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Original article

Antimicrobial activity of *Zataria multiflora* Boiss. essential oil incorporated with whey protein based films on pathogenic and probiotic bacteria

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Summary The antimicrobial potential of whey protein isolate (WPI) edible films containing 1–4% (v/v) *Zataria multiflora* Boiss. essential oil (EO) on food-borne pathogenic bacteria (*Escherichia coli, Salmonella enteritidis, Staphylococcus aureus* and *Bacillus cereus*) and probiotic bacteria (*Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus casei* subsp. *casei*) was evaluated. WPI films incorporated with 2% (v/v) of this EO inhibited the growth of all tested pathogenic bacteria and gram-negative bacteria were more sensitive than gram-positive bacteria. Incorporation of the EO at higher than 2% (v/v) showed significant antimicrobial effects ($P < 0.05$) for *S. enteritidis* and *L. acidophilus*. The growth of all probiotic lactic acid-producing bacteria also inhibited when 2% of the EO was added. Comparison of an image processing-based method with conventional method for measuring of inhibitory effects of edible films exhibited high correlations ($R^2 \geq 0.876$) between the two methods. These results revealed that *Z. multiflora* Boiss. EO is a good antimicrobial additive for some food applications when included into WPI edible films.

Keywords Edible films, image processing, probiotic, *Zataria multiflora* Boiss.

Introduction

One of the main reasons for packaging of many foods is to prevent surface growth where a large portion of spoilage and contamination occurs. For the same reason application of antimicrobial agents to packagings has met major advances in food packaging, which plays an important role in reducing the risk of pathogen contamination, as well as extending the shelf life of foods (Appendini & Hotchkiss, 2002; Gennadios, 2002; Cha & Chinnan, 2004).

In line with safety improvement, good deal of researches has been focused on incorporation of antimicrobials with natural compounds into the edible films (Cha & Chinnan, 2004). Edible films and coatings from casein, whey protein and total milk proteins have been discussed in detail. In general, the resistance to water vapour transmission of protein films is limited because they are highly polar polymers with a high level of hydrogen bonding and hydroxyl groups (Gennadios, 2002). Therefore, the incorporation of agents such as fatty acid esters, to decrease the water vapour permeability (WVP), is necessary when foods are coated with these films. However, it is shown that whey protein isolate (WPI) films can sustain their structural integrity at the high a_w of food surface and serve as effective carriers of antimicrobials (Zinoviadou *et al.*, 2009; Pintado *et al.*, 2010).

In recent years, the food industry showed an increasing interest in natural antimicrobials such as plant essential oils (EOs) because of greater consumer awareness and concern regarding synthetic chemical additives (Holley & Patel, 2005). A wide range of microorganisms have been subjected to investigations using EOs as antimicrobial reagents (Hammer *et al.*, 1999; Holley & Patel, 2005; Pranoto *et al.*, 2005; Seydim & Sarikus, 2006; Gómez-Estaca *et al.*, 2009). The phenolic components are chiefly responsible for the antibacterial properties of EOs (Burt, 2004). *Zataria multiflora* Boiss. (ZMB) is a spice plant belonging to the Laminaceae family that geographically grows mainly in Iran, Pakistan and Afghanistan (Ali *et al.*, 2000). This plant, known as Avishan-e-shirazi (in Iran), is used as a flavour

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agent in a variety of Iranian foods (Basti *et al*., 2007; Gandomi *et al*., 2009). The main constituents of the EO of this plant are phenolic compounds such as carvacrol and thymol (Shaffiee & Javidnia, 1997; Ali *et al*., 2000; Sharififar *et al*., 2007) that received the approval of FDA for use as food additives and could be applied in antimicrobial food packagings (Suppakul *et al*., 2003). Although some medicinal and antimicrobial effects of EO and extract of this plant have been reported (Hosseinzadeh *et al*., 2000; Ramezani *et al*., 2004; Basti *et al*., 2007; Sharififar *et al*., 2007; Zarei *et al*., 2007; Gandomi *et al*., 2009), their possible application as components of edible films remained untouched.

On the other hand, food products can be added with some beneficial bacteria that required for the desired taste and texture or should be viable during storage of the product before reaching the consumer as probiotic bacteria. The most well-known probiotics are lactic acid-producing bacteria (LAB) (Marth & Steele, 2001). To minimize the risk of loosing the useful microorganisms, it is clear that antimicrobial food packaging would also need to be evaluated for the activity against probiotic bacteria related to the potential health or nutritional benefits. However, the antimicrobial impact of several EOs in culture medium or when included into the edible films, on some strains of LAB, have been reported (Ouattara *et al*., 2000; Sagdic *et al*., 2003; Seydim & Sarikus, 2006; Gómez-Estaca *et al*., 2009). The aim of this work was to test the efficacy of ZMB EO as natural antimicrobial additive incorporated with edible films based on WPI, covering both foodborne pathogenic bacteria and some of the beneficial LAB.

Materials and methods

Microorganisms and cultures

Lyophilized cultures of *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus rhamnosus* (ATCC 7469), *Lactobacillus plantarum* (ATCC 8014) and *Lactobacillus casei* subsp *casei* (ATCC 39392) were obtained from Iranian Research Organization for Science and Technology (Tehran, Iran). All lactobacilli were activated in de Man Rogosa Sharpe (MRS) broth (Merck-Darmstadt, Germany) at 37 °C followed by streaking on MRS agar slants and incubation under the same conditions. The cultures were stored at 4 °C and sub-cultured monthly.

In addition, four pathogenic bacteria used in this study were *Escherichia coli* (NCTC 12900), *Salmonella enteritidis* (RITCC 1621), *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (isolated from food). They were obtained from the Food Hygiene Department of Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran and grown aerobically in Brain Heart Infusion (BHI) agar (Merck-Darmstadt, Germany) at 37 °C for 24 h and kept at 4° C.

Essential oil production

ZMB was collected from Shiraz (a city in Iran) and identified by Institute of Medicinal Plants, Tehran, Iran. The EO was obtained from air-dried aerial parts of the plant by steam distillation method as described by Basti *et al*. (2007) and analysed using gas chromatography mass spectrometry (GC–MS). It contained carvacrol as the main component (71.12%).

Preparation of antimicrobial edible film

Production of WPI films was based on the method of Kim & Ustunol (2001) with slight modifications. Whey protein isolate (Armor Proteines, Saint- Brice en Cogles, France) was dissolved in distilled water to obtain film forming solutions of 5% (w/v) concentration and glycerol (Sigma Chemical Co., St. Louis, MO, USA) (4% w/v) was added. After mixing and adjusting the pH to 8.0 with 2.0 N NaOH, the solutions were heated to 90 ± 2 °C while being stirred continuously. Following the addition of candelilla wax (Sigma-Aldrich Inc., Steinheim, Germany) (0.6%, w/v) during the last 5 min of heating, the solutions were homogenised for 1 min at 11000 r.p.m. (rotor diameter = 7.5 mm) using a homogenizer (Ultra-Turrax T25 basic; IKA-WERKE GMBH & Co., Staufen, Germany), filtered through cheesecloth and cooled. ZMB EO at 1%, 2%, 3% and 4% (v/v) concentrations were added to the film solutions. After homogenising for 2 min at 13000 r.p.m. (rotor diameter = 7.5 mm), solutions were shook at room temperature for 1.5 h to remove air bubbles. The solutions (12 mL) were cast onto 8.2 cm-dia Petri dishes and allowed to dry in an oven at 35 °C for 36 h. Dried films were peeled and stored to evaluation. Thickness of the films was determined with a micrometer (Digimatic Micrometer, Mitutoyo Ltd, Andover, UK; having a sensitivity of 0.01 mm) at twelve random locations of the film sheets. Average film thickness was 0.16 mm.

Determination of antimicrobial activity of the films

The antimicrobial activity of the films was tested following a plate diffusion assay. For all tested microorganisms, 10 mL of BHI agar (1%) was inoculated by 100 μL of bacterial cultures containing approximately 10^7–10^8 CFU mL^{-1} of the test bacteria. The edible films were cut into 9 mm diameter discs and then laid onto the plate surface. The plates were incubated aerobically at 37 °C for 24 h whereas for *L. acidophilus* they were incubated at 37 °C for 48 h in a CO_2 chamber. Diameter measurements of the clear zone around the film discs (inhibition zones) were made. Two methods were used for quantifying the antimicrobial activity of the films, which included (i) measuring the diameter of the zones with a calliper (mm). (ii) It was performed with ImageJ

1.38x software (National Institutes of Health, Bethesda, Maryland, USA) for digital image analysis (mm).

Images of the plates were taken using a colour digital camera (model IXUS 950 IS, Canon Inc., Tokyo, Japan) with no flash. The maximum resolution of the camera was 8.0 Megapixel. Feret's diameter for the inhibition zones and plates was measured using ImageJ software. Assuming the plate's diameter was 82 mm, the diameter of the inhibition zone was calculated as

Inhibition zone (mm) $= (82 \times D_Z)/D_P$

where D_Z is Feret's diameter of the zone on the surface of agar plate, D_P is Feret's diameter of the same agar plate. Analyses were replicated nine times.

Statistical analysis

Sigma Stat for windows software (Sigma Stat 1.0, 1994, Jandel Coporation, San Rafael, CA, USA) was used for statistical tests. One way ANOVA procedure followed by least significant difference (LSD) test was performed to determine any significant differences among the treatments at a 95% confidence interval. Hypothesis of differences between results of quantitative methods (calliper/image processing) was tested using paired *t*-test. The correlation between both methods was determined using linear regression analysis.

Results and discussion

Comparison of two quantitative methods

According to the results shown in Table 1, no significant difference was observed between image processing-based method and calliper method for the most examined bacteria. In this experiment, there was a high correlation ($R^2 \geq 0.876$) between the results obtained from both methods. These findings indicate that it is reasonable to use image processing-based method to quantify antimicrobial activity of edible films.

For testing the effectiveness of antimicrobial packagings in the food, agar plate method has been used in the same of those used to evaluate antimicrobial alone (Appendini & Hotchkiss, 2002; Fisher & Phillips, 2008). In this method, the diameter of the clear zones surrounding the films is measured, mostly with a calliper (Cagri *et al*., 2001; Sagdic *et al*., 2003; Seydim & Sarikus, 2006; Pintado *et al*., 2010). Over the past 30 years, there has been much interest in the automatic processing and analysis of digital images. Computer vision is being used increasingly by the food industry because it provides an alternative for automated, non-destructive and cost-effective techniques for determination of food quality (Brosnan & Sun, 2004). The current study showed another application of these techniques for assessment of food safety.

Antimicrobial effects of films against pathogenic bacteria

Table 2 shows the antimicrobial activity of WPI films containing ZMB EO against pathogenic bacteria. It is

Table 2 Antimicrobial activity of WPI film incorporated with *Zataria multiflora* Boiss. essential oil (EO) against pathogenic bacteria using the image processing method

Bacteria	Concentration of essential oil (%) in film solution	Inhibition zone (mm)
Escherichia coli	0 (control)	0.00^{d}
	1	12.9^{c}
	2	16.0^{b}
	3	16.6^{ab}
	4	19.0^{a}
Salmonella enteritidis	0 (control)	0.00^{e}
	1	12.6^{d}
	2	15.2^{c}
	3	17.2^{b}
	4	19.1^{a}
Staphylococcus aureus	0 (control)	0.00^{c}
	1	0.00^{c}
	2	12.65^{b}
	3	13.90^{a}
	4	14.33^{a}
Bacillus cereus	0 (control)	0.00^{b}
	1	0.00^{b}
	2	19.09^{a}
	3	18.3^{a}
	4	21.79^{a}

WPI, whey protein isolate.

Values (n = 9) with different letters indicate significant differences ($P < 0.05$).

Table 1 The significances of the differences between calliper and image processing-based methods (paired student's *t*-test) and the significant correlations ($P < 0.01$) between two methods by performing linear regression

Bacteria	*Escherichia coli*	*Salmonella enteritidis*	*Staphylococcus aureus*	*Bacillus cereus*	*Lactobacillus acidophilus*	*Lactobacillus rhamnosus*	*Lactobacillus casei*	*Lactobacillus plantarum*
Student's *t*-test	$P = 0.0003$	n.s.	$P < 0.0001$	n.s.	n.s.	n.s.	n.s.	$P = 0.0053$
Linear regression	$R^2 = 0.969$	$R^2 = 0.976$	$R^2 = 0.998$	$R^2 = 0.984$	$R^2 = 0.904$	$R^2 = 0.938$	$R^2 = 0.972$	$R^2 = 0.876$

n.s., non-significant.

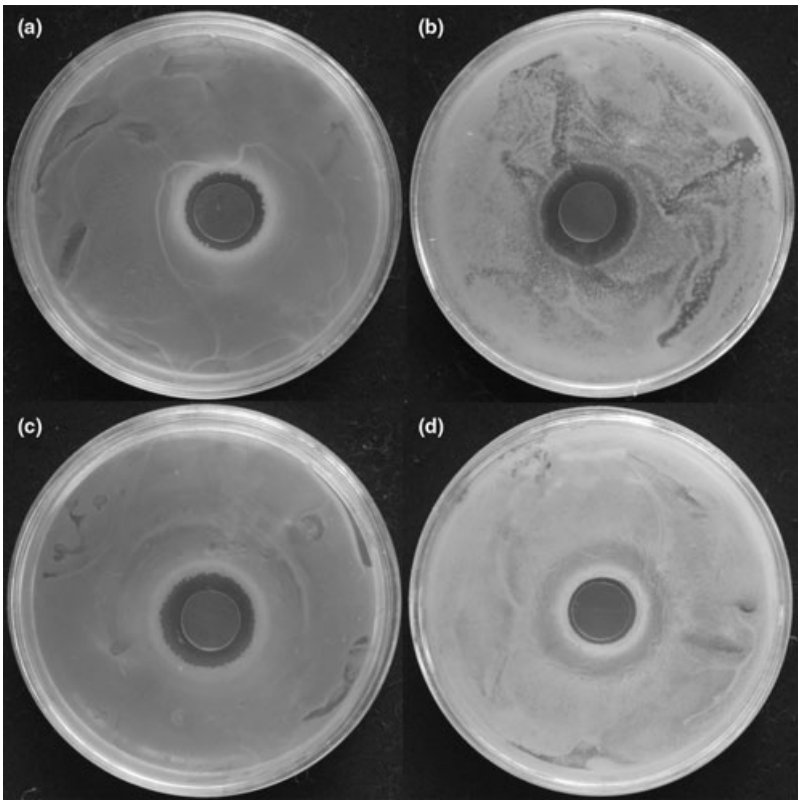

Figure 1 Inhibitory zones of whey protein isolate (WPI) film incorporated with 3% (v/v) *Zataria multiflora* Boiss. essential oil (EO) against (a) *Salmonella enteritidis*, (b) *Bacillus cereus*, (c) *Escherichia coli* and (d) *Staphylococcus aureus*.

clear that EO at 1% (v/v) is able to form a clear inhibition zone on *E. coli* and *S. enteritidis*, which are gram-negative bacteria, while the zone was not observed on tested pathogenic gram-positive bacteria at 1% level. For *S. enteritidis*, by increasing EO concentration, the zone of inhibition increased significantly ($P < 0.05$). According to the results, the inhibitory effects of WPI films were not affected by increasing EO concentrations beyond 3% for *E. coli* and *S. aureus* and 2% for *B. cereus* ($P > 0.05$). Figure 1 shows the images of the antimicrobial activity of WPI films incorporated with 3% EO against pathogenic bacteria.

It was found that the major components of ZMB EO with Iranian origin are phenolic compounds such as carvacrol that are responsible for the antimicrobial properties of this EO (Basti *et al*., 2007; Misaghi & Basti, 2007; Moosavy *et al*., 2008). Carvacrol is able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP (Burt, 2004). The direct application of ZMB EO demonstrated that $\log_{10}$ probability percentage of growth initiation of *Salmonella typhimurium* and *S. aureus* are affected by the values of EO (Basti *et al*., 2007). In this study, the EO was incorporated into a biopolymeric matrix and shown that they have the same effect as application of EO alone. Sharififar *et al*. (2007) reported that the EO of endemic ZMB strongly inhibited the growth of *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *Bacillus subtilis* ATCC 6051 and *Salmonella typhi* ATCC19430 especially the gram-negative strains. This is in agreement with our results (Table 2) that indicate that gram-negative strains are more sensitive than gram-positive bacteria to WPI films incorporated with EO.

In a previous study, WPI films containing 4% oregano EO (from Laminaceae) was shown to have the greatest zone of inhibition against *S. enteritidis* and *S. aureus* (Seydim & Sarikus, 2006). The study showed that the zone of inhibition increased significantly for *S. enteritidis*, *E. coli* O157:H7 and *S. aureus*, when the EO concentration was increased. In another work, it was pointed out that the use of WPI films containing the highest level of oregano oil (1.5% w/w in the film forming solution) resulted in a significant reduction of pseudomonad population during the entire beef's storage period (Zinoviadou *et al*., 2009). In general, it can be concluded that a higher concentration of EO is required to achieve the same antimicrobial effect in edible films *in vitro*. The composition of film material can also affect the migration mechanism of the antimicrobial agent into the food structure.

Antimicrobial effects of films on probiotic LAB

The results of inhibitory effects of edible films incorporated with ZMB EO against LAB is presented in Table 3. The bacteria selected here are probiotics which are important to be viable both in food products and in human gastrointestinal tract. As shown in Table 3, incorporation of EO at higher than 1% (v/v) started to exhibit a clear inhibitory zone on *L. rhamnosus* and *L. casei* subsp. *casei*, whereas a clear zone observed at 1% (v/v) level against *L. acidophilus* and *L. plantarum*. According to the results, *L. acidophilus* was found to be more sensitive to WPI films containing ZMB EO when compared with the other lactobacilli. Only for *L. acidophilus*, incorporation of the EO at higher than 2% (v/v) revealed significant antimicrobial effects ($P < 0.05$). It has been reported previously that an increase in concentration of oregano EO when incorporated into the WPI films was not effective for *L. plantarum* (DSM20174) above 2% (w/v) concentration (Seydim & Sarikus, 2006). These findings are in agreement with our results. In the work of Zinoviadou *et al.* (2009), 1.5% of oregano EO incorporated into the WPI films inhibited completely the growth of LAB population and increased the shelf life of fresh beef. Inhibitory activity of oregano, sage and thyme (all belong to Laminaceae family) extracts on *L. acidophilus* and *L. plantarum* has also been shown in the previous studies by Sagdic *et al.* (2003).

Table 3 Antimicrobial activity of WPI film incorporated with *Zataria multiflora* Boiss. EO against probiotic LAB using the image processing method

Bacteria	Concentration of essential oil(%) in film solution	Inhibition zone (mm)
Lactobacillus acidophilus	0 (control)	0.00[c]
	1	12.4[b]
	2	19.6[b]
	3	30.5[a]
	4	35.5[a]
Lactobacillus rhamnosus	0 (control)	0.00[b]
	1	0.00[b]
	2	13.24[a]
	3	16.5[a]
	4	15.38[a]
Lactobacillus casei subsp. *casei*	0 (control)	0.00[b]
	1	0.00[b]
	2	14.50[a]
	3	15.49[a]
	4	14.77[a]
Lactobacillus plantarum	0 (control)	0.00[b]
	1	12.8[a]
	2	13.2[a]
	3	13.5[a]
	4	13.9[a]

EO, essential oil; LAB, lactic acid-producing bacteria; WPI, whey protein isolate.

Values ($n = 9$) with different letters indicate significant differences ($P < 0.05$).

In conclusion, the results of the present work show that active compounds of ZMB EO could be immobilised in the WPI film and subsequently released, thereby inhibiting the target microorganisms. As a result of the inhibitor effects of this natural EO on the beneficial bacteria, such edible films are recommended only for non-probiotic food applications. With increased expectations for food products of high quality and safety standards, application of accurate and fast methods such as image processing technique for assessment of antimicrobial activity of edible films is very promising.

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