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HEALTH SCIENCES

In-vitro Antibacterial and Antibiofilm Activity of *Cinnamomum verum* Leaf Oil against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae*

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Abstract: Phytomedicines are becoming more popular in treatment of infectious diseases worldwide. *Cinnamomum verum* essential oil (EO) has been used as a therapeutic alternative for various diseases. This study aimed to evaluate the antibacterial and antibiofilm activity of the *C. verum* leaf EO against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae*. Effect of EO vapor on planktonic cells was determined using microatmosphere technique. CLSI M7-A10 method was employed in Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) experiments. Effect of EO on established biofilms was quantified and visualized using XTT and Scanning Electron Microscope (SEM). *In-vitro* toxicity was evaluated using Human Keratinocytes (HaCaT). Chemical analysis of EO was done using Gas Chromatography- Mass Spectrometry (GC-MS). All tested strains were sensitive to cinnamon oil vapor. EO exhibited 0.5 and 1.0 mg/mL MIC and MBC against all test strains. Minimum Biofilm Inhibitory and Biofilm Eradication Concentrations ($MBIC_{50}$ and MBEC) were 1.0 and 4.0 mg/mL. SEM indicated cellular shrinkages, cell wall damages, and decreased biofilm densities. Cinnamon oil didn't show any toxicity on HaCaT cell at any concentration tested. Eugenol was the most abundant compound in *C. verum* oil. *C. verum* EO shows an antibacterial and antibiofilm activity with minimal toxicity on host.

Key words: Bacteria, biofilms, *Cinnamomum verum*, essential oil, natural product, planktonic.

INTRODUCTION

Microorganisms have a great significance on human health. With the arbitrary use of available antimicrobial therapeutics and patients' non-compliance, antibiotic resistance is becoming an emerging health issue all over the world (Darwish et al. 2014, Karakonstantis & Kalemaki 2019). Plant material derived natural compounds with medicinal properties, especially antimicrobial natural products are widely used among different communities for treatment purposes (Oliveira et al. 2020). The easy accessibility, high efficacy, low toxicity to the human body and low antimicrobial resistance are some contributory factors for the high popularity of herbal natural antibiotics as therapeutic alternatives (Salam & Quave 2018, Tsao & Zhou 2007).

Cinnamomun verum/ true cinnamon/ Ceylon cinnamon is an indigenous plant of Sri Lanka and one of the widely using culinary species throughout the world including Sri Lanka, India and South American countries

(Jayaprakasha & Rao 2011). The leaves, bark, flowers, roots and fruits are used to extract volatile essential oils by steam distillation and the chemical composition is depending on the parts of the tree used to extract the oil (Haddi et al. 2017). Oils extracted from *C. verum* leaves exhibit various medicinal properties such as anti-parasitic effect, anti-cancer, anti-glycaemic and anti-lipaemic effect, activity against neurodegenerative and cardiovascular pathologies, blood pressure regulation, anti-oxidant and anti-inflammatory effect (Haddi et al. 2017, Rao & Gan 2014, Ranasinghe et al. 2013, 2016).

Biofilms are surface attached microbial cells, embedded in an extracellular matrix derived from microbial cells and host components (Weerasekera et al. 2016). Biofilms show increased resistance to antibiotics using various mechanisms including activation of efflux pumps, drug modification, neutralization and hydrolyzation (Sharma et al. 2019). On the other hand, extracellular matrix composed with polymeric substances acts as an external physical barrier and reduces the permeability of antibiotics (Sharma et al. 2019). Majority of human microbial infections are biofilm infections and the resistant nature of biofilms makes the treatment and eradication of biofilms more difficult. So, the invention of novel effective antibiofilm agents with high efficacy is becoming a necessity. Though, herbal antimicrobials are considered as a potential alternative in prevention, treatment and control of microbial biofilm infections in clinical setting, intensive studies should be carried out to identify effective compounds with high therapeutic efficacy and low toxicity.

Pseudomonas aeruginosa, *Staphylococcus aureus* and *Klebsiella pneumoniae* are three major etiological bacterial agents which are responsible for a number of infectious diseases in human body. *P. aeruginosa*, a non-capsulate and non-spore forming gram negative bacterium, is responsible for nosocomial infections especially in critically ill patients and those with immunocompromised diseases, respiratory tract infections in patients with cystic fibrosis, and wound infections in patients with diabetes (Wijesinghe et al. 2018a, Bassetti et al. 2018). On the other hand *S. aureus* is a predominant causative bacterial agent of systaemic infections, infective endocarditis, skin and soft tissue, osteoarticular, pleuropulmonary and medical device-associated infections (Lowy 1998). *K. pneumoniae* is one of the well-known human nosocomial pathogen which causes urinary tract infections, community-acquired pneumonia and hepatic abscesses (Nordmann et al. 2009, Siu et al. 2012). Role of above three bacterial species in chronic skin wound infections is significant (Howell-Jones et al. 2005) and identification of effective anti-infectives for treatment purposes is becoming a major research interest in the last few years.

The current study is designed to evaluate the efficacy of plant natural product, *C. verum* leaf essential oil as an antibacterial and antibiofilm agent against *P. aeruginosa, S. aureus* and *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Three bacterial strains, *P. aeruginosa* (ATCC 25619)*, S. aureus* (ATCC 29213) and *K. pneumoniae* (ATCC 13883) obtained from Microbiology and Immunology Area, Piracicaba Dental School, UNICAMP, Brazil were used for this study.

The stocks of standard bacterial strains were maintained in Brain Heart Infusion agar (BHI, Kasvi, Brazil) slants at 4°C. Before using for experiments, organisms were subcultured in

freshly prepared BHI agar plates and incubated aerobically at 37°C for 24 h.

The standard inocula of bacteria were prepared by adjusting the turbidity by comparing with 0.5 McFarland scale, which was equivalent to the absorbance of 0.08-0.10 at 600nm, corresponding to 1.5 x 10^8 CFU/mL.

Preparation of essential oil dilutions

C. verum leaf EO was purchased from Romik Lanka Marketing Services, Sri Lanka (WCC/3569) for this study. The essential oil was diluted in Tween 80 (0.05%) solution and BHI broth to obtain 32 mg/mL working concentration.

Antibacterial effect of *C. verum* leaf essential oil vapor

Effect of *C. verum* leaf EO vapor on planktonic bacterial cells were qualitatively evaluated using Microatmosphere technique explained by Serban et al. (2011) with few modifications (Serban et al. 2011).

Standard suspensions of *P. aeruginosa, S. aureus* and *K. pneumoniae* were prepared in sterile normal saline (NS) by dissolving a small portion of isolated colony and adjusting the absorbance to 0.5 McFarland standard turbidity. Quality controlled fresh BHI agar plates were then inoculated with prepared standard bacterial cell suspensions separately with a sterile cotton swab to get a confluent growth.

A sterile, 2 cm diameter filter paper disc embedded with the 100 µL of working solution of EO was attached to the inner surface of the lid of the Petri dish. Plates were then sealed with a parafilm and plates were incubated overnight at 37°C aerobically. Control plates without filter paper disks were also prepared. Plates were examined for any growth inhibition of inoculated bacteria on agar surface after 24 h incubation.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined using the CLSI M7-A10 broth micro dilution method with few modifications (CLSI 2015). Standard cell suspensions of all test strains were prepared in sterile BHI broth using 24 hours old fresh bacterial cultures. Working solution (32 mg/mL) of EO was added to first well column of 96 well sterile flat bottomed microtiter plate and serially diluted with sterile BHI broth, to prepare serial dilutions of EO along the rows (100 µL/well). The plates were then inoculated with prepared standard cell suspensions separately (100 µL/well).

Negative control (Growth control): 100 µL of BHI broth + standard cell suspension. Positive control: Chlorhexidine digluconate (CHL, Sigma-Aldrich, USA).

The plates were then incubated for 24 h at 37°C and were examined visually for the presence or absence of turbidity of the content. To determine the MBC, 5 µL of solutions from each well were plated in fresh BHI agar plates and incubated at 37°C for another 24 h aerobically. The lowest concentration of essential oil requires to completely kill the bacterial population (no growth on BHI agar surface after overnight incubation) was defined as MBC.

Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$) and Minimum Biofilm Eradication Concentration (MBEC)

To form biofilms in, 96-well sterile flat bottomed microplate was seeded with standard inoculum of test organisms (100 µL/well), followed by incubation for 24 h at 37˚C. The plate was carefully washed once with sterile distilled water (DW) and 100 µL of the EO dilutions were

added to the treatment wells separately. 100 µL of BHI broth was added to the negative control wells. The plate was then incubated for 24 h at 37°C (Silva & Fernandes 2010).

Biofilm viability of treated biofilms was quantified using XTT viability assay. Briefly, the plates were washed with sterile DW and then incubated with 80 µL/well of the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2HTetrazolium-5-Carboxanilide, Sigma-Aldrich) and menadione (Sigma-Aldrich) solution (4 µL of menadione in 10 mL XTT) for 2 h. The absorbance of resulting solution was measured at 490 nm using microtiter plate reader (Versa MAX, molecular Devices, USA) (Wijesinghe et al. 2020).

The CFU assay was done to determine the MBEC. Briefly, adhered and 24 h treated biofilms were completely scraped and serially diluted in Phosphate Buffered Saline (PBS). 100 µL of prepared dilutions was spread on BHI agar medium separately. Agar plates were then incubated at 37°C for 24 h and the number of colonies was counted.

Scanning Electron Microscopy (SEM) for determination of post-exposure architecture of established biofilms

For visualization of post-exposure architecture of bacterial biofilms, 24 h established biofilms were formed on sterile 10mm diameter glass cover slips and treated with different concentrations of EO for 24 h as explained previously. Treated biofilms were subsequently fixed with 2% glutraldehyde for 2 h and dehydrated with ethyl alcohol. After overnight critical point drying, the specimens were coated with gold and the biofilms were visualized with the SEM (JEOL, JSM 5600LV) in high-vacuum mode at 10 kV (Wijesinghe et al. 2018b).

Cytotoxicity

Cytotoxicity of *C. verum* EO on HaCaT (normal human keratinocyte cell line) was determined using MTT viability assay described by Monks et al. (1991) with few modifications (Monks et al. 1991). Briefly, 100 µL/well of $6.5x10^4$ cells/mL were added to 96- well culture plates and incubated for 24 h at 37°C with 5% CO_2. Then the medium was replaced with different concentrations of cinnamon EO containing fresh medium. The treated plates were again incubated for another 24 h at 37°C with 5% CO_2. After the incubation, viability of treated HaCat cells were determined using MTT assay as follows. Wells of microplate were filled with 100 µL of 5 mg/mL MTT per well. Plates were then incubated at 37°C for 4h followed by addition of 100 µL of Dimethyl Sulfoxide (DMSO) per well. The absorbance was measured at 492 nm using a microplate plate reader.

Determination of chemical composition of the EO

The chemical analysis of *C. verum* EO was performed using HP-6890 (Agilent, USA) gas chromatograph with HP-5975 (Agilent, USA) selective mass detector. The experimental conditions were as follows (Wijesinghe et al. 2020).

Capillary Column: 30 m x 0.25 mm x 0.25 µm;

Temperatures: injector (220°C), detector (250°C), column (60°C), 3°C/min, 240°C;

Ionization source: 70 eV;

Flow rate of carrier gas (highly dried Helium): 1.0 mL/min.

NIST-11 (National Institute of Standards and Technology) mass spectral database and NIST mass spectral search program (Version 2.0g) were employed to identify the analytes.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 16 was used in statistical analysis. Multiple means were compared using one way ANOVA and two way ANOVA. The level of significance was taken at 5% ($p < 0.05$).

RESULTS

Antibacterial effect of *C. verum* leaf EO vapor

All three test strains showed a zone of growth inhibition on agar surface after the 24 h exposure to the cinnamon leaf EO vapor, which indicates the antibacterial effect of *C. verum* leaf EO vapor against planktonic cells of *P. aeruginosa*, *S. aureus* and *K. pneumoniae*.

MIC and MBC

The MIC and MBC values corresponding to the *C. verum* EO and the control antibacterial agent CHL are represented in Table I. *C. verum* exhibited the similar antibacterial efficacy on all three test strains with 0.5 and 1.0 mg/mL MIC and MBC respectively. The MBC/MIC ratio of the test revealed that the *C. verum* leaf EO has a bactericidal effect on all three bacteria tested.

$MBIC_{50}$ and MBEC

Minimum EO concentrations required to reduce the biofilm viability by 50% (compared to the viability of negative control/ biofilms without treatments) were defined as $MBIC_{50}$. $MBIC_{50}$ point was determined using XTT assay readings of test biofilms and control biofilms. Percentage reduction of biofilm viability after treatment of 24 h established biofilms with different concentrations of *C. verum* leaf EO and CHL was calculated (Figure 1). Similarly, MBEC is defined as the minimum concentration of treatment required to kill the 24 h mature biofilm completely. Microbial biofilms were subjected to CFU assay in order to determine the minimum concentration required to completely kill the biofilm (MBEC). CFU assay readings are presented in Figure 2. $MBIC_{50}$ and MBEC of *C. verum* EO and CHL on three test strains are indicated in Table II.

Scanning Electron Microscopy (SEM) for determination of post-exposure architecture of established biofilms

Ultrastructure of 24 h established biofilms of *P. aeruginosa*, *S. aureus* and *K. pneumoniae* after treating with $MBIC_{50}$ concentration of *C. verum*

Table I. **Results of MIC and MBC of test bacteria. Experiment was made in triplicates with three individual experiments. CHL was used as positive control.**

Organism	*C. verum* EO			Control (CHL)		
	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
P. aeruginosa (ATCC 25619)	0.5	1.0	2.0	0.125	0.25	2.0
S. aureus (ATCC 29213)	0.5	1.0	2.0	0.125	0.25	2.0
K. pneumoniae (ATCC 13883)	0.5	1.0	2.0	0.125	0.25	2.0

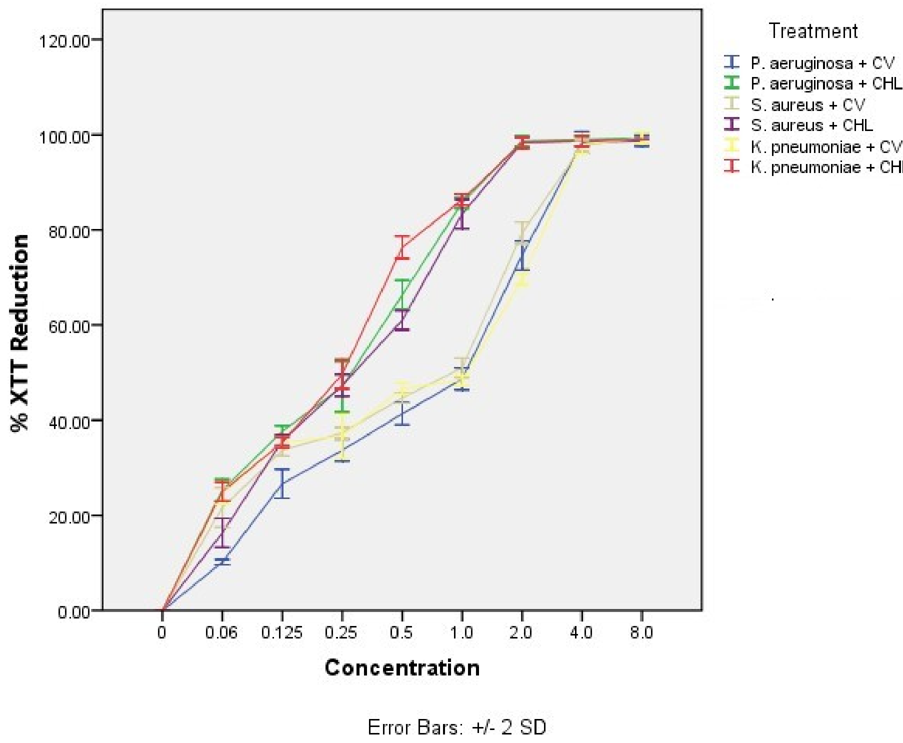

Figure 1. Percentage reduction of XTT metabolic activity of *P. aeruginosa* (ATCC 25619), *S. aureus* (ATCC 29213) and *K. pneumoniae* (ATCC 13883) 24h established biofilms after treatment with different concentrations of *C. verum* EO and Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD). CV: *C. verum* leaf oil, CHL: Chlorhexidine digluconate.

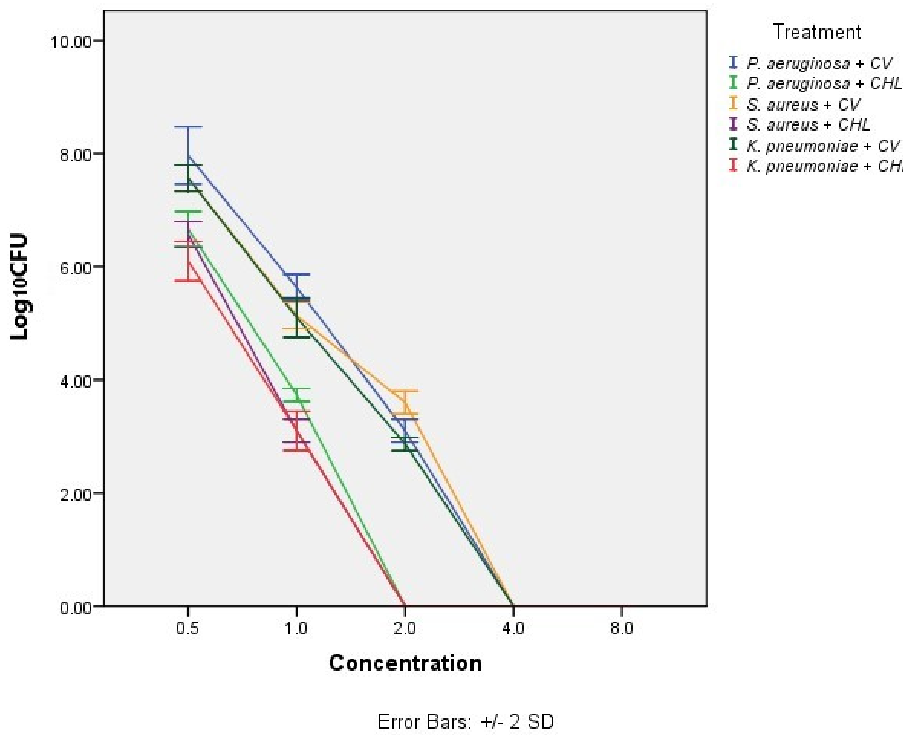

Figure 2. Log CFU values of 24h established *P. aeruginosa* (ATCC 25619), *S. aureus* (ATCC 29213) and *K. pneumoniae* (ATCC 13883) biofilms after the treatment with different concentrations of CV: *C. verum* leaf oil and CHL: Chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

Table II. Minimum Biofilm Inhibitory Concentrations ($MBIC_{50}$) and Minimum Biofilm Eradication Concentration (MBEC) for 24h established biofilms of *P. aeruginosa* (ATCC 25619), *S. aureus* (ATCC 29213) and *K. pneumoniae* (ATCC 13883). CHL: Positive control Chlorhexidine digluconate.

	***P. aeruginosa* (ATCC 25619)**		***S. aureus* (ATCC 29213)**		***K. pneumoniae* (ATCC 13883)**	
	C. verum	**CHL**	***C. verum***	**CHL**	***C. verum***	**CHL**
$MBIC_{50}$ (mg/mL)	1.0	0.25	1.0	0.25	1.0	0.25
MBEC (mg/mL)	4.0	2.0	4.0	2.0	4.0	2.0

leaf oil and CHL was qualitatively evaluated using SEM (Figure 3).

Both *C. verum* leaf EO and CHL caused bacterial cell shrinkages, cell wall damages and deformities, along with decreased biofilm cell density.

Cytotoxicity

In-vitro antiproliferative activity of *C. verum* leaf EO and CHL was determined using immortalized human keratinocyte cell line (HaCaT). There was no significant change in HaCaT cell viability at all concentrations of *C. verum* EO and CHL (0-1000 mg/mL) tested. This indicates the non-toxic nature of the treatment on human cells.

Determination of chemical composition of the EO

Based on GC-MS, Eugenol was identified as the most abundant chemical compound. The relative abundance of eugenol (Figure 4) was 77%. Benzyl benzoate (5%), Trans caryophyllene (3%), Eugenol acetate (3%) and Linalool (2%) were found as minor components.

DISCUSSION

Though there are number of Cinnamon species including *C. cassia* (Chinese Cinnamon), *C. burmannii* (Indonesian Cinnamon), *C. citriodorum* (Malabar Cinnamon), *C. loureiroi* (Vietnamese Cinnamon) and wild species all over the world (Chen et al. 2014), *C. verum* (Syn. *C. zeylanicum*/ Ceylon Cinnamon) is considered as “True Cinnamon” since it contains relatively small amount of toxic compound, coumarin and high amount of cinnamaldehyde in its’ EOs (Ranasinghe et al. 2013).

Apart from the use as a common culinary spice, cinnamon has been employed as a phytomedicinal therapeutic alternative against many human disease conditions in different cultures. The current study was conducted to evaluate the antibacterial efficacy of *C. verum*/ Ceylon cinnamon leaf EO against three common pathogenic bacterial species, namely *P. aeruginosa*, *S. aureus* and *K. pneumoniae*. Though there are published data on antibacterial activity of cinnamon bark oil, the antibacterial and antibiofilm activity of *C. verum* leaf EO on human pathogenic bacterial strains is not widely studied. The results obtained from the present study indicated that *C. verum* EO has an efficient antibacterial and antibiofilm activity on all the tested strains.

Microatmosphere technique was employed in antibacterial screening of *C. verum* leaf EO. Results demonstrated the antibacterial potency of EO vapor against planktonic bacterial species. The essential oil extracted from *C. verum* leaves is highly volatile and it vaporizes at the normal body temperature. So the actual effect may not be caused by liquid phase EO in *in-vivo* conditions. Hence it’s important to evaluate

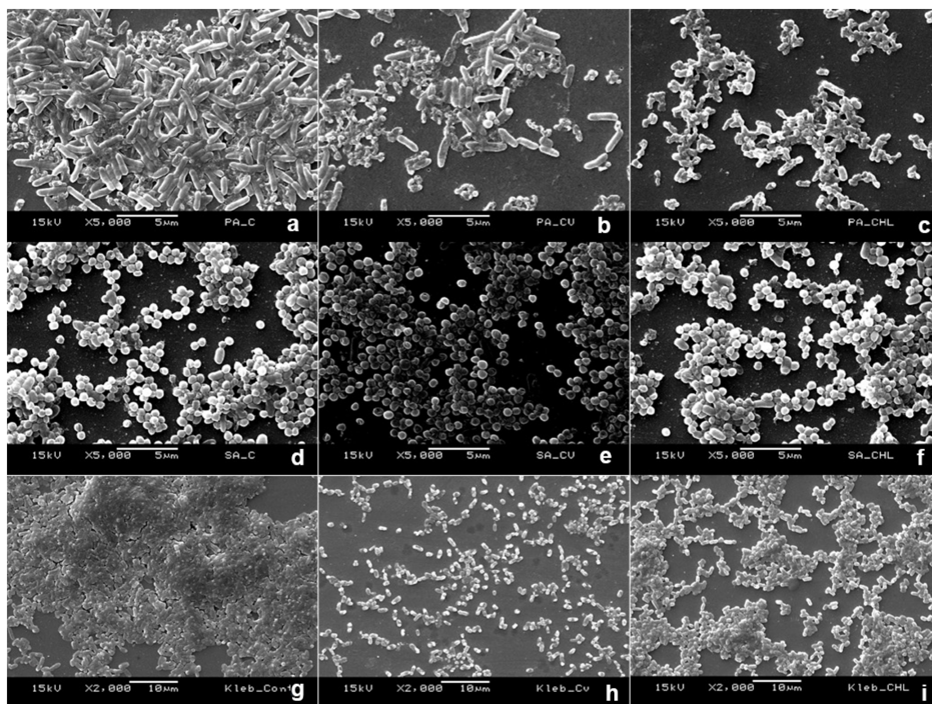

Figure 3. **SEM images of *P. aeruginosa* (a, b and c), *S. aureus* (d, e and f) and *K. pneumoniae* (g, h and i) 24h established biofilms after 24h treating with 1.0 mg/mL *C. verum* leaf EO (b, e and h) and 0.25 mg/mL CHL (c, f and i). a, d and g: Negative control biofilms.**

the efficacy of vapor phase of the volatile oil to predict the efficacy *in-vivo*. On the other hand, high volatility of the oil can cause negative or false results during routine antimicrobial screening tests including disk diffusion and well diffusion due to poor water solubility and poor diffusion through the medium. In this study, Microatmosphere technique was employed in antimicrobial screening to avoid above errors and to get an accurate readings (Sabir et al. 2017).

In the present study, *C. verum* leaf oil vapor demonstrated an antibacterial activity by exhibiting a growth inhibition with *C. verum* essential oil vapor.

Minimum Inhibition of all tested bacterial strains was achieved with 0.5 mg/mL (MIC) and minimum concentration required to kill the planktonic bacterial population completely was 1.0 mg/mL (MBC). Importantly, MBC/MIC ratio of the current study was 2.0 for all three test strains. According to the classification of antimicrobial agents published by Pankey & Sabath in 2004, *C. verum* leaf oil demonstrates a bactericidal effect on *P. aeruginosa*, *S. aureus* and *K. pneumoniae* (Pankey & Sabath 2004).

Further, based on the classification of antimicrobial potency of a natural product by Durate et al. 2005, (Duarte et al. 2005), *C. verum* EO shows a high antimicrobial activity against test strains with a MIC value of 0.5 mg/mL. Similarly, a study conducted by Oussalah et al. in 2006 observed a high antibacterial effect of *C. verum* leaf EO against meat isolates of *Pseudomonas putida* (Oussalah et al. 2006). At the same time, another study carried out by Oussalah et al. in 2007 observed a weak antibacterial activity of Cinnamon leaf EO on four pathogenic bacterial strains, *Escherichia* coli O157:H7, *Salmonella Typhimurium*, *S. aureus* and *Listeria monocytogenes* in planktonic state (Oussalah et al. 2007). The concentration of active compound in crude extract of oil, interferences of other compounds, species used to extract oil, methods used for antimicrobial screening and

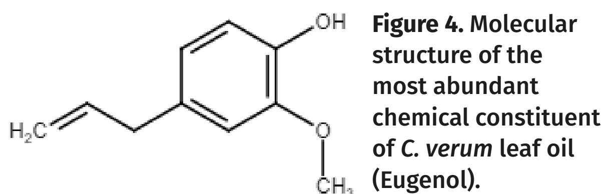

Figure 4. Molecular structure of the most abundant chemical constituent of *C. verum* leaf oil (Eugenol).

many other factors can influence this type of discrepancies of observations.

Since biofilm infections are becoming more prevalent and significantly affect the treatment efficacy, identification of novel strategies as well as evaluating the efficacy and applicability of available antimicrobials in biofilm eradication or prevention is a top priority when the microbial infections are concerned. Further, the available statistics emphasize the necessity of utilizing nontoxic, cost effective and accessible therapeutics in treatment of infectious diseases. So the potential applications of herbal medicines/ phytomedicines in microbial infections control, especially biofilm control should be widely studied. The present study evaluated the biofilm removal and eradication effects of true cinnamon leaf EO on three pathogenic bacterial biofilms.

50% reduction in biofilm viability of 24 h established bacterial biofilms was achieved at 1.0 mg/mL EO concentration whereas complete eradication of mature biofilms were noted at 4.0 mg/mL concentration. Though there are some published evidences on antibiofilm activity of Cinnamon bark EO, no data were found on antibiofilm activity of cinnamon leaf EO on bacterial biofilms.

SEM was conducted to evaluate the structural changes of the preformed bacterial biofilms with the EO exposure. SEM images revealed the characteristics of *C. verum* EO antibacterial action by indicating cell wall damages and deformities, cell shrinkages as well as leakages of intracellular compartment. The active and most abundant chemical constituent in Ceylon cinnamon leaf EO, Eugenol can cause above cellular alterations by acting upon cell wall and extracellular matrix. Since Eugenol is a lipophilic molecule, it can actively penetrate the phospholipid bilayer of cell membrane, alter its fluidity and permeability and ultimately disrupt it. Further, some published data suggests that Eugenol can facilitate the protein leakage of cell membranes in both gram positive and gram negative bacteria. The damaged cell walls and cellular leakages eventually cause death of microbial cell (Wijesinghe et al. 2020, Marchese et al. 2017, Pavesi et al. 2018).

Similar types of chemical profiles as current study were obtained from few chemical analysis experiments carried out previously. Paranagama et al. in 2001 analyzed the chemical constituents of *C. zeylanicum* leaf EO using GC-MS and identified Eugenol (76%) as the most abundant component followed by minor components, Cinnamaldehyde (3%), Linalool (3%) and ß caryophyllene (3%) etc (Paranagama et al. 2001). Similarly another study identified Eugenol (74.9%), β-caryophyllene (4%), Benzyl benzoate (3%), and few other components in *C. verum* leaf oil (Schmidt et al. 2006).

Since the toxicological assessment of a novel therapeutic agent is essential prior to its clinical application, this study assess the *in-vitro* toxicity of Cinnamon leaf EO on human cells, using the HaCaT cell line model. Interestingly, no cytotoxic/ anti proliferative effect was noted after the treatment with the highest possible concentration/ neat concentration (1000 mg/ mL) of *C. verum* leaf EO. Since there are lack of published scientific evidence on toxicity of cinnamon leaf oil on human cells, further studies were recommended with different human *in-vitro* cell lines and *in-vivo* experimental models to get the complete understanding of the toxicity of the EO before therapeutic applications.

With all discussed observations, we can conclude that *C. verum* leaf essential oil shows a high antibacterial and antibiofilm activity against *P. aeruginosa*, *S. aureus* and *K. pneumoniae* without causing any toxicity to human cells.

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All authors contributed extensively to the work presented in this paper and helped to draft the manuscript. All authors read and approved the final manuscript.