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Assessment of Antibiofilm Activity of Extracts from Selected Biological Sources

Deepa Madathil* and N. Rajendran

School of Bio Sciences and Technology, VIT University, Vellore, Tamilnadu - 632014, India.

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An environmental biofilm can be defined as a surface-attached microbial community growing embedded in a self-produced matrix of extracellular polymeric substances. Since biofilm producing bacteria are resistant to conventional therapy, there has been an increased interest in the valuable properties of medicinal plants and natural compounds which have been established to possess antibiofilm activities. The present study analyses the effectiveness of different bioactive compounds from Indian medicinal plant extracts and seaweeds in inhibiting the formation of these biofilms. Phytochemical analysis of plant samples was performed in order to identify the phyto-constituents responsible for antibacterial activity. Antibacterial activity of plant extracts were estimated by measuring the zone of inhibition produced. The results of antimicrobial study indicated that the extracts of the common plants *Cadaba fruticosa, Cassia alata, Terminalia chebula, Acalypha indica* and medicinal plants *Cuminum cyminum, Murraya koenigii* and *Mentha longifolia* possessed significant ability to inhibit the biofilm formation by five of the eight isolated strains. The molecular characterisation of the biofilm forming bacteria was performed and the 16S rRNA sequences were submitted to the GenBank. These results will be useful for the prevention of biofilm formation in the environment, thereby minimizing the environmental impact of persistent chemicals.

Key words: Environmental biofilms, Inhibition, 16S rRNA, BLAST, Phylogenetic analysis**.**

Bacterial biofilms are formed when unicellular organisms come together to form a community that is attached to a solid surface and encased in an exopolysaccharide matrix. It has been suggested that this matrix, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community (Mah *et al.*, 2001). The colonization of the biofilm bacteria corresponds to the occurrence of microorganisms in a particular environment. Colonization of biotic or abiotic surfaces results from two relatively distinct microbiological processes that is bacterial adhesion or biofilm formation. The initial bacterial adhesion can either be reversible or irreversible. Largely, a biofilm is defined as the sessile development of microbial cells. The engagement into a sessile mode of growth arises subsequent to the irreversible bacterial adhesion (Zhao *et al*., 2013); though, not all single adhered bacterial cells essentially engage into sessile development. Considering the differences in the molecular physiology of the bacterial cells, the distinction between single adhered and biofilm cells is of importance (Buncic *et al*., 2013). Biofilms have been known to cause accelerated deterioration of buildings, ships, pipes etc. This has led to extensive economic losses and chemical antifouling agents cause serious environmental damage.

As biofilms offer resistance to many antibiotics, the discovery of novel agents which could prevent their formation or adherence would be of great use. Biofilms growing in natural and

* To whom all correspondence should be addressed.
E-mail: deepa.m@vit.ac.in

industrial environments are resistant to bacteriophage, to amoebae, and to the chemically diverse biocides used to combat biofouling in industrial processes (Costerton *et al.*, 1999). Development of bacterial resistance to synergistic antibiotic combinations, such as those found in plants, may be slower than for single drug therapies (Quave *et al.*, 2008). In recent years, much of the research has been focused to identify alternative medicines to treat the infections caused by the drug resistant organisms. Various chemicals have been tested for their antibiofilm activities. Unfortunately, those chemicals cannot be used as drug molecules to treat the diseases associated with the biofilm (Cowan *et al.*, 1999). Furthermore, the environmental impacts of these chemicals are needed to be assessed. Hence, the alternative to the chemical antibiofilm agents is a natural source (Carneiro *et al.*, 2011). Plant derived molecules have found potential applications in pharmaceutical industry.

The current research comprises of the isolation and molecular characterisation of the bacterial strains isolated from the biofilms present in the natural environment. The present study was also involved in the determination of the phyto-constituents and antibacterial effects of the 17 different plants *Croton bonplandianus, Cassia fistula, Cadaba fruticosa, Cassia alata, Terminalia chebula, Gelidiella aerosa, Justicia gendarussa, Acalypha indica* (common plants) *Trigonella foenum-graecum, Azadirachta indica, Ocimum sanctum, Anethum graveolens, Phyllanthus emblica, Cuminum cyminum, Murraya koenigii, Mentha longifolia* and *Curcuma longa* (medicinal plants) and one seaweed *Gracilaria edulis.* Since these medicinal plants and seaweeds are known to possess antimicrobial properties against pathogens, they were selected to be tested against the biofilm forming bacteria from the environment.

MATERIALS AND METHODS

Sample collection

Biofilm samples were collected from the surfaces of concrete walls, boats and wooden structures in Besant Nagar, Pattinapakkam, Kasimedu and Ice house regions of Chennai, Tamil Nadu, India. The 16 samples thus obtained were incubated for 24 hours in nutrient broth. The cultures were serially diluted and plated using spread plate method and incubated overnight at 37°C. In order to obtain pure cultures, individual colonies were chosen and streak plated. The cultures were screened for their ability to form biofilms using crystal violet assay.

Biofilm forming ability

The biofilm forming ability of the bacterial strains was estimated using the crystal violet assay (Tendolkar *et al.*, 2004). 0.2ml of cultures grown overnight were transferred to a microplate and incubated at 37°C for 24 hours for complete growth of bacteria and biofilm formation. The medium used for cell suspension was removed and the cells adhered to the microplate were washed twice with phosphate buffer (pH 7.4), and the phosphate buffer was then drained. The surface- attached cells were fixed with 95% ethanol and stained with 1% crystal violet for 5 minutes. The excess stain was removed and the absorbance of the microplate was measured at 564nm. Bacterial strains with high absorbance at 564nm were considered as good biofilm forming bacteria. They were further chosen for microbial and biochemical characterization.

Biochemical identification of organisms

The biofilm forming organisms from the results of microtitre plate assay were taken for further identification (MacFaddin, 2000). Gram staining, catalase activity, oxidase activity, motility factor, nitrate and indole activities were done for the selected organisms. Growth condition at 50°C was also checked. Hydrolysis activity for casein (Brown and Foster, 1970) and starch (Bird and Hopkins, 1954) were studied. Carbohydrate fermentation test, carbohydrates such as glucose, lactose, sucrose, mannitol, maltose, arabinose, xylose, trehalose and salicin (Barker, 1956) were also analysed.

16S rRNA – based identification

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, forward primer (518F) CCAGCAGCCGCGGTAATACG, reverse primer (800R) TACCAGGGTATCTAATCC 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 at 72°C). The PCR product was purified (QIAquick PCR purification kit, Qiagen, Madrid, Spain) and analyzed by DNA sequencing (3730 DNA sequencing analyzer, ABI).

Collection and identification of plant samples

Plant samples of various Indian medicinal plants were collected from Chennai, Tamil Nadu and were identified. The samples were taken for extraction using ethanol, methanol, chloroform, ethyl acetate and water as listed in Table 1.

Phytochemical analysis

The 36 extracts from 17 plants and one seaweed were screened for the presence of various phytoconstituents. Tests for the presence of

Table 1. List of medicinal plants, their respective parts used and solvents taken for extraction

Extract No.	Plant name	Part used	Solvent
1	*Croton bonplandianus*	Leaf	Ethyl Acetate Methanol
2	*Cassia fistula*	Leaf	Ethyl Acetate Methanol
3	*Cadaba fruticosa*	Leaf	Ethyl Acetate Methanol
4	*Cassia alata*	Leaf	Chloroform Ethanol
5	*Terminalia chebula*	Fruit	Water Methanol
6	*Trigonella foenum-graecum*	Leaf	Ethyl Acetate Ethanol
7	*Gelidiella aerosa*	Leaf	Ethyl Acetate Ethanol
8	*Gracilaria edulis*	Leaf	Ethyl Acetate Ethanol
9	*Azadirachta indica*	Leaf	Ethyl Acetate Ethanol
10	*Ocimum sanctum*	Leaf	Ethyl Acetate Ethanol
11	*Justicia gendarussa*	Leaf	Ethyl Acetate Ethanol
12	*Acalypha indica*	Leaf	Ethyl Acetate Ethanol
13	*Anethum graveolens*	Seeds	Ethyl Acetate Ethanol
14	*Phyllanthus emblica*	Leaf	Ethyl Acetate Ethanol
15	*Cuminum cyminum*	Leaf	Ethyl Acetate Ethanol
16	*Murraya koenigii*	Leaf	Ethyl Acetate Ethanol
17	*Mentha longifolia*	Leaf	Ethyl Acetate Ethanol
18	*Curcuma longa*	Fruit	Ethyl Acetate Ethanol

carbohydrates (Sofowora, 1993), tannins (Harborne, 1993), saponins (Smolenski *et al.*, 1969), flavonoids, phlobatannins, terpenoids, anthraquinones, cardiac glycosides (Sonali *et al.*, 2010), alkaloids (Ayoolal *et al.*, 2010), quinones, phenols, coumarins (Suresh Kumar *et al.*, 2009), glycosides, proteins (Manasboxi *et al.*, 2010), steroids and phytosteroids (Kolawole *et al.*, 2006) were performed as described.

Antibacterial activity against strains

The plant samples were collected shade dried and powdered. The powdered samples (100gm) were extracted three times by cold percolation method with 300 ml of ethyl acetate or ethanol at room temperature for 72hrs. The filtrates were concentrated under reduced pressure at 40ºC and stored in refrigerator at 2-8ºC for use in subsequent experiments. Antibacterial activity of all extracts was determined against the selected biofilm forming bacteria using Kirby-Bauer disc diffusion method (Bauer, et al.,1966). The organisms isolated from the biofilms were spread on the test plates - Mueller Hinton agar using sterile swabs. Sterile wells were made with the help of a sterile cork borer in aseptic conditions. Samples (2mg) were added to the wells. Stock solutions of the extracts were prepared using DMSO (Dimethyl sulphoxide). 1mg of the crude extract was dissolved in 1ml of 10% DMSO and used for analysing the anti-bacterial properties. The test plates were incubated for 24 hours. The zone of inhibition (in mm diameter) was measured and it was considered as the activity of the extract against the test organisms

RESULTS AND DISCUSSION

Biofilm forming ability and biochemical identification of bacterial strains

The samples of bacteria collected from boats, concrete walls and wooden structures from Besant Nagar, Pattinapakkam, Kasimedu and Ice house in Chennai, Tamil Nadu, formed isolated colonies at dilutions between 10^{-3} to 10^{-7}. The isolated bacteria were then analysed further. The absorbance of the biofilm forming bacterial samples was shown in Table 2. The bacterial colonies which showed high absorbance were classified biochemically and listed in Table 3. Those bacterial strains with high absorbance at 564 nm were considered for microbial and biochemical characterization to identify the genus and species of the isolated bacteria as shown in Table 4a, 4b & 4c.

Molecular characterisation and phylogenetic analysis

The 16S rRNA sequences obtained from the eight biofilm cultures were analysed for the presence of similar sequences in GenBank using the BLAST algorithm. The eight sequences obtained from the PCR amplification were submitted to the GenBank database with the following accession numbers Biofilm A - KF234563.1 – *Bacillus subtilis* strain DR1 16S ribosomal RNA gene, partial, Biofilm B - KF234564.1 – Bacterium DR2 (2014) strain 16S ribosomal RNA gene, partial, Biofilm D - KF234565.1 – *Bacillus cereus* strain

Table 2. Determination of biofilm intensity measured at 564nm

S. No.	Bacterial strain	Absorbance	Biofilm intensity
1	A	0.589	High
2	B	0.679	High
3	C	0.215	Low
4	D	0.548	High
5	E	0.617	High
6	F	0.847	High
7	G	0.314	Low
8	H	0.753	High
9	I	0.641	High
10	J	0.912	High
11	K	0.183	Low
12	L	0.149	Low
13	M	0.220	Low
14	N	0.271	Low
15	O	0.271	Low
16	P	0.256	Low

Table 3. Biofilm forming bacterial strains

Serial No.	Biofilm ID	Organism
1	Biofilm A	*Bacillus subtilis*
2	Biofilm B	*Brevibacillus brevis*
3	Biofilm D	*Bacillus cereus*
4	Biofilm E	*Bacillus subtilis*
5	Biofilm F	*Bacillus spaericus*
6	Biofilm H	*Bacillus spaericus*
7	Biofilm I	*Brevibacillus brevis*
8	Biofilm J	*Bacillus circulans*

DR3 16S ribosomal RNA gene, partial, Biofilm E - KF234566.1 – *Bacillus subtilis* strain DR4 16S ribosomal RNA gene, partial, Biofilm F - KF234567.1 – Bacterium DR5 16S ribosomal RNA gene, partial, Biofilm H - KF686736.1 – *Lysinibacillus sphaericus* strain DR6 16S ribosomal RNA gene, partial, Biofilm I - KF686737.1 – Bacterium DR7 16S ribosomal RNA gene, partial, Biofilm J - KF686738.1 – *Bacillus* sp. DR8 16S ribosomal RNA gene, partial. An attempt was made to study the phylogenetic relationship among these and other 16S rRNA sequences of bacteria forming environmental biofilms like *Bacillus thuringiensis, Halobacillus* sp., *Viridibacillus arenosi, Bacillus tequilaensis, Bacillus amyloliquefaciens* etc. as shown in Fig. 1.

Phylogenetic analysis

A comparison of the 16S rRNA gene sequences with the non-redundant collection (GenBank, DDBJ, EMBL and PDB) of sequences was performed using BLAST (Zhang, 2000). A number of sequences of the genus *Bacillus* were aligned with the 16S rRNA gene sequences with greater than 90% sequence similarity. A multiple sequence alignment for these homologous sequences was performed using the algorithm described in ClustalW (Thompson, 1994). An evolutionary distance matrix was generated from these nucleotide sequences in the dataset. Further a phylogenetic tree was then drawn using the Neighbour joining method (Saitou, 1987). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 6.06 (Tamura, 2007). It is evident from phylogenetic analysis of the 16S rRNA sequences that the isolates represent a strain in genera *Bacillus*.

Phytochemical analysis

Qualitative tests were done to assess the presence of various phytochemicals in the 36

Table 4a. Morphology and biochemical tests for the biofilm A, B & D

Strain Test	Biofilm A	Biofilm B	Biofilm D
Growth on NA	White opaque, 2-3 mm colony	White opaque, 2-3 mm colony	White opaque, 2-3 mm colony
Gram	GPR	GPR	GPR
Catalase	Postive	Postive	Postive
Oxidase	Negative	Negative	Negative
Motility	Motile	Motile	Motile
Nitrate	Postive	Postive	Postive
Indole	Negative	Negative	Negative
Cell diameter	0.8	0.9	1.3
Chain of cell	-	-	+
Spore shape	Elliptical	Elliptical	Elliptical
Spore position	Central	Central	Central
Sporangium	Not swollen	Swollen	Not swollen
Parabasal crystals	Neg	Neg	Neg
Growth at 50°C	Neg	Neg	Neg
Egg yolk reaction	-	+	+
Casein hydrolysis	+	+	+
Starch hydrolysis	+		+
Arginine dihydrolase	-		
Glucose			
Lactose		-	-
Sucrose		+	
Mannit		Acid no gas	Acid no gas
Maltose		Acid no gas	Acid no gas
Arabin			
Xylose			
Trehal		Negative	
Salicin	*Bacillus subtilis*	*Brevibacillus brevis*	*Bacillus cereus*

extracts of the 17 medicinal and common plants and one seaweed collected for the study. The summary of the phytochemicals present in plants as per the different extracts were presented in Table 5a, 5b & 5c. The results indicated that carbohydrates, tannins, flavonoids, phenols and coumarins were strongly present in the ethyl acetate and methanol extracts of *Croton bonplandianus*, whereas the ethyl acetate and methanol extracts of *Cassia fistula* had higher proportions of carbohydrates, tannins, phenols and coumarins. Carbohydrates, flavonoids, quinones and terpenoids were highly present in the chloroform and methanol extracts of *Cadaba fruticosa*. Tannins, flavonoids, phenols and coumarins were predominantly present in the chloroform and ethanol extracts of *Cassia alata*. Carbohydrates, tannins and phenols were present in the methanol and aqueous extracts of *Terminalia chebula*.

The ethyl acetate and ethanol extracts of *Gelidiella aerosa* had strong presence of carbohydrates, tannins, flavonoids, phenols and coumarins. Carbohydrates, tannins, flavonoids and phenols were present in the ethyl acetate and ethanol extracts of *Gracilaria edulis*. Ethyl acetate and ethanol extracts of *Justicia gendarussa* contained tannins and alkoloids. Carbohydrate, flavonoid, alkaloid and phenols were present in the ethyl acetate and ethanol extracts of *Acalypha indica*. Carbohydrates, tannins, flavonoids, phenols and coumarins were predominantly present in ethyl acetate and ethanol extracts of *Trigonella foenum-graecum*, *Anethum graveolens* and *Azadirachta indica*. Saponins, flavonoids, alkaloids, phenols and coumarins were present in the ethyl acetate and ethanol extracts of *Ocimum sanctum*. The extracts of *Cuminum cyminum*, *Murraya koenigii*, *Mentha longifolia*, *Phyllanthus emblica* and *Curcuma longa* failed

Table 4b. Morphology and biochemical tests for the biofilm E, F & H

Strain Test	Biofilm E	Biofilm F	Biofilm H
Growth on NA	White opaque, 2-3 mm colony	White opaque, 2-3 mm colony	White opaque, 2-3 mm colony
Gram	GPR	GPR	GPR
Catalase	Postive	Postive	Postive
Oxidase	Negative	Negative	Negative
Motility	Motile	Motile	Motile
Nitrate	Postive	Postive	Postive
Indole	Negative	Negative	Negative
Cell diameter	0.8	1.0	1.0
Chain of cell	-	-	-
Spore shape	Elliptical	Elliptical	Elliptical
Spore position	Central	Subterminal	Subterminal
Sporangium	Not swollen	Swollen	Swollen
Parabasal crystals	Neg	Neg	Neg
Growth at 50°C	Neg	Neg	Neg
Egg yolk reaction	-	+	+
Casein hydrolysis	+	_	_
Starch hydrolysis	+		
Arginine dihydrolase	-		
Glucose			
Lactose			
Sucrose		Neg	Neg
Mannit			
Maltose			
Arabin			
Xylose		Neg	Neg
Trehal			
Salicin	*Bacillus subtilis*	*Bacillus spaericus*	*Bacillus spaericus*

to show the presence of any of the photochemical.

There are several phytochemicals present in these plant samples and both direct and indirect mechanisms are probably involved in the inhibition of bacterial biofilms (Vettam *et al*, 2007). The phytochemicals present in extracts which show anti-biofilm forming properties should be analysed further in order to pinpoint the phytochemical responsible for this property. It is widely acknowledged that plant extracts with alkaloids, flavonoids and phenols, called phytoalexins, have antimicrobial properties (Rauha *et al*, 2000). Plant volatile oils containing terpenoids have also known to promote the antibacterial characteristics of plant

Table 4c. Morphology and biochemical tests for the biofilm I & J

Strain Test	Biofilm I	Biofilm J
Growth on NA	White opaque, 2-3 mm colony	Dry flat spreading opaque colonies, 2-3mm
Gram	GPR	Gram positive rods
Catalase	Postive	Positive
Oxidase	Negative	Negative
Motility	Motile	Motile
Nitrate	Postive	Negative
Indole	Negative	Negative
Cell diameter	0.9	0.8 micron
Chain of cell	-	Positive
Spore shape	Elliptical	Elliptical
Spore position	Central	Sub terminal
Sporangium	Swollen	Swollen
Parabasal crystals	Neg	Negative
Growth at 50°C	Neg	Negative
Egg yolk reaction	+	
Casein hydrolysis	+	
Starch hydrolysis		
Arginine dihydrolase		
Glucose		Positive
Lactose	-	
Sucrose		
Mannit	Acid no gas	Positive
Maltose	Acid no gas	
Arabin		Negative
Xylose		
Trehal	Negative	Positive
Salicin		Positive
	B.brevis	*B.circulans*

Table 5a. Summary of the phyto-constituents

Phytochemical test	*C.b.*		*C.f.*		*Ca.f.*		*C.a.*		*T.c.*		*T.g.*	
Solvent	EA	M	EA	M	EA	M	C	ET	W	M	EA	ET
Carbohydrates	++	++	+	+	++	++	-	++	++	++	+	+
Tannins	+	++	+	++	-	++	++	++	++	+	+	++
Saponins	-	+	-	-	-	-	-	+	-	-	-	+
Flavonoids	+	++	-	++	++	++	++	++	++	-	+	++
Alkaloids	++	++	++	++	-	++	++	-	-	+	++	++
Quinones	-	-	-	-	++	++	-	++	++	-	-	++
Glycosides	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	++	-	-	-	-	-	-	-	-	-	+	-
Terpenoids	-	++	-	-	++	++	-	++	-	+	-	-
Phenols	++	++	+	+	-	++	+	++	++	-	+	++
Coumarins	++	++	++	++	-	-	++	++	++	-	+	++
Steroids and Phytosteroids	-	-	-	-	-	-	-	-	-	-	-	-
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-

C.b. - *Croton bonplandianus*, C.f. - *Cassia fistula*, Ca.f. - *Cadaba fruticosa*, C.a. - *Cassia alata*, T.c. - *Terminalia chebula* and T.g. - *Trigonella foenum-graecum*.
E.A-Ethyl acetate, ET-Ethanol, M-Methanol, W-Water and C-Chloroform. [++ suggests strongly present, + present and – absent].

extracts (Dorman and Deans, 2000). The likelihood that plant extracts containing these phyto-constituents would be effective antibacterial agents.

Antibacterial activity of plant samples

Universal spices like clove, cinnamon, bishop's weed, chilli, horse radish, cumin, tamarind, black cumin, pomegranate seeds, nutmeg, garlic, onion, tejpat, celery, cambodge, have been recorded to have potent antimicrobial activities against a range of pathogens such as *B. subtilis*, *B. cereus*, *Listeria monocytogenes*, *Salmonella anatum*, *Escherichia coli* and *Saccharomyces cerevisiae* (Bindu Sadanandan *et al.*, 2014). The traditional

Table 5b. Summary of the phyto-constituents

Phytochemical test	*G.a.*		*G.e.*		*A.i.*		*O.s.*		*J.g.*		*Ac.i.*	
Solvent	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET
Carbohydrates	+	++	++	+	+	+	-	-				
Tannins	+	++	+	+	++	+	-	-	-	-	+	+
Saponins	-	+	-	+	-	+	+	+	++	+	-	-
Flavonoids	++	+	+	-	++	++	++	++	-	-	-	-
Alkaloids	++	+	++	+	++	++	++	++	-	+	+	++
Quinones	++	+	++	++	++	+	-	-	++	++	++	++
Glycosides	-	-	-	-	-	-	-	-	+	-	-	-
Cardiac glycosides	-	-	-	-	++	-	-	-	-	-	-	-
Terpenoids	-	-	-	-	++	-	-	-	-	-	-	-
Phenols	+	+	+	+	+	++	++	++	+	-	-	-
Coumarins	++	+	+	-	-	++	++	++	-	+	++	+
Steroids and Phytosteroids	-	-	-	-	-	-	-	-	-	+	-	++
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-

G.a. - *Gelidiella aerosa*, G.e. - *Gracilaria edulis*, A.i. - *Azadirachta indica*, O.s. - *Ocimum sanctum*, J.g. - *Justicia gendarussa* and Ac.*i.* - *Acalypha indica.*
EA-Ethyl acetate and ET-Ethanol. [++ suggests strongly present, + present and – absent].

Table 5c. Summary of the phyto-constituents

Phytochemical test	*A.g*		*P.e.*		*C.c.*		*M.k.*		*M.l.*		*C.l.*	
Solvent	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET
Carbohydrates	++	++	-	-	-	-	-	-	-	-	+	-
Tannins	+	++	-	+	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	+	-	-	-	-	-	-	-
Flavonoids	+	++	-	-	-	+	-	+	-	+	+	+
Alkaloids	-	-	-	-	-	+	-	+	+	-	-	-
Quinones	+	++	-	-	-	-	-	-	-	-	+	+
Glycosides	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	-	-	+	-	+	+	+	-	+	-	-	+
Terpenoids	-	-	-	-	-	-	+	-	-	-	+	-
Phenols	+	++	-	+	-	-	-	-	+	-	+	-
Coumarins	-	++	-	-	-	+	-	+	-	+	-	+
Steroids and Phytosteroids	-	-	-	-	+	-	+	-	-	-	-	-
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	+

A.g. - *Anethum graveolens*, P.e. - *Phyllanthus emblica*, C.c. - *Cuminum cyminum*, M.k. - *Murraya koenigii*, M.l. - *Mentha longifolia* and C.l. - *Curcuma longa*.
EA-Ethyl acetate and ET-Ethanol[++ suggests strongly present, + present and - absent].

Table 6a. Antibacterial activity of selected plants

Organism/	*C.b.*		*C. f.*		*Ca. f.*		*C.a.*		*T.c.*		*T.g.*	
extract	EA	M	EA	M	EA	M	C	ET	W	M	EA	ET
A	R	R	R	12	R	12	20	10	R	R	R	R
B	R	R	10	14	R	R	18	R	R	R	R	R
D	R	R	8	R	R	14	R	R	R	14	R	R
E	R	R	R	10	8	12	19	8	R	12	R	R
F	R	R	R	R	R	12	24	10	R	12	R	R
H	R	8	R	R	R	14	R	R	R	18	8	R
I	R	R	8	13	R	R	19	R	R	R	R	R
J	R	R	R	R	R	14	8	R	R	12	R	R

C.b. - *Croton bonplandianus,* C.f. - *Cassia fistula,* Ca.f. - *Cadaba fruticosa,* C.a. - *Cassia alata,* T.c. - *Terminalia chebula* andT.g. - *Trigonella foenum-graecum*. EA-Ethyl acetate, ET-Ethanol, M-Methanol, W-Water and C-Chloroform.*R* – Resistance 'absence of zone'. Inhibition zone were measured in mm.

Table 6b. Antibacterial activity of selected plants

Organism/	*G.a.*		*G.e.*		*A.i.*		*O.s.*		*J.g.*		*Ac.i.*	
extract	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET
A	R	R	R	R	8	10	R	R	R	R	8	10
B	R	R	R	R	R	8	R	R	R	R	R	R
D	R	R	R	R	R	R	R	R	R	R	R	6
E	R	8	R	R	8	10	R	R	R	R	11	10
F	R	8	R	R	8	R	R	R	R	R	10	11
H	R	8	R	R	8	R	R	R	R	R	10	10
I	R	R	R	R	R	R	R	R	R	R	R	R
J	R	R	R	R	R	10	R	R	R	R	R	8

G.a. - *Gelidiella aerosa,* G.e. - *Gracilaria edulis,* A.i. - *Azadirachta indica,* O.s. - *Ocimum sanctum,* J.g. - *Justicia* gendarussa and Ac.*i.* - *Acalypha indica.* EA-Ethyl acetate and ET-Ethanol.
R – Resistance 'absence of zone'. Inhibition zone were measured in mm.

Table 6c. Antibacterial activity of selected plants

Organism/	*A.g.*		*P.e.*		*C.c.*		*M.k.*		*M.l.*		*C.l.*	
extract	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET	EA	ET
A	R	R	R	R	R	R	R	R	R	R	R	R
B	R	R	12	R	8	R	10	R	10	14	8	R
D	R	R	R	R	R	8	8	R	R	12	R	R
E	R	R	R	10	R	8	8	8	R	8	R	R
F	R	R	R	R	R	12	R	R	R	R	R	R
H	R	R	R	R	R	12	R	R	R	R	R	R
I	R	R	12	R	8	R	10	R	10	12	R	R
J	R	R	12	12	8	8	8	8	7	10	R	R

A.g. - *Anethum graveolens,* P.e. - *Phyllanthus emblica,* C.c. - *Cuminum cyminum*, M.k. - *Murraya koenigii,* M.l. – *Mentha longifolia* and C.l. - *Curcuma longa.* EA-Ethyl acetate and ET-Ethanol.
R – Resistance 'absence of zone'. Inhibition zone were measured in mm.

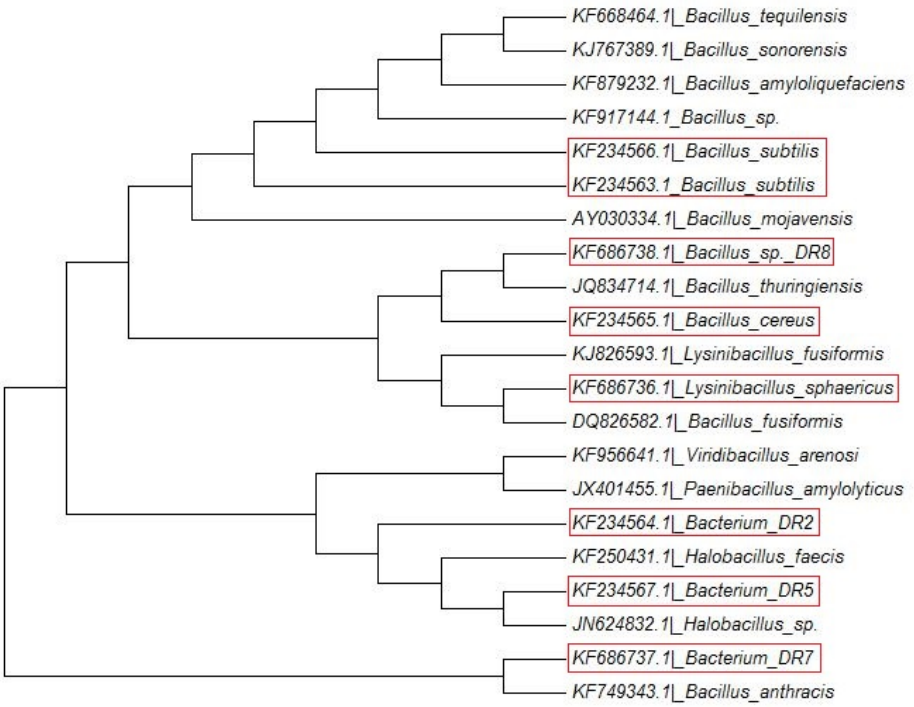

Fig. 1. Phylogenetic tree of the biofilm forming sequences with the similar sequences from BLAST (The species identified in the present study were highlighted in the boxes).

use of spices as food preservatives, disinfectants and antiseptics has been well established (De *et al.*, 1999). Aqueous extracts of garlic also has antibacterial activity against various bacteria (Khusro *et al.*, 2013).

The antibacterial activities of the selected plant extracts were measured using the well diffusion method and the results were tabulated (Table 6a, 6b & 6c). The plant extract that forms a zone of inhibition with the largest diameter is considered as the best antimicrobial agent. In this study, the plant extracts were tested against the biofilm forming bacteria isolated from the environment.

Chloroform extracts of *Cassia alata* had the maximum antibacterial activity against the biofilms forming *Bacillus subtilis*. This plant sample was also effective against *Brevibacillus brevi* and *Bacillus spaericus*. *Cassia fistula* and *Cadaba fruticosa* plant extracts in methanol showed significant inhibition of *Bacillus cereus* and *Brevibacillus brevi* biofilms respectively. The ethanol extracts of *Mentha longifolia* showed significant inhibition of *Brevibacillus brevis* and *Bacillus cereus*. The results showed that *Cassia alata* efficiently prevented biofilm formation and can be a potent antimicrobial agent against biofilm forming bacteria with an inhibition zone of up to 24mm in diameter. Also the extracts of the common plants *Cadaba fruticosa, Terminalia chebula, Acalypha indica* and medicinal plants like *Cuminum cyminum, Murraya koenigii* and *Mentha longifolia* possessed properties to significantly inhibit the biofilm formation. From the selected plants for the study, it was evident that only few extracts exhibited good antimicrobial activity owing to the resistance posed by the biofilm forming bacteria.

CONCLUSION

Biofilms have been observed on a variety of surfaces and in a variety of niches, and are considered to be the prevailing microbial lifestyle in most environments. A particular characteristic of biofilms is their extreme tolerance to either to

antimicrobial treatment or preventive measures. An alternative method for detecting the anti-biofilm forming agents is from the natural source like medicinal plants and it has gained a lot importance due to the recent development in the field of biotechnology. The present study made an attempt to know the potential effect of 17 plants and 1 seaweed, their phytoconstituents and antimicrobial activity against the organisms isolated from the environmental biofilms. The molecular characterisation of the biofilm extracts was performed and the 16S rRNA sequences were submitted to the GenBank public database. The antimicrobial study revealed that the extracts of the common plants *Cadaba fruticosa, Cassia alata, Terminalia chebula, Acalypha indica* and medicinal plants like *Cuminum cyminum, Murraya koenigii* and *Mentha longifolia* possessed properties to significantly inhibit the biofilm formation. The extracts of the seaweed *Gracilaria edulis* did not show any inhibitory effect on the biofilm formation. This differential effect may be specific to the solvent system used or possibly the concentration of the target bioactive compounds may not be sufficient to inhibit the growth of biofilm formation. This study can be extended to evaluate in particular the active components of the plant extracts and to identify the most active components by using GC-MS and FT-IR and to detect the potential anti biofilm compounds.

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REFERENCES

1. Ayoola, G.A., Coker, H.A.B., Adesegun, S.A., Adepoju-Bello, A.A., Obaweya, K., Ezennia, E.C., Atangbayila, T.O. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in South Western Nigeria. *Trop J Pharm Res*., 2008; **7**(3): 1019-1024.
2. Barker, H. A. Bacterial fermentations, 2nd ed., John Wiley & Sons.1956; 1-27.
3. Bindu, Sadanandan., PrernaLal, Rhonchamo, Humtsoe., A. Mishra. Antibacterial activity of garlic against *Bacillus Subtilis*. *International Review of Applied Biotechnology and Biochemistry*., 2014; **2**(1): 107-119
4. Brown, M. R. W., Scott, Foster, J.H. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. *J. Clin. Pathol.*, 1970; **23**: 172-177.
5. Buncic, S., Nychas, G. J., Lee, M. R., Koutsoumanis, K., Hebraud, M., Desvaux, M., Chorianopoulos, N., Bolton, D., Blagojevic, B., Antic, D. Microbial pathogen control in the beef chain: recent research advances. *Meat Sci.*, 2013; **97**(3): 288-97.
6. Santos, H.S., Arruda, F.V., Bandeira, P.N., Albuquerque, M.R., Pereira, M.O., Henriques, M., Cavada, B.S., Teixeira, E.H. Casbane Diterpene as a promising natural antimicrobial agent against biofilm-associated infection. *Molecules*., 2011; **16** (1): 190-201
7. Costerton, J.W., Stewart, P.S., Greenberg, E.P. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science*., 1999; **284**: 1318-22.
8. Cowan, M.M. Plant products as antimicrobial agents. *Clinical Microbiol Rev.*, 1999; **12**(4):564-582.
9. De, M., De, A.K., Banerjee, A.B. Antimicrobial screening of some Indian spices. *Phytother Res.*, 1999; **13**(7):616-8.
10. Dorman, H.J.D., Deans, S.G. "Antimicrobial agents from plants: Antimicrobial activity of plant volatile oils." *J Appl Microbiol*., 2000; 88:308-316.
11. Harborne, J.B. (ed): Phytochemical Methods: A guide to modern techniques of plant analysis, 3rd Edition. Chapman and Hall, New York, 1973 pp. 279.
12. Hopkins, R.H., Bird, R. Action of ±-amylase on amylase. *Biochem J*. 1954; 56:86–96.
13. Khusro, A., Aarti, C., Preetamraj, J. P., Panicker, S.G. In vitro Studies on antibacterial activity of aqueous extracts of spices and vegetables against *Bacillus licheniformisstrain* 018 and *Bacillus tequilensisstrain* ARMATI. *Int. J. Curr. Microbiol. App. Sci.* 2013; **2**(9): 79-88.
14. Kolawole, O.M., Oguntoye, S.O., Agbede. O., Olayemi, A.B. Studies on the efficacy of *Bridelia ferruginea Benth* bark extract in reducing the coliform load and BOD of domestic waste water. *Ethnobotanical Leaflets*, 2006; **10**: 228-238.
15. MacFaddin, J.F. Biochemical tests for identification of medical bacteria, 3rd ed., Lippincott Williams and Wilkins, U.S.A, 2000.
16. Mah Thien-Fah C, and George A O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, **9**(1): 34-39.

17. Manasboxi, Rajesh, Y., Rajakumar, V., Praveen, B., Mangamma, K. Extraction, phytochemical screening and in-vitro evaluation of anti-oxidant properties of C*ommicarpus chinesis* (aqueous leaf extract). *Int J Pharm. Bio. Sci.,* 2010; 537-547.
18. Quave, Cassandra, L., Lisa, Plano, R.W., Traci, Pantuso, Bradley, Bennett. Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*. *J Ethnopharmacol.*, 2008; **118**(3): 418–428.
19. Rahua, J.P., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kiyala, T., Pihlaja, K., Vuorela, H., Vuorela, P. "Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds." *Int. J. Food Microbiology*. 2000; **56**: 13 (3)-12.
20. Saitou, N., Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987; **4**(4): 406-25.
21. Sambrook, J., Fritschi, E.F., Maniatis T. Molecular cloning: a laboratory manual, 3rd ed., New York, 1989.
22. Smolenski, S.J., Silinis, H., Farnswoth, N.R. Alkaloids screening. V. Lloydia., 1974; **37** (3): 506-536.
23. Sofowora, A. Medicinal plants and traditional medicine in Africa. 2nd Ed. Sunshine House, Ibadan, Nigeria: 1993; 134–156.
24. Sonali, Jana., Shekhawat, G.S. Phytochemical analysis and antibacterial screening of *in vivo* and *in vitro* extracts of Indian medicinal herbs: *Anethum graveolens*, *J. Med. Plants Res.*, 2010; **4**(4): 206-212.
25. Sureshkumar, C.A., Varadharajan. R., Muthumani, P., Meera, R., Devi, P., Kameswari, B. Pharmacognostic and preliminary phytochemical investigations on the stem of *Saccharum spontaneum*, *J. Pharm. Sci. & Res.*, 2009; **1**(3): 129-136.
26. Tamura, K., Dudley, J., Nei, M., Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol.*, 2007; **24**(8): 1596-1599.
27. Tendolkar, P. M., Baghdayan, A. S., Gilmore, M. S. & Shankar, N. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. *Infect Immun.*, 2004; **72** (10): 6032–6039.
28. Thompson, J.D., Higgins, D.G. and Gibson, T.J., CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; **22**(22):4673-80.
29. Vattem, D.A., Mihalik, K., Crixell, S. H., McLean, R.J.C. Dietary phytochemicals as quorum sensing inhibitors. *Fitoterapia*. 2007; **78**(4):302-10.
30. Zhang, Z., Schwartz, S., Wagner, L., Miller, W. A greedy algorithm for aligning DNA sequences. *J Comput Biol*. 2000; **7**(2):203-14.
31. Zhao, K., Tseng, B. S., Beckerman, B., Jin, F., Gibiansky, M. L., Harrison, J. J., Parsek, M.R., Wong, G.C. Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. *Nature*. 2013; **497** (7449): 388–391.