Antimicrobial Activity of Edible Electrospun Chitosan/Cellulose Acetate/Gelatin Hybrid Nanofiber Mats Incorporating Eugenol

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Abstract

Antimicrobial nanofiber mats were successfully fabricated via electrospinning. Polymer solutions of chitosan, cellulose acetate and gelatin were blended at a volume ratio of 4:1:5. Eugenol at concentrations of 0 to 10.0% (v/v) was directly incorporated into the mixed polymer solutions. Electrospinning was performed at 23 kV with a flow rate at 0.7 ml/h and collector distance of 10 cm. The average diameters of fibers incorporated with eugenol ranged from 152.32±41.48 to 288.92±77.69 nm. Fibers with larger diameters and junctions appeared when the concentration of eugenol was increased. Eugenol release was observed within 300 min. The burst release of eugenol at 0.1, 0.75, and 1.5% (v/v) reached equilibrium after 60 min while the burst release at 3.0, 5.0 and 10.0% (v/v) continued to increase gradually. The phase transition temperatures of nanofiber mats incorporated with eugenol ranged from 129.69 to 161.84 °C. The thermal characteristic demonstrated that the melting point decreased in accordance with the increase of incorporated eugenol. The nanofiber mats with eugenol at less than 5.0% (v/v) showed better thermostability than mats incorporated with eugenol concentrations greater than 5.0% (v/v). Antibacterial activity was tested against *Salmonella* Typhimurium and *Staphylococcus aureus*. The results demonstrated that the edible electrospun CS/CA/Gel nanofiber mats incorporated with eugenol could effectively retard the growth of both bacteria. Our results suggest that eugenol incorporated nanofibers have potential applications as antimicrobial materials in active food packaging, air filtration, antibacterial textiles, wound dressing, drug delivery and others.

Keywords: antimicrobial nanofibers, electrospinning, edible electrospun, eugenol, chitosan
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1. Introduction

Public concern on health issues has increased due to foodborne pathogen contamination and environmental problems associated with plastic packaging. *Salmonella* Typhimurium and *Staphylococcus aureus* are the cause of foodborne diseases in both developed and developing countries [1- 3]. *Salmonella* Typhimurium belongs to the enterobacteriaceae living in human digestive tract and causes salmonellosis [4]. According to a report from the CDC (2013), it is estimated to cause 1.2 million cases each year in the USA with more than 23,000 hospitalizations. Among these, 450 deaths were recorded. Foods are the major source of these illnesses [1]. Likewise, *S. aureus* can cause gastroenteritis, accounting for an estimated 241,000 cases per year in the USA [5] due to the consumption of enterotoxin contaminated food [2, 3]. *Staphylococcus aureus* is a normal flora on the skin and in nasal passages. In addition, it can be found in the air, dust, sewage, water and on environmental surfaces [3, 6]. Improper preparation, handling and storage are the cause of foodborne contamination. 'To prevent the growth and spread of foodborne pathogens, the use of antimicrobial material from natural substances could reduce the growth of microorganisms and extend the shelf-life of food. Additionally, it should be biodegradable, eco-friendly, nontoxic and safe for consumers [7-9].

Nanofiber mats can be produced via electrospinning. The outstanding properties of nanofibers are a large surface area to volume ratio, superior mechanical performance, flexibility in surface functionality and high porosity [10-12]. They can be used for applications such as filtration membranes, scaffolds in tissue engineering, drug delivery, wound dressing and food packaging [13-15]. Electrospinning is an interesting process for production of nanofibers [10, 11, 15]. The device contains a high voltage power supply, blunt-ended stainless needle, syringe pump and ground collector [11, 16, 17]. When polymer solution is placed in the syringe connected to the stainless needle, a positive charge is connected to the stainless needle and the negative charge is connected to the ground collector. A droplet of the polymer solution at the end of the needle changes from a semicircle to a conical shape when a high voltage is applied. These phenomena can occur due to charge-charge repulsion [10, 11, 18]. The electrostatic repulsion force overcomes the surface tension of the polymer solution causing the polymer to be ejected towards the ground collector. The solvent evaporates and a continuous fiber is laid on the collector as a nonwoven membrane [12, 19, 20].

Chitosan (CS), cellulose acetate (CA) and gelatin (Gel) were natural polymers selected for this study as they are edible, biocompatible, biodegradable, eco-friendly with low toxicity. Chitosan is a derivative of chitin made of glucosamine and N-acetyl glucosamine [21, 22]. It is antimicrobial due to the availability of the positive change on the C-2 of the glucosamine monomer, which reduces the permeability of bacterial membrane [15]. However, chitosan is hard to electrospin as it mainly gives rise to droplets of nanometer and micrometer sizes [23, 24]. Therefore, gelatin was used as copolymer to improve the electrospinnability of the chitosan. Gelatin contains abundant amino and carboxyl hydrophilic groups that can be ionized by acidic agents or hydrolyzed to carry positive or negative charges [14, 25]. Gelatin can be used alone or as a blend to fabricate nanofibers for a range of applications [25-27]. However, electrospun nanofiber mats from chitosan-gelatin hybrid are often susceptible to moisture and rapidly dissolve when submerged in water. Consequently, cellulose acetate was used to improve the physical properties due to its thermostability, chemical resistance, biodegradability and so on [28, 29]. Electrospun nanofibers containing an antimicrobial agent can be used as food coating membranes, filter membranes and food packaging with antimicrobial properties [15, 30, 31]. Eugenol (4-allyl-2-methoxyphenol), which is categorized as GRAS (Generally Recognized as Safe) by the FDA, was used as the antimicrobial agent [4, 32]. Eugenol inhibits microorganisms by disruption of the cytoplasmic membrane, proton motive force, electron flow, active transport and protein synthesis [4, 8, 32-34]. Previous studies have demonstrated success in producing electrospun antimicrobial nanofibers [15, 30, 35, 36]. However, no edible

electrospun nanofibers have been reported. The aim of this research was to fabricate edible antimicrobial nanofiber mats with incorporated eugenol via electrospinning. The morphology of the electrospun CS/CA/Gel nanofiber mats, the release of eugenol and the thermal properties were determined. Additionally, the antimicrobial activity of the mats against foodborne pathogens was also investigated. The edible antimicrobial material may have potential applications as active food packaging and in biomedical products.

2. Materials and Methods

2.1 Materials

Shrimp chitosan powder (deacetylation 95.04%) was purchased from Taming Enterprises Co. Ltd., China. Gelatin powder (250 bloom) was purchased from Xiamen Huaxuan Gelatin Co. Ltd., China. Cellulose acetate (average Mn ~ 30,000) and eugenol 99% (v/v) were purchased from Sigma-Aldrich Co. Ltd, USA. Glacial acetic acid (>99%) was purchased from QRec, New Zealand. Peptone, Tryptic Soy Broth (TSB), and Tryptic Soy Agar (TSA) were used as bacterial culture media and purchased from Becton Dickinson and Company, USA. Bacterial strains of *Salmonella* Typhimurium ATCC 13311 and *S. aureus* ATCC 25923 were obtained from the Department of Medical Science, the Ministry of Public Health, Thailand.

2.2 Electrospinning of CS/CA/Gel nanofibers with incorporated eugenol

The preparation of the nanofiber mats was described previously in Somsap *et al.* [37]. Briefly, chitosan (CS), cellulose acetate (CA) and gelatin (Gel) were dissolved separately in acetic acid 80.0% (v/v) under continuous stirring at room temperature until completely dissolved. Then, CS (5.0% wt), CA (18.0% wt), and Gel (30.0% wt) were blended at a volume ratio of 4:1:5. Final concentration of polymer solution was 18.8% wt. Eugenol at concentrations of 0, 0.1, 0.75, 1.5, 3.0, 5.0, and 10.0% (v/v) of polymer solution was added. In the electrospinning process, the polymer solution with incorporated eugenol was loaded into a 10 ml plastic syringe with 18 gauge metal needle. The positive electrode was clasped to the needle while the negative electrode was clasped to the ground collector. The flow rate of polymer solution was controlled at 0.7 ml/h by a syringe pump (New Era NE-300, USA). A high voltage DC power supply (Gamma High Voltage Research, USA) was applied at 23 kV. The distance between needle and collector was 10 cm. Electrospinning was performed at room temperature and all electrospun nanofiber samples were dried overnight to remove residual solvent.

2.3 Scanning electron microscopy

The morphology of the electrospun nanofibers was observed using scanning electron microscopy (SEM JSM-7800F, JEOL, MA, USA) after coating the nanofiber samples with a thin gold layer to provide electrical conductivity. In total, 50 counts were used to calculate the average diameter of the nanofibers.

2.4 Eugenol release characteristics from electrospun nanofiber mats

The mats were cut into pieces of approximately 50 mg and immersed in 10 ml of distilled water. Eugenol release studies were carried out at room temperature. Samples of 5 ml were taken from distilled water after every 10 min until 300 min. After sampling, 5 ml of fresh distilled water was

added to sustain incubation. The amount of eugenol present in the water samples was determined by UV-vis spectrophotometry (Unicam, England) at a wavelength of 280 nm. The amount of eugenol release was determined from a standard calibration curve. The results were presented in terms of cumulative release as a function of time.

2.5 Differential scanning calorimetry

Thermal analysis was carried out by differential scanning calorimetry (Mettler Toledo, DSC 1 Module, Switzerland) at 30 to 180 ºC and a heating rate of 5 ºC/min. The samples, weighing about 3 mg each, were placed in an aluminum pan with holes in the lid.

2.6 Antibacterial activity

Salmonella Typhimurium and *S. aureus* were cultivated in sterilized TSB and incubated overnight at 37 °C. The culture was then diluted with sterile peptone solution 0.1% (w/v) to a concentration of 4 $\log_{10}$ colony forming units / milliliter (CFU/ml). Next, 5 ml of bacterial suspension was transferred to a flask containing 45 ml of sterilized TSB. The bacterial suspensions employed for the tests contained between 2 to 3 $\log_{10}$ CFU/ml. Antibacterial activity assays were conducted using a modified ASTM dynamic shake test [38]. The mats were cut into 50 mg rectangles and sterilized under ultraviolet (UV) radiation for 2 h (each side for 1 h) then placed into bacterial suspension flasks. The bacterial suspensions were shaken at 200 rpm in an orbital shaker for 0, 15, 30, 60, 120, 240, 480, 720, and 1,440 min. At the end of each period, 1.0 ml of bacterial suspension was transferred from the flask and serial dilutions were plated on TSA plates using the spread plate method [15, 38]. The numbers of viable bacteria were determined by counting colonies after 24 h of incubation at 37 °C. Bacterial suspension without mat was also used as control. The results were analysed by the number of viable bacteria as a function of contact time and compared with the control. The percentage reduction of bacteria was calculated using Equation (1) as follows :

$$\text{Reduction of bacteria (\%)} = (B\text{-}A)/B \times 100 \quad (1)$$

Where A and B are the numbers of surviving bacteria in the test samples and blank control after the specific contact time, respectively.

2.7 Statistical analysis

Results were subjected to statistical analysis using the SPSS package. One-way ANOVA with three replicates was applied. Duncan's multiple range test was used to analyze differences at a *p*-value ≤ 0.05.

3. Results and Discussion

3.1 Morphology of nanofibers

CS/CA/Gel nanofibers incorporating eugenol were successfully fabricated. The morphology of these nanofibers is shown in Figure 1. The results from Figures 1A, D, E from our previous study [39] were also used for comparison. The average fiber diameters incorporating eugenol at 0, 0.1, 0.75, 1.50, 3.0, 5.0 and 10.0 % (v/v) (n=50) were 152.32±41.48 nm, 156.11±17.10, 231.16±54.96 nm, 233.11±53.21 nm, 246.05±84.36 nm, 203.47±99.71 and 288.92±77.69 nm, respectively.

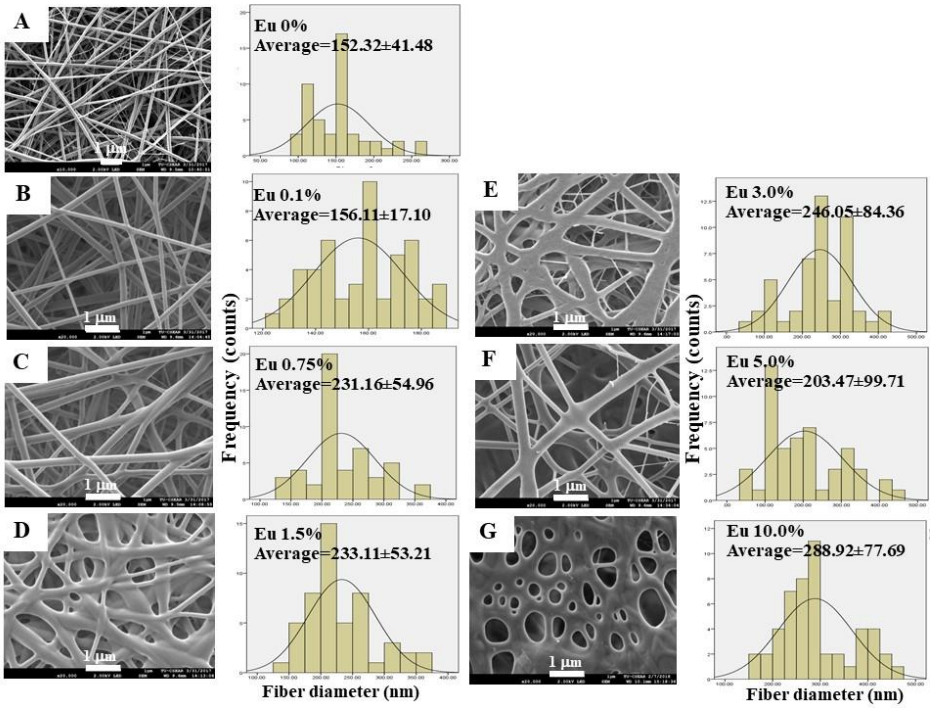

Figure 1. SEM images of morphology and fiber diameter distribution of CS/CA/Gel nanofibers containing different amounts of eugenol: 0% (A), 0.1% (B), 0.75% (C), 1.5% (D), 3.0% (E), 5.0% (F) and 10.0% (G). Magnification:10,000x (A) and 20,000x (B-G)

When the concentration of eugenol was higher, larger fiber diameters with junctions were observed. The control (eugenol 0.0%) exhibited smooth fibers with the smallest average diameter while the largest average fiber diameter was obtained at 10.0% (v/v) of eugenol. The diameter of fibers was later decreased when the concentration of eugenol was increased to 5.0% (v/v). No significant difference (p>0.05) in fiber diameter was found between samples containing 0.75, 1.5, 3.0 and 5.0% (v/v) eugenol. However, a significant difference (p<0.05) was observed at 10.0% (v/v) incorporation. Eugenol insertion into the nanofibers caused them to expand, accounting for the difference. As eugenol is a volatile organic compound, it induced melting of the CS/CA/Gel blend, producing junctions between the nanofibers. The electrospinning process and morphology of the electrospun nanofibers depend on the solution properties (concentration, viscosity, surface tension and conductivity) and processing conditions [11, 17]. When the processing condition was fixed, the solution properties influenced the fiber diameter and morphology. Increasing the concentration of eugenol leads to larger fiber diameters with junctions. Likewise, a polyurethane solution containing 10.0 wt% of olive oil has been reported to produce nanofibers with junctions because of the low volatility of the oil [2]. This might also be related to the electric conductivity of the polymer solution. When the processing condition was fixed, the addition of essential oil into the polymer solution decreased electrical conductivity, causing elongation of the jet as the electric force was insufficient, producing large nanofibers with junctions [11, 40-41]. Rieger and Schiffman [15] reported that electrospun chitosan/PEO nanofibers incorporating cinnamaldehyde essential oil at 0, 0.5 and 5.0% had average fiber diameters of 55±8 nm, 52±9 nm and 38±9 nm, respectively. A smaller fiber diameter was obtained when the concentration of cinnamaldehyde was increased. Incorporation of

antimicrobial agents directly into polymer solution prior to electrospinning is a simple method for loading antimicrobial agents or drugs [7, 42]. However, this process could have an effect on the electrospinnability and morphology of the nanofibers because of changes in the viscosity, surface tention and conductivity of the solution [42].

3.2 Release of eugenol from CS/CA/Gel electrospun nanofiber mats

The release of eugenol from the electrospun nanofiber mats at different time periods is shown in Figure 2. As the mats absorbed water, they expanded, producing burst release of eugenol. The cumulative release of eugenol from 0.1, 0.75, 1.5, 3.0, 5.0 and 10.0% (v/v) incorporated samples at 60 min were $1.21x10^{3}\pm1.2x10^{-3}$ µg, $1.35x10^{3}\pm1.1x10^{-1}$ µg, $1.45x10^{3}\pm1.4x10^{-1}$ µg, $2.61x10^{3}\pm3.2x10^{1}$ µg, $2.70x10^{3}\pm5.8x10^{-2}$ µg and $3.35x10^{3}\pm5.1x10^{-2}$ µg, respectively. At 300 min, it was $1.37x10^{3}\pm8.2x10^{-3}$ µg, $1.60x10^{3}\pm8.2x10^{-4}$ µg, $1.69x10^{3}\pm8.2x10^{-4}$ µg, $3.25x10^{3}\pm1.4x10^{-3}$ µg, $3.74x10^{3}\pm4.5x10^{-3}$ µg and $6.23x10^{3}\pm8.8x10^{-2}$ µg, respectively. The burst release of eugenol at 0.1, 0.75, and 1.5% (v/v) reached a plateau after 60 min indicating that equilibrium had been reached. The burst release of eugenol at 3.0, 5.0, and 10.0% (v/v) continued to increase gradually.

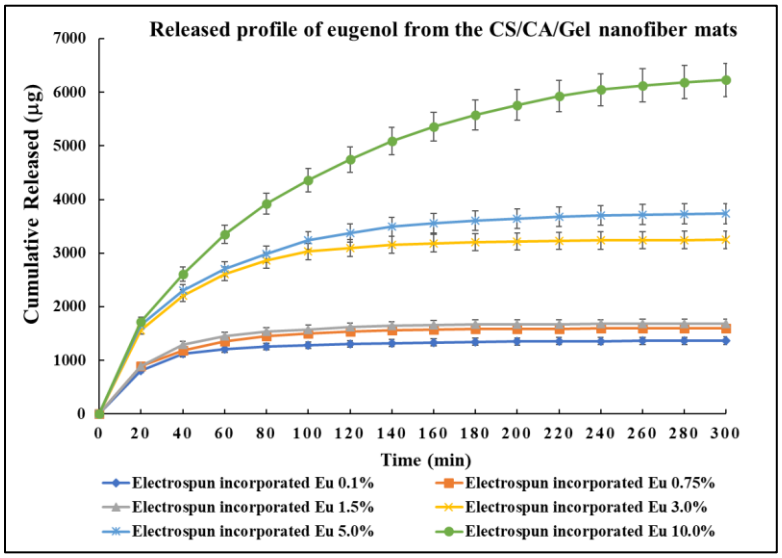

Figure 2. Released profile of eugenol from CS/CA/Gel electrospun nanofiber mats at different time periods

As the eugenol concentration was increased, the cumulative release of eugenol from the CS/CA/Gel nanofiber mats increased. For the cumulative release at 0.1, 0.75 and 1.5% (v/v), neither significant difference ($p>0.05$) in cumulative release were found between samples with 0.1, 0.75 and 1.5% (v/v) nor between samples with 3.0% and 5.0% (v/v). However, a significant difference ($p<0.05$) was found between the two groups and between samples with 10.0% (v/v) eugenol and all other samples.

The release rate of active agents from electrospun nanofibers depends on the method of inclusion into the polymer solution. A previous study reported that the direct inclusion of cinnamaldehyde into polymer solution presented the releasing phenomena into the aqueous solution within 180 min [15]. The release mechanisms can be classified into three categories; swelling-

controlled, diffusion-controlled, and reaction-controlled [15, 43, 44]. In this study, the electrospun chitosan/ gelatin nanofiber mats could easily be dissolved in water. When the networks of chitosan/gelatin hybrid are composed of cellulose acetate, the solubility is reduced because cellulose acetate is water- insoluble. This causes the CS/ CA/ Gel nanofiber mats to swell, eliminating degradability in water. The factors affecting drug release from the matrix depend on the solubility of the drug, the geometry of the particle and drug diffusion through the polymer and /or erosion of polymers [45]. The expansion of the matrix depends on the water absorption, which regulates the degree of swelling. When the matrix is inflated, the porosity of the coating material is larger. This will result in better diffusion [43]. In this study, the presumed release mechanism of eugenol from the CS/CA/Gel nanofiber mats is the diffusion through the matrix polymer network. However, the release behavior is intimately related to the swelling behavior [46]. Moreover, the release rate depends on the type and composition of the polymer and structure of the electrospun nanofiber mats [42].

3.3 Thermal property of electrospun nanofiber mats

The thermal properties of the mats were investigated using DSC analysis (Figure 3). The phase transition temperatures of samples incorporating eugenol at 0%, 0.1%, 0.75%, 1.5%, 3.0%, 5.0 % and 10.0% (v/v) were 152.71, 161.79, 161.84, 155.72, 153.15, 140.37 and 129.69°C, respectively. It was found that the melting point of samples with eugenol concentrations of 0.1 and 0.75 % was increased (~162°C) when compared to the control (152.71°C). The melting point of the 5.0% and 10. 0% eugenol samples was then decreased to 140. 37°C and 129. 69°C, respectively. This demonstrated that mats incorporating eugenol at less than 5.0% (v/v) had better thermostability. Additionally, the melting point correlated with time. As the eugenol concentration was increased, both melting point and time decreased.

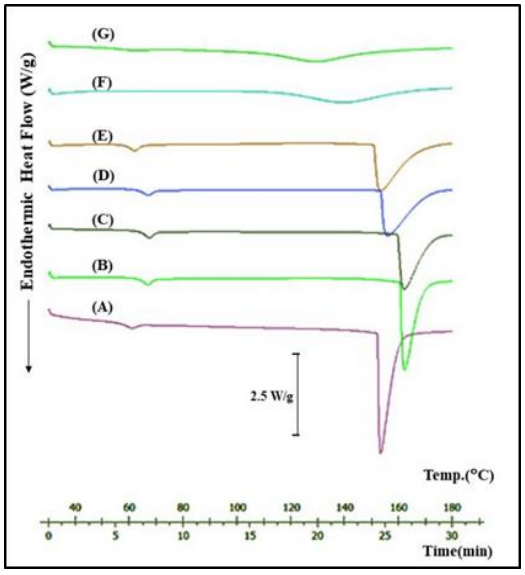

Figure 3. DSC thermograms of CS/CA/Gel electrospun nanofiber mats containing different amounts of eugenol: 0% (A), 0.1% (B), 0.75% (C), 1.5% (D), 3.0% (E), 5.0% (F) and 10.0% (G)

3.4 Antibacterial activity of nanofiber mats

Antibacterial activity was evaluated by dynamic shake test using two strains of foodborne pathogenic bacteria: gram negative (*Salmonella* Typhimurium) and gram positive (*S. aureus*). Figures 4 and 5 show the growth and reduction of bacteria after different contact times. Control AA (without eugenol or nanofiber mat) was the growth control each bacteria test, and control A (nanofiber mat without eugenol) was used in the comparison of CS/CA/Gel nanofiber mats containing different amounts of eugenol.

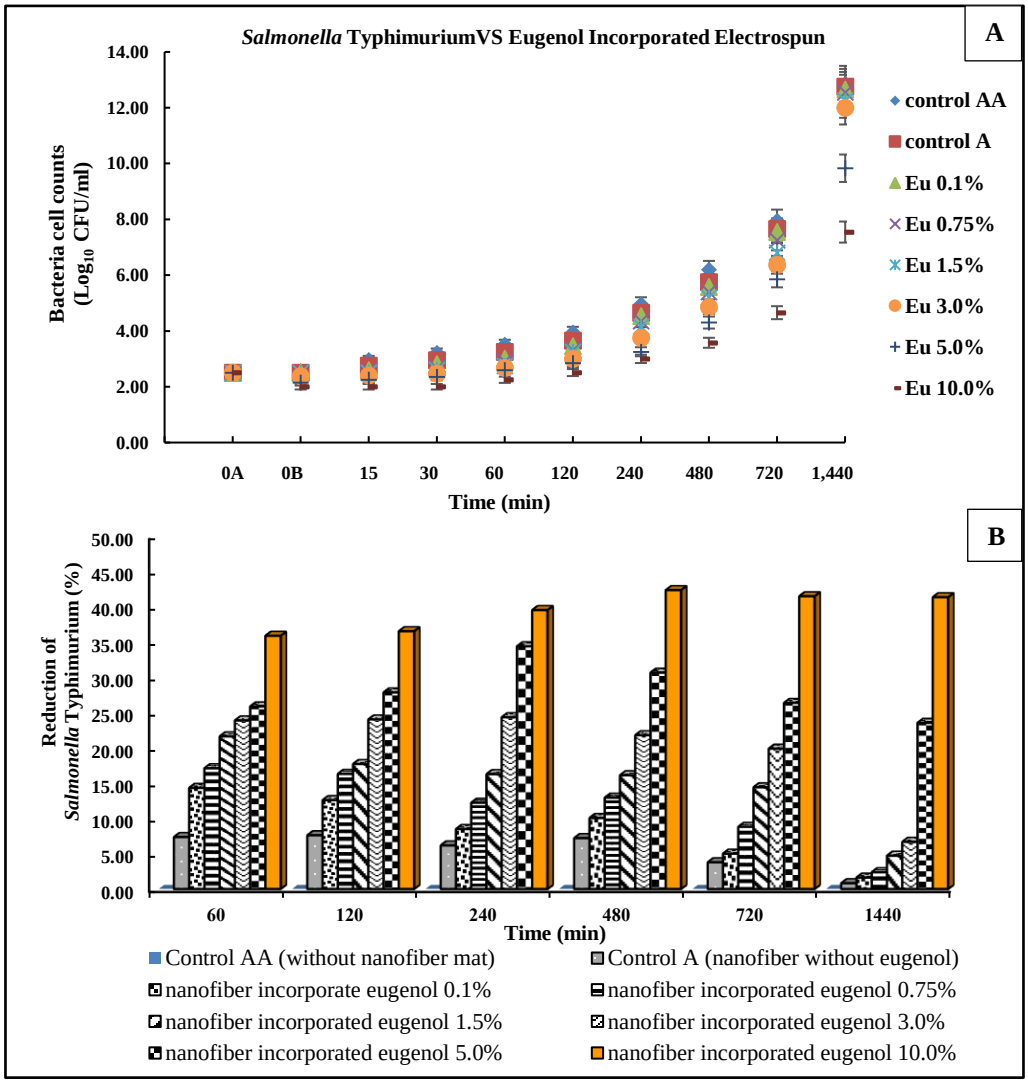

Figure 4. Growth and reduction of *Salmonella* Typhimurium at different time periods

Time 0A min is the initial population of each test with approximately 2 – 3 Log_{10} CFU/ml. and Time 0B min is the population of each test after contact with nanofiber mats incorporating eugenol for 15 sec. In Figure 4 A, the initial population of *Salmonella* Typhimurium was 2.51 log_{10} CFU/ml. At 60 min, the growth of *Salmonella* Typhimurium reached 3.51 log_{10} CFU/ml in control AA and 3.25 log_{10} CFU/ml in control A. As for the test samples, bacterial growth on mats containing eugenol at 0.1%, 0.75%, 1.5%, 3.0%, 5.0% and 10.0% (v/v) was 3.01, 2.91, 2.75, 2.67, 2.60 and 2.25 log_{10} CFU/ml, respectively. At 1,440 min, bacterial growth reached 12.86 log_{10} CFU/ml in control AA and 12.75 log_{10} CFU/ml in control A. As for the test samples, bacterial growth on mats containing eugenol at 0.1%, 0.75%, 1.5%, 3.0%, 5.0% and 10.0% (v/v) contained colonies of 12.65, 12.55, 12.25, 12.00, 9.83, and 7.54 log_{10} CFU/ml, respectively.

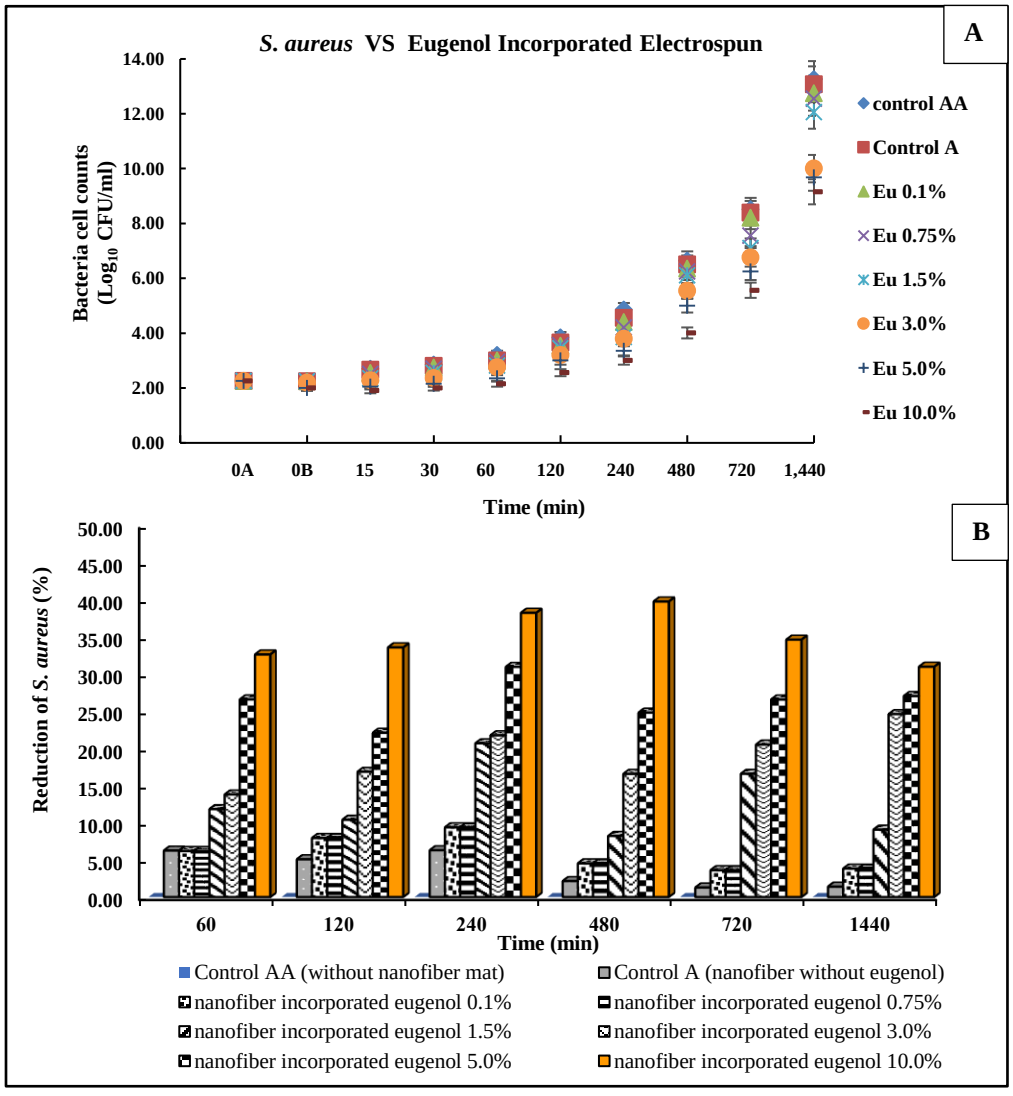

Figure 5. Growth and reduction of *S. aureus* at different time periods

From Figure 4 B, in every time period the growth of *Salmonella* Typhimurium reduced as the eugenol loading of the samples increased. At 1,440 min, reductions of 0.81, 1.61, 2.37, 4.71, 6.67, 23.53, and 41.33% were observed at eugenol concentrations of 0.0% (control A), 0.1%, 0.75%, 1.5%, 3.0%, 5.0% and 10.0% (v/v), respectively.

From Figure 5 A, the initial population of *S. aureus* was 2.25 $\log_{10}$ CFU/ml. At 60 min, the growth of *S. aureus* reached 3.20 $\log_{10}$ CFU/ml in control AA and 3.00 $\log_{10}$ CFU/ml in control A. As for the test samples, mats containing eugenol at 0.1%, 0.75%, 1.5%, 3.0%, 5.0% and 10.0% (v/v) contained colonies of 3.00, 2.96, 2.82, 2.76, 2.35 and 2.16 $\log_{10}$ CFU/ml, respectively. At 1,440 min, the colonies reached 13.26 $\log_{10}$ CFU/ml in control AA and 13.08 $\log_{10}$ CFU/ml in control A. As for the test samples, mats containing eugenol at 0.1%, 0.75%, 1.5%, 3.0%, 5.0% and 10.0% (v/v) contained colonies of 12.75, 12.55, 12.06, 10.00, 9.68 and 9.16 $\log_{10}$ CFU/ml, respectively. From Figure 5 B, in every time period the growth of *S. aureus* reduced as the eugenol loading of the samples increased. At 1,440 min, reductions of 1.38, 3.81, 5.31, 9.05, 24.57, 27.01 and 30.95% were observed at eugenol concentrations of 0.0% (control A), 0.1%, 0.75%, 1.5%, 3.0%, 5.0% and 10.0% (v/v), respectively. The results demonstrated that up to 240 min, the populations of both *Salmonella* Typhimurium and *S. aureus* increased gradually and then increasing more rapidly in all samples. The mat incorporating eugenol at 10.0% (v/v) showed the maximum growth reduction compared to control A.

Previous studies have suggested that eugenol may disrupt the cytoplasmic membrane of bacteria as well as increasing its permeability [4, 47]. The cell membrane of Gram-negative bacteria is sensitive to the hydrophobic property of eugenol. Eugenol can penetrate the lipopolysaccharide of the bacterial membrane and alter the cell structure. This phenomenon can cause leakage of intracellular components and lead to the death of bacterial cell [4, 32, 48, 49]. Moreover, the chitosan offer positively charged free amino group, which can interact with the negative bacterial cell wall causing membrane to rupture. Consequently, intracellular proteins were released due to membrane deterioration [15, 24, 50].

4. Conclusions

The results of this study showed that eugenol essential oil could directly be incorporated into a CS/CA/Gel polymer solution without the use of surfactant. Edible electrospun nanofiber mats were succesfully fabricated via electrospinning. The release of eugenol was related to eugenol diffusion through the polymer and/ or erosion of the polymer. Thermal analysis demonstrated better thermostability when the concentration of eugenol was less than 5.0% (v/v). The CS/CA/Gel edible electrospun nanofiber mats with incorporated eugenol showed enhanced activity against *Salmonella* Typhimurium and *S. aureus.* This approach may effectively extend the shelf-life of food with potential applications in active food packaging and others products.

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