

Time-Kill Kinetic Effect of Sodium Citrate, Sodium Nitrite and Cinnamaldehyde Against Biofilm Forming *Escherichia coli* O157:H7

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Abstract. Food safety is a significant concern of every sector of the food industry. Survival of *Escherichia coli* O157:H7 with biofilm-forming potential in commercial food premises is a possible danger to consumers' health, especially in societies where most of the population depend on it for their daily meals. Preservation of fresh food quality being of utmost importance, new innovative means of inhibiting pathogenic microorganisms in foods are being evaluated to be effective at destroying microorganisms and preserving the physical and organoleptic properties. This study aimed to inhibit biofilm formation of *Escherichia coli* O157:H7 by food additives; sodium citrate, sodium nitrite and cinnamaldehyde. The isolate obtained was subjected to Gram's staining and various biochemical identifications and later confirmed by the latex agglutination test. The *Escherichia coli* O157:H7 was further subjected to a biofilm formation potential test on Congo red media. Antimicrobial susceptibility testing was conducted to obtain the susceptibility/resistance pattern of the isolate to the food additives. The MIC, MBC and time-kill kinetics effect was determined following CLSI 2017 guideline. The highest growth inhibition zone of 31 mm was exhibited by cinnamaldehyde, followed by sodium nitrite with 26 mm and sodium citrate with 13 mm. The MIC was determined to be 2.5 mg/ml for sodium citrate, 0.25 mg/ml for sodium nitrite and 0.125 µl/ml for cinnamaldehyde. Sodium citrate was found to be bacteriostatic between 6-8 hrs with 72.9% reduction, sodium nitrite and cinnamaldehyde exhibit both bacteriostatic and bactericidal effects between 2-24 hrs with percentage inhibition of 65-90% and 63-100%, respectively. This study showed that sodium citrate, sodium nitrite and cinnamaldehyde exerted strong antimicrobial properties indicating their potential as suitable preservatives.

Keywords: food additives; inhibitory effect; time-kill kinetics; biofilm-forming *Escherichia coli* O157:H7.

INTRODUCTION

Food safety is a significant concern of every sector of the food industry. Survival of *Escherichia coli* O157:H7 with biofilm-forming potential in commercial food premises is a possible danger to consumers' health, especially in societies where most of the population depend on it for their daily meals. The development of biofilms by the food-borne pathogens attached to surfaces in the food processing environments results in the deterioration of products, persistence of pathogenic bacteria and transmission of food-associated dis-

eases [1]. In addition, bacteria in biofilms are more resistant to antimicrobials than their planktonic counterparts, making their elimination from food and the food processing facilities a significant challenge. Therefore, their presence creates excellent and urgent concern for the current food industries (small- and large-scale industries) and public health [2]. Food processing aims to provide safe products with good organoleptic characteristics. This is achieved by using a diverse range of food additives or preservatives to improve and maintain the food quality (nutritional value, texture, safety, taste and appear-

ance) and safety, thus extending the shelf life of food products. Therefore, eliminating biofilms or minimization of biofilms formed by food-borne pathogens using food additives is essential for consumers to provide safe and suitable quality products. This, in turn, justifies the need for this study. Preservation of fresh food quality being of utmost importance, new innovative means of inhibiting pathogenic microorganisms in foods are being evaluated to be effective at destroying microorganisms and preserving the physical and organoleptic properties.

MATERIALS AND METHODS

The bacterial isolate (*Escherichia coli* O157:H7) was confirmed through Gram's staining and various biochemical identifications and later confirmed by the latex agglutination test (Plate 1) [3].

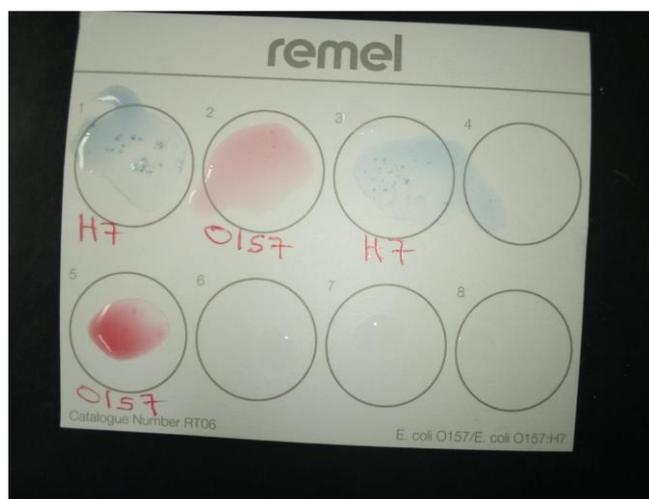


Plate 1 – Latex agglutination test for *Escherichia coli* O157:H7

The confirmed *Escherichia coli* O157:H7 was further subjected to a biofilm production assay to determine the phenotypic expression of colonies in the Congo Red Agar medium (Plate 2).

Briefly, the Congo Red Agar was prepared by dissolving 37 g Brain Heart Infusion agar (TITAN BIOTECH LTD), 36 g sucrose and 0.8 g congo red (BDH LTD) in 1 litre of distilled water. The solution was sterilized by autoclaving at 121 °C for 15 min. After inoculation, the plates were incubated for 18 h at 37 °C, where the biofilm-producing isolates originate as black colonies [4, 5].



Plate 2 – Biofilm formation potential of *Escherichia coli* O157:H7 congo red agar

The inocula of the potent biofilm-forming strains were prepared and subcultures on TSA, which was incubated at 37 °C for 24 h. Colonies of each isolate were then transferred into TSB using a sterile cotton swab to match the turbidity standard of 0.5 McFarland (15×10^8 CFU/ml).

The food additives (sodium citrate, sodium nitrite and cinnamic aldehyde) were purchased and prepared in solutions. The solution was then diluted to different concentrations based on the application amount for food preservation (2 %, 1 %, 0.5 %, 0.25 % and 0.125 %) with an appropriate solvent [6].

In-vitro antimicrobial activity of food additives was conducted against isolated *Escherichia coli* O157:H7, using the agar well diffusion method as described by the Clinical and Laboratory Standards Institute [6]. The standardized isolate was streaked over the entire surface of Mueller Hinton agar plates with a sterile cotton swab. Then, a hole with a diameter of 6 is punched aseptically with a sterile cork borer, and a volume of 100 μ l of different concentrations of food additives (Sodium citrate, Sodium nitrite, Cinnamic aldehyde) was introduced into the well, Augmentine disc 30 μ g (Oxoid) was used as a positive control in the assay. The plates were then incubated at 37 °C for 24 h. The size of the clear zone was measured in mm. This assay was done in triplicates.

The recommendation of [6] performed the Minimum Inhibitory Concentrations and Minimal Bactericidal Concentrations determination. These were performed in a 96-well microtiter plate using a two-fold micro broth dilution technique using an inoculum that matched McFar-

land's turbidity standard (1.5×10^8 CFU/ml). The MIC is the lowest food additive concentration that completely inhibits visible growth. The MBC was determined by subculturing the wells onto the MHA plate that showed no visible increase for the MIC.

Time-kill kinetics assay of the solutions of the food additives was carried out following the procedure described by [7]. The keys were prepared for concentrations equal to 1X MIC, 2X MIC, and 4X MIC. An inoculum size of 1.0×10^8 CFU/ml was added and incubated at 37 °C. Aliquots of 1.0 ml of the medium were taken at time intervals of 0, 1, 2, 3, 4, 5, 6, 12, and 24 h and were inoculated aseptically into 20 ml nutrient agar and incubated at 37 °C for 24 h. A control test was performed for the organism without the additives. The colony-forming unit (CFU) of the microorganisms was determined. The procedure was performed in triplicate (three independent experiments), and a graph of the log CFU/ml was plotted against time.

The results were expressed as mean \pm SD two-way ANOVA test was used to compare results among and within groups for any significant dif-

ference in efficacy of food additives against biofilm-forming *Escherichia coli* O157:H7 using SPSS version ($P < 0.05$).

RESULTS AND DISCUSSION

The isolate was confirmed to be *Escherichia coli* O157:H7 due to the appearance of colourless colonies on sorbitol MacConkey agar and agglutination with a latex agglutination kit.

The isolate obtained was confirmed to be biofilm producer on congo red agar due to the formation of dark colonies on the media indicating slime production. Hence, *Escherichia coli* O157:H7, which commonly contaminates food and contact surfaces in food industries, can develop a biofilm.

In vitro antimicrobial effects of food and preservatives have been elucidated as to the susceptibility or how resistant an organism is to it. In this study, the antimicrobial susceptibility pattern of sodium citrate, sodium nitrite and cinnamaldehyde showed (Table 1) a high zone of inhibition (mm) of 13.00 ± 0.88 , 26.00 ± 0.58 and 31.67 ± 0.88 , respectively, at 2% concentration each.

Table 1 – Antimicrobial susceptibility pattern showing mean zone of growth inhibition (mm) \pm SEM of food additives against *Escherichia coli* O157:H7

Food additives	Concentrations (mg/ml)				
	2 %	1 %	0.5 %	0.25 %	Control
Sodium citrate	13.00 ± 1.15	10.00 ± 0.58	9.00 ± 0.58	7.67 ± 0.33	24.6 ± 0.85
Sodium nitrite	26.00 ± 0.58	20.33 ± 0.88	14.67 ± 0.67	10.33 ± 0.88	24.8 ± 0.52
Cinnamaldehyde	31.67 ± 0.88	27.33 ± 0.33	25.33 ± 0.33	17.67 ± 0.88	25.6 ± 0.89

Notes: Zones of growth inhibition = diameter of well plus zones of growth inhibition; diameter of well = 6 mm. The mean growth inhibition zone was determined from three independent results ($n = 3$); SEM = standard error mean.

The most miniature zone of inhibition recorded for each additive was 0.25% concentration where sodium citrate had 7.67 ± 0.33 mm, sodium nitrite; 10.33 ± 0.88 mm and cinnamaldehyde 17.67 ± 0.88 mm. Among the three additives used, cinnamaldehyde has the highest zone of inhibition (31.67 ± 0.88) mm. At the same time, sodium citrate showed the smallest area of inhibition (7.67 ± 0.33) mm thus, showing that cinnamaldehyde has a strong antimicrobial effect against *Escherichia coli* O157:H7. This finding is in line with the work of [8], who reported antimicrobial susceptibility of cinnamaldehyde against avian *Escherichia coli* inhibition zones diameters rang-

ing from 16 to 44 mm. Similarly, the report made by [9] on microbiological profiling of food additives and evaluation of their antibacterial efficacy showed a clear zone of inhibition of 8–22 mm measured on agar well diffusion.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of sodium citrate, sodium nitrite and cinnamaldehyde recorded showed (Table 2) that all the three additives have varying MIC and MBC that can inhibit and cause a lethal effect on biofilm-forming *Escherichia coli* O157:H7.

Table 2 – Minimum inhibitory concentration and minimum bactericidal concentration of food additives against *Escherichia coli* O157:H7

Food additives	Concentrations (mg/ml)						MIC	MBC
	0	0.625	1.25	2.5	5	10		
Sodium citrate	0	0.625	1.25	2.5	5	10	2.5 mg/ml	5 mg/ml
	+	+	+	-	-	-		
Sodium nitrite	0	0.625	0.125	0.25	0.5	1	0.25 mg/ml	1 mg/ml
	+	+	+	-	-	-		
Cinnamaldehyde	0	0.625	0.125	0.25	0.5	1	0.125 µl/ml	0.25 µl/ml
	+	+	-	-	-	-		

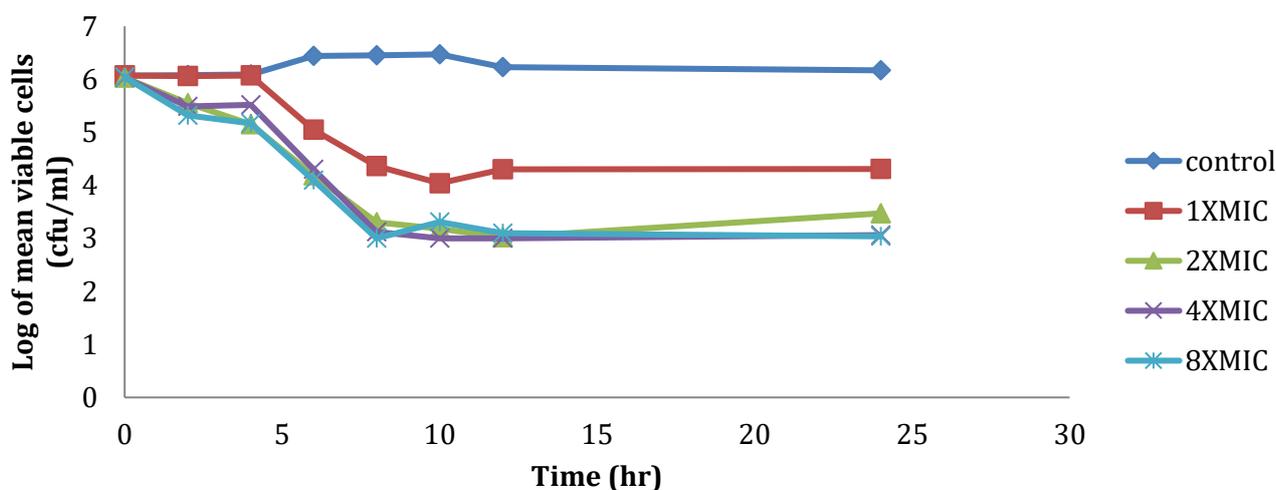
Notes: MIC – Minimum inhibitory concentration; MBC – Minimum bactericidal concentration; + growth activity (MIC/MBC decrease); – no growth (MIC/MBC increase).

The result showed that sodium citrate had MIC at 2.5 mg/ml and MBC at 5 mg/ml, sodium nitrite had MIC at 0.25 mg/ml and MBC at 1 mg/ml while cinnamaldehyde had MIC at 0.125 µl/ml and MBC at 0.25 µl/ml. Compared to the three additives, cinnamaldehyde had the most potent inhibition and lethal effect at a lower concentration than sodium nitrite next to cinnamaldehyde, followed by sodium citrate as the weak with higher concentration. The MIC and MBC value for sodium citrate increase twice the MIC and MBC value of sodium nitrite and cinnamaldehyde. Thus, cinnamaldehyde and sodium nitrite have strong bactericidal and bacteriostatic effects, while sodium nitrite has weak bacteriostatic and bactericidal effects. This finding agrees with the work of [5]. The MICs of sodium citrate and cinnamaldehyde against *S. aureus* at 5 mg/ml and 0.5 µl/ml and MBC at 40 mg/ml and 2 µl/ml, respectively. The effect of cinnamaldehyde in fruit juice was reported to be at 0.25 to 1.5 µl/mL con-

centration, which completely inhibited the growth of *S. enterica* and *Escherichia coli* O157:H7 with MIC value of 0.25 µl/ml [10].

The time-kill kinetics profile of sodium citrate, sodium nitrite and cinnamaldehyde against *Escherichia coli* O157:H7 showed (Figure 1–3) a varying pattern of reduction in the number of viable cells within 24 hrs at different concentrations with a significant difference at $p=0.05$ when compared to control (growth of organism without antimicrobial agents).

Time-kill kinetic profile of sodium citrate at 1X MIC showed (Figure 1) no reduction in some viable cells with steady growth from 2-4 hrs compared to the control. At 6–8 hrs, there was a reduction in the number of viable cells followed by a gradual rise from 10 up to 24 hrs. At 8X MIC, a reduction in the number of viable cells was observed from 2 hrs of incubation with a gradual decrease in cell number up to 24 hrs.

Figure 1 – Time-kill kinetic effect of sodium citrate against biofilm forming *Escherichia coli* O157:H7

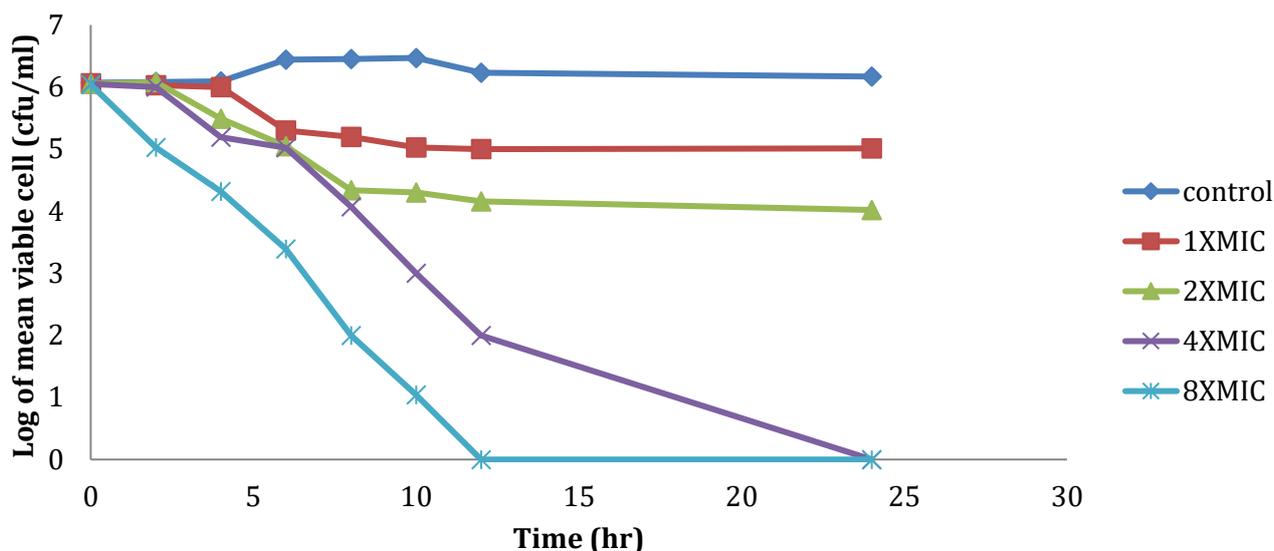


Figure 2 – Time-kill kinetic effect of sodium nitrite against biofilm-forming *Escherichia coli* O157:H7

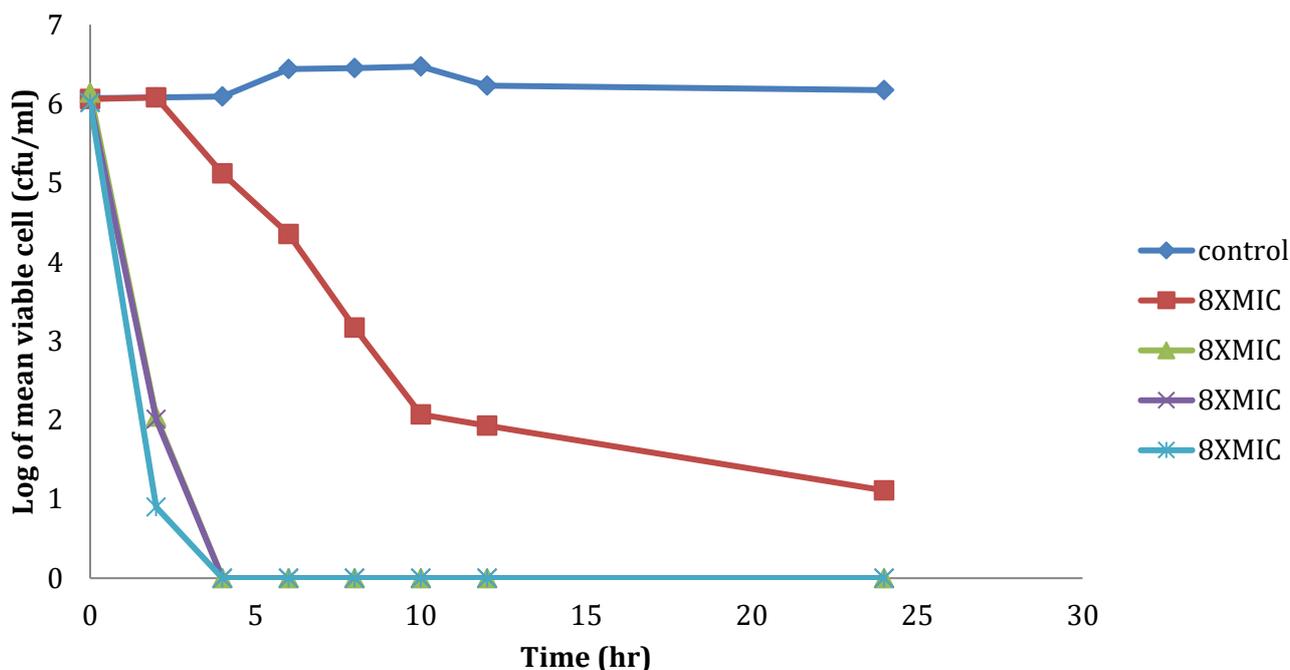


Figure 3 – Time-kill kinetic effect of cinnamaldehyde against biofilm-forming *Escherichia coli* O157:H7

The time-kill kinetic profile of sodium nitrite at different test concentrations showed (Figure 2) a decline in the number of viable cells between 6–24 hr at 1X MIC while reducing the number of the cell at the highest concentration used (8X MIC) was observed from 2–10 hrs. No growth was seen when subcultured and incubated between 12-24 hrs, thus making it bactericidal. Cinnamaldehyde showed (Figure 3) a more vital time-kill profile than sodium citrate and sodium nitrite by

exhibiting bacteriostatic and bactericidal effects against the test isolate within 24 hrs at varying tests concentrations. At 1X MIC, there was a gradual decrease in viable cell count. Complete inhibition of growth was observed between 4–24 hrs. This work agrees with [10] on the effect of cinnamaldehyde in fruit juice was reported to be at 0.25 to 1.5 µl/ml concentration, which completely inhibited the growth of *S. enterica* and *Escherichia coli* O157:H7 with MIC value of 0.25 µl/ml.

Similarly, [8] reported a 99.9 % reduction of *Escherichia coli* O157:H7 at 8X MIC of cinnamaldehyde but slower in action. Bacterial sensitivity to certain antimicrobials may be partly attributed to the increased fluidity of the cytoplasmic membrane. Stringent control of membrane fluidity is crucial for membrane-associated functions such as active transport of solutes, passive permeability to hydrophobic molecules and protein-protein interactions. The cytoplasmic membrane is the primary cellular site where cinnamaldehyde exerts its antibacterial action; therefore, cinnamaldehyde, by exerting a membrane-fluidizing effect, may further disrupt those membrane-associated functions. Although the precise mechanism of antibacterial activity of cinnamaldehyde is inconclusive, it is believed that cinnamaldehyde inactivates bacteria by inhibiting ATPase at sub-lethal concentrations and disrupting the cytoplasmic membrane at lethal concentrations. The finding shows that sodium citrate, sodium nitrite, and cinnamaldehyde are bacteriostatic and bactericidal molecules.

CONCLUSION

The isolate was identified as *Escherichia coli* O157:H7 through biochemical characterization and latex agglutination test. The antimicrobial susceptibility test revealed that the isolate is susceptible to the additives, showing cinnamaldehyde

stronger antimicrobial effect against *Escherichia coli* O157:H7. The minimum inhibitory concentration of sodium citrate required to inhibit the growth of *Escherichia coli* O157:H7 was obtained at 2.5 mg/ml, sodium nitrite at 0.25 mg/ml and cinnamaldehyde at 0.125 µl/ml. The minimum bactericidal concentration was obtained at 5 mg/ml, 1 mg/ml and 0.25 µl/ml respectively. Sodium citrate was found to be bacteriostatic between 6–8 hrs with 72.9 % reduction, sodium nitrite and cinnamaldehyde exhibit both bacteriostatic and bactericidal effects between 2–24 hrs with percentage inhibition of 65–90 % and 63–100 %, respectively. From this finding and comparative analysis, it is concluded that food additives (sodium citrate, sodium nitrite and cinnamaldehyde) can inhibit and can be used to control biofilm formation of *E. coli* O157:H7.

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Conflict of Interests

The authors declare no conflicting interest.

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