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ANTIBACTERIAL AND ANTIOXIDANT PROPERTIES OF EDIBLE CHITOSAN COATINGS INCORPORATED WITH ESSENTIAL OILS

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ABSTRACT

The antibacterial and antioxidant activities of edible chitosan coatings (ECC) with or without essential oils (EO) as Ceylon cinnamon type (CO), cinnamon bark (CBO), carvacrol (CAR), rosemary (RMO), tea tree (TTO) and olive (OLO) was investigated. Each of the mentioned oils was used at three different concentrations, 0.5, 1.0 and 1.5% (v/v) in order to test their antibacterial and antioxidant capacity. ECC solution did not show antibacterial activity without addition of EO. The incorporation of CO, CBO and CAR in high concentrations into ECC significantly decreased the activity against *S. aureus, S. typhimurium* and *E. coli* O157:H7 (p<0.05) compared to EO alone. Even though of this reduction, ECC with essential oils conserved antibacterial properties. On the other hand, the addition of CO, CBO and CAR potentiated the antioxidant activity of the ECC (p<0.05). The edible coatings with essential oils (ECEO) of CO, CBO and CAR at a concentration of 1.5% showed enhanced antioxidant and antibacterial activities.

KEYWORDS: Chitosan, essential oils, edible coatings, antioxidant, antibacterial.

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INTRODUCTION

Food-borne diseases are widespread and represent a growing public health problem, both in developed and developing countries. Each year, 178 million people in the United States suffer from acute gastroenteritis [1], and 25 to 30% of this type of illness are caused by *Salmonella, Escherichia coli* O157:H7 *and Staphylococcus aureus* are others pathogens [2]. Currently, food industries use synthetic preservatives in order to minimize food-borne diseases; unfortunately this strategy is not well accepted by the consumers who demand food with low levels of synthetic chemical preservatives owing to side effects [3]. Concomitantly, consumers also demand long shelf-life foods and food free of pathogens. Therefore, search for an antimicrobial active system has been encouraged by means of incorporation of antimicrobial substances into foods, on top of them or into packaging coating [4]. Chitosan and essential oils (EO) have been used as natural antimicrobial compounds to preserve the quality of food [5, 6]. The former is comprised of β-(1→4)-linked N-acetyl-D-glucosamine and D-glucosamine repeat units obtained by deacetylation of chitin. Chitosan is nontoxic, bio-compatible and biodegradable. These characteristics give chitosan a wide range of applications such as in pharmaceutics, biotechnology, biomedicine, agriculture and food processing [7]. Furthermore, chitosan is one of the most promising coating materials owing to the property of film-forming, and for its antimicrobial activity [8]. The edible films made of chitosan give rise to a semi-permeable barrier that controls gas exchange and reduces water loss of different horticultural products [9]. Additionally, these types of films behave as antimicrobial agents against pathogens and spoilage bacteria found in foods [10]. It has been proposed that the positive charge of the amino group of glucosamine unit interacts with the negative charges in the microbial cell membrane, thus altering its structure [11]. On the other hand, EO and their components have been used to provide foods with flavor and fragrance. Moreover, these oils are gaining relevance as Generally Recognized as Safe (GRAS) and good acceptance by consumers. EOs are also important natural resources that have a potential as antibacterial, antifungal and antioxidant [12]. The antibacterial activity of EO is associated with the terpenoid and fenolic compounds. This activity mainly depended of the proportions in which they are present and the interactions among them. The direct application of EO into food requires high concentration to achieve good antimicrobial activity. Under these conditions, products can be storage for an extended period of time [13]. Chitosan has been used to improve the physicochemical and antimicrobial activity of films, mainly because chitosan give rise to edible coatings. Because coatings are applied as a film-forming solution on foods and there are few studies related to the incorporation of EO into edible coatings made of chitosan and their effect to pathogens and their antioxidant activity. The aim of this study was to generate a model of edible coatings based on chitosan with incorporated EO, and *in vitro* determinations of antibacterial and antioxidant activities.

MATERIALS AND METHODS

Materials

CO (cinnamon oil) Ceylon type, CAR (carvacrol, 98% purity) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were purchased from Sigma-Aldrich. CBO (cinnamon bark oil), TTO (tea tree oil) and RMO (rosemary oil) were purchased from SARC supply solutions, USA. OLO (olive oil) was obtained from a local market. The chitosan was prepared from shrimp chitin (*Penaeus vannamei*). All other reagents used in this study were of analytical grade.

Characterization of chitosan

The chitosan was obtained by thermo-alkaline deacetylation of chitin. One g of chitin was homogenized with 15 mL of NaOH (50% w/v) at 95ºC per 2 h [14]. Size-exclusion chromatography

(SEC) was used for the analytical characterization of the molecular weight of chitosan. For this purpose, a sample of chitosan was solubilized in 0.25 M acetic acid to a final concentration of 4 g/L. This solution was injected in a HPLC-SEC, equipped with an ultrahydrogel^{TM} 500 column (7.8 x 300 mm) and light scattering detector PL-ELS 1000 (Polymer Laboratories, US). The separation was carried out with 0.25 M acetic acid as the mobile phase, at a flow rate of 0.3 mL/min by a HP Series 1100 isocratic pump. The molecular weight was expressed in kDa [15]. The grade of N-acetylation (DA) of chitosan was determined by Fourier Transformed Infrared (FTIR) in a Perkin-Elmer ATR-FTIR spectrometer system (Connecticut, USA), in absorbance mode. The samples were dried at 50°C overnight. ATR-FTIR spectra were acquired at 24°C and 35% RH at resolution of 4 cm^{-1} with 10 scans. The DA of the sample was estimated by the relationship of net absorption of the bands centered at 1320 and 1420 cm^{-1} [DA= $(31.9A_{1320}/A_{1420})$-12.2] as reported by Brugnerotto et al [16].

Preparation of chitosan and edible coatings with essential oils incorporated (ECEO)

The procedure for preparation of edible coating solution was as follow: chitosan (2 g) were dissolved in 1% v/v acetic acid (100 mL) and stirred for 4 min at 15500 rpm in an Ika Ultra-turrax T-18 basic, Germany. Thereafter, glycerol was added (500 µL/g of chitosan) into the solution and homogenized. CO, CBO, CAR, TTO, OLO and RMO oils were incorporated into edible chitosan coating solution (ECC) at the following concentrations: 0 (control), 0.5, 1.0, and 1.5% (v/v) as described by Hosseini et al [17]. Solutions were homogenized as previously described and kept at 5ºC overnight; then solutions were used to determine their antibacterial and antioxidant activities.

Preparation of essential oil (EO) solutions

Essential oils (CO, CBO, CAR, TTO, OLO and RMO) were dissolved in Tween 80 (1:8) following a modified procedure from that reported by Lorente et al [18]. Deionized water was added and homogenized in vortex-2 genie (Scientific Industries, USA), until the concentrations of 0 (control), 0.5, 1.0, and 1.5% (v/v) and then the solutions were kept at 5ºC overnight.

Bacterial strains and growth conditions

Escherichia coli O157:H7 (ATCC 43890), *Staphylococcus aureus* (ATCC 65384), and *Salmonella typhimurium* (ATCC 14028) were used. Strains were kept in tryptone soy broth (TSB) with glycerol (20%) and storaged at -40ºC until use. A loopful of bacteria was transferred to 10 mL of TSB and incubated at 37ºC overnight. Other loopful of the overnight culture was again transferred to TSB. The culture was grown at 37ºC until the desired amount of colony forming unit per mL (CFU/mL) for inhibitory studies.

Antibacterial activity

Agar diffusion method was used to determine the bacterial sensitivity exposed to ECEO and EO. An inhibition zone assay was performed adding 100 µL of inoculate containing 10^8 CFU/mL of each tested strain and streaked out over the surface of Muller-Hinton agar plates (Difco). Different solutions were added (70 µL) into agar wells (5-6 mm diameter) and incubated at 37ºC for 24-48 h as described by Coma et al [19]. Diameter of the inhibition halo was measured with a caliper (nearest 0.02 mm). Sensitivity was classified according to diameter of the halo as: not sensitive (< 8 mm), sensitive (9-14 mm), very sensitive (15-19 mm) and ultra sensitive (> 20 mm) [12]. Two types of controls were used, acidic water (pH= 4.0) and distilled water. Inhibition zone assays were performed by triplicate.

Antioxidant activity (DPPH radical assay)

Free radical-scavenging activities were measured according to the method of González-Aguilar et al [20] with modifications, as follows: aliquots (200 µL) of different concentrations of ECEO, EO and ECC (control) were added into a solution of the DPPH (0.2 mM). The mixture was shaken vigorously and incubated for 30 min at room temperature.

Absorbance was measured at 515 nm in a spectrophotometer (Thermo Spectronic Genesis 20, USA). The radical-scavenging activities of samples were expressed as an inhibition percentage (Ip%) of DPPH and it was calculated according to the Eq. *(1)*:

(Ip%)= $[(A_b—A_a)]/A_b)x100$ *(1)*

Where A_b is the absorbance value of the blank sample and *Aa* the absorbance of sample.

Statistical analysis

Each measurement was conducted in triplicate according to a completely randomized design. The experimental dates were subjected to an analysis of variance (ANOVA) and correlation using the NCSS Statistical Analysis System (Kaysville, Utah, USA). The results were expressed as the mean value±standard deviation (SD). For analysis of variance and significant differences were analyzed by Duncan's multiple range tests at 0.95 confidence level.

RESULTS AND DISCUSSION

The degree acetylation of chitosan obtained was 34% (1320 and 1420 cm^{-1} absorbance relation). FTIR spectra at 650-4000 cm^{-1} of chitosan shown in the Fig 1, where it appears two approximated peak at 1655 and 1625 cm^{-1}, corresponding to C=O, and C=N groups (amide I). The broad band in range 3000-3650 cm^{-1} has been attributed to OH stretching vibrations. All bands obtained to the chitosan functional groups were similar to the reported by Brugnerotto et al [16]. The chitosan obtained was of average molecular weight of 128 kDa (Mw) (74% area predominant) with a large polydispersity (>4) as shown in table 1.

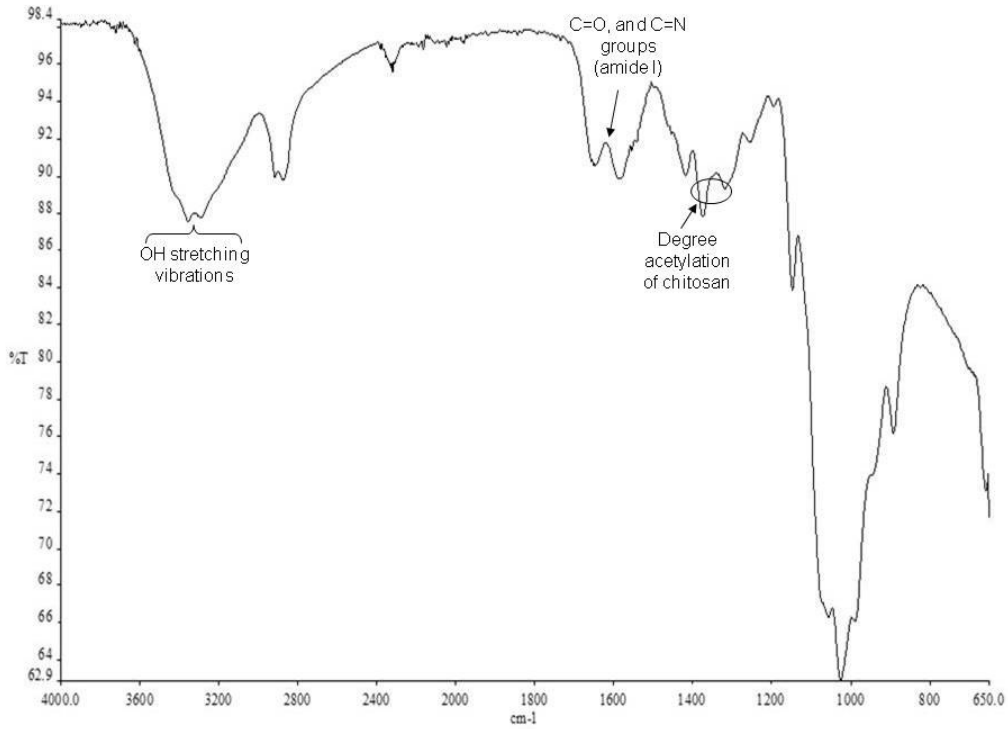

Figure 1
FTIR spectra of chitosan

Table 1
Characteristics of the chitosan used in this study according to their average molecular weight (Mw), polydispersity index (PD) and area percentage.

Chitosan fractions	Mw	PD	Area (%)
High	1652.6	1.29	15.45
Medium	128.0	4.12	74.02
low	1.00	1.66	10.52

It has been reported that the antimicrobial activity of chitosan depends on DA and Mw [21, 22]. Besides, bacteria, yeast and mold display different microbial sensitivity to this biopolymer. For example, the yeast, *Pichia guillermondii*, was more sensitive to chitosans with high Mw (319 kDa) and low DA (0.68%) than the mold *Penicillium digitatum.* Whereas, yeasts and molds were moderately inhibited with low Mw chitosan (107 kDa and 33.8% DA) [22]. Chitosan generally showed higher inhibitory activity for gram-positive bacteria than for gram-negative bacteria. Nevertheless the minimum inhibitory concentrations of chitosan and its molecular weights are different with individual bacteria [21]. The antimicrobial property of chitosan microcapsules of essential oil cross-linked to cellulose was also reported and its effectiveness was slightly higher for the gram-positive bacteria *Staphylococcus epidermidis* than the gram-negative *E. coli* O157:H7. Such behavior was explained by structural differences in the bacterial outer membrane in which chitosan interact electrostatically with lipopolysaccharides and proteins of gram-negative bacteria in an ionic-type binding with the amino group of chitosan at acidic pH [23].

Antibacterial activity

Herein, the combined antimicrobial action of chitosan and essential oils were tested. These chemical properties have been also related with their antioxidant capacity [11, 24]. The bacteria were insensitive with the controls, acidic water, distilled water and ECC, previously has been reported that chitosan could interact with lipopolysaccharide and other lipids from the cell membrane of *E. coli* O157:H7 and *Salmonella spp* promoting loss of barrier function of this organelle [25]. The different outcomes could be due to the high level of inoculum used herein ($1x10^8$ CFU/mL), this amount of inoculum could exceed the inhibition activity of ECC [19]. Moreover, has been previously reported that chitosan unable to diffuse through of agar media [26, 27]. Although other studies have reported an antibacterial effect for RMO [28] and TTO [29], the present study displayed no activity against any tested bacterial strains regardless the concentration of ECEO and EO. These results might be attributed to the lack of volatile compounds of TTO (γ-terpinene and terpinen-4ol) and RMO (α-pinene, 1,8-cineole and camphor), influenced by physical processes used in this study (homogenization) [30, 31]. Other causes must be the high level of inoculum that could inhibit the action of the active compounds from the tested EO.

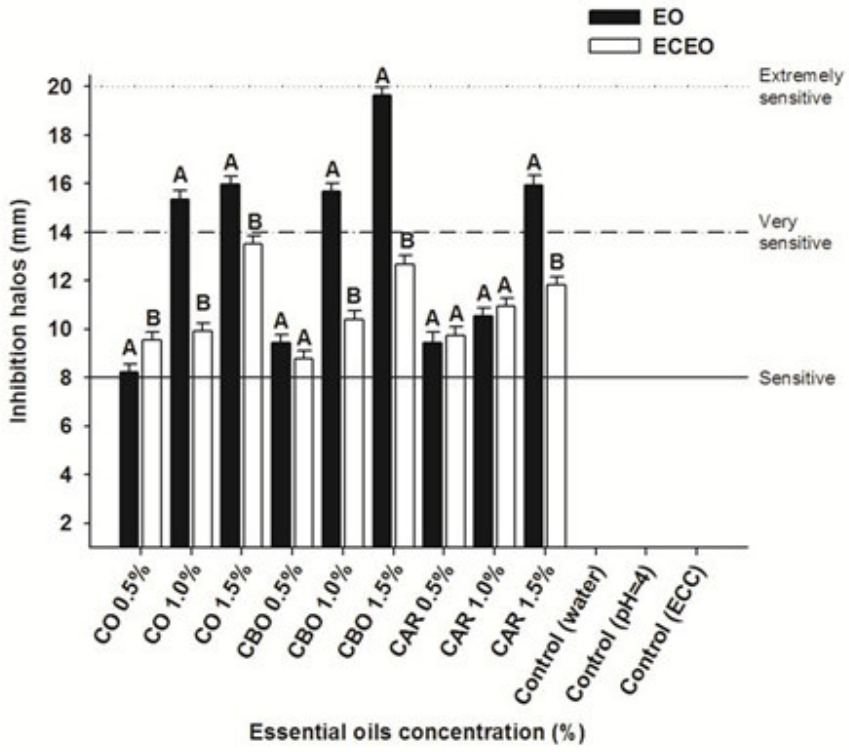

Figure 2

Antibacterial activity (inhibition halos) of chitosan solution with or without essential oils and Essential oils along against S. aureus. Different letters indicate significant differences between groups as determined by Duncan test (p< 0.05).

The incorporation of CO, CBO and CAR into ECC significantly decreased the activity of *S. aureus* in all concentrations (p<0.05), except for the lowest concentrations of CBO 0.5%, CAR 0.5% and CAR 1.0% (Fig 2). The effects were similar when testing the samples against *Salmonella* (Fig 3) and *E. coli* O157:H7 (Fig 4) of CAR at concentrations of 0.5 and 1.0% with no significant differences (p>0.05). ECC did not show antibacterial activity without addition of EO. The decreased antibacterial activity of ECEO compared to EO might be explained by chemical interactions between amino groups of chitosan that could block the active site of bacteria [32]. On the other hand, reduction in activity could be caused by the oils retention into the matrix, preventing their release. Previous report on a model of edible coatings made of carbohydrates such as β-cyclodextrin, showed that the coating could encapsulate the EO of thymus and release most 75% of the active compound into the environment under high relative humidity [33].

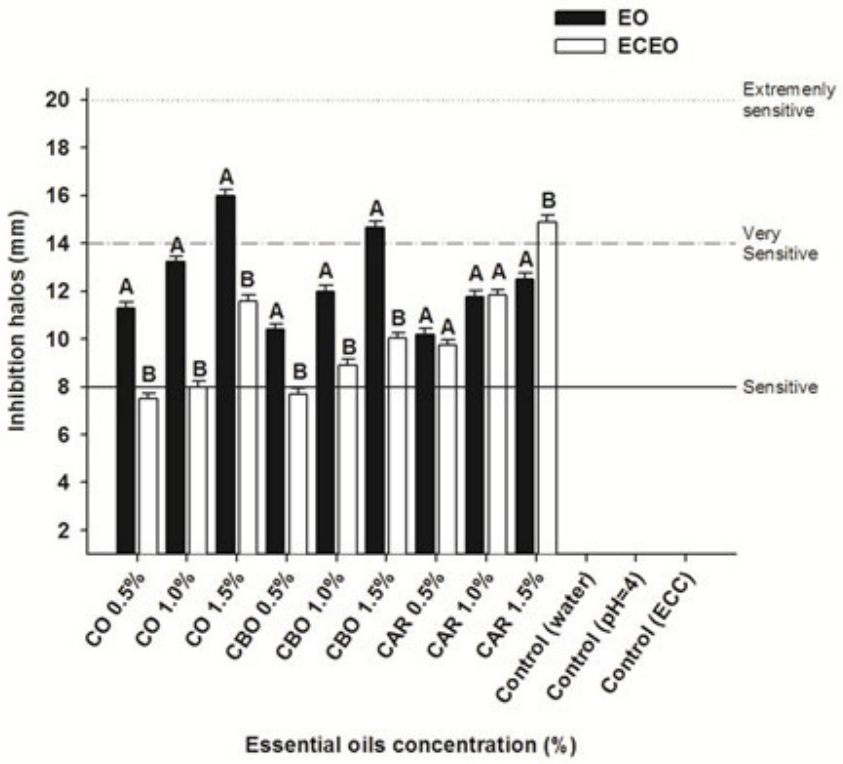

Figure 3

Antibacterial activity (inhibition halos) of ECEO and EO at different concentrations against Salmonella. Different letters indicate significant differences between groups as determined by Duncan test (p<0.05).

The ECEO treatments showed a strong inhibitory activity against *S. aureus* with 1.5% CO (13.5 mm), 1.5% CBO (12.6 mm) and 1.5% CAR (11.8 mm). The inhibitory activity against *Salmonella* was 1.5% CAR (14.9 mm) and 1.0% (11.8 mm), 1.5% CO (11.5 mm) and 1.5% CBO (10.0 mm). The inhibitory activity against *E. coli* O157:H7 was 1.5% CAR (16.5 mm), 1.5% CO (13.5 mm), 1.0% CAR (12.2 mm) and CBO 1.5% (11.0 mm). These results clearly pointed out that the antibacterial activity is proportional to the concentration of EO. Our results are similar to those of Seydim and Sarikus [34] whom reported a correlation between the concentrations of EO added to edible coatings and the antimicrobial activity against different food pathogens. Previous studies on the antibacterial activity of cinnamaldehyde and carvacrol (major active compound from oregano) oil applied against *Salmonella, E. coli* and *Staphylococcus* without a vehicle, such as edible coatings, showed remarkable antibacterial activity [35]. In regard to edible coatings studied herein, ECEO of CAR, exerted a major grade of sensitivity at all concentrations than the CO, which is in agreement with Rojas-Graü et al [36] on edible coating made of apple solution with CO and oregano oil for its evaluation against *E. coli* O157:H7. The authors found that oregano oil at concentrations as low as (0.1%) is more sensitive than 0.5% of CO which suggests a 5-fold reduction of *E. coli* O157:H7. The mechanism of action of EO has not been studied in great detail, particularly because of the large number of different groups of chemical compounds found in EO. In the case of CO and CBO, the cinnamaldehyde (3-phenyl-2-propenal) is the major component (≈60%). The antimicrobial capacity of cinnamaldehyde is given by the binding of its carbonyl group with acetyl-CoA carboxylases of the target bacteria to disrupt metabolism [5, 37].

The mechanism of action of CAR has been studied in *Bacillus cereus,* finding that interacts on target membranes changing the permeability of H^+ and K^+, thus promoting dissipation of ion gradients and therefore affecting essential processes of the cell and consequently cell death [38].

Antioxidant capacity

In the present study it was found that ECC shows a 17.7% of antioxidant activity. The free radical scavenging activity of chitosan solution has been attributed to the nitrogen, on the C-2 position, capacity to bind various free radicals simultaneously [39]. Apparently, this mechanism is related to the fact that the free radicals can react with the hydrogen ion from ammonium ions to form a stable molecule [40]. This antioxidant activity of chitosan has also been related to their molecular weight as stated by Youn et al [41], who reported that the antioxidative effect of chitosan is greater at high molecular weight (Mw) than low. On the other hand, Kyung and Thomas [24] report that 30 kDa chitosan showed the highest scavenging activity when compared to 90 and 120 kDa chitosan.

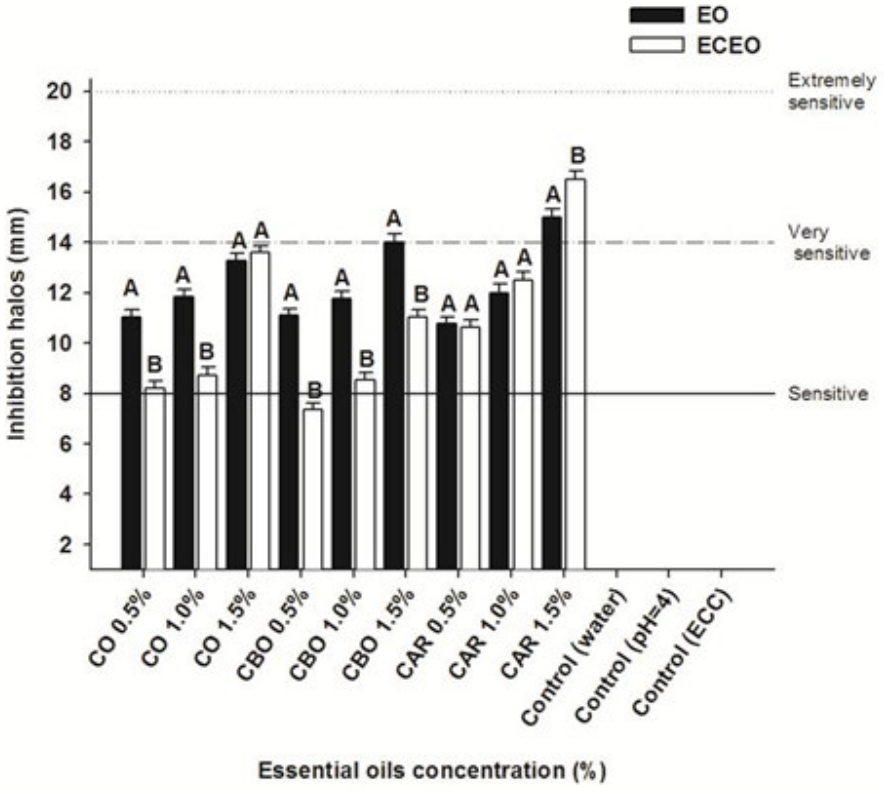

Figure 4

Antibacterial activity (inhibition halos) of ECEO and EO at different concentrations against E. coli* O157:H7*. Different letters indicate statistical significant differences between groups as determined by Duncan test (p<0.05).

The figure 5 shows the percentage of antioxidant activity (DPPH-radical-scavenging) of the ECEO, EO and ECC. Application of 1.5% CO resulted in a 94% of antioxidant activity; 1.5% CBO in a 95% and 1.5% CAR in an 86%. These results are similar to those reported by [42] in which the antioxidant capacity and relative content of phenolics EO of commercial interest were evaluated; they reported an 88% of the antioxidant activity for CBO and 79.2% for CAR. These authors also found that the main molecule responsible for the antioxidant

capacity of CO is eugenol, which explains the observed 93% of activity. This percent of activity might be attributed to the hydrogen donor capacity as stated by Baroty et al [43] who reported a positive correlation between volatile phenolic and the antioxidant activity. CAR is the main compound of oregano oil. Its antioxidant activity has been attributed to the phenolic ring that is able to donate a hydrogen atom from the phenol hydroxyl groups when reacting with radicals in order to produce stabilized phenoxyl radical [44]. The ECEO showed a significant reduction in the antioxidant activity when compared to EO (p<0.05). Both CO and CBO showed a lower loss of activity percentage than CAR. This phenomenon could be attributed to the easy release of cinnamon compounds or to a molecular rearrangement of the scavenger zone of free radicals. Instead, the low activity of CAR could be due to retention or partial encapsulation of EO, induced by the carboxylate group (-$NH3^+$ $^-$OOCH) [4]. Nevertheless, the antioxidant activity of ECEO was significantly higher than ECC (p<0.05). This data indicates a possible increment on the antioxidant properties of ECC along with the antibacterial activity. Moreover, ECEO of CO, CAR, and CBO at a concentration of 1.5%, showed more than 60% of DPPH radical scavenging activity. The high concentration (1.5%) of EO and ECEO from TTO showed the lowest radical scavenging activity, 8.5 and 17.7%, respectively; RMO showed 11.4 and 3.1% and OLO was 8.4 and 13.4%. There was a significant reduction in the antioxidant activity of ECC compared to that of EO at all concentrations of RMO and TA (p<0.05). OLO was the only treatment that did not showed changes in the antioxidant capacity at concentrations of 0.5 and 1.5% (p>0.05). Instead 1.5% OL showed a significant difference (p<0.05).

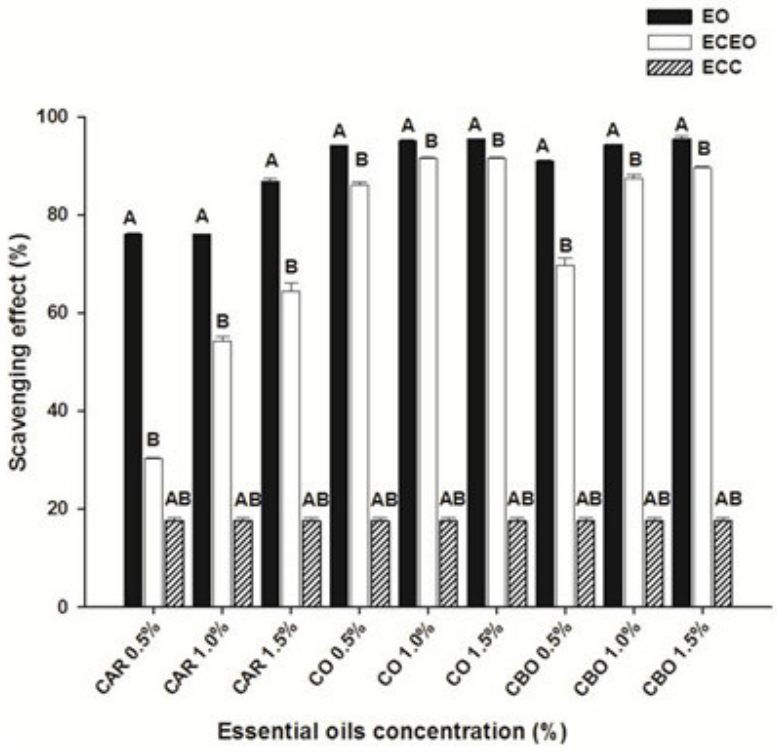

Figure 5

DPPH-radical-scavenging of ECEO and EO at different concentrations. Different letters indicate statistical significant differences between groups as determined by Duncan test (p<0.05).

The percentage of antioxidant activity (expressed as % of DPPH radical scavenging) of the EO and antibacterial activity are shown in Fig 6. This figure indicates a linear correlation of the antioxidant capacity for *S. aureus* (R^2=0.89), *Salmonella* (R^2=0.97) and *E. coli* O157:H7 (R^2=0.97). Moreover, the antioxidant activity of the ECEO versus antibacterial activity (Fig 7) showed a reduction in the correlation *S. aureus* (R^2=0.82), *Salmonella* (R^2=0.67) and *E. coli* O157:H7 (R^2=0.65).

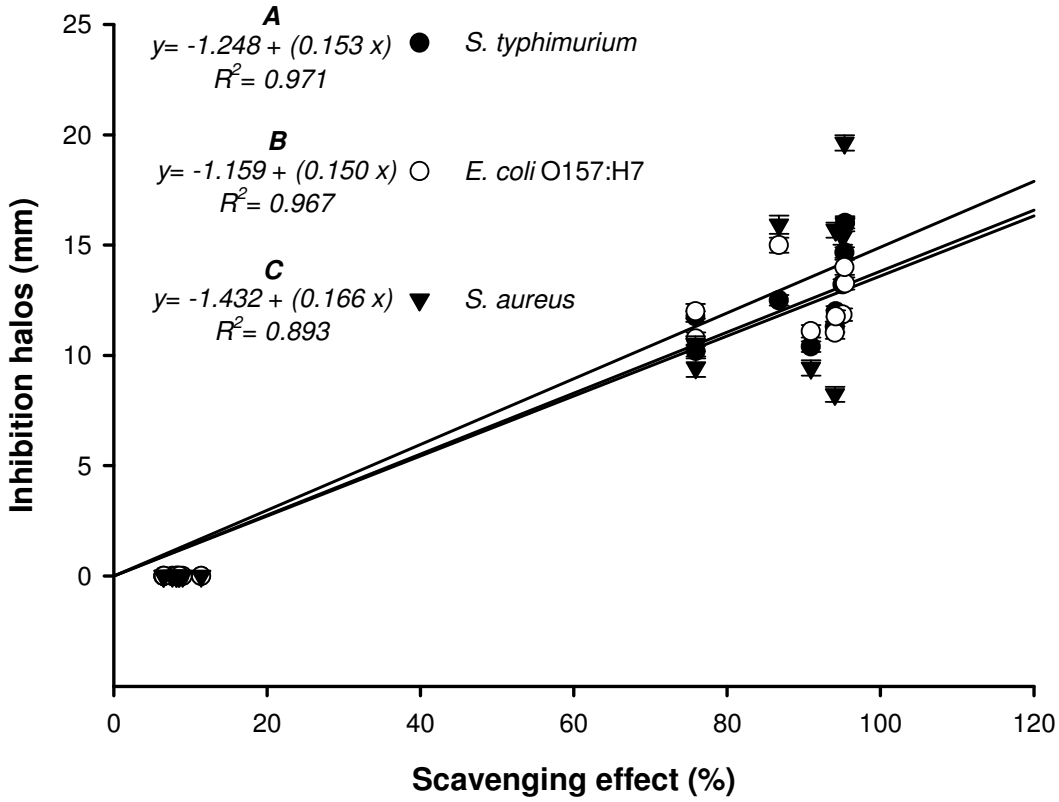

Figure 6
Relationship between scavenging effects, expressed as percent of EO and the inhibition halos of Salmonella, E. coli* O157:H7 *and S. aureus.

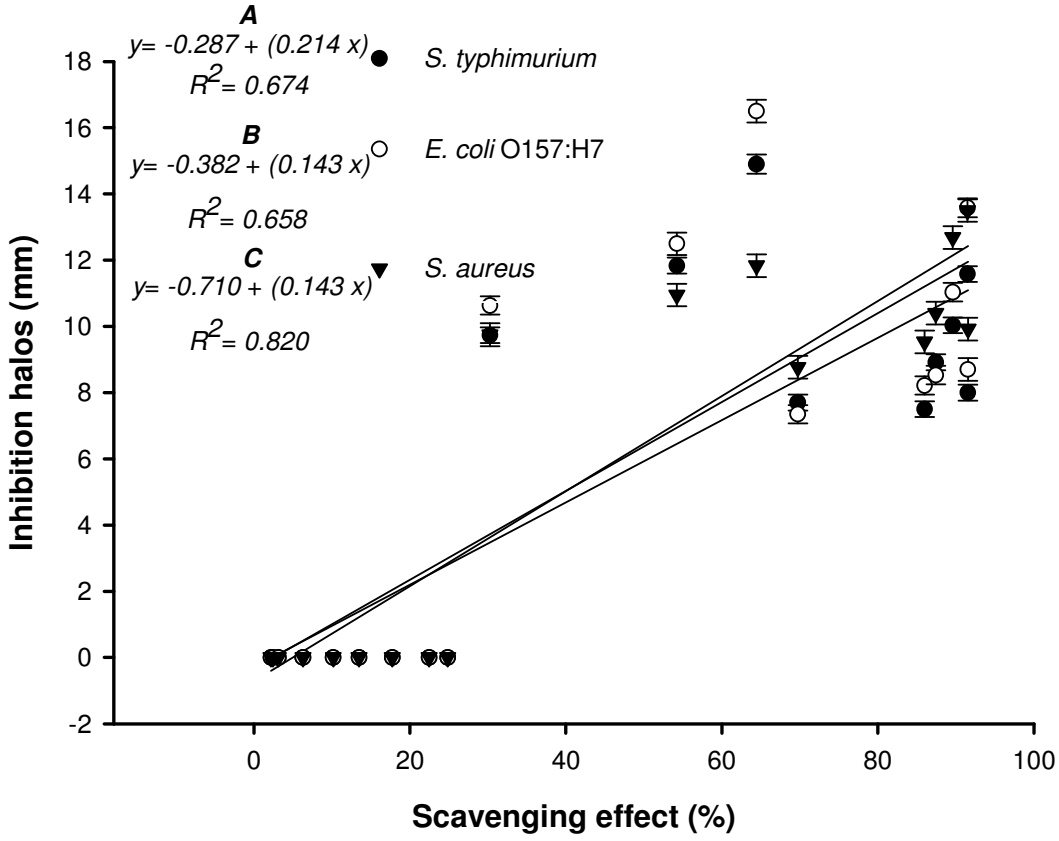

Figure 7
Relationship between scavenging effects, expressed as percent of ECEO and inhibition halos of Salmonella, E. coli* O157:H7 *and S. aureus.

CONCLUSION

In the present study, ECEO showed low antibacterial activity compared to EO, however, ECEO exerted a significant antibacterial activity against *S. aureus, Salmonella* and *E. coli* O157:H7. The ECEO of CO, CBO and CAR at the concentration of 1.5% showed a significant antioxidant and antibacterial activity. So, the ECEO could be used in food models as an alternative to extend the shelf life and safety of these products.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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