



Research Article

IN VITRO ANTI BIOFILM ACTIVITY OF *PIPER LONGUM* AND *PIPER NIGRUM* AGAINST CLINICAL ISOLATES OF *STREPTOCOCCUS PYOGENES* ISOLATED FROM PHARYNGITIS PATIENTS

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ABSTRACT

Streptococcus pyogenes is the persistent cause of purulent infections in humans. Biofilm formation is one of the important aspects of its pathogenicity. *S. pyogenes* biofilm communities favor significant resistance to antibiotic therapy. Analyzing novel sources against biofilm forming pathogens is therefore an important alternative treatment measure. In the present study we attempted to determine the antibiofilm activity of medicinal plants *Piper longum* and *Piper nigrum* against streptococcal biofilms. The chloroform, ethyl acetate, methanol and petroleum ether extracts of *P. longum* and *P. nigrum* grains were assessed for their anti-biofilm property in clinical isolates of *S. pyogenes* and a reference strain *S. pyogenes* MTCC 1924. The antibacterial activity test revealed that chloroform and petroleum extracts inhibited the test bacteria than other extract. The solvent extracts of both the plants showed an MIC at 2 - 4 mg/ml, depending on the solvent used. Growth curve analysis revealed that all the plant extracts did not have any effect on the bacterial growth at sub MIC level. The antibiofilm assay of various plant extracts showed varying degree of inhibition Ethyl acetate > Methanol > Chloroform > Petroleum Ether in a dose (0.5-2 mg/ml) dependant manner without affecting the bacterial growth. The methanol and ethyl acetate extracts of *P. longum* and *P. nigrum* showed significant ($p < 0.05$) biofilm inhibition in the range of 85 % to 96 % whereas the BIC of chloroform and petroleum ether extracts were significant 71 % to 84 % at a concentration of 2 mg/ml respectively. Streptolysin S activity of the isolates treated with plant extracts showed significant ($p < 0.05$) decrease as compared to control. Light microscopic and CLSM images proved the inhibition of *S. pyogenes* biofilm at varying concentrations.

Keywords: Indian medicinal plants, Biofilm Inhibitory Concentration, Streptococcal biofilms, Streptolysin S.

INTRODUCTION

Streptococcus pyogenes is one of the most important human pathogens associated with extensive human morbidity worldwide¹. *S. pyogenes* is classified as Group A streptococcus (GAS), Gram-positive, facultative anaerobic bacterium. It is associated with primary infections of skin, throat and mucosal surfaces. Usually the bacterial infection is self limited; antibiotics are administered to relieve discomfort and reduce complications². *S. pyogenes* produces three types of exotoxins which are responsible for causing fever and scarlet fever rashes³. Host immunity to *S. pyogenes* results in the development of antibodies specific to M protein⁴. *S. pyogenes* can be considered as an opportunistic pathogen. It is a part of the normal flora of the respiratory tract in many people, but usually does not cause complications until the person's natural defenses to disease is compromised. *S. pyogenes* is somewhat unique in that it does not cause just one disease, but is capable of causing many different diseases. *S. pyogenes* is the most common bacteria to cause sore throat⁵. Biofilm is a structured community of microorganisms encapsulated within a self developed polymeric matrix and adherent to a living or inert surface. Biofilms are also often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions and an extracellular matrix of polymeric substances. Single-celled organisms generally exhibit two distinct modes of behavior⁶. Biofilm formation is evident in allowing the bacteria to become resistant to antibiotic treatment and cause failure of antimicrobial therapy by significantly impairing the antibiotics function⁷. Failure of antibiotic treatment against *S. pyogenes* infections have increased and are known to be associated with biofilm formation which acts as a barrier against the action of antimicrobials⁸. To date penicillin remains as the antibiotic of choice for *S. pyogenes* infections as it has narrow spectrum, safety and cost

effective⁹. Although combination therapy provided the answer to antibiotic resistance for a while, there have been reports of emerging resistance to drugs in combination and multi-drug resistance in some bacteria¹⁰. As there is an increase in antibiotic resistance of *S. pyogenes* efforts are exerted to identify novel substances with good antibacterial properties. Drugs derived from natural sources are of major interest and thus they are focused for activity against biofilm producing microorganism. Natural drugs with good efficacy could help in limiting the emergence and transmission of these organisms. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illnesses since ancient times¹¹. Plant compounds are widely accepted due to the perception that they are safe and they have a long history of use in folk medicine as immune boosters and for the prevention and treatment of several diseases¹². Over the years, the use of medicinal plants, which forms the backbone of traditional medicine, has grown with an estimated 80 % of the populations, mostly in developing countries, relying on traditional medicines for their primary health care¹⁴. The resistant biofilms remain largely unexplored although they have been shown to be more resistant to antimicrobial agents than their planktonic counterparts¹⁵. *Piper nigrum* (common name: peppercorn) is a natural spice widely used in the Ayurvedic medicine. It is used in treatment for asthma, cough and diabetes and heart problems¹⁶. *Piper nigrum* (black pepper) and *Piper longum* (long pepper) are the plants of piperaceae family. The plants are traditionally used for treatment in malaria in India and are also reported to have many medicinal properties¹⁷. Owing to their exceptional medicinal properties the plants are widely used in Ayurvedic treatments for asthma, cough and diabetes and heart problems¹⁶. In this study, we assessed the antibiofilm properties of *P. nigrum* and *P. longum*

solvent extracts against *S. pyogenes* isolated from throat swabs of pharyngitis patients.

MATERIALS AND METHODS

Plant Collection and Preparation of Plant Extracts

Dried grains of *Piper longum* and *Piper nigrum* were obtained from local market in Coimbatore, Tamil Nadu, India. Voucher specimens of *P. longum* (Voucher No: 1112) and *P. nigrum* (Voucher No: 1113) have been deposited in the Botanical Survey of India, Southern Circle, Tamil Nadu, India Agricultural University, Coimbatore, Tamil Nadu, India. The pepper grains were washed, shade dried, finely powdered, sieved and stored until use. Five grams of powdered *P. longum* and *P. nigrum* were soaked in 50 ml of solvents such as methanol (M), chloroform (C), petroleum ether (P.E) and ethyl acetate (E.A) respectively overnight. The extracts were then filtered through Whatman No.1 filter paper. Removal of solvents from filtrate was done using rotary vacuum evaporation. Following vacuum evaporation, the dried extracts were collected and stored. Plant extract was dissolved in 100 % DMSO (v/v) and were diluted using sterile water prior to use.

Bacterial Strain and Culture Conditions

A total of 25 isolates were obtained from throat swabs of pharyngitis patients at Karpagam faculty of medical science and research institute, Coimbatore, Tamil Nadu, India. All the isolates were screened for *S. pyogenes* using Streptococcus selection agar (Himedia, Mumbai, India). *S. pyogenes* MTCC 1924 (IMTECH, Chandigarh) was used as reference strain. All the isolates were cultivated in Todd Hewitt's Broth (THB) (Himedia) for routine analysis. Glycerol stock was maintained at -20°C for further use. The *S. pyogenes* isolates were assayed for biofilm formation by inoculating in 100 µl THB in 96 well microtitre plates and incubation at 37 °C for 48 h. Isolates having biofilm formation potential alone were subjected for further analysis.

Antibacterial Activity Assays

The antibacterial activity potential of *P. longum* and *P. nigrum* solvent extracts were performed through agar well diffusion method in Muller-Hinton agar (MHA) (Himedia, India) by following the methods specified by Clinical and Laboratory Standards Institute (CLSI, Wayne, PA)¹⁸. Briefly, 100 µl of test bacterial suspensions such as KU-SP11, 16, 17, 18, 25 and MTCC 1924 with the cell density equivalent to 0.5 McFarland standard units (1×10^8 CFU/ml) were uniformly spread over the surface of MHA plate. The plates were kept undisturbed for 10 min for the absorption of excess moisture. 2 mg/ml of *P. longum* and *P. nigrum* extract were added to wells. Then, the plates were incubated at 37 °C and the zone of inhibition was measured after 24 h. Sterile distilled water was used as negative control and Penicillin G (0.03 mg/ml) was used as antimicrobial control. The minimal inhibitory concentration (MIC) assay of the plant extracts were performed according to the CLSI 2006 guidelines¹⁸. The bacterial suspensions (1×10^6 CFU/ml) were added to THB supplemented with different solvent extracts of *P. longum* and *P. nigrum* at concentrations ranging from 8 mg/ml – 0.0625 mg/ml and incubated at 37 °C for 24 h. The lowest concentration that produced inhibition of visible growth after overnight inhibition was recorded as MIC value.

Growth Curve Analysis

Growth curve analysis was done according to Issac Abraham et al (2011)¹⁹. Briefly, 1 % of overnight test pathogens (0.5 OD at 600 nm) were inoculated in 50 ml of LB broth separately supplemented with 2 mg/ml of *P. longum* and *P. nigrum* solvent extract. The flasks were incubated at 37 °C with 170 rpm agitation for 12 h in a rotator shaker. Cell density was measured using UV-visible spectrophotometer at every 1 h interval.

Biofilm Inhibition Assays

Biofilm Biomass Quantification Assay

Quantification of biofilm biomass was performed using Microtitre plate assay (MTP)²⁰ with slight modification. Briefly, 1 % overnight cultures (0.5 O.D at 600 nm) of test pathogens were added in 1 ml of fresh LB medium in the presence and the absence of *P. longum* and *P. nigrum* solvent extracts (0.5-2 mg/ml) separately. The samples were incubated at 37 °C for 16 h. After incubation, MTPs were emptied of free-floating planktonic cells and the wells were gently rinsed with sterile water. The biofilm was stained with 0.4 % crystal violet (CV) (Himedia, Mumbai, India) solution. After 15 min, CV solution was discarded completely and wells were filled with 1 ml of 95 % ethanol for destaining. The biofilm biomass was then quantified by measuring the absorbance at OD 650 nm using multiplate ELISA reader (Biotek-ELX-800, India).

Hemolysis Assay

Streptolysin S (SLS) Assay

SLS-haemolysis assay were performed according to Loridan and Alouf (1986)²¹ with slight modifications. Briefly, Sheep blood was procured and centrifuged at 3000 rpm for 3 min. The serum was collected and then washed with PBS buffer for 3 times by centrifugation. Then, 900 µl of 10 Mm Tris buffer was added and 100 µl of culture supernatant (control and treated) was added with 100 µl of sheep RBC and incubated for 20 min at -4 °C. It was then centrifuged at 3000 rpm for 5 min. The resulting supernatant absorbance was read at 540 nm using UV-visible spectrophotometer.

In Situ Microscopic Observation of Bacterial Biofilms

Light Microscopic Analysis

Visualization of biofilm by light microscopy was performed according to Nithya et al, (2010)²⁰. Briefly, the biofilm were allowed to grow on glass pieces (1 x 1 cm) placed in 24-well polystyrene plates supplemented with different solvent extracts of *Piper longum* and *Piper nigrum* (2 mg/ml) and incubated for 24 h at 37 °C. The slides were stained using crystal violet and were placed on slides with biofilm pointing upwards. The slides were observed under light microscopy at magnification of $\times 40$. Visible biofilms were documented with an attached digital camera (Nikon eclipse Model: E300).

Confocal Laser Scanning Microscopic (CLSM) Analysis

Confocal laser scanning microscopy was performed according to Nithya et al., (2010)²⁰ with slight modification. The test bacterial isolates were allowed to form biofilms on the glass slides as mentioned in the light microscopic analysis. After 16 h, biofilms in the glass slides were stained with 30 µl of 1 % acridine orange for 1 min. The excess stain was washed out and the stained glass slides were visualized with CLSM (Model LSM 710, Carl, Zeiss, Jena, Germany) at a magnification of $\times 20$.

Statistical Analysis

Statistical analysis was performed using SPSS (V.16, Chicago, USA). Values were expressed as mean \pm S.E. One way ANOVA test was used to compare parameters and values were considered significant if $p < 0.05$.

RESULTS

Effect of Plant Extracts on *S. pyogenes* Growth

All the four extracts of *P. longum* and *P. nigrum* were evaluated for their antibacterial activity by agar well diffusion assay. The methanol, ethyl acetate extracts of *P. longum* and *P. nigrum* showed no antibacterial activity against all test pathogens as there was no zone of inhibition at 2 mg/ml concentration. The chloroform,

petroleum ether extracts of *P. longum* and *P. nigrum* showed antibacterial activity as there was zone of inhibition (Table 1) at 2 mg/ml concentration which was also confirmed by MIC assay (Figure 1). The solvent extracts of *P. longum* and *P. nigrum* revealed varying degree of inhibition. The MIC of chloroform and petroleum ether was observed at 2 mg/ml, whereas the MIC of methanol and ethyl acetate at 4 mg/ml against the test bacteria. Hence a concentration below 2 mg/ml (Sub-MIC) was taken for further studies.

Growth Curve Analysis

The antibacterial activity of *P. longum* and *P. nigrum* solvent extracts were further evaluated by the growth curve analysis. The results indicate that all the extracts had no effect on the growth of the tested pathogens at 1 mg/ml concentration which was evident from absence in the cell densities between the treated and the untreated cultures. Results shown in Figure 2 (a-f) reveal that the *P. longum* and *P. nigrum* extracts did not possess any antibacterial activity at tested concentration against all test bacteria as compared to control.

Streptolysin- S (SLS) Assay

The bacterial isolates treated with different solvent extracts of *P. longum* and *P. nigrum* at 2 mg/ml were tested for Streptolysin S hemolysis assay. Streptolysin S (SLS) is a toxic immunogenic protein produced and secreted by most *S. pyogenes* group A strains. It is cytolytic or cytotoxic toward erythrocytes and other types of eukaryotic cells. Figure 3 represents the lysis of intact erythrocytes with the release of haemoglobin when the cell free liquid of *S. pyogenes* isolates treated with *P. longum* and *P. nigrum* extracts were added. Thus from the spectrophotometric observation, it is confirmed that absorbance of the control was less, which indicated the release of haemoglobin due to lysis of erythrocytes by SLS, whereas the plant extract treated test pathogens showed high absorbance values indicating intact erythrocytes. Hence it is confirmed that *Piper longum* and *Piper nigrum* solvent extracts were able to significantly ($p < 0.05$) reduce SLS activity of *S. pyogenes*.

Effect of Plant Extracts on Bacterial Biofilm Formation

The effects of different concentrations (0.5-2 mg/ml) of methanol, ethyl acetate, chloroform and petroleum ether extracts of *P. longum* and *P. nigrum* on biofilm-forming ability of test bacteria were determined. Evidently, a pronounced biofilm inhibition percentage was observed in the following order as ethyl acetate > methanol > chloroform > petroleum ether extracts of *P. longum* and *P. nigrum* against all test bacteria at a lowest concentration of 0.5 mg/ml. Figure 4a and 4b validated that methanol and ethyl acetate extracts of *P. longum* and *P. nigrum* have promising anti biofilm activity against the *S. pyogenes* isolates and MTCC 1924 at lowest BIC, respectively as compared to control. Significant ($p < 0.05$) inhibition of biofilm was observed in methanol, ethyl acetate extracts when compared to chloroform and petroleum ether extracts of *P. longum* and *P. nigrum*. To analyze the full potential of methanol and ethyl acetate extracts of both the plants in biofilm inhibition, susceptibility analysis was carried out at sub inhibitory concentrations by diluting the solvent extracts in two fold. The sub BIC (0.5 BIC) concentration of methanol and ethyl acetate extracts showed significant ($p < 0.05$) reduction in the biofilm inhibition of about 32-47 % (data not shown) in PN and 41-62 % in *P. longum*. The biofilm inhibitory potential of methanol and ethyl acetate extracts of both the plants at 2 mg/ml concentration, on the test bacteria was also confirmed by microscopic visualization (Figure 5A-5D). The CLSM analysis was performed to determine the architecture of the *S. pyogenes* biofilm biomass treated with *P. longum* and *P. nigrum* solvent extracts. The CLSM images revealed evident differences in biofilm matrix between treated and the untreated biofilms of all the test pathogens (Figure 6A and 6B).

DISCUSSION

Piper longum and *Piper nigrum* used in this study had been used as food sources all over the world. Although, the primary purpose of spices is to impart flavour and piquancy to food, the medicinal, antimicrobial and antioxidant properties of spices have also been exploited²². The antimicrobial activity of spices is documented and interest continues to the present²³. Though these plants serve as food and are also used for medicinal purposes little is known about the anti-biofilm property of these plants. This study provides an insight on the anti biofilm capabilities of these plants against the clinical isolates of *S. pyogenes*. Research has shown that, various spices exhibit antimicrobial activity against a wide range of microorganisms, however the efficacy differs depending on the type of spice, solvent used for extraction, composition and concentration of secondary metabolites and test organisms used. Table 1 shows the summary of the antibacterial results obtained from this study. It was observed that among the solvent extracts of *Piper longum* and *Piper nigrum*, chloroform and petroleum ether showed good inhibition against the test bacteria at 2 mg/ml (sub MIC concentrations). Interestingly, methanol and ethyl acetate extracts of *Piper longum* and *Piper nigrum* did not show inhibition against the test bacteria. The difference in the sensitivity is consistent with previous research which elucidated that isolates of the same species obtained from different sources exhibit different sensitivities to antimicrobial agents²⁴. It is evident from the results (Figure 1a and 1b) that among the four solvent extracts of *P. longum* and *P. nigrum*, chloroform and petroleum exhibited good antibacterial activity against the *S. pyogenes* isolates KU-SP11, KU-SP16, KU-SP17, KU-SP18, KU-SP25 and *S. pyogenes* MTCC 1924. The chloroform and petroleum ether extracts of *P. longum* showed good antibacterial activity against the test bacteria, compared to *P. nigrum*. The solvent type greatly influenced the antimicrobial activities of the extracts. The minimum inhibitory concentration (MIC) assay revealed that chloroform and petroleum ether extracts of *P. longum* and *P. nigrum* had minimum inhibitory concentration greater than 2 mg/ml, while the methanol and ethyl acetate extracts had MIC value of 4 mg/ml (Figure 1a). These results were evident from the antibacterial activity at sub MIC concentration of 2 mg/ml as shown in Figure 1b. Our results corroborate with that of Kuethe *et al.* (2008)²⁵, where greater inhibition of biofilm by methanol extracts have been attributed to the presence of phenolics and terpenoids well known for exhibiting antimicrobial activity and hence greater activity. This trend in sensitivity is consistent with other research findings, where Gram-positive bacteria have been reported to be more susceptible to solvent extracts than Gram-negative bacteria and yeasts²⁶. The Gram-positive cell membrane is single layered and hence offers little or no resistance to the entry of substances including antimicrobial agents than the gram-negative bacteria²⁷. Bupesh *et al.*, (2007)¹⁵ showed that organic leaf extracts of *M. piperita* possessed broad spectrum antibacterial activity against a wide range of both Gram-negative and Gram-positive bacteria. This activity has been attributed to the presence of potent compounds that include menthol, menthone, menthyl acetate and menthofuran in different solvent extracts used for the study. Figure 2 (a-f) validated that, all the solvent extracts of *P. longum* and *P. nigrum* did not interfere with the bacterial cell growth at tested concentrations (1 mg/ml) as the cell densities did not differ between untreated control and test bacteria. The results elucidated that the solvent extracts of *P. longum* and *P. nigrum* interfered at any step during the biofilm development but evidently did not inhibit the bacterial growth at tested concentration. According to Vattem *et al.* (2005)²⁸, the spices with well known antibacterial properties could also potentially possess antipathogenic activities, which may not be related to growth inhibition of the microorganism; which is well correlated with our results. The methanol and ethyl acetate extracts of *P. longum* and *P. nigrum* used in the present study is found to be ineffective on bacterial growth, but affects the cell-cell communication. As, the subsequent concentrations of plant extracts used for further studies were well below the MIC, it is less expected that it would impose a selective pressure for the development of resistance.

Table 1: Antibacterial activity assay of *P. longum* and *P. nigrum* solvent extracts on *S. pyogenes* isolates

Plant source	Plant Extract (2 mg/ml)	Zone of inhibition (mm)					
		MTCC 1924	KU-SP11	KU-SP16	KU-SP17	KU-SP18	KU-SP25
<i>P. longum</i>	Methanol	-	-	-	-	-	-
	Chloroform	18	12	21	12	15	25
	Petroleum Ether	23	21	20	20	23	24
	Ethyl Acetate	-	-	-	-	-	-
	Distilled water(-ve control)	-	-	-	-	-	-
	Penicillin-G*	31	28	33	34	28	25
<i>P. nigrum</i>	Methanol	-	-	-	-	-	-
	Chloroform	24	22	22	21	24	21
	Petroleum Ether	20	23	24	20	17	12
	Ethyl Acetate	-	-	-	-	-	-
	Distilled water(-ve control)	-	-	-	-	-	-
	Penicillin-G*	30	31	25	28	27	28

* Antibiotic used as positive control (0.03 mg/ml)

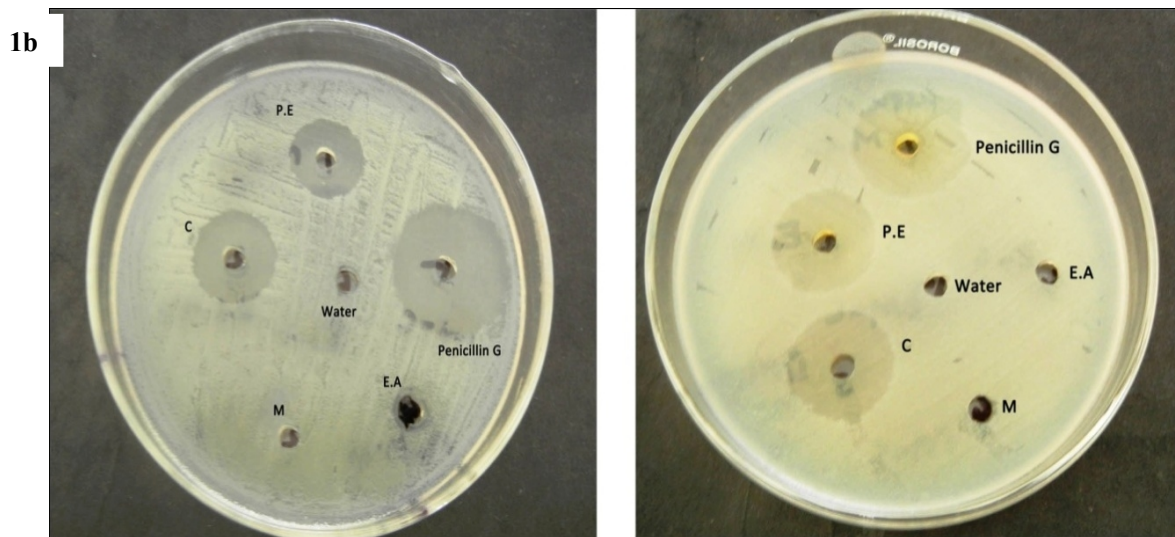
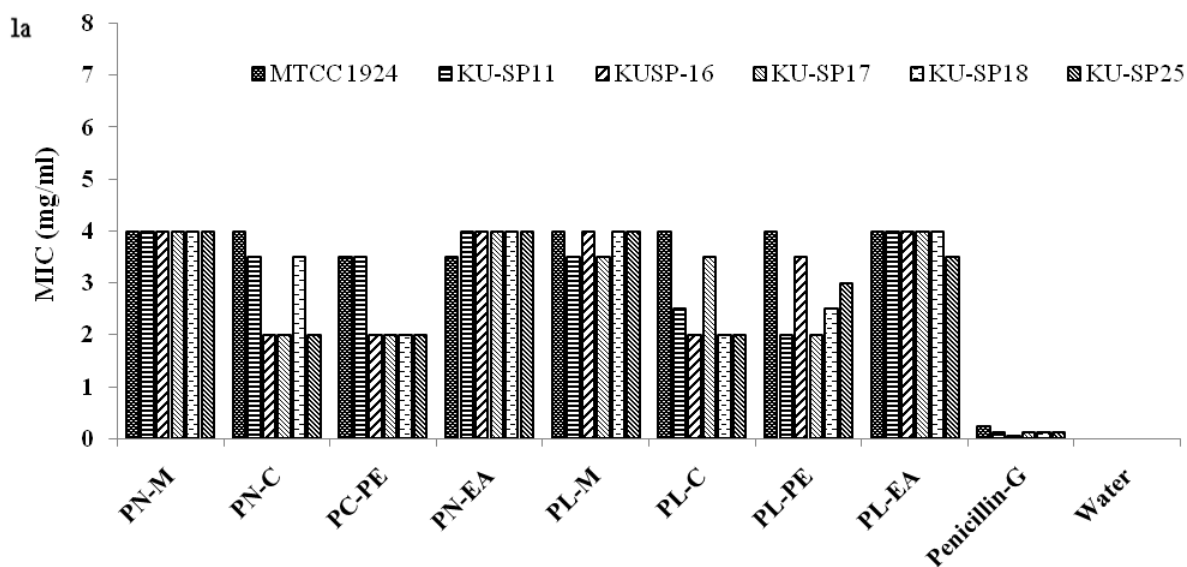
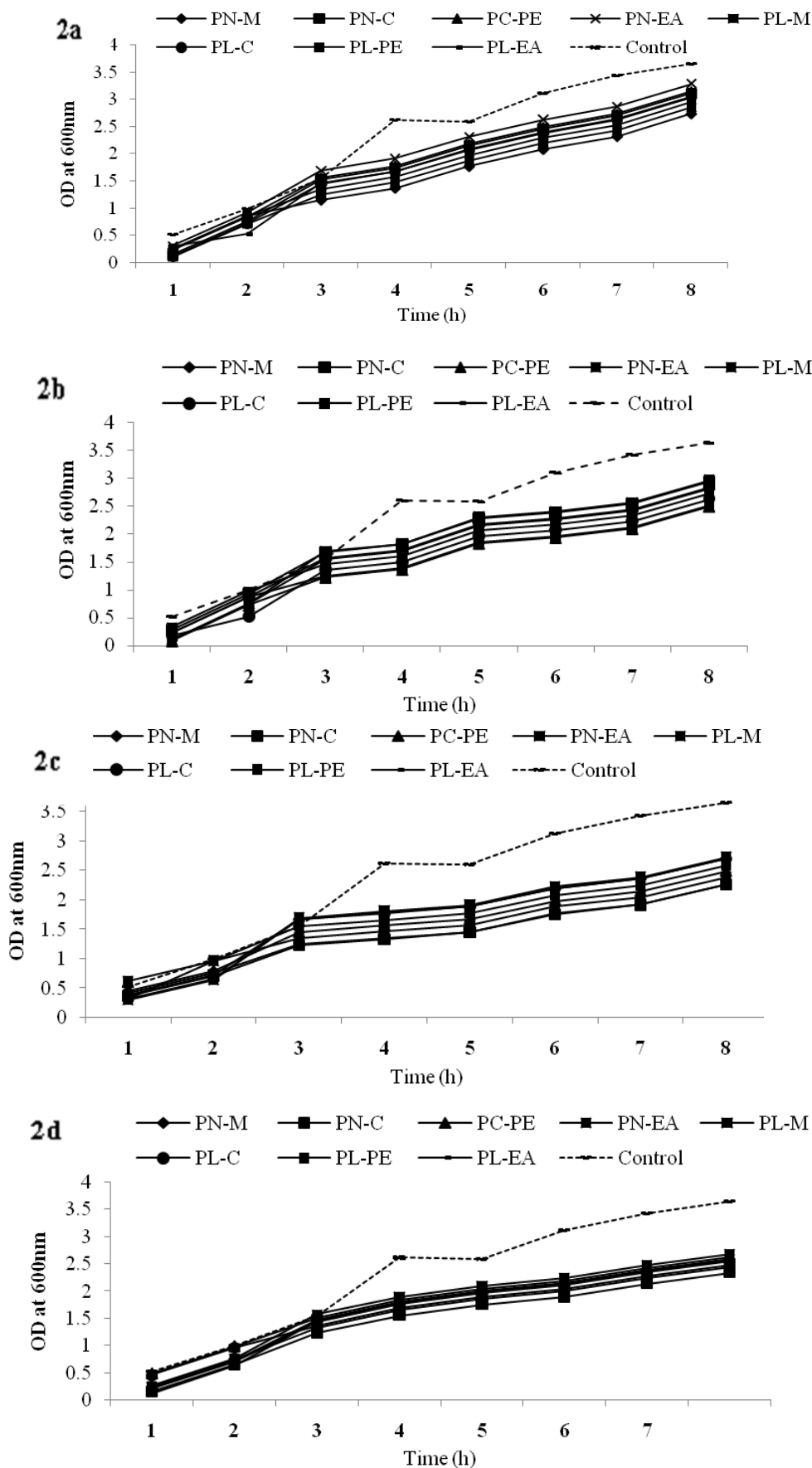


Figure 1: a) Minimum Inhibitory concentration assay of different solvent extracts (0.0625 – 8 mg/ml) in two fold dilution against *S. pyogenes* isolates. b) Antibacterial activity of *P. longum* and *P. nigrum* against KU-SP25 at 2 mg/ml concentration (P.E- Petroleum Ether, C- Chloroform, M-Methanol, E.A- Ethyl Acetate, Penicillin G- positive control, Distilled water-negative control)



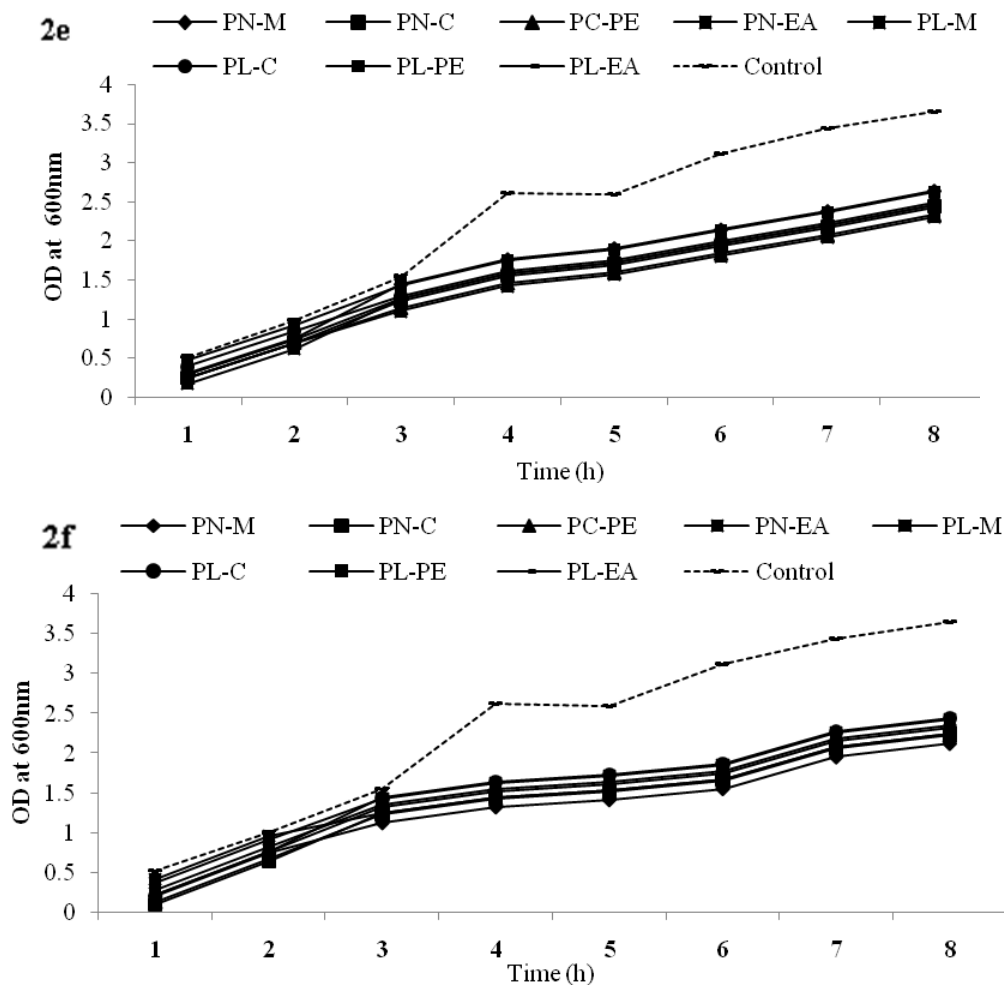


Figure 2: Influence of *P. longum* and *P. nigrum* on the growth of *S. pyogenes* isolates used in the experiment 2a) *S. pyogenes* MTCC 1924, 2b) KU-SP11, 2c) KU-SP16, 2d) KU-SP17, 2e) KU-SP18 and 2f) KU-SP25. The cell densities were quantified at 600 nm. (PN = *P. nigrum*, PL = *P. longum*, C= chloroform, PE=Petroleum ether, EA=Ethyl acetate, and M= Methanol)

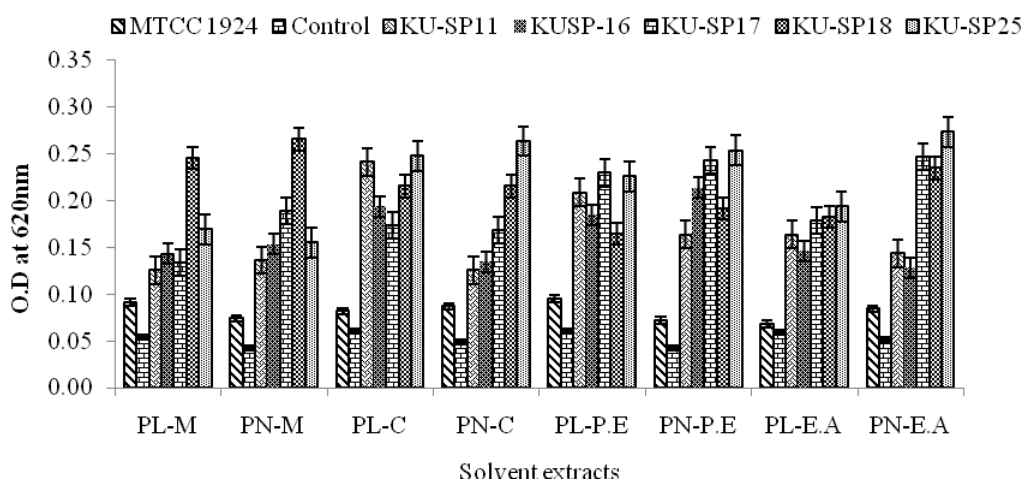


Figure 3: Determination of streptolysin S (SLS) inhibition by *P. longum* and *P. nigrum* solvent extracts. Values are mean \pm S.E. All isolates showed significant ($p < 0.05$) decrease in SLS activity as compared to control

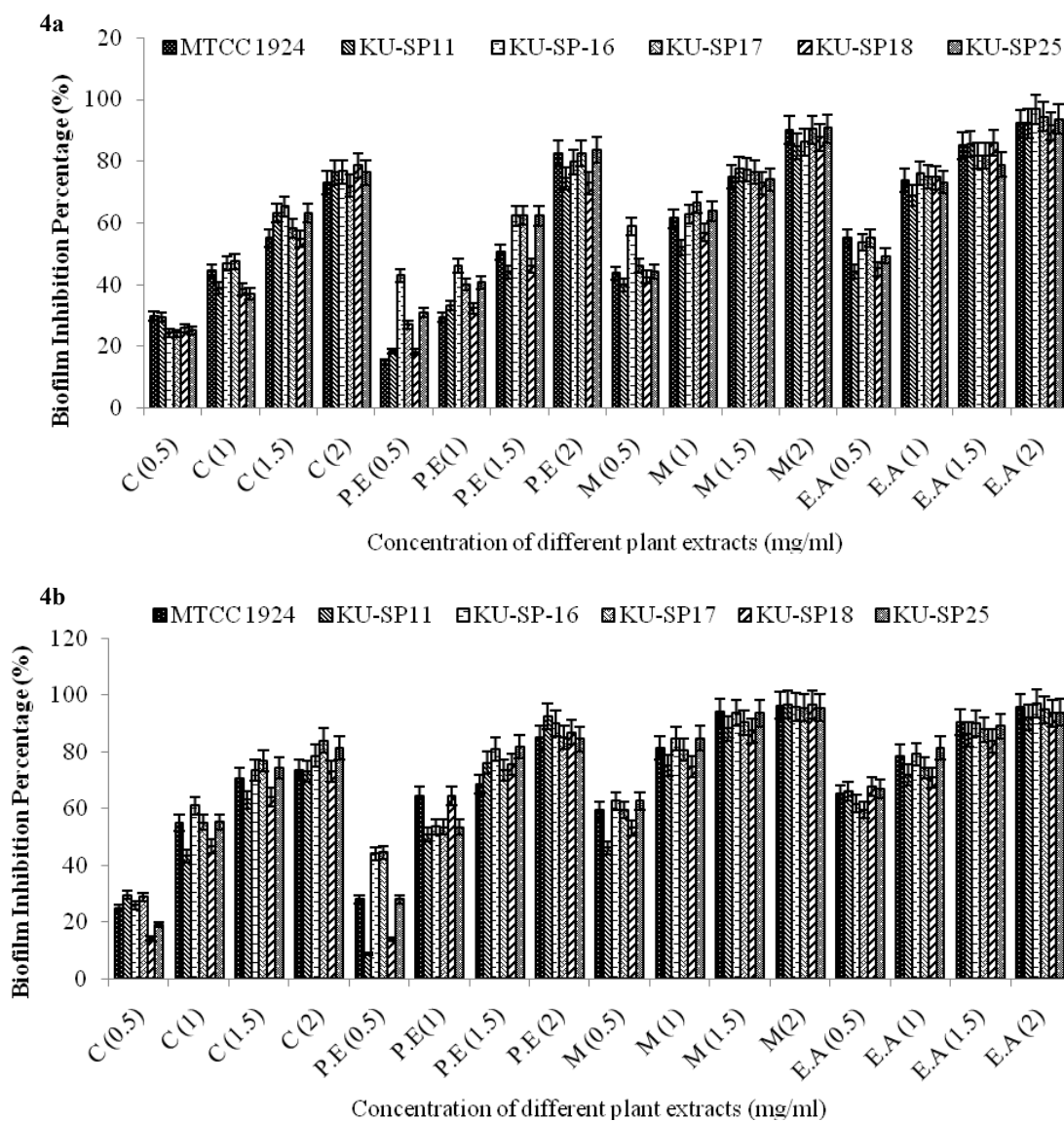


Figure 4: a) Percentage inhibition of biofilm formation of *S. pyogenes* by varying concentrations (0.5-2 mg/ml) of *P. nigrum* solvent extracts. b) Percentage inhibition of *S. pyogenes* isolates biofilm formation by varying concentration of *P. longum* solvent extracts. Mean values of triplicate independent experiments and S.E are shown.

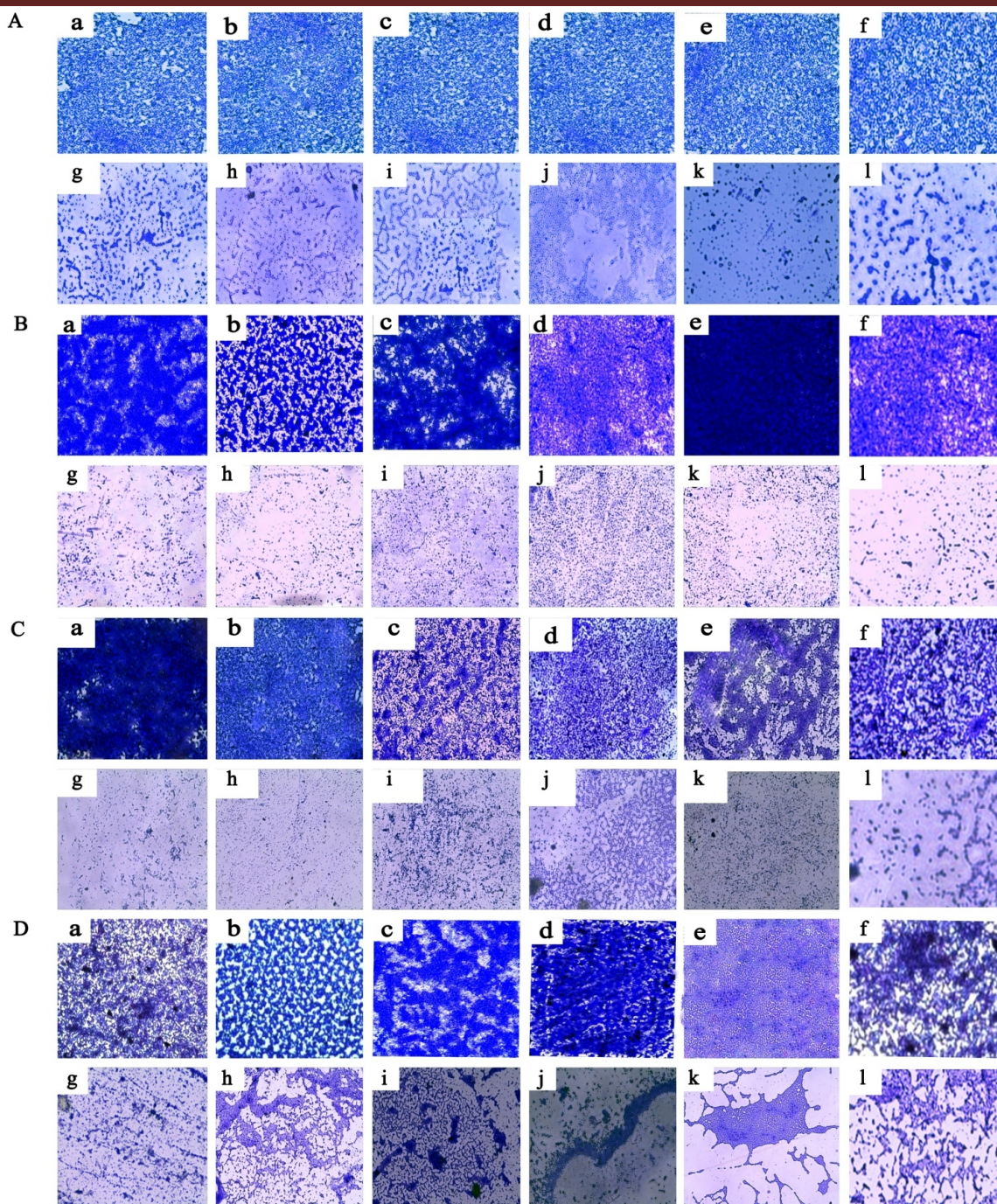


Figure 5: Light microscopy images (x 400) of *S. pyogenes* biofilms MTCC 1924, KU-SP11, KU-SP16, KU-SP17, KU-SP18 and KU-SP25 respectively, grown in the absence and/or presence of A) *P. longum* methanol extract, B) *P. longum* ethyl acetate extract, C) *P. nigrum* methanol extract, D) *P. nigrum* ethyl acetate extract (2 mg/ml). (a-f: Control, g-l: plant extract treated) (Images were taken using Nikon eclipse E200LED MV series microscope)

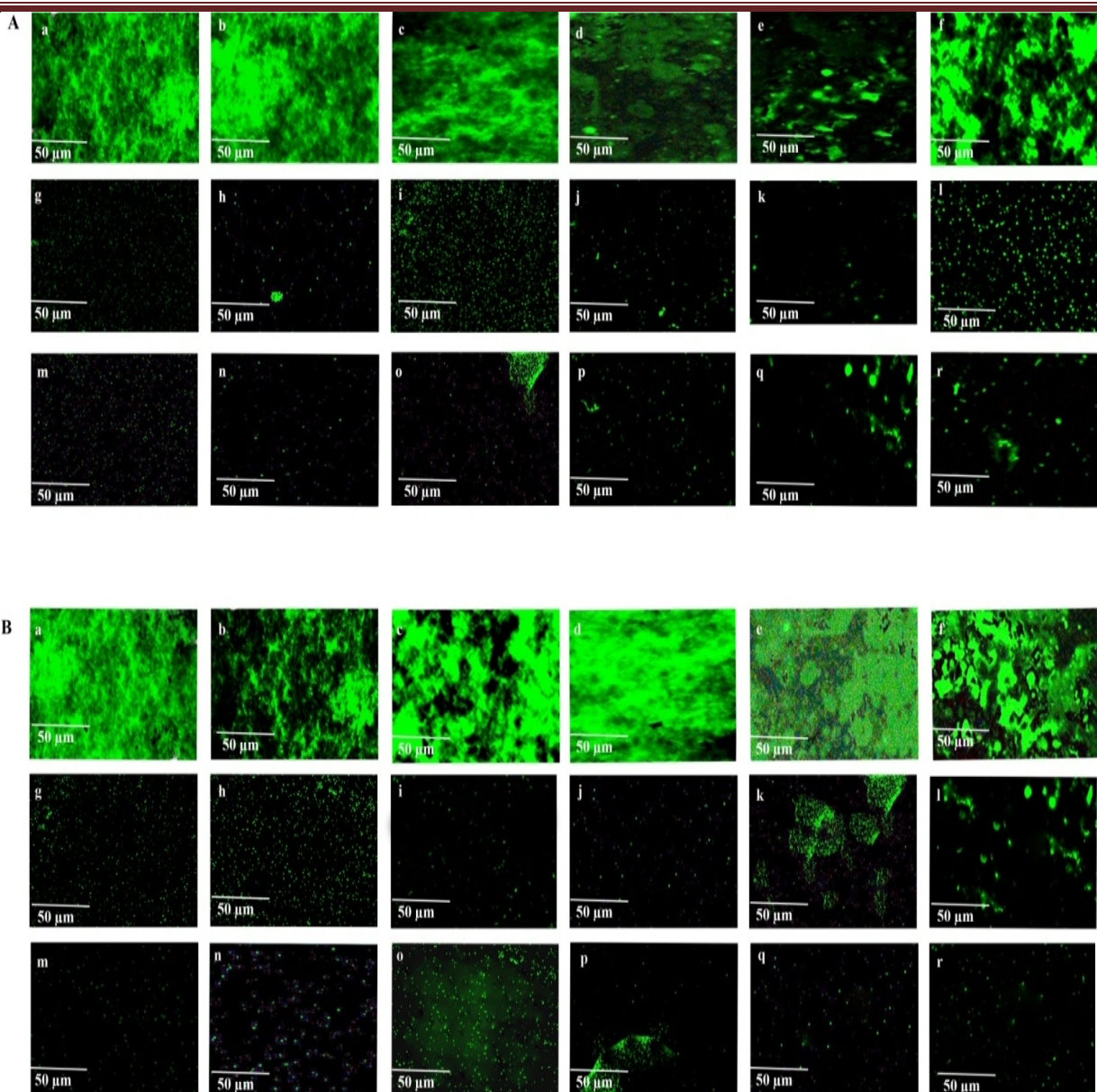


Figure 6: A-Confocal laser scanning microscopy (CLSM) images of (a-f) control, (g-l) *P. longum* methanol extract treated, (m-f) *P. longum* ethyl acetate extract treated (2 mg/ml) biofilms of *S. pyogenes* MTCC 2924, KU-SP11, KU-SP16, KU-SP17, KU-SP18 and KU-SP25 respectively. B- Confocal laser scanning microscopy (CLSM) images of (a-f) control, (g-l) *P. nigrum* methanol extract treated, (m-f) *P. nigrum* ethyl acetate extract treated (2 mg/ml) biofilms of *S. pyogenes* MTCC 2924, KU-SP11, KU-SP16, KU-SP17, KU-SP18 and KU-SP25 respectively

Streptolysin S (SLS) is an oxygen-stable oligopeptide primarily responsible for the characteristic zone of beta-hemolysis surrounding Group A streptococcal colonies grown on blood-agar medium²⁹. SLS production occurs in the cell free supernatants when *S. pyogenes* is cultured in the liquid media. Although SLS is not the only essential factor for the virulence of *S. pyogenes* the activity of SLS, an in-depth study of SLS has been more elusive³⁰. According to Kreikemeyer *et al.*, (2007)³¹ the virulence factors of *S. pyogenes* clinical isolates depends on the heterogeneity and the molecular characteristics of the isolates. In the present study we analyzed the effect of *P. longum* and *P. nigrum* solvent extracts against the SLS activity, which was considered as one of the virulence factors of *S. pyogenes*. Interestingly, it was observed that the test bacteria treated with plant extracts showed significant ($p < 0.05$) decrease in the SLS activity as compared with control (Figure 3). Further characterization of the activity at molecular level would elaborate a details mechanism on the inhibitory action. Quorum sensing (QS)

mechanism influences both the initiation and maturation of bacterial biofilm³². In the present study, exposure of test bacteria with *P. longum* and *P. nigrum* solvent extracts at 2 mg/ml significantly ($p < 0.05$) reduced the biofilm biomass, (Figure 4a and 4b). Evidently, it was observed that among all the solvent extracts used for study. Moreover, methanol and ethyl acetate extracts showed 50 % inhibition of the biofilm biomass at lowest concentration of 0.5 mg/ml. From Figure 5 (A-D) it is obvious that methanol and ethyl acetate extracts of *P. longum* and *P. nigrum* have inhibited the formation of micro colonies during the process of biofilm formation by the bacterial pathogens such as KU-SP11, KU-SP16, KU-SP17, KU-SP18 and KU-SP25 as compared to control. This evidently showed that biofilm formation was inhibited at the beginning of the attachment stage. It can therefore be postulated that treatment of the bacterial pathogens with plant extracts produced an unfavourable condition that repel the cells back into the fluid phase, thereby reducing surface adhesion. Milk constituents and chitosan have been

reported to inhibit cell attachment³⁴. Some researchers have also demonstrated the success of coating medical devices with biocides such as silver to reduce microbial adhesion and the subsequent disease pathogenesis³³. The present report is in agreement with the previous reports of You *et al.*, (2007)¹³, in which the extracts of *Streptomyces albus* significantly inhibited biofilm formation of *Vibrio sp.* by preventing their initial adherence. Therefore, it is envisaged that the active compound present in *P. longum* and *P. nigrum* possibly interacted with the expression of genes responsible for initial attachment, which facilitated the observed reduction in the biofilm formation. CLSM analysis of *P. longum* and *P. nigrum* extract treated biofilms displayed disintegrated architecture and reduced density than that of the untreated control biofilms of the test bacterial pathogens. It has been evidently shown that bacterial biofilm formation is mediated crucially by auto inducer peptides that also lead to the maturation of biofilm and so, inhibition of auto inducers peptides prevent biofilm formation³². As shown in Figure 6 (A and B), the biofilms of all tested bacterial pathogens were disturbed when treated with methanol and ethyl acetate extracts of *P. longum* and *P. nigrum*. These characteristics of the plant extracts could be employed for biofilm inhibition in combination with conventional antibiotics for improved drug delivery efficiency and disease management. Our results are in correlation with previous reports where the secondary metabolites from marine bacteria disturbed the biofilm formed by *Vibrio* spp²⁰.

CONCLUSION

The use of natural products as alternatives or complementary to conventional therapy has gained interest due to the perception that herbal products may be safe. Research on the efficacy of many natural products is currently under way with efforts to validate the reported pharmacological effects and also to identify active constituents that are responsible for many of the reported biological activities. Research on the antimicrobial activity of plants has been almost exclusively focused on the planktonic form of microorganisms. Less attention has been given to microbial biofilms as models in research although they have been implicated in most clinical infections and are more resistant to antimicrobial agents than the planktonic forms. Our study presents the data on the biofilm inhibition efficacy of *Piper longum* and *Piper nigrum* solvent extracts against the clinical isolates of respiratory tract pathogen *Streptococcus pyogenes*. In consideration of the non toxic nature and non antimicrobial properties, these plants could be used as a candidate medicine to prevent the onset of infections. However, further study on the extracts using advanced imaging techniques and molecular characterization of the principal compound responsible for anti biofilm property will enable a deeper insight on these medicinal plant in a new perspective.

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