JOURNAL OF NUTRITION FASTING AND HEALTH

In vitro Antimicrobial and Antioxidant Properties of Edible Coating Enriched with *Cinnamomum verum* Essential Oil

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ARTICLEINFO

Article type:
Research Paper

Article History:
Received: 15 Nov 2023
Accepted: 14 Jan 2024
Published: 15 Jan 2024

Keywords:
Edible coating
Cinnamomum verum
Essential oil
Antimicrobial
Antioxidant

ABSTRACT

Food coatings are a promising strategy to improve the safety and shelf life of food products by inhibiting or retarding the growth of harmful microorganisms. The current study assessed the *in vitro* antibacterial and antioxidant characteristics of a coating based on natural ingredients, including whey protein isolate (WPI), nanochitosan (NCH), bacterial nanocellulose (BNC), and cinnamon essential oil (CEO). The in vitro antibacterial assay of the edible coating solution was performed against four food-born pathogens, consisting *Staphylococcus aureus*, *Listeria monocytogenes, Escherichia coli*, and *Salmonella* Typhimurium. The antioxidant potency of the edible coating solution was evaluated by measuring its capability to scavenge free radicals.

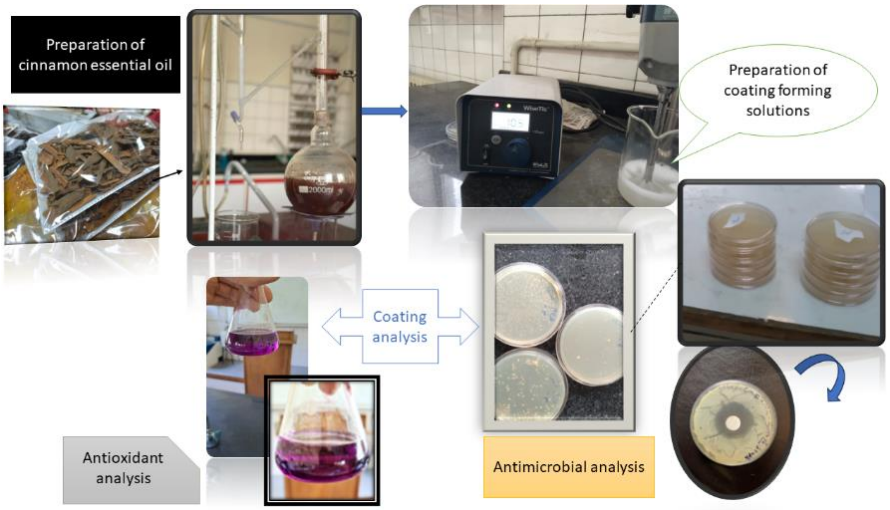
The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the coating decreased as the CEO concentration increased. The most significant difference in MIC and MBC was observed between the pure coating and the essential oil enriched coating group, which had the maximum essential oil concentration (1.5%). For *Salmonella* Typhimurium bacteria, this difference was 20% for MIC and 15% for MBC. For *Escherichia coli*, it was 15% for MIC and 20% for MBC. For *Staphylococcus aureus*, it was 20% for MIC and 20% for MBC. For *Listeria monocytogenes*, it was 15% for MIC and 20% for MBC.

The antibacterial charachtristics of the coating were evaluated using the disc diffusion technique. The results showed that the coating exhibited considerable antibacterial efficacy against all tested pathogens. The coating also exhibited significant antioxidant activity (up to 5.7% more than the control group).

These findings suggest that the coating based on WPI, NCH, BNC, and CEO has potential applications to improve the food safety.

► Please cite this paper as:
Sheerzad S, Khanjari A, Gandomi H, Akhondzadeh Basti A, Khorrami R. In vitro Antimicrobial and Antioxidant Properties of Edible Coating Enriched with Cinnamomum verum Essential Oil. J Nutr Fast Health. 2023; 11(4): 331-339. DOI: 10.22038/JNFH.2024.76257.1482.

GRAPHICAL ABSTRACT



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Introduction

Food security faces significant obstacles due to the ongoing worldwide population expansion. To meet the needs of future generations, the food supply must be increased. This can be achieved through three main strategies: increasing production, improving distribution, and reducing food waste. One of the most practical strategies to reduce food waste is to protect food samples from microbial, chemical, and oxidative spoilage [1]. Implementing specific methods, such as increased production, improved distribution, and the use of edible coatings, can effectively reduce food spoilage and enhance global food security in the ongoing battle against food loss.

Edible coatings, composed of edible biopolymers derived from food industry wastes or underutilized sources, offer a promising approach for preserving and packaging different food. These coatings improve the shelf life of food products by forming a thin layer on the surface, delaying microbial spoilage, inhibiting moisture loss, and inhibiting lipid, protein, and pigment oxidation [2, 3].

Whey protein isolate (WPI), a group of globular milk proteins, is an excellent matrix for food coatings because of its nutritional and functional properties. The high protein content (90-95%) and minimal fat, lactose, and minerals of WPI make it an ideal barrier against moisture, oxygen, and other gases. Moreover, WPI's emulsifying and foaming properties contribute to uniform and appealing coatings, while its ability to carry active ingredients like antioxidants or probiotics improves the functional properties and nutritional value of food [4].

Nanochitosan (NCH), a derivative of chitosan, a natural, non-toxic biopolymer derived from chitin, plays a crucial role in food coatings due to its unique properties. It possesses superb film-forming capability, antimicrobial and antioxidant activity, and oxygen barrier characteristics, making it an ideal ingredient for extending food shelf life. Furthermore, the capacity of NCH to absorb heavy metal ions decreases food oxidation and improves food safety [5].

Nanocellulose (NC), a biodegradable and renewable nanofiller derived from the breakdown of cellulose fibers, offers a sustainable and versatile alternative for food coatings. Similar to NCH and WPI, NC exhibits remarkable properties that enhance the functionality of food packaging materials. Its abundance, with plants producing around 75 billion tons annually, makes it a readily available resource. NC has three forms: bacterial nanocellulose (BNC), cellulose nanofibrils and cellulose nanocrystals. These nanomaterials have gained traction in packaging applications due to their ability to synergistically enhance the barrier, thermomechanical, and rheological characteristics of nanocomposites. The strong intermolecular and intramolecular hydrogen bonding in NC renders it insoluble in most solvents, contributing to its high strength. nanocellulose is transparent and possesses a reactive surface rich in hydroxyl groups, enabling surface functionalization for diverse applications [6].

In recent years, a paradigm shift has emerged in consumer preferences, favoring natural antioxidants derived from plant and spice extracts over synthetic antioxidants due to concerns regarding the potential toxic effects of the latter. This trend aligns with the growing demand for healthy, chemical-preservative-free foods. Dut to their phenolic content, many herbs and spices exhibit potent antioxidant effects, making them attractive alternatives for food preservation [7].

New approaches to food preservation have been made possible by the growing consumer desire for natural antioxidants and the increasing awareness of essential oils as effective antimicrobials. These strategies provide safer and healthier substitutes for artificial additives. Essential oils (EOs), naturally occurring compounds derived from plants, have gained prominence in food applications related to their remarkable antifungal, antiviral, antioxidant, and antibacterial properties. Cinnamon, a spice gained from the inner bark of Cinnamomum tree species, is particularly noteworthy for its antimicrobial properties. Its essential oil (CEO) possesses two key compounds, cinnamaldehyde and eugenol, which effectively inhibit microbial growth. Moreover, the broad-spectrum antimicrobial activity of cinnamon oil makes it suitable for various food products. These properties position cinnamon oil as a safe and natural alternative to conventional antimicrobial agents [8].

This research aimed to develop and investigate a novel edible coating boosted with the antibacterial and antioxidant characteristics of a mixture of biopolymers and essential oil, as a

healthy and natural alternative to synthetic preservatives.

Materials and Methods

Extraction of CEO

The dried cinnamon bark sample (120 g) was crushed. After that, the ground sample was added into a Clevenger-type apparatus to obtain EO by steam distillation process, as indicated in the European Pharmacopoeia. The collected CEO was dehydrated by sodium sulfate and then kept in opac tubes at four degrees centigrade for further analysis. The components of the collected CEO were identified and mentioned in our previous study [9].

Preparation of WPI/NCH/BNC/CEO Coating

The coating solution consisted of 9 g of WPI powder (w/v), 2 g of NCH (w/v), 1 g of BNC (w/v), and 5 ml glycerol (v/v), which was dissolved in 100 ml distilled water. The solution was maintained at 90°C in a thermostatic bath with continuous agitation for 20 min to promote WPI denaturation and enhance cross-linking among the compounds. After cooling the solution, CEO was incorporated at varying amounts (0.5, 1, and 1.5% (v/v)) and homogenized (Wisd homogenizer, Korea) at 24,000 rpm for two min [10]. One group was prepared without CEO and served as a control (CBW). The other three groups, containing the specified CEO concentrations, were designated as CBW+0.5% CEO, CBW+1% CEO, and CBW+1.5% CEO, respectively.

Microbiological Analysis

In vitro Antibacterial Assay

The antibacterial efficacy of the prepared coatings was evaluated against *Escherichia coli* O157:H7 ATCC 43895 and *Salmonella* Typhimurium ATCC 14028 as Gram-negative bacteria and *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 19117 as Gram-positive bacteria. To prepare inocula, the bacterial cells of each bacterium were transferred to tubes containing 10 ml Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h. Then, the optical density (OD) of each tested bacterial suspension was adjusted to 0.1 at 600 nm using a spectrophotometer. The resulting cultures (OD 600 nm = 0.1) were diluted in peptone water (0.1% w/v) to obtain a suspension containing about 7 log CFU/ml bacterial cells. The bacterial cell count of the inocula was measured by plating on BHI agar [11].

At first, each bacterial solution (0.2 ml) was added to each Erlenmeyer flask. Next, CEO solutions were made by using tween 80 (Merck, Germany) and distilled water, such that by adding 0.2 ml of each solution to the Erlenmeyer flasks, including liquid culture medium and test bacteria, to prepare concentrations of 0%, 0.015%, 0.03%, 0.045%, 0.09%, and 0.18%. Concentrations higher than this amount were not studied due to the poor solubility of CEO in tween 80 and distilled water. The Erlenmeyer flasks were then incubated in a shaking incubator at 37°C and 140 rpm for 24 h. The minimum inhibitory concentration (MIC) was identified as the lowest concentration of CEO at which there was no visible turbidity after 24 h. The experiment was repeated at lower concentration levels, including 0.01% and 0.005%, to find the MIC if no turbidity appeared at the 0.015% concentration level. After the MIC measurement, the minimum bactericidal concentration (MBC) was identified by transferring 0.1 ml of the of the Erlenmeyer flasks containing no visible turbidity to petri dishes containing the BHI agar medium for each type of bacteria. After 24 h of incubation at 37°C, the bacterial growth was monitored. The first concentration at which no colony was seen was regarded as the MBC [12].

The antimicrobial activity of CBW and CBW+CEO coatings containing 5% DMSO was evaluated by determining the MIC and MBC of coating solutions against the aforementioned foodborne pathogens. Various proportions of coating solutions in BHI broth (5%, 10%, 15%, …50% v/v) of were prepared. After that, prepared solutions were inoculated with 7 log CFU/ml of each tested bacterium and then incubated in a shaking incubator at 37°C for 24 h under continuous shaking (75 rpm). The MIC of coating solutions was defined as the last tube in the dilution series that exhibited no visible turbidity or signs of growth. The MBC of coating solutions was identified by plating 0.1 ml of the contents of each tube without turbidity onto BHI agar and incubating them at 37°C for 24 h [13, 14].

Disc Diffusion Assay

The assessment of antibacterial efficacy was conducted employing the disk diffusion assay as indicated by the clinical and laboratory standards institute's guidelines [15, 16] as follows: 0.1 ml of each bacterial suspension containing

approximately 7 Log CFU/ml was inoculated on the surface of Mueller-Hinton agar plates. Afterward, sterile Whatman paper discs (6.4 mm in diameter) impregnated with 10 µL of coating solutions were put onto the surface of the inoculated Mueller-Hinton agar. Then the plates were incubated at 37°C for 24 h. The diameter of inhibition zones (DIZ) was determined using ImageJ software (version 1.54) [17].

In vitro Antioxidant Activity

The antioxidant potency of the coating solutions was determined by their capability to scavenge DPPH free radicals. In brief, 0.5 mL of various coating solutions were dissolved in 1 mL of methanol and subsequently integrated into 2 mL of a methanolic solution of DPPH (100 mmol/L). The resulting mixtures were agitated and incubated at room temperature in the absence of light for 30 min, following which the absorbance was determined at 517 nm against blank. The antioxidant potency was quantified using the following formula:

Antioxidant activity (%) = [(A_{517} control - A_{517} sample) / A_{517}control] × 100

(A_{517} sample is the absorbance of the mixture of DPPH solution plus coating solution and A_{517} control is the absorbance of pure DPPH solution) [18].

Statistical Analysis

Data analysis was performed utilizing SPSS software (version 27, SPSS, Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was conducted, followed by Tukey's test, to discern any statistically significant variations among the mean values.

Result and Discussion

Chemical Composition of Cinnamon Essential Oil

The dominant constituents of the used CEO were cinnamaldehyde, δ-cadinene, and cis-cinnamaldehyde, respectively. It was documented that cinnamaldehyde is the most abundant component of CEO [19-21].

Other researchers have documented similar compounds; however, notable distinctions exist in concentration levels [22-24]. These variations may be related to divergent environmental factors such as weather conditions, soil composition, seasonal variations, geographic and geological factors influencing the growth of plants, as well as disparities in the age and stage of maturity. Additionally, distinctions may arise from variances in the processing of plant materials before EO extraction and variations in the extraction methods employed [25].

Microbiological Analysis

In vitro Microbiological Analysis

The CEO's MICs and MBCs against the investigated bacterial strains are presented in Table 1. In this study, the MIC and MBC values of CEO against tested bacteria ranged from 0.3 to 0.9 µl/ml and 0.45 to 0.9 µl/ml, respectively. The lowest MIC was observed in *S. aureus* and *L. monocytogenes*, while the highest MIC was recorded in *S.* Typhimurium. Conversely, the lowest MBC value was noted in *S. aureus*, while the highest MBC value was observed in *L. monocytogenes*, *E. coli*, and *S.* Typhimurium.

Table 1. MIC and MBC of CEO

Bacteria	*S.* Typhimurium	*E. coli*	*S. aureus*	*L. monocytogenes*
MIC (µl/ml)	0.9	0.45	0.3	0.3
MBC (µl/ml)	0.9	0.9	0.45	0.9

CEO exhibited intense antibacterial activity against all tested bacterial species. The findings of the current study are in the same boat as those of Prabuseenivasan et al. (2006) and Oulkheir et al. (2017), who also noted that CEO exhibited strong activity against selected bacterial strains [13, 26].

On the other hand, some researches has demonstrated the potent and consistent inhibitory effects of CEO against various pathogens [27]. Several investigations have indicated that the antimicrobial potency of EOs can be related to their characteristic hydrophobic nature and the properties of their components. This hydrophobicity allows essential oils to interact with the lipids of bacterial cell membranes, disrupting cell structures and increasing permeability [28, 29]. The resultant vast leakage from bacterial cells or the release of essential molecules and ions finally results in cell death [30].

The effectiveness of coating solutions in inhibiting four food-borne pathogens is detailed in Table 2. The CBW coating solution exhibited MIC and MBC values ranging from 30% to 35% and 35% to 45%, respectively, against the

following organisms in BHI broth: *S.* Typhimurium (35% MIC and 45% MBC), *E. coli* (30% MIC and 40% MBC), *S. aureus* (30% MIC and 35% MBC), and *L. monocytogenes* (30% MIC and 35% MBC). The incorporation of 0.5% EO with CBW further affected MIC and MBC values for all food-borne pathogens, ranging from 5% to 10%, except MBC values for *L. monocytogenes*, which exhibited no changes. Moreover, at higher concentrations of EO (1% and 1.5%), the MIC and MBC values declined. The impact of EO on MIC and MBC was generally consistent across these organisms, except for the MBC of *S.* Typhimurium and *E. coli* at a concentration of CBW+ 1.5% CEO, which showed no effect on their MBC value when compared to CBW+ 1% CEO. The antimicrobial potency of chitosan is proposed to result from mechanisms such as membrane leakage resulting from interactions between positively charged chitosan and the negatively charged bacterial cell surface, nutrient and essential metal chelation, and chitosan penetration into bacterial cells, thereby hindering DNA transcription [14].

Compared with other EOs, such as Apricot (*Prunus armeniaca*) kernel and *Ferulago angulata* EO, the synergistic effect of CEO on the antimicrobial activities of chitosan was found to be more pronounced [18, 31].

However, nanotechnology's utilization significantly augmented the coating solutions' antibacterial potency, as demonstrated in Table 2. This enhancement was contingent on the specific coating formulation; higher concentrations of CEO were more profoundly affected by nano emulsification. The heightened water dispersibility, coupled with a reduction in droplet size after homogenization, facilitated a more efficient and rapid penetration of antimicrobial constituents via the bacterial cell membrane, thereby amplifying their efficacy [32, 33]. Additionally, the application of severe mechanical stress caused the chitosan molecular chains to break into shorter chains, facilitating easier passage through the bacterial membrane and consequently increasing antibacterial activity [34].

Table 2. MIC and MBC of coatings

Coating Combination	*S.* Typhimurium		*E. coli*		*S. aureus*		*L. monocytogenes*	
	MIC (%)*	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)
CBW	35	45	30	40	30	35	30	35
CBW+ 0.5% CEO	30	40	25	35	20	30	25	35
CBW+ 1% CEO	20	30	20	20	15	20	20	25
CBW+ 1.5% CEO	15	30	15	20	10	15	15	15

*% of coating solution in BHI broth.

Antibacterial Assessment by Disc Diffusion Assay

The findings of the antibacterial potency of the coating solutions against the tested foodborne pathogen bacteria using the disc diffusion assay are displayed in Table 3. All treatments resulted in the creation of inhibition zones, except for coatings without CEO and CBW containing 0.5% CEO for gram negative bacteria. The integration of higher concentrations of CEO increased the DIZ, and the maximum inhibition zones were determined for CBW containing 1.5% CEO, with average inhibition zone sizes of 16.56, 18.2, 19.22, and 21.14 mm for *S.* Typhimurium, *E. coli*, *L. monocytogenes*, and *S. aureus*, respectively.

Table 3. Inhibition zone (mm)

Coating / Bacteria	CBW	CBW+ 0.5% CEO	CBW+ 1% CEO	CBW+ 1.5% CEO
E. coli	0^{aA*}	0^{aA}	15.9 ± 0.42^{aB}	18.2 ± 0.28^{aC}
L. monocytogenes	0^{aA}	15.34 ± 0.4^{bB}	16.81 ± 0.38^{bC}	19.22 ± 0.31^{bD}
***S.* Typhimurium**	0^{aA}	0^{aA}	15.19 ± 0.3^{aB}	16.56 ± 0.23^{cC}
S. aureus	0^{aA}	16.19 ± 0.44^{bB}	18.37 ± 0.41^{cC}	21.14 ± 0.39^{dD}

*Values are means ± standard deviations. Means with different lowercase letters within the same column are significantly different ($p < 0.05$). Means with different capital letters within the same row are significantly different ($p < 0.05$).

According to the documents, the antibacterial activity of food coatings devolves on some factors, consisting of the type and physicochemical characteristics of integrated antibacterial agents, the composition and concentration of coating materials, and the

technique of preparation of the coating solution [35, 36]. It was documented that flavonoids, aromatics, and esters are the major antibacterial constituents of CEO [37].

Additionally, the antibacterial findings of CBW containing CEO indicate successful incorporation of CEO into the coatings, and its release from the discs immersed with coating solutions during antibacterial tests.

In these cases, a synergistic effect between NCH and BNC is observed. The stiff, slender and rod-shaped particles of BNC have been predicted to damage the bacterial cell membrane seriously. Microbial cells are vulnerable to the cationic effect of chitosan because of this damaged membrane [38-40]. Therefore, these coatings can have good antimicrobial properties even with a small amount of BNC.

In the current research, gram-positive bacteria exhibited greater susceptibility to CBW coatings containing various concentrations of CEO. These findings align with existing literature, which generally suggests that gram-negative bacteria are more resistant to plant EOs (including CEO) than gram-positive bacteria [41].

The chemical composition analysis of CEO suggests that its antibacterial activity is primarily attributed to its high cinnamaldehyde content, a conclusion consistent with previous research [9]. The antimicrobial potency observed was variable and dependent on several factors, including the type of incorporated EO, concentration of EO, microbial group susceptibility, and sample storage time. Higher EO concentrations in the formulations corresponded to increased effectiveness of the coatings. It is noteworthy that, in the context of using EOs in NCH edible coatings, an increase in concentration often determines the difference between the presence or absence of effectiveness.

Furthermore, when combined with essential oils, BNC and WPI exhibit enhanced antimicrobial activity due to synergistic effects. Essential oils, characterized by their volatile and lipophilic nature, can readily penetrate bacterial cell membranes and disrupt internal structures. The tightly packed structure and nanofibrils of BNC further facilitate the penetration of essential oils into bacterial cells (24). Meanwhile, WPI's antimicrobial peptides and enzymes contribute additional antimicrobial activity. Combining BNC, WPI, and essential oils can result in a broad-spectrum antimicrobial effect against a wide range of microorganisms [42, 43].

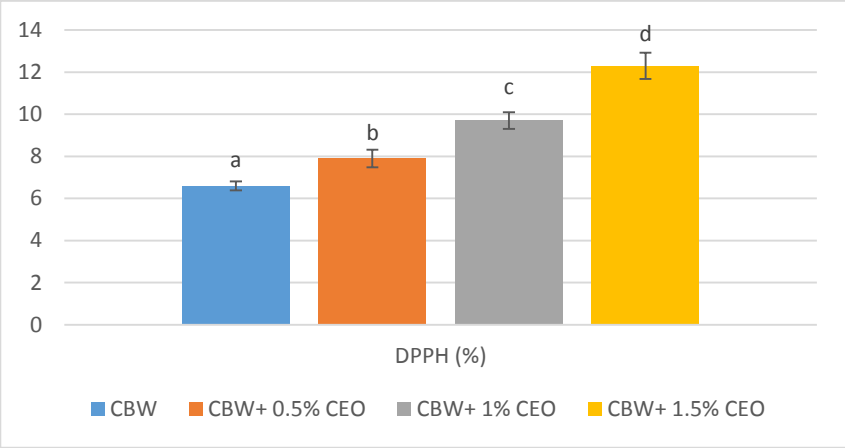

Figure 1. Effect of different concentration of CEO on antioxidant activity of CBW coating
* Different lower case above each graph column indicates significant difference ($p < 0.05$). values are given as mean ± SD.

Antioxidant Activity

Antioxidant activity measurement promotes the production of healthy meals, aligns with consumer health preferences, and provides valuable insights into food quality. In this research, the antioxidant effect of CEO was assessed, and the results are illustrated in Figure 1. Chitosan solution without EO exhibited a 6.6% scavenging activity on DPPH. The antioxidant activity of chitosan increased to 7.9, 9.7, and 12.3 by adding 0.5, 1, and 1.5% CEO, respectively, indicating a concentration-dependent scavenging activity of the chitosan solution on DPPH. The antioxidant potency of CEO may be

related to higher amounts of terpenoid compounds and ample amounts of cinnamaldehyde, which possess high antioxidant activities [44].

Regarding CEO, the results of the current study are consistent with those of Lalami et al., Moarefian et al., and Subki et al., who noted excellent antioxidant activity of CEO [17, 45, 46]. Moreover, the reduction in droplet size of NCH, WPI, BNC, and CEO emulsion after homogenization contributes to a increased specific surface of chitosan-EO, promoting easier and more effectual free radical scavenging. In consistent with these findings, Noori et al. (2018) declared a meaningful increase in the antioxidant potency of sodium caseinate coating enriched with ginger EO through ultrasonic nano emulsification [47].

Furthermore, bacterial nanocellulose (BNC) and whey protein isolate (WPI) exhibit antioxidant activity by scavenging free radicals, protecting cells from oxidative damage, and enhancing the antioxidant activity of other compounds. The hydroxyl groups and hydrogen bonding ability of BNC contribute to its antioxidant properties, while the amino acids and sulfhydryl groups in WPI play a role in its antioxidant activity. Combining BNC and WPI can further enhance antioxidant activity due to synergistic interactions between these materials [48, 49].

Conclusions

The antibacterial assays revealed the susceptibility of four major food-borne pathogens to CBW+ CEO, with this combination exhibiting heightened activity against common food-borne pathogens as the percentage of CEO increased. The addition of higher concentrations of CEO resulted in increased antibacterial efficacy, as evidenced by the expansion of the widths of the inhibition zones. The MIC and MBC of the coating diminished as the concentration of CEO increased, indicating its potential for inhibiting the growth of tested bacteria. Notably, the presented coating demonstrated significant antioxidant effects comparable to the control group, with the capability to scavenge free radicals. These findings underscore the potential of WPI, NCH, BNC and CEO as antibacterial and antioxidant agents. The research acknowledges the necessity of more studies to examine the potential and merits of these components in the food industry. To determine how well the coatings inhibit microbiological growth and food spoilage, these studies should involve evaluating the coatings over an extended period of time on foodstuffs.

Acknowledgments

The research council of the Faculty of Veterinary Medicine, University of Tehran, financially supported this article.

Conflict of Interest

The authors declare no conflict of interest.

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