



# Effects of *Citrus sinensis* Essential Oil and Intrinsic and Extrinsic Factors on the Growth and Toxin-Producing Ability of *Clostridium botulinum* Type A

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**Abstract**

**Background:** Considering the high fatality of botulism, the control of *Clostridium botulinum* and its neurotoxins has clinical importance. In this regard, using chemical preservatives, natural essential oils (Eos), and changes in the growth predisposing factors of bacteria are suitable methods to control the growth and toxin producing of *C. botulinum* in foods.

**Objective:** The current survey was done to assess the effects of *Citrus sinensis* EO and intrinsic and extrinsic factors on the growth and toxin producing of *C. botulinum* type A.

**Materials and Methods:** In this experiment with a factorial design, *C. sinensis* EO (0.0%, 0.015%, 0.03%, and 0.045%), nisin (0, 500, and 1500 IU/mL), nitrite (0, 20, and 60 ppm), pH (5.5 and 6.5), storage temperature (25 and 35°C), and sodium chloride (NaCl, 0.5% and 3%) were used to assess bacterial growth in the brain heart infusion medium. Finally, the mouse bioassay method was also used to assess toxicity.

**Results:** *Clostridium sinensis* EO with a concentration of 0.045%, as well as the reduction of pH and temperature could significantly delay the growth of bacteria ( $P \leq 0.05$ ) in contrast to the use of NaCl and nisin alone. However, all concentrations of sodium chloride (NaCl), nisin, and *C. sinensis* EO ( $< 0.045\%$ ) in interaction with each other, especially in combination with nitrite, showed good synergistic effects.

**Conclusion:** These results suggested that using certain concentrations of *C. sinensis* EO and nisin, along with other suboptimal factors caused a significant decrease in the nitrite contents of foods with a significant reduction in the growth and toxin-producing ability of *C. botulinum*.

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## Background

Botulinum toxin is recognized as a serious cause of food-borne botulism produced by the *Clostridium botulinum*.<sup>1,2</sup> This disease has high clinical and microbial importance due to the high mortality rate.<sup>1,2</sup> Thus, it is essential to decrease the risk of this toxin in diverse kinds of food samples.

Using high temperature, along with the boost concentrations of sodium chloride (NaCl) and chemical preservatives such as nitrite is a traditional method for decreasing the risk of *C. botulinum* in foodstuffs.<sup>3</sup> Nitrite and its precursor, nitrate, are known to positively affect the flavor, appearance, quality, and safety of cured meat. In particular, nitrite can guarantee the safety of food products through inhibiting the growth of microorganisms, especially *C. botulinum*.<sup>4</sup>

Nevertheless, concerns are growing with respect to the risks of nitrite application in meat products, including the formation of nitrosamines (i.e., mutagenic and

carcinogenic compounds).<sup>5</sup> The use of Hurdle technology is a possible approach for decreasing the concentrations of nitrite in foodstuffs, along with other processing technologies and compounds.<sup>6</sup>

In addition, nisin is a small and stable peptide with antimicrobial effects on different Gram-positive bacteria and the only authorized bacteriocin used as a food preservative.<sup>7</sup> In Hurdle technology, this peptide can be used effectively as a natural preservative.<sup>6</sup> Further, natural essential oils (EOs) are the mixtures of different extracted chemicals from the plants. Their relatively low cost, antimicrobial activities, low toxicity, and natural origin make them as a suitable option as food preservatives.<sup>8</sup>

*Citrus sinensis*, also known as orange, is an ancient medicinal plant with highly beneficial and therapeutic activities.<sup>9,10</sup> Citrus EOs have potential antimicrobial properties against spore-forming and food poisoning bacteria and fungi. Moreover, *C. sinensis* EOs and their major components are generally recognized as safe in

the food industry. Several studies have also reviewed the antioxidant and antimutagenic properties of orange Eos.<sup>9,10</sup>

Considering the above-mentioned explanation, this study aimed to determine and compare the effects of nitrite, nisin, and *C. sinensis* (sweet orange) EO alone and in combination with each other, affected by different levels of pH, NaCl concentration, and temperature on growth and toxin-producing ability of *C. botulinum* type A.

## Materials and Methods

### Plant Materials

The *C. sinensis* cv. *Valencia* fruits were obtained from Ramsar (Mazandaran, Iran) in May 2017 and identified at Citrus Research Institute of Iran. A Clevenger apparatus was used to expose fresh orange peels to hydro-distillation for 3 hours.<sup>11</sup> Then, gas chromatography (Agilent Technologies, USA) was carried out to determine EO composition. The procedure was done on an ionization energy of 70 eV, followed by performing mass spectrometry in the electron ionization mode.

### Nisin

Nisin was purchased from Sigma Aldrich (Sigma, USA) Company (containing 2.5% active nisin). To prepare a nisin concentration of 10 000 IU/mL, nisin (1 g) was added to a 100-mL volumetric flask, followed by adding 0.02 M hydrochloric acid to reach 100 mL. Additionally, to separate insoluble whey proteins, the solution was centrifuged for 15 minutes at 5000×g. Afterward, it was sterilized via filtration through a 0.22- $\mu$ m disposal and nonpyrogenic syringe filter (Millipore, Bedford, MA). The stock solution was diluted in sterilized 0.02 N HCl during the experiments to obtain nisin working solutions of 500 and 1500 IU/mL, respectively.<sup>12,13</sup>

### Nitrite

Sodium nitrite was purchased from Merck Company (Millipore, Germany).

### Test Organism

In this study, *C. botulinum* type A (RS5) was provided by the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

### Experimental Design

The experiment with a factorial design included different *C. sinensis* EO concentrations (i.e., 0.0, 0.015%, 0.03%, and 0.045%), nisin contents (0, 500, and 1500 IU/mL), nitrite contents (0, 20, and 60 ppm), pH values (5.5 and 6.5), storage temperatures (25 and 35°C), and NaCl concentrations (0.5% and 3%). It was carried out to study the growth of *C. botulinum* type A in the brain heart infusion (BHI) medium over 32 days (time to detection).

### Inoculum Preparation

After three consecutive passages of lyophilized bacteria in

the BHI broth (Merck Millipore, Germany), *C. botulinum* type A spores were cultured in the egg yolk agar (HiMedia, India) and BHI agar (Merck Millipore, Germany) via surface plating in an anaerobic jar (HiMedia, India) containing gas packs and an indicator strip (Merck Millipore, Germany) and then incubated at 35°C for 3 weeks. Next, the colonies were washed and gathered with the phosphate buffer at a pH of seven using the sweeping method. After the centrifugation of spores containing suspensions for 15 minutes at 5000×g, they were washed with sterile water and suspended in 50% ethanol (Merck Company, Germany). Finally, the spores were determined via plating on the BHI agar and stored at 4°C until further use.<sup>14,15</sup>

### Broth Substrate Preparation

BHI powder was dissolved in distilled water inside a screw-capped flask (250 mL) through mild heating. Considering the experimental design, salt (NaCl, Merck Millipore, Germany) and sodium nitrite were added in different quantities. During the study (32 days), dimethyl sulfoxide (DMSO 5% (v/v), Merck Millipore, Germany) and 0.05% (w/v) agar-agar (Merck Millipore, Germany) were added to the broth substrate as the emulsifier and stabilizer, respectively. Furthermore, DMSO and agar-agar were added to combinations without EO (0.0%) at the same quantities to consider possible effects on the growth of the bacteria.<sup>16</sup>

Then, HCl (1N, Merck, Germany) was used to adjust the pH in the combinations (6.5 and 5.5) and a pH meter (PHS, 3e, China) was used for pH measurements. After adding the flask content into screw-capped tubes (9 mL per tube), it was autoclaved for 15 minutes at 121°C. The aliquots of nisin concentrations (stock solution), as well as sterilized EOs were added to the tubes after cooling in order to prepare final concentrations (active nisin concentrations). No changes were reported after the re-measurement of the pH in the final combinations (broth).

### Inoculation and Storage of Broth Substrate

The spore suspension (1 mL) was inoculated into the substrate, which was collected in the previous stage. The final concentration of the spores of the substrate was equal to  $4 \times 10^4$  CFU/mL. To prepare anaerobic conditions, 1 mL of sterilized paraffin was added to each tube and the inoculated tubes were finally stored at 25°C and 35°C for 32 days. Visible growth (turbidity) was monitored at 5 intervals (i.e., 2, 4, 8, 16, and 32) and time was recorded during the experiments.

### Toxin Detection by Mouse Bioassay

Mouse bioassay was used for toxin detection according to the Food and Drug Administration protocol. This method is considered the gold standard due to high sensitivity in toxin detection (0.01 ng/mL).<sup>17,18</sup> After removing the turbid tubes from the incubators, cold centrifugation was

carried out for 15 minutes at 7000×g. Next, supernatant liquids were frozen at -20°C until injection into 6-8 weeks old mice weighing 18-25 g. The frozen samples were placed at room temperature for half an hour before use. Then, 2 mL of each tube sample was transferred to another tube and heated at 100°C in a water bath for 10 minutes. One mouse from each pair was used for intraperitoneal injection of 0.5 mL of an unheated fluid while the other mouse received 0.5 mL of the heated fluid. Eventually, the mice were monitored for the signs of botulism for 48 hours.<sup>14,18</sup>

**Statistical Analysis**

The present study evaluated the main and interactive effects of EO, nitrite, and nisin, with respect to salt concentration, pH, and temperature, on the growth and toxin-producing ability of *C. botulinum* using the analysis of variance test in SPSS for Windows (version 21.0, SPSS Inc.) and  $P < 0.05$  was considered as a significant level.

**Results**

Table 1 signifies the chemical composition of EO (Gas chromatography-mass spectrometry results). Limonene is recognized as the main EO component (92.85%).

Table 2 presents data related to the effects of nitrite, nisin, and *C. sinensis* EO (separately or in combination), with respect to salt concentration and temperature, on growth time and turbidity. Based on the results, the growth of bacteria was strongly affected by pH reduction. More precisely, no turbidity was observed in the tubes within 32 days, and growth was completely inhibited with a pH reduction from 6.5 to 5.5. Decreasing temperature from 35 to 25°C also delayed the growth time significantly ( $P \leq 0.05$ ). On the other hand, increasing salt concentration from 0.5% to 3% alone did not cause a major difference in the time of turbidity ( $P \leq 0.05$ ). However, when 3% salt concentration was associated with other preservatives, especially nitrite, it could delay the growth of bacteria for a longer time.

According to the results (Table 2), the evaluated concentrations of nisin (500 and 1500 IU/mL) alone had no significant effects on the prevention of the growth of

**Table 1.** Essential Oil Composition of *Citrus. sinensis* cv. Valencia Identified by GC-MS

| Compound  | Percentage |
|-----------|------------|
| α-pinene  | 0.93       |
| Sabinene  | 0.42       |
| β-myrcene | 2.32       |
| Limonene  | 92.85      |
| Deconol   | 0.4        |
| Linalol   | 0.28       |
| Sum       | 97.2       |

Note. GC-MS: Gas chromatography-mass spectrometry.

**Table 2.** Turbidity Time (Day) of *C. botulinum* Type A With the Inoculum Level of  $4 \times 10^4$  CFU/mL During 32 Days Storage in the BHI Broth as Affected by Temperature and Preservatives at pH Value of 6.5

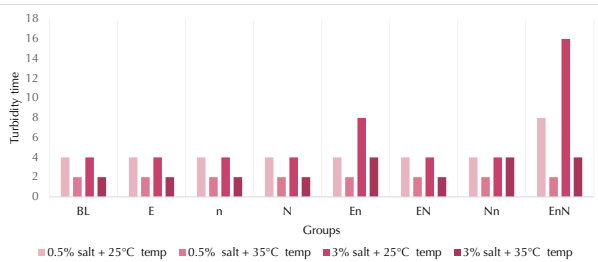
| EO (%) | Nitrite (ppm) | Nisin (IU) | Salt 0.5% |       | Salt 3% |       |
|--------|---------------|------------|-----------|-------|---------|-------|
|        |               |            | 25 °C     | 35 °C | 25 °C   | 35 °C |
| 0      | 0             | 0          | 4         | 2     | 4       | 2     |
|        |               | 500        | 4         | 2     | 4       | 2     |
|        |               | 1500       | 4         | 2     | 4       | 2     |
|        |               | 0          | 4         | 2     | 4       | 2     |
|        |               | 500        | 4         | 2     | 4       | 4     |
|        |               | 1500       | 8         | 4     | 8       | 4     |
|        | 20            | 0          | 8         | 4     | 16      | 8     |
|        |               | 500        | 8         | 4     | 16      | 16    |
|        |               | 1500       | 16        | 8     | 32      | 16    |
|        |               | 0          | 4         | 2     | 4       | 2     |
|        |               | 500        | 4         | 2     | 4       | 2     |
|        |               | 1500       | 4         | 2     | 8       | 4     |
| 0.015  | 20            | 0          | 4         | 2     | 8       | 4     |
|        |               | 500        | 8         | 2     | 16      | 4     |
|        |               | 1500       | 8         | 4     | 16      | 8     |
|        |               | 0          | 16        | 8     | 16      | 16    |
|        |               | 500        | 16        | 8     | 32      | 16    |
|        |               | 1500       | 32        | 8     | 32      | 16    |
|        | 60            | 0          | 4         | 2     | 4       | 2     |
|        |               | 500        | 4         | 2     | 8       | 4     |
|        |               | 1500       | 4         | 2     | 8       | 4     |
|        |               | 0          | 4         | 2     | 8       | 4     |
|        |               | 500        | 8         | 2     | 16      | 4     |
|        |               | 1500       | 8         | 4     | 16      | 8     |
| 0.03   | 20            | 0          | 4         | 2     | 8       | 4     |
|        |               | 500        | 4         | 2     | 8       | 8     |
|        |               | 1500       | 8         | 4     | 8       | 8     |
|        |               | 0          | 8         | 4     | 16      | 8     |
|        |               | 500        | 16        | 8     | 16      | 16    |
|        |               | 1500       | 32        | 16    | 32<     | 32    |
|        | 60            | 0          | 32        | 16    | 32<     | 32    |
|        |               | 500        | 32<       | 32    | 32<     | 32<   |
|        |               | 1500       | 32<       | 32<   | 32<     | 32<   |
|        |               | 0          | 8         | 4     | 8       | 4     |
|        |               | 500        | 8         | 4     | 8       | 8     |
|        |               | 1500       | 16        | 8     | 16      | 8     |
| 0.045  | 20            | 0          | 16        | 16    | 16      | 16    |
|        |               | 500        | 16        | 16    | 32<     | 32    |
|        |               | 1500       | 32<       | 32    | 32<     | 32<   |
|        |               | 0          | 32<       | 32    | 32<     | 32<   |
|        |               | 500        | 32<       | 32<   | 32<     | 32<   |
|        |               | 1500       | 32<       | 32<   | 32<     | 32<   |

Note. BHI: Brain heart infusion; EO: Essential oil; *C. botulinum*: *Clostridium botulinum*. \*There was no growth in the pH of 5.5 in all tubes until after day 32 (32<) thus its information is not presented in the table.

*C. botulinum* type A ( $P \leq 0.05$ ) although a good synergistic effect was found in interaction with nitrite and EO. Then, the effect of increasing concentration from 500 to 1500 IU/mL was determined when nisin was associated with certain concentrations of EO and nitrite.

Figure 1 displays the turbidity time of *C. botulinum* type A with the inoculum level of  $4 \times 10^4$  CFU/mL during 32 days storage in the BHI broth as affected by salt, temperature, and minimum preservative values at pH 6.5.

Figure 2 depicts the turbidity time (day) of *C. botulinum*



**Figure 1.** Turbidity Time (day) of *C. botulinum* Type A With the Inoculum Level of  $4 \times 10^4$  CFU/mL During 32 Days Storage in the BHI Broth as Affected by Salt, Temperature, and Minimum Preservative Values at pH 6.5.

Note. BL: Blank; E: Essential oil 0.015%; n: Sodium nitrite 20 ppm; N: Nisin 500 IU; En: Essential oil and nitrite; EN: Essential oil and Nisin; EnN: Essential oil, nitrite, and Nisin.

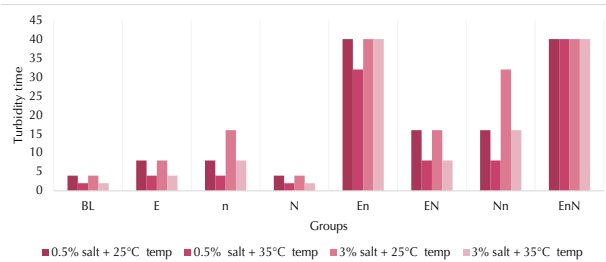
type A with the inoculum level of  $4 \times 10^4$  CFU/mL during 32 days storage in the BHI broth as affected by salt, temperature, and maximum preservative values at pH 6.5. The present results indicated that 20 ppm of sodium nitrite alone could not prevent the germination and growth of bacteria, but the growth of bacteria was obviously postponed by increasing the concentration of nitrite to 60 ppm. However, the synergistic effect of nitrite with salt, nisin, and *C. sinensis* EO was also significant ( $P \leq 0.05$ ). According to the results, concentrations  $\leq 0.03\%$  of *C. sinensis* EO did not influence the growth time of *C. botulinum* type A. By increasing EO concentration to 0.045%, bacterial growth occurred with a significant delay compared to lower concentrations ( $P \leq 0.05$ ). Nonetheless, the subinhibitory concentrations of *C. sinensis* EO, especially 0.03% concentration, in combination with other inhibitory agents, showed good synergistic effects.

In this study, despite the use of moderate concentrations of preservatives and synergistic effects, the growth of *C. botulinum* type A was completely inhibited during 32 days of the study in certain combinations.

The mice receiving non-heated samples died within 48 hours after injection, showing botulism symptoms such as muscle weakness, fuzzy hair, and respiratory failure (wasp-like narrowed waist) although all those mice, which had received heated specimens, survived, therefore, factors used in this study prevented toxin formation by inhibiting bacterial growth.

## Discussion

The properties of EOs, including flavor and antimicrobial activities, are directly related to their chemical constituents. Limonene is the main component of *C. sinensis* EO, and myrcene, alpha and beta pinene, and sabinene are among the other major constituents.<sup>19-22</sup> In our study,  $\beta$ -myrcene and limonene were recognized as the main components of *C. sinensis* EO, which is consistent with the results of the mentioned studies. Many factors such as genetic variations, climate, postharvest factors (e.g.,



**Figure 2.** Turbidity Time (day) of *C. botulinum* Type A With the Inoculum Level of  $4 \times 10^4$  CFU/mL During 32 Days Storage in the BHI Broth as Affected by Salt, Temperature, and Maximum Preservative Values at pH 6.5.

Note. BL: Blank; E: Essential oil 0.045%; n: Sodium nitrite 60 ppm; N: Nisin 1500 IU; En: Essential oil and nitrite; EN: Essential oil and Nisin; EnN: Essential oil, nitrite, and Nisin.

storage conditions), and extraction methods can affect the chemistry of Eos.<sup>23</sup>

Previous studies examined the effects of *Citrus* EOs on food spoilage organisms and food-borne pathogens. For instance, Settanni et al evaluated the antimicrobial activity of some *Citrus* EOs via hydro-distillation against *Staphylococcus aureus*, *Salmonella enteric*, and *Listeria monocytogenes*. In their study, greater effectiveness against Gram-positive bacteria was found in most EOs compared to *Salmonella*. This finding can be attributed to the resistant outer membrane of Gram-negative bacteria.<sup>24</sup> In this regard, Randazzo et al found that the incorporation of lemon EOs in chitosan films could be effective in controlling *L. monocytogenes*, particularly under refrigerated applied conditions.<sup>25</sup>

However, few studies examined the effects of EOs, especially *C. sinensis* EO, on *Clostridium botulinum*. For example, Ismaiel and Pierson evaluated the effects of thyme, clove, pimenta, black pepper, Origanum, onion, garlic, and cinnamon oil on the germination and growth of *C. botulinum* types A, B, and E. At 150 and 200 ppm, all oils completely prevented germination and the spores of type A were more sensitive than those of types B and E.<sup>26</sup>

In another study, Nevas et al examined the antibacterial effects of 13 Finnish EOs against 12 bacterial strains. *C. botulinum* types B and E, besides *Clostridium perfringens*, were the most sensitive bacteria. They concluded that due to the sensitivity of *C. botulinum* to EOs, it can be used to reduce the concentration of nitrite in meat products.<sup>27</sup> Similarly, Chaibi et al studied the antibacterial activity of 9 EOs, prepared by hydro-distillation, on the vegetative cells and spores of *Bacillus cereus* and *C. botulinum* 62A. The spores showed less resistance compared to vegetative cells. *Bacillus* spores were more sensitive than that of clostridium. Orange EO had bacteriostatic effects and did not show bacteriocidal properties at evaluated concentrations.<sup>28</sup>

In our study, *C. sinensis* EO with a concentration of 0.045% caused a major delay in the growth of *C. botulinum*

type A at both temperatures and salt concentrations. However, the EO could not prevent bacterial growth for more than eight days. Considering the effect of salt concentration and pH on *C. botulinum*, Lalitha and Gopakumar found that the spores of *C. botulinum* types A and B could grow in cooked meat at 30°C and produce toxins up to 8% salt. They further reported that the lag phase and time to toxicity increased by decreasing the storage temperature. Overall, salt tolerance decreased by decreasing pH and storage temperature.<sup>29</sup>

Likewise, Jensen et al investigated the growth probability of proteolytic *C. botulinum* at different temperatures. The log percent probability of growth was 2 at 20°C and 30°C and 2.7 at 37 °C<sup>30</sup>. In addition, Baker et al concluded that in vacuum packaging at 30, 20, 16, and 8°C, toxin-producing ability occurred on days 0.5, 1, 2, and 9 in *C. botulinum* type E, respectively. Contrarily, under MA packaging, no toxicity was observed at 4°C even after storage for 60 days.<sup>31</sup>

In the present study, decreasing temperature from 35 to 25°C led to a delay in growth and toxin-producing ability. Further, the pH reduction from 6.5 to 5.5 in all studied conditions completely prevented *C. botulinum* growth and toxin-producing ability, which indicates the higher importance of pH in controlling food-borne botulism compared to the other factors. However, many studies showed that pH less than 5 and even less than 4.8 is needed for the effective prevention of *C. botulinum* growth. This difference in pH between our study and some other studies can be related to differences in the inoculum dose and bacterial strain.

Based on the results of our study, when salt (sodium chloride) was used alone, no significant difference was found between 0.5% and 3% salts ( $P \leq 0.05$ ). It cannot be concluded that an increase in salt concentration does not have any effects on the growth of *C. botulinum*. However, it can be interpreted that both concentrations of the salt are inadequate to prevent growth.

The increase in salt concentration, when accompanied by an increase in other preservatives, especially nitrite, had a significant effect on postponing growth and toxin-producing ability. These findings are consistent with those of the study by Meng et al. They demonstrated that increasing the concentration of sodium chloride from 1% to 2%, accompanied by an increase in sodium lactate and a reduction in temperature, had beneficial effects on delaying toxin-producing ability.<sup>32</sup>

Regarding the effects of nisin on *C. botulinum*, Scott and Taylor stated that nisin could be a proper alternative to nitrite. However, more nisin is needed in processed meat probably owing to nisin binding to meat particles.<sup>33</sup> On the other hand, Okereke and Montville concluded that nisin alone cannot guarantee the health of food, but works well with other inhibitors such as sodium chloride.<sup>34</sup> According to a study by Khanipour et al, potassium sorbate, salt, and nisin exhibited major inhibitory effects

on the growth of *C. sporogenes* (nontoxicogenes surrogates of *C. botulinum*) at high pH (>4.5) and high moisture (>95%). The combinations of preservatives were found to be more effective than their independent applications.<sup>35</sup> Moreover, in a study by Zhang et al, the synergistic effects of D-limonene nano-emulsion with nisin were confirmed against four food microorganisms.<sup>36</sup>

In the present study, nisin was unable to prevent *C. botulinum* growth at the concentrations of 500 and 1500 IU/mL whereas, in combination with salt, *C. sinensis* EO, and nitrite, good synergistic effects were reported in this regard. The results also showed the synergistic effect of nitrite and EO on the test organisms. These findings are in line with those of the study by Cui et al, which used a combination of sodium nitrite and spice extracts, including sage, cloves, and nutmeg. The antibotulinal efficacy of this combination could reduce the amount of nitrite in the meat model system to approximately 10 ppm.<sup>37</sup>

## Conclusion

In meat curing, nitrite is described as a multifunctional food additive. However, there is growing concern over the carcinogenic effects of nitrite in humans. One approach is to use low concentrations of sodium nitrite, along with other natural compounds which are acceptable to food organizations and consumers. The findings of this study showed that by using certain concentrations of *C. sinensis* EO and nisin, along with other suboptimal factors, the nitrite quantity needed to control the growth of *Clostridium botulinum* could be reduced to a satisfactory level. However, the efficacy of this complex in the food matrix should be considered in terms of antimicrobial properties and other nitrite functions.

## Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

## Ethical Approval

The experiment was conducted according to the protocol approved by the Islamic Azad University, Science and Research Branch, Tehran, Iran.

## Authors' Contributions

All authors contributed equally in this article, especially in designing, laboratory analysis, statistical analysis, and manuscript writing.

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