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BACTERICIDAL AND ANTI-BIOFILM ACTIVITY OF SELECTED ETHNOMEDICINAL PLANT EXTRACTS AGAINST *STREPTOCOCCUS PYOGENES*

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ABSTRACT

Streptococcus pyogenes, armed with multiple virulence factors, is a known notorious human pathogen responsible for various invasive and non-invasive diseases. The traditional usage of medicinal plants to treat human infections is an ever-increasing source of bioactive molecules. The present study demonstrates the antimicrobial activity against standard bacterial strains and the inhibitory effects against *Streptococcus pyogenes* biofilm of fifteen ethnomedicinal plant extracts. Plants like *Holarrhena pubescens* (256 µg/ml), *Rhododendron arboreum* (64 µg/ml), *Hydrocotyle javanica* (128 µg/ml), and *Mimosa diplotricha* (128µg/ml) showed potential antibacterial activity. These also exhibit a concentration-dependent reduction (83 – 92%) in the biofilm formation of *S. pyogenes* (SF370), significantly reducing micro-colonies and adherent cells, as revealed by crystal violet and microscopic assays. The AlamarBlue assay reveals that these crude plant extracts do not possess any bacteriostatic or bactericidal activity at their respective MBIC level against *S. pyogenes*. The GC-MS analysis revealed sesquiterpenes, unsaturated fatty aldehydes, and many other bioactive molecules in the plant extracts. This analysis has strengthened the

ethnomedicinal information on these plants towards antimicrobial properties and contributes to anti-biofilm activity.

Keywords: MBIC, *Streptococcus pyogenes*, Biofilm Inhibition, Resazurin, MIC, GC-MS

1. INTRODUCTION

Bacteria are living either as singular planktonic or sessile aggregates of multicellular form called biofilms. Biofilm is a re-organized form of planktonic cells encased in a self-produced matrix of Extracellular Polymeric Substances (EPS) attached to a surface **[1]**. Biofilms are intractable to antibiotic therapy, being one of the causes of antimicrobial resistance (AMR) and insistent infections by clinically significant pathogens. *Streptococcus pyogenes* (Group A *Streptococcus*) is an overwhelming bacterial pathogen responsible for significant human morbidity and mortality globally. This exclusive human pathogen is causing mild self-limiting infections in the respiratory tract and skin, including tonsillitis, pharyngitis, impetigo, and pyoderma **[2]**. However, it can cause invasive infections by invading deeper tissues leading to life-threatening streptococcal toxic shock syndrome, septicemia, pneumonia, necrotizing fasciitis, and endocarditis. Repetitive infections may trigger post-infections and immune sequelae complications like post-streptococcal glomerulonephritis, acute rheumatic fever, rheumatic heart disease, etc. **[3]**. The problem of antimicrobial resistance (AMR), its emergence, and spread are of great concern to the international health community. Misuse and overuse of antibiotics have led to multidrug resistance among *S.pyogenes* strains also **[4]**. The efficiency of biofilm formation by *S. pyogenes* has worsened its pathogenesis and antibiotic resistance. About 96% of the clinically isolated *S. pyogenes* strains from Beijing in 2011 were erythromycin resistant. Clinical strains with multidrug resistance to tetracycline, clindamycin, and fluoroquinolone were also found **[5]**. Anti-biofilm agents are considered potential alternatives as they inhibit biofilm formation and strengthen the action of antimicrobial drugs. These agents interfere with the bacterial communication system and regulatory mechanism needed for biofilm formation without imposing selection pressure on microbial cells, thereby lowering the risk of resistance development **[6]**.

Biofilms are responsible for 80% of infections caused by microbes **[3]**. The increased resistance of bacterial biofilms to antibiotics leads to treatment failures and has rejuvenated the search and advancement of natural anti-biofilm agents. Plant-based compounds are mostly

preferred as they are safe and have a long-standing tradition of using to treat and prevent infectious diseases by folklore medicinal practices **[7]**. Many plant-based antimicrobial compounds of interest have been well researched to develop novel therapeutic compounds.

However, they have seldom been explored for their anti-biofilm activity. Focussing should move towards the anti-virulent potential of metabolites from medicinal plants at sub-lethal concentrations rather than simply checking the effectiveness of their antimicrobial properties **[6]**. Such studies will reveal the purpose of using medicinal plants in treating infections by folklore. Plants are a rich source of various secondary metabolites (phytochemicals), such as flavonoids, terpenoids, polyphenols, tannins, and alkaloids, which often possess antimicrobial properties **[8]**.

With this background, the present study explores the antimicrobial and anti-biofilm potential of fifteen ethnomedicinally essential plants that have been traditionally used in different parts of the world to cure many ailments. Different standard bacterial strains were used to analyze the antimicrobial potential of these plant extracts, and *S. pyogenes* was used to evaluate their anti-biofilm potential. Ethnomedicinal uses of these plants are appreciable with huge applications, but the active principles of these treatments have not yet been divulged scientifically. Moreover, the inhibitory efficacy of these plant extracts on the biofilm formation of *S. pyogenes* remains unexplored. Hence, the present study was undertaken to explore the *in vitro* potential and identify the most efficient among these herbal plant extracts against the biofilm formation potential of *S. pyogenes*.

2. MATERIALS AND METHODS

2.1. Collection of Plant material

Fresh and healthy plants were collected from the Southern Western Ghats of Kerala. Plant materials collected were: *Boerhavia diffusa* L. (BD), *Clitoria arborea* Benth. (CA), *Coscinium fenestratum* (Gaertn.) Colebr (CF), *Croton sparsiflorus* Morong (CS), *Holarrhena pubescens* Wall. ex. G. Don (HP), *Hydrocotyle javanica* Thunb. (HJ), *Hyptis capitata* Jacq. (HC), *Mentha piperita* L. (MP), *Mimosa diplotricha* C. Wright ex Sauvalle (MD), *Lantana camara* L. (LC), *Rhododendron arboreum* Sm. ssp. *nilagiricum* (Zenker) Tagg (RA), *Persicaria glabra* (Willd.) M.Gómez (PG), *Solanum pubescens* Willd. (SP), *Spermacoce verticillata* L. (SV), and *Vitex negundo* L. (VN). The leaves were washed thoroughly in tap water, followed by successive washing in distilled water. Washed plants were cut into small pieces and air-dried at room temperature (25±2ºC) under shade. Finally, the dried material was

grounded to a coarse powder in a mechanical grinder and was stored in air-tight containers for further analysis.

2.2. Preparation of Plant extracts

Soxhlet apparatus (Borosil, India) was used for hot extraction. A known amount of powdered plant material (10g) was sequentially extracted by a series of solvents with increasing polarity using hexane, chloroform, ethanol, and methanol. The extract was collected and evaporated *in-vacuo* in a Rotavapour (Heidolph, Germany).

2.3. Bacterial Strains and Culture Conditions

The test organisms used for the antimicrobial study were procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-IMTECH, Chandigarh, India, and American Type Culture Collection (ATCC), USA, include bacteria like *Escherichia coli* (MTCC 443), *Proteus vulgaris* (MTCC 426), *Serratia marcescens* (MTCC 97), *Salmonella enterica* ser. *typhi* (MTCC 733), *Vibrio cholerae* (MTCC 3904), *V. fluvialis* (MTCC 4432), *Bacillus subtilis* (MTCC 441), *Enterococcus faecalis* (*MTCC* 439), *Staphylococcus aureus* (MTCC 7443), and *Streptococcus pyogenes* SF370 (ATCC700294D-5). Fungal strain *Candida albicans* (MTCC 3017) was also used. The standard bacterial strains were sub-cultured in Mueller-Hinton broth (Hi-Media, Mumbai, India), while *S. pyogenes* in Todd-Hewitt broth (Hi-Media, Mumbai, India) supplemented with 0.5% yeast extract and 1% glucose (THYG broth). The *Candida* strain was sub-cultured in Potato dextrose broth. The OD of the test pathogen was adjusted to 0.5 McFarland standard of 0.1 at OD_{600} nm from overnight culture equal to 1×10^8 CFU/ml for antimicrobial testing according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The bacterial broth cultures were incubated at 37°C and fungus at room temperature. Overnight culture of *S. pyogenes* in THYG broth (~2.3×10^5 CFU/ml) was considered standard cell suspension for biofilm studies **[4]**. Excess turbidity was adjusted with respective broths in all cases.

2.4. Antimicrobial susceptibility testing

Known quantities of dried extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml (w/v). From this stock, 10 µl (1mg/ml) was applied to 6 mm filter paper disc and used for antimicrobial testing following disc diffusion assay **[9]**. Test bacterial suspensions prepared as mentioned were spread uniformly over the surface of MHA or THYG agar plates. The seeded agar plates were kept undisturbed for 10 min for absorption of excess moisture. Standard

ciprofloxacin (CIP) (0.005mg/mL) was used as a positive control for antibacterial, while Nystatin (NYS) (100 units/disc) was for antifungal assays. The DMSO was used as a negative control. All bacterial plates were incubated at 37ºC and *Candida* plates at 25ºC. The zone of inhibition was measured as mm diameter after 24 h.

2.5. Determination of Minimum Inhibitory Concentrations

The Minimum Inhibitory Concentration (MIC) of the plant extracts against *S. pyogenes* was determined by the broth microdilution method in THYG broth in 96 well microtiter plates (Thermo Scientific Nunc, USA) following the CLSI guidelines **[6]**. Two µl of each crude plant extract with increasing concentrations (10-1024 µg/mL) were added to the 96 well microtiter plates containing 196 µl of THYG broth. The concentration of DMSO was maintained as 1% in all wells. The test pathogens at 1% concentration were added to this **[10]**. The microtiter plates were incubated at 37ºC for 16-18 h, and the Optical Density at 600 nm read against the broth containing 1% DMSO as blank control. The lowest concentration that inhibited the visible bacterial growth like that of control was taken as the MIC **[6]**. Penicillin (Hi-Media, Mumbai, India) was used as a positive control.

The Resazurin Microtiter assay method was used for the secondary confirmation of MIC. Resazurin solution was prepared by dissolving 33.75 mg in 5 mL of sterile distilled water. All the steps followed are the same as above. After incubation, 20 µl of Resazurin solution was added to the wells, and plates were wrapped with aluminium foil and incubated for 4-6 h. The colour change was then assessed visually. A change from blue to pink indicates resazurin reduction due to bacterial growth. The lowest concentration that prevented the colour change was taken as MIC **[11]**.

2.6. Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by withdrawing 10 µl aliquot from the well plates where no visual growth of the bacteria was observed. The aliquots were plated on THYG agar and were incubated at 37ºC for 24 h **[12]**. All the experiments were done in triplicates.

2.7. Determination of Minimum Biofilm Inhibition Concentration (MBIC)

The minimum concentrations that showed maximum reduction in biofilm formation compared to control were considered the MBIC. The MBIC was determined by the broth microdilution method in 24-well polystyrene microtiter plates with 1mL of THYG broth. The positive plant extracts at an increasing concentration (2 - 512 µg/mL) were added to THYG broth

containing 1% *S. pyogenes* cell suspension. The plates were then incubated at 37ºC for 24 h, and the cell density was read at 600 nm. The planktonic cells were washed off with distilled water, plates were allowed to dry, and the biofilm formed was stained with 1 mL of 0.4% (w/v) crystal violet (Hi-Media, Mumbai, India) for 10 min. Washed twice with distilled water and dried. The biofilm bound to crystal violet was extracted using glacial acetic acid (20%) for 10 min and was read at 570 nm (Tecan Spark 10M, Austria). The percentage inhibition was measured as: % of inhibition = (Control OD_{570}-Treated OD_{570}) / Control OD_{570}) X 100 **[4]**.

2.8. Light microscopic analysis

The *S. pyogenes* biofilm was grown on glass pieces (1x1 cm) in the presence and absence of plant extracts at its MBIC concentration. Briefly, the glass pieces were placed in 24-well polystyrene plate wells containing 1ml THYG broth and 1% standard bacterial cell suspension as inoculum. Plates were incubated at 37ºC for 24 h. The glass pieces were washed twice with sterile distilled water and stained with 0.4% crystal violet for 10 minutes. Excess stains on the glass pieces were washed with sterile distilled water, air-dried, observed under Optical light microscope (Olympus: CX43, Japan) at 400X magnification, and documented using an attached digital camera (Magcam, model: DC 10, India) **[4]**.

2.9. Cell viability assay

The cytotoxic effects of the plant extracts at its MBIC against *S. pyogenes* were analyzed using AlamarBlue cell proliferation assay kit (Bio-Rad, USA). *S. pyogenes* was grown in 1ml THYG broth in the presence or absence of the plant extracts individually and incubated at 37ºC for 24 h. The cell pellet was collected and resuspended in 1ml of 0.9% saline followed by the addition of 100 µl of AlamarBlue (1mg/mL). Multiwell plates were incubated in the Dark for 4-6 h. OD was read at 570 and 600 nm. The percentage difference in reduction of AlamarBlue was determined according to the manufacturer's instruction between treated and control samples **[13]**.

2.10. GC-MS analysis

The plant extracts with activity against the *S. pyogenes* biofilm formation was subjected to GC-MS analysis (Shimadzu, QP2010S). Gas chromatography column (ELITE-5MS) (ID 0.25mm, thickness 0.25 µm, 30 m length) was coupled to a mass detector (with 5% diphenyl and 95% 140 dimethylpolysiloxane) using standard GC-MS parameters. The injector was set in the split injection mode having 260ºC of temperature. The initial temperature was set at 80ºC (3 minutes), which was programmed to increase to 280ºC at a rate of 5°C/minute. The major peaks were compared with the MS reference database

of NIST 11 (National Institute of Standards and Technology, Gaithersburg, USA) and WILEY 8 **[6]**.

3. RESULTS

3.1. Antimicrobial activity of plant extracts

The antibacterial activities of fifteen plant extracts were evaluated against eleven pathogenic strains. The extracts exhibited varying degrees of inhibitory activity against the tested bacteria and fungus comparing to positive and negative controls (**Table 1)**. Methanolic plant extracts exhibited more antimicrobial activity when compared with other solvents, except *M. diplotricha,* which showed more potency in ethanolic extract. The extracts of *R. arboreum* (RAME)*, H. pubescens* (HPME) and *M. diplotricha* (MDEE) showed the broader spectrum of activity, forming inhibition zones against eight organisms (both Gram positive and Gram negative) tested. The maximum zone (18 mm) of antibacterial activity was observed with the hot methanol extract of *H. pubescens* (HPME) against *S. pyogenes*. The hot methanol extracts of *H. javanica* (HJME) and *R. arboreum* (RAME) and hot ethanol extract *M. diplotricha* (MDEE) showed 17 mm, 16 mm inhibition, and 14 mm zone of inhibition respectively against *S. pyogenes*. The antifungal activity against *Candida albicans* was shown by methanol extract of *H. pubescens* (HPME) with an inhibition zone of 17 mm. Methanol extracts of *C. arborea* (CAME)*, S. verticillata* (SVME)*, V. negundo* (VNME)*,* and ethanol extract of *M. diplotricha* (MDEE) also showing activity against *Candida albicans* with inhibition zones of 11 mm, 8 mm, 9 mm and 10 mm respectively.

3.2.Determination of MIC and MBC

Both broth microdilution method and Resazurin microtiter assay methods were applied to find the Minimum Inhibitory Concentrations (MIC). **Table 2** summarizes the MIC values of each extract tested and also the MBC values. The methanol extracts of *R. arboreum* (RAME) and *C. fenestratum* methanol extract (CFME) showed vigorous activity with the lowest MIC of 250 µg/ml. The MIC of methanol extracts of *H. javanica* (HJME), *H. pubescens* (HPME), *H. capitata* (HCME), *C. arborea* (CAME) and *S. verticillata* (SVME), and ethanol extract of *M. diplotricha* (MDEE), were found to be 500 µg/ml. The Resazurin-based assay confirmed the above MIC values.

3.3.Inhibition of biofilm formation in *S. pyogenes*

The plant extracts which showed potential MIC and MBC (≤ 500 µg/ml) such as methanol extracts of *H. javanica* (HJME), *R. arboreum* (RAME), *H. pubescens* (HPME), *C. fenestratum* (CFME), *H. capitata* (HCME), *C. arborea* (CAME), *S. verticillata* (SVME) and ethanol extract of

M. diplotricha (MDEE) were analysed for biofilm inhibition. The efficiency of these extracts was investigated for *S. pyogenes* biofilm inhibition in the presence or absence of the crude extracts at concentrations ranging from 2 to 512 µg/ml. Only four plant extracts showed biofilm inhibitory activity below its MIC level. Among these, *H. javanica* (HJME) extract showed 92% of biofilm inhibition at 128 µg/ml concentration. Similarly, *R. arboreum* (RAME) at a concentration of 64 µg/ml gave 91% inhibition; *H. pubescens* (HPME) (256 µg/ml) and *M. diplotricha* (MDEE) (128 µg/ml) gave 83% and 86% inhibition respectively. These concentrations were considered MBIC since, beyond this concentration, no significant increase in anti-biofilm activity was witnessed. A concentration dependent biofilm inhibition efficiency of these plants without inhibiting the growth of the *S. pyogenes* are shown in the graph **(Figure 1A-D)**. The four plant extracts showed potential inhibition of biofilm formation of *S. pyogenes* in a concentration dependent manner, without inhibiting the growth of the tested concentrations. This shows that the four plant crude extracts are not possessing bacteriostatic or bactericidal activities at their MBIC concentration.

3.4.Microscopy

Light microscopic analysis of slides in bacterial cell suspensions also revealed that there is a significant reduction in microcolony formation and biofilm architecture with the treatment of selected plant extracts compared to untreated ones **(Figure 2)**.

3.5.Cell viability assay

The cell viability and cytotoxicity assays using AlamarBlue were done to check the effect of the selected plant extracts on cell proliferation. The results show no significant variation in the percentage reduction of AlamarBlue between treated and control cells **(Figure 3)**. All four plant crude extracts, namely, RAME, HJME, HPME, and MDEM studied were not possessing bacteriostatic or bactericidal activities at their MBIC concentrations.

3.6.GC-MS analysis

The four plant extracts with potential activity against the *S. pyogenes* biofilm formation were subjected to gas chromatography–mass spectrometry (GC-MS) analysis using standard parameters. The peaks obtained were compared with the MS reference databases and identified major bioactive compounds present in the crude plant extracts **(Table 3)**. Major constituents include terpenes and sesquiterpenes, contributing to the bioactive potential of these plants. Methanol extract of *Hydrocotyle javanica* (HJME) shows the presence of Oxalic acid allyl tetradecyl ester (20.86%), Palmitic acid vinyl ester (18.56%) and Quinic acid

(10.87%) as the major compounds. In the methanol extract of *Rhododendron arboreum* (RAME) n-Capric acid vinyl ester (40.02%) and Nonadecane (27.94%) are the major compounds. Phytol acetate (17.3%), 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*, R*-(E)]]- (T-phytol) (13.01%) and Fumaric acid 4-isopropylphenyl pentadecyl ester (9.32%) are present in the methanol extract of *Holarrhena pubescens* (HPME); while Palmitic acid vinyl ester (87.11%) and Mome inositol (10.84%) in the ethanol extract of *Mimosa diplotricha (*MDEE). The results are also validating the use GC–MS spectroscopy as an extremely effective method for determining the presence or absence of a wide variety of phytochemicals in bioactive plant extracts.

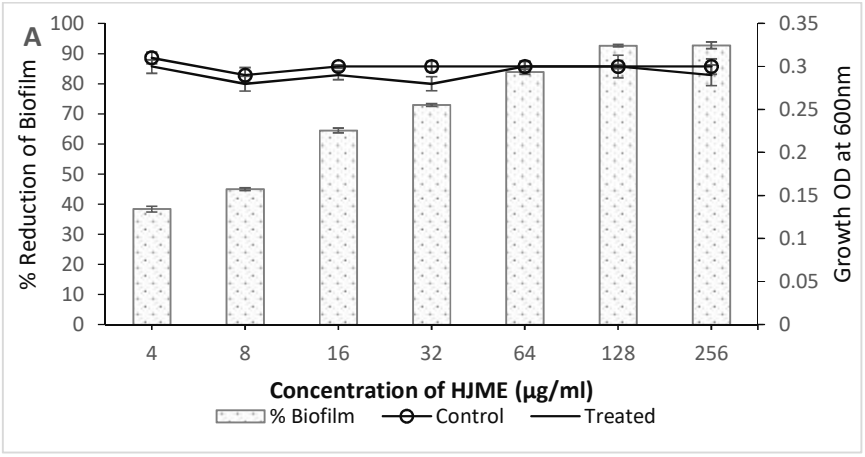
Table 1: Antimicrobial activity of fifteen crude plant extracts. Zones of inhibition diameter (mm) of plant extracts at 1 mg/ml concentration

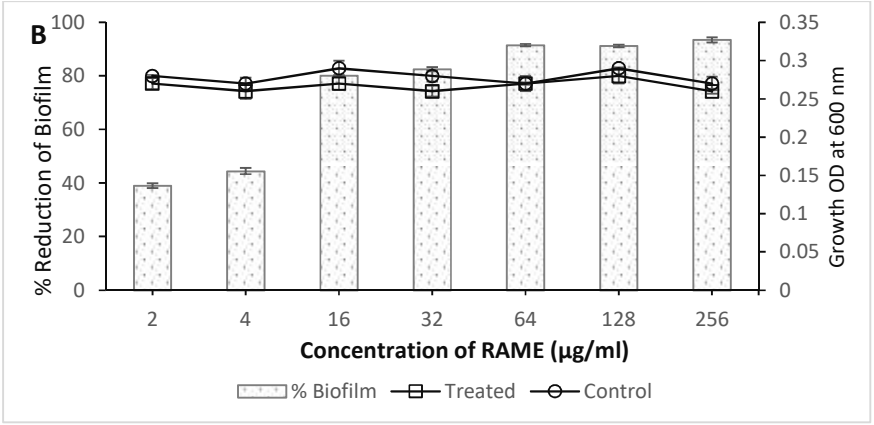
Plant Extracts (MeOH/EtOH)	Zones of inhibition (mm diameter)										
	E. coli MTCC 443	*V. cholerae* MTCC 3904	*V. fluvialis* MTCC 4432	*S. typhi* MTCC 733	*P. vulgaris* MTCC 426	*S. marcescens* MTCC 97	*B. subtilis* MTCC 441	*S. aureus* MTCC 7443	*S. pyogenes* ATCC 700294D-5	*E. faecalis* MTCC 439	*C. albicans* MTCC 3017
HJME	-	14	12	-	-	12	10	10	17	10	-
RAME	9	15	12	-	13	-	12	16	16	10	-
HPME	15	-	11	17	-	-	9	8	18	9	17
CFME	14	10	-	-	-	-	-	-	9	-	-
PGME	-	9	10	-	-	8	10	-	7	-	-
MPME	14	-	-	-	-	-	-	-	-	10	-
HCME	9	9	-	-	9	-	8	8	9	-	-
SPME	-	-	-	9	-	-	-	11	9	-	-
BDME	9	-	-	9	-	10	10	12	8	-	-
LCME	10	-	-	-	10	-	-	10	7	-	-
CSME	-	8	-	-	-	-	-	9	-	-	-
CAME	10	-	9	-	8	-	9	-	8	9	11
SVME	9	8	-	-	-	-	-	-	8	9	8
VNME	12	10	11	-	12	9	-	-	8	-	9
MDEE	12	12	-	-	9	-	11	12	14	8	10
Negative Control (DMSO)	-	-	-	-	-	-	-	-	-	-	-
Positive Control (CIP/NYS)	25	22	20	18	20	24	25	15	18	18	32

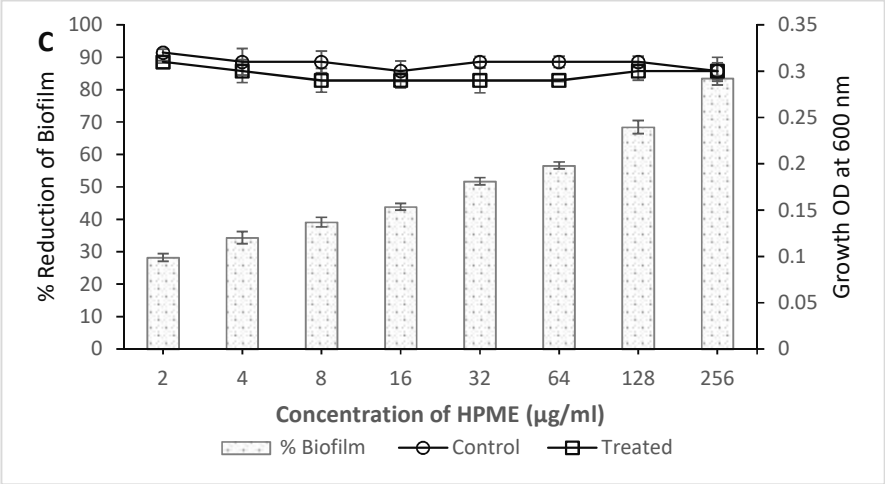
The activity scored in terms of diameter (mm) of Inhibition zones; No activity (-); Ciprofloxacin (CIP) positive control for antibacterial assay; Nystatin (NYS) for anti-fungal assay; Plant extracts using Methanol (MeOH): *Hydrocotyle javanica* (HJME), *Rhododendron arboreum* (RAME), *Holarrhena pubescens* (HPME), *Coscinium fenestratum* (CFME), *Persicaria glabra* (PGME), *Mentha piperita* (MPME), *Hyptis capitata* (HCME), *Solanum pubescens* (SPME), *Boerhavia diffusa* (BDME), *Lantana camara* (LCME), *Croton sparsiflorus* (CSME), *Clitoria arborea* (CAME), *Spermacoce verticillata* (SVME), *Vitex negundo* (VNME); Plant extract using Ethanol (EtOH): *Mimosa diplotricha* (MDEE); MTCC: Microbial Type Culture Collection and Gene Bank (CSIR-IMTECH, Chandigarh); ATCC: American Type Culture Collection (USA).

Table 2: Minimum inhibitory concentration (MIC, µg/ml), minimum bactericidal concentration (MBC, µg/ml) of crude plant extracts against *S. pyogenes*

Plant name	MIC (µg/ml)	MBC (µg/ml)
Hydrocotyle javanica **methanol extract (HJME)**	**500**	**500**
Rhododendron arboreum **methanol extract (RAME)**	**250**	**250**
Holarrhena pubescens **methanol extract (HPME)**	**500**	**1024**
Coscinium fenestratum **methanol extract (CFME)**	**250**	**250**
Persicaria glabra **methanol extract (PGME)**	**>1024**	**>1024**
Mentha piperita **methanol extract (MPME)**	**>1024**	**>1024**
Mimosa diplotricha **ethanol extract (MDEE)**	**500**	**1024**
Hyptis capitata **methanol extract (HCME)**	**500**	**1024**
Solanum pubescens **methanol extract (SPME)**	**>1024**	**>1024**
Boerhavia diffusa **methanol extract (BDME)**	**1024**	**>1024**
Lantana camara **methanol extract (LCME)**	**>1024**	**>1024**
Croton sparsiflorus **methanol extract (CSME)**	**1024**	**>1024**
Clitoria arborea **methanol extract (CAME)**	**500**	**>1024**
Spermacoce verticillata **methanol extract(SVME)**	**500**	**>1024**
Vitex negundo **methanol extract (VNME)**	**>1024**	**>1024**
Penicillin	**0.008**	**0.016**







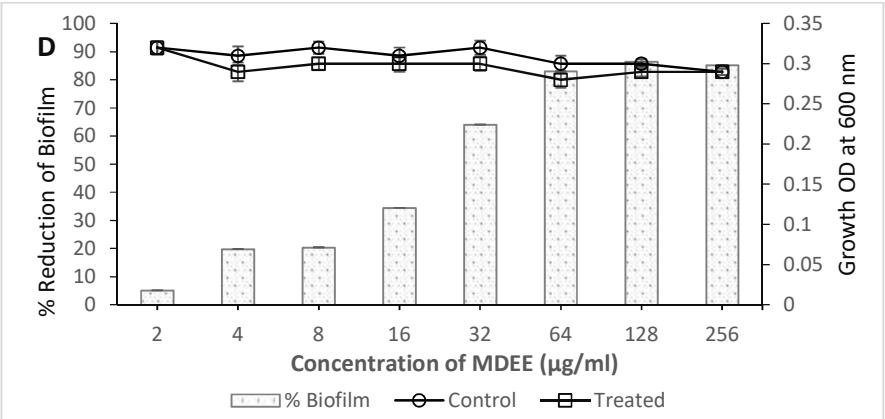


Figure 1: Effect of crude plant extracts on growth and biofilm development in *S. pyogenes.*

(A) Graphs displaying percentage reduction in biofilm formation at different concentrations (2- 256 µg/ml) of methanol extract of *Hydrocotyle javanica* (HJME) showing 92% inhibition at 128 µg/mL, (B) Methanol extract of *Rhododendron arboreum* (RAME) showing 91% inhibition at 64 µg/mL, (C) Methanol extract of *Holarrhena pubescens* (HPME) showing 83% inhibition at 256 µg/mL, and (D) Ethanol extract of *Mimosa diplotricha* (MDEE) showing 86% of inhibition at 128 µg/ml concentration. Error bars represent the standard error of the mean (±SEM) of triplicate experimental value

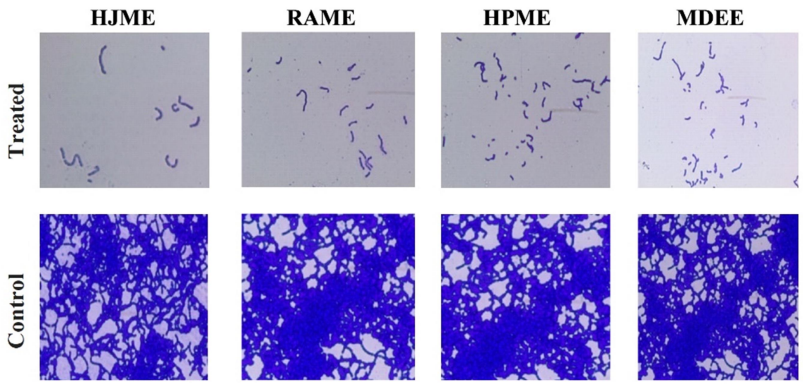


Figure 2: Micro Photographs of Biofilms formed Slides of *S. pyogenes* SF370 (ATCC 700294D-5) in the presence (treated) and absence (control) of plant extracts.
Light microscopic images of crystal violet stained *S. pyogenes* biofilms at 400X magnification showing inhibition in micro-colony formulation in treated samples with crude plant extract at its respective MBIC, compared to controls (untreated). HJME -Methanol extract of *Hydrocotyle javanica,* RAME - Methanol extract of *Rhododendron arboreum* , HPME - Methanol extract of *Holarrhena pubescens*, and MDEE - Ethanol extract of *Mimosa diplotricha*. (Colour figure)

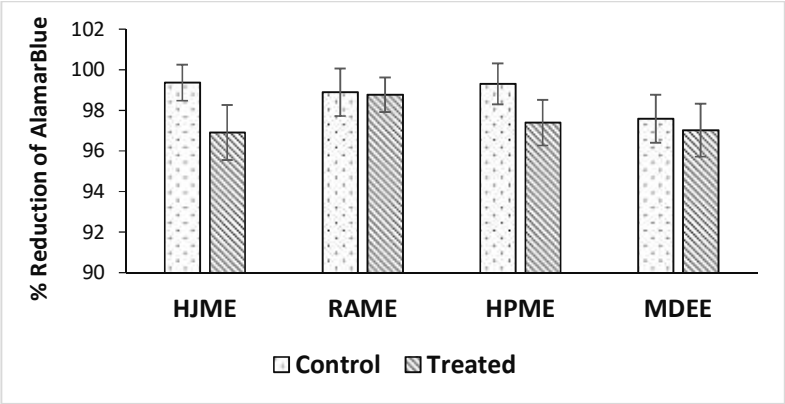


Figure 3: Effect of crude extracts on the metabolic profile of *S. pyogenes* SF370 (ATCC 700294D-5) at their Minimum Biofilm Inhibitory Concentration MBIC.
Graph displaying the percentage reduction of AlamarBlue in treated and control samples of all the four plant extracts at their MBIC concentrations. HJME - Methanol extract of *Hydrocotyle javanica,* RAME - Methanol extract of *Rhododendron arboreum* , HPME - Methanol extract of *Holarrhena pubescens*, and MDEE - Ethanol extract of *Mimosa diplotricha*). Error bars represent the standard error of the mean (±SEM) of triplicate values

Table 3: List of Major Phytochemicals identified through GC-MS analysis.

Peak No.	R. Time	Area%	Name of the Metabolites
Methanol extract of *Hydrocotyle javanica* (HJME)			
1	17.338	1.41	Caryophyllene
2	18.133	3.23	(E)-.beta.-Famesene
3	18.946	1.91	1,5,9-Cyclododecatriene, 1,5,9-trimethyl-
4	20.799	0.76	(+)-Nerolidol
5	21.448	0.81	Caryophyllene oxide
6	22.466	10.87	Quinic acid
7	25.625	1.23	(-)-Loliolide
8	26.784	7.64	Phytol, acetate
9	28.557	4.32	Methylpalmitate
10	31.789	5.71	Cis-Linoleic acid methyl ester
11	31.915	3.68	Methyl linolenate
12	32.132	2.53	Isophytol, acetate
13	44.476	3.29	Cyclohexyl cinnamate
14	46.501	18.56	Palmitic acid vinyl ester
15	46.575	20.86	Oxalic acid, allyl tetradecyl ester
16	49.434	13.18	2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane

Methanol extract of *Rhododendron arboreum* (RAME)			
1	**21.368**	**0.30**	**E-14-Hexadecenal**
2	**22.167**	**2.43**	**(1R,3R,4R,5R)-(-)-Quinic acid**
3	**22.340**	**0.39**	**Cis-isoapiole**
4	**25.807**	**0.30**	**9-Eicosene, (E)**
5	**28.557**	**0.75**	**Methyl palmitate**
6	**31.892**	**0.68**	**Methyl 7-octadecenoate**
7	**32.137**	**0.25**	**Phytol, acetate**
8	**32.346**	**0.28**	**Methyl stearate**
9	**35.341**	**0.87**	**Alpha.Gamma-dipalmitin**
10	**38.263**	**0.90**	**1,3-diolein**
11	**38.637**	**0.48**	**Glycerin 1,3-distearate**
12	**43.415**	**0.98**	**Squalene**
13	**43.607**	**2.65**	**Glycerol trilaurate**
14	**43.867**	**3.66**	**3,3-Diethyltridecane**
15	**44.313**	**40.02**	**n-Capric acid vinyl ester**
16	**44.617**	**27.94**	**Nonadecane**
17	**44.842**	**3.30**	**1,2-dipalmitoylcephaline**
18	**45.181**	**3.35**	**Delta.-butylvalerolactone**
19	**48.043**	**3.98**	**Cholest-5-en-3-ol, butanoate**
20	**48.913**	**6.48**	**Lupeol**
Methanol extract *Holarrhena pubescens* (HPME)			
1	**21.370**	**3.04**	**E-14-Hexadecenal**
2	**22.394**	**2.38**	**Megastigmatrienone**
3	**24.928**	**8.17**	**Gamma-hydroxyisoeugenol**
4	**25.811**	**2.58**	**1-Octadecene**
5	**26.793**	**6.78**	**Phytol, acetate**
6	**28.559**	**4.23**	**Methylpalmitate**
7	**30.106**	**9.02**	**2-Propenal, 3-(3,4-dimethoxyphenyl)-**
8	**31.893**	**4.29**	**ELAIDINSAEUREMETHYLESTER**
9	**32.136**	**17.30**	**Phytol, acetate**
10	**32.349**	**2.79**	**Methyl stearate**
11	**48.936**	**4.71**	**Oleic acid, 3-(octadecyloxy)propyl ester**
12	**49.141**	**1.20**	**Heptadecane, 4-methyl-**
13	**49.274**	**5.07**	**2,3-bis(tetradecanoyloxy)propyl stearate**
14	**49.565**	**1.99**	**Tetradecyl oleate**
15	**49.633**	**2.59**	**Myristic acid vinyl ester**
16	**49.700**	**9.32**	**Fumaric acid, 4-isopropylphenyl pentadecyl ester**
17	**49.767**	**13.01**	**2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [r-[r*,r*-(e)]]- (t-phytol)**
18	**49.883**	**1.53**	**14-beta-h-pregna**
Ethanol extract of *Mimosa diplotricha (*MDEE)			
1	**15.559**	**10.84**	**Mome inositol**
2	**39.050**	**0.59**	**Tricosanoic acid, 2-methoxy-, methyl ester**
3	**39.092**	**1.45**	**Octadecanoic acid, ethenyl ester**
4	**40.305**	**87.11**	**Palmitic acid vinyl ester**

4. DISCUSSIONS

One of the great wisdoms of Indian culture is its traditional ethnobotanical knowledge. Scientific validation of this information will lead to modern drug discovery as it was well proven **[14]**. Plants exhibited antimicrobial properties as a self defence mechanism and due to the presence of four major classes of phytochemicals: phenolics and polyphenols, terpenoids and essential oils, lectins and polypeptides, and alkaloids **[15]**. Recent scientific works have revealed that many phytochemicals can disrupt biofilms or inhibit their formation **[16]**. In this manuscript, we have screened fifteen plants for their antimicrobial activity and potential for the inhibition of biofilm formation in *S. pyogenes*. Hot extracts of fifteen plants were analysed for their antimicrobial activities, and those with potential MIC and MBC values against *S. pyogenes* were further evaluated for their capacity to inhibit biofilm formation. Methanol extracts of *Hydrocotyle javanica* (HJME), *Rhododendron arboreum* (RAME), *Holarrhena pubescens* (HPME)

and ethanol extract of *Mimosa diplotricha* (MDEE) are showing significant antimicrobial activity and also inhibiting the biofilm formation of *S. pyogenes,* below the level of their minimum inhibitory concentration. These plants are known for their ethnobotanical uses for the treatment of different types of ailments, including bacterial infections. The aqueous methanol (80%) extract of *H. javanica was* reported to have different biomolecules with antibacterial potential **[17]**. The flower, leaf, stem, and roots of *R. arboreum* are the ingredients of many traditional medicines. The Earlier reports suggest that methanolic crude extracts of this plant is having significant activity against *B. subtilus, S. typhi* and *S. aureus* etc. **[18]**. Siddiqui **[19]** reported the antimicrobial activity of the methanolic extract of the bark of *H. pubescens. M. diplotricha* is also reported to have antimicrobial properties **[20]**. Though these plant extracts are reported to have antimicrobial properties, the antibacterial activity against *Streptococcus pyogenes* has not been reported*,* except that of *R. arboreum* **[21]**. The antibiofilm potential of these plants, especially against *S. pyogenes* has not been explored so far. However, *H. pubescens is* reported to have antibiofilm activity against *Vibrio cholerae* and is effective in modulating its virulence factors **[22]**.

Inhibitory effects of a plant extract or biomolecules on biofilm formation are often assessed by their MBIC (minimum biofilm inhibitory concentration) values **[23]**. Based on the results obtained, it was evident that *H. javanica*, *R. arboreum, H. pubescens* and *M. diplotricha* significantly inhibited the biofilm formation of *S. pyogenes* in a concentration-dependent manner without inhibiting the bacterial growth. The essential oils from *Origanum vulgare* and *Salvia officinalis* showed an MBIC of 500 µg/mL against *S. pyogenes* **[24]**. The methanol extract of *Hemidesmus indicus was* also found to be active against *S. pyogenes* biofilm formation at 150 µg/mL of MBIC **[25]**. Our results also suggested that the plant extracts interfered during *S. pyogenes* biofilm formation at some stage rather than destroying them after formation. The results of the light microscopic analysis confirmed changes exerted by these plant extracts on microcolonies formation.

The bioactive molecules considered as promising anti-biofilm agents should not affect the metabolic activity of the pathogens. The AlamarBlue cell viability assays demonstrate cell viability and cytotoxicity using the efficiency of reducing resazurin dye by living cells. The minimum biofilm inhibition concentrations of the four plant extracts were subjected to AlamarBlue assay to assess their effect on

the viability of bacterial cells. From the results, it is evident that there is only a meager difference between the metabolically active cells of treated and untreated samples, suggesting that the selected plant extracts target the biofilm formation rather than acting as a bacteriostatic or bactericidal agent at their MBIC.

Mass spectroscopy coupled with gas chromatography (GC-MS) has an essential role in the phytochemical analysis of medicinal plants containing bioactive metabolites **[26]**. We have performed GC-MS analysis of the active extracts of the above plants to validate their bioactive potential. The GC-MS analysis revealed the presence of many biologically active compounds in them like ethanol extract of *H. javanica* (HJME) such as Caryophyllene, Quinic acid, (-)- Loliolide, Phytol, Methyl palmitate, Cyclohexyl cinnamate, Oxalic acid allyl tetradecyl ester etc. The GC-MS of methanol extract of *R. arboreum* (RAME) shows the presence of Capric acid vinyl ester, Nonadecane, derivatives of Quinic acid, derivatives of Phytol, Methyl stearate, derivatives of dipalmitin, Squalene, Glycerol trilaurate, and Lupeol etc. Methanol extract of *H. pubescens* (HPME) shows the presence of Phytol acetate, derivatives of phytol, derivatives of Fumaric acid, Megastigmatrienone, Gamma-hydroxyisoeugenol, 1-Octadecene, Methylpalmitate, Methyl stearate, and Myristic acid vinyl ester etc. The GC-MS analysis ethanol extract of *Mimosa diplotricha (*MDEE) reveals the presence of Mome inositol, Tricosanoic acid 2-methoxy- methyl ester, Octadecanoic acidethenyl ester and Palmitic acid vinyl ester. Anti-biofilm effect of β-caryophyllene on *Streptococcus mutans* **[27]**; Quinic acid on *Pseudomonas aeruginosa* **[28]**; Phytol in combination with cefotaxime on *Acinetobacter baumannii* **[29]** were reported earlier. Methanol extract of *Euphorbia hirta* displayed potent biofilm inhibition activity against *P. aeruginosa* (MBIC, 128 ug/mL) and GC-MS analysis shows the presence of compounds like phytol, linoleic acid, palmitic acid, squalene and megastigmatrienone **[30]**. The ethanolic extract of *Melia dubia* leaf contains Myristic acid methyl ester in GS-MS analysis and found effective in downregulating the transcriptional regulator, SdiAto controls the biofilm and virulence factors of uropathogenic *E. coli* **[31]**. Manickam **[32]** has reported that the crude fatty acids have inhibitory activity against the biofilm formation of *S. pyogenes*. Many compounds belonging to monounsaturated fatty aldehydes have been revealed in the GC-MS analysis. Moreover, the presence of sesquiterpenes and other

important bioactive compounds may also account for the anti-biofilm activity of the four plant extracts.

5. CONCLUSION

The present study disclosed the therapeutic value and the inhibitory potency of the four plant extracts, the methanol extracts of *Hydrocotyle javanica*, *Rhododendron arboreum*, *Holarrhena pubescens,* and ethanol extract of *Mimosa diplotricha* against *S. pyogenes.* These plantsare showing significant antimicrobial activity and also inhibiting the biofilm formation of *S. pyogenes,* below the level of their minimum inhibitory concentrations. The GC-MS analaysis revealed the presence of many known bioactive compounds, presumed to be regulating the biofilm formation by *S. pyogenes*.

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