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In-vitro antibiofilm activity of selected medicinal plants against *Staphylococcus aureus* biofilm on chitin flakes as substrate

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ABSTRACT

The aim of the study is to assess the antibiofilm activity of ethanolic extracts of few Indian traditional herbal plants against *Staphylococcus aureus* biofilm along with phytochemical analysis of plant extracts and identification of active compounds. Broth micro-dilution method was followed for minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) study of the plants against *Staphylococcus aureus* ATCC 25923. Chitin was used as a substrate for biofilm formation and antibiofilm activity of plant extract was investigated. Scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FT-IR) of the chitin flakes were analysed to determine the inhibitory effect of the plant extract on *Staphylococcus aureus* biofilm. *Curcuma longa* showed the best antibacterial activities where MIC and MBC were 0.4 mg/ml and 1.60 mg/ml respectively. Viability cell counts of both planktonic and sessile condition of *Staphylococcus aureus* showed that *C.longa* ethanolic extract have an antibiofilm activity. The major compounds of *C.longa* extract was found to be curcumin, which responded for antibiofilm activity against S. aureus. *Curcuma longa* rhizome and its active compound curcumin can be a potential natural source of antibiofilm agents against infectious biofilm forming *Staphylococcus aureus*.

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INTRODUCTION

Herbal medicines are commonly used and available resources of primary health care in combating several kinds of diseases ranging from microbial diseases, stomach infection, cold, cough, ulcer for thousands years in traditional medicines including Indian folk medicine. These herbal medicines have unlimited ability to synthesize bioactive compounds as secondary metabolites with various pharmacological properties (Cragg and Newman, 2001; Jesonbabu *et al.*, 2012). These medicinal plants can be used as a promising alternative therapies for the treatment of various infections as these are with negligible side effects as higher doses of conventional antimicrobial agents can cause side effects; disturbance in the metabolism system and recurring infection for removing the biofilm of clinical isolates and non-susceptibility of clinical isolates against antibiotics (Donlan and Costerton, 2002; Asadpour and Ghazanfari, 2019).

Microbial biofilm are colonization of bacteria, engrained in a self-producing an extracellular polymeric substance (EPS) matrix, on any non-living or living solid surfaces (Vasudevan, 2014). Biofilm-allied microbial cells can irreversibly adhere to even

living tissues and indwelling medical devices as catheters, valves, prosthesis, and so forth (Parsek and Singh, 2003) . Being highly resistant to host immune defences and antibiotics, biofilm are considered as an important virulence factor that causes tenacious chronic and recurrent infections with up to 1,000 times more resistant to antibiotics than planktonic cells (free-floating) (Grant and Hung, 2013; Rasmussen and Givskov, 2006). An estimated 75% of bacterial infections become severely complicated to treatments and resistant to therapies due to the protected layer of extracellular matrix involved in biofilm formation (Sun *et al.*, 2013; Musk *et al.*, 2005). The augmented biofilm resistances to conventional treatments demand the need for a new control and alternative strategy (Simoes *et al.*, 2007).

Different standard methods like calgary method, microtiter plates as substrate are used for studying biofilm susceptibility tests (Ceri *et al.*, 1999).Chitin flakes, used as substrate in our previous study was an easy and cheap system where the growth pattern and biofilm susceptibility of both planktonic and sessile can be studied parallel in a single system (Baishya *et al.*, 2016). Chitin, a natural polymer with natural structural polysaccharide, is second most abundant in the world, with commercial concern because of their high nitrogen content (6.89%) and their exceptional properties such as bio compatibility, bio degradability, non-toxicity and adsorptive abilities (Kumirska *et al.*, 2010). In the present study phytochemical analysis of eight commonly used Indian traditional medicinal plants viz., Curcuma longa, Zingiber officinale, Ocimum sanctum, Mikania scandens etc., were done further to their influence on the viability on the planktonic cells and efficacy of biofilm removal from the substrate was evaluated in chitin flakes as substrate model.

MATERIALS AND METHODS

Preparation of medicinal plant extracts

The plants were collected from college premises or purchased from the local market (Table 1). The plant was identified from the Department of Botany, University of Calcutta, India. The leaves and rhizomes were dried at room temperature. The dried plants part were powdered using a grinder to coarse powder, packed into Soxhlet column and then extracted with 70% ethanol for 24 hours(h). The extracts were evaporated to dryness. The obtained crude extracts were stored in airtight container in the refrigerator at -4°C for further studies.

Phytochemical analysis of plant extracts

The extracts were subjected to preliminary phytochemical screening for the detection of major chemical groups. Plant extracts were taken and tested for tannin, flavonoids, phenolics, glycosides, alkaloids and steroids following standard protocol (Sofowora, 2008; Trease and Evans, 1989).

Bacterial strains and growth conditions

Experiments were performed with *Staphylococcus aureus* ATCC 25923. Microorganisms were maintained Tryptic soya agar (TSA) and a single colony was inoculated into Trypticase soya broth (TSB) medium and incubated at 37^0C for 24 h under aerobic conditions as appropriate. The strain was preserved in semi-solid agar at 40°C with 15% glycerol at -70 ^{0}C until use.

Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of plants extract was determined by the microdilution technique according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI, 2012). Two-fold serial dilutions of stock solution of each extract was done in brain heart infusion broth (BHI) to obtain different concentrations at a total volume of 100 μl per well in 96-well microtiter plates. The *S. aureus* strain at a concentration of 1X 10^6 CFU/mL attained at 100 μl was added to each well and incubated at 37^oC in suitable conditions. The medium and ethanol were used as the non-treated, negative controls, respectively.

The MIC was taken as the lowest concentration of the extracts that inhibited visible microbial growth. The aliquots from the wells corresponding to the MIC were sub cultured onto TSA plates, which were incubated at 37^0C for 24 h for the MBC, as the lowest concentration of the extracts where no visible growth were observed on the solid medium. All experiments were repeated thrice in duplicate.

Biofilm Formation *in vitro* on Chitin flakes by *Staphylococcus aureus* ATCC 25923

Tryptic Soya Agar (TSA) slant were incubated at 37°C overnight streaked with *Staphylococcus aureus* ATCC 25923 culture to grow at appropriate condition. Overnight grown culture was inoculated into TSB to yield a concentration of 1x10^6CFU/ml. Flask with TSB with chitin flakes were then inoculated with 500μl aliquot of the cells and was incubated at 37^0C for 72 h for the establishment of biofilm in the substrate i.e Chitin flakes (Banerjee *et al.*, 2015).

Effect of plant extracts on Pre-Formed Biofilm and Planktonic Cells

Table 1: Overview of the collected plants and its parts used for the study

Scientific name	Common Name	Family	Used part
Ixora coccinea	Jungle geranium	Rubiaceae	Flower
Allium sativum	Garlic	Amaryllidaceae	Rhizome
Mikania scandens	Climbing hempweed	Asteraceae	Leaf
Calendula officinalis	Pot marigold	Asteraceae	Flower
Curcuma longa	Turmeric	Zingiberaceae	Rhizome
Ocimum sanctum	Holy basil	Lamiaceae	Leaf
Alternanthera ficoidea	Joseph's coat	Amarathaceae	Leaf
Zingiber officinale	Ginger	Zingiberaceae	Rhizome

Table 2: Phytochemical screening results of ethanolic extracts of the plants

	Tannin	Flavonoid	Terpenoids	Phenolic	Steroid	Tripernoid	Glycoside	Saponin	Anthraquinone	Proteins
I.coccinea	++	-	++	++	-	+	-	-	-	-
A.sativum	-	-	++	-	+	-	++	-	+	-
M.scandens	+	+	+	+	-	+	-	-	-	-
C.officinalis	++	-	++	++	-	++	-	+	-	-
C.longa	+	+	++	+	+	-	-	+	-	-
O.sanctum	+	-	+	+	-	+	-	-	-	-
A. ficoidea	+	++	+	+	+	+	-	+	-	-
Z.officinale	-	-	++	-	-	+	-	-	-	-

Table 3: MIC and MBC of plant extracts against *Staphylococcus aureus* ATCC 25923.

Plants	Concentration of extracts (mg/ml)	
	MIC	MBC
Ixora coccinea	15.0± 1.2	45.0± 3.2
Allium sativum	11.4± 1.3	34.1± 3.2
Mikania scandens	1.4 ±0.2	6.2± 0.3
Calendula officinalis	14.0± 0.8	56.0± 1.6
Curcuma longa	0.4 ± 0.0	1.6± 0.12
Ocimum sanctum	30.0± 0.0	120.0± 1.2
Alternanthera ficoidea	29.8± 1.3	89.8± 3.8
Zingiber officinale	2.5±0.3	10.0± 0.4

Screening of the herbal extracts showed that *Curcuma longa* have the strongest anti-microbial activity against *Staphylococcus aureus* ATCC 25923 thus this extract was chosen for further part of the study. The flasks with 100ml of TSB with substrate (chitin flakes) were prepared to establish biofilm on the substrate (as mentioned previously) and was challenged with single dose concentrations (128*MIC, 256*MIC, 512*MIC, 1024*MIC, 2048*MIC) of *Curcuma longa* extract. Then incubated at 37°C, readings were taken at regular time intervals 2, 4, 6, 24 hours (h). Treated biofilms formed on chitin flakes were rinsed with phosphate buffer saline (PBS) (pH 7). The biofilm were removed from the chitin flakes by sonication for 5 minutes. Colonies were counted after the recovered medium was serially diluted and biofilm cultures were spotted on TSA plates and incubated overnight at 37^0C (Gomes *et al.*, 2012). For planktonic, OD were measured at 570nm. Scanning Electron Microscope (SEM) and Fourier-transform infrared spectroscopy (FT-IR) of the chitin flakes were analyzed.

Bioactive compound assay

Thin layer chromatography (TLC) fingerprinting *C. longa* extract

For TLC-fingerprinting study, 100 mg of C. longa extract, 5 mL of curcuminoid (the standard including curcumin, demethoxycurcumin and bisdemetoxycurcumin) were prepared in 1 mL of 95%

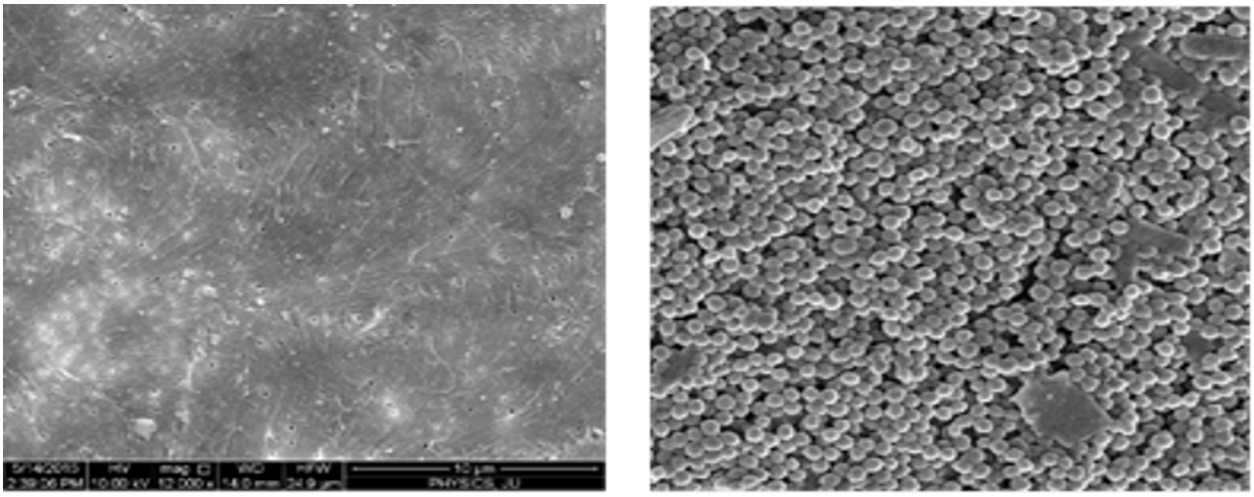

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Figure 1: (A) The substrate chitin flakes without biofilm formation (B) chitin flakes after 72 h biofilm of *Staphylococcus aureus* ATCC 25923

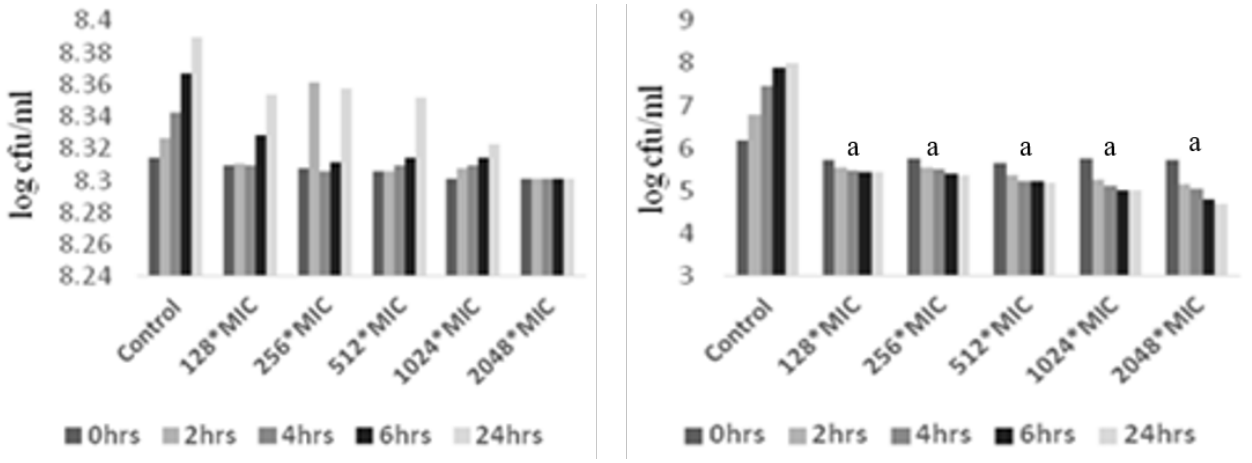


Figure 2: Effect of *C.longa extract* on eradication on planktonic cells (A) and *S. aureus* biofilm (B) at different time interval and different concentrations. 'a' indicates reduced value of log10 differed significantly from the control i.e. without treatment

ethanol. A 20μL of each sample was applied onto the alumina silica gel 60 F_{254} TLC plate, and was run through a solvent system using a mixture of Chloroform-ethanol-glacial acetic acid (95:5:1 v/v) as the mobile phase. The plate was air dried, and observed under UV light 365nm (Kinghorn *et al.*, 1996). The relative front values (Rf) was calculated.

TLC bioautography and UV spectrometric analysis of bands

TLC-bioautography was done using agar overlay method where the TLC plate were placed on Mueller Hinton Agar (MHA) plates with 10μl of *S.aureus* ATCC 25925 inoculum (10^6 CFU/ml) and incubated overnight at 37^0C (Rahalison *et al.*, 1991). The components of each band were scraped out, washed with methanol and were analyzed using UV spectrometry.

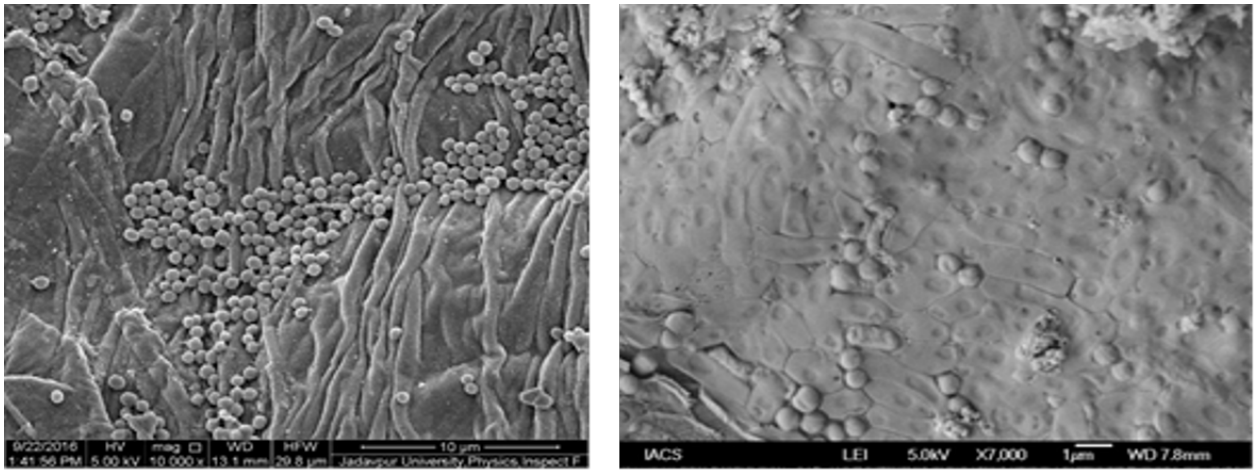
Statistical analysis

Data were expressed as mean and standard deviation (SD) by computational analysis from the experiments with triplicate independent experiments. One- way ANOVA followed by Turkey's posthoc was done for statistical analysis of the data on GraphPad Prism 8. $p< 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

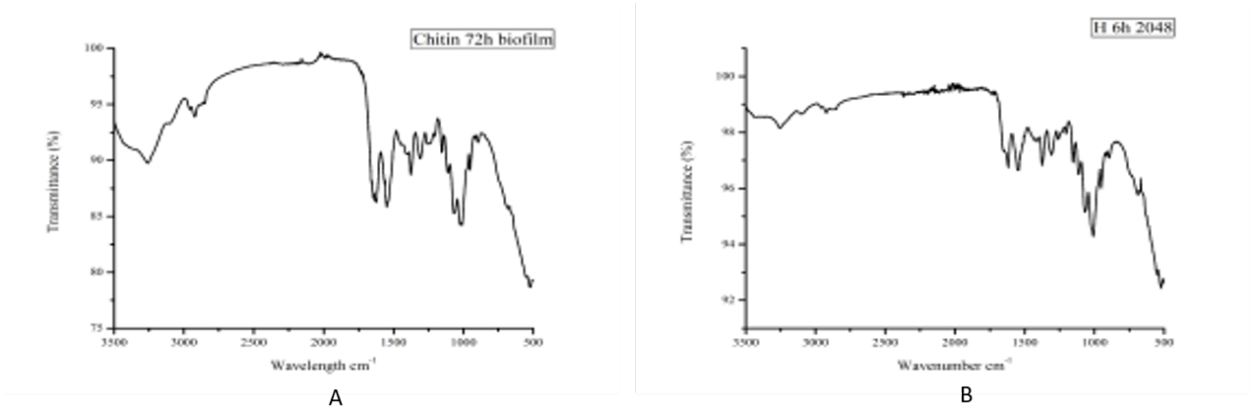
Phytochemical analysis of plant extracts

The results of the phytochemical analysis of the plant extracts have shown a notable variation. The detailed investigations of phytochemicals screen-



A B

Figure 3: Scanning electron microscopy (SEM) micrographs of the *S.aureus* ATCC 25923 biofilm structure. Presence of colonies and extracellular matrix on 72 h preformed biofilm on chitin flakes at 128*MIC treatment (A) 2048*MIC plant extract treated biofilm shows shrinkage of extracellular matrix following 24 hours incubation (B)



A B

Figure 4: FT-IR spectrum(500-3500 cm^{-1}) of chitin flakes with 72h *S. aureus* biofilm (A) preformed biofilm treated with 2048*MIC of *Curcuma longa* extract at 6 h (B)

ing of the selected plants are shown in (Table 2). Compounds like flavonoid, terpenoid, phenol, tannin were present in most of the selected plants. Curcuma longa extract shows the existence of tannin, flavonoid, terpenoid, phenol, saponin and steroid as its phytochemical compounds.

Minimal Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the plants extracts against *S. aureus* ATCC 25923 was shown in (Table 3). It was evident that the value of MBC for the sessile population is more than that of the MIC of the planktonic growth. The MIC and MBC value range 0.4 mg/ml and 1.60 mg/ml respectively for the ethanolic extract of *Curcuma longa* against *S.aureus* ATCC 25923 being the lowest MIC and MBC among the selected plants whereas *Ocimum sanctum* gave the highest MIC/MBC range at 30 mg/ml/ 120mg/ml.

Biofilm Formation by *Staphylococcus aureus* ATCC 25923

SEM microphotographs revealed the surface of microcolonies of the biofilm on the chitin flake substrate indicating probable presence of the EPS (Figure 1B) after 72 h biofilm formation in contrast to the surface of chitin flake without biofilm (Figure 1A).

Effect of plant extracts on Pre-Formed Biofilm and Planktonic Cells

The study next investigated the ability of *Curcuma longa* to eradicate 72 hours pre-formed biofilms on chitin flakes. When the concentration of the extract higher than the MIC (128*MIC, 256*MIC, 512*MIC, 1024*MIC, 2048*MIC), a statistically significant reduction was observed in the number of

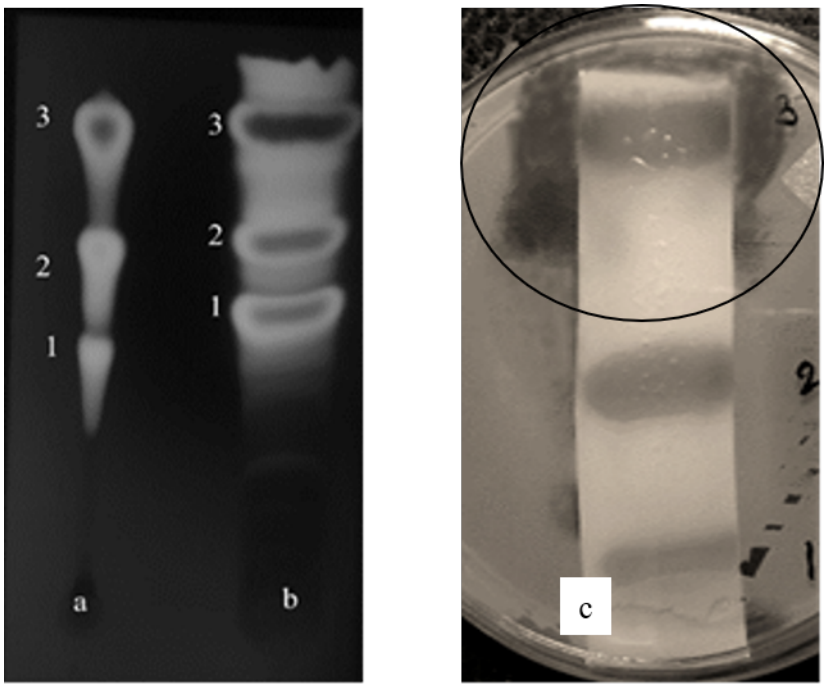


Figure 5: TLCprofile in UV 365nm of standard curcuminoids [Curcumin (3), demethoxycurcumin(2),bisdemethoxycurcumin (1)] (a) and ethanolic extracts of *C. longa* showing three similar bands(b). TLC-bioautography against *S. aureus* ATCC 25923 of *C.longa* extract (c). Zone of inhibition for the band marked by circle

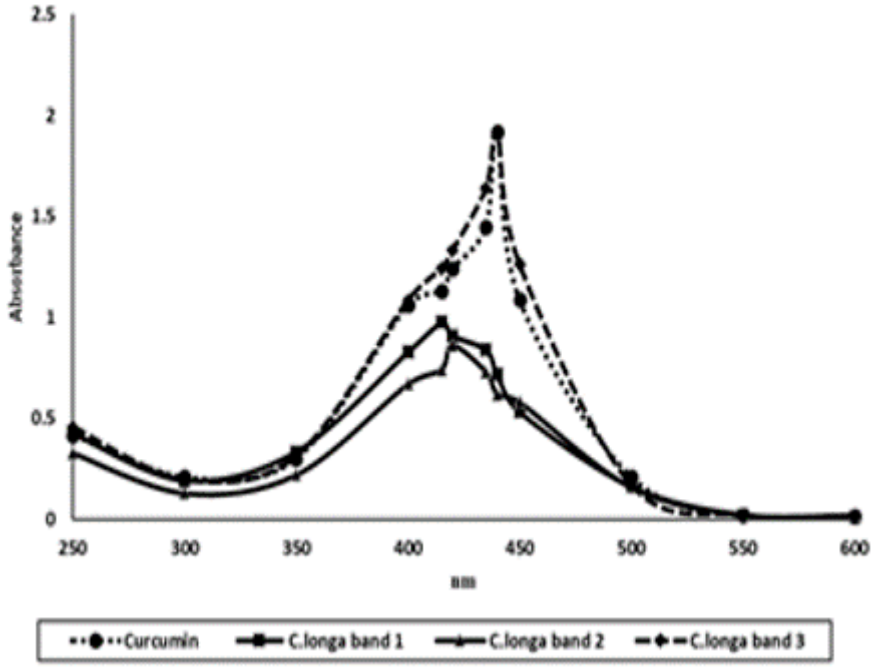


Figure 6: UV-Visible spectra showing three bands of *C.longa* extract and standard curcumin from curcuminoids

bacteria forming biofilm ($p < 0.05$) after 24h of treatment, in comparison to the control samples.

On the other hand, *S. aureus* supplemented with different concentrations of plant extracts was found to be turbid after an overnight incubation indicating that the doses 128*MIC to 1024*MIC, did not inhibits *S. aureus* growth (Figure 2A) and showed a similar growth rate in the presence and absence of plant extract, confirming that the concentrations did not interfere with *S. aureus* growth. However at 2048*MIC the growth was stationary. Significant decreases in the number of viable biofilm-forming cells were detected after treatment with the *Curcuma longa* extract at the higher MIC concentration. After 2h of activity, the number of bacteria reduced to 5.53 log CFU/ml for 128*MIC, while for 2048*MIC it reached 5.14logCFU/ml ($p < 0.05$). By comparison, after 24 h of treatment the number of bacteria reduced to 5.43 and 4.69 log CFU/ml for 128*MIC and 2048*MIC, respectively ($p < 0.05$). In the control the bacterial count increased from 6.20 to 8.00 log CFU/ml after 24 h (Figure 2B) ($p < 0.05$).

The morphology of plant extract treated 2048* MIC and untreated *S. aureus* biofilm architecture formed on the chitin flakes following 24 hours incubation was analysed using scanning electron microscopy (SEM). At 10,000X magnification, SEM analysis *S. aureus* appeared as large aggregates of cells with a thick, dense and fully established biofilm consisting of overlapped multi-layered bacterial cells (Figure 3A). Upon treatment with plant extract treatment of *Curcuma longa*, biofilm production was disrupted and a uniform layer of cells with negligible biofilm mass was detected on the flakes (at 7000X). The bacteria appeared as a monolayer of dispersed cells scattered on the surface (Figure 3B).

On comparing the control biofilm spectrum with the treated biofilm spectrum (2048*MIC of plant extract) showed remarkable differences. Exopolysaccharide sugar specific peaks intensity were seen to be reduced with respect to treated biofilm to that of control biofilm flakes sample. This difference in spectra might be due less production of extracellular polysaccharides in biofilm in the presence of *Curcuma longa* extract. A characteristic absorption maxima with some transformations in the shape, and the number of absorption peaks were shown between control (untreated) and highest treated set (2048*MIC) *S.aureus* biofilms region from 850-1300 cm^{-1}range of wave number signifies sugars present in the EPS matrix(Figure 4A). The spectral peaks at region 820, 945, 1012, 1090, 1175, 1250 cm^{-1} confirmed the presence of β-glucans and mannans with other sugars like arabinose and mannose in the EPS matrix. However, the FTIR spectra also exhibited bands corresponding to carbonyl C=O stretching of chitin at 1627 cm^{-1} , N-H deformation of amide II at 1558 cm^{-1} and peaks at around 3215 cm^{-1} attributes to -NH and -OH groups. On comparing the control biofilm spectrum with the treated biofilm spectrum showed remarkable reduction of exopolysaccharide sugar specific peaks (Figure 4B).

Thin layer chromatography (TLC) fingerprinting, bioautography and UV spectrometric analysis of bands of *C. longa* extract

There were three distinct bands of *C.longa* extract components appeared on the TLC-plate (Figure 5b) with Rf $\sim$ 0.64, 0.51 and 0.39 respectively. The separation of standard curcuminoid content curcumin, demethoxycurcumin and bisdemethoxycurcumin, depicted on (Figure 5a) with Rf value 0.65, 0.5, 0.375 respectively. TLC-bioautography of *C. longa* extract (Figure 5c), large inhibition zone against *S. aureus* ATCC 25923 was seen for the band with Rf at 0.64 while the other bands did not show any inhibited zone. The UV spectral showed peaks at 415, 420 and 440 nm for the *C. longa* bands 1, 2 and 3 respectively (Figure 6). The band 3 of *C.longa* was observed to be similar to curcumin from standard curcuminoid with its peak also at 440nm. Band 1 and 2 of *C.longa* extract resembles bisdemethoxycurcumin and demethoxycurcumin also having its maximum absorbance at 415nm and 420nm respectively.

Plants have been used from ancient time to cure diseases and illnesses and even in the prevention and treatment for biofilm related infections due to presence of naturally occurring phytochemical compounds (Liziana *et al.*, 2013; Karuppiah and Mustaffa, 2013) . The phytochemical screening results show the presence of different compounds in all the eight plants along with *Curcuma longa* that is similar to the result of former workers (Sawant and Godghate, 2013). In this study total of eight ethanolic plant extracts were tested against *Staphylococcus aureus* ATCC 25923, it has shown that ethanolic extract of *Curcuma longa* exhibited antimicrobial activity against *Staphylococcus aureus* ATCC 25923 with least MIC of 0.4 mg/ml. The results were comparable to the study of (Marasini *et al.*, 2015) . The study of preformed biofilm on chitin along with the planktonic phase, shows that there was no such significant reduction of growth of *S.aureus* in the planktonic cell after treatment of *Curcuma longa* extract (128*MIC to 1024*MIC) in contrast to the treated biofilm that shows significant ($p<0.05$) reduction.

These findings propose that the reduction of *S.*

aureus virulence by *Curcuma longa* extract may not only due to its antimicrobial activity against planktonic cells but due to presence of phytochemical compounds having antibiofilm property. Kong et al., 2018, reported a similar pattern of result for the same bacteria in planktonic cell growth in their study. SEM and FTIR analyses were done to discern the mechanism how the plant extract affect the preformed microbial biofilms at the cellular level. FTIR spectroscopy analyses the interaction between the infrared radiation and the sample of its molecular composition. This spectroscopy technique is one of the standard and sensitive method to monitor small changes in the composition of cells (Orsini *et al.*, 2000; Galichet *et al.*, 2001). In the present study, the highest treated concentration (2048*MIC at 6 h treatment) and the control *S.aureus* biofilms on the chitin flakes indicated that *Curcuma longa* extract has not only prevented biofilm formation but also disrupted the established biofilms and also changes the exopolysaccharides matrix in its SEM images which was also seen when *C. albicans* biofilm were treated with jujube honey (Ansari *et al.*, 2013). The major differences in the FTIR spectra at 850-1300cm^{-1} between the treated and untreated might be due to the differences in exopolysaccharide sugar composition which also reflects that there was less production of extracellular polysaccharides in biofilm in the presence of *Curcuma longa* extract.

Curcuminoid, i.e. curcumin, demethoxycurcumin and bisdemethoxycurcumin are the predominant constituents in *C. longa* rhizome which have been studied for its antioxidant, anti-inflammatory and antimicrobial activity (Masuda *et al.*, 2001; Julie and Jurenka, 2009). Our study demonstrated that the major compound of *C.longa* extract were curcumin, demethoxycurcumin and bisdemethoxycurcumin also reported by Pothitirat and Gritsanapan, 2005. The UV-Visible spectra of each bands of *C.longa* extract showed similar peaks with the standard components of curcuminoid, in other words band 1 of *C.longa* corresponds to bisdemethoxycurcumin, band 2 to be demethoxycurcumin and band 3 was for curcumin (Kadam *et al.*, 2018) . However the study showed that curcumin exhibited the strong antibacterial activity against *S. aureus* with a clear inhibition zone in the TLC-bioautography assay. Thus, it indicated that curcumin could overcome the problems associated with *S.aureus* biofilm resistance and its barriers.

CONCLUSIONS

This study suggested that *Curcuma longa* rhizome can be a potential natural source of antibiofilm agents against infectious biofilm forming *Staphylococcus aureus*. For further study, the mechanisms of antibiofilm activity of major constituent of the plant should be investigated in more detail as well as its activity in vivo to determine the therapeutic potential of *Curcuma longa* in biofilm associated infections.

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