Development and characterization of essential oils incorporated chitosan-based cues with antibacterial and antifungal potentialities

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Development and characterization of essential oils incorporated chitosan-based cues with antibacterial and antifungal potentialities

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ABSTRACT
Herein, we report the characterization of chitosan-based cues incorporated with two different essential oils, i.e., tea tree oil (TTO) and clove oil (CO). The essential oils incorporated chitosan-based cues offer considerable solutions to eliminate pathogenic infections and/or to reduce microbial contaminations in healthcare facilities. In this background, chitosan(CST)-based cues were incorporated with TTO and CO and final cues were designated as CST-TTO and CST-CO. The loading efficiency was optimal in CST-TTO (91.5%) followed by CST-CO (83.3%). The optimally yielded both, CST-TTO and CST-CO were tested for antibacterial activities against two Gram+ve (Staphylococcus aureus, and Bacillus subtilis) and two Gram-ve strains (Escherichia coli and Salmonella typhimurium). An initial bacterial cell count (1.5 x 10^8 CFU/mL) was considered as a control value, and 2-log reduction was considered to report the antibacterial activity. The antifungal activity was tested against two Aspergillus strains, i.e., Aspergillus niger and Aspergillus flavus. The growth reduction for Aspergillus strains was recorded from 1 x 10^8 conidia/mL (initial count) to 1 x 10^2 conidia/mL (optimal inhibition) in the presence of CST-TTO and CST-CO. In summary, the results suggest that the newly developed CST-TTO and CST-CO cues can be potential candidates for food and biomedical applications as they hold promising capability to restrict some pathogenic and food spoilage microbes.

1. Introduction
Incorporation of natural additives including phenolic compounds, vegetable extracts, and protein hydrolysates in active food packaging materials is a promising and emerging trend nowadays. Plant-derived essential oils (PEOs) are natural bioactive additives that can be incorporated into the edible films/bio-composites and approved by the US Food and Drug Administration (US FDA) as safe replacement of synthetic additives (Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2013). Numerous reports have demonstrated the inhibitory potential of PEOs toward a range of food spoilage, foodborne, and postharvest pathogenic microorganisms (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Burt, 2004; Dorman & Deans, 2000; Wu et al., 2014) due to their antibacterial, antifungal, antiviral, antioxidant, and insecticidal activities (Atarés, Bonilla, & Chiralt, 2010; Chouhan, Sharma, & Guleria, 2017; Peng & Li, 2014). Those activities are attributed to their chemical composition, mainly the presence of phenolic compounds (Gómez-Estaca, De Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010). Despite potential antimicrobial activities, direct food applications dictate that the higher concentrations of PEOs to achieve the desired antimicrobial effect, which might confer inappropriate flavors and smells/odors from these natural constituents to the food product which may be unpleasant for consumers (Gutierrez, Barry-Ryan, & Bourke, 2009; Seydim & Sarikus, 2006). This scenario has led to the researchers to focus on the incorporation of PEOs into edible films/bio-composites for food packaging applications.

Essential oil encapsulation in suitable delivery systems has displayed great promise in foods to address dosage limitations and improve the biological stability and durability of bioactive compounds (Van Long, Joly, & Dantigny, 2016). Many study dealings with the encapsulation of various PEOs into edible films/bio-composites have obtained good results in reducing levels of food pathogens (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Emiroğlu, Yemiş, Coşkun, & Candogan, 2010; Gómez-Estaca et al., 2010; Sánchez-González, Cháfer, Hernández, Chiralt, & González-Martínez, 2011; Seydim & Sarikus, 2006). Recently, Hossain et al. (2017) reported that PEOs based bio-nanocomposite played a key role in reducing fungal propagation and contamination in processed food. The low doses of PEOs then diminish the influence of the bioactive components on the unwanted flavor, aroma, and perception of the food (Hossain et al., 2019; Lu, Kelly, & Miao, 2016; Salgado et al., 2013).

Among biopolymers, chitosan is considered a valuable bio-candidate to design biodegradable and reusable materials due to its biocompatibility, biodegradability, nontoxicity, mucoadhesiveness, and ability to form cross-linked hydrogels. Chitosan has been extensively used in various fields including food packaging, biomedical applications, drug delivery systems, tissue engineering, cosmetics, and wound healing (Salgado, López-Caballero, Gómez-Guillén, & Montero, 2017). In the field of food packaging, chitosan has been employed in the form of films, coatings, sheets, and foams to extend the shelf life of perishable foods and combat the growth of spoilage and pathogenic microorganisms (Averbeck, Averbeck, & Idaomar, 2000; Seydim & Sarikus, 2006). Chitosan is widely employed in food packaging due to its excellent antimicrobial properties (Gómez-Estaca et al., 2010; Salgado et al., 2017; Seydim & Sarikus, 2006). However, the high cost of chitosan limits its potential applications in the food industry (Gómez-Estaca et al., 2010; Salgado et al., 2017). Therefore, the development of chitosan-based edible films/bio-composites with improved antimicrobial properties is considered a promising approach to expand its applications in the food industry.
biocompatible packaging composites owing to its antimicrobial, antioxidant, as well as, film-forming properties (Bilal et al., 2017, 2019; López-Caballero, Gómez-Guillén, Pérez-Mateos, & Montero, 2005). After cellulose, chitosan is the second most abundant and natural polysaccharide which exists in exoskeletons of crustaceans. Moreover, it has been approved as a food ingredient by the FDA (Ma et al., 2016). This study reports the synthesis and characterization of chitosan-based cues incorporated with two different PEOs, i.e., tea tree oil and clove oil. The resulting newly synthesized CST-based constructs were then tested for their antimicrobial activities against a range of pathogenic bacteria (food spoilers) and fungal stains.

2. Material and methods

2.1. Chemicals and/or reagents

Chitosan (MW 100–300 kDa with 82% degree of deacetylation), Potato Dextrose Agar (PDA), and peptone were obtained from a local supplier and distributor of Sigma-Aldrich (USA). All other chemicals/reagents were of standard analytical laboratory grade with the purity >98% and used without any further processing unless otherwise stated.

2.2. Essential oils

Two different food-grade essential oils, i.e., tea tree oil (Camellia sinensis) and clove oil (Syzygium aromaticum) were purchased from a local organic-based shop.

2.3. Microbial cultures

Two Gram+ve (Staphylococcus aureus, and Bacillus subtilis) and two Gram-ve strains (Escherichia coli and Salmonella typhimurium) were used to test the antibacterial activities of newly developed CST-TTO and CST-CO cues. Two Aspergillus strains, i.e., Aspergillus niger and Aspergillus flavus were used to test the antifungal activity. All microbial cultures used in this study were procured from Shanghai Luwei Technology Co., Ltd. China.

2.4. Culture maintenance

All the above mentioned bacterial cultures were grown overnight, each separately, in 50 mL sterile nutrient broth (liquid nutrient medium) at 30°C and 120 rpm. After 18 h incubation, each bacterial culture was diluted to yield an initial bacterial count of $1.5 \times 10^8$ CFU/mL (control value). While the fungal cultures were maintained in Kirk’s nutrient medium which was additionally supplemented with 1% sterile glucose. The freshly inoculated medium was incubated at 30°C and 120 rpm. After the stipulated incubation, the culture with $1 \times 10^8$ conidia/mL (control value) was used for further subsequent experiments.

2.5. Development of CST-TTO and CST-CO cues

One-pot synthesis approach was adopted to develop the CST (control), CST-TTO and CST-CO cues (test samples). Briefly, a film-forming solution of chitosan (CST) was prepared by dissolving 0.5% (w/v) CST ultrasonically (SB-5200DTN Ultrasonic Bath, Scientz) in 5.0% (w/v) acetic acid solution for 30 min under uninterrupted stirring (120 rpm) at 30 ± 3°C. Following that equal ratio of 0.5% (w/v) of both essential oils, each separately was incorporated into the CST solution and homogenized for 5 min. Both CST-TTO and CST-CO were cast into thin films by pouring the solutions into a prelabelled Petri plate followed by overnight incubation in a hot air oven at 60 ± 3°C to develop cues. The resultant CST-TTO and CST-CO cues were recovered and washed three times with distilled water to eliminate the excessive smell of acetic acid and used for further studies.

The percent loading efficiency (LE) is evaluated using Equation 1.

\[
\text{Loading efficiency (\%) } = \frac{W_f - W_i}{W_i} \times 100 \tag{1}
\]

where $W_f$ = final dry weight and $W_i$ = initial weight (chitosan without essential oils)

2.6. Antibacterial activity: qualitative evaluation

Agar well-diffusion assay was followed to test the qualitative-based antibacterial activities of CST, CST-TTO, and CST-CO cues against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Salmonella typhimurium. Briefly, the bacterial cultures with $1.5 \times 10^8$ CFU/mL freshly prepared suspension, each separately, were mixed with the agar medium (50°C) and then poured in sterile Petri dishes. Following that a sterile core bore was used to make 10 mm holes on inoculated agar plates and filled with the same diameter size of CST, CST-TTO, and CST-CO cues. All test plates were stipulated and then incubated overnight at 30°C. After the stipulated incubation of 24 h, the zone of inhibition was recorded, and the degree of inhibition was expressed with the following key order as maximal zone of inhibition with +++ as a total inhibition; limited zone of inhibition with ++ as a partial inhibition; marginal zone of inhibition with + as a slight inhibition; and no zone of inhibition with – as no inhibitory activity.

2.7. Antibacterial activity: quantitative evaluation

The antibacterial activities of CST, CST-TTO and CST-CO cues against two Gram+ve (Staphylococcus aureus, and Bacillus subtilis) and two Gram-ve strains (Escherichia coli and Salmonella typhimurium) were recorded by
follow ing the AATCC Anti-Bacterial Test method adopted from Iqbal (2015). All the test specimens were heat sterilized at 90°C for 30 min. Each of the bacterial culture suspension containing approximately 1.5 × 10^8 CFU/mL was spread onto the surfaces of the test cues and incubated under controlled conditions for 24 h (Iqbal, 2015). After 24 h incubation, all test specimens were washed twice using 50 mL phosphate buffer (pH, 7.0) and the washed suspension was used to record the viable cell number. The viable cell number in each of the washed suspension was determined by the conventional spread-plate method, and plate counter agar was used to calculate the CFU/mL by serial dilution. In comparison with the control (initial bacterial count of 1.5 × 10^8 CFU/mL), the CFU/mL values were used to calculate the antibacterial activities of the test constructs using Equation 2 reported by Iqbal (2015).

\[
\text{Log reduction} = \text{Log CFU Control sample} - \text{Log CFU Treated sample}
\]

Due to the intrinsic variability of the antibacterial test results, at least a 2-log reduction was considered necessary to claim an antibacterial activity, as reported by Elegir, Kindl, Sadocco, and Orlandi (2008).

### 2.8. Evaluation of antifungal activity

The vapor contact assays described by Hossain et al. (2016) was followed to test the antifungal activity of CST, CST-TTO and CST-CO cues against Aspergillus niger and Aspergillus flavus. The conidia from Aspergillus niger and Aspergillus flavus were developed and adjusted to 1 × 10^8 conidia/mL and used for subsequent antifungal activity assay.

### 2.9. Statistical analysis

All the results expressed are average data values of three independent measurements performed in triplicate ± standard deviation (SD). The SD values are presented as Y-error bars in Figures.

### 3. Results and discussion

#### 3.1. Loading efficiency (LE): CST-TTO and CST-CO cues

One-pot synthesis approach was used to incorporate the PEOs (TTO and CO) onto the CST-based thin film cues. Figure 1 illustrates the incorporation or loading efficiency of newly developed CST-based CST-TTO and CST-CO cues. The maximal percent loading efficiency (%LE) of 91.5% was recorded for CST-TTO followed by 83.3% for CST-CO prepared in 1:1 ratio (CST:TTO and CST:CO). The obtained results were also trailed by inserting a polynomial order 2 curve fit against each test specimen which confirms a maximal %LE in the CST-TTO. The optimally yielded PEOs incorporated CST-based thin films cues were further tested for their antibacterial, and anti-fungal potentialities.

#### 3.2. Antibacterial activity: qualitative evaluation

The results obtained after qualitative evaluation of antibacterial activity of CST, CST-TTO and CST-CO cues against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Salmonella typhimurium are summarized in Table 1. The maximal inhibitory activity was recorded for CST-TTO against Bacillus subtilis followed by Staphylococcus aureus and Escherichia coli. The least inhibitory activity of CST-TTO was recorded against Salmonella typhimurium. Whereas in case of CST-CO, the highest inhibitory activity was recorded against Bacillus subtilis and Staphylococcus aureus. The least inhibitory activity was observed against Escherichia coli while no inhibitory activity was found against Salmonella typhimurium. The CST alone showed inhibition against Bacillus subtilis and Staphylococcus aureus and no inhibitory activity was found against Escherichia coli and Salmonella typhimurium. As evident from the literature, the bacterial inhibitory activities of PEOs such as clove oil, lavender oil, oregano essential oils, and rosemary essential oils have been reported against pathogenic microorganisms including Listeria monocytogenes, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Lactobacillus plantarum, and Staphylococcus aureus (Campo, Amiot, & Nguyen-
3.3. Antibacterial activity: quantitative evaluation

Herein, the quantitative-based antibacterial potentialities of pristine (CST) and TTO and CO incorporated in CST-TTO, and CST-CO thin film cues were evaluated against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhimurium*. Owing to the intrinsic variability of antibacterial test results, a log factor with at least 2-log or less than 2-log reduction was considered necessary to claim an antibacterial activity, as reported by Elegir et al. (2008) and Iqbal (2015). More specifically, 2-log reduction, as compared to the control value (5-log), was considered necessary for bacteriostatic activity. Whereas less than 2-log reduction was claimed as the bactericidal activity of the test specimens against different test strains. Figure 2 illustrates the log reduction results of CST, CST-TTO and CST-CO cues against Gram+ve strains, i.e., *Staphylococcus aureus*, *Bacillus subtilis*. While the results against Gram-strains, i.e., *Escherichia coli* and *Salmonella typhimurium* are shown in Figure 3. As compared to the initial bacterial cell count (expressed as a log value 8), the test specimen CST-TTO displayed a significant reduction in the log values against Gram+ve and Gram-ve strains with bactericidal and bacteriostatic potentialities, respectively. The pristine CST sample was bacteriostatic against *Staphylococcus aureus*, and *Bacillus subtilis*. While no antibacterial activity of CST was recorded against *Escherichia coli* and *Salmonella typhimurium*. CST-CO was completely bactericidal against *Staphylococcus aureus*, and *Bacillus subtilis*, slight bacteriostatic against *Escherichia coli* and not active at all against *Salmonella typhimurium*. In this context, Wu et al. (2014) reported antimicrobial activity of *Hypophthalmichthys molitrix* skin gelatin-chitosan films incorporated with oregano essential oil against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus enteritidis*, and *Shigabacillus*. One reason behind the variable antibacterial activities, as evident in Figures 2 and 3, is because the Gram+ve strains and more sensitive or prone to the bioactive/antibacterial agents such as essential oils and phenolic-based bioactive compounds as compared to Gram strains (Burt, 2004; Iqbal, Kyazze, Locke, Tron, & Keshavarz, 2015a, 2015b, 2015c). This clear activity difference between Gram+ve and Gram-ve strains may also be related to the presence of additional outer membrane outside the cell wall in Gram-ve strains, which restricts the diffusion of antibacterial agents (Burt, 2004; Sánchez-González et al., 2011).

3.4. Evaluation of antifungal activity

The antifungal evaluation of pristine (CST), as well as TTO and CO, incorporated CST-TTO, and CST-CO thin film cues was performed against *Aspergillus niger* and *Aspergillus flavus*. The initial inoculation of each strain was $1 \times 10^8$ conidia/mL and the samples were incubated for four weeks under the temperature-controlled environment (30°C). At the end of each week, the samples were tested and the fungal growth curves of control (no added cues) and test specimens, i.e., CST, CST-TTO, and CST-CO are summarized in Tables 2 and 3. The maximum growth inhibition was found for CST-TTO against *Aspergillus*.
Aspergillus niger with $1 \times 10^2$ conidia/mL. CST-CO showed optimal inhibition of Aspergillus niger with $1 \times 10^3$ conidia/mL after 4-weeks of incubation (Table 2). In case of Aspergillus flavus, CST-TTO, and CST-CO suppressed the growth of $1 \times 10^8$ conidia/mL to $1 \times 10^7$ conidia/mL and $1 \times 10^4$ conidia/mL, respectively (Table 3). Very recently, Hossain et al. (2019) studied the antifungal activities of combined treatments of irradiation and essential oils encapsulated chitosan nanocomposite films in in vitro and in situ conditions against Aspergillus niger, Aspergillus flavus, Aspergillus parasiticus, and Penicillium chrysogenum with up to 51–77% growth reduction.

### 4. Concluding remarks

In conclusion, CST-based along with essential oils incorporated thin film cues, i.e., CST, CST-TTO, and CST-CO were developed and characterized for potential antibacterial and antifungal activities. The above-discussed results suggest that the thin film cues hold maximum incorporation of TTO and CO with 83.3–91.5% LE. Comparable antibacterial activities were recorded via qualitative and quantitative assays with highest activities against Gram+ve strains. Moreover, notable fungal growth reduction potential was also recorded for both, CST-TTO and CST-CO cues, against Aspergillus strains from $1 \times 10^8$ conidia/mL (initial count) to $1 \times 10^3$ conidia/mL (optimal fungal inhibition).

### Disclosure statement

No potential conflict of interest was reported by the authors.

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