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INHIBITORY POTENTIALS OF SELECTED MEDICINAL PLANTS AGAINST BIOFILM FORMING BACTERIA

***Florida Tilton, P. C., Kunthavai and Lakshmipriya, K.**

Biozone Research Technologies Pvt. Ltd., Chennai, India

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ABSTRACT

The current study was an attempt at elucidating the inhibitory potentials of certain plants against biofilm forming bacteria. Plants chosen for analysis were *Leucusaspera, Cassia alata, Cadabafruticosa, Carica papaya, Terminaliachebula, Barringtoniaacutangula, Wrightiatinctoria* and *Eupatorium triplinerve*. In this study biofilm forming bacteria were isolated from pipelines and water filter candles fromin and around Chennai. Biofilm forming bacteria was identified by Microtitre plate assay. Further confirmation of bacterial species was obtained by biochemical tests and 16S rRNA analysis. *Paenibacilluspolymyxa, Campylobacter jejuni* and *Escherichia coli* were three bacterial species which showed high biofilm forming ability. Extracts of *Cadabafruticosa, Barringtoniaacutangula, Terminaliachebula, Wrightiatinctoria* showed antibacterial effect against these bacteria.

INTRODUCTION

Microorganisms have a unique characteristic feature of forming clusters on some solid surfaces for it survival. Single species or multiple species can lead to formation of biofilm irrespective of the surface; biotic or abiotic respectively. Mixed species biofilm are prevalent (George *et al.,* 2000). Single species bio films were mostly found in medical implant devices (Archibald *et al.*, 1997 and Adal *et al.*, 1996). Certain environmental conditions like nutrient depletion causes bacteria to initiate Biofilm formation (Poulsen, L.V., 1999). Once the bacteria gets attached to the surface, they undergo changes to adapt themselves for life on the surfaces. Biofilm are held together by Exopolysacchride or Extra Polymeric substances (EPS) made up of polysaccharide, proteins and extracellular DNA (Jakubovics *et al.*, 2013). Main adherence factor that influences all the cellsto form a matrix is EPS. Such matrix can trap water, ions, High and low molecular weight mass molecules. Main constituent in biofilm formation is water which constitutes up to 97% (Zhang *et al*., 1998). Biofilm formation mainly depends on the substrate concentration. Three models have been proposed about biofilm structure such as heterogeneous structure of mosaics, ion Channel passage model and dense confluence model (Wimpenny and Colasanti, 1997). Bacteria adherence to the marine vessels attracts other microorganism to cause bio fouling, thus affecting the speed leading to increased fuel consumption. Biofilm formation is difficult to control and their occurrenceis prevalent in water areas mostly. Generally biofilm shows high antibiotic resistance (Mah and Toole, 2001). Alternative way for synthetic substances is natural substances obtained from plants which may have inhibitory effect on biofilm forming bacteria (Steinberg *et al.*, 1997). Natural compound obtained from plant extract has various activity like inhibition of peptidoglycan synthesis (Ogunlana, *et al.*, 1987), affects membrane structure (Cox, *et al.*,2000), modifies hydrophobicity of membrane surface (Turi *et al.*,1997) and quorum sensing of the bacteria(Gao *et al.*, 2003). In this study few plants were chosen namely *Leucusaspera, Cassia alata, Cadabafruticosa, Carica papaya, Terminaliachebula, Barringtoniaacutangula, Wrightiatinctoria, and Eupatorium triplinerve* and were studied for their anti-biofilm activity.

Corresponding author: Florida Tilton, P. C.,
Biozone Research Technologies Pvt. Ltd., Chennai, India

MATERIALS AND METHODS

Sample collection

Biofilm samples were collected from different placeslike Porur, Velachery, Avadi, Thiruvanmiyur and Ambatturin

Chennai. Collected samples were taken from pipelines, and water filter candles.

Isolation of bacteria

Bacterial species were isolated from the collected samples by serial dilution and agar plating method. The inoculated plates were incubated at 37°C for overnight. After incubation, the selected bacterial colonies with distinct morphology were picked. The isolated colonies were then restreaked onto nutrient agar plates to obtain single colony.

Microtitre Plate assay

Screening of biofilm forming organism was done bymicrotitre plate assay (Li *et al.*,2001 & Pratt *et al.*, 1998). The selected colonies were grown in Luria Bertani Media. Overnight grown cultures 200µl were then transferred to ninety six well microtitre plates and incubated at 27˚C for 24 hours without shaking. Absorbance was noted at 546 nm for overnight culture. Titre plates were washed twice with sterile distilled water. Plates was then stained with 0.01 % crystal violet for 30 minutes. Plates were washed with 95 % Ethanol then dried. To the dried plates Dimethyl Sulfoxide (DMSO) was added and absorbance was read at 546nm

Biochemical Analysis

Bacterial cultures showing positive results in microtitre plate assay were taken for further identification (Clarke *et al*., 1957; MacFaddin 2000; Wheelis, 2008 & Farmer JJ, *et al.*, 1987) for Gram staining, Catalase activity, oxidase activity, motility factor, nitrate and indole activity. Growth condition at 50˚C was also checked. Hydrolysis Activity for casein (Brown and Scott Foster, 1970) and starch (Bird and R. H. Hopkins, 1954) were studied. Carbohydrate fermentation test, Carbohydrates such as glucose, lactose, sucrose, mannitol, maltose, arabinose, xylose, trehalose and salicin (Barker HA. 1956) were studied.

***16S rRNA* Molecular Characterization**

The genomic DNA was extracted from the isolated strains using standard phenol: chloroform method (Sambrook *et al*.., 1989). 16S rRNA sequence was amplified using universal primers by PCR method. Amplification was carried out in a 20µl reaction setup containing 0.3µM of each primer, 0.2mM deoxynucleotide triphosphates, 100ng of template DNA sample and 1 U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for Thermal cycling reactions consisting of an initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (1 min at 94°C), annealing (45 s at 48°C), and extension (1 min at 72°C), with a final extension(10 min at72°C). The PCR product was purified (QIAquick PCR purification kit, Qiagen, Madrid, Spain) and analyzed by DNA sequencing (3730 DNA sequencing analyzer, ABI).

Preparation of Plant Extract

Healthy and fresh Leaves of *Leucusaspera, Cassia alata, Cadabafruticosa, Carica papaya, Terminaliachebula, Barringtoniaacutangula, Wrightiatinctoria,* and *Eupatorium triplinerve* were collected from Chennai. The leaves were dried without sunlight for a period of 15 days. Dried leaves were powdered and dissolved in respective solvents(Table 1). Extracts were filtered using What man no1 filter paper and dried in rotary evaporator under suitable pressure. Final product were utilized for further studies (Majorie, 1999).

Table 1. Plant extract preparation with different solvents

No.	Plant name	Part used	Solvent
1	*Leucusaspera*	Leaf	Ethyl acetate
2			Ethanol
3	*Cassia alata*	Leaf	Ethyl acetate
4			Ethanol
5	*Cadabafruticosa*	Leaf	Ethyl acetate
6			Ethanol
7	*Barringtoniaacutangula*	Leaf	Ethyl acetate
8			Ethanol
9	*Terminaliachebula*	Fruit	Ethyl acetate
10			Ethanol
11	*Carica papaya*	Leaf	Ethyl acetate
12			Ethanol
13	*Wrightiatinctoria*	Leaf	Ethyl acetate
14			Ethanol
15	*Eupatorium triplinerve*	Leaf	Ethyl acetate
16			Ethanol

Antibacterial activity

Plant Extracts were checked for their antibacterial activity against isolated organism. Mueller-Hinton agar plates were prepared (Aboaba *et al.*, 2006).Once the overgrown cultures were spread on to the plate, wells were punctured and plant extract approximately 50 µl were added. The plates were incubated and zone of inhibition was measured.

RESULTS AND DISCUSSION

Assesment of Biofilm formation (Microtitre Plate Assay)

Microtitre plates Assay were performed from the Biofilm forming bacterial isolates. Biofilm forming activity was determined the Optical Density value is greater than 0.6.Out of ten Isolates only three isolates shown high absorbance value Greater than 0.6 OD were considered as high Biofilm forming activity (Table 2). These isolates were then identified using biochemical characterization.

Table 2. Biofilm forming activity checked at 546 nm

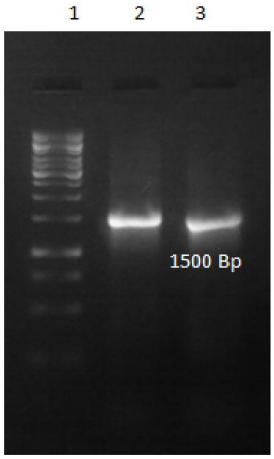
S. No.	Sample Name	Absorbance	Biofilm intensity
1	A	0.589	Low
2	B	0.579	Low
3	C	0.215	Low
4	D	0.648	High
5	E	0.517	Low
6	F	0.847	High
7	G	0.314	Low
8	H	0.453	Low
9	I	0.641	High
10	J	0.183	Low

Biochemical Characterization

Isolates which showed high Biofilm forming activity were taken for Biochemical analysis. Morphological, Fermentation activity, Hydrolysis activity, Growth conditions were checked. Table 3summarises the result for bacterial identification. Three bacterial isolates were identified as *Paenibacilluspolymyxa, Campylobacter jejuni*and*Escherichia coli*.

Table 3. Bacterial identification

S. No.	Isolate	Organism
1	D	*Paenibacilluspolymyxa*
2	F	*Campylobacter jejuni*
3	I	*Escherichia coli*



Lane 1- 1kb Ladder

Lane2&3- 16S rRNA gene (1500bp)

Figure 1. 16S rRNA gene isolated from bacterial species *Campylobacter jejuni*

extract. *Barringtoniaacutangula* ethyl acetate and ethanol extracts was found to inhibit *Campylobacter jejuni. Terminaliachebula* and *Cadabafruticosa* ethanol extract showed activity against *Paenibacilluspolymyxa and Escherichia coli. Wrightiatinctoria* ethanol extract showed activity against *Campylobacter jejuni.* The results were tabulated in Table 4.

Conclusion

Biofilm formation leads to biofouling i.e accumulation of microorganism like fungus, marine sea weeds, phytoplankton in areas exposed to water. Accumulation of such microbes leads to corrosion of the system affecting its efficiency. Many synthetic antifouling agents used so far were less efficient. Natural compounds from various plants have ability to inhibit biofilm formation. In this study out of eight plants *Cadabafruticosa, Barringtoniaacutangula, Terminaliachebula and Wrightiatinctoria* were found to inhibit biofilm forming *Paenibacilluspolymyxa, Campylobacter jejuni* and *Escherichia coli*. These plant extract inhibit biofilm formation at concentration of 3µg/µl. Isolation and characterization of active compounds, which exhibits inhibition of biofilm formation might be a used as effective alternative in biofilm control.

Table 4. Antimicrobial effect of plant extract against Bacterial isolates

No.	Plant name	Solvent	*Campylobacter jejuni*	*Paenibacilluspolymyxa*	*Escherichia coli*
1	*Leucusaspera*	Ethyl acetate	R	R	R
2		Ethanol	R	R	R
3	*Cassia alata*	Ethyl acetate	8	R	R
4		Ethanol	R	R	R
5	*Cadabafruticosa*	Ethyl acetate	R	R	R
6		Ethanol	12	14	16
7	*Barringtoniaacutangula*	Ethyl acetate	24	R	R
8		Ethanol	10	R	R
9	*Terminaliachebula*	Ethyl acetate	R	R	R
10		Ethanol	12	14	14
11	*Carica papaya*	Ethyl acetate	R	R	R
12		Ethanol	R	R	R
13	*Wrightiatinctoria*	Ethyl acetate	R	R	R
14		Ethanol	8	R	R
15	*Eupatorium triplinerve*	Ethyl acetate	R	R	R
16		Ethanol	R	R	R

(Note: Numerical value in the above table indicates the size of zone of incubation in mm)

Molecular Characterization

Further confirmation of bacterial species using 16S rRNA analysis was carried out. 16S rRNA gene is most widely used in identifying bacterial Phylogeny. Gene specific primers were used to amplify the gene. Amplified 16S rRNA gene was screened using 1 Kilobaseladder as marker (Figure 1). Obtained PCR product was purified and sequenced. The obtained 16S rRNA sequence was subjected to BLAST analysis and the results showed 99% similarity for *Campylobacter jejuni* species.

Antibacterial Activity test

The susceptibility of biofilm forming bacteria to plant extracts was studied. Hence their anti-bacterial activity against *Campylobacter jejuni, Escherichia coli* and *Paenibacilluspolymyxa* was analysed. Plant extraction was done sequentially with two solvents for each plant. Clear Zone of inhibition was obtained for *Cadabafruticosa* ethanol

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