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Anti-biofilm effect of phytochemicals on Entero Haemorrhagic *Escherichia coli* biofilm

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Abstract

The study was designed to investigate the biofilm forming capacity of Enterohaemorrhagic *Escherichia coli* (EHEC) isolates and the effect of phytochemicals *viz*., trans-cinnamaldehyde and eugenol in reducing the growth of EHEC on meat contact surfaces. A total of 40 EHEC isolates confirmed from 132 *E. coli* recovered from meat contact surfaces such as cutting and knife were used as the study material. To study the antibiofilm effect of phytochemicals, EHEC biofilms were artificially created on fiber and wooden boards and tested against two concentrations of each of trans-cinnamaldehdye and eugenol extracts respectively. Out of the 40 EHEC isolates only two isolates recovered from wodden cutting board had shown biofilm forming ability. Further, minimum inhibitory concentration (MIC) of phytochemicals was determined against biofilm producing EHEC and the MIC for trans-cinnamaldehyde and eugenol was found to be 3.5 per cent and 5.5 per cent respectively. A concentration of 3.5 per cent of trans-cinnamaldehyde and 5.5 per cent of eugenol showed reduced growth of EHEC biofilm whereas concentrations of 4.0 per cent of trans-cinnamaldehyde and 6.0 per cent of eugenol completely inhibited biofilm forming EHEC. The study demonstrated that cinnamaldehyde and eugenol as promising phytochemicals against EHEC biofilms. However, in order to apply these phytochemicals further studies are required to ascertain their toxicity and potential side effects against mammalian cells.

Keywords: Enterohaemorrhagic *Escherichia coli*, meat contact surfaces, trans-cinnamaldehyde, eugenol, biofilm, antibiofilm

Introduction

Apart from occurrence and lethal syndrome, Enterohaemorrhagic *Escherichia coli* (EHEC) possess an ability to colonise and form biofilms both on biotic and abiotic surfaces. Biofilms could be described as group of organisms enclosed in a self-produced extracellular polymeric matrix which is attached to a hydrophobic surface. The formation of biofilms and subsequent encasement of bacterial cells in a complex matrix can enhance resistance to antimicrobials and sterilizing agents making these organisms difficult to eradicate and control (Lajhar et al., 2018). Further the biofilms helps the organism to evade the effect of sanitizers and disinfectants in a food processing industry. These biofilms also acts as a source of recontamination during food processing (Kot et al., 2015). However, there is limited information regarding the composition of biofilm matrix in spite of the EHEC serotypes continue to gain importance as pathogen of concern (Crim et al., 2015). Nowadays, large number of chemical sanitizers are used at higher doses in order to prevent the attachment of microbes on surfaces and subsequent biofilm formation. This has resulted in increased antimicrobial resistance of the biofilm flora and the

control of which has become a matter of concern. But concerns over the use of these chemicals have led to the use of natural substances which are generally regarded as safe.

Low-molecular-weight phenolic compounds are among the most typical components of essential oils with antimicrobial activity. They could have an activating or inhibiting effect on microbial growth according to their structure and concentration (Galvao et al., 2012). Moreover the antimicrobial residues and consumers demand for least processed food leads to the search of naturally occurring edible antimicrobials or phytochemicals.

The trans-cinnamaldehyde and eugenol are one of the naturally occurring compounds, which are derived from bark of cinnamon trees and clove oil respectively. It produces antimicrobial and anti-biofilm properties by forming a coating over the site of application. Even though EHEC is a widely studied organism there is dearth of information regarding its biofilm forming ability on meat contact surfaces. Also there is only limited data available on anti-microbial and antibiofilm effect of phytochemicals *viz*., trans-cinnamaldehyde and eugenol on EHEC. By considering all these factors the present study was undertaken.

Materials and Methods

Bacterial strain and growth condition

Out of 132 *E. coli* isolates recovered from meat contact surfaces *viz.*, cutting board and knife from retail meat shops, a total of 40 EHEC isolates were confirmed as per procedure described by Meng et al.,(2001), formed the material for the present study. The standard culture was maintained in Brain Heart Infusion (BHI) broth with one per cent glycerol by sub culturing at regular intervals and periodically tested for their purity, morphological and biochemical characteristics.

Surface preparation

Two types of surfaces, wooden board and fiber were used as matrices for artificial creation of biofilm. Fiber and wooden board were cut in the form of flat coupons of size 3.7 ×1.8 cm. Coupons were first cleaned with 99 per cent acetone and washed with distilled water and cleaned with 70 per cent ethanol. After that coupons were washed with sterile distilled water and autoclaved at 121ºC for 15 min. Coupons were dried by keeping at 60ºC for about 1 h.

EHEC biofilm formation on test surfaces

From the overnight inoculated culture of EHEC one milli litre was spread on wooden and fiber coupons each. Coupons were kept for incubation at 37ºC for 24 h. After incubation, coupons were aseptically transferred to a sterile test tube, washed three times with physiological saline solution to remove loosely attached cells. Serial dilution was done with normal saline solution. The bacterial count was enumerated by standard spread plate technique.

Biofilm production assay for EHEC

Biofilm production in terms of slime production by isolates was determined by cultivation on Congo Red Agar (CRA) plates (Dadawala et al., 2010). A loop full of isolate was streaked onto CRA plates. The plates were incubated at 37°C for 24 h. to 48 h. The production of rough black colonies by bacterial cultures indicated the ability of the isolates to produce biofilm.

Minimum inhibitory concentration of transcinnamaldehyde and eugenol

Trans–cinnamaldehyde and eugenol was purchased from Sigma – Aldrich Chemical Pvt Limited, Banglore, having more than 84.72 per cent deacetylation, ash 0.45 per cent, moisture 8.35 per cent, pH 7.4 and mesh size 24. The MIC of phytochemicals *viz*., 95 per cent of trans-cinnamaldehyde and 98 per cent of eugenol were estimated using disk diffusion method (Gutierrez-Larrainzar et al., 2012). The different concentrations of both were prepared using dimethyl sulfoxide. Stock solution of trans-cinnamaldehyde and eugenol was prepared by mixing 1.05 ml and 1.02 ml into 9.95 ml and 9.98 ml DMSO respectively. From the stock solution the working solution of each was prepared.

A filter paper discs (5 mm in diameter) impregnated with the extracts were placed on test organism-seeded plates. 10µl plant extracts were added into each filter paper disc and Streptomycin sulphate (10 µg /disc) were used as positive control and methanol solvent (100 µg / ml) were used as negative control. The antibacterial assay plates were incubated at 37°C for 24h. The diameters of the inhibition zones were measured in mm.

Effect of phytochemicals *viz*., trans-cinnamaldehyde and eugenol in reducing the growth of EHEC on meat contact surfaces

Three or four colonies of each EHEC isolates were selected from pure culture and transferred into sterile nutrient broth and incubated at 37ºC for 24 h. The optical density value of the culture was adjusted to10^5 cfu/ml and smeared onto meat contact surfaces (wood). The wood was treated with two concentrations of phytochemicals *viz*., trans-cinnamaldehyde and eugenol for a contact period of five min. Finally, the wood was washed with normal physiological saline solution and 100 µl of aliquot was spread onto MacConkey Sorbitol Agar w/ Cefixime and Tellurite (CT-SMAC) plates. A control wood without treatment with trans-cinnamaldehyde and eugenol were also kept to compare the effect. Enumeration of cells from both control and treatment groups was done in order to compare the effect of both the phytochemicals.

Effect of tran–cinnamaldehyde and eugenol on EHEC biofilm

For biofilm assays, a one per cent (w/v) trans–cinnamaldehyde and eugenol solution was prepared in DMSO. From this solution, 3.5 and 4.0 per cent of trans–cinnamaldehyde and 5.5 and 6.0 per cent of eugenol solutions were prepared separately. Sterilized fiber and wooden board coupons were immersed in one, 3.5 and 4 per cent of trans–cinnamaldehyde and 5.5 and 6.0 per cent of eugenol for 3 h and dried in a laminar air flow cabinet in order to make a coating over these surfaces. Trans–cinnamaldehyde and eugenol coated coupons were then inoculated with one ml of TSB containing bacterial culture and incubation was done at 37^0C for 24 h. Control coupons without trans–cinnamaldehyde and eugenol coating were also kept to compare the effect. After the incubation period, coupons were washed three times with physiological saline and EHEC cells attached to each surface was determined.

Enumeration of biofilm cells

Enumeration of biofilm cells on artificial matrices was done by the method described by Deka(2014) with necessary modifications. The test tubes which contained 10 ml of sterile physiological saline and biofilm attached coupons were vortexed for two min at low intensity in order to detach the biofilm cells. Washing was repeated thrice and serial dilutions were performed in order to separate the detached cells. The bacterial count was enumerated by standard spread plate technique. After serial dilutions, 100 µl aliquot with an appropriate dilution was plated on CT-SMAC agar plates and incubated at 37^0C for 24 h. The biofilm cell mass was determined for treatment and control groups and count was expressed as $\log_{10}$ cfu of the coupon surface area.

Statistical analysis

The data obtained were subjected to statistical analysis using the SPSS version 24.0. In the present study to compare the effect of trans–cinnamaldehyde and eugenol on EHEC biofilms Mann Whitney U test was used. This test compares the efficiency of treatments with trans–cinnamaldehyde and eugenol on EHEC.

Results

Biofilm forming ability of EHEC isolates

The biofilm forming ability of EHEC isolates was studied using congo red assay (CRA). An overnight grown culture in nutrient broth was streaked onto CRA plates and kept for incubation at 37ºC for 24 to 48 h. Biofilm forming ability of EHEC isolates were confirmed by presence of rough black colour colonies whereas red colour colonies were considered as negative. In the present study two isolates (5%) out of 40 EHEC isolates showed biofilm forming ability (Table.1).

Table. 1. Biofilm positive EHEC isolates from retail meat shops

	Sources	EHEC Biofilm formation-retail meat shops(RS)				Overall occurrence	
		RS1	RS2	RS3	RS4	No.	%
1.	Cutting board	0	2	0	0	2	5
2.	Knife	0	0	0	0	0	0
Total		0	2	0	0	2	5

Effect of phytochemicals *viz*., trans-cinnamaldehyde and eugenol in reducing the growth of EHEC on meat contact surfaces

The minimum inhibitory concentration of phtochemicals was studied by disk diffusion method. The effect of phytochemicals was confirmed by zone of inhibition at each concentration. Minimum inhibitory concentration was obtained at 3.5 per cent level and 5.5 per cent level of trans-cinnamaldehyde and eugenol with a zone of inhibition at 10.67 ± 1.20 mm for trans-cinnamaldehyde and 7.67 ± 0.33 mm for eugenol, respectively. The zone of inhibition was increased with higher concentration for both phytochemicals as shown in Table 2 which pointed out the MIC was found to be at 3.5 per cent and 5.5 per cent concentration of trans-cinnamaldehyde and eugenol, respectively. Further investigation regarding the effect of phytochemicals on wood, after the treatment of 3.5 per cent concentration of trans-cinnamaldehyde, there was reduction of the EHEC organisms on wood up to 0.6666 ± 0.6666 mean $\log_{10}$ cfu which was statistically significant different when compared to control of 5.4255 ± 0.2010 mean $\log_{10}$ cfu whereas at a concentration of 4.0 per cent of trans-cinnamaldehyde and 5.5 per cent of eugenol, there was complete elimination of the EHEC organism from meat contact surfaces which showed significant difference from that of control (Table.3).

Table 2. Minimum inhibitory concentration (MIC) of trans-cinnamaldehyde and eugenol

Sl. No	Treatment	Zone of inhibition (mm)			
		Concentrations (%)			
		3.5	4.0	4.5	5.0
1.	Transcinnamaldehyde	10.67 ±1.20	27.33 ± 2.72	35.67 ±2.34	39 ± 1.15
2.	Eugenol	7.67 ± 0.33	18.33 ± 1.45	25.33 ± 1.86	30.33 ± 0.88

Table 3. Mean log count of Enterohaemorrhagic *E. coli* coupons treated with trans-cinnamaldehyde and eugenol

Sl. No.	Treatment	Concentrations (%)	EHEC Mean ± SE log10 cfu/ml
1.	Control	0	5.4255 ± 0.2010a
2.	Transcinnamaldehyde	3.5	0.6666 ± 0.6666b
		4.0	0 ± 0b
3.	Eugenol	5.5	0 ± 0b
		6.0	0 ± 0b

Figures bearing different superscript differ significantly.

Anti-biofilm effect of phytochemicals on EHEC biofilm

To study the anti-biofilm effect of phytochemicals on EHEC biofilm, the biofilm were produced on two artificial matrices *viz*., wooden and fibre using the EHEC isolates obtained from different retail meat shops. The effect of trans-cinnamaldehyde and eugenol on EHEC biofilm was studied by determining the count of EHEC cells attached to phytochemical coated surfaces. Comparative analysis was done along with control group which recorded 3.5 per cent of trans-cinnamaldehyde showed significant reduction in EHEC count in both the coupons with 3.5143 ± 0.1296 mean $\log_{10}$ cfu in wooden coupons and 3.9482 ± 0.3837 mean $\log_{10}$ cfu in fiber coupons (Table 4). There was complete elimination of EHEC at 4.0 per cent level from both the coupons. There was reduction in EHEC count at 5.5 per cent level of eugenol in both the coupons with 3.4782 ± 0.1126 mean $\log_{10}$ cfu and 2.2447 ± 1.1236 mean $\log_{10}$ cfu in wooden and fiber coupons (Table 4). Complete elimination of EHEC was recorded at 6.0 per cent level of eugenol in both the coupons.

Up on statistical analysis by Mann Whitney U test, there was significant difference in both plant extract in the specified percentages on wooden and fibre.

Table 4. Mean log count of 24 h. Enterohaemorrhagic *E. coli* biofilm cells treated with trans-cinnamaldehyde and eugenol

Sl. No.	Treatment	Concentration (%)	Mean log10 cfu ± SE	
			Wodden	Fibre
1.	Control		4.7499 ± 0.0550^{a}	4.7139 ± 0.2214^{a}
2.	Trans-cinnamaldehyde	3.5	3.5143 ± 0.1296^{b}	3.9482 ± 0.3837^{b}
		4.0	0^{b}	0^{b}
3.	Control		4.7499 ± 0.0550^{a}	4.7139 ± 0.2214^{a}
3.	Eugenol	5.5	3.4782 ± 0.1126^{b}	2.2447 ± 1.1236^{b}
		6.0	0^{b}	0^{b}

Figures bearing different superscript differ significantly.

Discussion

In order to analyse the biofilm forming ability of the 40 EHEC positive isolates recovered from132 *E. coli* isolated from meat contact surfaces of four retail meat shops, the congo red assay was standardized. The results revealed that out of 40 EHEC positive isolates only two isolates obtained from cutting board surfaces of retail meat shops had the biofilm forming ability. The results obtained in this study was in accordance with work done by Perumal and Ignacimuthu, (2000), in which only five isolates out of 100 EHEC positive samples has shown biofilm forming ability from Arunachal Pradesh, India. From the present study, even though the number of isolates which had biofilm forming ability was less, there will be higher chances of genomic evolution and virulence dependent attachment of EHEC on surfaces and further contributing towards biofilm formation (Sheng et., 2016). Further horizontal gene transfer could occur among the organisms which can result in transfer of gene encoding biofilm formation. Lower prevalence of biofilm forming isolates in the present study could be pointed out towards the hydrostatic changes in wood matrices, direct contact to air as the EHEC biofilms were formed primarily when oxygen level was low and frequent disturbance to the cutting surface matrices.

India is a country which is having rich biodiversity and traditional flora which could be used in traditional medicine and reduction of infectious microbial growth without having harmful residual effect. Phytochemicals used in this study was trans-cinnamaldehyde and eugenol which has potential antimicrobial effect and anti-bactericidal activity (Siti Nur Ashakirin, 2017). In the present study, Minimum inhibitory concentration (MIC) of phytochemicals has been standardized in order to study the anti-biofilm effect and effect of these phytochemicals to reduce the growth of EHEC on meat contact surfaces. Initially the MIC of trans-cinnamaldehyde was standardized at 3.5 per cent after evaluating different concentrations of trans-cinnamaldehyde. In the present study at 3.5 per cent concentration of transcinnamaldehyde, there was clear zone of inhibition with 10.67 ± 1.20 mm. Hence the MIC value for trans-cinnamaldehyde was standardized as 3.5 per cent. Minimum inhibitory concentration of eugenol was found to be 5.5 per cent after evaluating the different concentrations of eugenol. At 5.5 per cent concentration of eugenol, a clear zone of inhibition was recorded with value of 7.67 ± 0.33 mm. Hence the MIC value for eugenol was standardized as 5.5 per cent. A concentration of 3.5 per cent and 4.0 per cent of trans-cinnamaldehdye brought about 5 $\log_{10}$ cfu/ml reduction of EHEC organisms on wooden surface. However, concentrations of eugenol at 5.5 per cent and 6.0 per cent showed total elimination of EHEC organism on wood. The concentration obtained for both the extracts showed significant difference from the control sample. Cinnamaldehyde and eugenol are spice oil components, active against both gram positive and gram negative bacteria (Gill and Holley, 2004). Ethanolic extract of cinnamon bark inhibited the growth of *E. coli* and enhances the food safety (Muthuswamy et al., 2008).

The present study focused on antibiofilm effect of phytochemicals. Because of application of fiber and wooden materials in food processing industry, these two materials were selected for artificial creation of biofilm. Since the minimum inhibitory concentration of trans-cinnamaldehdye was 3.5 per cent, two different concentrations of *viz*., 3.5 and 4.0 per cent of trans-cinnamaldehyde were included in the study. Overnight EHEC culture in TSB was diluted to 100 times before inoculating onto test coupons because researchers reported a better biofilm formation in nutrient deprived media. The study revealed that at a concentration of 3.5 per cent of trans-cinnamaldehyde there was reduction of EHEC biofilm cells up to 3.5143 ± 0.1296 mean $\log_{10}$ cfu on wooden coupons and 3.9482 ± 0.3837 mean $\log_{10}$ cfu on fiber coupon when compared to control 4.7499± 0.0550 mean $\log_{10}$ cfu of wood and 4.7139 ± 0.2214 mean $\log_{10}$ cfu of fiber. There was total elimination of EHEC biofilm cells at 4.0 per cent concentration and was significantly different between two concentrations. The observation was made such that 0.3 per cent of trans-cinnamaldehdye had effect on biofilm cells that were decreased to lower than 20 per cent of the control group. The activities of essential oils derived from the trunk bark of *Cinnamomum zeylanicum* (EOCz) and *Cinnamomum cassia* as well as cinnamaldehyde on bacterial biofilms biofilm is sensitive to all concentrations of the plant extract (Firmino et al., 2018). At room temperature films containing cinnamaldehyde and other plant extracts or their combination (25 + 75%) exhibits strongest bactericidal effect, whereas at lower temperatures there is a lower killing rate (Nostro et al., 2015). In the present study, at concentration of 5.5 per cent, there was reduction of EHEC biofilm cells up to 3.4782 ± 0.1126 mean log10 cfu on wooden coupons and2.2447 ± 1.1236 mean $\log_{10}$ cfu on fiber coupon when compared to control 4.7499± 0.0550 mean $\log_{10}$ cfu of wood and 4.7139 ± 0.2214 mean $\log_{10}$ cfu of fiber. There was total elimination of EHEC biofilm cells at 6.0 per cent concentration.

Present investigation revealed that there was a higher occurrence of *E. coli* and EHEC from cutting board surface and knife swabs from retail meat shops which possessed a potential public health threat. It was also proved that EHEC has the ability to form biofilm even though low number of isolates had biofilm forming ability. Uses of phytochemicals at proper concentrations may significantly help to mitigate or eliminate EHEC from meat contact surfaces thereby promoting public health. Usage of phytochemicals should be promoted thereby reducing the usage of antibiotics and hazardous chemicals and preventing their residual effect.

Conclusion

Consumer and occupational group awareness is necessary to prevent zoonotic implications of EHEC. Hence this study demands nation-wide educational programmes to the butchers regarding hygienic practices and its benefits in assuring the safety of the meat to the consumer. Further the study necessitates investigation on uses of phytochemicals and its spectrum on different foodborne bacterial pathogens of public health significance. Control of foodborne diseases requires a multifaceted one health approach which involves multiple disciplines in order to eliminate hazardous food pathogens from farm to fork.

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