

Detection of antibacterial effects of various fruit species on motile *Aeromonas* species

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Abstract

The aim of this study is to determine the antimicrobial effects of extracts prepared from orange (*Citrus sinensis* L.), lemon (*C. limon* L.) and pomegranate (*Punica granatum* L.) peels on motile *Aeromonas* species including *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* using disc diffusion and broth dilution techniques. In addition, the antibacterial effects of edible films and coatings prepared from orange, lemon and pomegranate peels on these bacteria were also investigated with the well agar diffusion technique in the current study. The essential oils (EOs) used in the study were obtained from the fruit peels by the distillation method. To test the antibacterial susceptibilities of essential oils, sterile discs were placed on the surface of the Petri dishes which were inoculated by bacteria. The dishes were incubated at 37 °C for 24 h. At the end of the incubation period, the zone diameters observed around the discs were measured. Florfenicol, flumequine and oxytetracycline were used as control antibiotics in the study. Antibacterial susceptibilities of fruit peels were also determined by broth dilution technique. The Minimum Inhibition Concentration (MIC) values of the fruit peels were found and the lowest concentration where turbidity which was not observed was accepted as MIC for that bacterial species. Carrageenan, xanthan, starch and carop were used as matrix in the preparation of edible films and coatings. According to the results of the study, it was found that EO extract prepared from lemon peels was more effective on *A. hydrophila*, *A. caviae* and *A. allosaccharophila* than EO extracts prepared from pomegranate and orange peels in disc diffusion test. Also, it was found that edible films prepared from pomegranate peels had the most antibacterial effect on the three bacterial species.

Keywords: Fruit, motile *Aeromonas* species, essential oil, edible films and coatings

1. Introduction

Bacteria are important pathogens of cultured fish and they cause serious economic losses. Therefore, bacterial pathogens pose major threat to fish production worldwide (Wamala *et al.*, 2018). A group of bacteria cause superficial disorders such as skin or gill infections, while others lead to systemic infections in fish (Briede, 2010; Wamala *et al.*, 2018). Important bacterial species which are isolated from freshwater fish include *Aeromonas* spp., *Streptococcus* spp., *Flavobacterium* spp., *Edwardsiella* spp., *Pseudomonas* spp., *Vibrio* spp. and *Mycobacterium* spp. (Wamala *et al.*, 2018). The genus *Aeromonas* belongs to the family *Aeromonadaceae*. Members of the genus are Gram-negative, rod-shaped, facultative anaerobic, cytochrome-oxidase positive and ferment glucose (Grim, 2013; Liu, 2015). As *Aeromonas* species can develop in a wide range of pH, salinity and temperature, they are widely distributed worldwide including marine environments, rivers, lakes, sediments, waste water and drinking water sources (Briede, 2010; Liu, 2015). Generally, members of the genus *Aeromonas* are isolated from fish, shellfish, pets, birds, meat and dairy products (Liu, 2015).

For this reason, researchers have directed to alternative methods such as treating bacterial fish diseases with different herbs and plant extracts with antimicrobial and/or immunostimulant properties have been used for therapeutic or protective purposes (Pandey *et al.*, 2012; Sudheesh *et al.*, 2012; Ngugi *et al.*, 2016; Thackenko *et al.*, 2016).

Today, there are at least 25 known species of the genus *Aeromonas*. However, *Aeromonas hydrophila*, *A. caviae* and *A. veronii* biotype *sobria* are the most frequently isolated species (Liu, 2015; Abd El-Tawab *et al.*, 2017). The members are divided into two groups as non-motile psychrophilic and motile mesophilic species (Grim, 2013). The non-motile psychrophilic species, *A. salmonicida* is pathogenic for cold blooded animals including fish while the motile mesophilic species, *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* were isolated from 85% of human clinical specimens which were affected by *Aeromonas* infections (Grim, 2013). In aquaculture, *A. salmonicida* strains cause furunculosis, especially in salmonid fish. Mesophilic *Aeromonas* species such as *A. hydrophila* and *A. veronii* have been reported to cause hemorrhagic septicemia in salmonid fish species and ulcerative lesions in cod and carp (Janda and Abbott, 2010).

Today, antibiotics are used to treat bacterial infections which have been observed in fish culture conditions. However, the use of antibiotics to control these infections is of limited and resistance development in the bacterial species can be observed (Pandey *et al.*, 2012; Sudheesh *et al.*, 2012).

Citrus fruits are the most cultivated fruit trees, reaching approximately 131 million in the world and their most important representatives include orange (*Citrus sinensis*, L.), lemon (*C. limon*, L.), tangerine (*C. reticulata*, L.) and grapefruit (*C. paradisi*, L.) (Ergüt, 2015). Pomegranate (*Punica granatum*, L.) is a fruit only found in the Mediterranean basin and South Asia. Extracts of all parts of the fruit are known as therapeutic because of antimicrobial effect (Gaber *et al.*, 2015).

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Antibacterial activities of orange, lemon and pomegranate and edible films and coatings against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* were investigated in this study. The effects of essential oils which were derived from fruit peels and films and coatings against the isolates were determined by disk diffusion and broth dilution methods with the formation or absence of the zone diameters.

2. Materials and Methods

2.1. Materials

2.1.1. Fruit samples used in the study

Pomegranate (*Punica granatum* L.), lemon (*Citrus limon* L.), orange (*Citrus sinensis* L.) fruits were used in the study. The fruits were washed with de-ionized water and then peeled. The peels were oven dried at 70 °C for 48 hours (Edogbanya *et al.*, 2019).

2.1.2. Bacterial species used in the study

Stock cultures of *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* strains were used for antimicrobial susceptibility tests. Bacterial cultures were inoculated onto Brain Heart Infusion agar (BHIA, Merck) plates. The plates were incubated at 25 ± 2 °C for 24 hours. Subcultures of the bacterial cultures were prepared.

2.1.3. Control antibiotics

Oxytetracycline (OTC 30 µg, Oxoid), florfenicol (FFC 30 µg) and flumequine (UB 30 µg) were used as control antibiotics.

2.2. Methods

2.2.1. Essential oils from the fruit peels

Essential oils (EOs) from the fruit peels were prepared according to the distillation method. Briefly, the fruit peels were pulverized using an electric grinder and the powdered fruit peels (15 grams of sample in 150 mL of ethanol) were extracted for 48 hours according to Tunç *et al.*, (2013). Then, the resulting mixture was filtered and evaporated in a rotary evaporator (Kılınc *et al.*, 2018).

2.2.2. Antimicrobial activities of essential oils

Antimicrobial activities of lemon, orange, and pomegranate essential oils were determined by disc diffusion and broth dilution methods (CLSI, 2006; Kılınc *et al.*, 2018; Küçük, 2018).

2.2.3. Disc diffusion method

Samples taken from 18-24 hours cultures of the bacterial species used in the study were adjusted to McFarland No: 1 (2.4x10⁸). Then, the samples were spread on the surface of the petri dishes containing Mueller-Hinton agar (MHA, Condo lab) using sterile swabs. The extracts of the fruit peels were then impregnated with sterilized discs as 30 µL. Florfenicol, flumequine and oxytetracycline were used as

control antimicrobials. The inoculated petri dishes were incubated at 37 °C for 24 hours. The zone diameters around the discs after incubation period were measured and recorded (Kılınc *et al.*, 2018).

2.2.4. Broth Dilution Method

Antimicrobial susceptibilities of the fruit peels were determined by macro dilution method. In order to prepare bacterial suspension, the turbidity of each bacterial strain produced up to logarithmic phase was adjusted to McFarland No.1. Subsequently, the bacterial solutions were diluted 1/100 with Mueller-Hinton broth (MHB) to a final concentration of 10⁶ cfu/mL (Erdağlı, 2011; Küçük, 2018). 1 mL of cation adjusted Mueller-Hinton Broth (CMHB) was added to each test tube to inoculate 10 µL of bacterial suspension in the each tube. Two fold dilutions were prepared starting from the first tube. The inoculated tubes were incubated at 24 ± 2 °C for 16-18 hours (CLSI, 2006; Küçük, 2018). At the end of the incubation period, the minimum inhibition concentration (MIC) values of the fruit peels were determined in % by determining the number of tubes from which the growth observed. The lowest concentration without turbidity was evaluated as MIC for that bacterial strain (Table 1-3). Nutrient broth cultures (without extracts) of the bacterial strains were used as controls (CLSI, 2006).

Table 1. Results of MIC values orange extract

	Orange (µg/mL)							
	7.8125	15.625	31.25	62.5	125	250	500	N.B
<i>A. hydrophila</i>	-	-	-	+	+	+	+	+
<i>A. caviae</i>	-	-	-	+	+	+	+	+
<i>A. allosaccharophila</i>	-	-	-	+	+	+	+	+

+ : where the microorganism grows; - : where the microorganism does not grow ; N.B: improvement in nutrient broth

Table 2. Results of MIC values lemon extract

	Lemon (µg/mL)							
	7.8125	15.625	31.25	62.5	125	250	500	N.B
<i>A. hydrophila</i>	-	-	-	+	+	+	+	+
<i>A. caviae</i>	-	-	-	+	+	+	+	+
<i>A. allosaccharophila</i>	-	-	-	+	+	+	+	+

+ : where the microorganism grows; - : where the microorganism does not grow; N.B: improvement in nutrient broth

Table 3. Results of MIC values pomegranate extract

	Pomegranate (µg/mL)							
	7.8125	15.625	31.25	62.5	125	250	500	N.B
<i>A. hydrophila</i>	-	-	-	+	+	+	+	+
<i>A. caviae</i>	-	-	-	+	+	+	+	+
<i>A. allosaccharophila</i>	-	-	-	+	+	+	+	+

+ : where the microorganism grows; - : where the microorganism does not grow ; N.B: improvement in nutrient broth

2.2.5. Production and determination of antimicrobial activities of edible films and coatings from fruit peels

To prepare edible film solution, each of the based polymers was homogenized in 1% (w/v) distilled water at 90 °C for 30 minutes in the magnetic stirrer. 3% glycerol as a plasticizer (active compound) and 3.2 grams to 100 mL for pomegranate, orange and lemon from the extracts of fruit peels in concentrations that the bacterial growth was not observed were added to edible film solutions (Kılınç *et al.*, 2018). Antimicrobial activities of edible films and coatings prepared from fruit peel extracts were determined using well agar diffusion method. The turbidity of the solutions of *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* was adjusted to Mc Farland No:1. After adjustment, the solutions were spread over the surface of the petri dishes containing MHA. 6 mm diameter wells were drilled on the surface of the medium. 50 µL of each of the edible films and coating solutions were added to these wells. The inoculated media were incubated at 37 ± 2 °C for 24 hours. At the end of this period, the zone diameters around the wells were measured and recorded (Kılınç *et al.*, 2018).

3. Results

3.1. Disc diffusion results of the EO extracts from fruit peels against *Aeromonas* species

According to disc diffusion test results, it was found that EO extract prepared from lemon peels was more effective than EO extracts prepared from pomegranate peel and orange peel against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila*. The inhibition zone diameters of lemon peel EO changed from 12 ± 1 to 15 ± 1 mm for the isolates; however, the EOs extracts from pomegranate peel and orange peel did not show antibacterial activities against *A. caviae* and *A. allosaccharophila*. EOs from orange peel and pomegranate peel showed antibacterial activity against *A. hydrophila*. Disc diffusion results of the assay are given in Table 4, Figure 1.

Table 4. Disc diffusion results of the EO extracts from fruit peels against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* in the study

Bacterial species	Inhibition zone diameters (mm)					
	Lemon	Orange	Pomegranate	O*	FFC	UB
<i>A. hydrophila</i>	15±1	18±2	22±2	21.6±2.3	37.3±2	25
<i>A. caviae</i>	15±1	ZN	ZN	25	R	53±2.6
<i>A. allosaccharophila</i>	12±1	ZN	ZN	R	32±1	R

*O: oxytetracycline, FFC: florfenicol, UB: flumequine, ZN: zone was not observed, R: Resistance

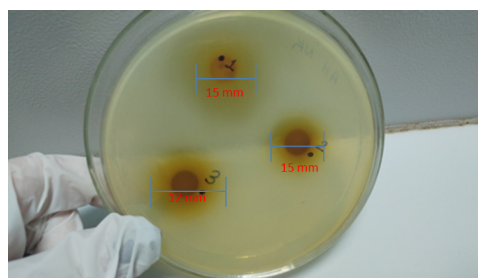


Figure 1. Disc diffusion results of the EO extracts from lemon peel against *Aeromonas hydrophila* (1), *A. caviae* (2) and *A. allosaccharophila* (3) in the study

It was determined that extract prepared from lemon peel showed antimicrobial effect on all bacterial species but extracts prepared from lemon peel, orange peel and pomegranate peel had antibacterial effect only against *A. hydrophila*. According to the antimicrobial susceptibility test results of extracts obtained from fruit peels, edible films and coatings solutions were produced from fruit peel extracts with the highest and lowest efficacy. The antibacterial effects of edible films and coatings against bacterial species are given in Tables 5,6,7. While edible film solutions prepared with carrageenan pomegranate, xanthan pomegranate and carob pomegranate were effective on *A. hydrophila* (17.5 ± 2.5 mm, 13.6 ± 1.2 mm and 18.3 ± 4.0 mm, respectively), edible film prepared with starch had no effect on this species. However, according to the results of edible films prepared with pomegranate carrageenan, xanthan, carob and starch, these edible films showed antibacterial activities on *A. caviae* (13.0 ± 0.8 mm, 16.3 ± 1.2 mm, 15.0 ± 2.0 mm and 18.6 ± 4.5 mm, respectively) and on *A. allosaccharophila* (14.0 ± 0.8 mm, 16.3 ± 1.2 mm, 15.0 ± 0.8 mm and 16.6 ± 2.3 mm, respectively) (Figure 2). Carrageenan orange coating solution was effective only against *A. allosaccharophila* (16.3±2.6 mm) and was not effective on the other *Aeromonas* species. Xanthan orange was effective against *A. hydrophila* (23.3±1.2 mm) and *A. allosaccharophila* (15.6±3.0 mm), but it did not show effect against *A. caviae*. Carob orange had effect on *A. caviae* (15.0 ± 0.8 mm) and starch orange showed antibacterial activity against *A. hydrophila* (15.0 ± 0.8 mm). Carrageenan lemon showed effect against *A. allosaccharophila* (15.3±0.95 mm) but it did not have antibacterial effect against other species. Xanthan lemon was effective against *A. allosaccharophila* (13.0 ± 2.4 mm) and carob lemon had effect against *A. hydrophila* (15.3 ± 2.4 mm). Starch lemon did not show effect against the bacterial species tested in the study.

Table 5. Antibacterial activities of edible films with pomegranate peel against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* (well agar diffusion method)

Bacterial species	Inhibition zone diameter (mm)			
	*CP	XP	CRP	SP
<i>A. hydrophila</i>	17.5±2.5	13.6±1.2	18.3±4.0	-
<i>A. caviae</i>	13.0±0.8	16.3±1.2	15.0±2.0	18.6±4.5
<i>A. allosaccharophila</i>	14.0±0.8	16.3±1.2	15.0±0.8	16.6±2.3

*CP: Carrageenan Pomegranate, XP: Xanthan Pomegranate, CRP: Carob Pomegranate, SP: Starch Pomegranate.

Table 6. Antibacterial activities of edible coatings with orange peel against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* (well agar diffusion method)

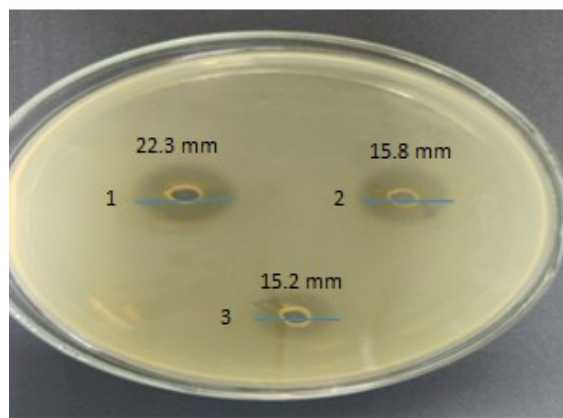
Bacterial species	Inhibition zone diameter (mm)			
	*CO	XO	CRO	SO
<i>A. hydrophila</i>	-	23.3±1.2	-	15.0±0.8
<i>A. caviae</i>	-	-	15.0±0.8	-
<i>A. allosaccharophila</i>	16.3±2.6	15.6±3.0	-	-

*CO: Carragenean Orange, XO: Xanthan Orange, CRO: Carop Orange, SO: Starch Orange

Table 7. Antibacterial activities of edible coatings with lemon peel against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* (well agar diffusion method)

Bacterial species	Inhibition zone diameter (mm)			
	*CL	XL	CRL	SL
<i>A. hydrophila</i>	-	-	15.3±0.5	-
<i>A. caviae</i>	-	-	-	-
<i>A. allosaccharophila</i>	15.3±0.95	13.0±2.4	-	-

*CL: Carragenean Lemon, XL: Xanthan Lemon, CRL: Carop Lemon, SL: Starch Lemon

**Figure 2.** Zone diameters of edible films prepared with carop pomegranate on *A. hydrophila* (1), *A. allosaccharophila* (2) and *A. caviae* (3) (well agar method)

4. Discussion

4.1. Disc diffusion results of the EO extracts from fruit peels against motile *Aeromonas* species.

The EO extracts of lemon (*C. limon*) had antibacterial activities against *A. hydrophila*, *A. caviae* and *A. allosaccharophila*. This result was agreed with a similar study with Nguyi *et al.*, (2016) who reported that EO extract of bitter lemon (*C. limon*) fruit peel had antibacterial activity against *Bacillus cereus*, *Campylobacter jejuni*, *E. coli*, *L. monocytogenes*, *Salmonella typhimurium*, *S. aureus* and *Vibrio vulnificus*. Edogbanya *et al.*, (2019) reported that lemon peel EO extract had no significant effect on *E. coli*, *P. aeruginosa* and *S. aureus*, but orange peel EO extract had a significant effect on all isolates. In this study, the EO from orange peel showed antibacterial activity against *A. hydrophila* but it did not have effect against both *A. caviae* and *A. allosaccharophila*.

Gaber *et al.*, (2015) reported that ethanol extracts of *P. granatum* peels have been found to be effective on *A. hydrophila*. In this study, the EO extract of pomegranate peel showed antibacterial activity against *A. hydrophila*. But, the EO extract did not show inhibitory effect against *A. caviae* and *A. allosaccharophila*.

In general, it has been reported that Gram-negative bacteria are less susceptible to antimicrobials due to the polysaccharide outer membrane that restricts the diffusion of hydrophobic compounds from fruit and plant-derived antimicrobials, and as a result, Gram-negative bacteria species show more resistance to plant-derived antimicrobials than Gram-positive bacteria species (Tajkarimi *et al.*, 2010; Tekçe and Gül, 2016). It has also been suggested that *A. hydrophila* is the most susceptible species to plant-derived antimicrobials among Gram-negative bacteria species, but the antimicrobial activity of herbs may vary according to the microorganisms tested (Tajkarimi *et al.*, 2010). In the current study, it was found that essential oil extracts prepared from lemon, orange and pomegranate peels showed antibacterial effects on *A. hydrophila* except *A. caviae* and *A. allosaccharophila*. This revealed that *A. hydrophila* was the most susceptible to herbal antimicrobials among Gram-negative species, as noted by Tajkarimi *et al.*, (2010).

4.2. Antimicrobial activities of edible films and coatings against *Aeromonas* species.

Kılınç *et al.*, (2018) examined the antimicrobial effects of edible films prepared from lemon, orange, red apple and green apple extracts on *E. coli*, *S. aureus*, *Enterococcus faecalis*, *B. cereus*, *S. epidermidis* and *S. typhimurium*. The researchers informed that xanthan lemon, carragenean lemon and carob lemon from edible films were more effective against the above-mentioned bacterial species. In our study, carragenean lemon and xanthan lemon were effective against *A. allosaccharophila*, whereas carob lemon was effective against *A. hydrophila*. Starch lemon did not show antibacterial effect against *A. hydrophila*, *A. allosaccharophila* and *A. caviae*. According to the results of the study reported by Kılınç *et al.*, (2018), carragenean lemon, xanthan lemon and carob lemon had antibacterial activities against *E. coli*, *S. aureus*, *Enterococcus faecalis*, *B. cereus*, *S. epidermidis* and *S. typhimurium*, whereas in the current study, carragenean lemon, xanthan lemon and carob lemon had been found to be effective on *A. allosaccharophila* and *A. hydrophila* (respectively). Carob orange only had effect on *A. caviae*. It was found that edible films prepared from pomegranate showed the most antibacterial activities on the three bacterial species.

5. Conclusions

There are some works on alginate and gelatin-based films and coatings containing fruit or plant or extracts due to advantages of its antimicrobial properties in seafood (Öztürk *et al.*, 2018; Alparslan *et al.*, 2019). However, studies on antibacterial effects of edible films and coatings prepared from fruit peels against the motile *Aeromonas* species from fish which cause diseases in fish have not been reported. For this reason, it was tried to reveal the deficiency in this area and the antibacterial activities of orange, lemon and pomegranate and edible films and coatings against *Aeromonas hydrophila*, *A. caviae* and *A. allosaccharophila* in the present study. According to the results of the study, it was found that EO extract

prepared from lemon peels was more effective on *A. hydrophila*, *A. caviae* and *A. allosaccharophila* than the EO extracts prepared from pomegranate and orange peels in the disc diffusion test. Also, it was found that edible films prepared from pomegranate peels had the most antibacterial effect on these bacterial species.

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