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Antimicrobial Effect of Gel-Type Nanoemulsion of Chitosan Coating Containing Essential Oils of *Zataria multiflora* and *Bunium persicum* on *Pseudomonas* Artificially Inoculated onto Salmon Fillets

Mehrdad Rezaeian
Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Saeid Khanzadi
Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Mohammad Hashemi
Medical Toxicology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

Mohammad Azizzadeh
Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

Corresponding author: Saeid Khanzadi

Tel: +985138805610

Email: khanzadi@um.ac.ir

Address: Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Azadi square, Mashhad, Iran.

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ABSTRACT

Background and objectives: Chitosan is a preservative that is commonly used in food packaging due to forming a film with antimicrobial activity. Many antimicrobial agents have been used to control the growth of different bacteria, fungi and yeasts in food products using chitosan coating. The present research was conducted to examine inhibitory effects of a coating incorporated with the essential oils of *Zataria multiflora* (ZEO) and *Bunium persicum* (BEO) on the growth of *Pseudomonas* artificially inoculated onto salmon fillets over a period of 12 days at 4 °C.

Methods: The antibacterial activity of BEO against *P. aeruginosa* was evaluated using the microdilution method via determining minimum inhibitory concentration and minimum bactericidal concentration. For the food model investigation, three *P. aeruginosa* strains were inoculated onto trout fillets as culture cocktail to assess their survival over 12 days of storage.

Results: The results indicated that ZEO and BEO had stronger inhibitory effect on *P. aeruginosa* in trout fillets when applied along with gel type nano-emulsion of chitosan solution. The separate use of each of these substances also significantly inhibited the growth of these pathogenic bacteria compared with the control. In addition, the use of chitosan coating without any antimicrobial agent affected the growth of *P. aeruginosa*.

Conclusion: The gel type nano-emulsion of chitosan coating containing ZEO and BEO can be applied on foodstuff, particularly fish and its products, as an antimicrobial agent.

Keywords: Fishes, Chitosan, Pseudomonas aeruginosa.

INTRODUCTION

The rainbow trout (*Oncorhynchus mykiss*) belongs to the Salmonidae family. The demand for rainbow trout has been increasing over the past decade. Spoilage risk of fresh fish products is higher than that of other foods. Fish muscle spoils due to biological reactions, such as oxidation of lipids, endogenous enzymes activity and metabolic activities of microorganisms that results in a short shelf life (1,2).

During recent years, a considerable number of studies have addressed the use of natural preservatives in food to enhance food safety and shelf life without any adverse effects (3). In this regard, edible coatings and films are of significant importance given their capability to act as a carrier of food additives such as antimicrobials, flavors, enzymes, antioxidants and colors (4, 5).

Chitosan is a preservative that is commonly used in food packaging due to forming film inducing antimicrobial activity (6). Many antimicrobial agents have been used to control the growth of different bacteria, fungi, and yeasts in food products using chitosan coating (7). There are various methods for applying preservatives in food products. Some of these methods include direct incorporation into food (8), addition of sachets into packages (9), direct application on the food surface (6) and addition into packaging materials (10).

Recently, edible coating has emerged as a delivery system for essential oils (EOs) as a food safety hurdle technology; however, they may raise some issues (11). The hydrophobic and volatile features of EOs as well as their sensitivity to oxygen and light reduce their stability during processing and storage. Upon addition, EOs can interact with hydrophobic components of food (e.g., fat and protein), which minimizes their sanitizing effect and/or efficiency against microorganisms (12). Plant-based EOs contain a high level of phenolic compounds (e.g., flavonoids and phenolic acids), exerting antimicrobial activity against various microorganisms (13). Gram-negative bacteria show higher resistance to the antibacterial effects of EOs compared to their Gram-positive counterparts. This higher resistance is caused by a barrier against hydrophobic compounds (e.g., EOs), which is created by the hydrophilic lipopolysaccharides in the outer membrane of these bacteria(14). However, this is not always the case (15, 16).

Zataria multiflora EO (ZEO) has high antioxidant and antimicrobial properties; therefore, they can be widely used in food preservation. This substance is also utilized for medical purposes as an antiseptic and antitussive agent for the treatment of respiratory tract infection and irritable bowel syndrome (17).

Seedlings of *Bunium persicum* contain volatile oils with antimicrobial, antifungal, antihistamine, antioxidant and medicinal properties (18). Nano-emulsions can be specifically applied for food products because of their unique features, including ease of preparation, high-grade functions and fine particle size. These features provide enhanced interactions of active compounds with bio-membranes and facilitate their transfer (19). Nano-emulsions are produced by multiple approaches, such as low- and high-energy techniques (19). One of the high-energy methods is ultrasonic emulsification that can be effectively applied to prepare nano-emulsions with small droplets and low size distributions (20). The emulsion-based systems developed by food-grade components are easily distributed in food to control the growth of various existing microorganisms (21). A large number of investigations have examined the use of chitosan as an edible coating or film loaded with EO-based nano-emulsion for inhibiting the growth of foodborne pathogens in meat and vegetable products (22-26). Nonetheless, there are a limited number of studies on the application of natural antimicrobials via coatings or films for controlling pathogenic bacteria in fish accounting for the outbreak of foodborne illnesses (27, 28). With this background in mind, the present study was designed to examine inhibitory activity of ZEO and BEO-based chitosan nano-emulsion on the growth of *Pseudomonas* inoculated in fish samples at 4 ˚C.

MATERIALS AND METHODS

The ZEO and BEO were purchased from the Iranian Institute of Medicinal Plants (Karaj, Iran) and Sigma-Aldrich Chemical Co. (St. Louis, USA), respectively. All media used in the study were purchased from Merck Inc. (Darmstadt, Germany). All reagents were of analytical grade and supplied from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

In addition, the *P. aeruginosa* strains (ATCC: 9027, 1707, 27853) were purchased from the Department of Food Hygiene at Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran.

ZEO and BEO were analyzed by gas chromatography (Agilent 7890A/5975C) equipped with a Chrome-pack CP-Sil 8 CB capillary column (50 m x 250 µm x 0.12 µm). The flow rate of helium was set to 5 ml/min. The column temperature was initially 50 °C, held for 2 min, and then gradually increased to 120 °C at a 4 °C/min rate, then to 200 °C at a 2 °C/min rate, and finally increased to 280 °C, held for 8 min as described previously (12).

The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of ZEO and BEO were determined using the microdilution method (29). To prepare bacterial suspensions, the *P. aeruginosa* strains were cultured in 9 mL of brain heart infusion (BHI) broth and then incubated at 37 °C for 24 h. After reaching turbidity equal to 0.5 McFarland standard (1.5×10^8 CFU/mL), the bacterial suspensions were diluted to obtain the desired bacterial density (1.5×10^7 CFU/mL).

Subsequently, ZEO and BEO were dissolved in distilled water (32 mg/ml) containing Tween 80 (0.2%, $w_{\text{ZEO and BEO}}$) as the stock solution of the ZEO and BEO emulsion. Half of the solution was subjected to ultra-turrax at 3,000 rpm and ultrasonic emulsification sonicator for 6 min (30) as the stock solution of nano-emulsion of ZEO and BEO. Both solutions were then prepared at a concentration range of 0.31-32- mg/mL. Next, 20 µl of the inoculums with 20 µL of various concentrations of emulsion and nano-emulsion of ZEO and BEO were added to wells of a 96-well plate containing 160 µL of BHI broth. The wells that contained no bacteria (180 µL of BHI broth and 20 µL of the EO) were considered as negative controls, while those without EO (180 µL of BHI broth and 20 µL of the inoculums) were regarded as positive controls. Therefore, each well had a final volume of 200 µL with inoculums having a final concentration of almost 1.5×10^6 CFU/mL. The ZEO and BEO had the final concentration range of 0.031-4 mg/mL. Later, the microplate was incubated at 37 °C for 18-24 h in a shaker incubator (GFL 3031) at 50-100 rpm. The lowest concentrations of ZEO and BEO that inhibited the growth of the bacteria were considered as the MICs. In addition, the lowest concentrations of the EOs that resulted in no visible bacterial growth on BHI agar were considered as MBCs.

The chitosan powder (2% w/v) was dissolved in sterile distilled water containing 1.5% glycerol as a plasticizer to prepare the solutions of chitosan. The solution was constantly stirred for 10 min to obtain a clear solution and then mixed with acetic acid (1%, w/v), ZEO (0.5% and 0.25%) and BEO (0.5% and 0.25%). The ZEO and BEO were poured into chitosan solutions in the presence of Tween 80 (0.2% w of EO) as an emulsifier. They were then stirred for 30 min to obtain a uniform, stable and clear emulsion. Gel type nano-emulsion of chitosan was formulated according to the protocol described previously (19) with slight changes. Then, the whole coating was subjected to ultraturrax for 3 min at 3,000 rpm and ultrasonic emulsification sonicator (50°C, pulse; 45 sec and rest; 15 sec) for 6 min (20). Particle size was measured using sonication with the dynamic light scattering device.

Fresh rainbow trout fish (*Oncorhynchus mykiss*) weighing 300±50 g was bought from a local fish farm (Mashhad, Iran) in the summer in 2018. After filleting the samples, they were immediately sent to the laboratory. The samples were washed thoroughly to remove blood and slime. After drying, the fillets were sliced into pieces weighing 25±1 g, followed by ethanol spray (95% v/v). Subsequently, the samples were burnt and trimmed to eliminate surface microorganisms (7). Each side of the samples was separately inoculated by 50 µL bacterial suspension (1.5×108 CFU/mL) to achieve a final concentration of about 10^6 CFU/g (7,31). The inoculated samples were categorized into seven groups and then treated by being immersed in coating nano-emulsion for 1 min, drained for 30 min, and stored at 4°C for 12 days. Finally, the analysis was performed on days 0, 2, 3, 6, 9, and 12 while stored at 4°C.

Changes in bacterial count (CFU/g) of trout fillet samples inoculated with *P. aeruginosa* during 12 days of storage were recorded after coating with chitosan, nano-chitosan, nano-chitosan containing BEO 0.25% (w/v), nano-chitosan containing ZEO 0.25% (w/v), nano-chitosan containing BEO 0.5% (w/v), and nano-chitosan containing ZEO 0.5% (w/v). For the enumeration of the inoculated bacteria,

10 g of samples were added to 0.1% sterile peptone water to obtain a final volume of 90 mL. Subsequently, the samples were subjected to homogenization for 2 min using a stomacher (Seward Medical, London, UK). After the preparation of decimal dilutions, 10 µL of serial dilutions of homogenates were cultured onto Pseudomonas agar base (31-34), followed by incubation at 37 °C for 24 h to count the bacteria.

Data obtained from tests in triplicate were statistically analyzed using repeated measures ANOVA and Dunnett's tests in SPSS software (version 21; SPSS, Inc. Chicago, IL, the USA). A p-value of less than 0.05 was considered as statistically significant.

RESULTS

The mean droplet size in both ZEO and BEO emulsions was 2700 nm which decreased less than 500 nm when nano-emulsion was prepared for MIC and MBC. The PDI mean and droplet size decreased after the preparation of different nano-emulsion coatings. In this regard, the mean droplet size decreased more in the sonicated chitosan coating than in chitosan coating. The highest particle size was observed in chitosan coating (2194 nm) and the lowest was observed in nano-chitosan+ ZEO (401.3 nm). The tested bacteria showed significant sensitivity to ZEO and BEO (Table 1). In this regard, the BEO emulsion had higher MIC and MBC values against *P. aeruginosa* compared to ZEO emulsion.

Table 1- Antibacterial properties of *Zataria multiflora* emulsion against the tested bacteria by micro-dilution method

Strains	Essential oil	MIC (mg/ml)	MBC (mg/
	Zatria multiflora	1.25	2.5
P. aeruginosa 9027			
P. aeruginosa 1707		1.25	2.5
P. aeruginosa 27853		2.5	2.5
P. aeruginosa 9027	*Zataria multiflora* nano-	0.62	1.25
P. aeruginosa 1707	emulsion	0.62	1.25
P. aeruginosa 27853		1.25	2.5
P. aeruginosa 9027	*Bunium persicum*	2.5	5
P. aeruginosa 1707		2.5	5
P. aeruginosa 27853		5	10
P. aeruginosa 9027	*Bunium persicum* nano-	1.25	2.5
P. aeruginosa 1707	emulsion	1.25	2.5
P. aeruginosa 27853		2.5	5

Table 2 shows the impact of seven different treatments on *P. aeruginosa* growth during 12 days of storage. The initial count of *P. aeruginosa* was 6.51±0.23 log CFU/g, which elevated in all samples during the storage. However, in nano-chitosan+ZEO 0.5% samples (6.25±0.10) and nano-chitosan+BEO 0.5% (6.27±0.33), there was a lower trend.

Table 3 shows the average reduction rate of *P. aeruginosa* count after the treatments. Based on the results, the highest reduction in *P. aeruginosa* count (1.03 log CFU/g) was observed in nano-chitosan+ZEO 0.05% samples when compared with other treatments and the control.

Table 2- Changes in bacterial count (CFU/g) of trout fillet samples inoculated with *P. aeruginosa* during 12 days of storage

Group	Day 0	Day 2	Day 3	Day 6	Day 9	Day 12
Control	6.51±0.23	6.90±0.40	7.18±0.21	7.74±0.11	8.32±0.45	9.17±0.41
Chitosan	6.46±0.12	6.86±0.07	7.01±0.13	7.63±0.13	8.28±0.22	8.83±0.07
Nano-chitosan	6.29±0.22	6.61±0.16	6.83±0.11	7.47±0.15	8.09±0.25	8.59±0.11
Nano-chitosan+BEO 0.25%	6.28±0.25	6.56±0.09	6.70±0.24	7.24±0.23	7.75±0.06	8.22±0.22
Nano-chitosan+ ZEO 0.25%	6.28±0.56	6.53±0.23	6.65±0.11	7.02±0.20	7.58±0.14	8.06±0.22
Nano-chitosan+BEO 0.05 %	6.27±0.33	6.40±0.23	6.64±0.15	7.02±0.12	7.47±0.13	7.88±0.26
Nano-chitosan+ZEO 0.05 %	6.25±0.10	6.34±0.24	6.42±0.24	6.68±0.09	6.83±0.16	7.06±0.20

Table 3-Average reduction rate of *P. aeruginosa* count after treatment

Group (J) / Mean difference I-J	Chitosan	Nano-chitosan	Nano-chitosan +BPEO 0.25%	Nano-chitosan + ZMEO 0.25%	Nano-chitosan +BPEO 0.05%	Nano-chitosan +ZMEO 0.05%
Group (I)						
Control	**0.1235**	**0.3238***	**0.5092***	**0.6139***	**0.6884***	**1.0361***
Chitosan		**0.2003***	**0.3857***	**0.4904***	**0.5649***	**0.9126***
Nano-chitosan			**0.1854**	**0.2901**	**0.3646***	**0.7123***
Nano-chitosan +BEO 0.25%				**0.1047**	**0.1792**	**0.5269***
Nano-chitosan +ZEO 0.25%					**0.0745**	**0.4222***
Nano-chitosan +BEO 0.05%						**0.3477***

DISCUSSION

Psychrophilic bacteria are species capable of living at 7 °C or below (35), enabling them to cause spoilage in aerobically stored fresh meat at chilled temperatures (36). The most important specifications that make *Pseudomonas* important in food spoilage are their proteolytic and lipolytic properties, which cause bad odor and taste in food as a result of protein and fat decomposition. These bacteria are aerobic and quickly grow on high-protein and fatty foods, such as meat, resulting in the formation of slime on the surface of food. Pseudomonads are psychrotrophic; therefore, they can multiply at refrigerator temperature (6, 37). This is the first study to report the MIC and MBC values of ZEO and BEO nano-emulsion. The MIC and MBC values of ZEO and BEO nano-emulsion were lower than those of emulsions. Nevertheless, the MIC and MBC values of ZEO nano-emulsion were lower than those of BEO nano-emulsion. This result can be due to the increased antibacterial activity of nano-emulsion form compared to that of the emulsion form (39). A previous study on the sensitivity of *Escherichia coli* to chitosan and nano-chitosan in acidic conditions reported no specific antimicrobial effect for chitosan at a pH of 5. However, they observed that nano-chitosan was more sensitive to this pH at 0.2% concentration of *E. coli* O157: H7 cells (40). In general, the reduction of bacterial count was considerable in the last two groups due to the great impact of nano-chitosan solution and the level of EO. In addition, the comparison of chitosan and nano-chitosan treatment groups in the mentioned study demonstrated a more decrease in bacterial count in the nano-chitosan group (40). In our study, the mean droplet size in both ZEO and BEO emulsions was 2700 nm which decreased less than 500 nm when nano- emulsion was prepared for MIC and MBC. Similarly, the results of the previous studies indicated a decrease in the droplet size of the GEO nano-emulsion (21). We also observed that the mean droplet size decreased more in the sonicated chitosan coating than in chitosan coating, which is in line with results of a previous study (35). In the present study, the BEO emulsion had higher MIC and MBC values against *P. aeruginosa* compared to ZEO emulsion. In a previous study, the BEO MIC and MBC values against *P. aeruginosa* were reported as 4 and 8 mg/ml, respectively (38). We evaluated the impact of seven different treatments on *P. aeruginosa* growth during 12 days of storage. The initial count of *P. aeruginosa* was 6.51±0.23 log CFU/g, which elevated in all samples during the storage. However, in nano-chitosan+ZEO 0.5% samples (6.25±0.10) and nano-chitosan+BEO 0.5% (6.27±0.33), there was a lower trend, which is in line with the results of a previous study (40). As shown in the present study, the small-sized particles and the high surface-to-volume ratio of nano-emulsions lead to higher bioavailability of non-polar components compared to that of conventional emulsions(41).

CONCLUSION

Based on the results, the gel-type nano-emulsion of chitosan solution with ZEO and BEO has anti-microbial effect against *P. aeruginosa*, a common foodborne pathogen. Furthermore, the enhancement of EO level to 0.5% could improve this effect. The results also demonstrated that nano-chitosan+ZEO 0.5% had the highest inhibitory effect against *P. aeruginosa*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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