Antibacterial and anti-biofilm activity of cinnamon and clove oils against uropathogens

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Abstract

Introduction. The aim of the study is to explore *in vitro* antibiofilm activity of cinnamon (*Cinnamomum zeylanicum*) and clove (*Syzygium aromaticum*) essential oils and their main phytoconstituents cinnamaldehyde and eugenol against clinical bacteria isolates of patients with urinary tract infection (UTI).

Materials and methods. Antibacterial activities of cinnamon and clove essential oils and their active constituents were determined using disc diffusion and minimal inhibition concentration methods.

Result and discussion: In the present research, *Escherichia coli* was studied as the main bacterial uropathogen accounting for 70% of bacterial isolates identified among twenty patients with UTI. The results showed that eugenol had the highest antimicrobial activity against *E. coli* strains, followed by cinnamaldehyde and clove oils. Further, fourteen *E. coli* isolates obtained from the urine samples were screened for their biofilm-forming activity on polystyrene surfaces. Our finding reported that 28.5% isolates were not able to form biofilm as was determined by crystal violet staining assay. Eugenol and cinnamaldehyde exhibited high biofilm inhibition activity with a percentage of inhibition ranged from 80.54 to 95.2 and 73 to 89.0%, respectively. The minimum inhibitory concentration values of cinnamon oil, clove oil, cinnamaldehyde, and eugenol for *E. coli* strains range from 0.68–0.823 mg/ml, 0.75–0.923 mg/ml, 0.55–0.72 mg/ml and 0.44–0.56 mg/ml, respectively. A low minimum inhibitory concentration value may be an indicator of the high efficacy of an antibacterial agent in inhibiting the visible growth of microorganisms. Eugenol showed the highest antibacterial activity with minimum inhibitory concentration of 0.44 mg/ml for 71.4% of isolates and minimum inhibitory concentration of 0.56 mg/ml for 24.8% of isolates.

Conclusion. The tested cinnamon and clove essential oils exhibited very strong antibacterial and antibiofilm activities against *Escherichia coli* isolates obtained from UTI patients and hence can be used as a promising alternative for antibiotics substitution.

Introduction

Gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Proteus species, Acinetobacter species, Enterobacter species, Klebsiella species,* and *Citrobacter species* are the most common bacterial species associated with urinary tract infection (UTI). These uropathogens are found to be drug-resistant and biofilm forming bacteria, which is a major factor in the pathogenesis of recurrent urinary infections.

Biofilm is the structure made by colonies of microorganisms that adhere and grow on the surface of the cell and non-cellular materials, which is eventually enclosed in an extracellular polymeric substance matrix (Hall et al., 2017; Jamal et al., 2018). This extracellular matrix is mainly composed of polysaccharides and can to adhere to both biotic and abiotic surfaces. Biofilm is the colony of microbes that are irreversibly attached to a range of surfaces in the food industry in just a minute. Recently, biofilm formation in food processing surfaces and equipment has become a great concern in the food industry because they are responsible for the corrosion of metal surfaces, equipment damage, and cross-contamination of processed food (Abdallah et al., 2014; Colagiorgi et al., 2017). The formation of biofilm is a self-protecting barrier for the growth of bacteria against environmental stresses, antimicrobial drugs, and the host human defense. Extensive research has been done to prevent and inhibit biofilm formation through the implementation of several synthetic and natural drugs. However, complete success in the detachment or destruction of biofilm has not yet been developed (Basavaruju et al., 2016). Synthetic drugs contain so many side effects like nausea, diarrhea, dizziness, lightheadedness, headache, or trouble sleeping. There is a need to search for natural sources which can heal the problem without causing any side effects to the patients. However, plant-based natural products have received much attention in preventing antibiofilm activity in the last decades. Hence it is a matter of much concern, and the research was done to acquire a new source of antimicrobials and biofilm-eradicating agents to combat the infections associated with biofilm-causing drug-resistant bacteria.

The aim of the present study is to evaluate the antimicrobial activity of natural antimicrobial agents (cinnamon and clove essential oils and their main phytoconstituents cinnamaldehyde and eugenol) against clinical bacteria isolates of UTI patients.

Materials and methods

Sample preparation and extraction of essential oils

The spices cinnamon (*Cinnamomum zeylanicum*) and clove (*Syzygium aromaticum*) were thoroughly cleaned and then dried in an oven at 55 °C. After drying, barks and buds were grounded into a coarse powder and placed inside a thimble made from thick filter paper, which was then subjected to solvent extraction technique by a soxhlet extractor. The solvent used for extraction was ethanol. The solvent was heated to reflux at a temperature above 100 °C for 5 and 10 hours. After the extraction, the products were collected and purified using a rotary evaporator at a fixed temperature of 50 °C (Wong et al., 2014).

Isolation, identification of bacterial strains

The bacterial strains were isolated from urine samples of twenty UTI patients from Janhit the hospital, Prayagraj, and were streaked on nutrient agar for single colony separation. Three single colonies were selected, streaked separately in MacConkey agar and Eosin

methylene blue (EMB) agar (HiMedia, Mumbai, India). The morphological (Gram's reaction, cell shape, and arrangement) and biochemical characterization (indole test, motility test, Vogues Proskeur test, methyl red test, catalase test, nitrate reduction test, triple sugar iron test, urease test)of recovered uropathogens were carried out according to Harley and Prescott (1997), and the results were compared with the pure maintained cultures. The most probable species was decided on the basis of PIBWN software.

Antibiotic assay

The disk diffusion technique did the antibiotic susceptibility test of *Escherichia coli* isolates. The antibiotics used were amoxicillin (30 µg/disc), amoxicillin/clavulanic acid (10 µg), piperacillin + tazobactam (10 µg), ticarcillin + clavulanic acid (10 µg), Cefepime, cefuroxime (30 µg) and ceftriaxone (CT, 30 µg). The isolates were inoculated into 10 ml of sterile nutrient broth and incubated at 37±1.0 ºC overnight. 100 µL inoculum was swabbed on the surface of agar plates, and then antibiotic-impregnated discs were immersed in it after cooling. The plates were then kept for incubation at 37 °C for 24 hours. An inhibition zone on the plates showed antimicrobial activity.

Antibacterial assay

The antimicrobial activity was evaluated by the agar well diffusion method **(**Bagamboula et al., 2004). Bacterial inoculums containing 10^6 CFU/ml were swabbed on the surface of sterile Mueller-Hinton agar plates using a sterile cotton swab and allowed to dry for 3–5 minutes. A sterilized borer (10 mm) impregnated by the tested essential oils (10 µl /disc) was used to prepare agar wells and then incubated in an upright position at 37±10 ºC for 24 hrs. Sterile blank paper discs impregnated with only sterile dimethyl sulfoxide (DMSO) served as the negative control, and antibiotic ciprofloxacin (5 µg/disc) was used as a positive control. The zone of inhibition was measured around each disc to evaluate the antibacterial activity and expressed in millimeters (mm).

Assessment of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined by the microdilution method proposed by (Jorgensen and Turnidge, 1999) with minor modifications. Different concentrations of samples ranging from 0.05 to 2.5% (v/v) were prepared in DMSO and transferred into 96 well microliter plate (100 µl/well. The test was performed in triplicates alongside a blank sample (without bacterial inoculum). The concentration at which no visible growth was observed was recorded as MIC value**.**

Biofilm production assay

100 µl of overnight grown bacterial suspension were poured into the wells of a 96-well microtiter plate and allowed to incubate for 24 h to 48 h at 37° C. Then, the supernatant culture was discarded, and wells were washed twice with phosphate-buffered saline to remove the loosely adhered. The adhered cell was stained with 200 µl 1% of crystal violet for 10 minutes. The excess strain was discarded, and the wells were washed with distilled water and air-dried. The plate was then read spectrophotometrically at 570 nm in an ELISA reader **(**Sa et al., 2012).

Biofilm Inhibition Assay

100 µl of the final concentration of essential oils (equivalent to MIC) was poured into the 96-well microtiter plate consisting of fresh 50 µl of bacterial culture (10^8 CFU/ mL) in each well and incubated at 37 °C for 24 h. The formation of biofilm was quantified by the crystal violet method as described earlier. The well with sterile water was considered a control. Inhibition of biofilm was assessed by the formula described by (Jadhav et al., 2013):

% Inhibition = 100 - (OD570 sample/OD570 control) x 100 Inhibition of biofilm

Statistical analysis

The experiments were done in triplicates, and their mean values were represented. All statistical analyses were done using analysis of variance (ANOVA) in SPSS Version 17.0. Differences were considered significant when $p< 0.05$. Graphs were prepared in Sigma.

Ethical consideration. Patient consent was obtained, and anonymity was preserved by assigning an identification number.

Results and discussion

Isolation and identification of bacteria

Twenty urine samples of UTIs patients were collected from Janhit hospital, Prayagraj. The result of present study revealed that 70% of bacterial isolates belonged to *E. coli*, and 30% belonged to *Klebsiella spp.* The bacterial species were identified on the basis of their morphological and biochemical characteristics (Table 1, 2). The results recorded from biochemical tests were subjected to PIBWIN software to identify the most probable organism. It was found that *E. coli* was the most prevalent UTI pathogen, followed by *Klebsiella spp*. Earlier studies also have recorded similar findings (Bitew et al., 2017; Gupta et al., 2011).

Table 1

Identification of clinical isolates

Features	Nutrient Agar		EMB Agar	
	Isolates 1, 3, 4, 5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	Isolates 2, 6, 7, 8, 9, 10	Isolates 1, 3, 4, 5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	Isolates 2, 6, 7, 8, 9, 10
Gram reaction	Gram –ve	Gram -ve	Gram –ve	Gram –ve
Shape	Circular	Circular	Circular	Circular
Color	Creamy white, shiny	Grayish white	Green metallic sheen	Pink to purple
Surface	Mucoid	Mucoid	Mucoid	Mucoid
Elevation	Flat	Dome-shaped	Convex	Convex
Opacity	opaque	Translucent- opaque	opaque	Translucent- opaque

Table 2

Biochemical characteristics of culture isolates

Biochemical tests	Isolates 1, 3, 4, 5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	Isolates 2, 6, 7, 8, 9, 10
Catalase test	Positive	Negative
Lysine test	Positive	Negative
Nitrate test	Negative	Negative
Citrate test	Negative	Negative
Methyl test	Positive	Negative
Voge's test	Positive	Negative
Indole test	Positive	Negative
Urease test	Positive	Positive
Peptone test	Positive	Negative
Motility test	Negative	Negative
Tryptophan test	Positive	Positive

Antibiotic sensitivity test of *E. coli* isolates

In the present work, the bacterial isolates *E. coli* were selected for further studies because of the high prevalence in urine samples among UTI patients. Results of the disc dilution test for antibiotic sensitivity of isolates to seven different antibiotics are represented in Table 3.

Table 3

Antibiotic resistant pattern of the *E. coli* isolated strains

Isolates	***AM***	**AMC**	**PTB**	**TCA**	**Cefepime**	**Cefuroxime**	**Ceftriaxone**	**Biofilm activity**
E1	R	R	S	R	R	R	R	M
E2	R	R	S	R	R	R	R	S
E3	R	R	S	R	R	R	R	M
E4	R	R	S	R	R	R	R	S
E5	R	R	S	R	S	S	S	N
E6	R	S	S	S	S	S	S	N
E7	R	S	S	S	S	S	S	N
E8	R	S	S	S	S	S	S	N
E9	R	R	S	R	S	S	S	M
E10	R	R	S	R	S	S	S	M
E 11	R	R	S	R	S	R	S	M
E12	R	R	S	R	S	R	S	M
E13	R	R	S	R	R	R	R	M
E14	R	R	S	R	S	R	S	M

Amoxicillin (AM, 30 μg/disc), amoxicillin/clavulanic acid (AMC, 10 μg/disc), piperacillin + tazobactam (PTB, 10 μg/disc), ticarcillin + clavulanic acid (TCA, 10 μg/disc), cefepime (30 μg/disc), cefuroxime (30 μg/disc g), ceftriaxone (CT, 30 μg/disc). R-resistant, S-sensitivity.

In this study, common antibiotics used to treat UTIs were selected. Our finding revealed that all the bacterial isolates were resistant to amoxicillin, and most of the bacterial isolates (78.57%) were resistant to amoxicillin/clavulanic acid and ticarcillin + clavulanic acid. This is attributed to the ability of strains to produce beta-lactamase. Previous studies also reported the resistance of these strains to aminopenicillins (Iroha et al., 2012). Similarly, Dibua et al. (2014) revealed that amoxicillin was not active against any Gram-negative strains of the urine specimens. Our finding observed that only piperacillin associated with tazobactam presented very good activity against *E. coli* isolates, consistent with previous studies that reported antibiotics combination increased sensitivity (Sagna, 2019). Inconsistent with other studies found that tested multidrug-resistant strains isolated from clinical samples were resistant to the majority of commonly used antibiotics in developing countries.

Antibacterial assay

Several studies reported the antibacterial activity of medicinal plants for the management and cure of UTIs. In the present study, the effect of essential oil and their phytocompounds were performed after 24 h of the incubation period of bacterial culture in Mueller Hinton broth (Sigma Aldrich, SA). Figure 2 depicts the antibacterial activity of spice oil and its active compound against *E. coli* clinical isolates, expressed as diameters of inhibition. The result of preliminary antibacterial activity revealed that cinnamon oil, clove oil, cinnamaldehyde, and eugenol were active against all the tested *E. coli* isolates with a zone of inhibition >12 mm. Eugenol has a high percentage of inhibitory action on 85.70% of isolates (n-10) with the inhibition zone of 27mm, followed by cinnamaldehyde with the inhibition zone of 22 mm within only two isolates. Clove oil exhibited a zone of inhibition of 19 mm for 50% of the isolate, and cinnamon oil showed a zone of inhibition of 15 mm for 42.8% of the isolates (Figure 2). The result of the study was consistent with the work done by Prabuseenivasan et al. (2006).

After preliminary screening, the antimicrobial agents were subjected to minimum inhibitory assay. The minimum inhibitory concentration (MIC) is the minimum concentration of the antibacterial agent that could inhibit 50% of the bacteria after 24 h incubation. Figure 3 illustrated the MIC values of cinnamon oil, clove oil, cinnamldehyde and eugenol for *E. coli* ranges 0.68–0.823 mg/ml, 0.75–0.923 mg/ml, 0.55–0.72 mg/ml, and 0.44–0.56 mg/ml, respectively. A low MIC value may be an indication of the high efficacy of the antibacterial agents in inhibiting the visible growth of microorganisms. Eugenol showed the highest antibacterial activity, with a MIC 0.44 mg/ml in 71.4% of isolates and a MIC of 0.56 mg/ml in 24.8% of isolates. Our finding reported that individual phytochemical compounds eugenol followed by cinnamaldehyde had highest antibacterial activity than their respective essential oil. This finding concurs with other studies which reported cinnamon and clove oil exhibited the lowest MIC values for *E. coli*. Another study by Millezi et al. (2019) showed the antibacterial and antibiofilm activity of cinnamon essential oil and eugenol against uropathogens.

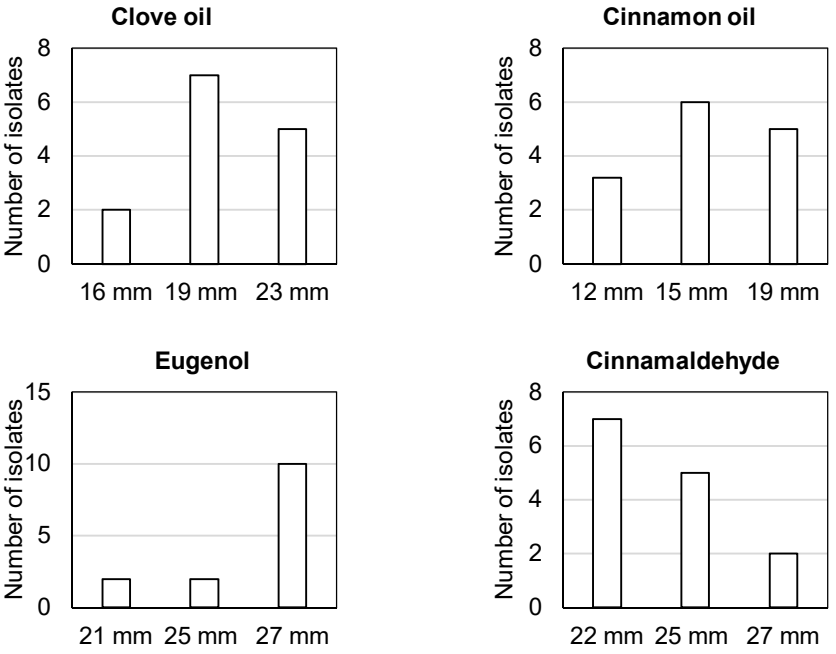

Figure 2. Zone of inhibition (mm) of essential oils against *E. coli* isolates

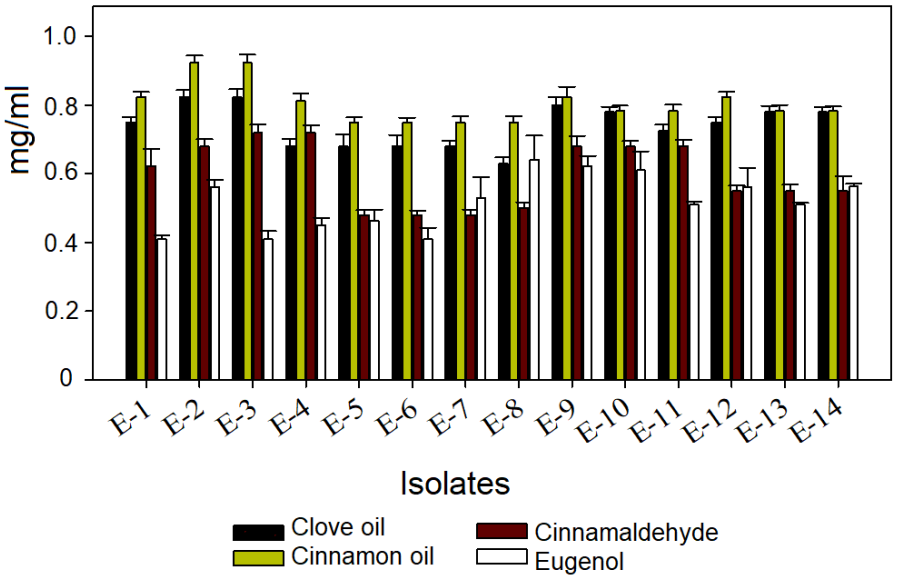

Figure 3. Minimal inhibition concentration (MIC) values (mg/mL) of essential oils against *E. coli* isolates tested with micro-dilution assay

Biofilm formation capacity of isolates

Previous studies reported that different isolates have different capacities to form biofilm. In this study, fourteen *E. coli* isolated from UTI patients were examined for their biofilm-forming abilities on polystyrene surfaces. Our results suggested that 57.8% (n-8) of the isolates have moderate biofilm formation ability, and only 14.2% (n-2) form strong biofilm formation ability with optical density 570 (OD570) values ranging from 0.146–0.18 and 0.26–0.29 respectively whereas 28.5% (n-4) were not able to form biofilm as determined by crystal violet staining assay. These strains were divided as strong (OD570 > 1), moderate (0.1 > OD570 < 1), and negative (OD570 < 0.1) based on their absorbance in crystal violet assay (Table 3). This result agrees with the researchers that showed each isolate have own capacity to form thickness due to the difference in the ability to produce autoinducers (Quorum sensing signaling molecules). Our finding reported that out of 14 isolates 10 isolates were able to form biofilm.

Anti-biofilm activity

The *in vitro* biofilm activity of essential oils and main active constituents on the prevention or reduction of biofilm development in 24 and 48 h preformed biofilms are presented in Figure 4.

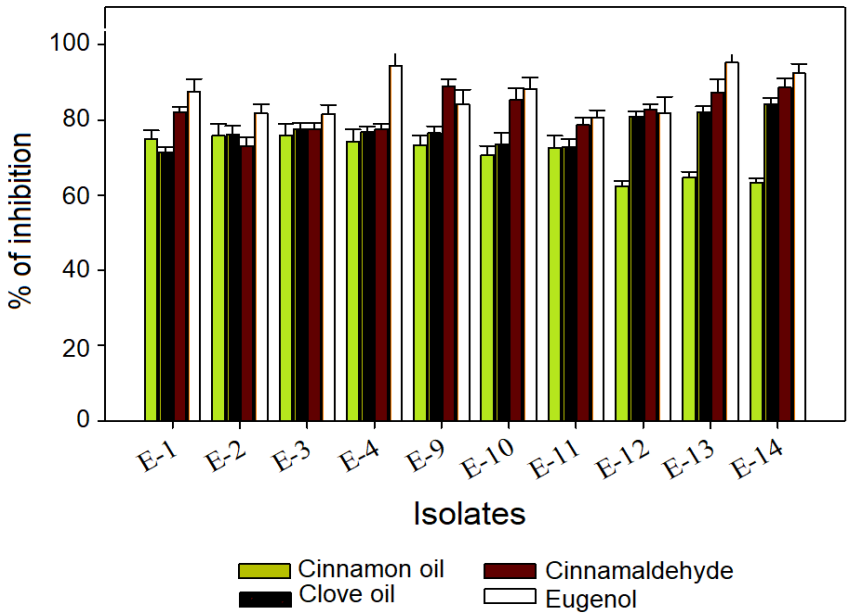

Figure 4. Antibiofilm activity (% of inhibition) of essential oils against *E. coli* isolates

Eugenol, followed by cinnamaldehyde, showed the highest antibiofilm activity, with the percentage of inhibition varying from 80.54% to 95.2% and 73% to 89.0%, respectively. The action of eugenol resulted in no biofilm formation by 90% of isolates; however, cinnamaldehyde provided inhibition for 80% of isolates, whereas the application of clove oil

and cinnamon oil resulted in weak biofilm formation by 60% to 70% of isolates. Cinnamon oil exhibited the minimum antibiofilm activity that ranges 62.4%-75.9%. The outcomes of the present work showed that eugenol and cinnamldehyde had highest antibiofilm effect followed by the clove oil and cinnamon oil. Amalaradjou et al. (2011) showed the ability of trans-cinnamaldehyde to inhibit uropathogenic *Escherichia coli* biofilm formation isolated from catheters and also demonstrated that trans-cinnamaldehyde prevented virulence factors of uroepithelial cells by down-regulating genes in the pathogen. Clove oil at sub-MIC levels was reported to repress QS-associated characters in reporter strains (Khan et al., 2000)

Conclusion

A urinary tract infection is the second most common infection that occurs in the human body and *Esherichia coli* is found to be the most prominent bacterial strain responsible for the disease. Most of the uropathogenic bacterial strains are found to be drug-resistant and have biofilm-forming capacity. The present study was done to discover the natural antibacterial and anti-biofilm agents from a huge reservoir of Indian medicinal plants. The study revealed that *E. coli* strains isolated from clinical samples were resistant to the majority of commonly used antibiotics. Eugenol, followed by cinnamaldehyde, has highest antibacterial and antibiofilm activity than their respective essential oils. In conclusion, our study suggested that eugenol and cinnamaldehyde can be used as plant-derived antibiotics for biofilm-associated urinary tract infections.

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