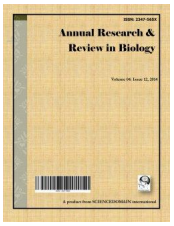

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Antibacterial Activity of Corn Starch Films Incorporated with *Zataria multiflora* and *Bonium persicum* Essential Oils

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Authors' contributions

This work was carried out in collaboration between all authors. Author MA designed the study, performed the statistical analysis and wrote the protocol. Author ZA wrote the first draft of the manuscript. Authors HH and EA managed the analyses of the study. Author MH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Nowadays biodegradable packaging such as edible coatings and films, are known as alternatives to plastic compounds and synthetic packaging since they are carriers for food additives (e.g. natural ingredients) and they do not cause any environmental contamination. Antimicrobial bioactive films are a special type of packaging carrying antimicrobial agents that can reduce the risks of food pathogens and consequently, increase the shelf-life of the foodstuff.
Materials and Methods: In this study, the antimicrobial effect of biodegradable starch film containing *Zataria multiflora* and *Bunium persicum* (concentration range from 1 to 20 mg/ml) essential oils was examined on four species of bacterial pathogens including *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas aeruginosa* using disk diffusion

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and plate count assay methods.
Results: The results of the disk diffusion method showed that the highest antimicrobial effect of *Zataria multiflora* and *Bunium persicum* essential oils was related to the concentration of 20 mg/ml, which in this concentration the maximum diameter of the inhibition zone for *S. aureus* (most sensitive bacteria) was recorded to be 31.3 mm and 22.36 mm respectively. Also, *E. coli* was determined as the most resistant bacteria with a diameter of the inhibition zone of 26.13 mm and 19 mm for *Zataria multiflora* and *Bunium persicum* respectively. The results of plate count assay showed that there was a significant difference in colony counts of *L. monocytogenes*, *S. aureus* and *P. aeruginosa* treated between films containing the lowest concentration of *Zataria multiflora* essential oil (1 mg/ml) and control samples ($P<0.05$).
Conclusion: In conclusion, the corn starch bioactive films incorporated with the essential oils of *Zataria multiflora* and *Bunium persicum* can be used as safe antimicrobial compounds in the food packaging industry.

Keywords: Starch film; thyme; black caraway; agar disk diffusion; plate count assay.

1. INTRODUCTION

In view of the severe environmental pollution caused by plastic food packaging, there is a considerable interest in edible and biodegradable films made from natural polymers [1]. Edible films are said to be thin edible materials that are used as coatings on foodstuff. These films can contain materials such as antioxidants, antimicrobial agents, paints and essential oils, unlike conventional packaging. Also, The advantage of edible Films is the ability to prevent the penetration of CO_2, moisture, oxygen to the surface of the food [2].

Edible films are made of polysaccharides, proteins or fats [3]. Starch is the most abundant polysaccharide in nature that has been replaced by plastic, due to its low cost and biodegradability potential [4]. starch can be provided from different sources in order to produce edible films such as: rice, potatoes, corn and wheat [5]. Corn starch is commonly applied in food industry for its gelation or water holding capacity [6]. Factors such as the source of the starch and the proportion of the constituents of the films affect the properties of the starch films [7].

Since chemical additives are hazardous, the application of natural additives has increased recently [8-10]. In recent years, essential oils (EOs) are being used as additives in biodegradable films [11,12]. EOs are extracted from plants with antimicrobials and antioxidants features [13,14]. Most of them are known as Generally Recognized as Safe (GRAS) [15]. Research has shown that antioxidant or antibacterial compounds of plants can be a good alternative to chemicals in food and feed industry, as well as in disease treatments [8,16-20].

Foodborne microbial diseases due to total cost of health care, loss of productivity, costs of investigations of an outbreak, loss of income due to closure of businesses or losses of product sales when consumers avoid of particular products are necessary. One of the possible strategies to reduce foodborne infections is the development of effective preservation methods capable of eradicating microbial contamination of foods [21]. Over the last few years, new challenges in food industry have emerged such as the increase of antimicrobial resistance of food borne pathogens to common preservatives and the consumers demand for natural products. Therefore, new approaches using natural or bio-based products as food preservatives need to be investigated [22].

Zataria multiflora Boiss of *Lamiaceae* family grows in different parts of Asia, especially in Iran and Pakistan. Thymol and carvacrol are the main compounds of *Z. multiflora* essential oils (ZMEO) with active antimicrobial effects. ZMEO has been registered by the European Commission and was used as flavors in foodstuffs and it has been determined that the use of this EO does not pose a threat to the health of consumers [23,24].

Bunium persicum Boiss is one of the most widely used plants in food industry and medicine. For example, black cumin seeds are known as flavors used in bread, cheese, sweets, meat products and different sauces. Terpenoid compounds in *B. persicum* essential oils (BPEO) such as cuminaldehyde, gamma terpinene, para

cymene as well as other active ingredients are bioactive compounds with antimicrobial and antioxidant activities [25].

There have been several studies on the antimicrobial effects of edible films containing essential oils [5,11,15,24]. But to the best of our knowledge no study has been done to investigate the antimicrobial properties of corn starch films containing essential oils of *Z. multiflora* and *B. persium.* Hence, due to the importance of the dangerous effects of chemical preservatives and food pathogens on consumer's health, we have evaluated *in vitro* antimicrobial effects of corn starch-based edible films containing *Z. multiflora* and *B. persicum* essential oils on some of the most important food borne pathogens.

2. MATERIALS AND METHODS

2.1 Materials

Corn starch and glycerol were purchased from Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany). All media cultures including Plate Count Agar (PCA) and Peptone Water (PW), were provided from Merck Company (Merck, Darmstadt, Germany).

2.2 Bacterial Strains

Lyophilized bacteria cultures of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 15224), *Listeria monocytogenes* (ATCC 13932) and *Pseudomonas aeruginosa* (ATCC 15442) were provided from the Iranian Research Organization for Science and Technology (IROST) (Tehran, Iran). Lyophilized bacteria were grown in BHI broth at 35°C for 24 h before the tests.

2.3 Isolation of Essential Oils

Dried seeds of black cumin (*B. persicum*) and flower shoots of thyme (*Z. multiflora*) were obtained from a local market in Zanjan, Iran and were then authenticated at the Institute of Medicinal Plants, Karaj, Iran. EOs were extracted by hydro-distillation method using a clevenger apparatus at 100°C for 3 h. The extracted oils were dried over anhydrous sodium sulfate, followed by filtering (0.22 m) and being stored at 4ºC before use. Gas Chromatography–Mass Spectroscopy (GC–MS) analysis of the EOs were performed as described by previous authors [12].

2.4 Preparation of Starch Film

Aqueous solutions of 3% (w/v) corn starch and 1.8% glycerol were prepared by heating at 90°C under agitation for 10 min to allow gelatinization. Afterwards the solution was cooled down to approx. 40°C and ZMEO and BPEO were added in concentration of 1 to 20 mg/ml (1, 2.5, 5, 10, 15 and 20 mg/ml) separately and then homogenized with a IKA Ultra-Turrax T-25 Digital Homogenizer (IKA, Germany) for 2 min at 2000 rpm till homogeneous mixture was obtained. Aliquots of 25 mL of the solutions were cast on petri dishes (r = 5 cm) and later dried at room temperature for 24 h [26].

2.5 Antibacterial Activity of Bio-degradable Films

Antibacterial activity of biodegradable films impregnated with *Z. multiflora* and *B. persicum* essential oils were evaluated using agar Disk diffusion and plate count assay methods.

2.5.1 Agar disc diffusion assay

To perform agar disk diffusion method, 0.1 ml of the overnight grown bacterial cultures (broth culture containing approximately, 1–2 $\times 10^8$ CFU/mL (according to density of 0.5 McFarland standard) were spread on Plate Count Agar (PCA-Merck, Darmstadt, Germany). Then disc cuts of starch films (colorless and flexible, with a thickness of about 1 mm) with a 6 mm diameter were placed on PCA plates containing the intended bacteria. Plates were incubated at 37°C for 24 h. The diameter of the inhibition zone was considered as an indicator of the antimicrobial activity. Chloramphenicol disc (30u/g) was used as positive control [27].

2.5.2 Plate count assay

Antibacterial activity of impregnated corn starch films with plate count assay was evaluated according to the described protocol by Sugumar et al. (2015). The pieces of the bioactive films (2.5c m) were transferred to the test tubes containing 10 ml of 0.1 OD (Optical density) adjusted bacteria at 600 nm (1–2 $\times 10^8$ CFU/mL). Tubs were then stored at 28°C– 30°C for 24 hours in an incubator equipped with shaker. A test tube with no film was considered as control.

Then serial dilutions were prepared using 0.1 mL of each sample and spread on Nutrient agar (Merck, Darmstadt, Germany) in duplicates. The plates were incubated at 37°C for 24 hours [28].

2.6 Statistical Analysis

Statistical analysis (ANOVA) was carried out using SPSS statistical software, Version 19.0 (SPSS Inc., Chicago, IL). All the experiments were performed in triplicate. Tukey's post hoc test was used to analyze differences between the treatments.

3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity Using Disk Diffusion Methods

The obtained results of the antimicrobial properties of biodegradable starch films impregnated with ZMEO and BPEO are presented in Table 1 and Table 2, respectively. According to the results, films containing ZMEO have good antimicrobial effects. The best inhibitory concentration of both EOs in starch film was 20 mg/ml. *S. aureus* and *E. coli* are the most sensitive and resistant bacteria to ZMEO with an inhibition zone of 31.3±1.8 mm and 26.13±0.08 mm, respectively. Several studies have been done on the antimicrobial effects of *Zataria multiflora* essential oil and edible films containing ZMEO [14,23,27,29,30]. Gharehbagh et al. evaluated the antibacterial effect of chitosan film incorporated with ZMEO (2%) and reported that the growth inhibition zones were 27.7 mm ,28 mm and 34 mm for *E. coli*, *S. aureus* and *L. monocytogenes* respectively [31]. Boroumand et al. reported that the inhibitory zone diameters of caseinate edible films contains essential oils for *E. coli* O_{157}:H_7 and *S. aureus* which were 17.25, 20.15 mm respectively, indicating a good effect for inhibition of the bacterial growth [32]. Also, in a study by Moradi et al., the results showed that chitosan film containing ZMEO, could significantly increase the diameter of the inhibition zone of *L. monocytogenes* growth compared to the films with no essential oil (P<0.05) [33]. The inhibition zone diameter of BPEO on *S. aureus* was 22.36±0.77 mm at a concentration of 20 mg/ml (Table 2). Antimicrobial effect of *B. persicum* is due to presence of strong antimicrobial compounds such as monoterpene, cuminaldehyde and thymoquinone. According to a study by Teneva et al., BPEO had good antimicrobial effect on gram-positive bacteria. The minimum inhibitory concentration and inhibition zone diameter of BPEO were 600 ppm and 8-10 mm for gram-positive bacteria, respectively [34]. Talei et al. studied the antimicrobial effect of cumin on six gram positive and negative bacteria. They have reported bacteriostatic effects of BPEO on *Staphylococcus epidermidis*, *S. aureus*, *E. coli* and *Enterococcus faecalis* [35]. Carvacrol and Thymol are the most common aromatic and antimicrobial compounds of some plants, especially in *Z. multiflora*. BPEO is composed of about 15% carvacrol, based on plant sources [23]. Both of these materials have the same chemical structure (phenolic compound) with strong antimicrobial properties. Heretofore, many studies have been carried out on the synergistic effects of various components of plants in various foods to increase their antimicrobial strength such as using carvacrol and thymol of ZMEO [36]. These compounds can penetrate into the bacterial cell membrane and have an important role in shaking the contents of the cell [37,38]. In the present study, phenolic monoterpene carvacrol and its isomer, thymol, were found to be the major antimicrobial.

The variation in inhibition zone diameter of different bacteria showed that effect of ZMEO and BPEO on gram-positive bacteria is greater due to the specific structure of these bacteria. In fact, the resistance of gram-negative bacteria to essential oils is due to the presence of cell walls in these organisms. Therefore, the results of the growth inhibition zone indicated that the concentration of *B. persicum* and *Z. multiflora* essential oil on gram-positive bacteria *(Listeria monocytogenes* and *Staphylococcus aureus)* was higher than that of gram-negatives.

The results of this study showed an increase in the concentration of essential oil in films, which led to an increase in diameter of the inhibition zone. For example, *S. aureus* had an inhibition zone diameter of 8.96 ± 0.30 mm in 1 mg/ml, and 11.13 ± 0.46 mm at 2.5 mg/ml, which significantly increased (P<0.05). In the present study, the inhibition zone diameters of *L. monocytogenes* and *S. aureus* when were treated with a concentration of 20 mg/ml of ZMEO were significantly higher than the inhibition zone diameter of chloramphenicol (P<0.05). In an study by Fazeli et al., they have shown a relationship between the essential oil of *Z. multiflora* and *Gentamycin* antibiotic on *S. aureus* [39].

Table 1. Antibacterial effect of edible starch film containing *Z. multiflora* essential oil using agar disc diffusion method (mean ± SD)

Bacteria	Diameter of inhibition zone (mm)						
	1 (mg/ml)	2.5 (mg/ml)	5 (mg/ml)	10 (mg/ml)	15 (mg/ml)	20 (mg/ml)	Chloramphenicol (30 u/g)**
L. monocytogenes	9.13±0.97[a]	10.96±0.3[a]	15.16±0.5[b]	19.6±0.7[c]	27.86±1.02[d]	[e]30.43±1.0	29±0.9[d]
S. aureous	8.96±0.3[a]	11.13±0.5[b]	16.53±0.5[c]	20±0.1[d]	26.9±0.5[e]	31.3±1.8[f]	25.43±1.4[e]
E. coli	7.16±0.5[a]	9.43±0.4[b]	12.03±0.2[c]	16.9±0.4[d]	20.6±0.6[e]	26.13±0.1[f]	29.23±1.6[g]
P. aerogenosa	7.23±0.6[a]	8.86±0.6[a]	12.56±1.4[b]	16.5±0.7[c]	20.6±1.0[d]	28.33±1.3[e]	26.8±0.3[e]

Values followed by different small letters within the same rows are significantly different (P<0.05) according the Tukey's test

Table 2. Antibacterial effect of edible starch film containing *B. persicum* essential oil using agar disc diffusion method (mean ± SD)

Bacteria	Diameter of inhibition zone (mm)						
	1(mg/ml)	2.5(mg/ml)	5(mg/ml)	10(mg/ml)	15(mg/ml)	20(mg/ml)	Chloramphenicol (30 u/g)**
L. monocytogenes	8.83±0.2[a]	9.76±0.64[a]	12.46±0.75[b]	15.96±o.15[c]	18.2±0.26[d]	22.13±0.4[e]	29±0.9[d]
S. aureous	8.03±0.15[a]	9.96±0.35[b]	12.76±0.32[c]	15.06±0.20[d]	18.06±0.2[e]	22.36±0.77[f]	29.23±1.6[g]
E. coli	7.16±0.2[a]	8.23±0.35[a]	11.16±1.1[b]	13.86±0.47[c]	16.56±o.58[d]	19±0.6[e]	25.43±1.4[e]
P. aerogenosa	7.03±0.51[a]	8.16±0.28[b]	12.36±0.50[c]	14.4±0.45[d]	16.2±0.2[e]	19.4±0.6[f]	26.8±0.3[e]

Values followed by different small letters within the same rows are significantly different (P<0.05) according the Tukey's test

3.2 Antibacterial Activity Using Plate Count Assay Methods

The results of the antimicrobial properties of starch films impregnated with ZMEO and BPEO using plate count assay are presented in Fig. 1 and Fig. 2, respectively. These results showed that there was a significant difference between the number of colony counting of *L. monocytogenes*, *S. aureus* and *P. aeruginosa* under the influence of the film, using different concentrations of ZMEO, and by increasing the concentration of the essential oil in the film, the number of bacteria reduced (P<0.05). In 1 mg/ml BPEO, the reduction of *Listeria* mononcytogenes, *E. coli* and *Pseudomonas*

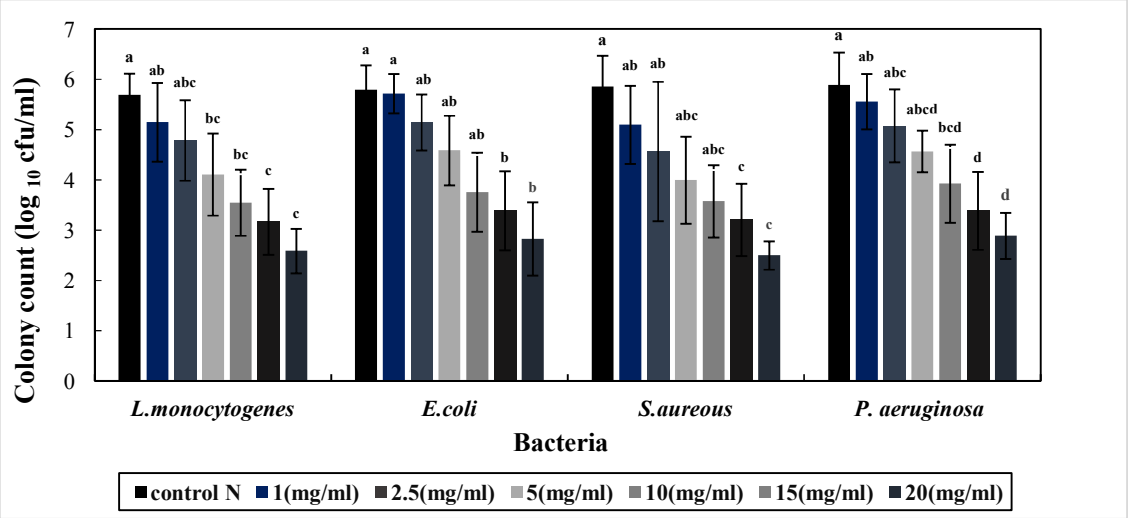

Fig. 1. Antimicrobial effects of biologically active starch film containing *Z. multiflora* essential oil on different bacterial species using plate count assay

Values followed by different small letters within each bacteria are significantly different (P<0.05) according the Tukey's test

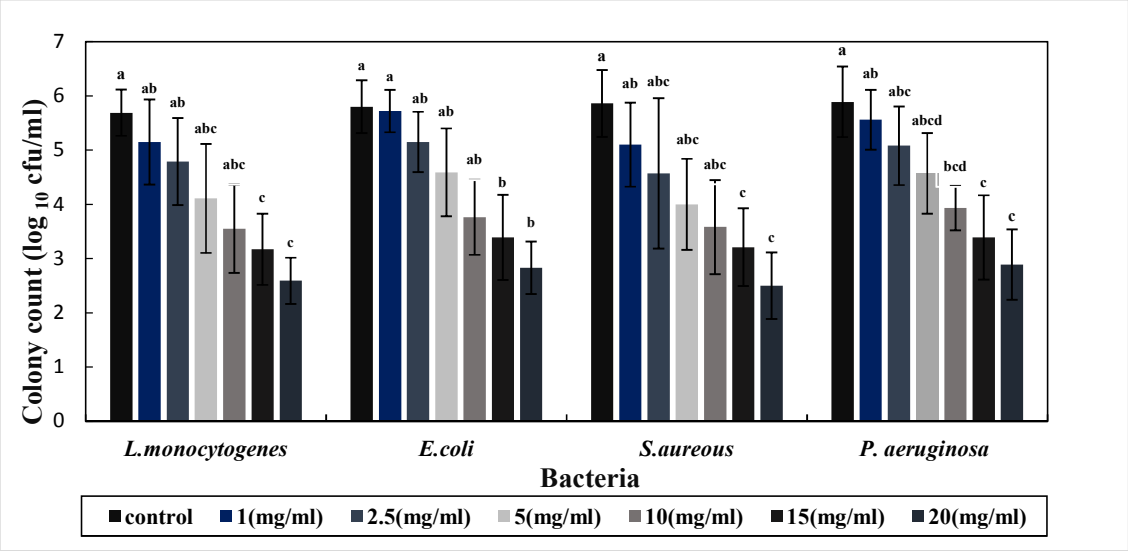

Fig. 2. Antimicrobial effects of biologically active starch film containing *B. persicum* essential oil on different bacterial species using plate count assay.

Values followed by different small letters within each bacteria are significantly different (P<0.05) according the Tukey's test

strains, in comparison with the control group (without film), had a significant difference (P<0.05). In the case of *Z. multiflora*, in the concentration of 20 mg/ml, there is a significant difference between *E. coli* and other bacteria (P <0.05). Also, there was no significant difference between the two essential oils for all bacteria in 1 and 2.5 mg/ml concentrations (P <0.05).

There are several approaches to control food microbial growth, although application of antimicrobial films is a known effective one (36). Volatile antimicrobial compounds used in food packaging through migration, and non-volatile compounds through dissolution can display antimicrobial activities [37]. The superiority of edible films is that they can carry food additives like flavors, antimicrobial compounds, enzymes, and paints. Adding antimicrobial and antioxidant compounds to edible films, besides the fact that they improve physical and mechanical properties, could also control chemical degradation, improve color, and control the growth of food microorganisms [38]. In this method the antimicrobial compounds are not added to the foodstuff directly, thus the possibility of alteration in their composition is not observed. Another advantage of this method is the gradual release of antimicrobial agents into the foodstuff and the protection of volatile antimicrobial compounds of essential oils.

4. CONCLUSION

The result of the study showed that biodegradable starchy films containing natural essential oils have good inhibitory effect on food pathogens and can be used as an active ingredient in food industry to enhance safety and to prolong foods' shelf life.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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