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Production of Shiga-Toxin by *Escherichia coli* O157:H7 isolated from Raw Cow Milk obtained within Kaduna Metropolis, Nigeria

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Abstract

Shiga toxin-producing Escherichia coli O157: H7 (STEC) is a food-borne pathogen that is associated with human illnesses from mild gastroenteritis to severe life-threatening hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Its pathogenesis can be enhanced by the occurrence of certain virulence genes and the ability to produce biofilm. The present study investigates the spectrum of such pathogenesis on the basis of the occurrence of shiga-toxigenic E. coli O157:H7 in raw cow milk obtained from cattle herds. Furthermore, the inhibitory potential of some selected essential oils against their biofilm formation was determined. Results revealed isolated shiga-toxigenic E. coli O157:H7 harboured the stx1 and stx2 virulence genes. Inhibitory assessments of cinnamon, clove, eucalyptus, and tea tree oils also revealed impairment of biofilm formation at 1%, 2% and 3% concentrations, with cinnamon having a higher inhibitory impact. The presence of E. coli 0157:H7 in raw cow milk suggests that they are not restricted to contaminating raw meat only, but can be a contaminant of dairy products as well. The combined effect of stx1, stx2, and biofilm formation is of public health concern as plasmid transfer among strains could result in antimicrobial resistance. Phytochemicals from plants such as cinnamon, clove, eucalyptus, and tea tree could play vital biofilm inhibitory roles in decreasing the virulence of E. coli 0157:H7.

Keywords: Escherichia coli O157:H7, Cow, Milk, and Shiga-Toxin,

INTRODUCTION

Shiga toxin-producing *Escherichia coli* O157: H7 (STEC) are food-borne pathogens that are associated with human illnesses, which could be life-threatening (Farroh *et al.*, 2013). They have emerged through the production of Shiga toxins, *stx1* and *stx2* (Musa, 2021; Parsons *et al.*, 2020). The production of Shiga toxins is a key factor contributing to the development of Hemolytic uremic syndrome and can result in both acute, potentially life-threatening illness and lifelong, chronic illness (Ericson *et al.*, 2022). The pathogen generally spreads from feces and foods like meat and milk, especially from dairy cattle (Farroh *et al.*, 2013). Food-borne illnesses from dairy product consumption include infections with *Salmonella enterica, Staphylococcus* sp., *Listeria monocytogenes,* and *Escherichia coli* O157:H7 (Umoh, 2020).

Milk is undoubtedly a nutritious basic food. It is rich in water, proteins, carbohydrates, fats, lactose, calcium, riboflavin, and other B vitamins (Muhammed *et al.*, 2022). Cow's milk is produced on an industrial scale and is by far the most commonly consumed form of milk. The composition and proportions of milk vary with animal breed, feed, age, and phase of lactation (Taylor and Kabourek, 2003). Raw milk has a high water activity (aw = 0.99) and an almost neutral pH (King *et al.*, 2023). Milk is referred to be a high-risk food as it is highly nutritious and serves as an ideal medium for microbial proliferation (Tasci, 2021). Common bacterial pathogens of public health concern today in fresh milk and its derivatives include *Bacillus cereus, Yersinia enterocolitica, Listeria monocytogenes, Salmonella* sp., and *Escherichia coli* (Laba and Udosek, 2023).

Many efforts have been made to reduce biofilm formation on food industry surfaces, but these efforts were mainly based on new disinfectants with different efficacies (Shrestha *et al.*, 2023). Several alternative products to classic disinfectants (chlorine, quaternary ammonium), such as plant-derived antimicrobials of essential oils (EOs) and their compounds, display more significant antimicrobial action in shorter action times (Carrascosa *et al.*, 2021). Thus, there is an urge for a safe and effective antibiofilm approach and eradication. Natural products have been suggested as potentially useful and encouraging antimicrobial and anti-biofilm

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agents (Lu *et al.*, 2019). Essential oils are promising as safe products to ingest with reduced undesired side effects, such as modified taste, mucosal desquamation, and tooth discoloration, and finally they present efficient and safe alternatives for dental caries management (Bahjat, 2019).

The presence of foodborne pathogens in a country's food supply affects the health of the local population. The World Health Organisation's (WHO) position is that food safety must be recognized as a public health function and access to safe food as a basic human right (Mensah, 2022). The food safety strategy aims to contribute to the reduction in morbidity and mortality due to contaminated food. In view of the growing concern about food-borne pathogens across the country, especially in dairy activities of the local herders. The research is aimed at determining the occurrence of Shiga-toxin *Escherichia coli* O157:H7 in raw cow milk obtained from cattle herds within Kaduna metropolis, Nigeria.

MATERIALS AND METHODS

Description of Sampling Site

Pastoral communities produce the bulk of the milk consumed in the rural and urban areas of Nigeria (Ugwu, 2020). The area covered in this study within Kaduna metropolis includes Igabi LGA (Malalin Gabas, Rafin Guza, Kawo), Kaduna North (Ungwan Rimi), and Kaduna South (Tudunwada) between March and May 2022 from cattle herds. Kaduna metropolis is located between Latitudes 10° 20' N and 10°37'N of the Equator and Longitudes 7° 22' E and 7°31'E of the Greenwich meridian. The metropolis cuts across Kaduna North, Kaduna South, as well as parts of Igabi and Chikun Local Government Areas of Kaduna State (Akpu and Tanko, 2017).

Collection of Milk Samples

Fresh milk samples were collected from Cattle as described by Yakubu *et al.* (2021) with little modification. The milk samples were collected in sterile sample bottles between March and May 2022 directly from cattle herds and transported in a cold chest to the Department of Microbiology Laboratory, Kaduna State University.

Isolation and Screening for Shiga Toxigenic *E. coli* 0157:H7

Milk sample (100 µL) was cultured on Eosin Methylene Blue agar media (EMB) following a tenfold serial dilution, which was then incubated at 37°C for 24 hours. To confirm *E. coli* 0157:H7, the isolates were sub-cultured on Sorbitol MacConkey agar (SMAC) and incubated at 37°C for 48 hours (Hessain *et al.*, 2023). All presumptive colonies that stain red with the Gram reaction were subjected to a panel of conventional biochemical tests. All presumptive colonies that stained red with the Gram reaction were subjected to a panel of conventional biochemical tests, including the indole test, methyl red, Voges Proskauer, and Citrate utilization tests (IMViC) (Yakubu et al.. 2021)

Indole Test

The test organisms were inoculated into the tubes of peptone water (5ml), and one test tube was left un-inoculated as a control. The tubes were then incubated at 37oc for 48 h. After incubation, 1ml of KOVAC's reagent was added to all the tubes, including the control. The tubes were shaken gently and allowed to stand for 1-2 minutes, after which they were observed for the formation of a cherry-red ring. Indole production was detected by KOVAC's or Ehlrich's reagent, which contains p-dimethylaminobenzaldehyde that reacts with indole to produce a red colored compound (Wartu *et al.*, 2019).

Methyl-Red Test

A colony of the test organism from EMBA was inoculated into 0.5mL of sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. Appearance of a bright red color was taken as a positive methyl red test (Senthikumaran *et al.*, 2014).

Voges-Proskauer Test

Two drops each of alpha-naphthol and potassium hydroxide were added to the glucose phosphate broth (Voges-Proskauer broth), which had been inoculated with bacteria. The test tube was observed for colour change. A cherry red colour indicates a positive result, while a yellow-brown colour indicates a negative result. A reversal in the order of the reagents being added may result in a weak-positive or false-negative reaction (Senthikumaran *et al.*, 2014).

Citrate Utilization Test

The preparation and sterilization of Simmons citrate agar tubes were completed, and all the isolates were then inoculated through stab inoculation. The tubes were subsequently incubated at 37 °C for 24 hours. A positive result was observed as the indicator changed from green to an intense Prussian blue colour. The colour change from green to Prussian blue indicates the utilization of citrate. This test was designed to assess the ability of microorganisms to utilize citrate as their sole source of carbon (Olutiola *et al.*, 1991).

Triple Sugar Iron Agar (TSI)

A sterile straight wire loop was used to pick a well-isolated colony. The TSI was inoculated by first stabbing through the center of the medium to the bottom of the tube and then streaking it on the surface of the agar slant. The cap of the tube was left loosely before it was incubated at 350C in ambient temperature for 18 to 24 hours. Result: H_2S production shows blackening. H2S: *Salmonella typhi*. (Senthikumaran *et al.*, 2014).

Amplification of Shiga Toxin Gene Region by Polymerase Chain Reaction (PCR):

Extraction of DNA was carried out using the Wizard Genomic DNA Purification Kit (Promega USA) as stated by the manufacturer's instructions. The primers used are shown in Table 1.

The PCR reaction mixtures consist of master mix and the conditions were profiled for 30 minutes, consisting of about 30-40 cycles, with denaturation at about 94°C for the period of 30 seconds, annealing at 51°C for the period of 45 seconds, and extension at 72°C for the period of 1-5 minutes. The resulting products were run on agarose gel electrophoresis. Amplicon bands were visualized using gel imaging system photographs over a shortwave UV light source (Ultraviolet Products, Inc., San Gabriel, Calif.).

Table 1: List of primer sequences used in PCR analysis for detecting *E. coli* virulence genes

Primers	Sequence (5' to 3')	Target gene	Size (bp)	Reference
stx1-F	ATAAATCGCCATTCGTTGACTAC	*stx1*	180	(Paton& Paton 1998)
stx1-R	AGAACGCCCACTGAGATCATC			
stx2-F	GCGGTTTTATTTGCATTAGC	*stx2*	115	(Wang et al. 2022)
stx2-R	TCCCGTCAACCTTCACTGTA			

Biofilm Production by *E. coli* 0157:H7 Isolate

Each bacterial isolate was inoculated in 5 mL of TSB supplemented with 1% glucose, aerobically in plastic tubes, and incubated at 37 °C for 48 hours. The content of the tubes was emptied upon incubation and washed three times with PBS to remove planktonic bacteria (Knobloch *et al.*, 2002). The tubes were subsequently stained with 1 mL of crystal violet solution and incubated for 15 minutes. The stain was removed by washing in water. The tubes were left in an inverted position to dry, after which 5 mL of 96% ethanol was added to each tube and incubated for an additional 15 min. One (1) mL of the ethanol/crystal violet solution was transferred into plastic cuvettes, and absorbance was measured at 600 nm (Hassan *et al.*, 2022). Uninoculated tubes were used as a negative control. The amount of biofilm formed was scored as 1-weak/none (0.077 – 0.154), 2-moderate (0.154 – 0.308), and 3-high/strong (> 0.308) and further calculated in percentage inhibition. The experiment was performed in duplicate.

Evaluation of Microbial Biofilm Inhibition using Essential Oils

Four different pure essential oils, namely, cinnamon, clove, eucalyptus, and tea tree oils, were purchased from a 100% pure essential oil production company (Blomera Essential Oils, Lagos, Nigeria). These oils were chosen following a literature survey and their use in food and pharmaceutical industries (Bolouri *et al.*, 2022). Evaluation of the ability of different concentrations of essential oils to inhibit biofilm production by a monospecies of *E. coli* 0157:H7isolate was performed on plastic disposable tubes. A qualitative method for the detection of biofilm formation was performed as described by Christensen *et al.* (2000). Briefly, different concentrations (1, 2, and 3%) of essential oils were prepared in BHI broth. Two (2) mL of each concentration were inoculated with 0.1 mL bacterial culture and incubated for 24 hrs. Biofilms were stained with crystal violet and results were compared qualitatively and quantitatively. The assays were conducted in duplicate. The percentage (%) inhibition of

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biofilm was determined from the formula described by (Somrani *et al*, 2024):

% Biofilm Inhibition = [{(OD_{600} Control- OD_{600} Sample)} X 100]/ OD_{600} Control

Where: % Inhibition = Percentage Biofilm inhibition, OD control = Absorbance of control at 600 nm, OD sample = Absorbance of test sample at 600 nm

RESULTS

Milk samples cultured were observed for colony morphology and colour of *E. coli* isolates on EMB Agar (Plate 1a). The medium contains selective agents that inhibit gram-positive bacteria and differentiate gram-negative bacilli, including *E. coli*, to form a black metallic sheen on the medium as shown in (Plate 1a). Colonies from EMB with green metallic sheen were sub-cultured onto SMAC agar, which is selective for *E. coli* O157:H7. The colonies with morphological features on the medium were suspected to be *E. coli* O157:H7, forming colorless colonies on SMAC (Plate 1b).

The isolated bacteria exhibited different reactions to various biochemical test reagents (Table 2). They tested positive to indole, methylene red and catalase test while negative to Voges Proskuer and citrate utilisation test respectively. The colour change from pink to yellow indicating that sugars such as glucose, sucrose, lactose and maltose has been fermented by production of gas bubble.

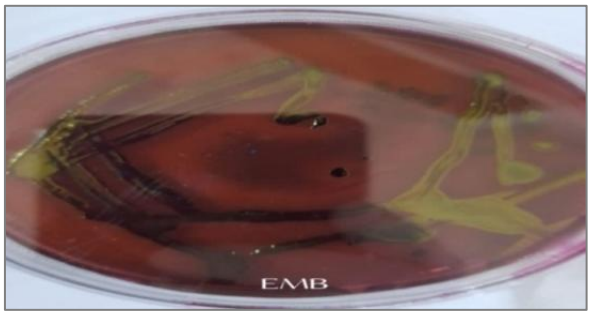

Plate 1a: Colonial Morphology of *E. coli* on EMB Agar

Plate 2b: Confirmed *E. coli* O157:H7 on SMA Agar

Detection of Virulence Genes of Shiga Toxin-Producing *E. coli* O157:H7

Gel electrophoregram of *stx1* and *stx2* shiga toxin gene detected from pathogenic *E. coli* O157:H7 isolate with the size of the amplicon at approximately ~ 180 bp and 115 bp from a shiga toxigenic *E. coli* O157:H7 isolated from a Milk sample is as presented in Plate 2 and Plate 3, respectively.

Table 2: Biochemical Characterization of Shiga Toxigenic *E. coli* O157:H7 isolated from Sorbitol MacConkey Agar

S/N	Isolate Code	Gram React.	MR	Citrase	VP	Indole	Glucose	Lactose	Sucrose	Fructose	Galactose	H2S	Gas	Suspected Organism
1	4R	- Rod	+	-	-	+	+	+	-	-	+	-	+	*E. coli*
2	4K	- Rod	-	+	+	-	+	+	+	-	+	-	+	*K. pneumonia*
3	20T	- Rod	-	+	+	-	+	+	+	+	+	-	+	*E. aerogenes*
4	13UR	- Rod	+	-	-	+	+	+	-	-	+	-	+	*E. coli*
5	20UR	- Rod	+	-	-	+	+	+	+	-	-	-	+	*E. coli*

Key: SN= Serial Number, **MR=**Methyl Red, **VP** =Voges Proskeuer

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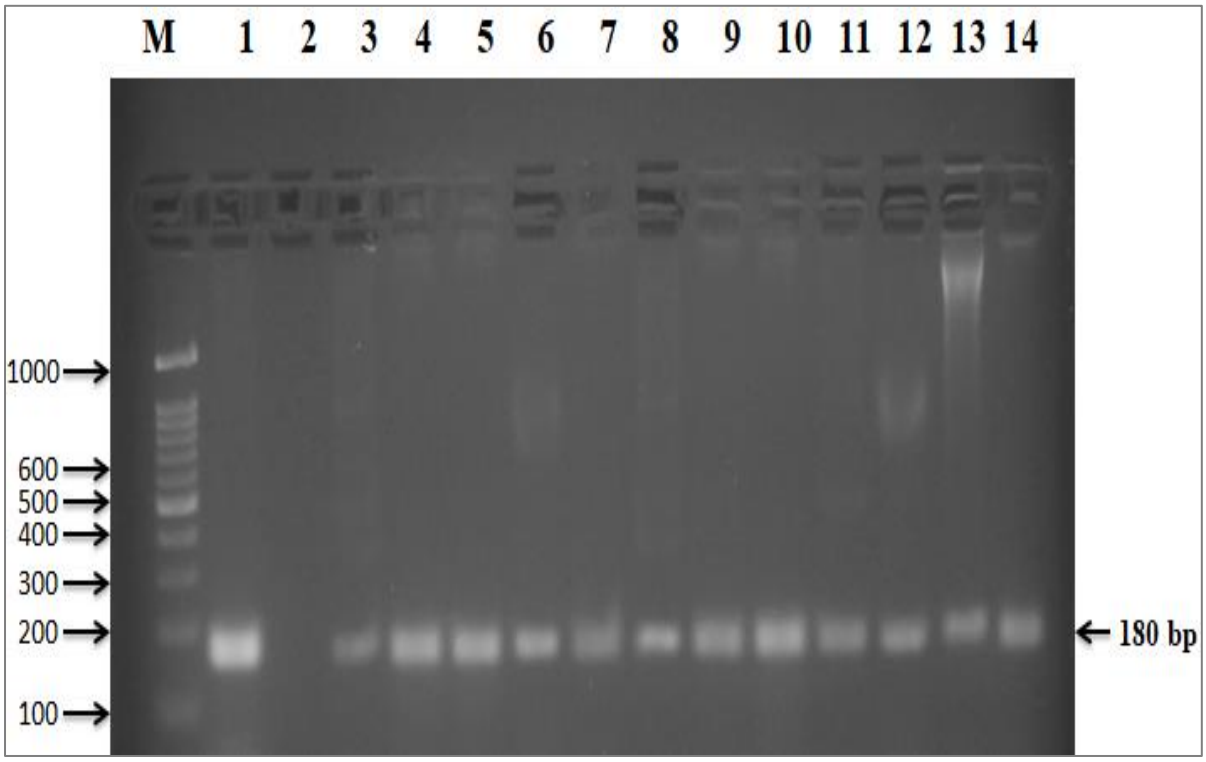

Plate 2: Gel Electrophoregram of *stx1* Shiga Toxin Gene from Pathogenic *E. coli* O157:H7 (lanes 3); **Key: bp:** Base pair. **M:** Molecular ladder, **Lane 1**: Positive control, **Lane 2**: Negative control, **Lane 3-14**: *stx1* Virulence Gene Detected from *E. coli* O157:H7

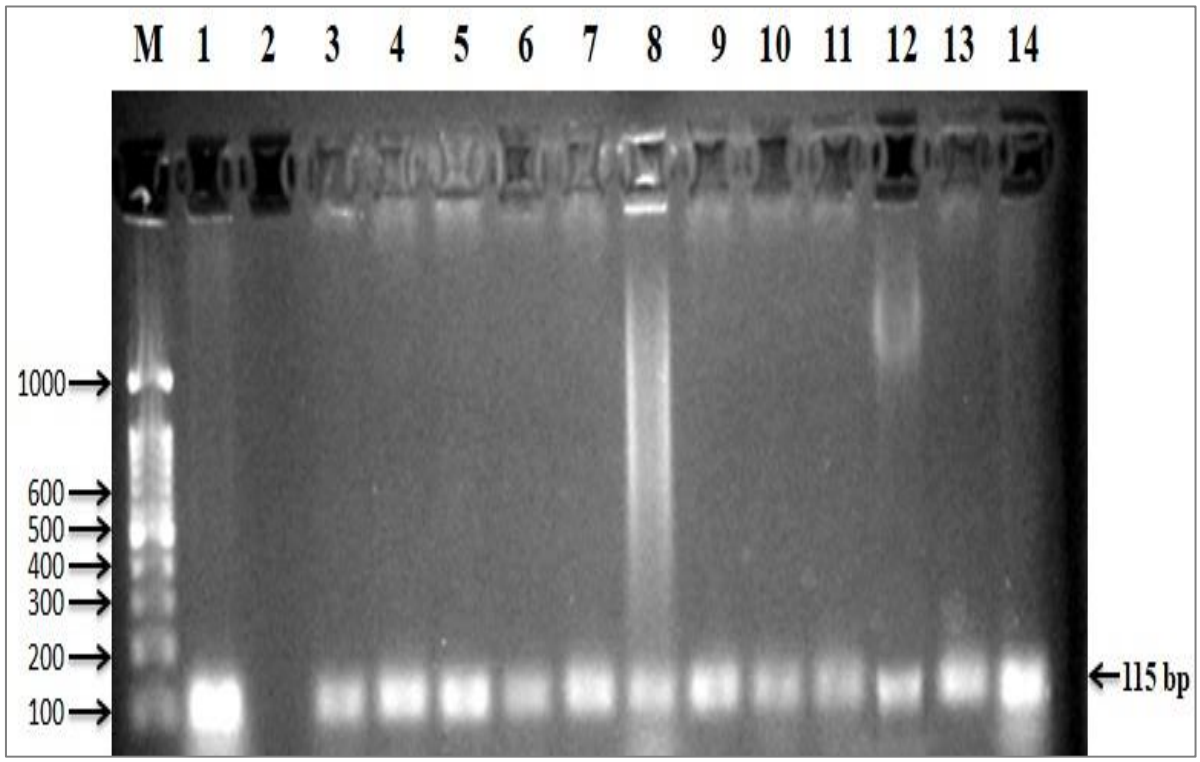

Plate 3: Gel Electrophoregram of *stx2* Shiga Toxin Gene from Pathogenic *E. coli* O157:H7 (lanes 3); **Key: bp:** Base pair, **M:** Molecular ladder, **Lane 1**: Positive control, **Lane 2**: Negative control, **Lane 3-14**: *stx2* Virulence Gene Detected from *E. coli* O157:H7

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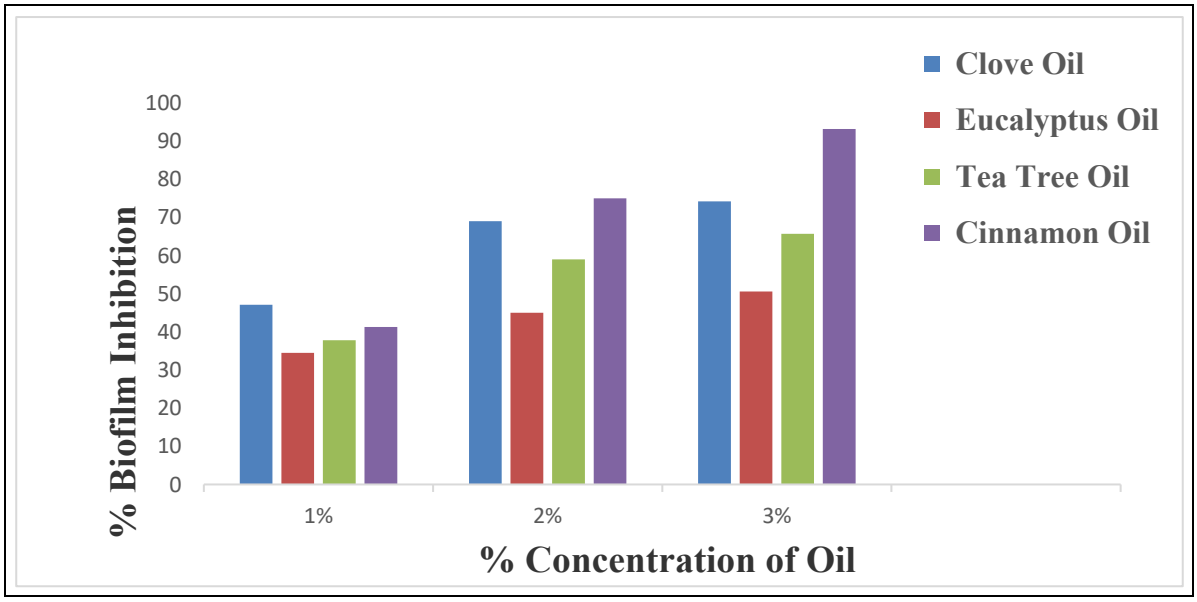

Figure 1: Antibiofilm Activity of Different Essential Oils on *E.coli* O157:H7 Isolate

Table 3: Spectrophotometric Detection of Biofilm

Biofilm Classification	OD Range	Obtained OD	Number of Isolate
None Adherent	<0.077		
Weak	0-077 - 0.154		
Moderate	0.154 - 0.308		
Strong	>0.308	1.032	1(100%)

Key: **OD** = Optical Density, < = Less than, > = Greater than

Biofilm Formation by *E. coli* O157:H7 Isolate

Escherichia coli O157:H7 Isolate was categorized as a strong biofilm producer by spectrophotometric detection as shown in Table 3. The strain showed strong biofilm formation. It has been indicated that STEC may form biofilms in a wide range of strengths. Results obtained were averaged and expressed as numbers, and the interpretation of the biofilm results was done according to Stepanovic et al. (2000). The results showed biofilm production varied greatly when compared to the antibiofilm activity in Figure 1. The results indicated that the pathogenicity of *E. coli* strains could be much higher based on the level of biofilm production as determined by the microplate assay.

Antibiofilm Activities of Essential Oils using Quantitative Method

Relationship of antibiofilm activities of the different oils on *E. coli* O157:H7 is demonstrated in Fig. 1. There was difference in the response to different concentrations of different oil on the tested *E. coli* O157:H7 isolates with clear demonstration of decreased production of biofilm as the concentration of oil increases from 1%, 2% and 3% with increased in percentage inhibition.

Clove oil has been shown to be effective at a 3% concentration and decreases biofilm inhibition as the concentration of oil decreases. Tea tree oil has been confirmed as an antibiofilm agent that inhibited biofilm formed by *E. coli* O157:H7 as the concentration of oil increases from 1, 2, and 3% with an increase in biofilm inhibition. Eucalyptus oil showed a partial antibiofilm activity against a biofilm formed by the isolate of *E. coli* O157:H7. Cinnamon oil clearly showed a decrease in biofilm progression with an increase in oil concentration. However, an increase in concentration (1%, 2%, and 3%) increases the percentage inhibition of biofilm formed by *E. coli* O157:H7.

DISCUSSION

Typical growth of *E. coli* produces a greenish metallic sheen. Colony morphology of bacterial species obtained on the agar with pink, purple, or metallic sheen, as seen under a light microscope, confirmed *E. coli* O157:H7 as reported by Huang *et al.* (2021). On the other hand, *E. coli* 0157:H7 does not ferment sorbitol. *E. coli* O157:H7 does not ferment sorbitol due to a lack of ability to produce ß-D-glucuronidase

(GUD) and is also more resistant to cefixime and tellurite. Based on one or more of these properties, several solid growth media, including sorbitol-MacConkey (SMAC) agar and its cefixime-tellurite-supplemented formula (CT-SMAC), have been developed (Farrokh *et al.*, 2012). Shiga toxin-producing *E. coli* O157:H7 (STEC O157:H7) is a significant pathogen associated with foodborne infections, which cause severe illness in humans. Its implications in virulence and antimicrobial resistance contribute to the pathogen's severity and the challenges in controlling its spread (Swamy & Thakur, 2020; Wang *et al.*, 2022). Shiga Toxins (Stx1 and Stx2) O157:H7 produces potent shiga toxins, which inhibit protein synthesis in host cells, leading to cell death. These toxins are central to the pathogen's ability to cause severe diseases like hemorrhagic colitis. Stx2 is generally more virulent than Stx1 (Vasconcelos *et al.*, 2018). The transmission of STEC O157:H7 is primarily through contaminated food, particularly undercooked ground beef, unpasteurized dairy products, and contaminated vegetables. It can also spread through water, direct contact with animals, and person-to-person transmission (Bach *et al.*, 2002). The pathogen is responsible for significant foodborne outbreaks globally (Kavanaugh and Ribbeck, 2022). The combination of its virulence and resistance mechanisms exacerbates public health challenges in industrial settings.

The activity of essential oils is determined by their constituents, active ingredients, functional groups, and their synergistic interactions. The mechanism of action varies with the type of essential oil or bacterial strain used. It is well known that Gram-positive bacteria are more susceptible to essential oils compared to Gram-negative bacteria (Upadhyay *et al.*, 2021; Huang *et al.*, 2021). This is because Gram-negative bacteria have a complex rigid lipopolysaccharide (LPS) outer layer. The relationship between essential oils and biofilm formation is key to developing novel strategies to control biofilm associated with foodborne infections. Essential oils interfere with the early stages of biofilm development by preventing the adhesion of microbes to surfaces (Upadhyay *et al.*, 2021). Different Essential oils were used to induce the inhibition of microbial biofilm by *E. coli* O157:H7 in the present study.

Clove oil was active against the biofilm of *E. coli* O157:H7 isolates as the concentration increases. The inhibition revealed the effectiveness of the oil against the *E. coli* O157:H7biofilm. This may be due to the presence of eugenol, which is the major compound, accounting for at least 50%. The remaining 10–40% consists of eugenyl acetate, ß-caryophyllene, and α-humulene. The active ingredient of clove oil is eugenol, a widely used antibacterial agent in dentistry, and has been proven to reduce halitosis (Katyayani *et al.*, 2017).

Components like eugenol (from clove oil) and cinnamaldehyde (from cinnamon oil) have been shown to prevent bacterial adhesion to surfaces. They do this by interfering with cell surface proteins and weakening microbial attachment, thereby preventing biofilm formation on various surfaces (Swamy & Thakur, 2020).

Eucalyptus (*Myrtaceae*) species produce large amounts of various complex mix of volatile compounds (terpenes, phenolics, alcohols, aldehydes, etc.) (Swamy & Thakur, 2020). The value of Eucalyptus oil for medical purposes is due to the content of the specific oil ingredient: 1, 8-cineole (cineole or eucalyptol) (Leite *et al.*, 2022). The main components of eucalyptus EO are 1,8-cineole (63.1%), p-cimene (7.7%), a-pinene (7.3%), and a-limonene (6.9%) (Kim *et al.*, 2021). The present study also indicated that eucalyptus oil was active against the isolate *E. coli* O157:H7, with a percentage increase, and partially active against a monospecies *E. coli* O157:H7 isolate at a lower percentage (Mota *et al*, 2021).

Tea tree oil is derived from the native plant, *Melaleuca alternifolia*. Tea tree oil is composed of terpene hydrocarbons and their alcohols, which constitute α-pinene (21.64 %), γ-terpinene (21.09 %), terpinen-4-ol (17.31 %), limonene (9.37 %), and o-cymene (6.54 %). Tea tree oil was active against the sample isolate *E. coli* O157:H7 as a monospecific isolate under investigation. The wide range of action allows them to be effective against mixed-species biofilms, which are common in industrial settings. The tea tree essential oil has antibacterial activity against microorganisms, but this activity is evaluated only in high concentrations. They do this by interfering with cell surface proteins and weakening microbial attachment, thereby preventing biofilm formation on various surfaces (Leite *et al.*, 2022; Thosar *et al.*, 2023). All selected essential oils demonstrated an antibiofilm activity, with cinnamon being the most active in biofilm inhibition, followed by clove, tea tree, and finally eucalyptus oil.

CONCLUSION

Due to the emergence of biofilm formation from bacterial species, the study identified *E. coli* O157:H7 and detected *stx1* and stx2 genes at 180 bp and 115 bp fragments. The *E. coli* O157:H7 formed a strong biofilm as a monospecies. Thus, Essential oils have become a promising alternative in inhibiting the problem biofilm formed by *E. coli* O157:H7. It is well known that bacterial biofilms have a significant impact in industrial and environmental settings. In food processing and packaging, essential oils are being explored as natural preservatives to prevent biofilm formation on equipment and surfaces, reducing contamination (Rinaud *et al.*, 2021).

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