



ISSN: 0975-833X

RESEARCH ARTICLE

ANTIBIOFILM ACTIVITY OF *PIPER LONGUM* ETHANOL EXTRACT AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS

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ARTICLE INFO

Article History:

Received 20th March, 2015

Received in revised form

27th April, 2015

Accepted 15th May, 2015

Published online 27th June, 2015

Key words:

Piper longum,
FT-IR analysis,
Antibiofilm,
MRSA,
Throat isolate

ABSTRACT

The study was carried out to ascertain the functional groups present in *Piper longum*, and to assess its ability to disrupt biofilms caused by throat infectious methicillin resistant *Staphylococcus aureus* (MRSA) strains. Ethanol extract of *Piper longum* was subjected to fourier transform infrared (FT-IR) analysis which revealed the presence of different pharmacologically active functional groups. Two strains of methicillin resistant *Staphylococcus aureus* (MH4 throat isolate and MTCC 96 standard strains) were used. Minimum inhibitory concentration (MIC) was performed by micro dilution assay on a 96 microtitre well plate (OD 600 nm) and was found to be 1 mg/ml for both isolate MH4 and MTCC 96. This was further confirmed by agar well diffusion assay and growth curve analysis. Biofilm inhibition assay was performed at sub-MIC concentrations using microtitre plate (24 well plates) assay. The highest biofilm disruption was observed at 0.5 mg/ml concentration for both strains. This study revealed potential antibiofilm activity exhibited by the ethanol extract of *Piper longum* against the ever rising methicillin resistance in *Staphylococcus aureus* infections.

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Citation: Isoe Moraa Everlyne, *¹Teepica Priya Darsini, D., ²Kulandhaivel, M. and ¹Srinivasan, P., 2015. "Antibiofilm activity of *Piper longum* ethanol extract against methicillin resistant *Staphylococcus aureus* strains", *International Journal of Current Research*, 7, (6), 17030-17035.

INTRODUCTION

Staphylococcus aureus is a Gram-positive coccal bacterium that is a member of the firmicutes, frequently found in the human respiratory tract and on the skin. *S. aureus* is the most common bacterial species to cause staph infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies Kylutmans *et al.* (1997), Cole *et al.* (2001). *S. aureus* continues to be a dangerous pathogen for both community-acquired as well as hospital-associated infections. Several serious diseases are caused by biofilm-associated *S. aureus* infections in which quorum sensing system is thought to play a major role Jeremy *et al.* (2004). The pathogenicity of these bacterial infections range from soft tissues, skin, respiratory, joint endovascular bone, to wounds. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical

wound infections. One successful strategy of regulation of virulence developed by the bacteria to overcome host defenses involves synchronizing the expression of virulence factors as a function of the population density in a process known as quorum sensing Miller *et al.* (2001). Quorum sensing in *S. aureus* leads to biofilm formation which aids the pathogen in drug resistance and enhanced virulence. Furthermore the biofilm formation in MRSA has become prevalent globally. Biofilms are complex architecture of microbial cells that are highly resistant against unfavourable conditions and antibiotics. Formation of biofilm is recognized as an important virulence factor for the opportunistic pathogens and "true" pathogens. Todar *et al.* (2005) Bacteria use quorum sensing to coordinate behaviors like biofilm formation, which can occur within a single bacterial species as well as between diverse species. Single-celled organisms generally exhibit two distinct modes of behaviour which are; biofilms and planktonic forms as described by Patterson *et al.* (2010). Medicinal plants have served a basis for the treatment of many diseases including bacterial diseases. Although a lot of research on plants and the active constituents is currently underway, the focus is mainly on the antimicrobial properties against planktonic bacteria.

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The resistant biofilms remain largely unexplored Bupesh *et al.* (2007). *Piper longum* commonly known as long pepper is a flowering vine native to Indo-Malayan region. It belongs to the *Piperaceae* family and cultivated for its fruits, which is used as a spice and seasoning. The crude drug “Piperis Longi Fructus,” of the *P. longum* is commonly used in folk medicine to treat bronchial trouble and is used as a carminative and analgesic. Parmar *et al.* (1997) and Jung *et al.* (2010) The *P. longum* contains 1% volatile oil, alkaloids, resin, piperlonguminine and piperine, a waxy alkaloid N-isobutyl deca-trans-2-trans-4-dienamide and a terpenoid substance. There is a number of reports on the medicinal properties of *P. longum* but using the traditional claim on its use for bronchial treatment, thus, the current study was undertaken to examine the effect of the crude ethanol extract on the virulence of this biofilm forming MRSA MH4 and MTCC96 rather than the bactericidal activity.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were: *Staphylococcus aureus* MTCC 96 (IMTECH, Chandigarh, India) reference strain for antimicrobial testing and *S. aureus* MH4 isolated from throat swabs of pharyngitis patients attending Karpagam Medical Center. Routine tests were carried out on both strains and confirmed them to be catalase positive, coagulase positive, biofilm forming, tetracycline sensitive and methicillin resistant. For *S. aureus* liquid cultures, Lysogeny Broth (Bertani, 2004) was used and for cultures on solid support, Tryptic Soy Agar (TSA, Difco, Laboratories, Detroit, MI, USA) was used. Cultures were stored at -80 °C in Brine Heart infusion (BHI) supplemented with 15 % glycerol.

Plant Material

Collection and authentication

Piper longum fruits were purchased from a market in Coimbatore, Tamilnadu, India, authenticated by botanical survey of India, Coimbatore and a voucher specimen was deposited there. (Voucher no.1112)

Processing and extraction

Piper longum fruits were powdered using domestic grinder and a portion (50 grams) of the powder was extracted with 500 ml (1:10) using 90 % ethanol by cold maceration method for two weeks with occasional shaking after which the extract was filtered through Whatman No. 1 filter paper. The extract was then reduced to dryness by a vacuum rotary evaporator (Buchi type, Flawil/Schweiz, Switzerland) at 40 °C as described by Harborne (1998) and Teepica *et al.* (2013), with slight modifications. The resulting dry crude extract (3.67 grams) was stored at 4 °C for further use.

Fourier transform infrared analysis

Ten micrograms of the ethanol crude extract was used for the analysis using Fourier transform infrared spectroscopy

SHIMADZU 8000 series at a range of 400-4000 cm⁻¹ which gave characteristic peaks and the functional groups were determined.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined according to the protocol given by the Clinical and Laboratory Standards Institute (CLSI, 2006). Different concentrations (8 mg/ml to 0.0625 mg/ml) of *Piper longum* ethanol extract were prepared from a stock solution of 1 mg/ml of the crude extract redissolved in dimethyl sulfoxide (DMSO (100 % v/v)). Double dilution method was followed on 96 microtiter well plates. The extracts were added to 1 % of the test strains (0.4 OD at 600 nm) in Lysogeny broth (LB) medium and incubated at room temperature for 24 hours. The MIC (OD 600 nm) was determined by considering the lowest concentration at which microbial growth inhibition was obtained. The MIC well (showing no turbidity) was taken and blotted on a fresh LB medium and observed for growth to determine the BIC. All other experiments were carried out at sub- MIC to ensure that antibacterial activity of the plant does not interfere with the antibiofilm activity. The experiments at all concentrations were carried out in triplicates.

Agar well diffusion assay

This was done as per the method specified by the Clinical and Laboratory Standards Institute (CLSI, 2006) Mueller Hinton agar (High media, India) was used. The antibacterial activity agar well assay was done by punching the wells and then spreading the test strains uniformly on the agar plate with 100 µl an equivalent of 0.5 Mc Farland Standards (1 x 10⁸ CFU). Two concentrations of the *Piper longum* ethanol extracts were used, above and below MIC (2 mg/ml and 0.025 mg/ml respectively) to compare the difference on their activities. Tetracycline (30 µg/ml) was used as positive control and 100 % (v/v), DMSO served as the negative control. The plate was then incubated at 37 °C overnight and observed for zone of inhibition.

Growth curve analysis

A concentration (0.5 mg/ml) of *Piper longum* below MIC was taken for growth curve analysis. One percentage of overnight culture (0.4 OD at 600 nm) was inoculated in 100 ml conical flask containing 50 ml of LB medium and it was incubated at 37 °C. Cell density was measured at one hour interval for 14 hours, using UV-visible spectrophotometer (Model Shimadzu UV 2450).

Biofilm inhibition assays in 24 well MTP

Quantification of biomass inhibition was performed according to Balaji *et al.* (2013), using microtitre plate assay. Different concentrations (0.5 mg/ml - 0.0625 mg/ml) of *Piper longum* ethanol extracts were added to overnight *S. aureus* culture (0.4 OD at 600 nm) in LB medium and cultured at 37°C overnight without agitation. Positive control was maintained with bacterial culture and without plant extract and negative control with plant and without *S. aureus* culture. The wells were then washed with sterile distilled water to remove the planktonic

cells, and stained with 0.4 % (w/v) crystal violet (Himedia India) for five minute. Excess crystal violet was rinsed off and the adhered biofilms were resuspended in 1 ml of 95 % ethanol. The optical density (OD) of the intensity of crystal violet was measure at 600 nm using ELISA reader.

In situ observation of biofilm

Light microscopic analysis was carried out according to Nithya *et al.* (2011). One millilitre (1ml) of lysogeny broth (LB) was added into the 24 well microtitre plates containing one centimetre square (1 cm²) glass slides. About 50 µl of overnight cultures (0.4 OD at 600 nm) were added to the centre of the slides followed by addition of 50 µl of different concentrations (0.5 mg/ml - 0.0265 mg/ml) of the plant extract. Positive control was left without plant extract. The 24 well microtitre plate was then incubated at 30 °C for 16 hours after which the glass slides were removed, washed off planktonic cells and stained with 0.4 % (w/v) crystal violet staining solution. Excess stain was washed and the slides were air dried and observed under light microscope (Nikon Eclipse E 300 Japan)

Statistical analysis

The experiments were carried out in triplicates and the values were calculated using one-way analysis of variance (one-way ANOVA) and Statistical Package for the Social Sciences (SPSS) software Version 16.0, Chicago, USA). The calculated probability or 'p' value less than 0.05 was considered significant ($p \leq 0.05$). The values in results were represented as mean \pm standard error.

RESULTS

FT-IR analysis

Fourier transform infrared spectroscopy (FT-IR) was performed on Shimadzu FT-IR spectrometer 8000 series, between 4000 - 400 cm⁻¹. The analysis revealed the presence of different functional groups including; Alkyl halides, alcohols, carboxylic acids, esters and ethers, nitro compounds, alkanes, aliphatic amines, primary amines, aromatics, alkenes, and alcohols and phenols (Table 1 and Fig 1). The major groups were represented by alcohols and phenols at 3311.78 cm⁻¹ due to O-H bonds, primary amine at 1639.49 cm⁻¹ due to N-H bond, aliphatic amines at 1028.06 cm⁻¹ due to C-N bond and alkyl halides at 584.43 cm⁻¹ due to C-CL bonds.

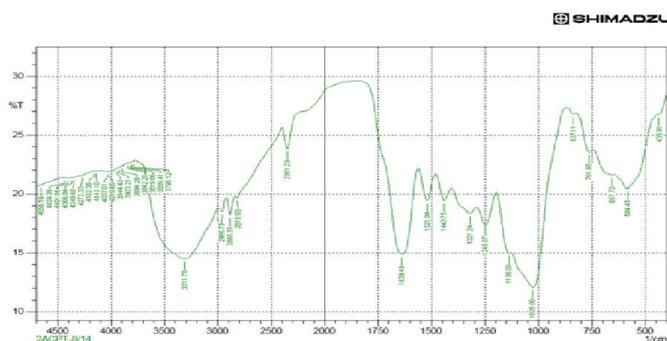


Fig. 1. FT-IR spectrum of *Piper longum* ethanol extract

Table 1. FT-IR analysis of *Piper longum* ethanol extract

Peak Values (cm ⁻¹)	Chemical bond	Functional groups
837	C-CL	Alkyl halides
1028	C-O stretch	Alcohols, carboxylic acids, esters and ethers
1138	C-N stretch	Aliphatic amines
1250	C-N stretch	Aliphatic amines
1321	N-O stretch	Nitro compounds
1443	C-C stretch	Aromatics
1522	N-O asymmetric stretch	Nitro compounds
1640	N-H bend	Primary amines
2351	-	Unknown
2820	H-C=O, C-H stretch	Primary amines
2886	C-H stretch	Alkenes
2961	C-H stretch	Alkenes
3312	O-H stretch	Alcohols, phenols

Minimum Inhibitory Concentration

The MIC for both *S. aureus* MH4 and MTCC 96 was observed at 1 mg/ml (Fig. 2). When the 0.5 mg/ml well was transferred to a fresh Mueller Hinton Agar (MHA) and incubated for another 24 hours, there was no visible growth. The subsequent tests were carried out at sub MIC (0.5 mg/ml - 0.0265 mg/ml). To further confirm this, antibacterial activity and growth curve analysis were carried out.

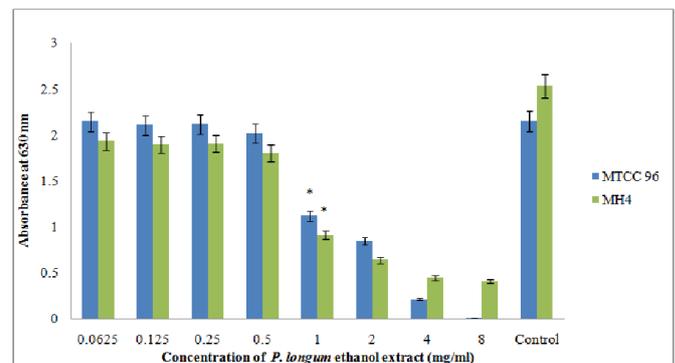


Fig. 2. Minimum Inhibitory Concentration (MIC) of *S. aureus* (MTCC&MH4) treated with *P. longum* ethanol extract in two fold dilution method. Values are mean (\pm) standard deviation of three replicates (n=3). Means followed by * are significant ($P < 0.05$)

Agar well disc diffusion assay

Two concentrations were taken for the assay; above and below MIC (2 mg/ml and 0.025 mg/ml respectively).

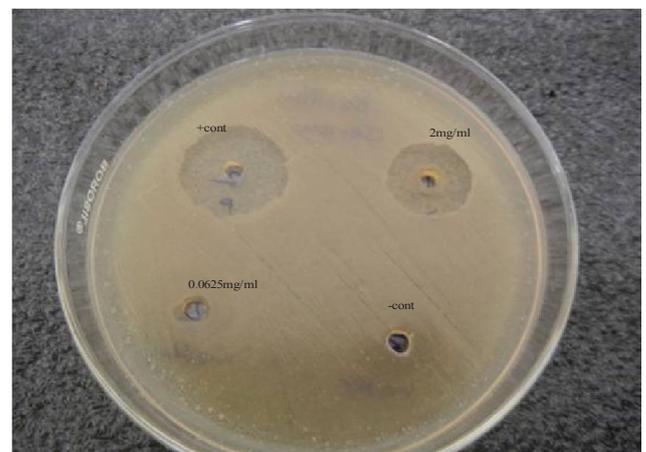


Fig. 3. Agar well diffusion assay

Tetracycline (30 µg/ml) was used as positive control and dimethyl sulfoxide (100 % v/v) as negative control. The concentration above MIC showed a clear zone of 20 mm, while at sub-MIC concentration (0.0625 mg/ml) there was no zone of inhibition, this indicated that the sub-MIC concentration did not possess antibacterial activity and thus the sub-MIC concentrations were ideal for antibiofilm inhibitory assay. There was an inhibition zone of 27 mm for positive control and no zone of inhibition for negative control (Fig 3).

Growth Curve analysis

The growth curves of both *S. aureus* strains (MH4 and MTCC 96) were carried out at sub-MIC concentration of 0.5 mg/ml and were compared with standard, which showed no difference in their growth pattern (Fig 4). This result further confirmed that at sub-MIC the extracts were not bactericidal.

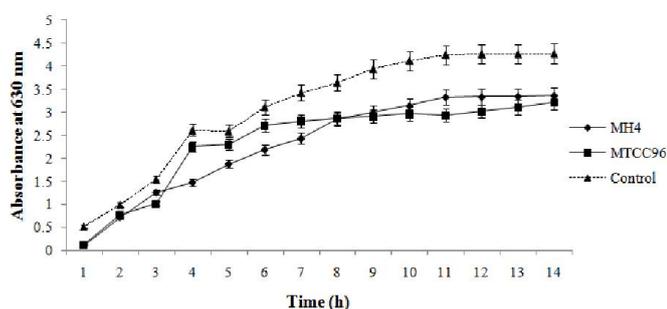


Fig. 4. Growth curve analysis of *P. longum* against *S. aureus*

Biofilm inhibition assay

Biofilm inhibition was observed to be highest at 0.5 mg/ml in both MH4 and MTCC 96 strains. At the three sub-MIC concentrations used (0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml) MH4 strain showed decreasing inhibition percentage and least inhibition was observed at the lowest concentration (0.0625 mg/ml). *Staphylococcus aureus* strain MTCC 96 showed a concentration dependent decrease in inhibition percentage from 0.25 mg/ml to 0.0625 mg/ml (Fig 5). The results indicated that the inhibition percentages were dose dependent.

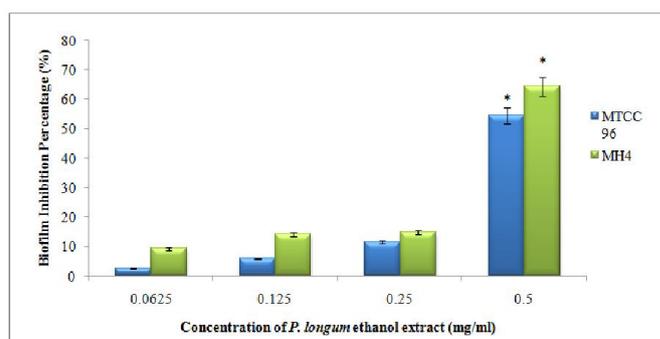


Fig. 5. Biofilm inhibition percentage of *S. aureus* (MTCC & MH4) treated with *P. longum* ethanol extract at sub MIC concentration. Values are mean (\pm) standard deviation of three replicates (n=3). Means followed by * are significant ($P < 0.05$)

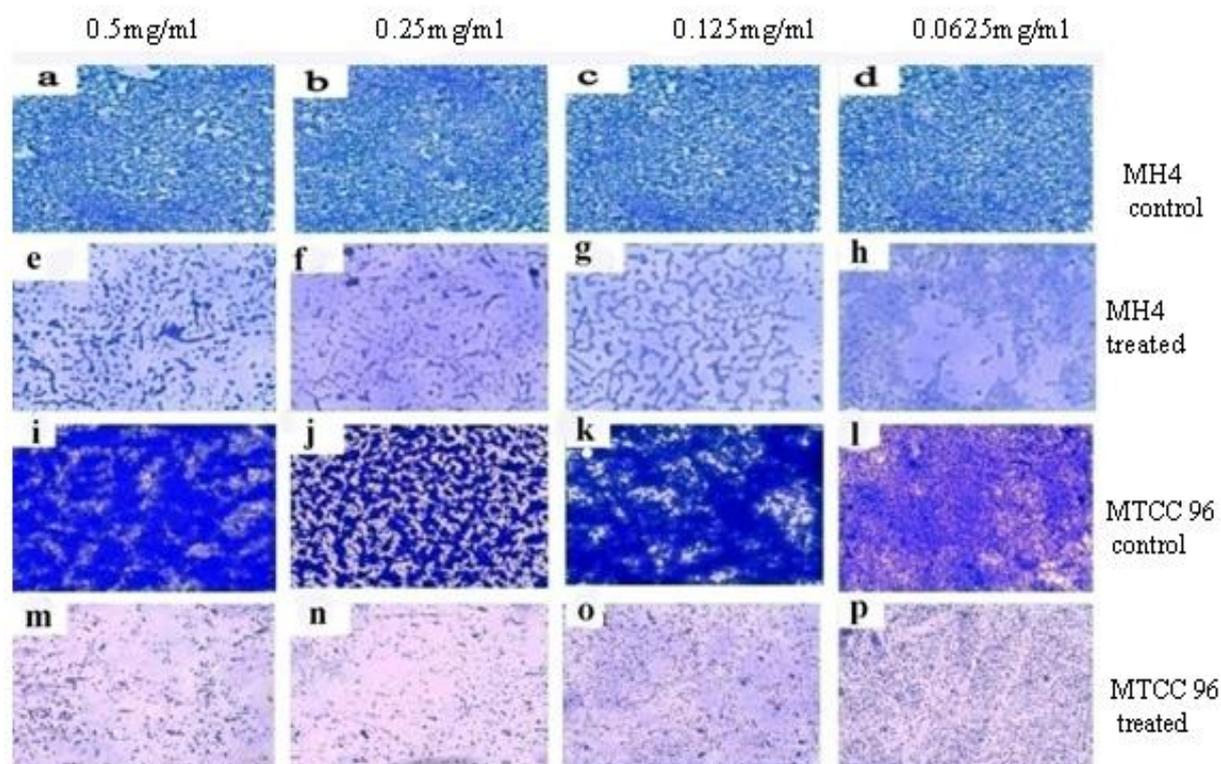


Fig. 6. Light microscopic images of *S. aureus* biofilms (MH4 and MTCC 96) control and treated with *P. longum* ethanol extract at sub-MIC concentrations (0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml respectively)

a, b, c, d indicates MH4 control (untreated)

e, f, g, h indicates MH4 treated with different concentrations of *P. longum*

i, j, k, l indicates MTCC96 control (untreated)

m, n, o, p = MTCC96 treated with different concentrations of *P. longum* (0.5 mg/ml to 0.0625 mg/ml respectively)

Light Microscopic Analysis (Magnification x 200)

The light microscopy clearly indicated that the biofilm populations in both MH4 and MTCC 96 treated were highly reduced. Both strains at the concentrations of 0.5 mg/ml and 0.25 mg/ml had lesser population than the concentrations 0.125 mg/ml and 0.625 mg/ml respectively. The results are shown in Fig. 6.

DISCUSSION

Methicillin resistant *Staphylococcus aureus* is on the rise all over the world. Studies have shown that it has the ability to form biofilms and thus its treatment and eradication is not easy. Conventional antibiotics usually target planktonic cells rather than this biofilm, thus the *S. aureus* easily evade treatment and survive. From the literature survey, there is less information regarding the biofilm inhibition approach. Plant phytochemical compounds have been proved to contain antimicrobial effect against pathogens. *Piper longum* which has a plethora of antimicrobial compounds was selected for our study. *P. longum* is known traditionally to be beneficial for the throat thus, it was considered appropriate for our study since, we focused on throat infectious MRSA strains.

The present study was carried out to analyse the functional groups present in the ethanolic extract of *P. longum* by using fourier transform infrared spectroscopy (FT-IR) and to study the effect of this extract on two biofilm forming methicillin resistant strains of *S. aureus*; MH4 a clinical throat swab isolated from pharyngitis patients and MTCC 96. FT-IR revealed the presence of alcohols, alkyl halides, carboxylic acids, esters and ethers, nitro compounds, aliphatic amines, aromatics, primary amines, alkanes, alkenes, alcohols, and phenols. This result collaborates with the earlier studies of Deepthi *et al.*, (2012) who isolated piperine from *P. longum* using 95% ethanol, in their study they characterised the isolated compound using FT-IR and similar constituents as those in our study were reported (C-O, O-H, C-H stretch and N-H bend this could be attributed to the presence of piperine. The MIC was 1 mg/ml for both the tested strains. Agar well diffusion assay as well as the growth curve confirmed the MIC of the test organisms. Biofilm inhibition percentage was highest at the concentration of 0.5 mg/ml for both MH4 and MTCC 96.

The results revealed that the inhibition was concentration dependent and it decreased with decrease in concentration. The light microscopy (Magnification x 200) revealed that the biofilm population was highly reduced. In the study carried out by Srinivasa *et al.*, (2001), it clearly depicted the strong activity of piperine against *S. aureus*, but their study was on planktonic cells. In turn, our study focused on the biofilm forming *S. aureus* MH4 and MTCC 96 isolates and showed promising antibiofilm activity. Similarly, Vinay *et al.*, 2013, carried out a study on the methanolic crude extracts of *P. longum* against Vancomycin-resistant *S. aureus*-pARI818, (MIC > 1200 µg/ml) and found it a new source of safe plasmid curing agent which causes antibiotic resistance reversal. In our study a high biofilm inhibition percentage was obtained at 0.5 mg/ml (500 µg/ml), which confirmed that *P. longum* ethanol

extract had a potential antibiofilm activity against methicillin resistant *S. aureus* strains.

The ability of *S. aureus* to form biofilm had led to the increased resistance to antibiotic treatments; hence the biofilm inhibition approach will add value to the eradication and treatment of these pathogenic strains. Plant photochemicals have shown potential antibiofilm activity and *P. longum* has been one in the claims. Our study revealed that the *P. longum* ethanol extract is packed with pharmaceutically active functional groups some of which were earlier reported in piperine, an antimicrobial compound isolated from *P. longum*. Further, this study proved that *P. longum* had strong biofilm disruption ability at concentration as low as 0.5 mg/ml. There is a traditional claim of its use in treatment of bronchial troubles; this study clearly indicated that *P. longum* had potential antibiofilm activity against methicillin resistant *S. aureus* strains that cause throat infections.

Acknowledgement

We acknowledge the Laboratory of Clinical Biotechnology and Herbal Medicine, Karpagam University for providing the light microscope for our study and the Karpagam University Infrastructural Facility laboratory (KUIF) for FT-IR analysis.

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