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The antimicrobial effect of grapefruit peel essential oil and its nanoemulsion on fish spoilage bacteria and food-borne pathogens

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ABSTRACT

In the study, improvement in antibacterial properties of grapefruit peel essential oil after the nanoemulsification process was investigated. The essential oil composition was characterized using GC-MS, with D-Limonene being the main identified compound (82.86%). The antibacterial properties against common food-borne pathogens (*Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumonia*and *Salmonella* Paratyphi A) as well as 6 fish-spoilage bacteria isolated from 4 spoiled fish species: mackerel, sardine, anchovy and rainbow trout, have been evaluated using paper disc diffusion, MIC and MBC methods. The grapefruit peel essential oil showed bacteriostatic properties against the majority of tested bacterial strains, however, only the growth of *Salmonella* parathypi A, *Vibrio vulnificus* and *Seratialique faciens* was inhibited by the essential oil in the concentration up to 25 mg/mL. Incorporation of essential oil into the nanoemulsion system resulted in increased bacteriostatic potency when the oil concentration in the nanoemulsion were considered, however, no bactericidal effect was observed, which was probably due to too low concentration of essential oil in the nanoemulsion system. The improvement in the nanoemulsion preparation method and formulation should be investigated to further improve its antibacterial properties.

1. Introduction

Contaminated food products, with microbiological contamination being the main cause, are responsible for over 600 million disease incidences and 420 000 death every year (WHO, 2020). Moreover, microbiological spoilage is one of the main causes for the generation of food waste (Saucier, 2016). Therefore, one of the main goals of the modern food technology is to find new and effective means to inhibit and eliminate the microbiological contamination. Although many different compounds with antimicrobial properties have been discovered, recently there has been growing interest in the use of natural food preservatives with antimicrobial properties. Such natural antimicrobials are often considered safer than their synthetic equivalents, which are more widely accepted by consumers and may prove effective against pathogens which have developed resistance to various synthetic antibiotics (Pisoschi et al., 2018). Plant-derived essential oils (EO) are one group of such natural food preservatives (Pandey, Kumar, Singh, Tripathi, & Bajpai, 2017). The effectiveness of EO is associated with the presence of multiple various antimicrobial compounds, with a typical EO containing 20–60 different bioactive compounds. This results in effectiveness specificity of each EO against various microorganisms (Swamy, Akhtar, & Sinniah, 2016). EO are often included into emulsion systems to reduce the impact of their high reactivity and hydrophobicity which impairs their use within the food industry. Recently, EO nanoemulsions have gained much attention, since the use of such nanosystems allows to minimize the impact of EO addition on sensory properties of the food product and to further improve the bioactive properties of EO, including antimicrobial properties (Donsì & Ferrari, 2016). However, it should be noted that the improvement in antibacterial efficiency of nanoemulsions varies depending on bacteria species. Moreover, in case of some bacteria species, the use of nanoemulsion can reduce the antibacterial efficiency compared to regular EO (Ozogul et al., 2020).

Grapefruit (*Citrus paradisi*) is a subtropical citrus fruit with an annual production estimated at around 8.3 million tons (FAO, 2017). The consumption of such amounts of grapefruit results in the generation of high quantities of grapefruit peels, which are normally treated as waste material. Meanwhile, grapefruit peel can be further utilized to produce valuable extracts (Najafinejad, Mohammadi, Mehdi Afsahi, & Sheibani, 2018; Pérez-Fonseca, Alcala-Canto, Salem, & Alberti-Navarro, 2016) or

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essential oil (Wu, Jin, Xu, & Yang, 2017). Grapefruit peel EO exhibits potent antibacterial and antioxidant properties (Deng et al., 2020; Uysal, Sozmen, Aktas, Oksal, & Kose, 2011) and can be effectively used as biopesticides against mosquito larvae (Okunowoa, Afolabia, Oyedejia, Matanmia, & Awodeleb, 2016). On the other hand, there is a limited information available about the antibacterial efficiency of grapefruit peel EO nanoemulsion. The existing research indicates that it can improve the shelf-life of rainbow trout fillets by inhibiting the oxidation progress, sensory scores or the growth of total mesophilic and psychrotrophic bacteria (Durmus, 2020; Uçar, 2020), however, not having effective influence on biogenic amine formation (Kosker, 2020). There is no research in which the effects of grapefruit EO nanoemulsion on common pathogenic and fish-spoilage bacteria would be examined.

Therefore, the aim of this study was to investigate and compare the efficiency of grapefruit peel EO and its nanoemulsion on common pathogenic gram-positive (*Staphylococcus aureus, Enterococcus faecalis*) and gram-negative (*Klebsiella pneumonia, Salmonella* Paratyphi A) bacteria, as well as on common fish-spoilage bacteria (*Photobacterium damselae, Enterococcus faecalis, Vibrio vulnificus, Proteus mirabilis, Serratia liquefaciens, Pseudomonas luteola*).

2. Materials and methods

2.1. Bacterial strain isolation and identification

The isolation and identification of fish spoilage bacteria (*P. damselae, E. faecalis, V. vulnificus, P. mirabilis, S. liquefaciens, P. luteola*) was performed in the same manner as described by Yazgan, Ozogul, and Kuley (2019). The bacteria were isolated from three spoiled fish species caught in the Mediterranean Sea: mackerel (*Scombers combrus)*, anchovy (*Engraulis enrasicolus)* and sardine *(Sardinella autira)* and from rainbow trout *Oncorhynchus mykiss)* obtained from a local farm near Adana, Turkey. *K. pneumonia* (ATCC700603)*, S. aureus* (ATCC29213) and *E. faecalis* (ATCC29212) were obtained from the American Type Culture Collection (Rockville, USA), while *Salmonella* Paratyphi A (NCTC13) was obtained from the National Collection of Type Cultures (London, UK).

2.2. Essential oil acquisition and composition

Grapefruits (*Citrus paradisi*) were obtained from BIOMESI (Adana, Turkey) and the EO was collected using the hydrodistillation method using a Clavenger type device consisting of a round-bottomed flask, tube for volatile oil determination and a reflux condenser. The EO was stored at 4 °C until used for analysis.

The identification of EO composition was performed by means of GC-MS (PerkinElmer Clarus 500, Waltham, USA) as described in detail by Yazgan et al. (2019). One μL of oil diluted in hexane was injected into the GC apparatus. The separation was carried out on a PerkinElmer SGE non-polar fused silica capillary column (60 m × 0.25 mm). The oven temperature was at set 60 °C for 10 min, after which it was increased to 250 °C and maintained for 10 min. The injector temperature was 220 °C. Helium, at a flow rate of 1.5 mL/min, was used as a carrier, gas helium was used as the carrier gas, while the flow rate was 1.5 mL/min. The electronic ionization energy was set to 70 eV with the temperature of 200 °C for ion source. The range of the scanned mass was 35–425 m/z with the interface line temperature of 250 °C. The chemicals were identified using the NIST-MS and WILEY-MS libraries.

2.3. Preparation of nanoemulsion

The grapefruit EO nanoemulsion was prepared by mixing grapefruit EO with Tween 80 and water using a ratio of 10:1:89 w/w. The emulsion was formed using an CY-500 ultrasonic homogenizer (Optic Ivymen System, Barcelona, Spain). The parameters of homogenization were: duration 15 min, amplitude 72 μm, 500W power and 20 kHz ultrasound frequency.

Table 1
Composition of grapefruit peel EO.

Compound	%
D-Limonene	82.86
β-Phellandrene	4.17
β.-Myrcene	2.51
o-Cymene	1.18
β-Pinene	0.76
(−)-Carvone	0.73
D-α-Pinene	0.72
cis-Carveol	0.62
Decanal	0.58
Limonene glycol	0.53
trans-2,8-*p*-Mentha-dien-1-ol	0.49
Caryophyllene	0.49
cis-p-Mentha-2,8-dien-1-ol	0.47
trans-Carveol	0.46
α.-Terpineol	0.45
Linalol	0.42
Limonene oxide, cis-Limonene 1,2-epoxide	0.40
Limonene oxide, *trans-*	0.38
Caryophyllene oxide	0.34
δ-Cadinene	0.29
Valencene	0.25
2-Pentadecyn-1-ol/trans,*trans*-2,6-Dimethyl-2,6-octadiene-1,8-diol	0.25
Octanal	0.24
Citronellal	0.20
(R)-lavandulyl acetate	0.20

2.4. Physical properties of nanoemulsion

All measurements of physical properties of the grapefruit peel EO nanoemulsion were performed at 25 °C. The average droplet size of the nanoemulsion and its polydispersity index were analyzed using the Malvern Panalytical Mastersizer 2000 (Malvern, UK). Thermodynamic stability was analyzed for 14 days using the method proposed by Shafiq et al. (2007). Viscosity of the nanoemulsion was measured via the ARES rheometer (TA Instruments, New Castle, USA). Surface tension of the nanoemulsion was measured with the Attension Theta goniometer (Biolin Scientific, Espoo, Finland).

2.5. Analysis of antimicrobial properties

The antibacterial properties of grapefruit peel EO and its nanoemulsion were analyzed using 3 analyses: the paper disc diffusion method, Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC). All microbiological analyses were performed on pure grapefruit peel EO (100%) and their nanoemulsions which contained 10% of EO. The disc diffusion method was performed as described by Yazgan et al. (2019). Three positive (30 μg tetracycline, 10 μg streptomycin and 5 μg neomycin) and one negative (Tween 80) controls were used. The nutrient agar (Merck, Darmstadt, Germany) was used as a standard test medium. The amount of both grapefruit peel EO and its nanoemulsion used in the assay was 50 μL. The incubation of bacterial plates was carried out for 18–24 h at 37 °C. The results were calculated as an average of four measurements.

The MIC and MBC analyses were performed according to the CLSI M07-A9 standard and as described in detail by Ozogul et al. (2020). The initial stock solution was adjusted to contain 50 mg/mL of grapefruit peel EO and its nanoemulsion, while the inoculum suspension used contained 6 log CFU of each bacterial strain. Two-fold dilution with Mueller Hinton Broth was used to obtain the final concentrations of the EO and its nanoemulsion with in the range of 0.19–50 mg/mL. Positive (tube with Mueller Hinton Broth and bacterial suspension) and negative (tube with Tween 80) control samples were also prepared. Incubation was carried out at 35 °C for 18–24h. Tubes without any visible growth were marked as MIC, while MBC was determined by subculturing the

Table 2
Antimicrobial activity of grapefruit essential oil and its nanoemulsion against foodborne pathogens and spoilage bacteria.

Bacterial strains		Grapefruit EO zone Diameter (mm)	Grapefruit EO Nanoemulsion Zone Diameter (mm)	Streptomycin	Neomycin	Tetracycline
Pathogen bacteria	*Staphylococcus aureus*	10.25 ± 0.56	7.88 ± 0.54 (78.8)[a]	14.75 ± 0.35	20.00 ± 0.57	20.75 ± 0.35
	Klepsiella pneumonia	7.50 ± 0.61	6.00 ± 0.00 (60.0)	10.5 ± 0.71	23.50 ± 0.71	27.00 ± 0.28
	Salmonella Paratyphi A	10.50 ± 0.61	6.25 ± 0.56 (62.5)	16.25 ± 0.64	20.73 ± 0.88	23.25 ± 0.35
	Enterococcus faecalis	6.50 ± 0.50	0.00 ± 0.00	3.7 ± 0.14	25.50 ± 0.71	30.50 ± 0.71
Spoilage bacteria	*Photobacterium damselae*	10.75 ± 0.75	15.66 ± 0.00 (156.6)	18.75 ± 0.35	22.25 ± 0.35	28.5 ± 0.42
	Enterococcus faecalis	0.00 ± 0.00	0.00 ± 0.00	19.25 ± 0.21	6.00 ± 0.42	16.75 ± 0.35
	Vibrio vulnificus	6.75 ± 0.25	0.00 ± 0.00	22 ± 0.71	25.00 ± 0.28	28.00 ± 0.71
	Proteus mirabils	0.00 ± 0.00	0.00 ± 0.00	2.2 ± 0.14	20.75 ± 0.35	18.25 ± 0.35
	Serratia liquefaciens	8.38 ± 0.54	0.00 ± 0.00	24.5 ± 0.71	23.00 ± 0.14	16.25 ± 0.07
	Pseudomonas luteola	14.24 ± 0.56	5.00 ± 0.00 (50.0)	22.25 ± 0.35	20.75 ± 0.35	18.125 ± 0.18

[a] Results in parenthesis show the power of nanoemulsion after considering the real concentration of EO in the nanoemulsion system (Zahi et al., 2017).

Table 3
MIC and MBC determination of grapefruit essential oil and its nanoemulsion on foodborne pathogens and spoilage bacteria.

Bacteria Strains		Grapefruit EO		Grapefruit EO nanoemulsion		Tween 80	
		MIC	MBC	MIC	MBC	MIC	MBC
Pathogen bacteria	*Staphylococcus aureuss*	25	>25	>25	>25	>25	>25
	Klebsiella pneumoniae	>25	>25	≥25	>25	>25	>25
	Salmonella paratyphi A	6.25	≥25	6.25 (0.625)	>25	>25	>25
	Enterococcus feacalis	>25	>25	>25	>25	>25	>25
Spoilage bacteria	*Photobacterium damselae*	>25	12.50	25 (2.5)	>25	>25	>25
	Enterococcus feacalis	25	>25	>25	>25	>25	>25
	Vibrio vulnificus	12.50	>25	>25	>25	>25	>25
	Proteus mirabilis	≥25	>25	≥25	>25	>25	>25
	Serratia liquefaciens	12.50	>25	12.50 (1.25)	>25	>25	>25
	Pseudomonas luteola	3.13	6.25	25 (2.5)	>25	>25	>25

[a]Results in parenthesis show the power of nanoemulsion after considering the real concentration of EO in the nanoemulsion system (Zahi et al., 2017).

MIC tubes onto the Mueller Hinton Agar. The plates without any visible growth were marked as MBC.

3. Results and discussion

3.1. Physical properties of the nanoemulsion and volatiles composition of grapefruit peel essential oil

The mean droplet size and polydispersity index of the grapefruit peel EO nanoemulsion was 204.5 nm and 0.168, respectively. The viscosity and surface tension were 1.48 cP and 40.21, respectively. The emulsion had good stability for at least 14 days.

GC-MS analysis of volatile components of the grapefruit peel EO identified 25 compounds (Table 1). D-Limonene was the main present compound constituting 82.86% of the total identified compounds, which is a similar level to that reported by other authors (Karioti, Skaltsa, & Gbolade, 2007; Njoroge, Koaze, Karanja, & Sawamura, 2005). D-Limonene exhibits potent anti-inflammatory, anticancerogenic and antioxidant (Yu, Yan, & Sun, 2017) as well as antimicrobial properties (Zahi, El Hattab, Liang, & Yuan, 2017). On the other hand, D-Limonene is prone to oxidation, which can be inhibited by its nanoemulsification (P.-H. Li & Chiang, 2012). Other compounds present in quantities higher than 1% included β-Phellandrene (4.17%), β.-Myrcene (2.51%) and *o*-Cymene (1.18%).

3.2. Antimicrobial properties

The results of the antimicrobial activity measured via the paper disc diffusion method are shown in Table 2. As expected, Tween 80, as a negative control, did not show an inhibition zone against any of the tested bacteria. The grapefruit peel EO exhibited antioxidant activity against most of the tested bacterial strains, aside from *E. feaecalis* and *P. mirabils,* both isolated from spoiled fish. On the other hand, the nanoemulsified form of EO demonstrated lower antimicrobial activity against all of the tested pathogenic and fish-spoilage bacteria, with the exception of *P. damselae*. Moreover, the grapefruit EO nanoemulsion did not exhibit any activity against *E. faecalis, V. vulnificus, P. mirabils,* or *S. liquefaciens*. Both EO forms had lower antibacterial activity than all compared antibiotics apart from *E. faecalis,* which was more susceptible to grapefruit EO than to streptomycin.

Grapefruit EO showed potent bacteriostatic effect against *Salmonella* Parathypi A and *P. luteola,* and a moderate bacteriostatic effect against *V. vulnificus, S. liquefaciens, S. aureus* and *E faecalis* (Table 3) The bactericidal effect could be observed only for *P. luteola.* A bacteriostatic effect of the grapefruit EO nonemulsion could only be observed against Salmonella Parathypi A, *P. luteola, S. liquefaciens* and *P. damselae,* but did not show bactericidal effect against any of the tested bacterial strains. Although gram-positive bacteria are usually more susceptible to plant essential oils, due to their less complex structure of the cell walls, such a trend was not observed in this study (Seow, Yeo, Chung, & Yuk, 2014).

Lower antibacterial activity of grapefruit EO nanoemulsion is

surprising, since in the majority of available reports, it is indicated that nanoemulsification improves the antimicrobial properties of EO (Donsì & Ferrari, 2016). Nonetheless, it should be noted that in this experiment, the grapefruit peel EO nanoemulsion contained only 10% of the EO, therefore, 10 times lower levels of bioactive EO compounds were present. Recalculating the results to include the real content of grapefruit peel EO (10-fold reduction), as done by Zahi et al. (2017), shows increased inhibition zone while applying the paper disc diffusion method for most pathogenic bacteria aside from *E. faecalis* (Table 2). Also, the bacteriostatic effect measured by MIC analysis shows improvement in the antimicrobial properties against all bacteria towards which such an effect was observed, aside from *E. faecalis* and *S. aureus*. However, the 10% EO level present in the nanoemulsion was too low to exhibit an inhibition zone for most of the analyzed fish-spoilage bacteria during the use of the paper disc diffusion method or to exhibit the bactericidal effect measured by MBC. Therefore, to improve the antimicrobial properties of the grapefruit peel EO nanoemulsion, further improvements should be applied. There are several physicochemical properties which may have influence on the antimicrobial properties of the nanoemulsions. Those include droplet size, electrical properties and the location as well as concentration of the antimicrobial compounds. Moreover, the effectiveness of nanoemulsion also depends on type of emulsifier or other additives (Prakash, Baskaran, Paramasivam, & Vadivel, 2018). Although low molecular emulsifiers such as Tween are usually correlated with increased antimicrobial properties (Donsì, Annunziata, Vincensi, & Ferrari, 2012; Prakash et al., 2018), some authors showed that they also depend on the type of bacteria strain, which shifted antibacterial properties towards various bacteria observed when Tween 80 was used (W. Li et al., 2015). The change in the nanoemulsion preparation method may also improve antibacterial properties. The nanoemulsion studied in this work had a mean droplet size of 204.5 nm. The droplet size of the nanoemulsion is closely related to the antimicrobial properties of the nanoemulsion since smaller droplets allow easier transport of the bioactive substances through bacterial membranes (Donsì & Ferrari, 2016). In contrast, Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, and Martín-Belloso (2014) reported that reducing sonification time improved the antimicrobial properties of lemongrass EO nanoemulsion, despite higher droplet size of the resulting nanoemulsion. Moreover, they observed that nanoemulsions obtained using the microfluidization method had significantly higher antibacterial properties against *E. coli*. This could be due to high temperature and degradation of volatiles present in the EO, since longer sonification duration resulted in an increase of the temperature on the outlet of the system.

4. Conclusions

The nanoemulsion of grapefruit peel EO has been successfully obtained and characterized with D-Limonene being the main constituent of the EO. Grapefruit peel EO showed bacteriostatic properties against most of the tested bacterial strains, however, only the growth of *Salmonella* parathypi A, *Vibrio vulnificus* and *Seratia liquefaciens* was inhibited by the EO in the concentration up to 25 mg/mL. The antibacterial properties of EO have been improved by incorporating it into the nanoemulsion system, when the EO concentration within the system has been considered. The nanoemulsion showed bacteriostatic properties against some of the tested bacteria strains, however, no bactericidal effect was observed. The improvement in the nanoemulsion preparation method and formulation should be investigated to further improve its antibacterial properties.

Author contribution

Yesim Ozogul: Formal analysis, designed the experiment, performed analyses and calculations and revised the manuscript. Fatih Ozogul: Formal analysisdesigned out the analyses and calculated the results. Piotr Kulawik: Writing - original draftwrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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