



## *Hemidesmus indicus*, a traditional medicinal plant, targets the adherence of multidrug-resistant pathogens to form biofilms

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### ABSTRACT

Aggregated mode of bacterial growth in the form of biofilm is a prime reason for nonspecific antibiotic resistance and several persistent and chronic infections in clinical settings. Hence, targeting the initial adherence, early & mature biofilms of the bacterial pathogens would be a suitable alternative to control multidrug-resistant bacterial infections. The root bark of *Hemidesmus indicus* is a well-known in the Ayurvedic medicinal system. In the present study, antibiofilm potential of methanolic extract of *H. indicus* root (HI) was evaluated against nosocomial bacterial pathogens such as methicillin-resistant and -susceptible *Staphylococcus aureus* and *Streptococcus pyogenes*. The minimum biofilm inhibitory concentration (MBIC) of HI was found to be 300 and 150 µg/ml for *S. aureus* and *S. pyogenes*, respectively. Further results from microscopic analyses and *in vitro* bioassays validated the biofilm inhibitory potential of HI. HI at MBIC failed to eradicate the preformed biofilms of the test pathogens. Chromatographic analysis divulges the presence of several functional metabolites in the HI responsible for its bioactivity. Thus, the present study suggests that HI targets the biofilm formation of the test pathogens by hindering the initial adherence of the cells to form biofilm. The extract of HI root can be used as a suitable alternate to treat biofilm mediated infection and would be considered in drug formulations to control the onset of biofilm mediated bacterial infections.

### 1. Introduction

Biofilm is a self-produced physical structure where the bacterial organisms wraps themselves in an exopolysaccharide matrix and resist the invading chemotherapeutics and host immune responses to cure the infections (Koo et al., 2017). The paradigm of growing togetherness among pathogenic bacteria makes the treatment strategy failure in various infections including upper respiratory tract infections, urinary tract infection, catheter-induced infection etc (Jacobsen et al., 2008; Hewer and Smyth, 2009). Biofilm mediated infections continue to challenge the treat strategy and enhance the morbidity, quality of life and can assist the progress of follow-up diseases such as Alzheimer's disease and cancer (Bjarnsholt et al., 2018; Allen, 2016). More, manifestation of such biofilm-associated infections in immunocompromised

patients leads to chronic diseases or even sometimes to death (Wu et al., 2015).

Although, a series of treatment methods have already been proposed to treat biofilm mediated infections (Bjarnsholt et al., 2018), all these treatment methods have their own limitations including ineffectiveness of antibiotics due to complex biofilm architecture, induction of resistance selection due to the continuous exposure to sub-inhibitory concentrations, short time effect, cytotoxicity, etc (Koo et al., 2017). To overcome these issues, interest in exploring the potential of traditional medicinal plants and its phytocompounds has been increased in recent years (Barbieri et al., 2017; Kannappan et al., 2017). The herb *Hemidesmus indicus* R. Br. (Apocynaceae) is native of India and commonly called as Anantamul, Nannari in local languages. Root of this plant has long been used in traditional medicinal system for treating fever, skin

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diseases, leucorrhoea, syphilis and rheumatism. *H. indicus* root contains diverse classes of metabolites which includes steroids, terpenoids, flavonoids, saponins, phenolic compounds, glycosides, carbohydrates and so on (Lakshmi and Rajendran, 2013). Syrup prepared from the roots is used as a flavoring agent and in the preparation of a sherbet which have cooling properties. *H. indicus* root has been studied for its anti-microbial and anti-inflammatory activities (Dutta et al., 1982; Gayathri and Kanabiran, 2009). Very recently, this plant has been reported for its properties like hypoglycaemic, hypolipidaemic and anti-quorum sensing (Zahin et al., 2010; Joshi et al., 2018). However, to our knowledge, no report states the potential of HI against the biofilm formation of multidrug-resistant bacterial pathogens, especially *S. aureus* and *S. pyogenes*.

## 2. Material and methods

### 2.1. Plant material and extract preparation

The root of *H. indicus* was procured from the local ayurvedic shop. The roots were thoroughly washed with tap water and then shade dried. The dried roots were milled to a fine powder and the extracts were made by following the previous method with little modifications (Choo et al., 2006). 1g of powdered root was soaked in 50 ml of methanol for 48 h. Further, the organic phase was separated and dried at 55 °C. The residue was weighed and dissolved in methanol at a concentration of 100 mg/ml and which was used for further assay.

### 2.2. Bacterial strains and culture conditions

Methicillin-resistant (MRSA, ATCC 33591) & methicillin-susceptible *S. aureus* (MSSA, ATCC 11632) and *S. pyogenes* SF370 were used in this study. *S. aureus* strains were grown in Tryptic Soya Broth (TSB) (Hi-Media, India) supplemented with 1% sucrose. Todd Hewitt broth supplemented with 5% yeast extract and 5% glucose (THYG; HI-Media, India) was used for *S. pyogenes*. All the bioassays were carried out at the cell density of 0.4 at O. D<sub>600 nm</sub> ( $1 \times 10^8$  CFU/ml) and all the strains were incubated at 37 °C for 24 h.

### 2.3. Determination of minimal biofilm inhibitory concentration

The MBIC was assessed through crystal violet staining method (Kannappan et al., 2017). Briefly, HI (50 – 500 µg/ml) was added in a 24-well polystyrene microtitre plate (MTP) containing growth medium with standard bacterial suspension. The plates were then incubated at 37 °C for 24 h. After incubation, the planktonic cells were removed from the wells and adhered biofilm cells were stained with 0.4% crystal violet solution (w/v). The biofilm adhered stain was eluted with 20% glacial acetic acid and quantified at OD<sub>570nm</sub> by UV-visible spectrometer (Hitachi U-2800, Tokyo, Japan). The minimum concentration of HI showing maximum percentage of biofilm inhibition without growth reduction was considered as MBIC. Percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = ((\text{Control OD}_{570\text{nm}} - \text{Treated OD}_{570\text{nm}}) / \text{Control OD}_{570\text{nm}}) \times 100$$

After incubation, HI was simultaneously analyzed for its effect on the growth of test pathogens using spectrophotometer at OD<sub>600nm</sub>

### 2.4. XTT reduction assay

In XTT reduction assay, metabolic activity of viable cells were assessed using XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) as substrate (Padmavathi et al., 2014). The test bacterial cells treated with and without HI was washed twice with sterile Phosphate buffered saline (PBS, pH 7.4) and resuspended in

an equal volume of the same. The cell suspensions were further incubated at 37 °C for 8 h in dark, along with XTT (Sigma, St. Louis, MO, USA)/menadione (Hi-Media, Mumbai, India) solution in the ratio of 12.5:1 respectively. After incubation, calorimetric changes were measured at OD<sub>490nm</sub> using spectrophotometer.

### 2.5. Microscopic visualization of bacterial biofilms

In order to envisage the effect of HI on biofilm architecture, test pathogens were incubated with  $1 \times 1 \text{ cm}^2$  glass slides in the wells of MTP with respective growth medium at 37 °C for 24 h in the presence and absence of MBIC of HI. For light microscopic observations, the slides were stained with crystal violet and imaged under light microscope after incubation at a magnification of 400× (Nikon Eclipse Ti 100, Japan). For Confocal laser scanning microscopic (CLSM) analysis, the glass slides

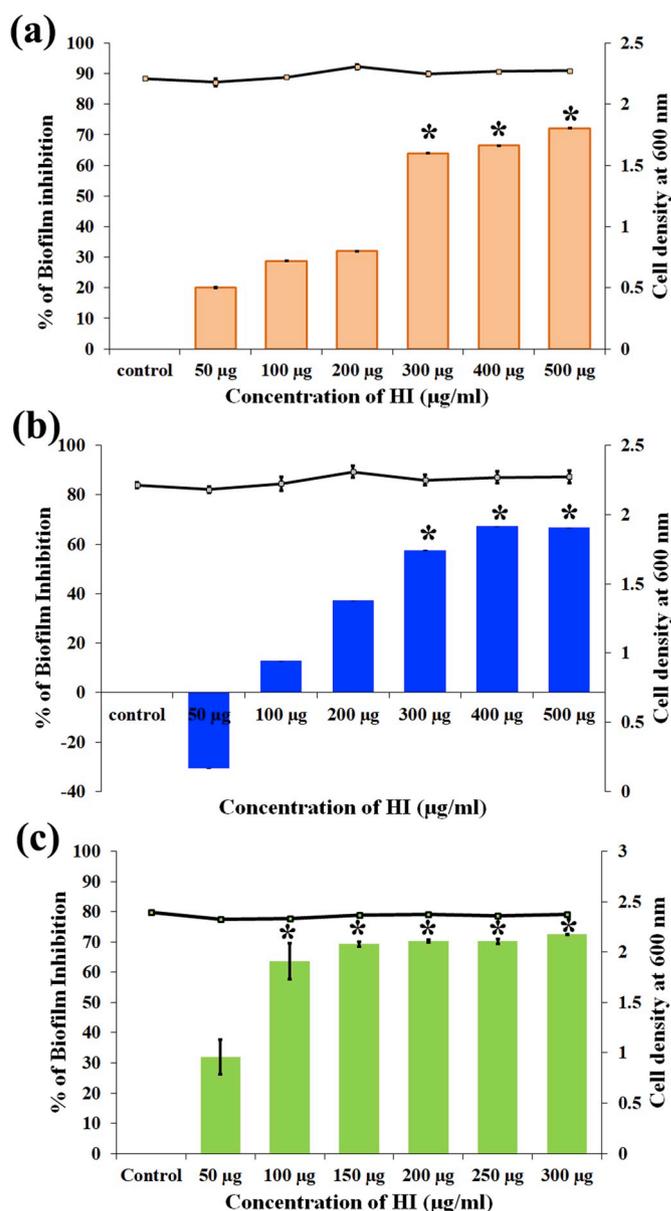
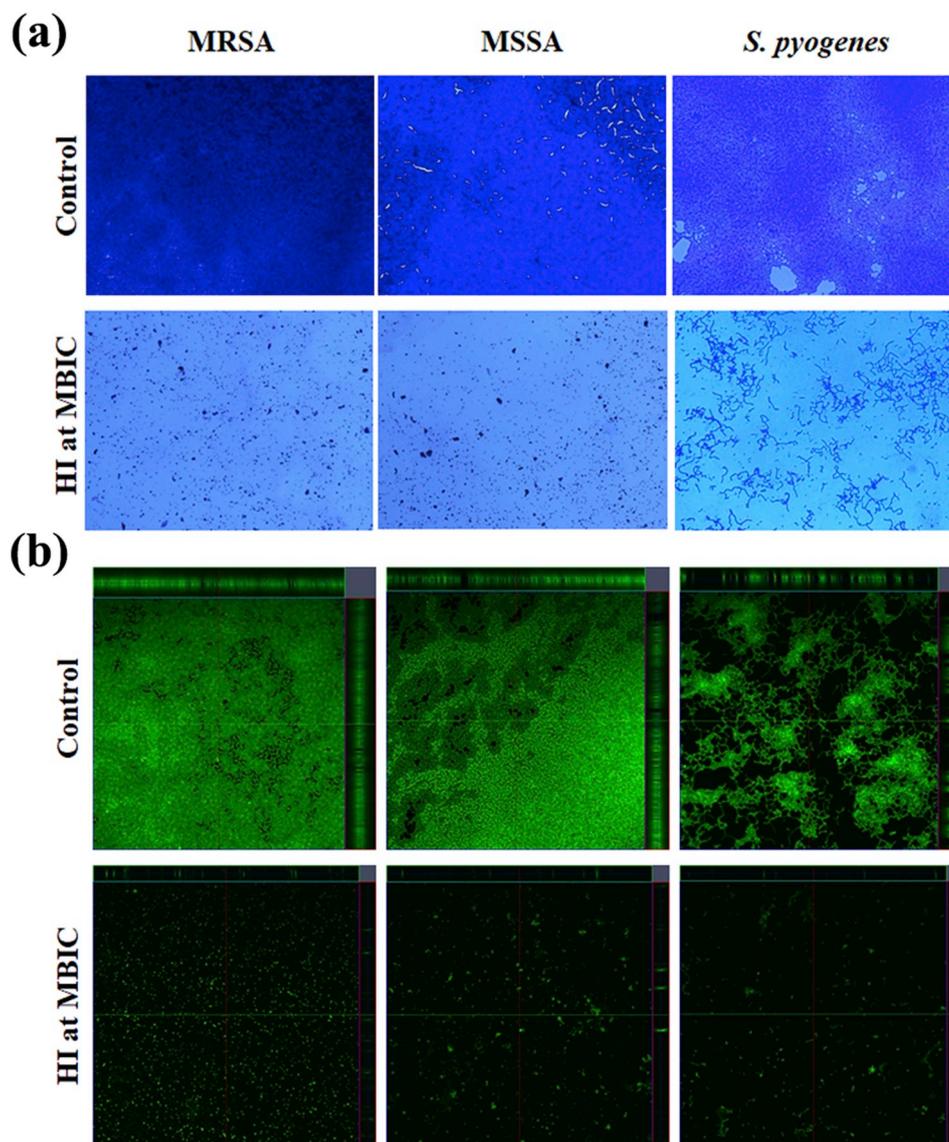


Fig. 1. Antibiofilm activity of HI at varying concentrations (50–500 µg/ml) on MRSA (a), MSSA (b) and *S. pyogenes* (c). The concentration of 300 and 150 µg/ml was identified as MBIC for *S. aureus* and *S. pyogenes*, respectively as it showed maximum biofilm inhibition without bacterial growth. Mean values of triplicate independent experiments and SD are shown. ANOVA was used to compare the treated and untreated controls. \* indicates significance at  $p \leq 0.05$ .



**Fig. 2.** Microscopic analyses of biofilm formed by the test pathogens. Representative light microscope (a) and confocal laser scanning microscope (b) images depicting the inhibitory potential of HI at MBIC against the biofilm formation of *S. aureus* and *S. pyogenes*.

were stained with 0.1% acridine orange (w/v) solution and imaged under CLSM (LSM 710, Carl Zeiss, Germany). The Z-Stack images were analyzed using COMSTAT software (generously gifted by Dr. Claus Sternberg, DTU Systems Biology, Technical University of Denmark) to obtain the biofilm biovolume, average thickness and surface to volume ratio of the biofilm formed in control and treated samples (Kannappan et al., 2017).

## 2.6. Mature biofilm disruption assay

For mature biofilm disruption assays, the test pathogens were allowed to form biofilm on the glass slides for 24 h at 37 °C. The slides with biofilm were later transferred to the MTP containing fresh growth medium along with and without HI at MBIC. After incubation, the slides were stained with crystal violet and imaged under light microscope as described above (Kannappan et al., 2017).

## 2.7. EPS inhibition assay

EPS inhibition assay was carried out as described previously with some little modifications (Santhakumari et al., 2016). Briefly, MTP

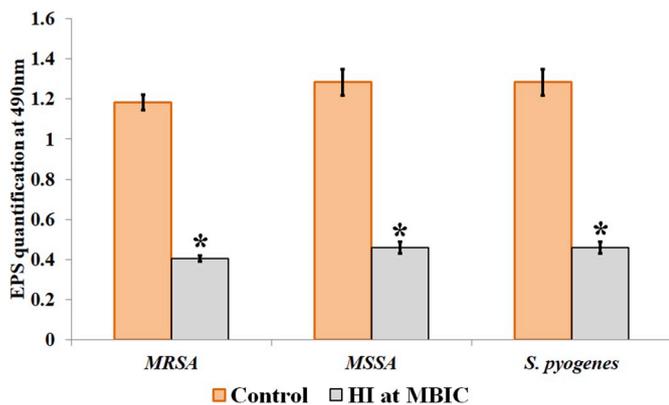
containing growth medium along with the test pathogens were incubated at 37 °C for 24 h in the presence and the absence of HI. After incubation, the cells were harvested and washed with 0.9% NaCl to which equal volume of phenol (5% v/v) and then 5 volume of concentrated H<sub>2</sub>SO<sub>4</sub> were added. The final reaction mixture was incubated in the dark for 1 h and the absorbance was measured at OD<sub>490nm</sub>.

## 2.8. Fourier transform infrared (FT-IR) spectroscopic analysis

Test pathogens treated with and without HI were washed thrice with sterile PBS (pH 7.4) and the cell pellet was freeze dried (Alpha 2–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). One hundred milligram of potassium bromide (IR grade) was mixed with 1 mg of freeze dried bacterial culture pellet to prepare KBr pellet. Prepared KBr pellet was analyzed by FT-IR spectroscopy (Nicolet™ iS5, Thermo Scientific, U.S.A). A total of 64 scans were taken with 4 cm<sup>-1</sup> resolution. The spectrum was scanned in the range of 4000–400 cm<sup>-1</sup>. All the IR spectra were plotted as absorbance and analyzed using OMNIC software (Nithyanand et al., 2015).

**Table 1**  
COMSTAT analysis of test pathogens treated with and without HI.

	Biofilm biomass ( $\mu\text{m}^3/\mu\text{m}^2$ )		Maximum thickness ( $\mu\text{m}$ )		Surface to volume ratio ( $\mu\text{m}^2/\mu\text{m}^3$ )	
	Control	Treated	Control	Treated	Control	Treated
MRSA	35.56	25.54	33.43	23.94	0.0328	0.0439
MSSA	50.97	25.28	47.78	23.70	0.0243	0.0443
<i>S. pyogenes</i>	38.84	24.01	36.90	22.81	0.0305	0.0464



**Fig. 3.** Effect of HI at its MBIC on the EPS production in test pathogens. The graph represents the quantitative data of EPS production in the control and HI-treated *S. aureus* and *S. pyogenes* cells. Mean values of triplicate independent experiments and SD are shown. Student-t Test was used to compare the treated and untreated controls. \* indicates significance at  $p \leq 0.05$ .

## 2.9. Blood survival assay

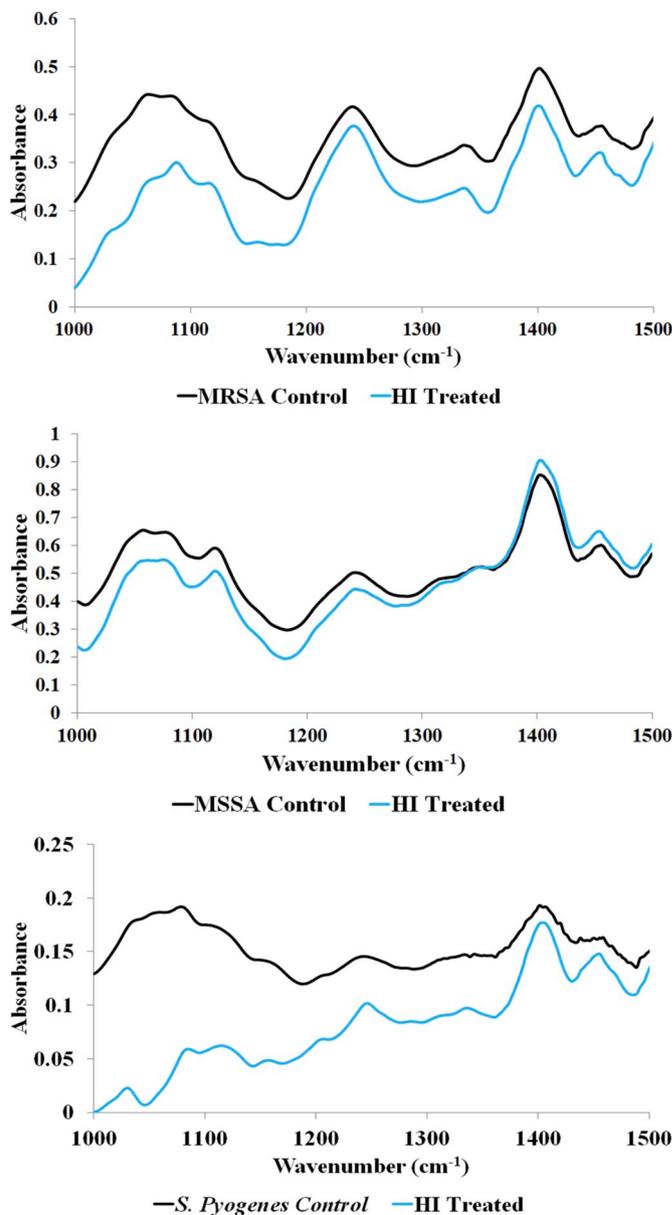
The effect of HI on surface-associated protein was assessed using a blood survival assay (Subramenium et al., 2015). Briefly, HI-treated and untreated test pathogens were allowed to grow overnight at 37 °C. Fifty microlitres of this overnight culture was added to 450  $\mu\text{l}$  of healthy human blood and mixed thoroughly. The mixtures were then rotated for 3 h at 37 °C. The viability of cells in control and treated samples were quantified by the spread-plate method on tryptic soya agar plates.

## 2.10. Successive solvent extraction of *H. indicus* root

Cold extraction method was followed to extract the cellular components of *H. indicus* root. 5 g of powdered root was successively extracted with 250 ml of solvents ranging from highly non-polar to polar for 48 h. After extraction, the organic layer was separated using Whatman No. 1 filter paper and kept in vacuum desiccator for complete dryness. The dried extracts were weighed and dissolved in methanol or in distilled water containing 0.02% Tween 20. Subsequently, all the extracts were assessed for its antibiofilm potential against MRSA.

## 2.11. Gas chromatography-Mass spectroscopy

The bioactive constituent of successive methanol extract was analyzed by GC-MS-138 QP2010 (SHIMADZU), using a Rxi®-5 ms capillary column [(5% diphenyl/95% 140 dimethyl polysiloxane), ID 0.25 mm thickness 0.25 139  $\mu\text{m}$ ]. The oven temperature of the column was maintained at 60 °C for 3 min, 250 °C for 8 min and 280 °C for 2 min. The sample was introduced into the column by a splitless mode of injection. The interface and ion source temperature was set at 250 °C and helium was used as a carrier gas with a delay time of 2 min. The scan range was 40–600 a.m.u. The total run time was 43 min. The major peaks were identified by comparison with the MS reference database of NIST software (National institute of standards and technology,



**Fig. 4.** FT-IR spectrum of MRSA, MSSA and *S. pyogenes* treated with and without HI at MBIC. FT-IR spectra showed variation in the region corresponding to the mixed profile of proteins and fatty acids (1500–1000  $\text{cm}^{-1}$ ) in the HI treated cells.

Gaithersburg, USA).

## 2.12. Statistics

All experiments were performed in experimental triplicate for thrice and the statistical analysis was performed using SPSS package (SPSS v20.0; SPSS Inc., Armonk, NY, USA). The differences between the untreated and treated samples were analyzed by Dunnett-ANOVA and Student-t test. Values were expressed as mean  $\pm$  standard deviation. The  $p$ -value  $\leq 0.05$  was set as significant.

## 3. Results and discussion

The crude methanol extract of the *H. indicus* was weighed and the dry yield was calculated as 2.4%. The extract was then dissolved in methanol and was used for further all assays.

Although *H. indicus* root has been studied for its potential against

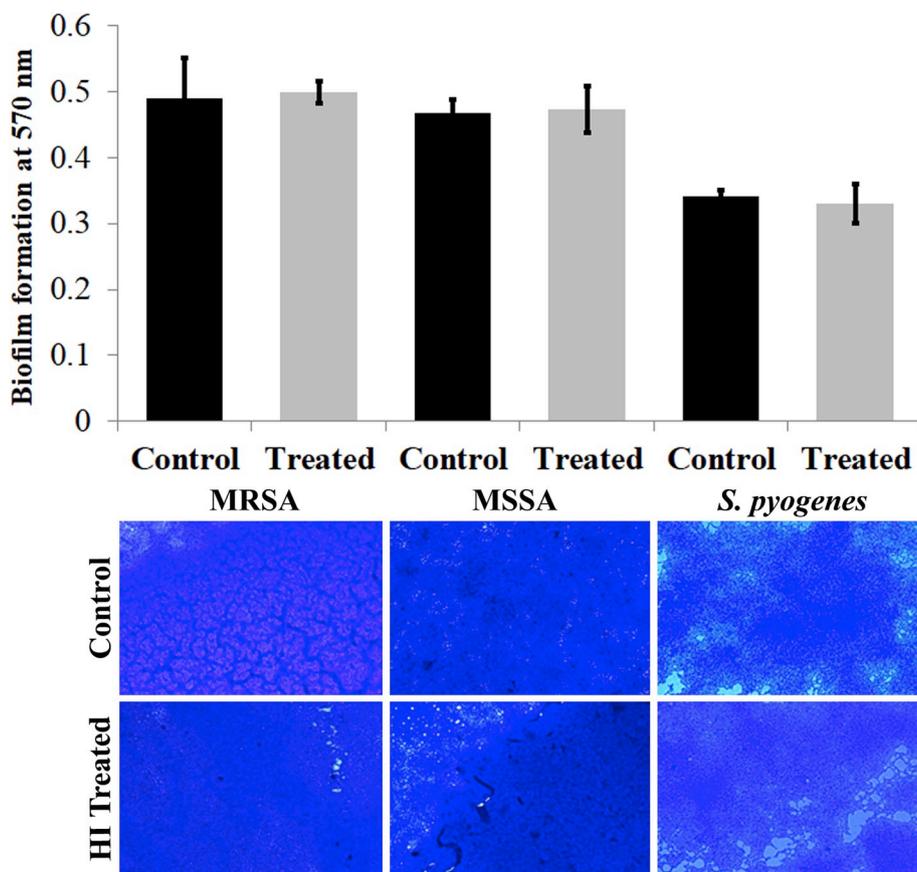


Fig. 5. Quantitative results showing the non-inhibitory potential of HI at MBIC on the preformed biofilms of test pathogens (A). Light microscopic images authenticate the quantitative results of crystal violet biofilm formation (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

several human ailments so far, its efficacy against the biofilm formation of the bacterial pathogens has not yet been studied. Hence, the present investigation has aimed to evaluate the efficacy of methanolic extract of *H. indicus* root (HI) to impede the biofilm formation of nosocomial bacterial pathogens. Primarily, the antibiofilm activity of the HI was assessed against the test pathogens. The HI inhibited the biofilm formation of the test pathogens in a concentration dependent manner and its MBIC was found to be 300 and 150  $\mu\text{g/ml}$  for *S. aureus* (Fig. 1A and B) and *S. pyogenes* (Fig. 1C), respectively. Hence, the 300 and 150  $\mu\text{g/ml}$  were fixed as MBIC for *S. aureus* and *S. pyogenes*, respectively and were used in all the assays. The result obtained in the present work is superior to the recent work with plant extracts, where the ethanol extract of *Piper betle* inhibited 50% of *Streptococcus mutans* biofilm formation at 390  $\mu\text{g/ml}$  concentration without growth inhibition (Teaupaisan et al., 2017). An antibiofilm agent, which reduces the virulence and biofilm formation without targeting bacterial viability, is expected to avoid selection pressure that promotes antibiotic resistance (Koo et al., 2017). To confirm the non-bactericidal activity of HI, cell density of the test pathogens cultured in the presence and absence of HI at MBIC was measured. As anticipated, no significant growth reduction was observed between the untreated and HI treated samples (Fig. 1A, B & C). In addition, XTT reduction assay also validated the non-antibacterial nature of HI (Fig. S1). Thus, the obtained results reconfirmed the potential of HI as an antibiofilm agent and not an antibacterial agent.

Microscopic observation of biofilms in the glass slides clearly revealed the impact of HI on the of test pathogens' biofilm formation. Light microscopic images showed a thick coating of biofilm in the control slides whereas a thin and discontinued biofilm with reduced number of microcolony formation was seen in HI treated slides (Fig. 2A). Further, CLSM analysis also revealed a prominent disintegration in the

biofilm architecture in the presence of HI at MBIC than that of the untreated biofilms of the test pathogens (Fig. 2B). In addition, COMSTAT analysis also evidenced that HI noticeably reduce the biofilm biomass & average thickness and increased surface to volume ratio compared to their respective untreated controls (Table 1). Moreover, pathogenic cells displayed an aggregated mode of growth upon HI treatment (Fig. S2). Concomitantly, phytocompound 3-furancarboxaldehyde thwarts the bacterial adhesion by means of inducing cellular aggregation (Subramenium et al., 2015). EPS confers the pathogens' adherence and three dimensional biofilm formations. It plays a crucial role in immune evasion and tolerance towards antimicrobial agents (Wu et al., 2015). HI interferes with the EPS production and reduces the EPS synthesis to a level of 66, 64 and 76% in MRSA, MSSA and *S. pyogenes*, respectively (Fig. 3). Consistent with our findings, previous studies with plant extracts have portrayed a weak biofilm formation with reduced EPS production in *S. aureus* and *S. pyogenes* (Elmasri et al., 2014; Nithyanand et al., 2015).

Recent past, FT-IR technique has been performed to analyze the difference in the chemical composition of prokaryotes and eukaryotes (Schmitt and Flemming, 1998). Likewise, in this study also, FT-IR analysis was done to monitor the changes in the cellular components of the test pathogens upon treatment with HI. Variation in the FT-IR spectra of HI treated and untreated control validated the results of *in vitro* assays. From Fig. 4, it was evidenced that the spectral difference was observed in the region containing mixed profile of cell membrane proteins and fatty acids ( $1500\text{--}1000\text{ cm}^{-1}$ ). Similar to the results of the present work, earlier work done by Elmasri et al. (2014) stated that sesquiterpenes from *Teucrium polium* targets biofilm formation of *S. aureus* by interfering fatty acid biosynthesis. Preformed biofilms are the real players in the pathogenesis of any particular pathogen. Hence,

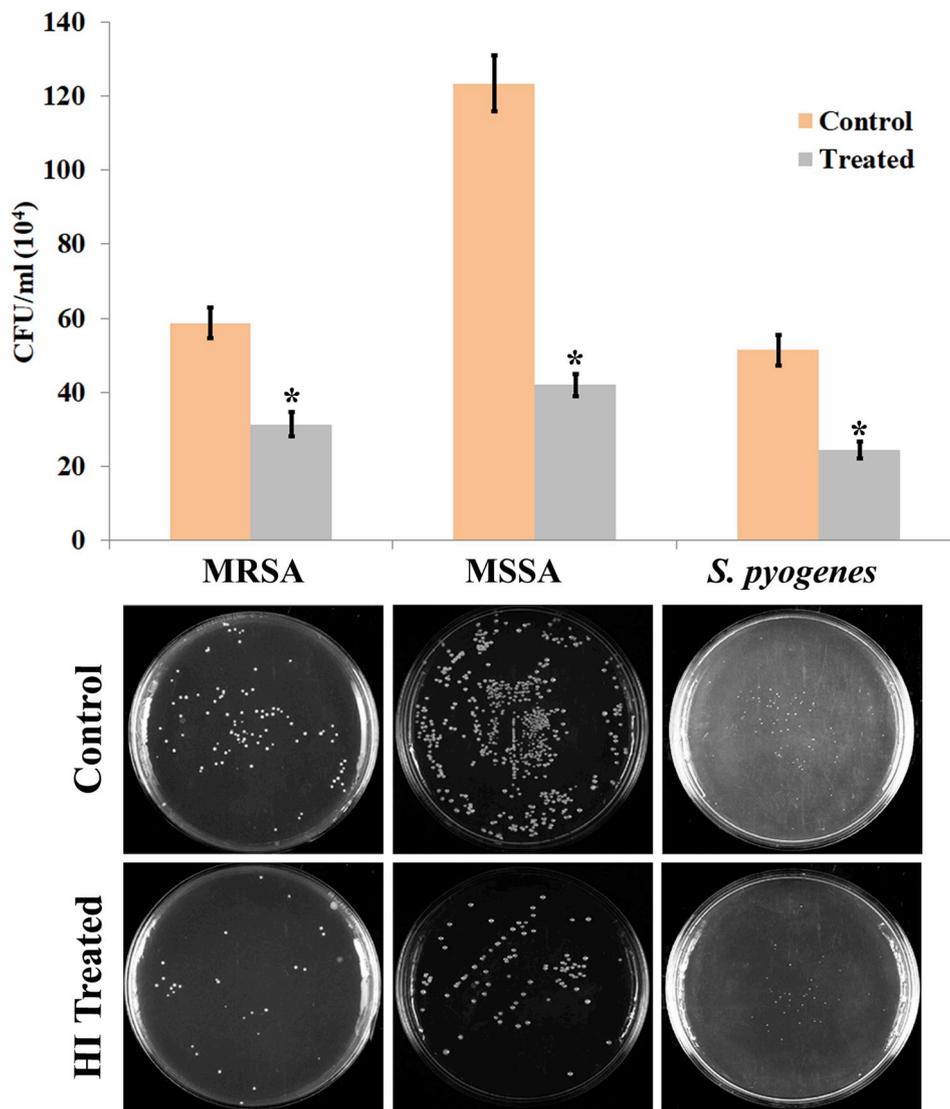


Fig. 6. Qualitative assessment of HI at MBIC on survival of the test pathogens in healthy human blood. Compared to the control, HI treatment sensitizes the test pathogens towards the healthy human blood and aids its clearance by host immune system. Mean values of triplicate independent experiments and SD are shown. Student-t Test was used to compare the treated and untreated controls. \* indicates significance at  $p \leq 0.05$ .

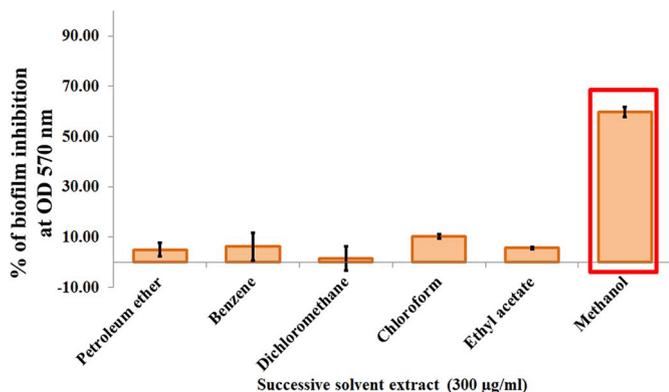


Fig. 7. Antibiofilm potential of different solvent extracts obtained through successive solvent extracts. Among the solvents tested, methanol is the only solvent showing predominant biofilm inhibitory effects against MRSA biofilm formation. Mean values of triplicate independent experiments and SD are shown.

Table 2

Compounds present in the successive solvent extract of HI as identified by GC-MS analysis.

Sl. No	Compound name	Retention time	Peak area
1.	2-Furancarboxaldehyde	10.058	3.08
2.	1,3-bis(1,1-dimethylethyl)-Benzene	10.169	5.33
3.	2-Hydroxy-4-Methoxy Benzaldehyde	11.413	9.13
4.	1-Chloro- 4- methoxy benzene	12.540	5.49
5.	1-Dodecanol	14.068	7.77
6.	2-Propenoic acid	20.395	25.28
7.	Hexadecanoic acid	25.795	3.92
8.	Propanoic acid	27.010	3.33

the effect of HI on the preformed biofilms of the test pathogens was assessed. HI had no significant effect over the preformed biofilms of the test pathogens, which was evidenced through light microscopic analysis (Fig. 5). On the whole, reduction in the spectral region of cell membrane fatty acids indicates that HI could possibly interfere with the fatty acid components present in the cell membrane thereby alter the cell-substrate interaction, adhesion and subsequent microcolony development of the test pathogens rather than the preformed biofilms.

The viability of pathogenic bacterial organism in healthy human blood is predominantly determined by its surface-associated protein, since it helps to evade phagocytosis (Courtney et al., 2006). The effect of HI on surface-associated protein was assessed using a blood survival assay. From Fig. 6, it is clearly evident that HI interferes in the function of surface-associated proteins and reduces the survival of test pathogens in the healthy human blood. The obtained result corroborates well with the results of EPS assay and cellular component analysis through FT-IR spectroscopy. Previously, Subramenium et al. (2015) reported that 3-furancarboxaldehyde treatment increased the rate of aggregation and reduced the biofilm formation in *S. pyogenes* with no significant survival difference in healthy human blood. This contradictory result is attributed to the presence of several other phytochemicals in HI that might act in some other mode to reduce the survival of the test pathogen in whole blood.

In order to further elucidate the active principle present in the HI, powdered *H. indicus* root sample was subjected to polarity based successive solvent extraction. Among the solvents tested, successive methanolic extract shown a promising antibiofilm activity against MRSA (Fig. 7). Hence, the successive methanolic extract was subjected to GC-MS analysis. From GC-MS analysis (Fig. S3), eight compounds were identified as major compounds (Table 2). 2-Furancarboxaldehyde, an isomer of 3-furancarboxaldehyde which has been reported to have *covR* mediated antibiofilm activity against *S. pyogenes* (Subramenium et al., 2015). Similar to the results obtained in the present study, *S. pyogenes* grown in the presence of 3-furancarboxaldehyde shown aggregated growth pattern. Meanwhile, the other major leads like 2-hydroxy-4-methoxy benzaldehyde was reported to inhibit the growth of food borne pathogens (Wang et al., 2010), whereas 1-dodecanol was reported to inhibit the *Candida albicans* hyphal formation (Davis-Hanna et al., 2008). Hexadecanoic acid and 1, 3-bis (1,1-dimethylethyl)-benzene hinders the quorum sensing mediated virulence factors and biofilm formation of certain Gram negative bacterial pathogens (Santhakumari et al., 2016; Padmavathi et al., 2014). However, there are no studies states the biofilm inhibitory efficacy of identified compounds against *S. aureus*. Thus, it is suggested that the compounds identified from the methanolic extract of HI may act synergistically to prevent the onset of biofilm formation by the test pathogens.

In conclusion, the present work delineates the antibiofilm efficacy of *H. indicus* root against the biofilm formation MRSA, MSSA and *S. pyogenes*. The HI effectively reduced the biofilm formation of test pathogens without impeding the test pathogens growth. The results of biofilm biomass quantification assay, followed by auto-aggregated growth, microscopic analysis and EPS quantification evidenced that HI targets the initial adherence of bacterial pathogens to form biofilm. GC-MS analysis of HI revealed the presence of functionally important compounds which could be responsible for its antibiofilm potential. Thus, the results of the present work revealed the antibiofilm potential of root extract from the traditional medicinal plant *H. indicus* and would be considered in drug formulations to control the onset of biofilm mediated multidrug-resistant bacterial infections.

## Conflicts of interest

Authors have no conflict of interest to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101338>.

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