Studies on anti-biofilmic activity of Neem &Tulsi

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Abstract

Natural products have played an important role asone of the major sources of new drugs for the pastdecade due to their incomparable structural diversity (Baker et al., 2007). With state-of-the-artmethodologies for separation and isolationprocedures, the search of new leads from plants, thatcan be used to develop drugs for human therapy inpersistent infections, has increased considerably andhas led to the discovery of compounds with inhibitoryactivities on biofilm formation in bacteria. This studyevaluated the role of Neem and Tulsi in inhibiting biofilmformation by *B. cereus*. Factors contributing toadherence and biofilm formation were also studied. Evaluation of the effect of its action was observed before and after the biofilm formation.Results demonstrated that Tulsi extracts has the antibacterial property rather than working as an anti-biofilmic agent but incase of Neem, its extract possess anti-biofilmic property which reinforce the possibility of employing Neem in theeradication of biofilm infections. As global scenario is changing toward the use of nontoxic plant productshaving medicinal value, hence extensive research is required on Neem for its better economic and therapeuticutilization. Althoughthese agents are effective and showed potential in the treatment of biofilm-associatedinfections, their mechanisms of action remainunclear.

Keywords: Neem and Tulsi extract, anti biofilmic property, antibacterial property

Introduction

Biofilms are currently identified as an assemblage of surface-associated microbial cells that are enclosed in hydrated extracellular polymeric substances (EPS) (Sauer,Rickard, & Davies, 2007), usually containing polysaccharides, proteins, phospholipids, teichoic and nucleic acids.Even mineral crystals, silt particles and blood components are found in the EPS of biofilms (Donlan, 2002). A biofilm community may comprise single and/or multiple species of bacteria and form a single layer or three-dimensional structures. (Sauer et al., 2007).

Pathogenic microorganisms can also attach to and grow on food surfaces, equipment and processing environments to form biofilms. For example, *Listeria monocytogenes* forms biofilms on floor drains, storage tanks, hand trucks, conveyor belts and other food-contact materials (Mafu, Roy, Goulet, &Magny, 1990). Several studies were focused on the attachment of bacterial pathogens to food surfaces such as, *L. monocytogenes* to beefsurfaces (Dickson, 1990) and *Salmonella sp.* to chicken skin (Campbell, Duckworth, Thomas, &McMeekin, 1987). The existence of pathogenic bacteria on food and food-contact surfaces increases the food safety risk. Therefore, the mechanisms of microbial biofilm formation in the food-processing industry have become a hot topic in the past several years. Reasonable analyses of biofilm formation have been carried out in some laboratories by various technologies, providing theoretic bases for implementing new strategies to eliminate biofilms in food plants.

Compounds that kill or inhibit the growth of bacteria have routinely been used to interfere with biofilm formation. However, the use of these compounds may select for the strains resistant to them and the application of these compounds at sub-inhibitory levels can cause biofilm stimulation. For these reasons, inhibitors that regulate biofilm formation without interfering with bacterial growth have received attention during the last decade. Landini et al. have introduced various biofilm inhibitors. Quorum sensing (QS) inhibition is the most extensively studied approach. QS is a mechanism that controls coordinated bacterial behaviors in response to the density of bacterial cells and is tightly linked to bacterial biofilm formation as well as to the production of virulence factors. Patulin, halogenated furanones, and analogs of 3-oxo-C12 homoserine lactone are widely known to inhibit QS in Gram-negative bacteria by competing with inherent QS signal molecules (i.e., *N*-acyl homoserine lactones [AHLs]) through binding to QS signal

molecule receptors, accelerating receptor turnover, and inhibiting LasR-dependent gene expression. Apart from QS inhibitors, enzymes that degrade QS signal molecules (e.g., acylase and lactonase) also interfere with bacterial QS and biofilm formation. Compounds that interfere with production of bis-(32 -52)-cyclic dimericguanosine monophosphate (c-di-GMP) or facilitate the degradation of this molecule are another category of biofilm inhibitors. c-di-GMP is a second messenger used for signal transduction by various bacteria and reportedly modulates lifestyles associated with biofilm formation. Sulfathiazole reduces the cellular level of c-di-GMP through inhibition of c-di-GMP biosynthesis. Another mechanism of biofilm inhibitors is related to the dispersal of cells from the biofilm. Some compounds such as dispersin B and Nitric oxide reduce biofilm formation by promoting such cell dispersal. In addition to these biofilm inhibitors, bacterial capsular polysaccharides, triterpenes, DNA-degrading enzymes, nanoparticles, etc. reportedly inhibit bacterial biofilm formation. Some biofilm inhibitors have been isolated from natural products, which is advantageous because these inhibitors are generally less toxic and more specific compared to synthetic compounds.

Microbial biofilms are highly resistant to antibiotics and host immune defences. Formation of biofilms by clinically relevant microbial pathogens is at the root of many chronic and recurrent infections, and biofilms have been estimated to account for 80% of all microbial infections in the human body. Biofilm-associated infections can be broadly divided into two types: infections associated with indwelling medical devices and native biofilm infections of host tissues. For the former type, bloodstream or urinary tract infections can be caused by infectious biofilms originally formed on the surfaces of indwelling medical devices, such as central venous catheters, mechanical heart valves, urinary catheters, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts, endotracheal tubes, contact lenses, intrauterine devices and dental unit waterlines. In these cases, pathogens may originate from the epithelial flora of patients, healthcare personnel or other sources in the environment, to form infectious biofilms on the surfaces of indwelling medical devices, and subsequently gain access to human organs or tissues via indwelling medical devices inserted into the human body. Native biofilm-associated infections are often chronic, opportunistic infections in otherwise sterile locations of the human body, and mainly include chronic lung infections of cystic fibrosis patients, chronic otitis media, native valve infectious endocarditis, chronic osteomyelitis, recurrent urinary tract infection, chronic wounds, dental caries and periodontitis.

Biofilm-associated infections can be caused by a single microbial species or by a mixture of species, with interactions between multiple species increasing their persistence. Pathogens frequently involved in biofilm-associated infections include Gram-positive bacteria (especially streptococci and staphylococci), Gram-negative bacteria (especially *Pseudomonas aeruginosa*, *Escherichia coli* and*Klebsiellapneumoniae*) and fungi (especially *Candida* sp. and *Aspergillus* sp.).

Materials and methods

Microorganism: A biofilm producing strain of *Bacillus cereus* was cultivated in Luria Bertani(LB) broth for 24h at 37 ºC and was used for subsequent studies.

Inhibitor of plant origin:Neem (*Azadirchtaindica*), purchased as pure Neem extract tablet from Patanjali Ayurveda and Tulsi (*Ocimumtenuiflorum*), purchasedas pure Tulsi extract tablet from Apollo Pharmacy were used as water soluble extract for checking their anti-bio filmic activities.

Production of biofilm: Freshly prepared LB broth(2 ml) was added to each of the wells andwas inoculated with overnight grown fresh culture of a unit OD.The strain was grown on coverslips kept in each well of a 12-well tissue culture plate(Nest BiotechnologyCo. Ltd.). First two wells were marked as positive and negative control, with original untreated bacterial culture and non-inoculated medium respectively.Rest of the wells were inoculated with 1% and 4% inoculum.Plates were covered and incubated at 37°C for 12-14 h.The wells were decanted and Crystal Violet assay was performed.

Measurement of Biofilm: The coverslips were put in 1ml crystal violet for 1min followed by washing with distilled water. The coverslips with bacterial growth were then observed under microscope (40X magnification). After destaining with 1ml of alcohol, the crystal violet stain was extracted from the coverslips adhered to the stained cells. The Optical densities (OD) of the extracted crystal violet from each of the wells was measured in UV-Visible spectrophotometer (Shimadzu, Japan) at wavelength 540 nm.

Test of anti-bio-filmic activities: The bacterial culture (2 ml) was treated with 200µl of Neem and Tulsi solutions independently and allowed to grow on the coverslip placed on well to detect the role of pretreatment by these inhibitors, while after bacterial growth and biofilm formation, these inhibitors were added to check the influence of the inhibitors on biofilm formation.

Statistical analysis: All the experiments were done in triplicate and their mean value with standard deviation was considered.

N.B. Before determining the optimum working concentrations, the kinetics of the biofilm formation by *Bacillus cereus* was checked with varying (1%, 2%, 4%, 6%, 8%, and 10%) concentrations of culture in 12 well Tissue culture plate (Fig 1)

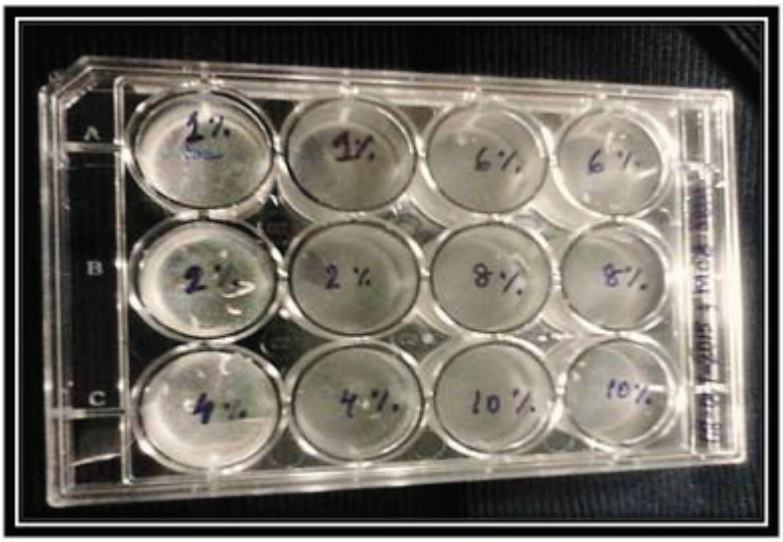


Fig 1: Tissue culture plate with coverslip for the growth of *Bacillus cereus*. Different wells have varied cell concentrations of 1%, 2%, 4%, 6%, 8% and 10%.

Results and discussion

The production kinetics revealed that the working strain achieved the maximum biofilm production at 4th hour of growth (Fig 2), afterwhich it slowly declined. Since the OD of biofilm measured was higher than 0.500, the strain could be designated as a high producer of biofilm (Maldonado *et al*, 2007)

The optimum concentration of cells for the biofilm formation was found to 4% inoculum (Fig 3), beyond which no further increase in biofilm production could be seen, which might be due to quorum sensing (QS) inhibition.

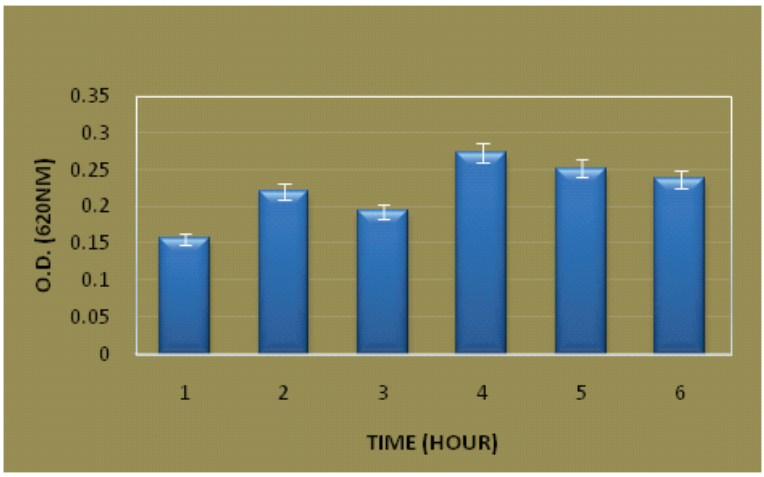


Fig 2.Kinetics of biofilm production.

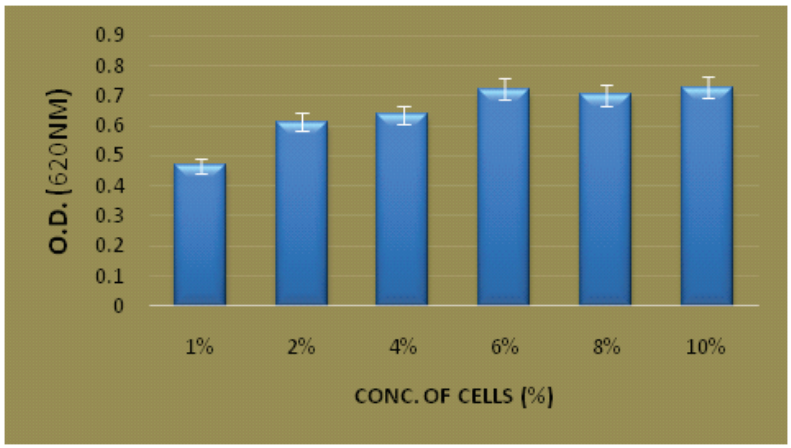
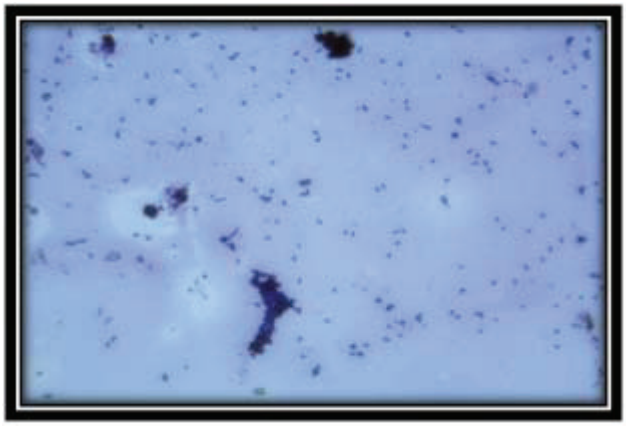
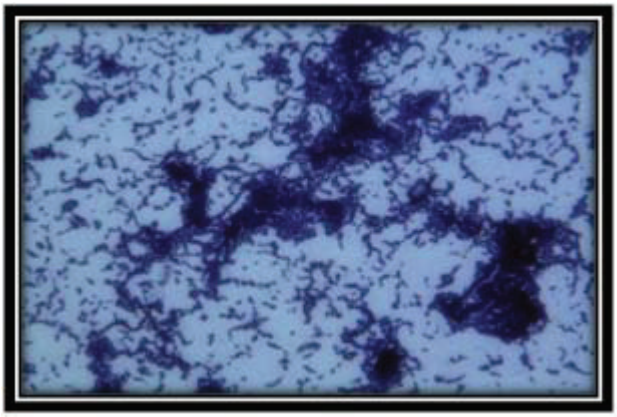


Fig 3.Effect of cell concentration on biofilm production.



A



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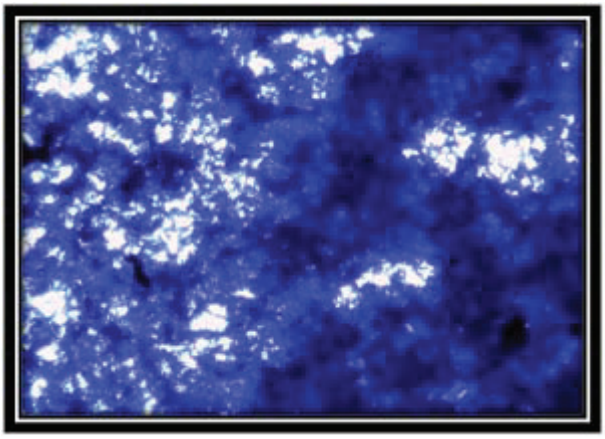

C

Fig 4.Photo-micrographic presentation of bio-film formation by *Bacillus cereus* (after 12th hour of incubation). Cell concentration: A: 1%, B: 2%, C: 4%.

To evaluate the role of NeemExtract (NE) and Tulsi Extract (TE) on biofilm, biofilm was developed in the absence and in the presence of NE and TE (Pre and post treatments).The effectiveness of Neem and Tulsi as an anti-biofilmic agent was determined from the O.D. values, which suggested that Neem exhibited a better anti-biofilmic efficacy against *Bacillus cereus* as compared to Tulsi. The results (Fig 5 and 6) showed that the Neem extract had visually appreciable activity against established biofilm formation, the maximum reduction obtained was of 88% (pre-treatment) and 91% (post treatment) in biofilm formation, when analyzed by Neem. Whereas the maximum reduction for Tulsi was determined to be 58.69% (pre-treatment) and 33.45% (post treatment). Maximum clearance on the Gram Stained smears was also found with the Neem as anti- biofilmic agent. Tulsi showed lower biofilm eradication capabilities.

Exposure to selected concentrations (5%, 10%, 15%, 20% and 25%) of NE and TE also changed the count of rod-shaped cells of *Bacillus cereus* along with significant reduction in exo-polysaccharide amount.

The results revealed that Tulsi extracts had the anti-bacterial property rather than working as an anti-biofilmic agent whereasNeem extract possessed anti-biofilmic property which reinforced the possibility of employing NE in the eradication of biofilm infections.

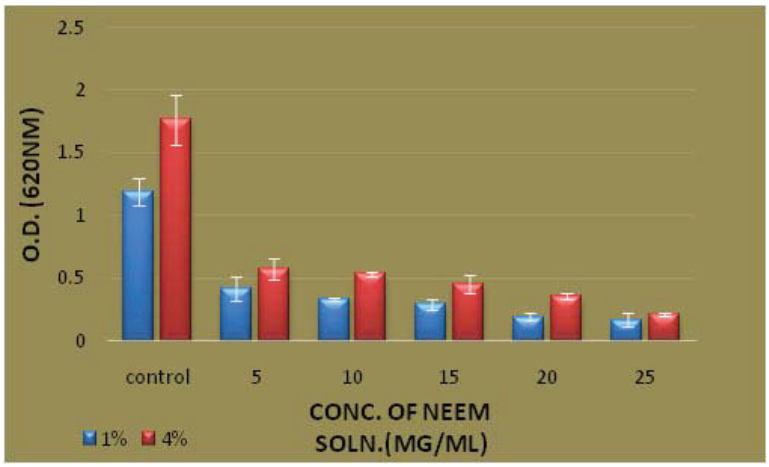

Fig 5 (a) Pre-treatment of Neem solutions (1% & 4% cell conc. of *B. cereus*)

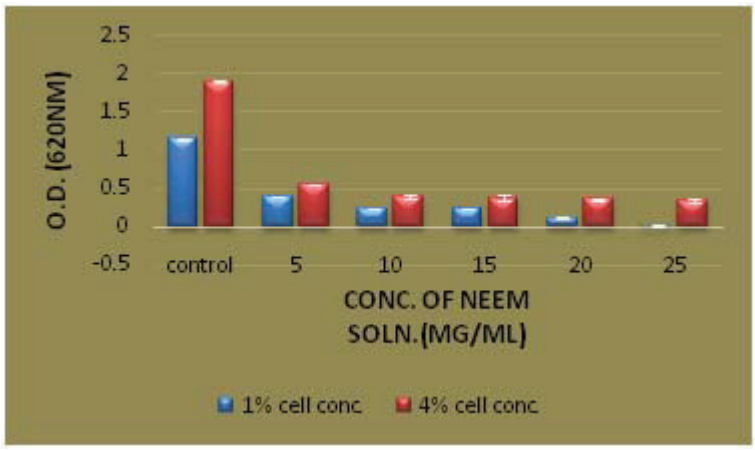

Fig 5 (b) Post-treatment of Neem solutions (1% & 4% cell conc. of *B. cereus*)

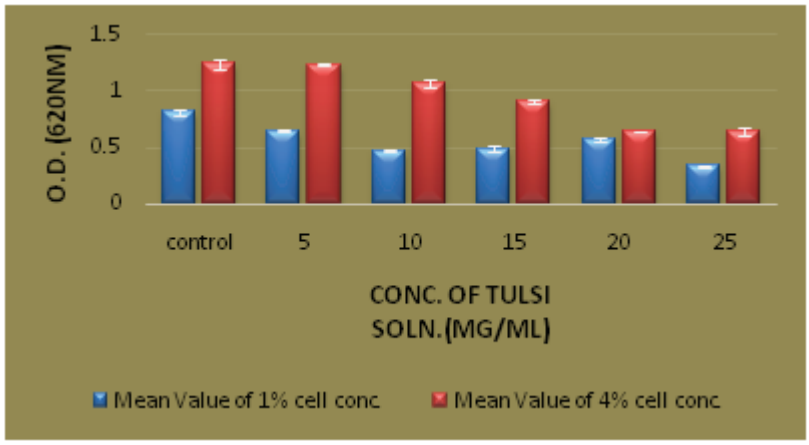

Fig 6 (a) Pre-treatment of Tulsi solutions (1% & 4% cell conc. of *B. cereus*)

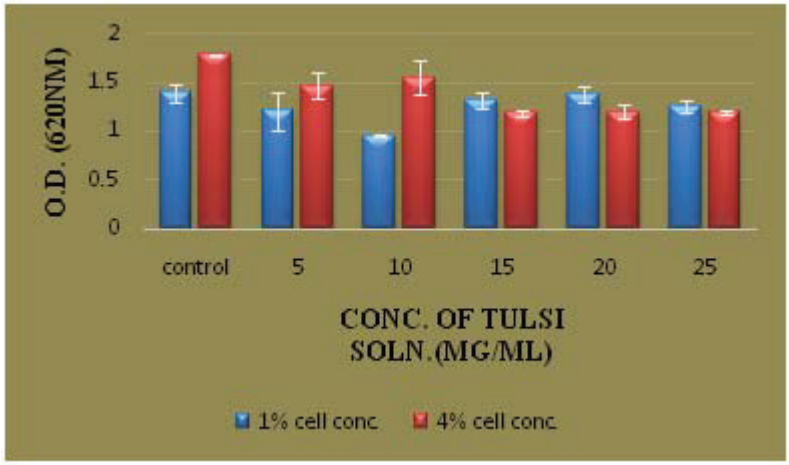

Fig 6 (b)Post-treatment of Tulsi solutions (1% & 4% cell conc. of *B. cereus*)

Conclusion

As global scenario is changing toward the use of nontoxic plant products having medicinal value, hence extensive research is required on Neem for its better economic and therapeutic utilization. Our results hold a great promise for the medical community, due to the observed reasonable efficiency of Neem on the reduction of the bacterial biofilm.

Acknowledgement

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