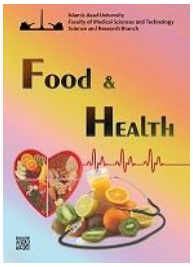

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Evaluations of the antibacterial effect of chitosan edible film contain ajwain essential oil on some foodborne pathogenic bacteria

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ABSTRACT

Biodegradable active packaging containing natural extracts derived from plants with antimicrobial properties is one alternative strategy that can be considered by the food packing industry to reduce the use of environmentally harmful synthetic polymers. Chitosan is a safe, natural, no allergen and biocompatible polymer with health benefits. The aim of this study was to investigate the effects of chitosan film containing different concentrations of ajwain essential oil on some foodborne pathogenic bacteria, including *Listeria monocytogenes* ATCC 19118, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 65138 and *Escherichia coli* O157:H7 ATCC 25922, and *Vibrio parahaemolyticus* ATCC 43996 with disk diffusion method was evaluated. The results showed that by increasing the concentration of essential oil, the inhibition zone's diameters were significantly expanded ($p<0.05$). The maximum and minimum antimicrobial effects of essential oil were observed against *S. aureus* (26.73mm) and *E. coli* O157:H7 (11.25 mm). The results of this study confirmed that the use of chitosan-based antimicrobial films incorporated with ajwain essential oil has a remarkable antibacterial effect and therefore it can be practical in active packaging in the food industry.

1. Introduction

Interest in edible films and coatings in recent years has been on the rise due to several advantages such as biodegradability, environmental friendliness, and extended shelf life. Edible films and coatings are generally manufactured from proteins, polysaccharides, and lipids used solely or in combination with each other (1). Chitosan a cationic polysaccharide made from the alkaline N-deacetylation of chitin is commercially prepared from shellfish processing waste (2). Chitosan produced by the deacetylation of chitin is widely used in phenolic preservation of some fruits such as pomegranate, litchi, and raspberries (3). Chitosan is effective against both gram-positive and gram-negative bacteria and fungi (2-4).

Essential oils are mixtures of compounds characterized by their capacity to generate flavor or aroma and are generally obtained from spices, aromatic herbs, fruits, and flowers (5). Ajwain (*Carum copticum*) is a grassy, annual an annual herbaceous essential oil-bearing plant belonging to the *Apiaceae* family, which grows in India, Iran, and Egypt (6). Hydrodistillation of ajwain fruits yields an essential oil consisting primarily of *thymol*, *gamma-terpinene*, and *p-cymene* as well as more than 20 trace compounds (predominately terpenoids), The ajwain fruit oil has several therapeutic effects, including diuretic, carminative, analgesic, anti-dyspnoea and, anti-inflammatory compounds, carminative, anti-spasmodic, stimulant, anti-dyspnea, analgesic, anti-asthma, anti-vomiting, antimicrobial and antioxidant activity, antiaflatoxigenic and antitermitic nature (6-10). To the best of our knowledge, there is a lack of data about using ajwain essential oil (AEO) in the edible film. Hence, the aim of this study was to investigate the effect of chitosan films containing different concentrations of AEO on some foodborne pathogenic bacteria.

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2. Materials and methods

2.1. EO extraction

Ajwain seeds were purchased from Shiraz (Iran). In the preparation phase, 150 grams of powdered seeds were mixed in 1 liter of distilled water. The oil extraction Hydro distillation was done in 3 h, using a Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia (6)[6]. The EO obtained were separated from the water and dried over anhydrous Na_2SO_4, and stored in dark glass bottles at 4 ºC prior to use (11).

2.2. GC and GC/MS analyses

Gas chromatography analysis was performed using HP 6890 gas chromatograph equipped with an FID and DB1 fused silica column (60 m × 0.25 mm i.d, film thickness 0.25 µm). The oven temperature was programmed at 250ºC at a rate of 4ºC/min. Injector and detector temperature were 250ºC and 265ºC, respectively. The carrier gas, helium, was adjusted to a linear velocity of 30 cm/s and 1 µl of sample dissolved in CH_2Cl_2 was injected. Gas chromatography/ mass spectrometry (GC-MS) analysis. GC conditions were the same as reported, and the same column was used. MS conditions were as follows: ionization voltage, 70 eV; the ion source temperature was 260ºC scan rate, 1 scan/s. Identifications of components of the oil were based on retention indices relative to normal alkanes and computer matching with the Wiley 275 library, as well as by comparison of the fragmentation patterns of mass spectra with those reported in the literature (12).

2.3. Preparation of edible film

Chitosan (medium molecular weight, Sigma-Aldrich Chemical Co.) solution was prepared with 1.5% (w/v) chitosan in 1% (w/v) acetic acid. To achieve complete dispersion of chitosan, the solution was stirred at room temperature overnight. The solution in beakers was placed into a hotplate/magnetic stirrer and glycerol was added to chitosan at 0.75 mL/g concentration as a plasticizer and stirred for 10 min. The resultant chitosan film solution was filtrated through a Whatman no. 3 filter paper to remove any undissolved particles. Then the ajwain essential oil (AEO), mixed with Tween 80 (Aldrich Chemical Co., Steinheim, Germany), and added to the chitosan solution. The final film solution consisted of 1.5% chitosan,1% acetic acid, 0.75% glycerol, 0.2% Tween 80 singly or incorporated with 0.5% and 1% ajwain essential oil (AEO). The final film-forming solution was homogenized under aseptic conditions at 21600 rpm for 1 min. The control solution was prepared without the addition of ajwain essential oil (AEO) (13).

2.4. Microbial strains

In this study, five microorganisms were used, namely *Listeria monocytogenes* ATCC 19118, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 65138 and *E. coli* O_{157}:H_7 ATCC 25922, and *Vibrio parahemolyticus* ATCC 43996. All these microorganisms were provided and authenticated by the Department of Food Hygiene, University of Tehran (Tehran, Iran).

2.5. Bacteria culture

Prior testing antimicrobial activity, each of the bacteria strain's inoculum was prepared by transferring cells from working culture to BHI broth. After 18 h incubation at 30 °C, the second subculture was prepared and incubated for 18 h at 30°C. Then sterile cuvette tubes containing 4ml brain heart infusion broth were provided in order to transfer different amount of the second culture to these tubes. The bacterial broth culture was adjusted to optical density (OD) (absorbance) of 0.1 at 600 nm, using a Spectronic 20 spectrophotometer (Milton Roy Company, USA), were cultivated on BHI agar plates, in order to count the number of bacteria.

2.6. Antimicrobial activity

Antibacterial activities of edible films were determined by Disk diffusion method. Films were cut into 10 mm discs by using a template. For all tested microorganisms, BHI agar plate was inoculated by100 µL of bacterial cultures containing approximately 1×10^7 CFU/mL of the tested bacterium. Then, the plates were placed in an incubator at 37˚C for 24 hours. After the incubation, the inhibition zone was then measured using caliper (14).

2.7. Statistical analysis

Statistical analyses of the obtained data were performed by Student's t-test and ANOVA using the SPSS, Inc., Chicago, IL (version 20) software package program. Probability (p) values of less than 0.05 were considered significant

3. Results and discussion

Upon GC/ MS analysis, the essential oil was found to contain 16 different compounds which the major compounds detected were shown in Table 1. The results derived from analysis of the ajwain essential oil showed essential oil yield at 2.99% (w/w). Among all individual constituents, which were identified by GC/MS, *Thymol*, *p-Cymene*, and γ-Terpinene stand as the major groups of compounds with 28.58, 25.14 and 21.85 percent respectively. The experimental results of the antimicrobial activity of chitosan film contain ajwain essential oil are presented in Table 2. It was also observed that the most susceptible bacterium to the antimicrobial effects of chitosan film at 1 % concentration of AEO was *Staphylococcus aureus (*26.73 mm) whereas *Escherichia coli* (16.40 mm) was the most resistant one. Hydrodistillation showed that ajwain seeds contained 2.99 % (w/w) EO and AEO had 16 compounds, representing 98.51% of total oils.

Table 1. Main components of ajwain essential oil

Component	Retention Index	Content (%)
α-Thujene	922	1.16
α -Pinene	931	0.48
Sabinene	965	0.16
ß-Pinene	971	2.86
Myrcene	979	1.52
p-Cymene	1013	25.14
Sylvestrene	1023	2.35
γ-Terpinene	1051	21.85
meta Cymenene	1073	0.14
Terpinolene	1079	0.22
Terpinene-4-ol	1175	1.42
α-Terpineol	1189	0.4
Carvone	1246	1.7
Thymol	1281	28.58
Carvacrol	1292	9.92
Dill apoile	1591	0.61
Total		**98.51**

The main constituents were thymol (28.58%), p-cymene (25.14%) and γ-terpinene (21.85 %) stand as the major groups of compounds. In most of the earlier reports with ajwain EO, thymol was identified as the chief component (6, 15-17) showed that the major components of AEO were thymol (49.0%), γ-terpinene (30.8%), p-cymene (15.7%). Oroojalian et al. (11) showed that AEO contained main compounds, including thymol (48.4%), p-cymene (21.8%), γ-terpinene (21.3%) and β-pinene (2.6%). Goudarzi *et al*. (6) reported that AEO contained main compounds, including thymol (36.7%), cymene (21.1%), γ-terpinene (36.5%). Moazeni *et al*. (16) reported thymol (50.07%), γ-terpinene (23.92%), and p-cymene (22.9%) were found to be the major AEO. These differences in chemical compositions of the oils could be attributed to environmental effects on the plants. EOs containing phenolic compounds (e.g. *thymol*, *carvacrol*, *γ-terpinene*, and *p-cymene*) are widely reported to possess high levels of antibacterial activity (16, 18). It has been reported that *thymol* might induce its antimicrobial action by perturbation of the lipid fraction of the microorganism plasma membrane, resulting in alterations of the membrane permeability and leakage of intracellular materials, Although *terpinene* was the second main constituent identified in the AEO, no strong antibacterial activity was reported from its gamma isomer (6). *P-cymene* is another major compound identified in AEO that is a hydrophobic molecule and causes swelling of the cytoplasmic membrane (6). It is not an effective anti-bacterial agent when used alone, however, in combination with other phenolic compounds such as *carvacrol*, it has shown a great antimicrobial activity (6, 19). By incorporating *cymene* in the lipid bilayer of bacteria, facilitating the transport of phenolic monoterpenes of EOs across the cytoplasmic membrane (6). Investigation of the mechanism of antibacterial action of some EOs revealed that these compounds increase membrane permeability. The oil components dissolve in the membrane, causing swelling and reducing membrane function, and lead to cell death (11). Results for antimicrobial activity as evaluated by the disc diffusion test, are shown in Table 2. AEO inhibited Gram-positive bacteria quite effectively, with *S. aureus* (26.73 mm) being more susceptible than *E. coli* (11.25 mm).

Table 2. The inhibition effect of chitosan antimicrobial film incorporated with ajwain essential oil on different types of bacteria.

Ajwain essential oil concentration (%)	*Staphylococcus aureus*	*Bacillus cereus*	*Listeria monocytogenes*	*Vibrio parahemoliticus*	*Escherichia coli* O_{157}:H_7
0	0^{Aa}	0^{Aa}	0^{Aa}	0^{Aa}	0^{Aa}
0.6	18.83 ± 1.72^{Ba}	17.47 ± 1.57^{Bab}	15.63 ± 0.57^{Bbd}	13.20 ± 0.86^{Bde}	11.25 ± 1.97^{e}
0.8	22.54 ± 1.5^{Ca}	21.35 ± 0.85^{Cab}	17.00 ± 2.48^{Bbc}	15.50 ± 1.14^{Cbc}	13.67 ± 2.35^{Cc}
1	26.73 ± 0.6^{Da}	23.13 ± 2.5^{Db}	21.42 ± 1.8^{Cb}	18.35 ± 0.37^{Dbc}	16.40 ± 0.27^{Dc}

[a-d] *Lowercase letters within the same column with different superscript are significantly different ($p<0.05$).*
[A-D] *Uppercase letters within the same row with different superscript are significantly different ($p<0.05$).*

The antibacterial activity of *C. copticum*, possibly relates to the high amount of thymol and γ–terpinene (6), reported that the essence exhibited significant antibacterial activities against *Salmonella* Thyphimorium, *Escherichia coli*, *Staphylococcus aureus* bacteria by agar diffusion method, except *P. aeuroginosa* (20), showed that AEO exhibited significant antibacterial activities against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Candida albicans*. According to a number of studies, Gram-positive bacteria are more sensitive to EOs than being Gram-negative bacteria. Due to the possession of an outer membrane, surrounding the cell wall of Gram-negative bacteria, it is logical to expect that these bacteria would be less susceptible to the antibacterial activity of EOs. This outer membrane may restrict the diffusion of hydrophobic compounds through its lipopolysaccharide covering. Gram-positive bacteria allow direct contact of the EOs' hydrophobic constituents with the phospholipid bilayer of the cell membrane, where they bring about their effect, either causing or increasing ion permeability and leakage of vital intracellular constituents, or impairment of bacterial enzyme systems (11).

4. Conclusion

The results of this study confirmed that the use of chitosan-based antimicrobial film incorporated with AEO oil has a remarkable antibacterial effect and therefore it can be practical in active packaging in the food industry.

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