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The Use of Chitosan and Whey Protein Isolate Edible Films Incorporated with *Zataria multiflora* Boiss. Essential Oil as an Active Packaging Ingredient Against Some Common Foodborne Bacteria

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ABSTRACT: In this study, antimicrobial activity of chitosan and whey protein isolate edible films incorporated with different concentrations of *zataria multiflora* Boiss essential oil ;ZEO (1%, 2%, 3% and 4% v/v) and *zataria multiflora* Boiss essential oil nanoliposome (0, 1, 2, 3 and 4%) against *Listeria monocytogenes* (ATCC 19118), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 65138), *Escherichia coli* O157:H7(ATCC 35218) and *vibrio parahaemolyticus* (ATCC 43996) were evaluated using *disc-diffusion* test. The circular disks containing ZEO and NZEO were cut and placed on bacterial lawn. Plates were incubated for 24 h at 35˚C. The inhibition zone diameter was measured by digital caliper. These inhibition zones were formed against *Listeria monocytogenes* (ATCC 19118), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 65138), *Escherichia coli* O157:H7(ATCC 35218) and *vibrio parahaemolyticus* (ATCC 43996). The results of this study indicated that by increasing the concentration of ZEO in both whey protein isolate and chitosan films, a significant increase was observed in inhibition zones' diameters ($P<0.05$). The greatest antimicrobial effect was achieved at 4 % concentration of essential oil against *Listeria monocytogenes* in both films. Whereas films containing NZEO did not show any significant antimicrobial activities at the highest concentrations. According to the results of the GC/MS analysis, *Zataria multiflora* Boiss. essential oil is a rich source of different antimicrobial compounds especially thymol and carvacrol which might be considered as the main reasons of the observed antimicrobial activities in the formulated biofilms. The results of this study indicated that whey protein isolate and chitosan edible films containing *Zataria multiflora* Boiss essential oil showed a good antimicrobial effect and this may be considered as an advantage to use them as an active packaging ingredient in food products.

Keywords: *Antimicrobial Activity, Chitosan, Whey Protein Isolate, Zataria multiflora Boiss.*

Introduction

Recent foodborne microbial outbreaks have created an overwhelming need for new approaches to inhibit microbial growth on food surfaces and yet ensuring the quality maintenance and safety margin of these products. In food products the microbial contamination has the highest intensity on the surfaces, thus controlling microbial growth on surface of these products is of great importance. A new method in food preservation is active packaging that refers to using films or coatings incorporated with antimicrobial agents. Implementation of films as an active packaging is more advantageous than direct addition of

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antimicrobials, because some components of foods can inhibit the effects of antimicrobials, furthermore when used in films, antimicrobials can migrate to food surfaces selectively and gradually. Films can be manufactured from various materials including proteins and polysaccharides (Cortez, 2012). Antimicrobial packaging using edible films incorporated with antimicrobial agents such as bacteriocins, enzymes, organic acids, essential oils and etc. has been found to be highly effective in inhibiting the growth of pathogenic microorganisms (Ramos *et al*., 2012). Chitin is a polysaccharide and is the predominant constituent in outer skeleton of insects and crustaceans. The chemical structure of chitin resembles that of cellulose but a hydroxyl group is substituted with an acetyl amine group on each monomer (Kumar *et al.*, 2004). Removal of acetyl groups in chitin results in formation of chitosan. Chitosan is not soluble in water but is soluble in diluted acid solutions (below its PK_a , which is almost 6.3) (Shepherd *et al*., 1997). Chitosan films are prepared by dipping or casting in diluted acid solutions (Assis *et al*., 2002). According to some studies chitin and chitosan have shown antimicrobial effects against wide range of microorganisms including algae, bacteria, yeast and fungi (Goy *et* al., 2009). however recently a study reported that chitosan solutions show strong antimicrobial effects but chitosan films, prepared from the very same solution, lack these effects (Foster *et al*., 2011). Chitosan is an edible and biodegradable film and most studies have reported that it can be used in active packaging (Rodríguez-Núñez *et al*., 2012).

Whey protein isolate (WPI) films are edible and biodegradable. These films have attracted the attention in recent decades because they are a by-product of cheese makering industries and therefore are easily available and reasonably inexpensive and they also demonstrate extremely good mechanical features and have an oxygen barrier function (Ramos *et al*., 2012).

Incorporation of an antimicrobial agent in films used in active packaging, inhibits the microbial growth on the surface of foods (Santiago-Silva *et al.,* 2008). Due to the growing awareness of consumers regarding synthetic chemical additives, there have been a considerable interest in using natural antimicrobials in food industry (Shakeri *et al.,* 2011). Essential oils are natural, volatile oily compounds that are derived from several parts of plants. Many studies have reported a truly remarkable antimicrobial properties for Essential oils. In nature these compounds protect the plants from several pathogenic microorganisms (Bakkali *et al*., 2008). Essential oils are usually prepared by steam and hydrodistillaton (Khanjari *et al*., 2013). According to the literature, essential oils that have more phenolic concentrations show stronger antimicrobial effects. Thymol and carvacrol are examples of phenolic compounds found in some essential oils (Burt, 2004). *Zataria multiflora* Boiss is a plant that is a member of Lamiaceae family. It grows in central and southern parts of Iran, Pakistan and Afaghanistan (Basti *et al*., 2007). The essential oil derived from this plant contains considerable amounts of phenolic compounds, especially thymol and carvacrol (Sharififar *et al*., 2007). This plant is known as Avishan-e-Shirazi and is used as a flavoring agent in a wide range of Iranian foods (Basti, 2007). It has extremely beneficial properties such as having anti-nociceptive, antimicrobial, anti inflammatory and spasmolytic effects (Avaei, 2015).

In order to overcome Essential oils' limitations (including thermal degradation, change of flavour or function, oxidation and etc.), it seems a good idea to use nanoparticles. Nanoparticles are also capable to delay and sustain the release of essential oil from the product (São Pedro, 2013)

The aim of this study is to evaluate the antimicrobial effects of chitosan and whey protein isolate edible films incorporated with *Zataria multiflora* Boiss essential oil on some foodborne pathogenic bacteria.

Materials and Methods

- *Extraction of the Essential Oil*

The essential oil was obtained from dried aerial parts of the plant by steam distillation method for three hours, using a Clevenger - type apparatus. Traces of water was removed using anhydrous sodium sulphate. Chemical composition of the essential oil was analysed using Gas Chromatography Mass Spectrometry (GC_MS). The obtained essential oil was stored in falcons covered with aluminum foil at 4°C.

- *Preparation of liposomes*

In order to prepare liposomes, phosphatidylcholine (10mg/ml) and cholesterol (5 mg/ml) were dissolved in ethanol (10ml) by stirring, the solution was then injected to 20ml of ultra pure water using magnetic stirring by syringe pump. The combined solution was subjected to magnetic stirring (400 rpm). After the formation of liposomes, the solution was stirred for another 15 minutes. Eventually the ethanol and water phases were removed using rotary evaporator. The suspension of nano liposomes was then kept at 4^0 C.

- *Test Organisms*

The Lyophilized cultures of *Listeria monocytogenes* (ATCC 19118), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 65138), *Escherichia coli* O157:H7(ATCC 35218) and *vibrio parahaemolyticus* (ATCC 43996) were grown twice in a tube containing 10 mL of BHI (Brain Heart Infusion) broth (Merck, KGaA, Darmstadt, Germany) at 35°C for 18 h (or 6h for *vibrio parahaemolyticus*) followed by streaking on BHI agar (Merck, KGaA) slant and incubation under the same condition. The cultures were stored at 40°C (or ambient temperature 22–25^0C) and subcultured monthly.

- *Preparation of Inoculum*

Microorganisms inoculums were prepared by transferring cells from working culture to tube of BHI broth (containing1% NaCl for *vibrio parahaemolyticus*). After 18h incubation at 35°C (or 6h for *V. parahaemolyticus*), the second subculture was prepared and incubated for 18h at 35^0C (or 6h for *V. parahaemolyticus*). The broth cultures were placed in a 13 ×100-mm sterile cuvett and the optical density (OD) was adjusted to the absorbance of 0.1, using a spectronic 20 spectrophotometer (Milton Roy Company, Ivyland, PA). This adjustment gave a cell concentration for all microorganisms. The number of the cells in the suspension was estimated by duplicate plating from 10-fold serial dilutions on BHI agar and counting the colonies after 24h incubation at 35°C.

- *Preparation of Chitosan based films*

Sigma aldrich chitosan (DDA;Degree of deacetylation;> 75%) was dissolved in an aqueous solution of acetic acid (1%v/v) to obtain a concentration of 2% (w/v). This solution was stirred on a hot plate with magnetic stirrer for 24 hours. The solution was then filtered using a whatman number three filter paper and undissolved particles were removed by vacuum filtration. Glycerol was added to the solution (0.5 ml/g) as a plasticizer. Tween 80 was also added to the solution at the level of 0.25% (v/v) as an emulsifier. The resulting solution was stirred for 30 minutes at room temperature. The pH was adjusted to 5.8 using 1.0 N NaOH. Different concentrations of nanoliposome essential oil were added to the solution (0%, 1%, 2%, 3% and 4% v/v). The solution was homogenized at 8000 rpm for three minutes and then it was carefully poured into glass sterile petri dishes and

dried for 36 hours at room temperature. The films were detached from the dishes and their thickness was measured using digital calipers. All the films were stored in a desiccator containing sodium bromide 48h prior to the testing (Pranoto, 2005)

- *Preparation of whey protein based films*

Whey protein isolate (Davisco) was dissolved in distilled water to obtain a concentration of 5% (w/v). Glycerol was added to the solution (5% w/v) as a plasticizer. The pH was adjusted to 8 using 1.0 N NaOH. The solution was heated to 90 °C and stirred persistently. During the last five minutes of heating the candelilla wax (0.6-0,8%) was added to the solution. The solution was then filtered using a whatman number three filter paper. Different concentrations of essential oil and nanoliposome essential oil were added to the solution (0%, 1%, 2%, 3% and 4% v/v). The solution was homogenized at 8000 rpm for three minutes and then was subjected to vacuum filtration in order to remove undissolved particles. The solution was carefully poured into glass sterile petri dishes and dried for 36 h at room temperature. The films were detached from the dishes and were stored in a desiccator containing sodium bromide 48h before testing (Shakeri *et al*, 2011).

- *Chemical analysis of Zataria multiflora Boiss essential oil*

This was carried out according to Eftekhar et al. (2011).

- *Determination of antimicrobial activity of the films*

The antimicrobial activity of chitosan and WPI films was evaluated using disc diffusion test. For tested microorganisms, 15 mL of BHI agar was inoculated by 100 µL of bacterial suspension containing approximately 1×10^7CFU mL^{-1} of the tested bacteria. Films were cut into 9 mm diameter discs and put on the Muller-Hinton plate surface and then plates were incubated at 37° C for 24 h. Inhibition zones' diameters around the films were measured by a digital caliper.

- *Statistical analysis*

Statistical analysis using SPSS 16 software and one way anoval test was applied.

Results and Discussion

- *Chemical composition of Zataria multiflora Boiss essential oil*

The chemical components of *Zataria multiflora Boiss* EO, analyzed by GC_MS technique, are summarised in Table 1. The main compound of EO was carvacrol that accounted for 73.64% of the total composition.

- *Antimicrobial activity of the films*

Table 2 shows the antimicrobial effects of whey protein isolate based films incorporated with *Zataria multiflora* Boiss. essential oil against the selected bacteria. The results indicated that increasing the concentration of EO resulted in a statistically significant increase in inhibition zones' diameters. The maximum and minimum antimicrobial effects (at 4 % concentration of EO) were observed against *Listeria monocytogenes* and *Escherichia coli* respectively. Figure 1 shows the zone of inhibition for each bacteria (at 2 % concentration of EO), cultured on whey based films incorporated with *Zataria multiflora* Boiss. essential oil.

Table 3 shows the antimicrobial effects of chiotsan based film incorporated with *Zataria multiflora* Boiss. essential oil against the selected bacteria. It is perfectly clear that by increasing the concentration of EO resulted in a statistically significant increase in inhibition zones' diameters. The greatest antimicrobial effect (at 4 % concentration of EO) was achieved against *Listeria*

monocytogenes and the lowest antimicrobial activity was observed against *Staphylococcus aureus* and *Escherichia coli*. Chitosan films alone, showed no obvious antimicrobial activity. Figure 2 shows the zone of inhibition for each bacteria (at 2 % concentration of EO), cultured on chitosan based films incorporated with *Zataria multiflora* Boiss essential oil. Chitosan and WPI films incorporated with NZEO did not show any inhibitory effects on all tested microorganisms.

Table 1. Chemical composition of *zataria multiflora* Boiss. essential oil as identified by gas chromatography–mass spectromy

Compound	Retention Index	percentage
Alpha-pinene	936	0.63
1-Octen-3-ol	982	0.29
3-Octanone	988	0.75
Beta-myrcene	992	0.56
3-Octanol	996	0.34
1-Phellanderene	1003	0.27
Alpha-tripenene	1017	0.76
Beta phellanderene	1031	0.33
1,8-Cineole	1033	0.52
Gamma-terpinene	1062	2.27
Alpha-tripinolene	1088	0.26
Linalool	1101	1.28
Thymyl methyl ether	1236	0.62
Carvacrol methyl ether	1248	3.15
Thymol	1294	4.31
Carvacrol	1320	73.64
Isopiperitone	1365	0.59
Carvacryl acetate	1381	1.7
Trans-caryophellene	1428	2.93
Alpha-humulene	1460	0.35
(+) Spathulenol	1587	0.58
Caryophellene oxide	1595	0.83
Sum		96.96

Table 2. Inhibition zones (mm) of whey protein isolate film incorporated with different concentrations of *zataria multiflora* Boiss. essential oil on *Listeria monocytogenes* (L.M), *Bacillus cereus* (B.C), *Staphylococcus aureus* (S.a), *Escherichia coli* O157: H7 (E.coli) and *vibrio parahaemolyticus* (V.P)

***Zataria Multiflora* EO (%) Bacteria**	**0**	**1**	**2**	**3**	**4**
Escherichia coli O157: H7	0	14.7± 0.6	26.7 ± 2.1	25.3 ± 2.1	27.7 ±1.5
Bacillus cereus	0	15.3 ± 1.5	25.7 ± 0.7	31.7 ± 1.5	33.3 ± 2.1
Listeria monocytogenes	0	10.7 ± 0.6	25.1± 0. 8	27.4 ± 0.5	41.0 ± 1.0
Staphylococcus aureus	0	17.2 ± 0.8	21.0 ± 1.0	27.7 ± 0.6	37.0 ± 2.6
vibrio parahaemolyticus	0	18.0 ± 0.9	25.3 ± 0.6	30.0 ±1.0	37.0 ± 1.0

Table 3. Inhibition zones (mm) of chitosan film incorporated with different concentrations of *zataria multiflora* Boiss. essential oil on *Listeria monocytogenes* (L.M), *Bacillus cereus* (B.C), *Staphylococcus aureus* (S.a), *Escherichia coli* O157: H7 (E.coli) and *vibrio parahaemolyticus* (V.P)

***Zataria Multiflora* EO (%) Bacteria**	**0**	**1**	**2**	**3**	**4**
Escherichia coli O157: H7	0	16.3 ± 0.6	27.7 ± 1.5	32.7 ± 1.5	39.1 ± 0.9
Bacillus cereus	0	21.3 ± 1.5	28.1± 0.9	31.7 ± 1.5	42.0 ± 2.0
Listeria monocytogenes	0	25.3 ± 1.5	34.0 ± 1.2	34.0 ± 2.6	43.3 ± 1.5
Staphylococcus aureus	0	21.7 ± 2.5	28.0 ± 0.9	31.7 ± 1.1	33.0 ± 1.0
vibrio parahaemolyticus	0	22 .0 ± 2.0	28.3 ± 0.6	33.0 ± 0.9	37.3 ±1.5

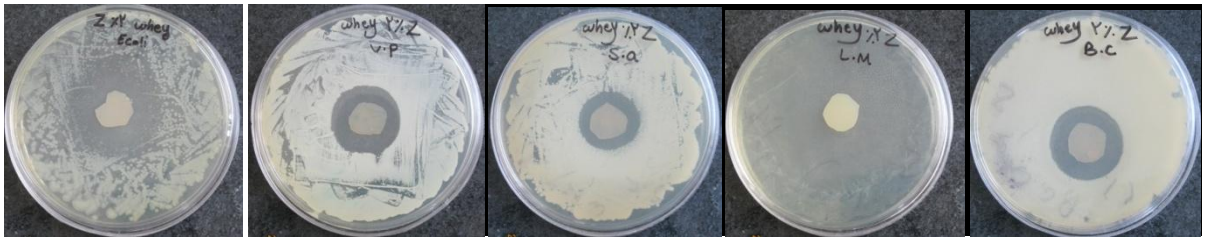

Fig. 1. Inhibition zones of Whey protein isolate films incorporated with 2% zataria multiflora Boiss. essential oil on *Listeria monocytogenes* (L.M), *Bacillus cereus* (B.C), *Staphylococcus aureus* (S.a), *Escherichia coli* O157: H7 (E.coli) and *vibrio parahaemolyticus* (V.P)

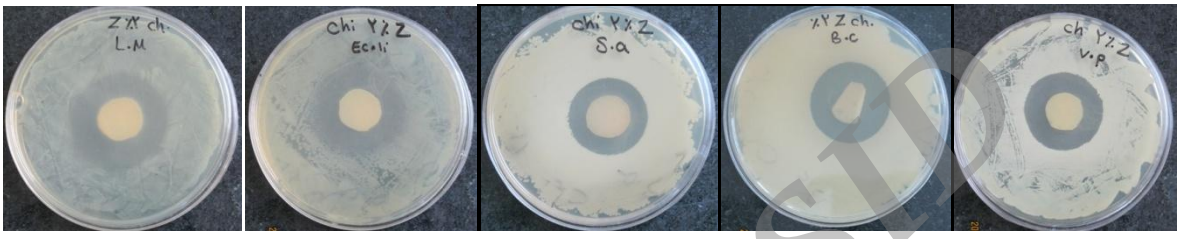

Fig. 2. Inhibition zones (mm) of chitosan films incorporated with 2% *zataria multiflora* Boiss. essential oil on *Listeria monocytogenes* (L.M), *Bacillus cereus* (B.C), *Staphylococcus aureus* (S.a), *Escherichia coli* O157: H7 (E.coli) and *vibrio parahaemolyticus* (V.P)

The results of this study showed that carvacrol is the main constitue of zataria multiflora Boiss essential oil, in contrast to the present study, Shakeri *et al*. (2011) reported thymol as the major compound of *Zataria multiflora* Boiss essential oil. Carvacrol is a monoterpenic phenol which is one of the key components of some aromatic plants such as oregano and thyme and has been used as a flavoring and food preservative agent in food industry. The results from various in vitro and in vivo studies have illustrated that carvacrol contains extensive range of biological features namely, antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory, hepatoprotective and vasorelaxant properties among others. Besides, carvacrol demonstrates its antimicrobial properties by means of causing damage to the structure and function of cellular membrane (Suntres *et al*., 2015). The chemical composition of essential oils varies in the same species of plant due to the season, climate and growing conditions and these differences make it unique (Khanjari *et al*., 2013).

According to the results, both whey and chitosan films incorporated with *Z. multiflora* Boiss showed stronger inhibitory effects against gram positive bacteria as compared to gram negative bacteria. These results showed close agreement with the findings of Hosseini *et al*. (2009). This finding could be due to different structures of cell walls of gram positive and gram negative bacteria. Peptidoglycan forms up to 95% of the cell wall of gram positive bacteria while it only composes 20% of cell wall in gram negative bacteria. The differences in structure resulted in more sensitivity in gram positive bacteria against phenolic compounds of essential oil (Nazzaro *et al.*, 2013).

The present study showed the maximum and minimum inhibitory effects of chitosan films incorporated with *Z. multiflora* Boiss against *Listeria monocytogenes* and *Escherichia coli* respectively. This finding was in definitive agreement with the work carried out by Mehdizadeh *et al*. (2012).

Foster *et al.* (2011) reported that chitosan solutions that were used to prepare films

showed noticeable antimicrobial activity but chitosan films prepared from the same solution lacked any obvious antimicrobial properties. This finding suggests that the final biomaterial morphology of chitosan can have an impact on its antimicrobial features. These findings were similar to the results of the present study.

Liolios *et al.* (2009) reported that the antimicrobial activities of thymol and carvacrol both were increased after nanoencapsulation. Unlike the findings by these researchers, our study indicated that nanoencapsulated forms of EO did not show any antimicrobial effects. This might be due to different type of EO or different way of preparing nanoencapsulated EO.

Nanoencapsulated forms of EO have a liposome coating and that is why they are released more slowly as compared to the free essential oils. They also have less content of EO as compared to free forms. These might be the reasons of not observing antimicrobial activity from nanoencapsulated forms of EO in present study.

Conclusion

The results of this study revealed that chitosan and whey edible films incorporated with *Z. multiflora* Boiss essential oil have an extremely good inhibitory effect against common food borne pathogenic bacteria and can be used as an active packaging ingredient in food industry in order to guarantee the safety and prolong the shelf life of the food. However further research works and studies are required in order to evaluate the organoleptic and other effects of this active packaging. It is hoped that these findings will open up new horizons in food safety and hygiene.

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