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Antibacterial Properties of Bioactive Starch Films Containing *Bunium Persicum* Seed's Essential Oil Nanoemulsion Fortified with Cinamaldehyde

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ABSTRACT

Background: The present study aimed to evaluate antimicrobial properties of corn starch edible films prepared with *Bunium persicum* essential-oil nanoemulsions (BPEOne) and BPEOne fortified with cinnamaldehyde (Fortified BPEOne with CIN) against some common food-borne pathogens.
Methods: Treatments (films containing BPEO, CIN, BPEOne, BPEO+CIN, BPEOne+CIN, Fortified BPEOne with CIN in 0.5, 1, 2, and 4 % concentrations) were prepared using starch solutions. In vitro antimicrobial activity of BPEOne films was evaluated using agar disk diffusion and plate count assay methods against *Salmonella enteritidis*, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*.
Results: The inhibitory effect of each treatment against some food-borne pathogen bacteria increased significantly with the enhancement of concentrations (P<0.05). The highest bacterial inhibitory effect was achieved in higher concentrations (2%, 4%). The antimicrobial activity of BPEO nanoemulsion was higher than pure BPEO-containing films. *S. aureus* and *S. enteritidis* were more susceptible and resistant bacteria to BPEO, BPEO nanoemulsion-containing films, respectively.
Conclusion: The films containing a combination of the BPEO (or its nanoemulsion) with CIN have better antimicrobial activity, however the fortification of nanoemulsions with other antimicrobial agents may not show the same results.

1. Introduction

Essential oils (EOs) and plant extracts have been widely used as antimicrobial compounds and good alternatives to chemical antimicrobials in the food industry [1]. Encapsulating of EOs and plant extracts in a variety of polymer coatings and application of more advanced technologies such as nanotechnology are preferred methods to reduce the inevitable effects of these bioactive materials in food [2]. An alternative to incorporate plant's EOs, extracts or their derivatives in food is adding them into biodegradable edible films [3]. Applying antimicrobial films and coatings as active packaging are one of the new important technologies to improve food safety and preserve food products [4]. Nowadays, edible films are made of polysaccharides, proteins, lipids, or a combination of them. These biomaterials are of particular importance in food packaging due to their natural materials, recyclability, and no environmental pollution [5]. Among these biomaterials, starch is of special importance since it is abundant in nature and affordable. Starch films have advantages such as flexibility and transparency as packaging materials [6]. Black cumin (*Bunium persicum*) seeds and cinnamon (*Cinnamomum zeylanicum*) are antioxidant and

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antimicrobial compounds used as flavoring agents and preservatives in food. *Bunium persicum* (BP) is a self-pollinated perennial plant of Apiaceae family which is known as an antihistamine, anticonvulsant, anti-helminth, anti-asthma and anti-respiratory disorders in traditional medicine. Benzaldehyde, gamma Trpinene, Cyclo heptane, Isopropyl cyclohexane, n-hexane and p-Cyimen are main constituents of cumin plants [7]. Cinnamon is obtained from the inner bark of some tree species from the genus Cinnamomum of Lauraceae family which is known as anti-diabetes and anti-bronchitis in traditional medicine. Cinnamaldehyde forms a major part of the cinnamon compound with eugenol and cinnamic acid. The antimicrobial effect of Cinnamaldehyde (CIN) has been proven against Gram-positive and Gram-negative bacteria due to the presence of the aldehyde group in its structure [7]. Both of these plants and their derivatives have been used in the food industry as spices, flavoring, antioxidants, and antimicrobial agents. Nanoscale range compounds have a high surface-to-volume ratio; therefore, when the particle size is reduced to this scale (<150 nm), the resulting materials exhibit more physical and chemical properties due to their higher contact surface. In addition, when they are added to compatible polymers, they can improve the mechanical properties, thermal stability, and antimicrobial activity of biopolymer coatings [8]. By encapsulating anti-microbial compounds with nanotechnology, the release of nanoparticles has been carried out in a controlled manner which can prevent EOs sublimation and undesirable effects on the sensory properties because of increasing the operating sites in the mass unit and reducing the dose of bioactive compounds. Nanoemulsification of EOs improves dispersing of the EOs in an aqueous phase. It enables encapsulation of EO with higher concentrations than their water solubility. In other words, the minimum inhibitory concentration of EOs increases in nanoemulsions due to their dispersing in an oil phase and interactions between the emulsifier and the microorganism′s cell membranes [9,10]. Antimicrobial effects of EO nanoemulsions in comparison with free EO have been reported in several studies[9,11,12]. The optimal effects of nanoemulsions in films have also been reported by several researchers in various studies. For instance, Chen *et al.* (2016) conducted a stdudy on biopolymer films containing thyme and CIN nanoemulsions [13]. As far as our knowledge allows, there are no studies to evaluate the antimicrobial effects of the combination nanoemulsion of BPEO (BPEOne) with CIN and fortified nanoemulsion of BPEO with CIN in edible films. Therefore, the purpose of this study was to determine the antimicrobial properties of produced biodegradable starch film containing nanoemulsions of BPEO and BPEO fortified with CIN against some important food-borne pathogens.

2. Materials and Methods

2.1 Materials

All chemicals (Corn starch, glycerol, Tween 80, Cinnamaldehyde) and culture media (Plate Count Agar (PCA), Brain Heart Infusion Broth (BHI Broth), and Chloramphenicol discs were purchased and provided from Sigma-Aldrich (Sigma Aldrich, St. Louis, USA) and Merck (Merck, Darmstadt, Germany) Companies. Lyophilized bacteria cultures of *Salmonella enteritidis* (ATCC 14028), *Escherichia coli* (ATCC 15224), *Listeria monocytogenes* (ATCC 13932), and *Staphylococcus aureus* (ATCC 25923) were provided from the Iranian Research Organization for Science and Technology (IROST, Tehran, Iran). Fresh cultures of lyophilized bacteria were provided using BHI broth at 35 °C for 24 h before the tests.

2.2 Isolation of Essential Oils

Dried seeds of BP were provided from a local market in Zanjan, Iran. The purchased seeds were identified taxonomically (IMPH-7036) in the Medicinal Plant Research Center, ACECR, Institute of Medicinal Plants, Karaj, Iran. Hydro-distillation method was used to extract EO using a Clevenger apparatus at 100 °C for 3 h. Each time 100 gram seeds were weighted and hydrodistillated. First, hydrodistillation was repeated six times and a total of 9 mL of EO was collected. Then, the separated EO was dried over anhydrous sodium sulfate, filtered (0.22 m) and stored at 4 ºC before use [7].

2.3 Gas chromatography-mass spectrometry (GC/MS) analysis

An Agilent 7890B gas chromatograph (Agilent, USA) combined with Agilent 240 Ion Trap mass spectrometer (MS) was used to detect the chemical composition and active ingredients of BPEO. Gas chromatograph equipped with a HP5-MS column (60 m × 0.25 mm ID × 0.25 μm film thickness) and mass spectral libraries (Wiley Registry 11th Edition/NIST 2017). The carrier gas was Helium (He) with a flow rate of 1 ml/min. The initial column temperature was 50 °C for 5 min. and gradually increased to 150 °C at a 10 °C/min rate and held for 2 mins. The temperature was increased to 300 °C at 20 °C/min for 5 min. Injected volume and split ratio were 1 μl and 1:50, respectively. Impact energy, decomposition velocity, analysis interval, and fragments for mass spectrometer (MS) were 70 eV, 1000, 0.5, and 45-450 Da. To identify the EO composition and compounds, the obtained mass spectrums were compared to those of the database (Wiley Registry 11th Edition/NIST 2017 data software) and also by comparison with data from previous studies based on their RI and mass spectral fragmentation [14,15].

2.4 Nanoemulsions preparation

2.4.1 Preparation of essential oil nanoemulsion of B. persicum

For this purpose, ten concentrations of BPEO and Tween 80 as a surfactant were mixed according to Table 1. The vials containing 6.4 gr mentioned EO were prepared with different volumes of Tween 80 and reached 80 mL with distilled water. All vials were stirred on a magnetic mixture for 5 min at

10000 rpm, separately. The next step was mixing and stirring the final solutions with an ultrasonic homogenizer (APU, Adeeco, Iran) at a total input power (400 W) and constant frequency (24 kHz) for 10 min at 14000 rpm speed [16,17]. The final formulation was set based on polydispersity index (PDI) and nano-droplet size. Seventh formula mentioned to final formulation (80 ml nanoemulsion with 8% concentration) (Table 1). Prepared nanoemulsion was stored in vials at a cool (4 °C) and dark place before use.

Table 1: Ten formulations to set the best nanoemulsion solution based on PDI and droplet size

Formula	BPEO (w/w%)	Tween 80 (w/w%)	Total volume	PDI±SD	Mean size±SD (nm)
1	6.4	10	80	1.000 ± 0.00	90.91 ± 3.12
2	6.4	9	80	0.867 ± 0.94	139.6 ± 4.14
3	6.4	8	80	1.000 ± 0.00	188.01 ± 0.9
4	6.4	7	80	0.541 ± 1.1	184.22 ± 0.9
5	6.4	6	80	0.444 ± 0.12	136.11 ± 1.11
6	6.4	5	80	0.366 ± 0.14	140.21 ± 1.24
7	6.4	4 (3.92)	80	0.283 ± 0.08	131.28 ± 8.08
8	6.4	3	80	0.332 ± 0.43	123.24 ± 0.61
9	6.4	2	80	0.330 ± 0.72	132 ± 2.12
10	6.4	1	80	0.351 ± 0.09	125.5 ± 2.11

2.4.2 Preparation of essential oil nanoemulsion of B. persicum fortified with cinnamaldehyde (BPEOne&CIN)

For this purpose, two vials containing 76 mL water, 3.92 gr tween 80 in the first vial and 4 mL ethanol (5% v/v as co-solvent) plus 6.4 gr mentioned EO and 6.4 gr CIN in the second vial were stirred on a magnetic mixture for 5 min in 10000 rpm, separately. The next step was mixing two vials and stirring the final solution with an ultrasonic homogenizer (APU, Adeeco, Iran) at a total input power (400 W) and constant frequency (24 kHz) for 10 min at 14000 rpm speed [16,17]. The prepared nanoemulsion was stored in vials at a cool (4 °C) and dark place before use.

2.4.3 Determination of nanoemulsion droplets size

A Dynamic Light Scattering (DLS) instrument (ZEN 3600, Malvern Panalytical Ltd, Malvern, UK) using a Zetasizer Nano-ZS laser diffractometer was applied to determine the nanoemulsion droplets size on the first day and in a 25°C temperature. Each sample was diluted with deionized water (1:20 v/v) before droplet size determination.

2.5 Preparation of starch films containing Essential oil and nanoemulsion

An aqueous solution of 3% (w/v) corn starch plus 1.8% glycerol was heated at 90 °C and agitated to allow gelatinization on a magnetic heater for 10 min. The next step was cooling down the solution to approximately 40 °C and addition of BPEO, CIN, and nanoemulsions to prepare 5 to 40 mg/mL concentrations (0.5, 1, 2, 4 %) of free and combinations of bioactive ingredients, separately. Different concentrations of BPEO and CIN were selected according to the reported minimum inhibitory concentrations (MIC) in several studies conducted against various food-borne pathogens [18,19]. All prepared solutions were homogenized with a Homogenizer (IKA T25-Ultra Turrax, Staufen, Germany) at 2000 rpm for 5 min to obtain homogeneous mixtures. Then, 25 mL of the aliquot was cast on Teflon petri dishes (ϕ=10 cm) and dried at room temperature for 18-24 h. To prepare films containing a combination BPEOne and CIN in 4%, 2%, 1%, and 0.5% concentrations, a combination of 2%, 1%, 0.5%, 0.25% of BPEOne (diluted with DMSO 5%) and 2%, 1%, 0.5%, 0.25% CIN was added to the starch solution, respectively. This formulation was considered to prepare other films containing. Nanoemulsion droplet size was determined again after homogenization of the prepared solution to detect probably changes in nanoemulsion droplet size [20].

2.6 Antibacterial Activity of Biodegradable Films

The antibacterial activity of biodegradable films impregnated with CIN and BPEOne was evaluated using agar disk diffusion and plate count assay methods. Before evaluating the antibacterial effects, all prepared films were placed in sterile zip packs and exposed to ultraviolet light (UV) radiation in a laminar hood for 2 h (1 h for each surface of the film) to remove probable microbial contaminations of films [21].

2.6.1 Agar disc diffusion method

The antimicrobial activity of prepared film samples was determined using the agar disc diffusion method. To perform this method, 0.1 mL of the overnight grown bacterial cultures (broth culture containing approximately, 1-2 $\times 10^8$ CFU/mL according to the density of 0.5 McFarland standard) were spread on Plate Count Agar (PCA-Merck, Darmstadt, Germany). Under sterile conditions, film samples with a 6 mm diameter (with 0.2-0.25 mm thickness) were aseptically cut into disc shapes was 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 an indicator of antimicrobial activity. A chloramphenicol disc (30 u/g) was used as a positive control [15].

2.6.2 Plate count assay method

The antibacterial activity of impregnated corn starch films was evaluated according to the described protocol by Sugumar *et al.* (2015) [22] using the plate count assay method. Under sterile conditions, the same pieces of the bioactive films (2.5 cm^2) were transferred to the test tubes containing 10 mL of 0.1 OD (Optical density) adjusted

bacteria at 600 nm (1-2 ×10^8 CFU/mL). Tubes were then stored at 28-30 °C for 24 h in an incubator equipped with a shaker. Test tubes without film and organism were taken as negative control and test tubes containing pure adjusted bacteria (1-2 ×10^8 CFU/mL) without film were mentioned as a positive control. Then serial dilutions were prepared using 0.01 mL (Drop plate method) of each sample and spread on Brain heart infusion agar (BHI agar) (Merck, Darmstadt, Germany) plates in duplicates and incubated at 37 °C for 24 h. The enumerations were calculated using a dilution factor. The viability percentage of bacterial cells was plotted after 24 h of incubation at 37 °C. Results were stated as mean log CFU/gr ± standard deviation [22].

2.7 Statistical Analysis

All experiments were performed in triplicate. Statistical analysis (one-way ANOVA) was carried out using SPSS statistical software, Version 19.0 (SPSS Inc, IL, USA). Tukey's post hoc test was used to analyze differences between the treatments (P<0.05).

3. Results and Discussion

3.1 Composition of B. persicum essential oil

Table 2 illustrates the chemical composition of BPEO. The average EO yield of BP seeds was 1.5 %. GC/MS analysis of BPEO composition showed 31 compounds of which benzaldehyde (17.83%), γ–terpinene (17.27%), cycloheptane (12.56%), Isopropyl cyclohexane (11.21%), n-hexane (8.38%) and p-cymene (7.45%) are the main constituents, respectively. Volatile and non-volatile antimicrobial compounds used in food packaging can show their antimicrobial effects as a result of migration and dissolution, respectively [7]. The highest percentage of compounds in BPEO were related to aromatic aldehyde (17.83%), monoterpene hydrocarbons (45.66%), and oxygenated monoterpenes (11.21%). The other compounds were presented in less percentage (Table 2). Obtained results in some cases were consistent with the results of other studies conducted on BPEO (Table 3) [23,27]. Differences in the chemical composition of essential oils depend on differences in cultivar, cultivation conditions, duration and conditions of storage, planting season, weather conditions, geographic area and part of plant, method and duration of extraction of EO [7,14].

3.2 Nanoemulsion droplet size

The average droplet size of the nanoemulsion solutions of the BPEO and fortified BPEO with CIN was 131.28 ± 8.08 (nm) and 201 ± 30.7 (nm), respectively (Fig. 1,2). Nanoemulsion particle size has a special importance to determine their characteristics which can affect the antimicrobial, physical and mechanical properties of films impregnated with nanoemulsion. Table 1 confirms that the bioactive substance/surfactant ratio is the main determinant factor of the particle size in the nanoemulsion. Nano-scaling of bioactive materials provides more contact surfaces and results in higher bioavailability, transparency, and higher solubility [28]. Nanoemulsion particle size changes because of the alteration in items used in order to optimize production conditions including the composition of biologically active material, processing method, physicochemical properties of the dispersed and constant phases, homogenizing speed rate, equipment types like homogenizer or magnetic stirrer and the environmental conditions such as temperature and time [29,30]. Based on our knowledge, there are no similar studies to provide BPEOne to compare particle size with the obtained results of the present study. However, diversity in nanoemulsion particle size was seen in the results of the other studies. Otoniet al. (2014), and Saranyaet al. (2012) reported particle size in CIN nanoemulsion 121.75±7.47 nm and *Thymus daenensis* nanoemulsion 143.2±2.6 nm, respectively [16,31].

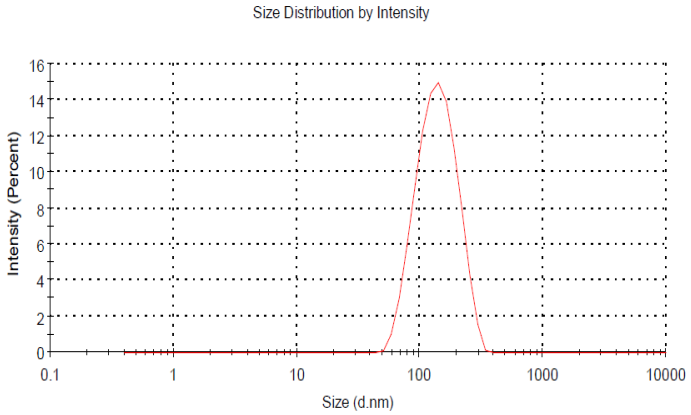

Figure 1: Droplet size of BPEO

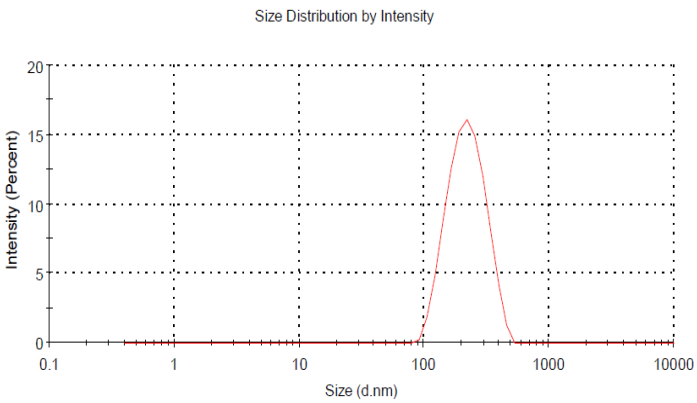

Figure 2: Droplet size of BPEO enriched with CIN

3.3 Antibacterial activity

3.3.1 Agar disc diffusion method

Antimicrobial activity and growth inhibition zone (GIZ) of different treatments using the disc diffusion method are presented in Figure 3. The antimicrobial effects of EOs are not limited to a specific mechanism due to the presence of different bioactive groups and compounds in their chemical composition.

Table 2: Chemical composition of *B. persicum* essential oil

No	Compound	Retention time (min)	Relative peak area (%)	Experimental Retention Index	Literature retention Index
1	Propane, 2-methoxy-2-methyl	4.8	0.18	510	512
2	Pentane, 3-methyl	4.9	1.04	554	560
3	n-Hexane	5.1	8.38	618	620
4	Cyclopentane, methyl	5.4	0.45	661	660
5	Bicyclohex-2-ene, 2-methyl-5-(1-methylethyl)	10.2	1.19	902	905
6	1-Isopropylcyclohex-1-ene	10.2	0.2	926	925
7	Trimethylbicyclohept-2-ene	10.4	2.45	948	950
8	Bicyclohexane, 4-methylene-1-(1-methylethyl)	11.0	2.27	897	900
9	Bicycloheptane, 6, 6-dimethyl-2-methylene	11.1	12.56	943	945
10	α-Phellandrene	11.5	1.21	969	970
11	4-Carene	11.6	0.66	1052	1050
12	p-Cymene	11.8	7.45	1042	1044
13	D-Limonene	11.8	1.52	1018	1019
14	β- Phellandrene	11.9	0.62	897	897
15	Eucalyptol	11.9	0.43	1059	1060
16	gamma-Terpinene	12.3	17.27	998	998
17	Cyclohexene, 3-methyl-6-(1-methylethylidene)	12.7	0.25	1052	1051
18	3-Cyclohexen-1, 4-methyl-1-(1-methylethyl)	13.9	0.71	1137	1036
19	3-p-Menthen-7-al	14.2	3.94	1175	1175
20	Benzaldehyde, 4-(1-methylethyl)	14.9	17.83	1230	1232
21	1-Cyclohexene-1-carboxaldehyde	15.3	0.4	1175	1173
22	3-Methyl-2-(2-methyl-2-butenyl)-furan	15.3	0.2	1115	1111
23	4-Isopropylcyclohexa-1, 3-dienecarbaldehyde	15.4	11.21	1186	1189
24	1, 4-p-Menthadien-7-al	15.5	4.61	1186	1190
25	1, 4-Cyclohexadiene-1-methanol, 4-(1-methylethyl)	15.8	0.36	1240	1242
26	3-Trimethylcyclohex-1-ene-4-carboxylic acid	16.1	0.18	1343	1345
27	3-Isopropyl-6, 8a-dimethyl-1	16.5	0.61	1481	1484
28	Caryophyllene	17.0	0.5	1494	1494
29	cis-.beta.-Farnesene	17.1	0.58	1440	1441
30	gamma.-Muurolene	17.5	0.5	1398	1395
31	Carotol	18.9	0.24 100%	1593	1596

Six main mechanisms are thought to be the mechanism of action for chemical compounds of the EOs against bacterial cells: damage to the structure of the cell wall and cytoplasmic membrane; degradation of membrane proteins; cell contents permeation; cytoplasm coagulation, and force discharge for the proton motivation in the microorganism cell [32]. Strong and extensive antimicrobial activity was reported for many EO nanoemulsions. Their formulations, surface charge, and droplet size are responsible for the convenient transfer of EO among the cell membrane and attachment of the EO to the sites of action [9]. Four roots were described for these interactions: 1- Enhancement of transporting due to the high surface area of nano-droplets and passive transfer of them through the outer cell membrane which causes disturbance in bacterial membrane physiology [16], 2- Assimilation ability of used emulsifier with phospholipid double layer of the cell membrane and more quickly release of EO, 3- Prolongation of the EOs' activity by sustained and continuous release of the EOs over time from the nanoemulsion droplets as the tank for EOs [33]. 4- Electrostatic interaction between nanoemulsions droplets and microbial cell walls which raises EOs concentration at the action sites [34]. Based on the obtained results, films containing CIN had a more inhibitory effect on bacteria while the BPEO-containing films were less effective. Similar results were obtained by Otoni *et al.* (2014) for an

edible film containing CIN nanoemulsion [31]. The differences in antimicrobial effect between the films can be related to the variations in the structure of the main bioactive ingredients and their mechanism of action against microorganisms. The CIN molecule consists of a benzene ring linked to an unsaturated aldehyde with inhibition ability of energy generation by preventing glucose consumption and absorbance and effects on membrane permeability [35]. The obtained result shows that the main ingredients in BPEO are monoterpene which commonly influence membrane activity [36]. The inhibitory effect of each treatment on bacteria increased significantly with the enhancement of concentrations (Figure 3). However, they did not show a good result in lower concentrations. Variations in the GIZ for different bacteria showed that all treatments were more effective against Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) than Gram-negative bacteria (*E. coli* and *S. entritidis*). In other words, Gram-positive bacteria were more sensitive to the antimicrobial effect of BPEO. The obtained results in this study are consistent with the results of Shojaee Garabagh et al. (2017) [37] and Aminzare et al. (2017) [7]. They showed by increasing the EO concentration, the inhibitory effect on bacteria was increased and both free and encapsulated forms of EO had higher inhibitory effects on Gram-positive bacteria than Gram-negative bacteria. The difference in the cell wall structure of these two groups of bacteria is the reason for the different sensitivity to antimicrobial agents [14]. The results of this study showed that in vitro antimicrobial activity of BPEO nanoemulsion was higher than pure BPEO. These results are consistent with the results of Sugumar et al. (2015) [22] and as well as the results of Otoni et al. (2014) [31]. Similar results were achieved by Acevedo-Fani et al. (2015) [38] performed on edible films containing nanoemulsion of essential oil.

Table 3: Constituents *of B. persicum* essential oil in different studies

Main components	Part of plant (Extract or EO)	part of Plant	Origins of plant	References
Benzaldehyde (17.83%), γ -Terpinene (17.27%), Bicycloheptane (12.56%), Isopropylcyclohexa (11.21%), n-Hexane (8.38%), p-Cymene (7.45%)	EO	Seed	Kerman, Iran	Present study
γ-terpinene (46.1%), cuminal (23.9%), p-cymene (15.9%), limonene (4.7%), 1,4-p-menthadien-7-al (4.5%)	EO, hydroalcoholic and polyphenolic extracts	Seed	Isfahan, Iran	[18,23]
Γ-Terpinene (45.7%), Cuminaldehyde (12.7%), Limonene (10.6%), Cuminyl alcohol (6.4%), p-Cymene (5.6%)	Supercritical fluid extraction	seed	Mazandaran, Iran	[18,24]
Cuminaldehyde (22.34%), carvacrol (19.88%), anisole (15.19%), o-Cymene (12.04%), γ – Terpinene (9.77%), α-Propylbenzyl alcohol (8.99%), β-Pinene (2.34%), D-Limonene (2.14%)	EO	seed	Iran	[7]
γ-Terpinene (46.1%), cuminaldehyde (15.5%), ρ-cymene (6.7%), limonene (5.9%), β –Pinene (2.5%)	EO	seed	Kerman, Iran	[18,25]
Caryophyllene (27.81%), γ-terpinene (15.19%), cuminyl acetate (14.67%), cuminaldehyde (5.96%), p-cymene (5.25%)	EO	seed	Iran	[18,26]
ρ -cymene (25.7%), γ–terpinene (23.9%), cuminaldehyde (22.6%), p-Mentha-l'3-dien-7-al and p-Mentha- 1,4-dien-7-al (21.9%), β –Pinene (0.5%)	EO	seed	India	[18,27]

3.3.2 Plate Count Assay Method

The antimicrobial activity of various treatments evaluated by plate count assay is presented in Figure 4. The films containing CIN alone had a more inhibitory effect on bacteria than other films. Compared to the control group, the bacterial count of *S. entritidis*, *E. coli*, *L. monocytogenes*, and *S. aureus* declined significantly, near to 0.58, 0.91, 0.92, and 1.03 Log CFU/mL, respectively (P <0.05). A significant count difference was seen for all bacteria other than *S. entritidis* between CIN-containing films with other treatments (*P*<0.05). BPEO and BPEO nanoemulsion-containing films had better effects on four pathogens at higher concentrations of essential oil (2% and 4%). BPEO nanoemulsion-containing films had higher antibacterial effects than BPEO-containing films. Films containing a combination of BPEO+CIN and BPEOne+CIN had the same effects and they had the most antimicrobial effect after CIN-containing film at the lowest effective concentration (1 %). The average number of bacteria for *S. entritidis*, *E. coli*, *L. monocytogenes* and *S. aureus* significantly decreased near to 0.36, 0.5, 0.64, and 0.33 Log CFU/ml, respectively compared to the control group (*P* <0.05). The results showed that the colony count of bacteria decreases further by increasing the concentration of EO. Similar results were reported by Sugumar et al. (2015) [22]. Several studies have also been conducted on the antimicrobial effects of EO′s nanoemulsion using the plate count assay method, but a similar study has not been carried out on films containing fortified essential oil nanoemulsion with other bioactive ingredients. A comparison of two nanoemulsion treatments of BPEOne+CIN (A) and fortified BPEOne with CIN (B) showed that the antimicrobial effects of nanoemulsion A was higher than nanoemulsion B at the same concentration. It seems that in the first treatment, there is no bond between BPEOne and CIN (both are free and each of them is separately affected) and both active components can easily interact with microbes and eliminate their structure. However, in the treatment B, the

nanoemulsion structure of the BPEO may act as a coating between the CIN and the microbial cells and antibacterial effect that has been seen was solely related to the BPEO and the effect of CIN was negligible or zero. The obtained results from the comparison of BPEOne treatment with A and B treatments can be attributed to the fact that the antimicrobial effect of B treatment was approximately same as the antimicrobial property of BPEOne (Figure 4). According to this result, nanoemulsion fortification with CIN is not recommended. A comparison of two treatments of BPEO+CIN (A') and CIN (B'), indicates that GIZ diameter of treatment A' was less than the GIZ of B' treatment for all bacteria which resulted from halving and decrease in the concentrations of active components used to prepare combined films treatments (Figure 3). Given that the antimicrobial activity of CIN is much higher than BPEO and due to the halving of the applied concentrations of bioactive compounds in films, a decrease in GIZ in treatment A than the treatment B is expected [39]. This subject also applies to the Plate Count Assay, and the count of colonies grown in the A films A was higher than B films B. *S. entritidis* and *S. aureus* were the most resistant and sensitive bacteria at the lowest effective concentrations of antimicrobial agents in the film treatments, respectively.

Figure 3: Antimicrobial effect of different treatments on bacteria using agar disk diffusion method: A. (*S. Entritidis*), B. (*E. coli*), C. (*L. monocytogenes*), D. (*S. aureus*). (mm)

- Non-identical small letters indicate a significant difference between treatments in each concentration (P <0.05).
- Non-identical capital letters indicate a significant difference between different concentrations in each treatment (P <0.05). CIN: Cinammaldehyde containing films; BP: B. persicum essential oil (BPEO) containing films; NanoBP+CIN: Nanoemulsion of BPEO+ CIN containing films; BP+CIN: BPEO+ CIN containing films; NanoBP&CiN: BPEO nanoemulsion fortified with CIN containing films; NanoBP: BPEO Nanoemulsion containing films; Control p: Control Positive (Chloramphenicol disc (30 u/g)).

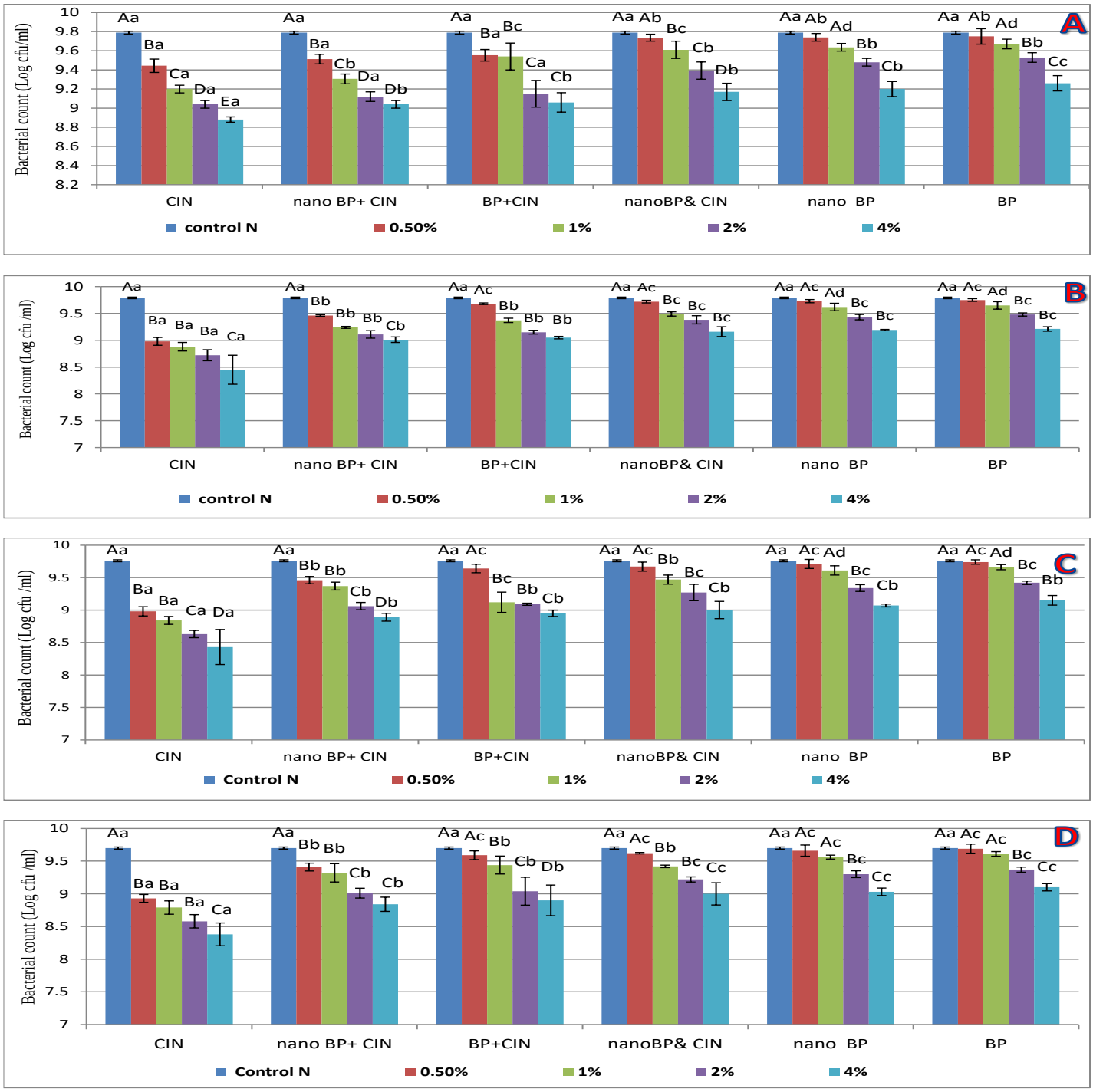

Figure 4: Antimicrobial effect of different treatments on bacteria using plate count assay method: A. (*S. Entritidis*), B. (*E. coli*), C (*L. monocytogenes*), D. (*S. aureous*). (Log CFU/mL)
- Non-identical small letters indicate a significant difference between treatments in each concentration (P <0.05).
- Non-identical capital letters indicate a significant difference between different concentrations in each treatment (P <0.05). CIN: Cinammaldehyde containing films; BP: B. persicum essential oil (BPEO) containing films; NanoBP+CIN: BPEO nanoemulsion + CIN containing films; BP+CIN: BPEO+ CIN containing films; NanoBPne&CiN: BPEO nanoemulsion fortified with Cinammaldehyde containing films; NanoBP: BPEO nanoemulsion containing films; Control N: Control Negative.

4. Conclusion

We can conclude from the data that the antimicrobial effects of BPEO were low. Moreover, an increase in antimicrobial properties was seen in the starch films containing nanoemulsion. The highest bacterial inhibitory effect was achieved in higher concentrations (2% and 4%). *S. aureus* and *S. enteritidis* were more susceptible and resistant bacteria to BPEO, and BPEO nanoemulsion-containing films, respectively. The films containing a combination of the BPEO (or its nanoemulsion) with CIN agents have better antimicrobial activity. However, fortification of nanoemulsions with antimicrobial agents is not recommended. It can be concluded, the incorporation of nanoemulsions into starch edible films may have supplementary applications in food packaging to preserve food products.

Authors' Contributions

Fariba Mardani: methodology; Investigation; Resources; data curation; writing-original draft preparation; project administration. Mehran Mohseni: data curation; writing-review and editing. Majid Aminzare: writing-review and editing; Supervision. Hassan Hassanzadazar: Conceptualization; methodology; formal analysis; Resources; writing-original draft preparation; writing-review and editing; Visualization; supervision.

Conflicts of Interest

The authors declare no conflict of interest.

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