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Comparative Antimicrobial Efficacy, Kinetic Destruction Pattern and Microbial Inactivation Dynamics of Extracted Cinnamon Essential Oil and Commercial Cinnamaldehyde against Foodborne Pathogens

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ABSTRACT

Background and Aim: The increasing demand for the discovery of next-generation antimicrobials necessitates the use of plant extracts as alternatives. This study investigates the antibacterial efficacy of extracted cinnamon essential oil (CEO) and commercial cinnamaldehyde (CN) against foodborne pathogens.

Materials and Methods: Kirby-Bauer disc diffusion method was used to screen the antimicrobial potency of CEO and CN. MIC and MBC were determined by the broth microdilution method. Kinetic destruction pattern was studied by time killing assay. CEO and CN mediated inactivation dynamics of *S. typhimurium* (ALM40) and *L. monocytogenes* were studied on the ground chicken meat model.

Results: Both CEO and CN showed remarkable antimicrobial efficacy against the test strains, with highest and lowest efficacy against *V. metschnikovii* and *E. coli,* respectively. The agents inhibited gram-positive and negative bacteria equally. CN showed higher efficacy than CEO although the results were very close. MIC of CEO and CN ranged from 0.625%-5% (v/v) and 0.078%-0.3125% (v/v), respectively. Heat treatment and pH alteration did not hamper the antibacterial potency of CEO. CEO and CN mediated destruction kinetics were faster in *L. monocytogenes* than *S. typhimurium* (ALM 40). Inactivation dynamics study showed CEO and CN to have slightly dose-dependent antimicrobial effects. Besides, storage conditions and time did not reduce the antimicrobial potency. The significant microbial reduction was observed in both CEO and CN treated meat samples than untreated controls. Notably, a complete reduction of viable count in meat model was observed in selective medium just after 24hrs storage.

Conclusion: Both CEO and CN showed promising antimicrobial effects to be used in combating foodborne pathogens.

Keywords: Cinnamon essential oil, Cinnamaldehyde, antimicrobial activity, foodborne pathogens

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1. Introduction

Food safety is a public health priority with paramount importance worldwide. Yearly, almost 1 in 10 people develops foodborne illness and 4, 20,000 die worldwide **(1)**. The main reason behind food spoilage is the occurrence of different pathogenic microorganisms (bacteria, viruses, parasites, yeasts and molds). Ever-expanding antibiotics resistance among these pathogens are raising concern among food safety professionals and public health sectors due to a lack of innovation in the development of new antibiotics **(2)**. Finding out a novel solution with next generation antimicrobials has become a public health emergency. Though chemical preservatives can prevent foodborne diseases, their continued

application causes bio-accumulation of chemical residues in the food and feed chain, increasing microbial resistance to the chemicals used, and renders negative health impacts. An eco-friendly and chemical or antibiotic-free food preservation approach is the top demand from consumers to minimize food poisoning outbreaks caused by foodborne pathogens and spoilage bacteria. To meet up this demand, researchers are struggling for many years to develop novel antimicrobials and food preservation technologies. But very few of these are devoid of harmful chemicals. Thus the interest of using new preservation techniques with a natural or "green" image is increasing day by day. One such possibility is the use of essential oils (EOs) as food preservatives to destroy harmful pathogens in food. Essential oils are secondary metabolites of plants that play critical role in plants defense mechanism **(3)**. Essential oils or their components have been demonstrated to not only possess broad-spectrum antibacterial properties **(4, 5)**, but also antiparasitic **(6)**, insecticidal **(7)**, antiviral, antifungal, and antioxidant properties **(8, 9)**. Although food industries primarily use essential oils as flavorings, they represent an intriguing source of natural antimicrobials for food preservation.

Cinnamon is one of the world's oldest and most frequently consumed spice that is obtained from the inner bark of several tree species from the genus Cinnamomum. Ayurveda (the Indian system of medicine) has documented the therapeutic benefits of cinnamon for over 6000 years **(10)**. Cinnamon contains various volatile components of multiple classes, namely monoterpenes, sesquiterpenes and phenylpropenes. Major active ingredient of cinnamon bark oil is cinnamalydehyde (precisely trans-cinnamaldehyde or 3-phenyl-2- propenal) **(11)** which is available commercially. There is a large bunch of previous report regarding the antibacterial, antifungal and antiviral activity of cinnamon essential oil and cinnamaldehyde **(12-17)** which renders the possibility for future formulation of organic food preservative using this agent. Cinnamon, according to Vasconcelos et al., can damage bacterial cell membranes, alter the lipid profile, inhibit ATPases, cell division, membrane porins, motility, and biofilm formation, and plays a role in anti-quorum sensing actions **(18)**. Many prior in vitro studies also validated the ability of cinnamon essential oil and cinnamaldehyde in inactivating diverse bacterial populations in various food stuffs, also at different storage conditions and periods **(19-23)**.

Despite of its reported advantageous features, commercial cinnamaldehyde is highly expensive, thus limiting its mass application. In contrast, extraction of cinnamon essential oil is more cost effective. To enrich the scientific community with more understanding regarding the comparative potentiality of cinnamon essential oil and cinnamaldehyde as a natural antimicrobial in food preservation, here we assessed the antibacterial efficacy of these agents against several commonly encountered food borne pathogens. We performed an in depth assessment to ensure that the agents are effective at various temperatures and pH, also show antibacterial activity against multi drug resistant (MDR) strains. As *Salmonella typhimurium* and *Listeria monocytogenes* are the two major causes of food borne illness worldwide **(24-27)**, we showed CN and CEO mediated inactivation of these bacteria in artificially inoculated ground chicken meat to validate their applicability for the future formulation of a natural food preservative.

2. Materials and Methods

2.1 Test Organisms

Sixteen bacterial species (10 gram positive and 6 gram negative) and another 8 multidrug resistant *Pseudomonas* spp. isolates were used in the current study based on their availability and importance as foodborne pathogens. Supplementary Table 1 enlists the list of test organisms and the name of repositories from where the strains were collected. The collected strains were stocked at -80℃ with 20% glycerol in cryogenic vials. Tryptic Soy Agar (TSA) slants (Nissui, Japan) were used to prepare working cultures and stored at 4°C. The cultures were periodically sub-cultured to fresh TSA slants to ensure cell viability.

2.2 Extraction of Cinnamon Essential Oil

The solvent-solvent extraction method was used to extract cinnamon essential oil (CEO). The method was adopted from **(20)**. Cinnamon barks were purchased from Dhaka new market, Bangladesh. Twenty grams of washed, dried and grounded barks were soaked in 80 mL n-hexane (Nacalai tesque Inc. Tokyo, Japan) and kept in shaker at room temperature for 48 hours. After that the mixture was squeezed with sterilized cheesecloth to separate the n-hexane fraction. The hexane from the fraction was evaporated at 40^0C using a drier, which left mass of organics called concrete. The remaining concrete was then dissolved in double amount of ethanol (95%) in a separation funnel and vigorously shaken for several hours to sediment the ethanol insoluble part. Finally, existing ethanol was removed by rotary evaporator and the mixture was filtrated by Whatman Filter Paper (Grade 1) to remove any remaining debris, leaving the absolute essential oil.

2.3 Screening the Antibacterial Activity of CEO and CN

The antibacterial activities of the extracted CEO and commercial CN (purchased from Nacalai Tesque Co, Kyoto, Japan) were evaluated using Kirby Bauer disc-diffusion assay against the selected food-borne pathogens and MDR isolates (Supplementary Table 1). From the 100% concentrated stock solutions of CEO and CN, 5% working solutions were prepared using 99% ethanol as solvent. Filter paper discs of six mm diameter were impregnated with 50 µL of 5% working solutions and dried overnight in hot air oven at 40˚C to evaporate the solvent properly and applied on Mueller-Hinton Agar (MHA) plates inoculated with the test organisms. Zone of inhibition (ZOI) in bacterial growth were measured in mm (including 6 mm disc) after 24hrs of incubation at 37˚C. A reference antibiotic, ciprofloxacin (5 µg/disc) was used as a positive control and disc containing ethanol only was used as a negative control.

Supplementary Table 1. List of test organisms used in this study.

Foodborne pathogens and spoilage bacteria	
Test organisms	**Source**
Escherichia coli ***Escherichia coli* DH5α** ***Salmonella typhi*** ***Pseudomonas aeruginosa*** ***Klebsiella pneumoniae*** ***S. aureus*** ***B. cereus*** ***Listeria grayi***	Centre for Advanced Research in Sciences (CARS), University of Dhaka, Bangladesh.
Escherichia coli* O157:H7 ATCC 12079** ***Salmonella typhimurium* (ALM 40)** ***Vibrio cholerae ***Vibrio parahaemolyticus* ATCC 17802** ***Vibrio metschnikovii*** ***Listerrria monocytogenes*** ***S.aureus* ATCC 25923** ***S. epidermidis***	International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)
Multidrug resistant (MDR) *Pseudomonas* spp. isolates	
Test isolates	**Source**
DMC-8b, BIHS-5, DMC-6, DMC-7, BIHS-7, DMC-24, BIHS-8, DMC-20b	Microbial Genetics and Bioinformatics Laboratory (MGBL), University of Dhaka, Bangladesh.

2.4 Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of CEO and CN

MIC and MBC of CEO and CN were determined by broth microdilution method followed by culturing on MHA, as described previously **(21)**. MIC was defined as the lowest concentration of CEO and CN at which no visible growth of the tested organisms were demonstrated on MHB after 24hrs incubation. Minimum bactericidal concentration (MBC) was defined as the lowest concentration that yielded no or only single colony in the MHA after another 24hrs incubation.

2.5 Determination of the Effect of Heat Treatment and pH Alteration on the Antimicrobial Activity of CEO

5% solutions of CEO were incubated at 37˚C, 50˚C, 75˚C and 100˚C temperatures for 30 minutes and cooled down. Another set of 5% solutions were adjusted to pH 2.0, pH 7.0 and pH 9.0 with 50mM Phosphate buffer followed by filtration with a membrane filter (0.45 µm). After that the antibacterial activity of heat treated and pH adjusted CEO were determined by disc diffusion method.

2.6 Time Killing Assay

CEO and CN were evaluated to investigate their kinetic destruction pattern against *Salmonella typhimurium* (ALM 40) and *Listeria monocytogenes*, two major causative agent of foodborne illness. Bacterial cells were grown on MHB for 6 hrs., followed by centrifugation and re-suspension in normal saline. 1ml of bacterial suspension was then transferred to MHB media containing 1xMIC concentration of CEO or CN. Finally, the bacterial concentration in the mixture was adjusted to 10^7 CFU/mL. To determine cell viability and bacterial growth in comparison with control (no plant extract), the prepared mixtures were inoculated on the TSA plates at predetermined time intervals (0h, 1h, 3h, 5h, 7h, 9h) and incubated for 24hrs at 37^0C. Number of viable cells were counted in

log CFU/mL after incubation which indicated the kinetic destruction pattern of CEO and CN.

2.7 Determination of CN and CEO Mediated Inactivation Dynamics of *S. typhimurium* (ALM 40) and *L. monocytogenes* in Ground Chicken Meat

Inactivation dynamics of *S. typhimurium* (ALM 40) and *L. monocytogenes* in ground chicken meat at different storage conditions were determined by a slight modification described by Hoque *et al*. (21) Nalidixic acid resistant *S. typhimurium* (ALM 40) was inoculated into 200gm ground chicken meat to obtain a final concentration of 8.85 or 8.44 log CFU/gm. *L. monocytogenes* at a final concentration of 7.73 or 7.35 log CFU/gm was used to inoculate in ground chicken meat. Inoculated meat samples were uniformly mixed with a sterilized spoon. 3xMIC and 5xMIC concentrations of CN and CEO were then added with the *S. typhimurium* (ALM 40) and *L. monocytogenes* inoculated meat samples, respectively. After proper mixing, meat samples were divided in small aliquots (5 g each) and packed in UV radiated Ziplock packs and stored at 4˚C and -20˚C for 14 days. Control samples with no CEO/CN treatment were also stored similarly. Microbiological analysis of control and treated sample was done on 1-14 days of post storage. Using a homogenizer, each samples were homogenized in sterilized peptone water for 3 minutes. The mixtures were diluted using tenfold serial dilutions (10^{-7}) and 0.1ml of each diluent were surface plated on Tryptone Soy Agar containing 50 µg/mL nalidixic acid (TSAN), which is a non-selective general purpose media; Bismuth Sulfite Agar containing 50 µg/mL nalidixic acid (BSAN) and Listeria Selective Agar containing 50 µg/mL nalidixic acid (LSAN) which are the selective media for *Salmonella* spp and *Listeria* spp, respectively. The plates were then incubated at 37˚C for 24 hours and then total viable cell was counted in both non-selective and selective media.

2.8 Statistical Analysis

ZOI were represented as means ± SD where SD means the standard deviation of the values obtained from three to four individual experiments. GraphPad Prism software was used to evaluate the significance among different data by analysis of variance (ANOVA). 5% level of significance was used to denote significant differences in the data.

3. Results

3.1 Antibacterial Effects of Extracted Cinnamon Essential Oil (CEO) and Commercial Cinnamaldehyde (CN)

CEO and CN were applied to 16 test organisms and 8 multidrug resistant isolates (Supplementary Table 1). All the test organisms showed positive response to CEO and CN except *E. coli* 0157:H7 to CEO (Table 1). ZOI ranged from 10.0 mm to 12.0 mm for 5% v/v CEO and 13.0 mm to 20.0 mm for 5% v/v CN. CEO and CN showed highest antibacterial activity against *V. metschnikovii and B. cereus,* providing a ZOI of 12 mm and 20mm respectively. The lowest CN activity was observed against *E. coli* (13 mm ZOI). *E. coli* O157:H7 withstood the antibacterial effect of CEO (no inhibition), but was highly affected by CN (14 mm ZOI). The standard antibiotic (CIP 5µg/disc) used in this study showed higher activity than both CEO and CN, reflecting that higher concentration (>5%) of CN and CEO will be required to achieve similar effect of the standard antibiotic. Although the antibacterial effect of CN was found to be higher than CEO, it is noteworthy that the difference was very low in case of ten test strains (*E. coli, E. coli* DH5α, *S. typhi, S. typhimurium* (ALM 40), *V. cholerae, V. metschnikovii* and *K. pneumoniae, S. aureus, L. monocytogenes and L. grayi*), differing from only 3-5mm ZOI. Also, the ZOI of CN against *V. parahaemolyticus, K. pneumoniae and B. cereus* were found to be very close to that of the standard antibiotic, differing from only 2-3mm. All these findings summarize a satisfactorily good level of antimicrobial activity of extracted cinnamon essential oil and its active ingredient cinnamaldehyde, even at 5% v/v concentration only.

Noteworthy, CN showed positive response against all the multidrug resistant isolates and provided 17 to 34 mm inhibition zone (Table 1). It was evident that CN showed higher antibacterial activity to MDR *Pseudomonas* spp. strains in comparison with sensitive *P. aeruginosa*. The zone diameter in these cases differed from 16mm in case of sensitive one to (17-34 mm) in case of MDR isolates. This gives an insight that possibly the MDR isolates get more sensitive to the natural antibacterial product cinnamaldehyde due to acquiring antibiotic resistance. Also, the extracted CEO was found to be more active against MDR *Pseudomonas* spp. isolate DMC-8b (19 mm ZOI) than sensitive *P. aeruginosa* (10 mm ZOI). Although our extracted CEO did not show sufficient antibacterial activity against six tested strains at 5% v/v concentration, positive antibacterial activity against DMC-8b and BIHS-8 provides an indication that the agent might be effective against MDR isolates at higher concentration.

3.3 Minimum Inhibitory Concentration of CEO and CN

MIC values of CEO against the test organisms ranged from 5% to 0.625% v/v (Table 2) which ensures that a satisfactorily low concentration of this agent is capable of inhibiting the growth of food borne pathogens. CN showed much more promising result compared to CEO. The MIC value of CN ranged from 0.078% to 0.3125% v/v which indicates a very desirable antibacterial activity. *V. cholerae, B. cereus* and *L. grayi*, showed a CN MIC value of 0.078% which is an up to scratch finding. Minimum Bactericidal Concentration (MBC) values of CEO and CN was found to be two fold lower than the respective MIC

values in all the tested cases. The value ranged from 1.25%-10% v/v in case of extracted CEO and 0.3125%-0.625%-v/v in case of commercial CN (Table 2).

Table 1. Antibacterial activities of extracted cinnamon essential oil (CEO) and commercial cinnamaldehyde (CN) against foodborne pathogens and spoilage bacteria.

Organisms	Zones of inhibition (in mm)		
	CEO (5% v/v)	CN (5% v/v)	CIP (5µg/disc)
Escherichia coli	10 ± 0.098	13 ± 0.56	22 ± 1.44
***Escherichia coli* O157:H7**	6 ± 0.0	14 ± 0.28	22.5 ± 0.28
***Escherichia coli* DH5α**	11 ± 0.5	15 ± 0.57	22 ± 0.5
Salmonella typhi	11 ± 0.763	15 ± 0.157	24 ± 1.0
Salmonella typhimurium (ALM 40)	10 ± 0.57	15 ± 0.763	24.5 ± 0.5
Vibrio cholerae	10 ± 0.5	14 ± 0.28	19.5 ± 0.5
Vibrio parahemolyticus	10 ± 0.57	16 ± 0.57	18 ± 0.28
Vibrio metschnikovii	12 ± 0.28	17 ± 0.57	23 ± 0.28
Pseudomonas aeruginosa	10 ± 0.5	16 ± 0.76	26.5 ± 0.5
Klebsiellla pneumoniae	11 ± 0.28	15 ± 0.57	18 ± 0.5
Bacillus cereus	11 ± 0.28	20 ± 0.28	19.5 ± 0.5
Staphylococcus aureus	10 ± 0.00	15 ± 0.5	22 ± 0.28
Staphylococcus aureus ATCC-25923	11 ± 0.28	17 ± 0.76	22.5 ± 0.5
Staphylococcus epidermidis	10 ± 0.28	16 ± 0.28	23 ± 0.57
Listeria monocytogenes	10 ± 0.28	14 ± 0.763	23 ± 0.56
Listeria grayi	12 ± 0.57	16 ± 0.28	27 ± 0.57
DMC-8b	19 ± 0.13	34 ± 0.047	0
BIHS-5	0	18 ± 0.23	0
DMC-6	0	17 ± 0.047	0
DMC-7	0	20 ± 0.047	0
BIHS-7	0	19 ± 0.094	0
DMC-24	0	17 ± 0.4	0
BIHS-8	10 ± 0.205	21 ± 0.082	0
DMC-20b	0	17 ± 0.5	0

The mean diameter of zone of inhibition including the diameter of the disc (6 mm) has been represented as (Mean ± Standard deviation) mm (n=3); *P*<0.05; CIP=Standard antibiotic ciprofloxacin (5µg/disc).

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of extracted cinnamon essential oil (CEO) and commercial cinnamaldehyde (CN).

Organisms	CEO		CN	
	MIC (% v/v)	MBC (% v/v)	MIC (% v/v)	MBC (% v/v)
Escherichia coli	5	10	0.3125	0.625
***Escherichia coli* O157:H7**	5	10	0.3125	0.625
***Escherichia coli* DH5α**	2.5	5	0.3125	0.625
Salmonella typhi	5	10	0.3125	0.625
Salmonella typhimurium (ALM 40)	5	10	0.3125	0.625
Vibrio cholerae	2.5	5	0.078	0.3125
Vibrio parahemolyticus	5	10	0.3125	0.625
Vibrio metschnikovii	0.625	1.25	0.3125	0.625
Pseudomonas aeruginosa	1.25	2.5	0.3125	0.625
Klebsiellla pneumoniae	5	10	0.3125	0.625
Bacillus cereus	1.25	2.5	0.078	0.3125
Staphylococcus aureus	5	10	0.3125	0.625
Staphylococcus aureus ATCC-25923	2.5	5	0.3125	0.625
Staphylococcus epidermidis	5	10	0.3125	0.625

Listeria monocytogenes	2.5	5	0.3125	0.625
Listeria grayi	1.25	2.5	0.078	0.3125

3.4 Effect of heat treatment and pH alteration on the antibacterial activity of CEO

CEO showed positive response after being treated with all the tested temperatures (37^0 C, 50^0 C, 75^0 C, 100^0 C) suggesting that it's activity was not destroyed at high temperatures even after 30 min treatment. An interesting finding was that the antibacterial effect of CEO against all the test strains increased with increasing temperature. The ZOI against the test strains ranged from 9-14mm at 37^0 C, 10-15mm at 50^0 C, 10-16mm at 75^0 C and 18-24mm at 100^0 C of heat treatment (Table 3). Here, it is remarkable that after 100^0 C heat treatment, our extracted CEO showed significantly higher level of antibacterial effect than the commercial CN itself at 37^{0}C. The zones of inhibition provided by CEO at 5% v/v concentration after 100^0 C heat treatment ranged from 18-24mm (Table 3), whereas the inhibition zone of CN at 5% concentration against the respective strains ranged from 13-15mm (Table 1).

Potential antibacterial activity of CEO was observed at all the tested pH condition (pH 2, 7 and 9) indicating a broad range of pH sustainability of this agent. Due to the alteration of pH, slight increase or decrease in the activity of CEO was noticed which also varied with different organisms. Highest activity was found in case of *Salmonella typhi* at pH 2 and pH 9. Lowest activity was observed against *S. typhimurium* (ALM 40) at pH 9. The ZOIs of CEO against six tested strains ranged from 17-19mm at pH 2, 14-19mm at pH 7 and 13-19mm at pH 9 (Table 3). Overall, acidic pH was found to be more favorable for exerting higher antibacterial effect, though the antibacterial activity was not diminished due to pH change.

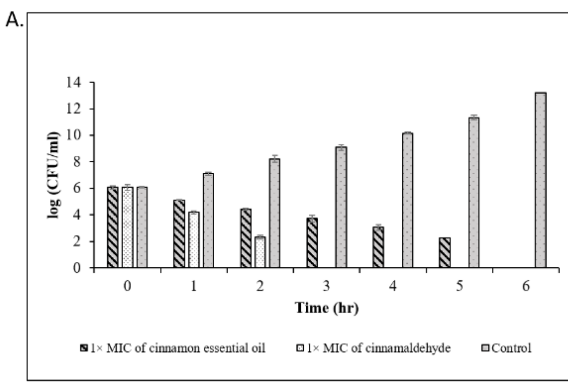
3.5 Kinetic destruction pattern of CEO and CN

S. typhimurium (ALM 40) and *L. monocytogenes* were challenged with extracted CEO and commercial CN at 1xMIC concentration to investigate whether the growth inhibiting effect of these antimicrobial agents is attributable to bacteriostatic or bactericidal activity with required time course. Bacterial viability was checked at every one-hour time points during incubation and viable cells were counted. In the absence of the CEO and CN, viable cells of *Salmonella typhi* and *Listeria monocytogenes* increased from 6.07 to 13.21 log CFU/ml and 6.96 to 10.15 log CFU/ml, respectively after 6hrs of incubation (Fig. 1). Significant decrease in the viable cells were observed in the presence of CEO and CN. Corroborating to the previous findings, CN came up with more desirable outputs in comparison with CEO. Within 3hr of incubation of *S. typhimurium* (ALM 40) with CN, complete eradication of the viable cells (6.07 log CFU/ml reduction in viable count) was observed, whereas CEO required 6hrs to exert the complete bactericidal effect. CEO and CN reduced the viable count of *L. monocytogenes* (from 6.96 log CFU/ml to 0) within 4hrs and 3hrs of exposure, respectively. However, the killing rate was found to be exponential in both cases ensuring both CEO and CN to be bactericidal at 1xMIC concentration.

Table 3. Effect of heat treatment and pH alteration on the antibacterial activity of extracted cinnamon essential oil (CEO).

Test organisms	Effect of Temperature (Zone of inhibition in mm)				Effect of pH (Zone of inhibition in mm)		
	37^0 C	**50^0 C**	**75^0 C**	**100^0 C**	**pH 2**	**pH 7**	**pH 9**
Escherichia coli	12 ± 0.58	13 ± 0.2	13 ± 0.08	18 ± 0.58	17 ± 0.5	17 ± 0.67	15 ± 0.3
***Escherichia coli* O157:H7**	ND	ND	ND	ND	17 ± 0.2	14 ± 0.3	17 ± 0.1
Salmonella typhi	10 ±0.6	10 ± 0.6	10 ± 0.2	20 ± 0.2	19 ± 0.44	15 ± 0.1	19 ± 0.03
***Salmonella typhimurium* (ALM 40)**	14 ± 0.08	15 ± 0.08	16 ± 0.58	24 ± 0.08	17 ± 0.4	18 ± 0.27	13 ± 0.15
Vibrio cholerae	9 ± 0.08	10 ± 0.58	11 ± 0.6	19 ± 0.58	ND	ND	ND
Klebsiella pneumoniae	9 ± 0.2	10 ± 0.2	12 ± 0.58	20 ± 0.08	ND	ND	ND
Staphylococcus aureus	ND	ND	ND	ND	18	19	19
Listeria monocytogenes	12 ± 0.58	14 ± 0.58	15± 0.08	22 ± 0.6	19	19	17

Mean diameter of zone of inhibition including the diameter of the disc (6 mm) has been represented as (Mean ± Standard deviation) mm (n=3); $P < 0.05$. ND: Not determined.

A.


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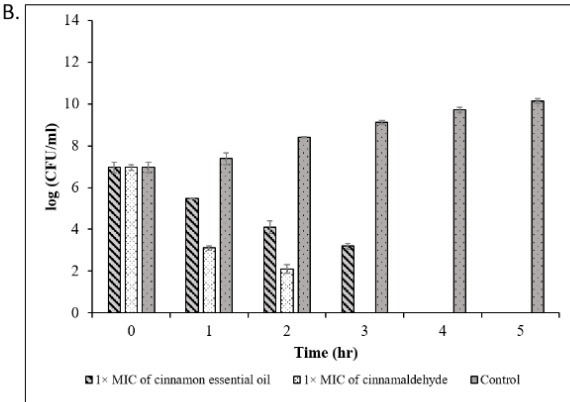

Figure 1. Kinetic destruction pattern of cinnamon essential oil (CEO) and cinnmaldehyde (CN) on the growth of *Salmonella typhimurium* (ALM 40) **(A)** and *Listeria monocytogenes* **(B)**. 1xMIC concentration of the agents were applied to observe viable cells during incubation at 37 °C. The results are represented as log CFU/ml where the counts are mean values ± standard deviation of three independent experiments.

3.6 Inactivation dynamics of *S. typhimurium* (ALM 40) inoculated in ground chicken meat due to the treatment with CN

Salmonella typhimurium (ALM 40) inoculated ground chicken meat was challenged by 3xMIC and 5xMIC concentration of commercial CN to inactivate the organism in meat when stored at 4°C and -20°C for 14 days. After artificially inoculating the meat sample with the test organisms, the viable count was 8.85 log CFU/gm in TSAN and 8.44 log CFU/gm in BSAN (Fig. 2). The viable count decreased rapidly in both cases after treatment with CN. While stored at 4⁰ C for one day, meat samples treated with 3xMIC concentration of CN showed a reduction of viable cell counts by 4.68 log CFU/gm in nonselective TSAN medium. 14 days storage of the sample in the same condition showed a decrease of viable count by 5.03 log CFU/gm in TSAN medium. After 14 days of storage in this condition, the final cell count was found 3.82 log CFU/gm in TSAN medium (Fig. 2A). However, after treatment with 5xMIC concentration of CN, *S. typhimurium* (ALM 40) count in TSAN decreased up to 4.94 log CFU/gm in one day while stored at 4⁰ C and thereafter a continual slight reduction was noticed through the entire storage period. Finally, the cell count was noticed to be 3.81 log CFU/gm in TSAN medium (Fig. 2B). In contrary, the microbial count in untreated control samples were reduced only (0.02-0.09) log CFU/gm after 14 days of storage. Here, an interesting finding was that no viable cell was recovered in the selective BSAN medium inoculated with CN treated meat samples just after one day of storage at 4⁰ C.

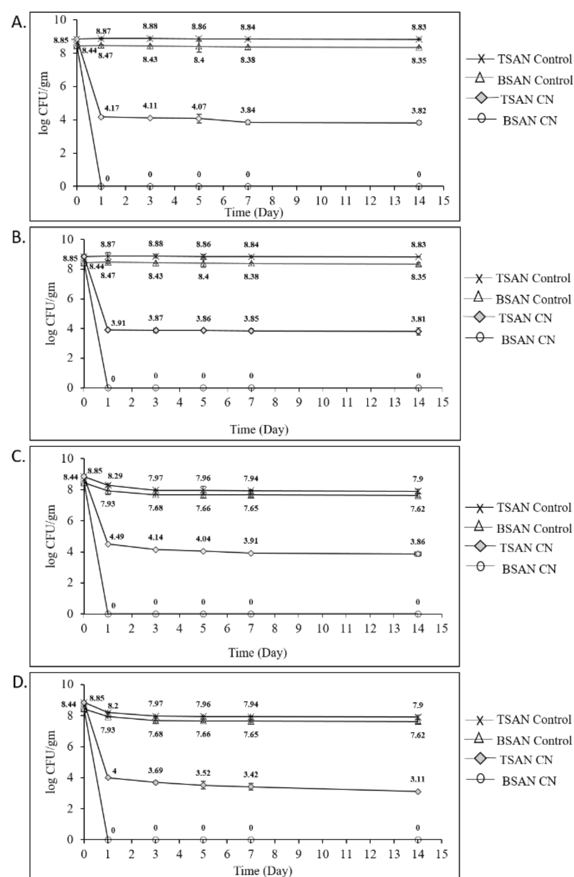

Figure 2. Inactivation dynamics of *Salmonella typhimurium* (ALM 40) in ground chicken meat treated with cinnamaldehyde (CN). A. 3xMIC concentration of CN and stored at 4⁰C. **B.** 5xMIC concentration of CN and stored at 4⁰C. **C.** 3xMIC concentration of CN and stored at -20⁰ C. **D.** 5xMIC concentration of CN and stored at -20⁰ C. The results are represented as log CFU/gm where the counts are mean values ± standard deviation of three independent experiments.

After one day of storage of the CN treated meat sample at -20⁰ C, the viable count in TSAN medium reduced by 4.36 and 4.85 log CFU/gm due to the exposure of 3xMIC and 5xMIC concentration of CN, respectively (Fig. 2 C and 2D). After 14 days of exposure in the mentioned condition, viable cell count in TSAN was reduced to 3.86 and 3.11 log CFU/ml, in case of 3xMIC and 5xMIC concentration of CN, respectively. Complete eradication of the test organisms was observed in BSAN medium after 1 day of exposure with each concentration. In contrast, untreated control samples showed only a

reduction of (0.82-0.95) log CFU/gm after the entire storage period.

The comparative study of the obtained results suggested that the activity of CN in meat sample is slightly dose-dependent. Microbial reduction by 3xMIC and 5xMIC concentration of CN differed only from 0.26 and 0.49 log CFU/gm in TSAN medium while stored at 4^0 C and -20^0 C, respectively (Fig. 2). But no difference between the efficacies of these two concentration was noticed while cultured the samples in selective BSAN medium. Both of the concentrations were able to cause great damage of the microbial cell and the cells could not recover while plated on BSAN medium, thus giving zero cell count in BSAN medium just after one day of storage. We also observed that storage condition does not affect much on the efficacy of CN. During storage at 4^0 C and -20^0 C, the viable cell reduction differed only (0.32-0.09) log CFU/gm. This insignificant difference can be explained by the slightly higher reduction of microbial cells in control samples while stored at -20^0 C. This finding suggest that food treated with CN can be stored equally good at both 4^0 C and -20^0 C refrigerator. There was rapid reduction of cell count in CN treated meat samples just after one day of storage and no further increase of the count was noticed when the storage period was extended to 14 days. The finding supports that prolonged storage period also does not hamper the efficacy of CN.

In this study, we used both nonselective TSAN and selective BSAN medium for the enumeration of *S. typhimurium* (ALM 40) on treated and untreated meat. Here, we found that, regardless of the CN concentration or storage condition, higher number of microbial population recovered on TSAN medium than on BSAN. Microbial counts ranged from (3.91-4.49) log CFU/gm higher due to the inoculation of treated meat samples in TSAN compared to inoculated on BSAN.

Overall, CN treated meat samples showed significant decrease in *S. typhimurium* (ALM 40) count in comparison with untreated one. Also, the viable count in selective BSAN medium reduced from 8.44 to 0 log CFU/ml just after one day of storage which ensures a highly reliable level of pathogen eradication in preserved meat.

3.7 Inactivation dynamics of *L. monocytogenes* inoculated in ground chicken meat due to the treatment with CEO

Ground chicken meat inoculated with *L. monocytogenes* was challenged by 3xMIC and 5xMIC concentration of extracted CEO and stored at 4ºC and -20ºC for 14 days. Initial viable count was 7.73 and 7.35 log CFU/gm in TSAN and LSAN, respectively (Fig. 3). Untreated samples exhibited a growth increase of 0.45 and 0.75 log CFU/gm during storage at 4ºC and -20ºC, respectively. One day of storage at 4^0C with 3xMIC and 5XMIC of CEO resulted in the reduction of 2.89-3.66 log CFU/gm viable count in TSAN medium (Fig. 3A and 3B). While stored at -20^0C, 3.14-3.62 log CFU/gm reduction was observed in TSAN due to 3xMIC and 5XMIC of CEO (Fig. 3C and 3D). 14-day storage at 4^0C reduced 4.73 and 4.23 log CFU/gm in TSAN due to 3xMIC and 5XMIC of CEO, while storage at -20ºC reduced 3.93 and 4.1 log CFU/gm, respectively. Storage condition (4^0C and -20^0C) affected only slightly (0.23 to 0.13 log CFU/gm) to the recovery rate of microorganisms in TSAN. Inactivation of *L. monocytgenes* in meat sample was found to be slightly dose-dependent, although the difference (0.17-0.77 log CFU/gm) was non-significant. Prolonged storage did not diminish the antimicrobial potential of CEO. No viable growth of *Listeria* was observed in selective LSAN media due to CEO treatment just after one day of storage. Both 3xMIC and 5xMIC of CEO was equally effective against *L. monocytogenes* inoculated meat samples stored at 4^0C and -20ºC which was confirmed by complete inhibition of *Listeria* growth in LSAN.

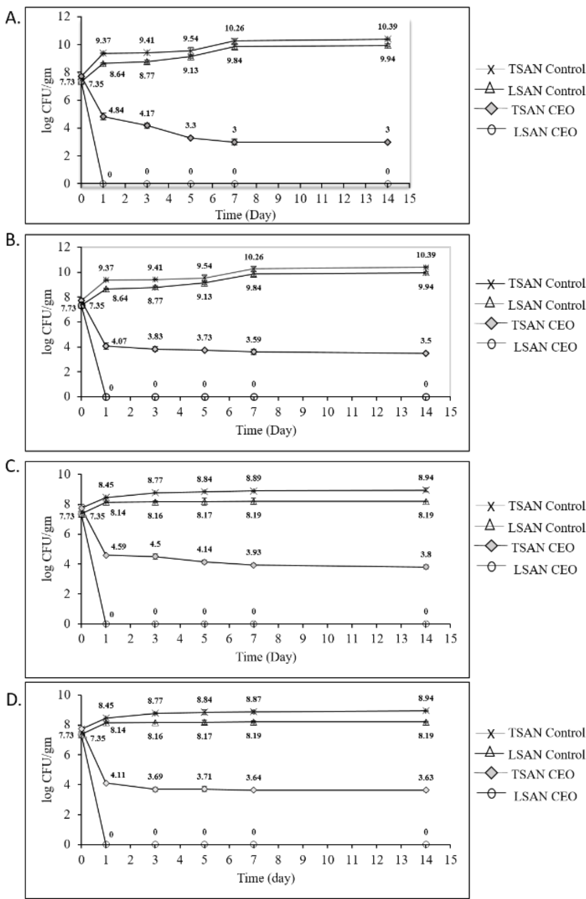

Figure 3. Inactivation dynamics of *Listeria monocytogenes* in ground chicken meat treated with cinnamon essential oil (CEO). A. 3xMIC concentration of CEO and stored at 4^0C. **B.** 5xMIC concentration of CEO and stored at 4^0C. **C.** 3xMIC concentration of CEO and stored at -20^0 C. **D.** 5xMIC concentration of CEO and stored at -20^0 C. The results are represented as log CFU/gm where the counts are mean values ± standard deviation of three independent experiments.

4. Discussion

Food borne pathogens are a threatening medical concern around the world nowadays, calling for progressively powerful food preservation methodologies. Negative concerns of the consumers toward synthetic or chemical food preservatives boost the demand for an alternative natural antimicrobial which leads to the notion of utilizing essential oils as food preservative. Several previous studies have archived the antimicrobial properties of different essential oils **(28, 29)**. The current study examined the antibacterial properties of extracted cinnamon essential oil (CEO) and commercial cinnamaldehyde (CN) against 16 food borne pathogens and 8 multidrug resistant *Pseudomonas* spp.

The results presented in Table 1 showed that both of the compounds under investigation exhibited significant antibacterial activity as evidenced by their zones of inhibition. *Vibrio metschnikovii* and *Listeria grayi* was found to be the most sensitive while *E. coli* and *S. aureus* to be the least. CN showed higher antibacterial activity than CEO against all the isolates possibly because of being a highly purified commercial product. Despite the fact that our extracted CEO did not undergo high level of purification like the commercial CN, still it demonstrated nearly equal antibacterial effect like CN (differing from only 3-5mm zone of inhibition) in case of ten test isolates (Table 1). These findings indicate that CEO can also offer expected level of antibacterial effect as its effectiveness is mostly attributed to its major ingredient cinnamaldehyde as previously stated **(30)**. Although the standard antibiotic (CIP 5µg/disc) showed higher activity than 5% CEO and CN, it is prudent to employ this natural product in exchange of antibiotics to combat the ever increasing antibiotic resistance of pathogenic microbes, which is a global concern in this century. Moreover, any antibacterial formulation carrying plant essential oils is expected to be much cheaper than the highly expensive antibiotics. CN showed high level of antibacterial activity against all the MDR isolates used in our study. CEO also showed antibacterial effect against two MDR isolates. The findings corroborated with Utchariyakiat et al. stating cinnamon bark oil and cinnamaldehyde to have strong antibacterial potential against MDR-PA isolates with MIC of 0.0562–0.225 % v/v and MBC of 0.1125–1.8 % v/v **(31)**. Moreover, they claimed that the combination therapy of cinnamon bark oil and cinnamaldehyde with colistin can be a beneficial alternative to antibiotic therapy. Noteworthy, growth inhibition of MDR *Pseudomonas spp.* by CN was higher than the sensitive *P. aeruginosa* (Table 1), which gives an insight that possibly the MDR isolates get more sensitive to the natural antibacterial product due to acquiring antibiotic resistance. Another previous finding reported that growth inhibition of MDR *K. pneumoniae* by natural compounds (extracts of Chamomile, Aquatic Pennyroyal and Nettle plants) was higher than seven different antibiotics **(32)**.

Results of MIC determination were likewise consistent with the antibacterial sensitivity profile indicating CN to possess higher antibacterial activity than CEO. MIC value of CEO ranged from 0.625% to 5% v/v (MBC 1.25% to 10% v/v) and CN ranged from 0.078% to 0.3125% v/v (MBC 0.3125% to 0.625% v/v). Several earlier studies reported the MIC values of cinnamaldehyde and their extracted cinnamon EO against food borne pathogens. Hili and colleagues have extracted the essential oil from *Cinnamomum zeylanicum* leaf oil and assayed its antimicrobial activity against three bacteria and four yeast strains. Their results showed that the leaf oil completely inhibited the growth of *E. coli*, *S. aureus*, and *P. aeruginosa* at the level of 500 µg/mL which is consistent with our findings **(33)**. Chang et al. showed that MIC of *C. osmophloeum* essential leaf oil was 500 µg/mL against *K. pneumoniae; Salmonella* spp. and 250 µg/mL against the other isolates (*E. coli, P. aeruginosa, V. parahemolyticus, E. faecalis, S. aureus, S. epidermidis*, and methicillin-resistant *S. aureus*) **(34)**. Our extracted CEO had a MIC value of 0.625% to 5%v/v (78.8-630.36 µg/mL) against the test isolates, which was consistent with other previous studies reporting MICs of comparable ingredients ranging from 75 µg/mL to 600 µg/mL **(30)**. This correspondence with earlier research confirms CEO's projected effectiveness against food-borne pathogens. CEO and CN both manifested similar bactericidal activity against both gram positive and gram negative microorganisms which indicates their broad spectrum applicability.

Heat treatment of CEO with various temperatures (37^{0}C, 50^{0}C, 75^{0}C, 100^{0}C) for 30 min revealed that the antibacterial effect of CEO was not destroyed by increasing temperature, rather the activity increased (Table 3). Hoque et al. also described similar findings (21). Highest antimicrobial activity of cinnamon at 60^{0}C was reported previously, although the antibacterial activity was not fully diminished due to increased heat treatment **(35)**. Strikingly, after 100^{0}C heat treatment, our extracted CEO showed significantly stronger antibacterial effect than the commercial CN itself (Table 3 and Table 1). This provides an indication of the heat induced activation of the active ingredient of cinnamon essential oil due to the destruction of interfering components present in extracted CEO. This finding implies that our extracted CEO (after 100˚C heat treatment) can be a reasonable alternative to commercial cinnamaldehyde, thereby reducing the additional cost of extracting cinnamaldehyde commercially from

cinnamon essential oil. Furthermore, due to CEO's great temperature tolerance, the oil can retain its antimicrobial activity at high temperatures of cooking and so can be employed as potential preservatives for the foods processed at high temperatures. Similar to heat treatment, pH changes had no effect on CEO's effectivenessand the activity was not diminished at various pH (pH 2, 7 and 9). In some cases, a little increase or decrease of activity was observed that also varied with the isolates. Various pH tolerance of CEO indicated the oil to be compatible for preserving both acidic and alkaline foods.

The results of time killing assay against *S. typhimurium* (ALM 40) showed that bacterial cells numbered 6.07 log CFU/mL were being killed within 6hrs and 3hrs after treatment with 1xMIC of CEO and CN, respectively. Senhaji *et al.* showed the kinetic destruction pattern of essential oil from *Cinnamomum zeylanicum* on the growth of *E. coli* O157:H7. At 0.05% concentration, the agent showed bactericidal effect after 30 min of incubation **(36)**. Time-killing graphs demonstrated by Utchariyakiat et al. showed the reduction of viable cells of *P. aeruginosa* PAO1 approximately 6 log CFU/mL after being treated with cinnamaldehyde and cinnamon bark oil for 1.3 and 2 h, respectively **(31)**. This difference in time killing graph obtained by our study and the mentioned others can be explained by the extensive antimicrobial resistance and heat tolerance of *S. typhimurium* **26, 37, 38)** which also supports *S. typhimurium* to be capable of tolerating essential oil treatment for longer period of time than other bacteria. Kinetics of *L. monocytogenes* destruction was faster (3hrs and 4hrs due to 1xMIC of CN and CEO) than *S. typhimurium*. Paudel and co-workers also reported higher log reduction of *L. monocytogenes* than *Salmonella spp.* in melon model **(39)**, thus reflecting the later one to be more antimicrobial resistant. 2x MIC and 4x MIC of ethanolic clove and negro pepper extracts was required to destruct 3 log units of *Listeria* spp. as obtained by previous study **(29)**. Our finding validates cinnamon to be more effective against *Listeria* than clove and negro pepper. Nozohour et al. also supported that trans-cinnamaldehyde is more effective against *Streptococcus equi* than other essential oil main components (1, 8 Cineole and Pulegone) **(40)**.

Being rich in essential nutrients, meat serves as a suitable source of microbial contamination as well as foodborne diseases. This is the reason to choose meat as our experimental model for studying bacterial inactivation dynamics using CEO and CN. Following the inactivation test, we observed that CEO and CN functioned in slightly dose-dependent manner which matches previous findings **(23, 31)**. We also noticed that neither the storage condition nor the storage time had a significant impact on the activity of the antimicrobials, ensuring that the agents may be used at various storage conditions and also for a prolonged period of time. However, in all cases of the test, complete reduction of viable counts was observed just after 1 day of treatment with both 3xMIC and 5xMIC of CN while cultured on selective BSAN medium. In agreement with our study, Yossa et al. showed up to six log reductions in the viable count of *S. typhimurium* in 2% cinnamaldehyde treated organic soil **(23)** and Al-Nabulsi et al. reported reduction of 2.87, 2.64 or 2.35 log10 CFU/ml in *Salmonella spp.* count due to 2.0% Cinnamon oil treatment at tahini stored at 37, 25 or 10^0C, respectively **(41)**. According to a prior study **(20)**, crude cinnamon EOs at 2.5% and 5% were not shown to be effective against *L. monocytogenes*. However, when the concentration was raised to 7.5% and 12.5% (3xMIC and 5xMIC of CEO) in our study, we observed inactivation of this species. The mentioned study concluded that clove EOs to be superior to cinnamon EOs for *Listeria* inactivation in ground beef, while we report increased concentration of CEO and only minute concentration of CN to be highly effective in preserving ground chicken meat.

A discrepancy between the count in nonselective TSAN and selective BSAN medium was observed in our study which might be related to the recovery of some injured cells in the nonselective medium. But the bacterial count in TSAN were also reduced sufficiently just after 1 day and remained in static phase during the whole preservation period. Though the nonselective medium facilitated the growth of some injured cells, it is unlikely that these cells can endure the harsh environment of human stomach and gut. And thus, this injured cells should carry a little risk to human health. Pointedly, CEO and CN treated samples showed significantly higher reduction of microbial cells than untreated ones in all cases.

Multiple previous studies reported on the inactivation of food pathogenes using plant extracts and essential oils. 2.38 and 2.78 logarithmic reductions of *S. typhimurium* on tomatoes treated with 4% sumac extract and 100 ppm oregano (respectively) were reported by Gündüz *et al.* **(42)**. The team of Vallejo *et al.* demonstrated that strawberry polyphenols can effectively inactivate *Listeria monocytogenes* and *Salmonella typhimurium* in strawberry juice **(43)**. Tassou *et al.* showed that 1%, 1.5% and 2% v/w concentrations of mint (*Mentha piperita*) essential oil killed *S. enteritidis* in tzatziki after being stored at 4°C for 6, 4 and 3 days, respectively **(28)**. Alongside, cinnamaldehyde and cinnamon oil is widely reported to be effective in inactivating *Listeria monocytogenes*, *E. coli*, *Vibrio parahaemolyticus* and many other bacterial strains in a variety of food models **(19-22)**. Considering all of the

previous findings, we can also claim CEO and CN to be a potential inactivating agent against *S. typhimurium and L. monocytogenes*, lending credence to their prospective application as a natural antimicrobial for meat preservation.

5. Conclusion

The findings of current study in agreement with preceeding data suggest that both CEO and CN are potential antimicrobial agents against food borne pathogens and can be implemented as a promising natural meat preservative. Considering overall cost-to-benefit ratio, extracted cinnamon essential oil can offer a rational alternative to commercial cinnamaldehyde.

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Conflict of Interest

Tohura Ahmed Suchi and Israt Dilruba Mishu contributed equally and would be recognized as co-first authors.

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