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Bacterial biofilm inhibition activity of ethanolic extract of *Hemidesmus indicus*

Mamatha A[1] & Nidhi Girish*[,2,+]
[1]Department of Pharmacognosy, KLE College of Pharmacy, Bengaluru
[2]Department of Life Science, Christ (Deemed to be University), Bengaluru 560 029
E-mail: [+]nidhi8sep@gmail.com

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Multi-drug resistance is one of the biggest nightmares in the field of healthcare today. Adding on to this, some bacteria like *Staphylococcus aureus* and *Pseudomonas aeruginosa* have the ability to form biofilms. These essentially are large colonies of bacteria that are held together by polysaccharides and other biomolecules which in turn facilitate in their adherence to solid substrate – both natural and synthetic. This further creates a life-threatening implication leading to nosocomial infections like pneumonia, Urinary tract infections (UTI), etc. increasing the co-morbidities and mortality of critically-ill patients. The combination of antimicrobial resistance, ability to form biofilms and threat of nosocomial infections calls for a need to investigate newer, safer alternatives.

Plant based medicaments have been used for centuries and they are a great alternative to synthetic drugs. In the present study, ethanolic extracts of *Hemidesmus indicus* was evaluated against clinically-important multi-drug resistant organisms. Percentage biofilm inhibition of plant extracts of *Hemidesmus indicus* by crystal violet assay method. Triplicate analysis was done and data obtained was statistically interpreted using Microsoft Excel. Alcoholic extracts of *Hemidesmus indicus* exhibited significant biofilm inhibitory activity against the common bacteria *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*. Further, isolation of the chief active constituent responsible for Anti-biofilm activity is in process.

Keywords: Biofilm inhibition, Drug Resistance, *Hemidesmus indicus*, Nosocomial infections
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IUPAC defines biofilms as 'Aggregates of bacterial colonies, where the cells are held together and adhere to a substrate, natural or synthetic by a self-produced extracellular matrix. Thus biofilm-mode of growth is one of the two physiological states bacteria can exist in, the other being free-floating planktonic state. Depending on environmental and genetic cues, the two forms are interchangeable. Conversion to biofilm form is called biofilm formation and conversion to planktonic form is called biofilm dispersal. Both these processes are highly regulated. Bacteria growing in biofilm mimic in vitro stationary phase and undergoes several morphological and metabolic changes. During this stage there is an increased production of signaling molecules, secondary metabolites like pigments and antibiotics. Typically the formation of biofilm takes place in 4 distinct stages: attachment, micro-colony formation, maturation and dispersal[8,9]

Quorum sensing is a highly regulated and effective means of communication seen in bacterial colonies. This involves the production of certain chemical messengers called auto-inducers which signals other bacteria of the same or adjacent communities. In gram-positive bacteria, the signaling molecules are called auto-inducer peptides (AIP) and in gram-negative they are called acyl-homoserine lactones (AHL)[5]. The formation of biofilms is regulated by quorum sensing molecules secreted by the bacteria. In *P. aeruginosa*, two gene clusters – las and rhl systems produce N-(3-oxododecanoyl) homoserine lactone and N-butyryl-homoserine lactone respectively. These chemicals induce exopolysaccharide production and eDNA synthesis and secretion which subsequently causing the formation of biofilms[14]. And in *S. aureus*, the AIP is produced by the agr D gene. This peptide activated Agr C and Agr A protein signals dispersal of biofilm[15].

The National Institutes of Health revealed that among all acute and chronic microbial infections, 65% and 80%, respectively, are associated with biofilm formation. One of the many mechanisms by

*Corresponding author

which bacteria are gaining antibiotic resistance is due to persistent biofilms[6].

Diseases occurring due to Biofilm implications include bacterial vaginosis, UTI, catheter infections, middle ear infections, cystic fibrosis[11], endocarditis, infections of permanent indwelling devices like joint prostheses, heart valves and inter-vertebral disc (NIH, 2002), dental plaques, gingivitis [18] and many others.

According to Centre for Disease Control and prevention (CDC), the most common organisms found in nosocomial infections are *Escherichia coli, Pseudomonas aeruginosa*[16]*, Staphylococcus aureus, Candida auris, Clostridium sordellii, Klebsiella* spp., Enterococci, etc., (https://www.cdc.gov/hai/organisms/organisms.html). All of which have the ability to form biofilms.

As the inhibition of biofilm formation strategies are insufficient specially in nosocomial infections, there is a strong need to develop other strategies for the control of microbial biofilms like surface modification, for example incorporation of antimicrobial agents to produce an intrinsically bactericidal surface or the design of antimicrobials specially from natural sources that are targeted at biofilm producing growth (high-diffusion–reaction molecules, agents inhibiting biofilm). Hence, an attempt is made to research upon the biofilm inhibitory activity of *Hemidesmus indicus* in-vitro by Crystal violet assay[17].

Hemidesmus indicus (Family: Apocyanaceae) also called Indian Sarsaparilla or Anantmool in Sanskrit, is a slender, lactiferous, twining, sometimes prostrate or semi-erect shrub. Roots are woody and aromatic. The stem is numerous, slender, terete, thickened at the nodes. This plant is shown to have a wide range of therapeutic activity[19]. Here we show the biofilm-inhibitory activity exhibited by extracts obtained from the leaves of this plant.

Methodology

Collection and identification of plant sample

Roots of *Hemidesmus indicus* was purchased from a local vendor in Bangalore. The sample was authenticated to be root of *Hemidesmus indicus* by Dr Mamatha A, Associate Professor, KLE College of Pharmacy, Bengaluru and voucher specimen deposited.

Pre-extraction and extraction of plant material

The collected sample was then shade dried for about 10 days, tossing every 6 hours. The sample was then pulverized in an electric blender to coarse powder, which was then stored in air tight containers until used.

Alcoholic (ethanolic) extracts of the samples were prepared by cold maceration method. It was done by soaking 10 g of the plant powder in 100 mL of ethanol separately for 48 h at room temperature with occasional stirring. Then the solution was filtered and the filtrate was concentrated by evaporation on water bath, allowed to dry and then stored in airtight container at 4°C.

Preparation of sample

0.5 g of the dried plant extract of *Hemidesmus indicus* was taken and dissolved in a very small amount of 70% ethanol. The volume was then made up to 10 mL using distilled water. The concentration was thus set at 5% (w/v).

Biofilm formation inhibition

100 µL of each of the bacteria – *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 1934 and *Staphylococcus aureus* MTCC 737 were inoculated with 4.9 mL of sterile nutrient broth. 3 sets of test tubes were used for each organism – one containing 700 µL of 5% alcoholic extract of *Hemidesmus indicus* was used as the test sample, tube two without any extract as negative control and tube three containing broth without inoculation as blank. The test tubes were then allowed to incubate at 37°C for 48 h in an incubator without shaking.

The broth was carefully discarded and the test tubes were washed with sterile distilled water 2-3 times without causing much agitation to remove any free-floating bacteria and debris. It was then stained using 0.1% aqueous crystal violet and incubated at room temperature for 20 min and washed again with distilled water to removed excess stain. The tubes were allowed to air dry overnight.

2 mL of ethanol was then used to dislodge the biofilm-bacteria. 600 µL of this solution was taken in a cuvette and volume was made up to 3 mL and the absorbance was measured at 570 nm using 'Systronic spectrophotometer – 169'.

% inhibition of biofilm formation was calculated using the formula

$$\%\ inhibition = \frac{Abs\ of\ negative\ control - abs\ test\ sample}{Abs\ of\ negative\ control} \times 100$$

Statistical analysis

The data obtained was analyzed using Microsoft Excel. The values are expressed as mean ± standard deviation for triplicate data (n=3). The means compared using independent sample T-test with $p<0.05$, i.e., 95% confidence.

Results

Discussion

Alcoholic extracts of *Hemidesmus indicus* was tested against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Ethanol was selected for extraction of the plant material as it is the most common solvent employed for extracting antimicrobial substances. The polarity of ethanol helps in isolating constituents in plants like tannins, flavones, Terpenoids and polyphenols, which tend to have anti-microbial property.

Percentage inhibition of biofilm formation of *Hemidesmus indicus* ethanolic extract was compared against two controls, ethanol and distilled water, by statistical analysis. Results obtained showed significant percentage inhibition – 89.61% for *Pseudomonas aeruginosa*, 90.58% for *Staphylococcus aureus*, 56.77% for *Escherichia coli* and 70.52% for *Bacillus subtilis*, proving that the sample under study is efficient over wide range of organisms. **(Table 1 & Table 2) (Fig. 1 & Fig. 2)**

Table 1 — Absorbance at 570 nm expressed as mean ±std dev (n=3)

Organism	Sample	Mean absorbance at 570 nm ± Std dev
Bacillus subtilis	Sample	0.103±0.005
	Negative control	0.348±0.004
Escherichia coli	Sample	0.075±0.003
	Negative control	0.797±0.006
Pseudomonas aeruginosa	Sample	0.078±0.004
	Negative control	0.751±0.003
Staphylococcus aureus	Sample	0.156±0.004
	Negative control	0.362±0.002

Table 2 — Percentage inhibition using *Hemidesmus indicus*

Organism	% inhibition of biofilm formation
Bacillus subtilis	70.52
Escherichia coli	90.58
Pseudomonas aeruginosa	89.61
Staphylococcus aureus	56.77

Biofilm inhibitory effect of the extract under study, may be due to presence of flavonoids present in the plant [22]. Flavonoids are capable of reducing biofilms synthesis because they can suppress the activity of the auto inducer – 2, a quorum sensing molecule responsible for cell-cell communication in a variety of Gram-positive and Gram-negative bacteria [20]. It may also be due to direct or indirect interference of the chemical constituents present on Quorum sensing mechanism [24].

Other possibilities as reported previously could be due to certain enzymes like despersin B and deoxy ribonuclease which play a major role in biofilm dispersal[10,12,23]. Enzymes which degrade the matrix formed in biofilms may be useful as anti-biofilm agents[10,13]. Recent studies have shown that a fatty acid messenger, cis-2-decenoic acid, can induce dispersion and inhibit the growth of biofilm[7]. Nitric oxide at sub toxic concentrations can trigger dispersal of biofilm [1] of several bacterial species and it is also reported that nitric oxide has the potential to treat patients suffering from chronic infections caused by biofilms[3,4].

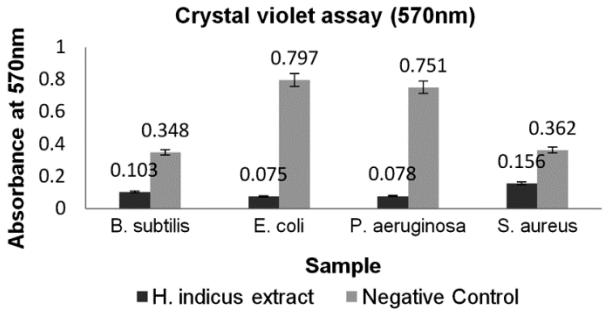

Fig. 1 — Absorbance at 570 nm expressed as mean ± std dev (n=3)

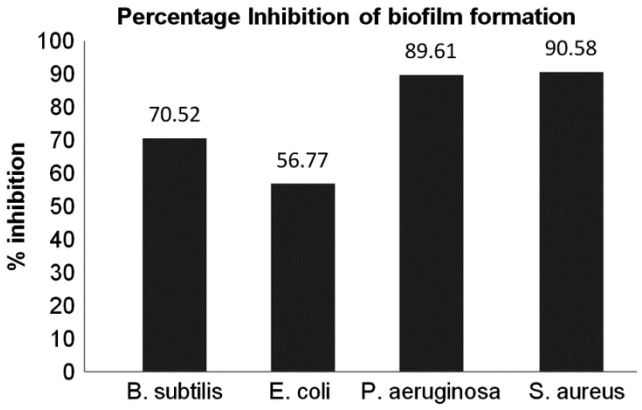

Fig. 2 — Percentage inhibition using *Hemidesmus indicus*

In conclusion, alcoholic extracts of *Hemidesmus indicus* exhibited significant anti-biofilm activity against the common bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* responsible for nosocomial infections. Further, isolation and bioactivity guided fractionation to identify and characterize the active principle responsible for anti-biofilm activity is in process. The potentiality of the drug would be much safer, economical and help in treatment of complications caused in nosocomial infection occurring to antibiotic resistance.

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