated to 500 ml sterile distilled water. In this way, it was diluted to an initial load of 10^7 CFU·ml^{-1} for inoculation of salad vegetables by dipping treatment.

Inoculation of samples with *E. coli*

The salad materials were weighed in equal amounts (3.5 g for each material of lettuce, parsley and dill, as a green salad model) and put into the inoculation liquid (prepared as 500 ml). The samples were kept in the inoculation liquid at room temperature for 15 min. After the inoculation step, the salad samples were placed on a sterile cheesecloth for a few minutes to remove excess water. Samples of 10 g were weighed into sterile jars. The jars were kept at 20 °C for 24 h to ensure that *E. coli* cells adhered to the salad sample surface [11].

Application of disinfection solutions

For disinfection of the samples, eight different wash immersion solutions were prepared: lemon peel extract (L), fresh mint extract (FM, via Soxhlet), lemon peel Clevenger extract (LC), dried mint Clevenger extract (MC), lemon peel hydrosol (LH), dried mint hydrosol (MH), lemon peel essential oil (LEO) and dried mint essential oil (MEO). The hydrosols and essential oils were prepared in concentrations of 5 % (v/v) and 0.2 % (v/v), respectively. A volume of 50 ml of the immersion solution was used for washing. For the control group sample, 50 ml of sterile distilled water was used for washing. Samples of 10 g prepared from vegetables inoculated with *E. coli* active culture were put into the washing solution and kept there for 10 min. This immersion time was based on previous studies [12]. At the end of the holding period, the sample was drained, excess water was removed with a sterile cheesecloth and after the treatment, 10 g of mixed salad samples were weighed into 90 ml of dilution liquid, after which serial dilutions were prepared.

Microbiological analysis

Total microbial counts were determined by the plate count method. For this purpose, serial dilutions were prepared by taking 10 g of each sample immersed in individual disinfectant solutions (L, FM, LC, MC, LH, MH, LEO, MEO) and diluting to 10^{-5}. Then, 1 ml from each prepared serial dilution was transferred to plates of plate count agar (PCA, Merck) and total microbial counts were determined by plate pour technique using incubation at 35 °C for 24 h [13]. At the end of the incubation period, typical colonies were detected and counted.

The spread plate method was used for determination of *E. coli* counts. Eosin methylene blue agar (EMBA, Merck) was used and 0.1 ml from serial dilutions diluted down to 10^{-5} were inoculated by the spread plate method on EMBA using incubation at 35 °C for 24 h. The number of *E. coli* colonies was determined [13].

Statistical analysis

SPSS Statistics 25.0 program (SPSS, Chicago, Illinois, USA) was used for statistical evaluation of the data. In the study, One Way Analysis of Variance (ANOVA) was applied to determine the significance level of the microbial reduction seen in the vegetable samples that were disinfected. Duncan's multiple comparison test, one of the post-hoc test methods, was used to determine the difference between groups. The statistical significance level in the whole study was $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

The essential oils of the lemon peel and dried mint leaf samples were analysed by GC-MS/MS. The identified major components, with their relative percentages and the retention times, are given in Tab. 1 for lemon peel and in Tab. 2 for mint samples. The lemon peel used in this study was obtained from *Citrus lemon* L. (sour lemon) that belongs to the *Rutaceae* family [14]. Lemon peel was subjected to the hydrodistillation method using Clevenger apparatus without drying. In the essential oil of lemon peel, 26 components representing 100 % of the oil were identified. D-Limonene was determined as the main component of the oil with a high percentage (65.6 %), followed by γ-terpinene (15.5 %) and β-pinene (7.5 %). The results are similar to previous studies [15].

Eighty-seven constituents were identified in the dried mint leaf essential oil, which corresponded to 92.3 % of the oil, with the major constituents being (–)-carvone (55.3 %), caryophyllene (7.3 %) and eucalyptol (5.9 %; Tab. 2). Mint (*Mentha* spp.) belongs to the *Lamiaceae* family of plants, which are known to be rich in essential oil [16]. Carvone is the most abundant compound particularly in spearmint oil (*M. spicata*), while menthol is the major component of mint oil (*M. piperita*) [17].

Tab. 1. Major components of lemon oil.

No.	Name	*RT* [min]	*LRI*	Peak area share [%]
1	D-Limonene	14.2	1196	65.6
2	γ-Terpinene	16.0	1240	15.5
3	β-Pinene	10.6	1114	7.5
4	β-Myrcene	12.7	1165	2.5
5	*p*-Cymene	16.9	1265	2.0
6	β-Phellandrene	11.1	1124	1.1
7	3-Thujene	8.1	1039	0.9
8	α-Citral/Geranial	35.4	1726	0.8
9	Geranyl acetate	36.4	1756	0.7
10	Nerol acetate	35.3	1722	0.7

The minor components < 0.7% are not shown.
RT – retention time, *LRI* – linear retention index.

Tab. 2. Major components of mint oil.

No.	Name	*RT* [min]	*LRI*	Peak area share [%]
1	(–)-Carvone	35.6	1728	55.3
2	Caryophyllene	30.8	1604	7.3
3	Eucalyptol	14.5	1209	5.9
4	D-Limonene	14.1	1196	5.2
5	Caryophyllene oxide	44.4		3.7
6	α-Terpineol	34.2	1695	3.0
7	Espatulenol	48.8		2.4
8	Neodihydrocarveol	36.2		2.2
9	Terpineol/δ-Terpineol	33.3		1.2
10	Carveol	40.2		1.2

The minor components < 1.2 % are not shown.
RT – retention time, *LRI* – linear retention index.

Antimicrobial effectiveness of mint and lemon peel extracts

In this research, total microbial counts and *E. coli* counts were determined for samples dipped into various washing solutions based on mint leaf and lemon peel extracts (Fig. 1, Fig. 2). Washing for 10 min with FM extracts caused a significant reduction in total microbial counts (7.42 log CFU·g^{-1}) and *E. coli* counts (7.36 log CFU·g^{-1}). MC solution caused reductions of 6.94 log CFU·g^{-1} in total microbial counts ($p > 0.05$) and 7.24 log CFU·g^{-1} in *E. coli* counts (significant, $p < 0.05$). It was previously reported that the mint extract added to tomato juice at 0.1% and 1.2% concentrations decreased the microbial flora by 4.77 CFU·ml^{-1} and 8.34 CFU·ml^{-1}, respectively [18]. Compatible with our results, it was determined that the application of dried mint leaf extract showed a dose-dependent inhibitory effect on *E. coli* [19] and total microbial counts [18].

In this study, two different extracts from the lemon peel were obtained by using Soxhlet and Clevenger extraction methods. Statistically, not any significant reductions ($p > 0.05$) for total microbial counts (6.67 log CFU·g^{-1}) and *E. coli* (6.95 log CFU·g^{-1}) counts were caused by lemon peel extracts (L). With the lemon peel extracts obtained via Clevenger method (LC), significant reductions were determined in total microbial counts (7.51 log CFU·g^{-1}) and *E. coli* counts (7.37 log CFU·g^{-1}) compared with control groups, respectively. In one previous study, it was stated that lemon peel extract at concentrations of 0.3 mg·ml^{-1} and 0.6 mg·ml^{-1} showed antimicrobial activity against *Staph. aureus* and *S.* Typhimurium, but resistance was reported against *E. coli* even when 1 mg·ml^{-1} concentration was applied [20].

Antimicrobial effects of essential oils

There has been interest in extracts and essential oils from aromatic plants for controlling pathogens in foods [9, 21]. Various by-products are also obtained during the essential oil production. Hydrosol is collected under the essential oil phase in Clevenger apparatus. Hydrosol can be produced easily and economically and can be used as an antimicrobial agent generally [22].

In this study, the antimicrobial activity of mint hydrosol was also investigated. Reductions of total microbial counts and *E. coli* counts of salad samples when treated with MH were not statistically significant (6.99 log CFU·g^{-1}, $p > 0.05$). No antimicrobial effect of MH was observed against *E. coli*. As it is known, essential oils show higher antimicrobial activity even at much lower concentrations compared to other extracts.

MEO caused significant reduction in total microbial counts (7.56 log CFU·g^{-1}) and *E. coli* counts (7.35 log CFU·g^{-1}) of salad samples ($p < 0.05$). The antimicrobial effect of mint essential oil on *E. coli* was shown also previously [23]. Houicher et al. also suggested *M. spicata* or *M. pulegium* essential oils as alternatives to synthetic preservatives in products to control food-

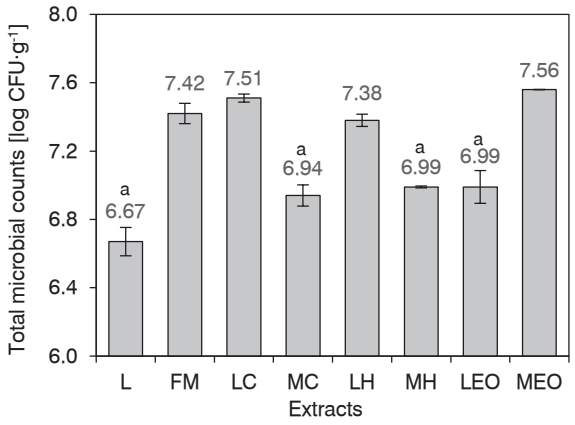

Fig. 1. Reductions in total microbial counts of the salad.

L – lemon peel Soxhlet extract, FM – fresh mint Soxhlet extract, LC – lemon peel Clevenger extract, MC – dried mint leaf Clevenger extract, LH – lemon peel hydrosol, MH – dried mint leaf hydrosol, LEO – lemon peel essential oil, MEO – dried mint leaf essential oil.
a – not different from the control group ($p > 0.05$).

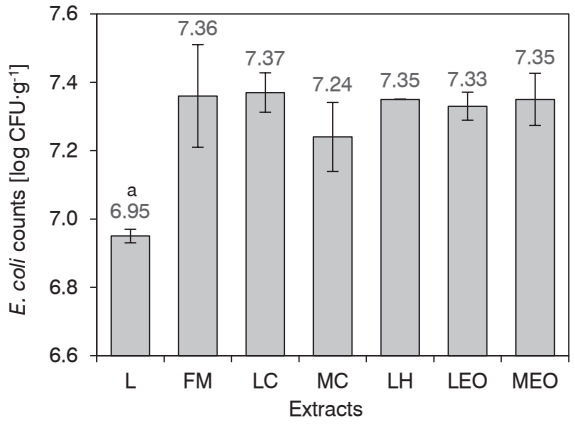

Fig. 2. Reductions in *Escherichia coli* ATCC 25933 counts of the salad.

L – lemon peel Soxhlet extract, FM – fresh mint Soxhlet extract, LC – lemon peel Clevenger extract, MC – dried mint leaf Clevenger extract, LH – lemon peel hydrosol, MH – dried mint leaf hydrosol, LEO – lemon peel essential oil, MEO – dried mint leaf essential oil.
a – not different from the control group ($p > 0.05$).

borne pathogens and to improve product safety [24]. In conformity with that, our study indicated that total microbial counts and *E. coli* counts of salad samples were effectively reduced by application of MEO washing solutions.

Dipping treatment of LH and LEO washing solutions caused decreases of 7.38 log CFU·g^{-1} and 6.99 log CFU·g^{-1} in total microbial counts, as well as 7.35 log CFU·g^{-1} and 7.33 log CFU·g^{-1} in *E. coli* counts on salad vegetables, respectively. However, no statistically significant difference was determined between the control group and LEO treatment group. Previous studies reported that lemon essential oil has an antimicrobial effect on *E. coli* O157:H7 [25]. In the study, it was shown that the essential oil and hydrosol obtained from the citrus plant showed inhibitory properties against *S. aureus* and *E. coli*. However, it was reported that the inhibition zones of hydrosol against bacteria were smaller than of the essential oil [26]. In another study, it was reported that sweet lemon peel hydrosol exhibited antimicrobial activity against *E. coli, Staph. aureus, Candida albicans, B. subtilis* and *Enterococcus faecalis*. It was stated that this effect might have been due to the high concentration of flavonoid and phenolic compounds in sweet lemon hydrosol [27].

D-Limonene is the main compound of the lemon peel essential oil that showed antibacterial activity against Gram-positive and Gram-negative strains. The reason for the antibacterial activity can be explained by its lipophilic toxin characteristic for cellular membrane structure [28]. Also, other mechanisms including disruption of microbial respiration and enzymatic system as well as cytotoxic effects may be responsible for their antimicrobial activity. These effects depend on the physico-chemical characteristics of the bacterial cell membrane [29].

Despite the limitations caused by the fact that the phenolic content of essential oils and hydrosols was not determined in this study, the results obtained showed that lemon hydrosol exhibited a good antimicrobial activity. In future studies, the antimicrobial compounds contained in essential oils and extracts obtained by various methods can be determined, and the extract with the highest antimicrobial activity can be identified.

CONCLUSION

Various decontamination processes need to be developed to ensure that salad vegetables have a low microbial load at consumption, which is an important factor from the public health safety point of view. It can be concluded that mint leaf and lemon peel extracts and essential oils have a potential of application as alternative sanitizers and improve the safety of certain fresh vegetables. In this study, no differences in visual quality of the salad vegetables were observed after the washing treatment with various solutions of extracts and essential oils. The use of chemical synthetic dis-

infectants has disadvantages, such as microbial resistance and environmental pollution. The development of natural antimicrobials may therefore be useful in this area. The natural antimicrobials, essential oils, hydrosols or related products may be effective, biodegradable and less toxic to the environment. These substances can be effectively used for disinfection and decontamination purposes in the production of "organic" foods, which have become common in recent years. In this respect, this study supports the use of mint essential oil and lemon peel Clevenger extract as natural disinfectants.

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