EFFECTS OF DIESEL EXHAUST PARTICLES ON *in vitro* BACTERIA BIOFILMS GROWTH AND EVALUATION OF ANTI-BIOFILM/ANTIMICROBIAL POTENTIAL OF A FEW SELECTED PLANT EXTRACT

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BIOTECHNOLOGY
BY

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DEPARTMENT OF BIOTECHNOLOGY

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Submitted
In partial fulfilment of the requirements for the
Degree of Master of Philosophy in Biotechnology

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CERTIFICATE

This is to certify that the dissertation entitled "Effects of diesel exhaust particles on *in vitro* bacteria biofilms growth and evaluation of anti-biofilm/antimicrobial potential of a few selected plant extracts" submitted to the Mizoram University for the award of Master of Philosophy in Biotechnology by Ruth Zomuansangi Registration no. MZU/M.Phil. /628 of12.06.2020, a Research scholar in the Department of Biotechnology, is a record of research work carried out by her during the period from 2019 to 2021 of study, under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma or other similar titles in this University or any other University or institution of higher learning.

It is further certified that the scholar fulfilled all the requirements as laid down by the University for submission of the M. Phil. dissertation.

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Place:

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Mizoram University

August 2021

I Ruth Zomuansangi, hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this dissertation did not form the basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to Mizoram University for the degree of Master of Philosophy in Biotechnology.

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CHAPTER 1

1. INTRODUCTION

The Diesel Exhaust Particles (DEP) is an imperative anthropogenic pollutant and is produced from the incomplete combustion of diesel fuels due to the increase in the number of diesel-fuelled transportation (Steiner et al.2016; Lawal 2018). The International Agency for Research on Cancer (IARC) in 2012 has grouped DEP as carcinogen group 1 based on the epidemiology study and is referred to be mutagenic and carcinogen to humans by the WHO (World Health Organization) in 2010 (Steiner et al.2016). The inhalation with PM2.5 emission faction and exposure to DEP is a risk factor for respiratory diseases, pulmonary diseases, otitis media, and cardiovascular diseases it as well increases the inflammation of the airway and reduces the lung function potential (Yang et al.2018; Shears et al.2019). The infection due to the DEP also depends on the duration of the exposure and the size of the particles inhaled, the finer the particles the more hazardous it gets (Lawal 2018). World-wide the vehicle emissions and pollution by the industrial are progressively known to be the major cause of air pollution that exerts adverse effects on human health (Landrigan et al.,2018). DEPs are a particulate component of diesel exhaust that includes sulfates, metal abrasion particles, silicates, ash particles, aerosols, and diesel soot (Steiner et al.,2016). The DEPs are respirable particles with a size range in diameter from 0.02 to 0.2 μm (Chen et al.,2018). DEP can adsorb many organic compounds on their surface including mutagenic and carcinogenic polycyclic aromatic hydrocarbons. DEP is respirable particles and during gas exchange deposits in the larynx, pharynx, distal lungs, nasopharynx, etc. (Shears et al.,2019).

Various studies in past suggest that DEP exposure increases microbial infections and aggravates respiratory infections (Chandel et al., 2018). In precise, there are strong signs to propose that DEPs exposure is linked to cardiovascular disease as well as respiratory disease (Attiq et al., 2018). Inhalation of DEP has been reported to reduce lung function, impair clearance of respiratory pathogens, and increase lung inflammation. The human nasopharynx microflora occupies in nasopharynx just after birth, and along with commensal bacteria forms a specialized structure called biofilm (Chen et al.,2018). The respiratory infectious bacteria and DEP interact in the nasopharynx and previous studies suggest that respiratory bacteria such as *Streptococci, Staphylococci, H. influenza* forms biofilms (Kadioglu et al.,2008). *Streptococcus pneumoniae* (*S. pneumoniae*), is a gram-positive bacterium colonized asymptomatically in the human nasopharynx by 40% in healthy children and 20% in healthy adults. However, in the elderly and infants, *S. pneumoniae* can distribute to the sterile site causing invasive opportunistic diseases leading to cause pneumonia, otitis media, meningitis, and septicemia (Gilley and Orihuela 2014; Sanchez et al.2011). The duration of the colonization of the *S.*

pneumoniae in the nasopharynx also decreases in number with age and varies from 2 weeks to 17 weeks. Although the colonization of the *S. pneumoniae* is asymptomatic it gets pathogenic as the bacteria invade other parts of the body such as the lungs, brain, and blood (Loughran et al.2019). It is quite not well known of the conditions that allow the bacterium to progress from a harmless organism to a potentially life-threatening pathogen; however, it is understood that exposure to a high level of pollutant is a known risk factor for the IPD (invasive pneumococcal disease). Some studies reported that respiratory tract infection risk increases by bacteria biofilms established in the nasopharynx, and these biofilms act as a reservoir of bacteria. Moreover, the effect of pollutants such as DEP is not well understood. Whether the presence of DEP increases bacteria biofilms and provides a surface for biofilm growth. Recent studies reported that urban particles and Asian sand dust (ASD) particles exposure increase *Streptococcus pneumoniae in vitro* biofilms and *in vivo* colonization and decrease human defense (Yadav et al.,2020 a; Yadav et al., 2020b). The bacteria biofilms are tolerant to antibiotics and the emergence of antibiotic-resistant bacteria further complicates the disease management. Therefore, to manage respiratory infection there is a need for unique bioactive compounds with antimicrobial/antibiofilm and anti-inflammatory/antioxidant activities. Mizoram, northeast India, near the Indo-Burma border (22º 40’ N; 93 º 0 3’ E) is one of the hot diversity zone habitats of many medicinal plants traditionally used by local people as medicine. Medicinal plants with bioactive compounds such as (stilbenes) are a potential source of antibiofilm/antimicrobial and antioxidant & COX-2 inhibitory compounds that could be explored to control bacterial infection and PM toxicity (Vestergaard et al., 2019).

Tithonia diversifolia is one of the utmost significant medicinal plants of the Asteraceae family which is widely in use in the Ayurvedic and Unani systems. The plant parts such as its roots, shoots, leaves are used in various diseases. It is also known as the Mexican sunflower, a shrub-like perennial (Ajao & Moteetee, 2017). The research of *T. diversifolia* by Pharmacological indicates its many active constituents like antimicrobial, anti-inflammatory (Owoyele et al., 2003, D.A. Chagas-Paula et al. 2011) anti-fungal, anti-malarial (Goffin et al., 2001, Madureira, 2002), and cancer chemopreventive activities (Bork PM et., al 1996).

The previous studies reported that medicinal plants such as stilbenes possess dual activities, such as antibiofilm/antimicrobial potential along with antioxidant & anti-inflammatory activity (Vestergaard et al., 2019; Yadav et al., 2019).

Particulate matter such as DEP and *S. pneumoniae* are some of the risk factors for respiratory infection, and how DEP exposure affects pneumococcal colonization/biofilms in the nasopharynx is not well known and studied. So, in the study, we elucidated the effect of

DEP on *pneumococci in vitro* growth of biofilms and evaluated the effects of *Tithonia diversifolia* plant extract to control *S. pneumoniae* growth in planktonic and in biofilms.

CHAPTER 2

2. REVIEW OF LITERATURE

India is severely affected by air pollution, and among the 30 most polluted cities worldwide 22 cities are in India (Yadav et al., 2017). Air pollutants such as DEP settles in the larynx, nose, pharynx, bronchi, trachea, and distal lung and increase microbial infection of the respiratory tract. Although, some researchers separated study the toxicity of particulate matters and bacteria (Ganesh et al., 2019; Yadav et al., 2017; Boovarahan et al, 2018). Studies suggested that in India the incidences of pneumonia are higher, and account for more deaths (three times in comparison to other countries) among children under age 5 than any other infectious disease, and the elevated incidences of pneumonia are attributed to household smoke exposure (Arlington et al., 2019). However, in India, no study was reported to study the effects and interaction of air pollutants on respiratory bacteria colonization /biofilms in the nasopharynx. The air-pollutant containing particulate matters such as DEP, urban particles, Asian sand dust particles, etc. are a major risk factor for respiratory tract infections (Park et al.,2006). A recent study reported that indoor air pollution exposure may be one of the factors to cause nasopharyngeal carriage of bacterial species and a major risk factor for respiratory tract infection. Further study showed that exposures to such pollutants are associated with an increased prevalence of specific nasopharyngeal bacteria during babyhood (Vanker et al., 2019). A similar study reported that solid fuel exposure is associated with childhood pneumonia (Adaji et al., 2019). Enhanced biofilm formation of *Streptococcus Gordonii* (Huang et al., 2014), *Staphylococcus aureus (Kulkarni et al., 2012), Pseudomonas aeruginosa* (Antunes et al., 2012) and *Candida albicans* (Semlali et al., 2014) has been detected in presence of cigarette smoke. It has been suggested that smoke alters gene expressions of bacteria and induces biofilm-related gene expression that elevates biofilms. In addition, *Staphylococcus aureus* adhesion to epithelial cells and reduced exposures to macrophages has been detected following cigarette smoke exposure (Kulkarni et al., 2012; McEachern et al., 2015). Woo et al., (2018) investigated the effect of different concentrations of fine particulate matter on biofilms and colonization to respiratory epithelial cells. The results of their study showed that fine particulate matters alter the hydrophobicity of bacteria surface and elevated bacteria in vitro biofilms formation and colonization to the human cell line (Woo et al., 2018). Hussey et al., (2017), studied the effects of black carbon on the in vitro and in vivo biofilm formation of *Staphylococcus aureus* and *Streptococcus pneumoniae.* The results of their study showed that exposure to black carbon stimulates structural, functional, and compositional in the biofilms of both *Streptococcus pneumoniae* and *Staphylococcus aureus* Hussey et al., (2017). In addition,

biofilms antibiotic tolerance were significantly affected and the bacteria colonization to the nasopharynx was increased and spread to the lungs in occurrence of black carbon particles (Hussey et al., (2017). Similarly, Mushtaq et al., (2011) detected increased *Streptococcus pneumoniae* adhesion in the human airway epithelial cells in the presence of urban particles and particulate matters stimulated adhesion was interceded by oxidative stress and PAFR. (Mushtaq et al., 2011). ASD exposure increase *TNF-α, MUC5AC, COX-2,* and *MUC5B* mRNA expression in middle ear epithelium cells and induces apoptosis- and oxidative stress-related gene expressions (Chang et al., 2015). A recent study shows that *in vitro* biofilms of *Streptococcus pneumoniae*, and establishment to Human middle ear epithelium cells (HMEEC), and *in vivo* colonization in middle ears mucosa of rats were amplified in presence of ASD particles (Yadav et al., 2020).

The natural Phyto-compounds a very good source of anti-biofilm/antimicrobial. Stilbenes family of *Vitis vinifera* (Parage et al.2012), plants containing active compounds such as resveratrol (Stilbene monomer) present in various families of plants including grapevines (*Vitis*), *Vitaceae*, and *Care* possess antioxidant and antimicrobial activities (Shimizu et al., 2000). ε-viniferin (dimeric resveratrol) including grapevines (*Vitis*), *Vitaceae*, and *Carex*, shows hepato-protective, P450 inhibitory antioxidants, and antimicrobial activities (Fiorentino et al., 2008). Furthermore, resveratrol alters the expression of virulence traits of the bacteria leading to inhibition of biofilm formation, reduced toxin production, interference with quorum sensing, and reduced motility (Vestergaard et al., 2019). The evolving research over the past few years purposed that quorum sensing regulates bacterial pathogenesis and biofilms formation by sporulation, controlling competence development, virulence induction, antibiotic synthesis, nutrient flux, and cell differentiation, along with other physiological events in pathogenic bacterial infections (Omwenga et al.,2017). Similarly, the anti-biofilm activities ε-viniferin and its derivatives have been reported contrary to Gram-negative bacteria such as *Escherichia* (Cho et al., 2013). Recently, our group study (Yadav et al., 2019) elucidated the anti-biofilm activity of natural and synthetic ε-viniferin and their derivatives against *Streptococcus pneumoniae* biofilms (Yadav et al., 2019). A previous study showed the antibiofilm potential of phytocompound eugenol and *eugenia caryophyllata* extract contrary to methicillin-resistant and sensitive *Staphylococcus aureus* clinical strain biofilms and *Streptococcus pneumoniae* (Yadav et al., 2015; Yadav et al., 2012). Many organic compounds such as coumarins, alkaloids, flavonoids, cinnamates, stilbenes, and xanthine possess the COX-2 inhibitory activity that involves inflammation (Chandel et al., 2018; Attiq et al., 2018). *Rubus ulmifolius* kill planktonic pneumococcal cells and disrupt pneumococcal biofilms

(Talekar et al.,2014). Mizoram, northeast India, near the Indo-Burma border (22º 40’ N; 93 º 0 3’ E) is one of the hot diversity zone habitats of many medicinal plants traditionally used by local against human pathogenic bacteria (Passari et al., 2016; Mishra et al., 2016). The northeast hot diversity zone plants could be explored to elucidate the antibiofilm/antimicrobial potential that could be further explored to develop a new drug against bacteria biofilms.

CHAPTER 3

3. METHODOLOGY

3.1 Bacteria Strain and Culture Medium

Streptococcus pneumoniae D39 strain used in this study was purchased from MTCC, Chandigarh, India. *S. pneumonia* strain D39 is an Avery strain, serotype 2, and well known for its invasive property (Avery et al. 1944). The whole genome sequence of D39 strain is available on NCBI and is well known strain used for research purpose.

S. pneumoniae was grown in brain heart infusion (BHI) broth, and for longer use and storage glycerol stocks of bacteria were prepared and maintained at −80°C until use. For growing the bacteria colonies blood agar plate (BAP) which is supplemented with 5% of sheep blood was used. The DEP used for this study was purchased from the National Institute of Standard and Technology (NIST), the USA as previously reported (Song et al.,2008; Yadav et al 2020).

3.2 Plant Extract Preparation

Tithonia diversifolia plant was collected from the roadside towards Mizoram University campus, Tanhril, Mizoram. The plant leaves were collected and dried in a hot air oven at 37°C and ground into a fine powder using a domestic mixer grinder. The powder obtained was then weighed and the extract was prepared by soaking it in methanol for 48 hours. The plant extract was prepared according the protocol reported previously by Singh et al., (2016). The plant extract after methanol treatment was filtered through a Whatman no. 1 filter paper. The filtration process was repeated three time followed by the evaporation to dry at 40°C with the help of the rotary evaporator (BUCHI, Switzerland) that resulted in the crude plant extract. The extracts were then kept at 4°C until further use (Singh et al,.2016).

3.3 *In vitro* evaluation of diesel exhaust particles on bacteria planktonic growth

In order to evaluate the effects of DEP on pneumococci growth planktonically, the D39 strain of S. pneumoniae was grown in broth medium supplied with different concentrations of DEP 50µg, 100µg, and 200µg . The growth of the bacteria was detected by measuring its optical density at 600nm (OD_{600}) at different periods by the procedure previously reported (Yadav et al.,2020). The planktonic growth experiment was repeated 3 times in replicates. The statistical significance was calculated with help of excel using the student's t-test, and *p-value less than 0.05 were considered significant.

3.4 *In vitro* evaluation of diesel exhaust particles on bacteria biofilm growth

In vitro bacteria, biofilm formation in the presence of DEP was studied using a static microtiter plate assay (Christensen et al. 2000, Yadav et al., 2015) with different concentrations of DEP from 50µg, 100µg, and 200µg. *S. pneumoniae* colony grown on BPA was then grown in BHI broth up till log-phase. The bacteria cell suspension was prepared by diluting cells 1:200 and bacteria suspension was inoculated in 24-well (1 ml) plates and 200ul in 96-well plate. The plate was incubated for 18 hours at 37°C. After the incubation period, the suspended medium was removed by slowly pipetting out, and biofilms at the bottom of the plate were washed twice with distilled water and stained with 0.1% crystal violet (CV) solution for 15 min. The stained biofilms were again washed with phosphate buffer saline (PBS) twice and dissolved in 1 ml (24-well plate and 200ul for 96-well plate) of ethanol. The absorbance of the biofilm was measured at 570nm using a spectrophotometer (Multiskan Skyhigh Microplate Spectrophotometer, Thermo Fisher Scientific). The biofilm growth experiment was repeated two times and the experiment was performed in triplicates. The statistical significance of the results was calculation using the student's t-test, by considering *p-value less than 0.05 as significant.

For viable bacteria detection within the biofilms, after washing the biofilms with PBS, it was dissolved in sterile water followed by sonication for 10 seconds to dissolve biofilms. The diluted biofilm suspension was serially diluted and spread on BAP to count the colonies. The colony counting units (CFU) of the biofilms were detected by colony formed counting after 24 hours incubation at 37°C (Yadav et al.,2019).

3.5 *In vitro* evaluation of metal ion constitutes of diesel exhaust particles on bacteria planktonic growth

DEP contains various components including metal ions. To study the effects of metal ion constituents of DEP on bacterial growth, planktonic bacteria were grown in a metal ion-free medium supplied with different concentrations of DEP from 50µg, 100µg, and 200µg. A metal ion-free medium was prepared by treating the BHI broth with Chelex 100 (Sigma, USA) for 12 hrs on a shaker followed by sterilization of medium via filter sterilization. Chelex 100 chelates metal ion present in the medium. Bacteria grown on BAP medium were further grown in BHI broth medium up till the log phase, and the cells were pelleted out by centrifugation and dissolved in a metal ion-free medium. The bacteria suspension in a metal ion-free medium

was supplemented with DEP at a concentration of 50µg to 200µg/ml and incubated at 37°C for 48hrs. After the incubation period, the absorbance was measured at 600nm at different time points (Yadav et al.,2020). To calculate the statistical significance, the planktonic growth experiment was performed in triplicates and repeated two times. The results were represented as mean and the statistical significance was calculated using the student's t-test, and *p-value less than 0.05 were considered significant.

3.6 *In vitro* evaluation of effect of metal ion constitutes of diesel exhaust particles on bacteria biofilm growth

The effect of metal ion present in DEP on pneumococci in vitro biofilm was evaluated using static microplate assay previously reported and used by our group (Christensen et al., 1986; Yadav et a., 2015). Briefly, the S. pneumoniae colonies grown on solid BAP were scraped and dissolved in broth, then the colonies grown in BHI broth medium up till log phase. The cells were pelleted by centrifuging and dissolved in sterile metal ion-free medium supplied with different concentration of DEP from 50µg, 100µg, and 200µg . The bacteria cell suspension in a metal ion-free medium were inoculated in 24-well (1 ml) plates and incubated at 37°C for 48 hours. After the incubation period, the medium was removed by carefully pipetting out, and biofilms were washed twice with sterile water. The biofilms at the bottom of the plate were stained with 0.1% CV solution for 15 minutes. The biofilms were washed with PBS twice by carefully pipetting, followed by dissolving in 1ml (24-well plate) of ethanol. After washing with PBS, the biofilms were dissolved in sterile water followed by sonicating for 10 seconds. The absorbance of the biofilms was measured at 570nm using a spectrophotometer.

The viable colonies of the bacteria within the biofilms were detected by colony-forming unit (CFU) counting of the pneumococci biofilms. After washing, the biofilm suspension was serially diluted followed by the spread on BAP. The colonies of pneumococci on BAP was counted after 24hours of incubation at 37°C (Yadav et al.,2020). The statistical significance calculation was carried out using student's t-tests, and *p values less than 0.05 were considered significant.

3.7 Minimum Inhibitory concentration (MIC) detection and antimicrobial activity of *Tithonia diversifolia* plant extract

Tithonia diversifolia plant extract MIC was detected using the microdilution method (Yadav et al.,2019). The S. *pneumonia* was grown in a BHI broth medium with different concentrations of plant extracts. The bacteria growth was detected by measuring the optical density at 600nm by the spectrophotometer MIC of the plant extract against pneumococci was the concentration of the extract at which no bacteria growth was visible.

3.8 Evaluation of the antimicrobial potential of *Tithonia diversifolia* plant extract in bacteria planktonic growth

An initial experiment with plant extract showed the inhibitory activity of plant extract on pneumococci growth, therefore, to examine the bactericidal or bacteriostatic effect of plant extracts against bacteria, a bacteria-killing experiment was carried (Yadav et al., 2019). *S. pneumoniae* D39 cell suspension was grown in BHI broth up till the early log phase, and the aliquots were treated with 2xMIC concentrations of plant extracts (10 µg), and the vehicle control samples were treated with DMSO, and the bacteria were grown further at 37°C. and viable bacteria were detected at the different periods (0, 6, 12, and 24 h) by the CFU counts.

3.9 Evaluation of the antibiofilm potential of *Tithonia diversifolia* plant extract in bacteria biofilm growth

To evaluate the effects of plant extracts on bacteria biofilms, *In vitro* S. pneumoniae D39 biofilms were grown in 24 well-plates and treated with different concentrations of plant extract. The alteration in biofilm biomass was detected by a static biofilm model using crystal-violet staining. The viable colonies of bacteria within biofilms were detected by CFU counts Yadav et al., 2019).

3.10 Evaluation of antibiofilm/antimicrobial mechanism of *Tithonia diversifolia* plant extract by crystal violet absorption assay

Tithonia diversifolia plant extract showed remarkable antimicrobial and anti-biofilm potential. The mode of action and mechanism of plant extracts antibiofilm potential was detected using crystal violet adsorption assay**.** The *S. pneumonia* was grown in BHI broth up till the early log-

phase and the cells were pelleted out by centrifuging at 45000g for 5 minutes at 4°C. The bacterial cells were then washed with PBS by carefully pipetting and treated with the plant extract and penicillin for 2 hours at 37°C. Penicillin was used as the vehicle control as it has no or very less effect (penicillin inhibits growing cells) on the bacterial cell membrane. Negative control was treated with DMSO (extract was dissolved in DMSO). The cells were harvested by centrifugation at 9300g for 5 minutes and treated with 10µl/ml CV solution (0.01%). The cell suspension was then incubated for 10 minutes and centrifugated at 13400g for 15 minutes. The OD of the cell suspension was detected at 590nm. (Yadav et al., 2019). The absorbance (OD600) value of the original solution CV solution was considered as 100%, and the percentage of CV absorption was detected by the follows formula:

$$\frac{OD\ value\ of\ the\ sample}{OD\ value\ of\ the\ crystal\ violet\ solution} \text{ X } 100$$

CHAPTER 4

4. RESULTS

4.1 Diesel exhaust particles exposure elevated the planktonic growth of the bacteria

S. pneumoniae D39 strain was grown planktonically in Brain Heart Infusion Broth (BHI) Media and with Diesel Exhaust Particles (DEP) at a different concentration from 50µg, 100µg, and 200µg and the vehicle control with no treatment of the DEP. The samples were then incubated at 37°C and the optical density was detected at different time intervals such as at 0, 2, 4, 6, 8, 10, 12, and 14hrs by the spectrophotometer at 600nm. The bacteria started to grow from 2-4hrs of incubation and the growth of the bacteria with the treatment of DEP is noticeably increased from 6hrs to 10hrs of incubation in comparison with vehicle control and reached its highest growth at 12hrs of incubation. The bacteria are then entering the early lag phase after the 14hrs of incubation. At 10 and 12hrs, pneumococcal growth was suggestively raised in the sample containing DEP matched to the vehicle control. The highest bacterial growth was detected in the sample containing 200µg of DEP. The error bar shown in the figure is the standard deviation from the mean value. The study was performed in triplicates and was repeated 2 times.

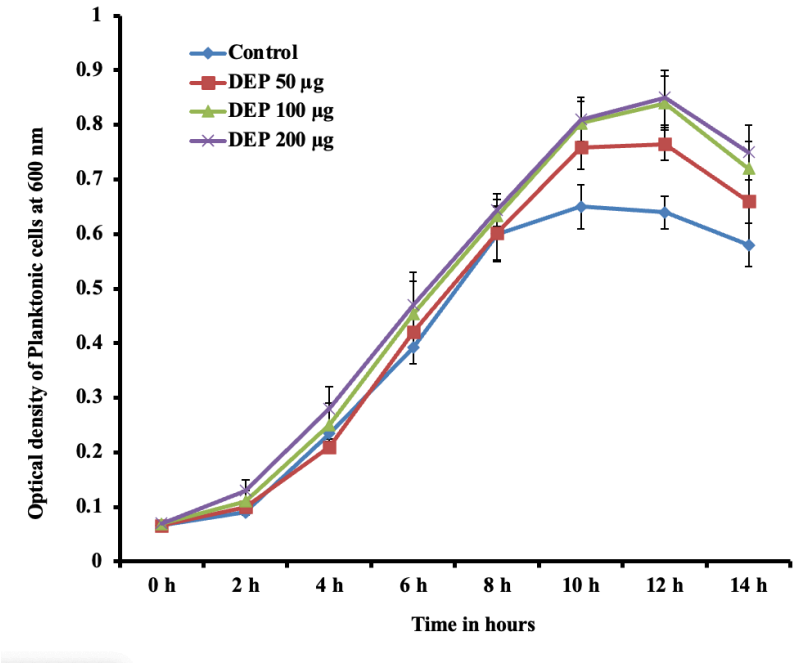


Figure 1: *Streptococcus pneumoniae* D39 strain, planktonic growth in BHI medium supplied with different concentrations of diesel exhaust particles (DEP). Bacteria growth of bacteria suspension was detected by measuring optical density at different time interval at 600nm. The growth of bacteria in presence of DEP was elevated in compare to the control. Error bar showing standard deviation from mean value. The study was performed in triplicates and repeated 2 times.

4.2 DEP exposure elevated *S. pneumoniae in vitro* biofilm growth

S. pneumoniae D39 strain *in vitro* biofilms were grown in BHI broth media with different concentrations of DEP (50µg, 100µg, and 200µg) along with the vehicle control with no treatment. The biofilms of *S. pneumoniae* were grown in a 24-well plate and, the samples were then incubated at 37°C and the optical density was detected after 18 hours of incubation. After incubation, the biofilm formed in the well was washed twice with decontaminated water and was stained with the crystal violet solution (0.1.%) and the optical density of the biofilm was measured by the spectrophotometer at 570nm. The biofilm growth of the bacteria with the treatment of DEP in different concentrations is significantly increased in comparison with vehicle control. The DEP treated 50µg shows a greater growth of the bacteria biofilms than the vehicle control and, 100µg and 200µg showed a significant ($p< 0.05$) growth of the bacteria biofilms (Figure 2 A). *Streptococcus pneumoniae* formed compact biofilms at the bottom of the plate in DEP supplemented sample in contrast to the control samples (Figure 2 B) The error bar is showing the standard deviation of the mean value. The study was performed in triplicates and was repeated 2 times. The statistical significance calculation was calculated using the student's t-test, and *p-value less than 0.05 were considered to be significant. The pre-established biofilm was diluted with the serial dilution method and was spread on a blood agar plate for the CFU count.

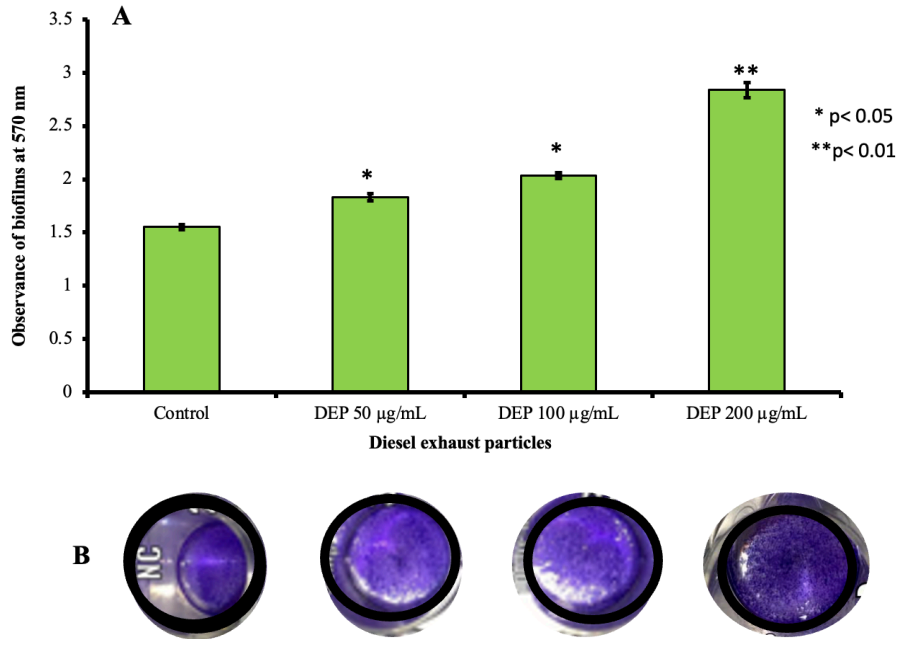


Figure 2: *In vitro* biofilm growth of *Streptococcus pneumoniae* D39 strain in brain heart infusion (BHI) medium supplied with different concentrations of diesel exhaust particles (DEP). **(A)** *In vitro* biofilms growth was significantly ($P< 0.05$) increased in presence of DEP. **(B)** Crystal violet-stained in vitro biofilms grown in 24-well tissue culture plate. Error bar showing standard deviation from mean value. Study was performed in triplicates and repeated 2 times. Statistical significance was calculated using students t test, and *p value less than 0.05 were considered significant.

4.3 DEP exposure elevated bacteria planktonic growth in metal ion free medium

S. pneumoniae was grown in metal ion-free BHI broth media treated with different concentrations of DEP at 50µg, 100µg, and 200µg. The control sample (vehicle control) was without treatment of the DEP. The bacteria samples were then incubated at 37°C and the optical density was measured at different time intervals 0, 12, 24, 36, and 48hrs by the spectrophotometer at 600nm. The planktonic growth of the bacteria in the metal ion-free media (chelex treated) was slower than the normal BHI medium as bacteria started to grow after 12hrs of incubation. In the treatment of the samples with DEP, bacteria growth was noticeably increased in comparison with vehicle control. In the presence of DEP pneumococcal planktonic growth was suggestively higher ($P<0.05$) (Figure 3). In presence of DEP at a concentration of 50, 100, and 200 ug/ml the pneumococcal growth was significantly increased. In 200ug of DEP, the bacteria growth was elevated at 12hrs of incubation, while in 50 and 100 ug DEP supplemented samples, growth started at 36 hrs. Error bar shows the standard deviation of the mean value. The study was performed in triplicates and repeated 2 times.

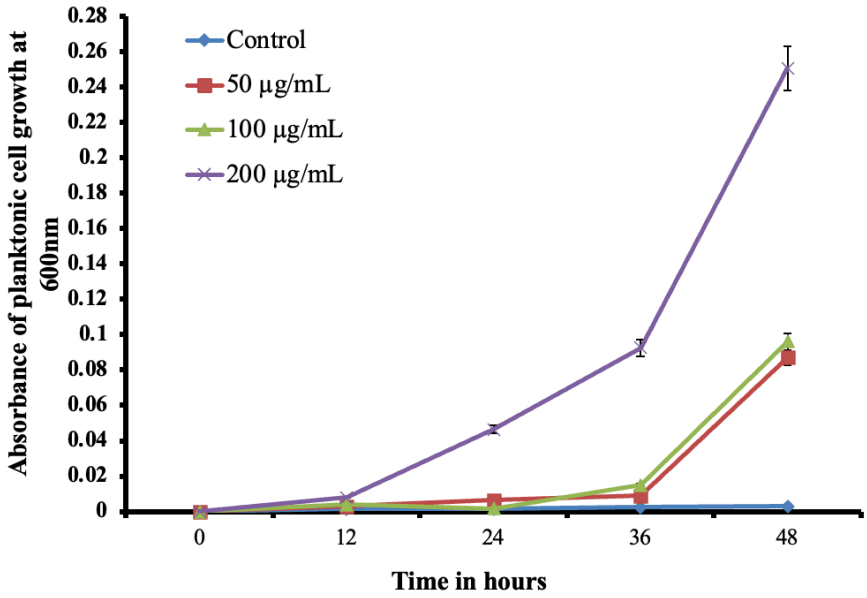


Figure 3: *Streptococcus pneumoniae* D39 strain planktonic growth in metal ion free medium BHI medium treated with chelex-100). Bacteria was grown with different concentration of DEP, and growth was detected by measuring optical density at 60nm at different time interval. In the presence of DEP pneumococcal planktonic growth were significantly ($P<0.05$) elevated. Error bar showing standard deviation from mean value. Study was performed in triplicates and repeated 2 times.

4.4 DEP exposure elevated bacteria in vitro biofilm growth in metal ion free medium

S. pneumoniae strain D39 was grown in a metal ion-free BHI broth media and treated with different concentrations of DEP 50µg, 100µg, and 200µg along with the vehicle control without treatment. The bacteria biofilms were grown in a 24-well plate and, the samples were then incubated at 37°C. After 48hrs incubation, the biofilm biomass was detected by staining biofilm by crystal violet. After incubation, the biofilm formed in the wells was washed twice by carefully pipetting with decontaminated water and stained with the crystal violet solution (0.1%), and the optical density was measured by the spectrophotometer at 570nm. The results showed that the biofilm growth of the bacteria with the treatment of DEP in different concentrations is noticeably increased in comparison with vehicle control. The biofilms growth with DEP treated 50µg shows a greater growth of the bacteria biofilms than the vehicle control and, 100µg and 200µg showed a significant ($p<0.05$) high growth of the bacteria biofilms (Figure 4). The error bar is showing the standard deviation of the mean value. The study was performed in triplicates and was repeated 2 times. The statistical significance calculation was calculated using the student's t-test, and *p-value less than 0.05 were considered to be significant.

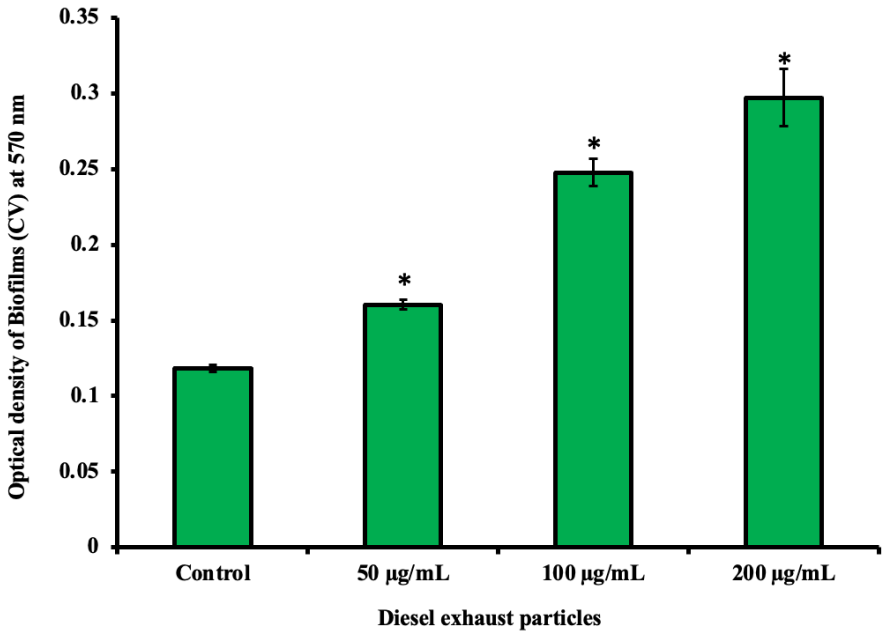


Figure 4: *In vitro* biofilm growth of *Streptococcus pneumoniae* D39 strain in metal ion free BHI medium (treated with chelex-100) for 48h, in presence or absence of diesel exhaust particles (DEP). In the presence of DEP pneumococcal *in vitro* biofilms were significantly ($P<0.05$) elevated. Error bar showing standard deviation from mean value. Study was performed in triplicate and repeated 2 times for statistical calculation. The statistical significance calculation was calculated using students t test, and *p value less than 0.05 were considered significant.

4.5 Minimum Inhibitory concentration (MIC) detection and antimicrobial activity of *Tithonia diversifolia* plant extract

The MIC detection of *Tithonia diversifolia* plant extract was carried out using the micro-dilution method by treating the *S. pneumoniae* with different concentrations of plant extracts ranging from 2.5µg, 5µg, 10µg, and 20µg. The vehicle control was treated with DMSO The bacteria with plant extracts were incubated at 37C for 24hrs, and the growth was measured by measuring optical density at 600nm. The result showed decreased or no growth of bacteria in presence of plant extract. The presence of plant extract significantly inhibited *Streptococcus pneumoniae* growth at all concentrations (2.5µg, 5µg, 10µg, and 20µg) tested in this study. The MIC is the concentration on which no visible growth of bacteria takes place. Our results showed low growth in samples treated with 2.5 µg/ml plant extract, however, no visible growth of bacteria was detected at 5µg plant extract (Figure 5). Therefore, the MIC of *Tithonia diversifolia* plant extract against *Streptococcus pneumoniae* D39 strain serotype 2 is 5µg. Error shown in figure 5 is the standard deviation from the mean value, and the study was performed in triplicate and repeated 2 times.

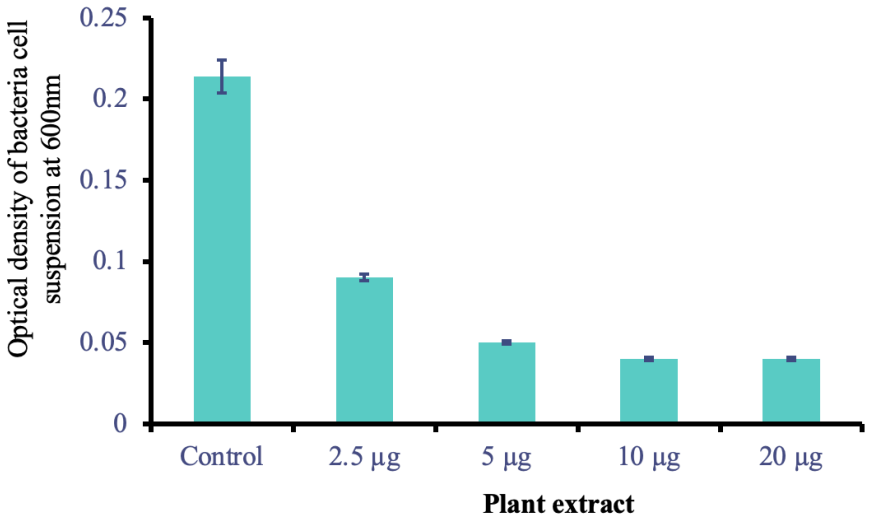


Figure 5: Minimum inhibitory concentration (MIC) detection of *Tithonia diversifolia* pant extract against Streptococcus pneumoniae D39 strain, using micro-dilution method. Error bar showing standard deviation from mean value. Study was performed in triplicate and repeated 2 times.

4.6 *Tithonia diversifolia* plant extract shows the antimicrobial potential against bacteria planktonic growth

The time-dependent killing experiment was carried out to study the effects of plant extract on bacteria. S. *pneumoniae* was grown-up in a BHI broth media till its early log phase and treated with the plant extract at the concentration of 5µg, 10µg, and 20µg. The vehicle control was treated with DMSO. The samples were incubated at 37°C and any alteration in the bacteria growth was measured by measuring the optical density after 18hrs by spectrophotometer at 600nm. The results showed that bacteria were further grown in the control sample and reached to log phase at 9 hrs. The growth of the bacteria with the treatment of the plant extract was noticeably decreased as the bacterial growth start to decline from 3hrs and from 6hrs the growth is linear whereas in the in-vehicle control the growth of the bacteria is elevated and reach its maximum growth at 18hrs of incubation. However, the bacterial growth was decreased with time in samples treated with plant extract. The bacteria cell suspension treated with 5, 10, and 20µg/ml showed decreased growth at 3hours and 6hours. The maximum decrease in bacteria growth was detected at early 3hours and hours, after 6hours bacteria growth decrease was not significant. In addition, the samples treated with 20µg showed a maximum decrease and it was at 6hours after treatment. The error bar shows the standard deviation of the mean value. The study was performed in triplicate and repeated 2 times for statistical calculation.

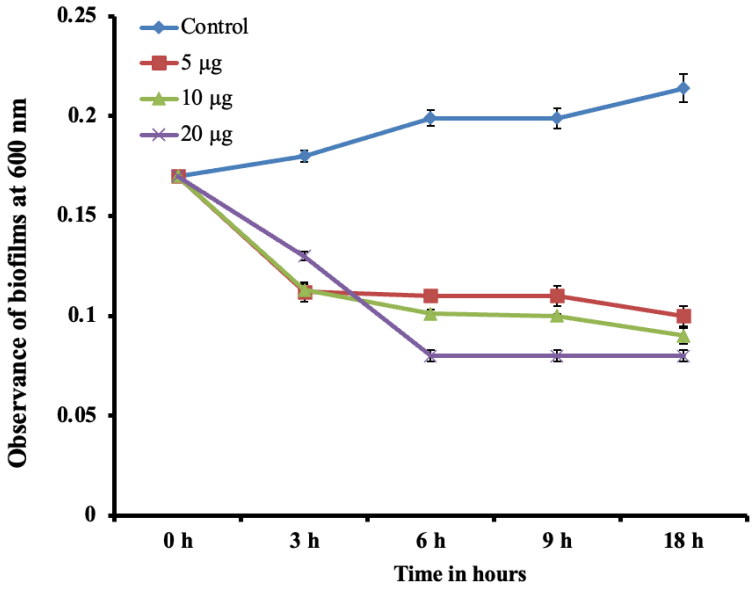


Figure 6: **Time dependent killing of Streptococcus pneumoniae.** Early log-phase grown pneumococci were treated with plant extract (5,10,20ug/ml), and any alteration in bacteria growth was detected by measuring optical density at different time interval. In vehicle control, bacteria were further grown to log-phase and attained max growth at 18 hrs. While in plant extract treated samples bacteria growth was declined. The bacterial growth was markedly decreased at 3 to 6 hours of plant extract treatment. Error bar showing standard deviation from mean value. Study was performed in triplicate and repeated 2 times for statistical calculation.

4.7 *Tithonia diversifolia* plant extract inhibits *Streptococcus pneumoniae* in vitro biofilms and eradicates pre-established biofilms

S. pneumoniae in vitro biofilm was grown in BHI media in 24 well plates and were treated with the plant extract at the concentration of 5µg, 10 µg, and 20 µg., the samples were incubated at 37°C for 18 hours. The optical density was measured by a spectrophotometer at 570nm. The results showed suggestively ($P<0.05$) decreased biofilms in samples treated with plant extract at all the concentrations tested. Indeed, no biofilms were detected at 5 µg and other concentrations of plant extract after 18 hours (Figure 7).

To study the effects of plant extract on pre-established biofilms, S. pneumoniae biofilms were grown for 15hours and then treated with plant extracts. The viable bacteria colony within biofilms were detected by CFU counts. The CFU counts results showed a significantly high number of viable bacteria within the biofilms of control samples, however, the viable bacteria were decreased within biofilms treated with plant extract (Figure 8A). The numbers of colonies on blood agar plate were few in biofilms treated with plant extracts (Figure 8B).

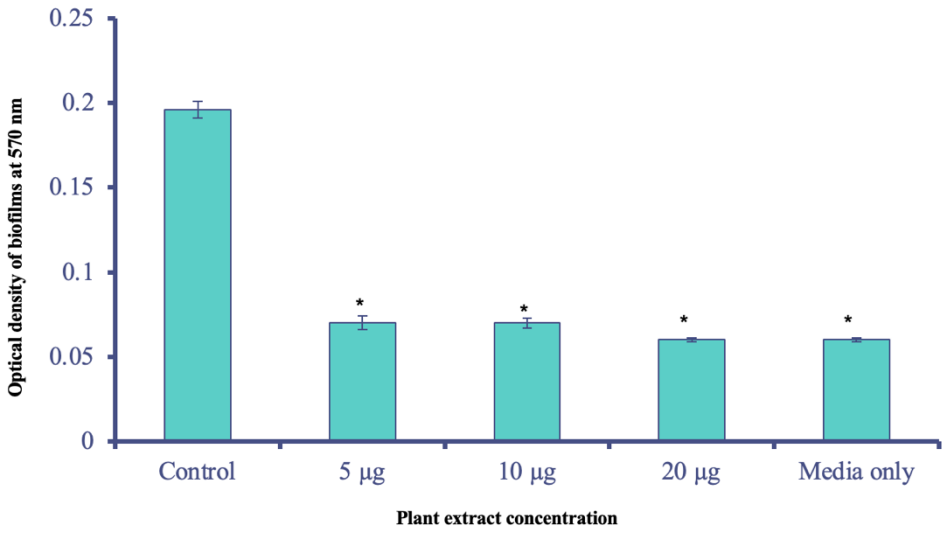
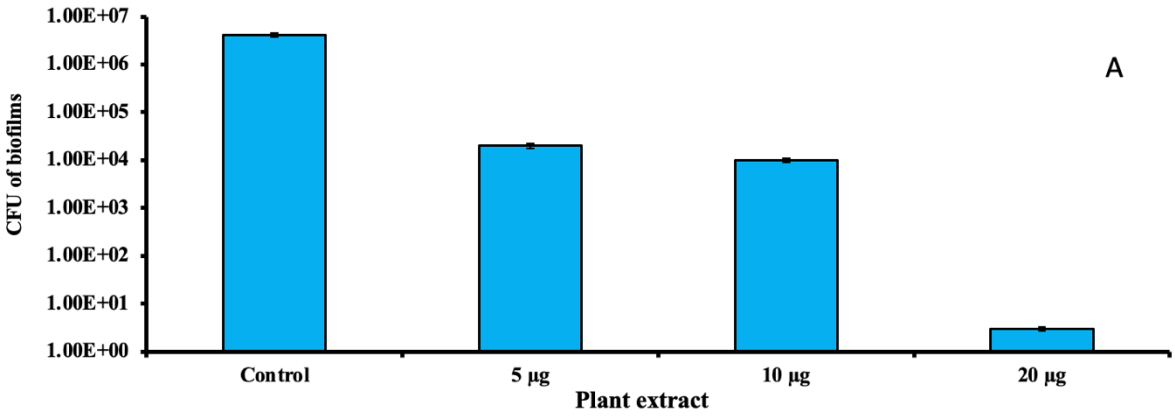


Figure 7: *In vitro* biofilm growth of *Streptococcus pneumoniae* D39 strain in BHI medium for 18h, in presence or absence of different concentration of plant extract. Biofilms were detected by cv microplate assay. In the presence of plant extract pneumococcal *in vitro* biofilms were suggestively ($P<0.05$) decreased. Error bar showing standard deviation from mean value. Study was performed in triplicate and repeated 2 times for statistical calculation. Statistical significance was calculated using students t test, and *p value less than 0.05 were considered significant.

(i) CFU Count



(ii) Different concentrations of plant extract spread on a blood agar plate for the CFU count

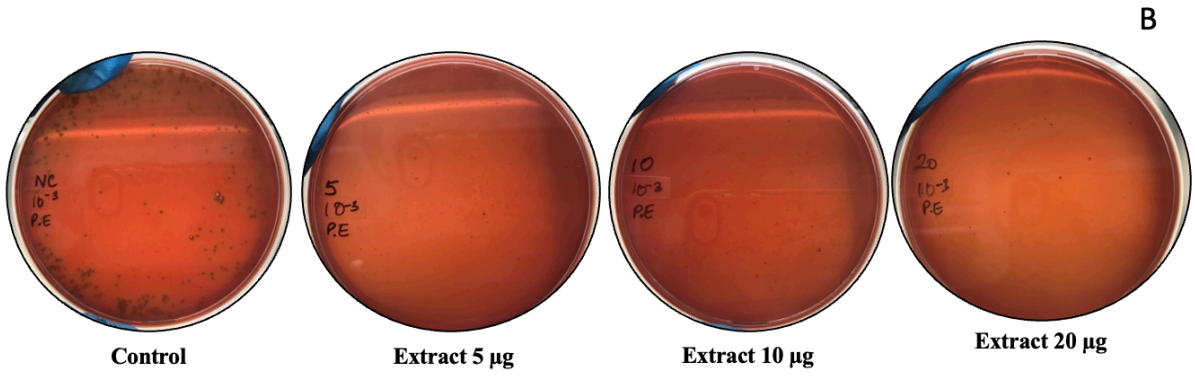


Figure 8: **Effects of tithonia diversifolia plant extract on *Streptococcus pneumoniae* pre-established biofilms**. *In vitro* biofilm was established for 18 hours, and treated with plant extract and viable cells within biofilms were detected using CFU counts. **(A)** Biofilm treated with different concentration of plant extract showed a significantly ($P<0.05$) decreased viable cells within the biofilms. **(B)**. *Streptococcus pneumoniae* vial cell counts within biofilms on blood agar plate. In control sample numerous colonies are visible, however, very less colonies are visible in biofilm samples treated with plant extract.

4.8 *Tithonia diversifolia* plant affects pneumococcal cell

The Crystal violet absorption assay was carried to elucidate the mechanism of antimicrobial activity of the plant extract. S. *pneumoniae* was grown till the log-phase and was treated with DMSO (as vehicle control), Penicillin (taken as a negative control as penicillin do not affect the bacteria cell membrane), and with the plant extract at 10 µg concentration. The CV assay showed that the control samples absorption approx. 20% CV, and penicillin treated bacteria also demonstrated approx. 25% of CV absorption. While plant extract treated bacteria showed approx. 80% CV absorption as shown in the graph plotted in figure 9. It implies that the bacteria cell membrane was compromised or disrupted by plant extract treatment.

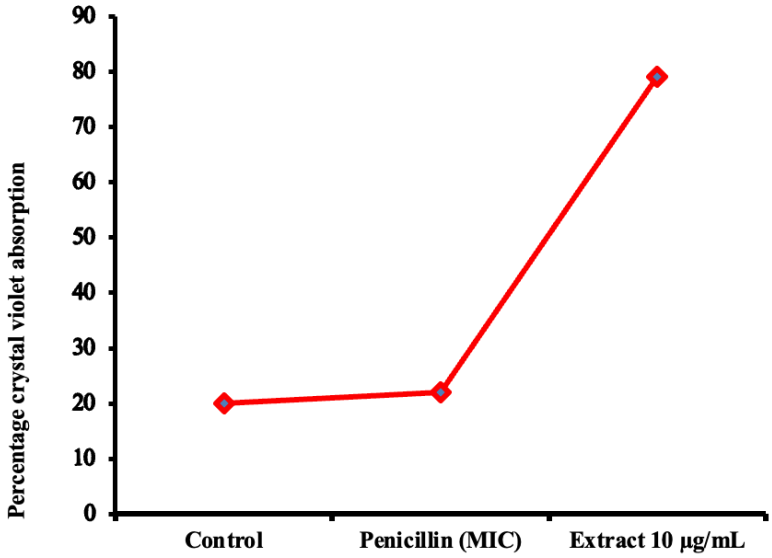


Figure 9: Crystal violet absorption assay to elucidate the mechanism of antimicrobial activity of plant extract. Log-phase *Streptococcus pneumoniae* were treated with DMSO (vehicle control), Penicillin (Negative control, as penicillin have no effect on bacteria cell membrane) and plant extract (10 µg). The cv assay showed that control samples absorption approx. 20% cv, and penicillin treated bacteria also demonstrated approx. 25% of cv absorption. While extracted treated bacteria showed approx. 80% cv absorption.

CHAPTER 5

5. DISCUSSION

Streptococcus pneumonia is a gram-positive, opportunistic pathogen and a very common bacteria that colonize the nasopharynx and these colonizing pneumococci form a biofilm community into the nasopharynx. These biofilms are particularly resistant to antimicrobial agents (Chao et al.2018). Biofilm formation is the essential step in pathogenesis as the biofilm is responsible for bacterial competence, persistence, a reservoir for invasive disease, and immune evasion (Reddinger et al. 2018). The toxic particulate matter (PM) deposits into the respiratory tract during the gaseous exchange and increases the chance of having an infection and disease (Yadav et al.2020). But it is still not well known the mechanisms how the particulate matter took its role in promoting infectious disease and invading human defense. In this study, we reported the effects of DEP particulate matter on *S. pneumoniae* Planktonic and Biofilm Growth. DEP causes adverse health diseases and infections that involve reactive oxygen species generation and, inflammation, and oxidative stress which can lead to DNA damage (Li et al.2015). The results of planktonic growth with different concentrations of DEP revealed elevated bacterial growth. The low growth of bacteria initially indicates that bacteria were adapting to growth in the presence of DEP, and it grows maximum at 10-12 hours of incubation. However, low growth in control (absence of DEP) indicates that DEP presence or constituents of DEP contribute to bacteria growth. Similarly elevated growth of *S. pneumoniae* was previously detected in presence of the Urban Particles (UP), and Asian Sand Dust (ASD) (Yadav et al.,2020a; Yadav et al.,2020b). The presence of black carbon also affects the physiology of bacteria and demonstrated enhanced growth (Hussey et al.,2017). Increased growth of biofilm in the presence of DEP could be due to the presence of particulate matter as the bacteria cling onto the bottom of the tissue culture plate increased. Probably the bacteria prefer to adhere and form robust biofilm on a rough surface, and the PM here acts as a substrate for the bacteria attachment and biofilm formation. Similar high biofilms were detected in the presence of UP by Yadav et al 2020a. They demonstrate that the bacteria physiology may be altered in the presence of urban particles, the constituents of UP favor in *vitro* biofilm growth (Yadav et al.,2020a). DEP can change the cell stiffness and adhesion force and could also decrease the protein and the DNA of the mammalian cell by the study reported by Q. Li et al.2015. In this study to better understand the *S. pneumoniae* growth alteration by the DEP, the BHI media was treated with the Chelex100 to chelate all the metal ions present in the BHI media and a metal ion-free media was prepared. The planktonic growth of the bacteria treated with the different concentrations of DEP in the metal ion-free media showed that the growth of bacteria was much slower than the bacteria grown in the normal BHI media. The bacteria in

presence of DEP were elevated from 12hrs of incubation and the vehicle control growth was linear and negligible. Whereas in the biofilm growth the bacteria on treating with different concentrations of DEP was significantly higher than the vehicle control. The results of this study give us insight into the effect of the DEP which can be causing adverse health diseases and infection mainly in the respiratory tract. High doses of DEP in many studies of *in vitro* fall inside the range of realistic exposure to air pollutant particulate matter at the flashpoints that may occur in the pulmonary interstitium of the exposed lung (Lawal 2017). Altogether, the elevated growth of pneumococci in both planktonic and biofilms indicates that DEP provides es favorable environment for bacteria growth and provides a suitable surface for biofilm formation. Similar results were reported by our group with urban particles and Asian sand dust particles (Yadav et al., 2020a; Yadav et al 2020b). Furthermore, the elevated growth in metal ion-free medium indicates that the DEP constituents specifically metal ions contribute to biofilm formation. It is reported that metal iron particularly iron is very important for pneumococci growth, virulence, and biofilm formation (Trappetti et al.,2011). And the previous study demonstrated that in absence of iron pneumococci biofilms were low. Particulate matter contains irons as one of the metal constituents that can be utilized by pneumococci for its growth and biofilm formation (Yadav et al., 2020a; Yadav et al 2020b; Hussy et al., 2018)

The formation of biofilm in the nasopharynx is crucial for infection establishment, therefore a dual activity compound that can inhibit bacteria growth/biofilms and having anti-inflammatory activity could be more effective to combat respiratory infections. Here we evaluated the antimicrobial/antibiofilm potential of the plant *Tithonia diversifolia* as it has been reported that the plant is having anti-inflammatory (Paula et al.2011) antimicrobial, anti-fungal, anti-malarial (Madureira, 2002), and cancer chemopreventive activities (Bork PM et., al 1996). The *S. pneumoniae* was treated with the methanolic plant extract of *T. diversifolia* and the growth under planktonic and biofilm growth were evaluated. Results indicate that *S. pneumoniae* growth was declining in the samples grown with plant extract. The antimicrobial effects of plant extract were concentration-dependent. Similarly, the in vitro biofilms of pneumococci were also decreased in samples treated with plant extract. The CFU counts decreased in pre-established biofilm on plant extract treatment indicates that plant extract diffused deep inside the biofilms and able to kill bacteria inside biofilms. It is reported that the matrix of biofilms prevents the diffusion of antimicrobial agents due to its polar nature, and the phenolic plant metabolites can overcome this barrier and kill bacteria (Roy et al.,2018). The crystal violet absorption assay was also followed by the method provided by Yadav et al.2019 to clarify the

mechanism of the antimicrobial activity of the plant extract and, the results show that the crystal violet was absorbed the most in the treatment with the plant extract implying that the bacteria cell membrane was compromised or disrupted by plant extract treatment.

6. CONCLUSIONS

The study showed that DEP exposure increased the *S. pneumonia* growth both in planktonic and in biofilm. And the elevated Pneumococcal biofilms in metal ion-free medium indicate that bacteria utilize DEP components for their growth, and DEP provides a favorable surface for biofilm growth. The inhibition of *S. pneumonia growth* on *Tithonia diversifolia extract treatment showed* the antimicrobial property of the plant extract. The eradication of pre-established biofilms on plant extract treatment indicates that *Tithonia diversifolia extract* can penetrate deep inside the biofilms across the matrix and kill bacteria. The increased absorption of crystal violet in *Tithonia diversifolia* extract-treated pneumococci showed that the target of action of plant extract is the cell membrane, which disrupted plant extract treatment.

ABBREVIATIONS

%	Percentage
*p	p-value
°C	Degree Celsius
µg	Micro Gram
BAP	Blood Agar Plate
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CV	Crystal Violet
DEP	Diesel Exhaust Particles
DMSO	Dimethyl sulfoxide
g	Gram
MIC	Minimum Inhibitory Concentration
ml	Milli Litre
nm	Nano Mitre
PE	Plant Extract
PM	Particulate Matter

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DEPARTMENT: BIOTECHNOLOGY

TITLE OF DISSERTATION: Effects of diesel exhaust particles on *in vitro* bacteria biofilms growth and evaluation of anti-biofilm/antimicrobial potential of a few selected plant extract

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COMMENCEMENT OF SECOND SEM/DISSERTATION :
(From conclusion od end semester exam)

APPROVAL OF RESEARCH PROPOSAL

BOS: 29/05/2020

SCHOOL BOARD: 12/08/2020

REGISTRATION NO. & DATE : Regn. No.: MZU/M.Phil. /628 of12.06.2020

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3	HSSLC	Physics, Chemistry, Biology, Mathematics, Computer Programming, Hindi, English	Banasthali Board	60.77%
4	HSLC	Hindi, English, Sanskrit, Social Science, General Science, Mathematics, Education for Home, Aesthetic Education	Banasthali Board	55.25%

Present Position

Pursuing the degree of Master of Philosophy (M.Phil.) in Biotechnology with entitled **"Effects of diesel exhaust particles on *in vitro* bacteria biofilms growth and evaluation of anti-biofilm/antimicrobial potential of a few selected plant extract"** under the supervision of Dr. H. Lalhruaitluanga and Co-supervision of Dr. Mukesh Kumar Yadav in the Department of Biotechnology, Mizoram University.

Publications:01

- Zothanpuia, Zomuansangi R, Leo VV, Passari AK, Yadav MK, Singh BP. Antimicrobial sensitivity profiling of bacterial communities recovered from effluents of the municipal solid waste dumping site. 3 Biotech. 2021 Feb;11(2):37. DOI: 10.1007/s13205-020-02548-z. Epub 2021 Jan 8. PMID: 33479592; PMCID: PMC7794261.

Webinars and Training Attended

- Participated in the 3 days training of **"How to operate the HPLC"** by waters company which was held in July 2019.
- Participated in the webinar **"National Institute of Food Technology Entrepreneurship and Management"** held by the Department of Agriculture and Environmental Sciences, Sonepat, Haryana, India.
- Presented a paper entitled **"Effects of Diesel exhaust particles on Streptococcus pneumoniae in vitro biofilms"** in the three days International Webinar organized by the Department of Biotechnology, Pachhunga University College (PUC), and Mizoram University (MZU) from 24th-26th June 2020.
- Participated in a program organized by Dept. of Biotechnology, Mizoram University and Dept. of Horticulture, Aromatic & Medicinal Plant (HAMP), Mizoram University.

Co-organized and funded by the Indian Council of Agricultural Research – National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, Uttar Pradesh.

DECLARATION

I hereby declared that all the information mentioned above is true to my knowledge and belief. I will solely be responsible if any of the information is found to be incorrect.

Place: Aizawl, Mizoram **RUTH ZOMUANSANGI**

Date:

ABSTRACT

EFFECTS OF DIESEL EXHAUST PARTICLES ON *in vitro* BACTERIA BIOFILMS GROWTH AND EVALUATION OF ANTI-BIOFILM/ANTIMICROBIAL POTENTIAL OF A FEW SELECTED PLANT EXTRACT

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCE

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BACKGROUND AND AIM

Microbes exist in adherent and free-floating forms. The adherent communities of microbes surrounded by an extracellular matrix are called Biofilms. The bacteria biofilms are responsible for various infections in humans and increase disease management costs and hospital stay time. The bacteria biofilms are tolerant to antibiotics and the emergence of antibiotic-resistant bacteria further complicates the disease management. The particulate matter (PM) present in air pollution is known to cause and aggravate respiratory infection, and a recent study reported that PM exposure increases bacterial biofilm mode of growth. Therefore, to manage respiratory infection there is a need for unique bioactive compounds with antimicrobial/antibiofilm and anti-inflammatory/antioxidant activities. Mizoram, northeast India, near the Indo-Burma border (22º 40' N; 93 º 0 3' E) is one of the hot mega diversity zones that habitats many medicinal plants traditionally used by the local people as medicine. Medicinal plants with bioactive compounds such as (stilbenes) are a potential source of antibiofilm/antimicrobial and antioxidant & COX-2 inhibitory compounds that could be explored to control bacterial infection and PM toxicity (Vestergaard et al., 2019).

Tithonia diversifolia is one of the utmost significant medicinal plants of the Asteraceae family which is widely in use in the Ayurvedic and Unani systems. The plant parts such as its roots, shoots, leaves are used in various diseases. It is also known as the Mexican sunflower, a shrub-like perennial (Ajao & Moteetee, 2017). The research of *T. diversifolia* by Pharmacological indicates its many active constituents like antimicrobial, anti-inflammatory (Owoyele et al., 2003, D.A. Chagas-Paula et al. 2011) anti-fungal, anti-malarial (Goffin et al., 2001, Madureira, 2002), and cancer chemopreventive activities (Bork PM et., al 1996).

The previous studies reported that medicinal plants such as stilbenes possess dual activities, such as antibiofilm/antimicrobial potential along with antioxidant & anti-inflammatory activity (Vestergaard et al., 2019; Yadav et al., 2019).

Particulate matter such as diesel exhaust particles (DEP) and *S. pneumoniae* are some of the risk factors for respiratory infection, and how DEP exposure affects pneumococcal colonization/biofilms in the nasopharynx is not well known and studied. So, in the study, we elucidated the effect of DEP on *pneumococci in vitro* growth of biofilms and evaluated the effects of *Tithonia diversifolia* plant extract to control *S. pneumoniae* growth (both in planktonic and in biofilms).

MATERIALS AND METHODS

Streptococcus pneumoniae D39 strain used in this study was purchased from Microbial type culture collection (MTCC), Chandigarh, India. *S. pneumonia* strain D39 is an Avery strain, serotype 2, and is well known for its invasive property (Avery et al. 1944). The whole-genome sequence of the D39 strain is available on NCBI and is a well-known strain used for research purposes.

S. pneumoniae was grown in brain heart infusion (BHI) broth, and for longer use and storage glycerol stocks of bacteria were prepared and maintained at −80°C until use. For growing the bacteria colonies blood agar plate (BAP) which is supplemented with 5% of sheep blood was used. The DEP used for this study was purchased from the National Institute of Standard and Technology (NIST), the USA as previously reported (Song et al.,2008; Yadav et al 2020).

Tithonia diversifolia plant was collected from the roadside towards Mizoram University campus, Tanhril, Mizoram. The plant leaves were collected and dried in a hot air oven at 37°C and ground into a fine powder using a domestic mixer grinder. The powder obtained was then weighed and the extract was prepared by soaking it in methanol for 48 hours. The plant extract was prepared according to the protocol reported previously by Singh et al., (2016). The plant extract after methanol treatment was filtered through a Whatman no. 1 filter paper. The filtration process was repeated three times followed by the evaporation to dry at 40°C with the help of the rotary evaporator (BUCHI, Switzerland) that resulted in the crude plant extract. The extracts were then kept at 4°C until further use (Singh et al,.2016).

To evaluate the effects of DEP on pneumococci growth planktonically, the D39 strain of S. pneumoniae was grown in a broth medium supplied with different concentrations of DEP 50µg, 100µg, and 200µg. The growth of the bacteria was detected by measuring its optical density at 600nm (OD_{600}) at different periods by the procedure previously reported (Yadav et al.,2020). The planktonic growth experiment was repeated 3 times in replicates. The statistical significance was calculated with help of excel using the student's t-test, and *p-value less than 0.05 were considered significant.

In vitro bacteria, biofilm formation in the presence of DEP was studied using a static microtiter plate assay (Christensen et al. 2000, Yadav et al., 2015) with different concentrations of DEP from 50µg, 100µg, and 200µg. *S. pneumoniae* colony grown on BPA was then grown in BHI broth up till log-phase. The bacteria cell suspension was prepared by diluting cells 1:200 and

bacteria suspension was inoculated in 24-well (1 ml) plates and 200ul in 96-well plate. The plate was incubated for 18 hours at 37°C. After the incubation period, the suspended medium was removed by slowly pipetting out, and biofilms at the bottom of the plate were washed twice with distilled water and stained with 0.1% crystal violet (CV) solution for 15 min. The stained biofilms were again washed with phosphate buffer saline (PBS) twice and dissolved in 1 ml (24-well plate and 200ul for 96-well plate) of ethanol. The absorbance of the biofilm was measured at 570nm using a spectrophotometer (Multiskan Skyhigh Microplate Spectrophotometer, Thermo Fisher Scientific). The biofilm growth experiment was repeated two times and the experiment was performed in triplicates. The statistical significance of the results was calculated using the student's t-test, by considering *p-value less than 0.05 as significant.

For viable bacteria detection within the biofilms, after washing the biofilms with PBS, it was dissolved in sterile water followed by sonication for 10 seconds to dissolve biofilms. The diluted biofilm suspension was serially diluted and spread on BAP to count the colonies. The colony counting units (CFU) of the biofilms were detected by colony formed counting after 24 hours incubation at 37°C (Yadav et al.,2019).

RESULTS

On growing *Streptococcus pneumonia* D39 strain planktonically in BHI medium treating with DEP at a different concentration from 50µg, 100µg, and 200µg, vehicle control was also taken where there is no treatment of the DEP. The optical density was measured at a different time interval at 600nm by a spectrophotometer. The observed results show that the samples where it was treated with DEP show a significant elevation of the bacterial growth is contrary to the vehicle control planktonically.

A similar study was performed where the bacteria *Streptococcus pneumonia* D39 strain was grown in BHI medium treating with DEP at a different concentration from 50µg, 100µg, and 200µg in a cell culture plate, and development of the biofilm was observed. The biofilm was visible after staining it with the crystal violet solution and the optical density was detected at 570nm. It was observed that after 18 hours of incubation, the samples where it was treated with DEP show an elevation of the growth of the bacteria contrary to the vehicle control. The

CFU count was also performed where greater numbers of the bacteria colony were found in higher the concentration of the treatment of DEP.

The BHI medium was then treated with a chelating agent Chelex-100 to produce a metal ion-free BHI medium then this metal-free ion medium was used for the study for both bacteria planktonic growth and biofilm formation. The metal ion-free media was used to grow the bacteria planktonically and as well as in biofilm growth and it was treated with different concentrations of DEP from 50µg, 100µg, and 200µg taking along the vehicle control with no treatment of the DEP. It was observed that in bacteria planktonic growth the incubation takes up to 48 hours and the sample treated with DEP shows a significant elevation of the growth of the bacteria planktonically whereas, in the biofilm growth it also shows a significant elevation of the growth of the bacteria in contrary to the vehicle control.

A minimum inhibitory concentration (MIC) detection of the plant extract *Tithonia diversifolia* was performed by treating the *S. pneumoniae* with different concentrations of plant extracts ranging from 2.5µg, 5µg, 10µg, and 20µg taking DMSO as the vehicle control and was incubated for 24hours. The optical density was measured at 600nm and the result suggests that the MIC of *Tithonia diversifolia* plant extract against *Streptococcus pneumoniae* D39 strain serotype 2 is 5µg.

The plant extract *Tithonia diversifolia* was then used to treat the bacteria *S. pneumonia* at different concentrations of 5µg, 10µg, and 20µg planktonically, taking DMSO as the vehicle control. After incubating for 18hours the optical density was measured at 600nm and it was observed that the growth of bacteria declines for the 6 hours of incubation whereas, on the other hand, the controlled growth of the bacteria was inclining.

The study was also performed for the bacteria biofilm growth where the bacteria *S. pneumonia* was treated with different concentrations of the plant extract 5µg, 10µg, and 20µg, taking DMSO as the vehicle control. The optical density was measured at 570nm and the observation suggests that the growth of bacteria on treating with the plant extract has declined or nullified the growth of the bacteria whereas, the growth of the bacteria in control was inclined.

A crystal violet absorption assay was carried to elucidate the mechanism of antimicrobial activity of the plant extract. S. *pneumoniae* was grown till the log-phase and was treated with

DMSO (as vehicle control), Penicillin (taken as a negative control as penicillin do not affect the bacteria cell membrane), and with the plant extract at 10 µg concentration. The CV assay showed that the control samples absorption approx. 20% CV, and penicillin treated bacteria also demonstrated approx. 25% of CV absorption. While plant extract treated bacteria showed approx. 80% CV absorption as shown in the graph plotted in figure 9. It implies that the bacteria cell membrane was compromised or disrupted by plant extract treatment.

DISCUSSION

Streptococcus pneumonia is a gram-positive, opportunistic pathogen and a very common bacteria that colonize the nasopharynx and these colonizing pneumococci form a biofilm community into the nasopharynx. These biofilms are particularly resistant to antimicrobial agents (Chao et al.2018). Biofilm formation is the essential step in pathogenesis as the biofilm is responsible for bacterial competence, persistence, a reservoir for invasive disease, and immune evasion (Reddinger et al. 2018). The toxic particulate matter (PM) deposits into the respiratory tract during the gaseous exchange and increases the chance of having an infection and disease (Yadav et al.2020). But it is still not well known the mechanisms how the particulate matter took its role in promoting infectious disease and invading human defense. In this study, we reported the effects of DEP particulate matter on *S. pneumoniae* Planktonic and Biofilm Growth. DEP causes adverse health diseases and infections that involve reactive oxygen species generation and, inflammation, and oxidative stress which can lead to DNA damage (Li et al.2015). The results of planktonic growth with different concentrations of DEP revealed elevated bacterial growth. The low growth of bacteria initially indicates that bacteria were adapting to growth in the presence of DEP, and it grows maximum at 10-12 hours of incubation. However, low growth in control (absence of DEP) indicates that DEP presence or constituents of DEP contribute to bacteria growth. Similarly elevated growth of *S. pneumoniae* was previously detected in presence of the Urban Particles (UP), and Asian Sand Dust (ASD) (Yadav et al.,2020a; Yadav et al.,2020b). The presence of black carbon also affects the physiology of bacteria and demonstrated enhanced growth (Hussey et al.,2017). Increased growth of biofilm in the presence of DEP could be due to the presence of particulate matter as the bacteria cling onto the bottom of the tissue culture plate increased. Probably the bacteria prefer to adhere and form robust biofilm on a rough surface, and the PM here acts as a substrate for the bacteria attachment and biofilm formation. Similar high biofilms were detected in the

presence of UP by Yadav et al 2020a. They demonstrate that the bacteria physiology may be altered in the presence of urban particles, the constituents of UP favor in *vitro* biofilm growth (Yadav et al.,2020a). DEP can change the cell stiffness and adhesion force and could also decrease the protein and the DNA of the mammalian cell by the study reported by Q. Li et al.2015. In this study to better understand the *S. pneumoniae* growth alteration by the DEP, the BHI media was treated with the Chelex100 to chelate all the metal ions present in the BHI media and a metal ion-free media was prepared. The planktonic growth of the bacteria treated with the different concentrations of DEP in the metal ion-free media showed that the growth of bacteria was much slower than the bacteria grown in the normal BHI media. The bacteria in presence of DEP were elevated from 12hrs of incubation and the vehicle control growth was linear and negligible. Whereas in the biofilm growth the bacteria on treating with different concentrations of DEP was significantly higher than the vehicle control. The results of this study give us insight into the effect of the DEP which can be causing adverse health diseases and infection mainly in the respiratory tract. High doses of DEP in many studies of *in vitro* fall inside the range of realistic exposure to air pollutant particulate matter at the flashpoints that may occur in the pulmonary interstitium of the exposed lung (Lawal 2017). Altogether, the elevated growth of pneumococci in both planktonic and biofilms indicates that DEP provides es favorable environment for bacteria growth and provides a suitable surface for biofilm formation. Similar results were reported by our group with urban particles and Asian sand dust particles (Yadav et al., 2020a; Yadav et al 2020b). Furthermore, the elevated growth in metal ion-free medium indicates that the DEP constituents specifically metal ions contribute to biofilm formation. It is reported that metal iron particularly iron is very important for pneumococci growth, virulence, and biofilm formation (Trappetti et al.,2011). And the previous study demonstrated that in absence of iron pneumococci biofilms were low. Particulate matter contains irons as one of the metal constituents that can be utilized by pneumococci for its growth and biofilm formation (Yadav et al., 2020a; Yadav et al 2020b; Hussy et al., 2018)

The formation of biofilm in the nasopharynx is crucial for infection establishment, therefore a dual activity compound that can inhibit bacteria growth/biofilms and having anti-inflammatory activity could be more effective to combat respiratory infections. Here we evaluated the antimicrobial/antibiofilm potential of the plant *Tithonia diversifolia* as it has been reported that the plant is having anti-inflammatory (Paula et al.2011) antimicrobial, anti-fungal, anti-malarial (Madureira, 2002), and cancer chemopreventive activities (Bork PM et., al 1996). The

S. pneumoniae was treated with the methanolic plant extract of *T. diversifolia* and the growth under planktonic and biofilm growth were evaluated. Results indicate that *S. pneumoniae* growth was declining in the samples grown with plant extract. The antimicrobial effects of plant extract were concentration-dependent. Similarly, the in vitro biofilms of pneumococci were also decreased in samples treated with plant extract. The CFU counts decreased in pre-established biofilm on plant extract treatment indicates that plant extract diffused deep inside the biofilms and able to kill bacteria inside biofilms. It is reported that the matrix of biofilms prevents the diffusion of antimicrobial agents due to its polar nature, and the phenolic plant metabolites can overcome this barrier and kill bacteria (Roy et al.,2018). The crystal violet absorption assay was also followed by the method provided by Yadav et al.2019 to clarify the mechanism of the antimicrobial activity of the plant extract and, the results show that the crystal violet was absorbed the most in the treatment with the plant extract implying that the bacteria cell membrane was compromised or disrupted by plant extract treatment.

CONCLUSION

The results of this study showed that DEP exposure increased the *S. pneumonia* growth both in planktonic and in biofilm. And the elevated Pneumococcal biofilms in metal ion-free medium indicate that bacteria utilize DEP components for their growth, and DEP provides a favorable surface for biofilm growth. The inhibition of *S. pneumonia growth* on *Tithonia diversifolia extract treatment showed* the antimicrobial property of the plant extract. The eradication of pre-established biofilms on plant extract treatment indicates that *Tithonia diversifolia extract* can penetrate deep inside the biofilms across the matrix and kill bacteria. The increased absorption of crystal violet in *Tithonia diversifolia* extract-treated pneumococci showed that the target of action of plant extract is the cell membrane, which disrupted plant extract treatment.

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