Research Article

Inhibition of Biofilm Forming Bacteria by Processed *Tamarindus indica* Seed Extracts

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Received: 22-07-2018; **Revised:** 02-08-2018; **Accepted:** 03-09-2018.

ABSTRACT

Biofilm is produced by a community of microorganisms that are attached to a substratum and embedded in a matrix of extracellular polymeric substances (EPS) produced by them. Biofilms help the microorganism increase its resistance against antibiotics making it harder to treat diseases caused by them. The inhibition of biofilm formation can play a major role in reducing the resistance of biofilm forming gram positive and gram negative bacteria against antibiotics. In the present study, bacteria were isolated from urine samples of UTI infected diabetic patient. The obtained culture was characterized and the identified gram negative organisms were checked for biofilm formation activity and its inhibition by variously processed seed extracts of *Tamarindus indica*. Tamarind seed extracts exhibited antimicrobial effect and was confirmed by agar well diffusion assay. It was found that cooked tamarind seed extract had the highest antimicrobial activity among all the sample extracts and was checked for their biofilm inhibition capabilities by biofilm inhibition assay and it exhibited anti-biofilm activity. Thus this study can be used to reduce the biofilm forming ability of the bacteria making it more vulnerable to antibiotics and hence allowing treatment of diseases to become more effective.

Keywords: Biofilm, Antibiofilm activity, *Tamarindus indica*, dehulling,

INTRODUCTION

Extracellular polymeric substance (EPS) which surrounds a mono or multi specie population of microbial cells adhered to a surface is called a biofilm[1]. The composition of the matrix varies from specie to specie and depends on the type of microbial species which have formed the biofilm[2]. A biofilm generally comprises of complex carbohydrates such as polysaccharides, nucleic acids and proteins. 90% of the dry weight of a biofilm is because of the extracellular polymeric substance, depending on particular microbial isolates, which is the main or most important component of a biofilm[3]. Apart from other functions this EPS is found to stop the entry of antibiotics to the microbial species embedded in the biofilms[4].

The weaponry of curative agents accessible to cure biofilm caused infections at present take into very little account the complexity of a biofilm and biological nature of interactions taking place within the biofilm. This becomes a problem, because biofilms resulting in persistent infections cannot be resolved with standard antibiotic treatments. Since the problem of bacterial group behaviors has not been considered until recently, the strategies required to cure infections caused by biofilms have not been devised. Therefore, understanding bacterial social behaviours and their molecular mechanisms in the development of biofilms will greatly facilitate the development of novel strategies in the prevention and treatment of biofilm infections.

The formation of a biofilm is also advantageous in acquiring transmissible, genetic elements at faster rates. And increased rate of conjugation is found to occur in bacteria present in biofilms. This suggests that evolution by horizontal transfer of genetic material may occur rapidly in a biofilm, making it the perfect milieu for emergence of new pathogens by acquisition of antibiotic resistance, virulence factors, and environmental survival capabilities.

Inability of the bacteria to escape the biofilm would make the biofilm a death trap when the nutrient supply is diminished and environmental conditions become unfavorable. Once the bacterium is encased in exopolysaccharide, however, abandoning the biofilm becomes a significant task. At such times, a polysaccharide lyase may provide the bacterium with an escape. This product hastens detachment of biofilm-associated cells.

Tamarind is used in treatment of cold, fever, diarrhoea, jaundice and in skin cleanse[5]. Tamarind seeds have a good composition of various amino acids like Methionine, Phenylalanine, Valine, etc. Tamarind seeds are also a good source of vitamins and various minerals like potassium, calcium and magnesium.

Tannins, flavonoids, alkaloids and other aromatic compounds are secondary metabolites of plants and they have a defence mechanism against microorganism and insects. This is what may confer the antimicrobial effect that certain seeds have[6], Tamarind seed is one of them. Broad spectrum of antimicrobial activity is shown by tamarind extracts and this can be used for control of infectious diseases. Human consumption is safe and hence it can be widely researched more for use in drugs[5].

Tamarind seed extracts were found to have antibiofilm forming properties. It has ability to lessen the formation

of biofilm by certain strains of bacteria like *Pseudomonas, Escherichia,* etc[7].

Leguminous plants are known to possess anti nutritional components and these can be removed by processing. The various processing techniques include cooking, autoclaving, germinating, dehulling and soaking in water. These have a biochemical change in the anti nutritional composition of the seeds, some increasing and others decreasing the levels. Dehulling, germinating and soaking decrease the levels of tannins and phenolics while autoclaving increases the level of phenolics[8].

The present study includes exposure of seeds of *Tamarindus indica* to various methods of processing and to determine the inhibitory effect of these processed seeds against biofilm forming organisms.

MATERIALS AND METHODOLOGY

Plant source

Using Random Sampling Technique (RST), the seeds of *Tamarindus indica* were collected from local areas of Bengaluru district, Karnataka State, India. The seed samples obtained were dried in sunlight for 24 hours. Any seed that was immature or damaged were removed and the remaining mature seeds were washed with normal water and stored in refrigerator until further use.

Plant sample processing

Tamarind seeds were collected and processed by different methods. The samples obtained were

Sample 1: Control

The seeds were not processed.

Sample 2: Soaked

The seeds were soaked in water for five days and were then dried at 60°C.

Sample 3: Dehulled

Seeds soaked for five days were hand pound to separate the hull from the seeds. The dehulled seeds were then dried at 60°C.

Sample 4: Cooked

The seeds were cooked for 30 minutes and the mucus was washed away from the seed coat and the seeds were dried at 60°C.

Sample 5: Autoclaved

The seeds were autoclaved and cooled. They were then dried at 60°C.

Sample 6: Germinated

The seeds were treated with 50% Sulphuric acid for thirty minutes after which it was washed and sowed into a medium containing coco pith and sand in the ratio of 1:1. Ten days later the seeds were cleaned, dried overnight at 60°C.

All the above samples were finely powdered after they were dried at 60°C.

Aqueous extract of processed seeds

Each of the 6 samples were ground in a mortar and pestle into fine powder. 5% and 10% extract of each sample was prepared using distilled water and stored in cold conditions.

Culturing of microbes

Multi drug resistant strains of bacterial cultures were isolated from UTI samples of a diabetic patient from Bhagwan Mahaveer Jain Hospital, Bengaluru. Two isolated strains were chosen based on their biofilm formation when incubated overnight in TSB medium.

The isolated strains were characterized by IMViC test, Oxidase test, Catalase test, Casein hydrolysis, Urease test, Starch hydrolysis and growth on EMB Agar plates.

Biofilm formation assay using micrometer plate method

Quantification of formation of biofilm by each UPEC was assayed by Microtiter plate method with some modifications[7]. All the assays were performed using triplicates.

Well diffusion assay

The organisms were inoculated into 20ml of nutrient broth. After overnight incubation at 37˚C, 1ml of the broth was spread over nutrient agar plates using sterile spreader and left to air dry. Using a sterile well puncher, wells were punched equidistant from each other and 10µl of 5% plant extract were added to wells. A positive and negative control was maintained. Ampicillin drug was loaded in the positive well and DMSO was loaded in the negative. The plates were incubated overnight at 37˚C and observed.

Biofilm Inhibition by plant sample extracts

To the microtiter plate 100µl of overnight incubated TSB broth containing bacterial culture was added. Each bacterial isolate was treated with 5% and 10% plant extract and left for overnight incubation at 37˚C. The next day, cells suspension was aspirated and the wells were washed with distilled water. 1% crystal violet was prepared and added to the wells to stain them and was incubated at room temperature for 30 minutes. The dye was then removed and the wells washed with 0.1M PBS. The wells were then emptied of PBS and further 95% ethanol was added to each well and incubated at room temperature. After 15 minutes, the plate was read at 600nm in ELISA plate reader.

RESULTS AND DISCUSSION

Microbial biofilms occupy about 99% of the surfaces. The competition between microbes for nutrients and other growth factors play an important role in the development of a biofilm. The chemical signals produced by the high density of organisms in the biofilm signals with the responding cells in the biofilm, thus by this factor the

complexity of the biofilm structure is increased. Inhibition of biofilms is important to reduce the anti-microbial effect induced by the films and the use of natural products like Tamarind seeds which has anti-microbial properties is more beneficial than use of chemically synthesized products.

Isolation and characterization of microbes

Multi drug resistant bacterial cultures were isolated from a diabetic patient and cultured and subcultured on McConkey Agar plates to obtain pure strains. Biochemical tests were carried out on the isolated strains.

Table 1: Biochemical Tests of Isolate 3 and Isolate 4

	Isolate 3	Isolate 4
MR	-	-
VP	+	+
Indole	-	-
Citrate utilisation	+	+
Oxidase	+	+
Catalase	+	+
Casein Hydrolysis	+	+
Urease test	+	+
Starch Hydrolysis	-	-

Primary biofilm formation assay

Primary biofilm attachment assay was performed in various concentrations for isolate 3 and isolate 4 (Fig 1). The culture was diluted from 10^8 CFU/ml to 10^3CFU/ml by serial dilution. It was noted that higher concentrations of dilutions had higher values, hence indicating higher attachment to well walls. Lower concentrations of dilutions showed lower attachment. The first three dilutions showed the highest levels of reading which was read using an ELISA plate reader. This can be visualised in Fig 2a and Fig 2b. It can be understood that the levels of attachment are directly proportional to concentration of the isolate.

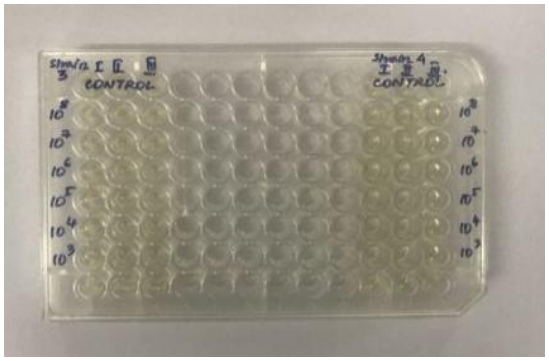


Figure 1: Primary biofilm formation assay in microtitre plate

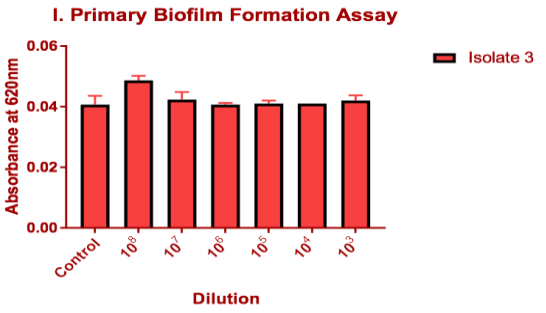


Figure 2a

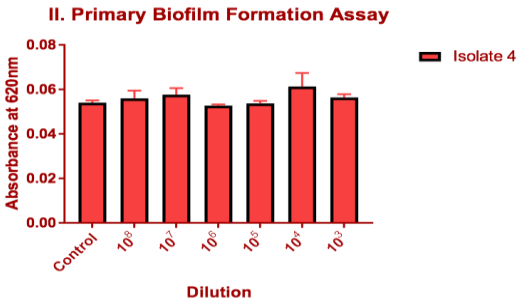


Figure 2b

Figure 2: Graphical representation of primary biofilm formation assay by isolate 3 (Fig 2a) and Isolate 4 (Fig 2b)

Secondary Biofilm formation assay

Secondary biofilm formation assay was carried out after 24 hours of incubation in microtiter wells (Fig 3). Higher the incubation time provided, more biofilm is formed. Formation of biofilm also depended on the number of bacteria present in the wells. Therefore the formation of biofilms depended greatly on the concentration of bacteria as well as time of incubation. The wells were read using ELIZA plate reader. The values obtained have been graphically represented for both the isolates in Fig 4a and 4b. Both the isolates indicated high biofilm formation.

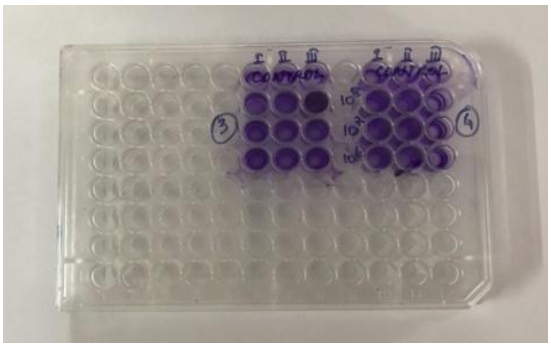


Figure 3: Secondary biofilm formation assay in microtitre plate

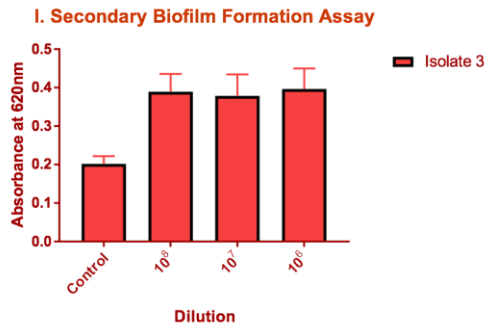


Fig 4a

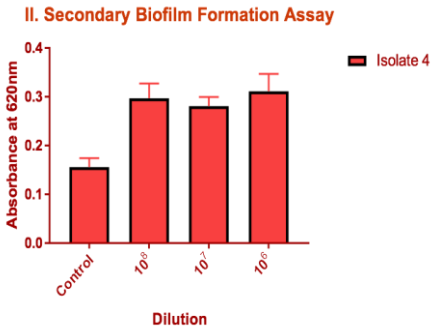
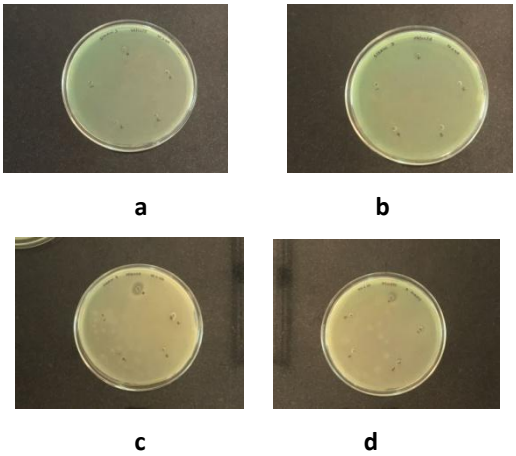


Fig4b

Figure 4: Graphical representation of secondary biofilm formation assay by isolate 3 (Fig 4a) and isolate 4 (Fig 4b)

Well diffusion assay

To plates with overnight growth culture, 10µl of 5% plant extract were added to wells. A positive and negative control was maintained. Ampicillin drug was loaded in the positive well and DMSO was loaded in the negative. The plates were incubated un-inverted overnight at 37˚C and observed the next day. It was observed that in isolate 3 and isolate 4, the cooked seed extract that is sample 4, had the highest antimicrobial property by displaying largest zone of inhibition among all the other samples. Therefore further on tests were done using samples 4.



a **b**

c **d**

Figure 5 : The well diffusion plates along with positive and negative control for isolate 3 (Fig 5a and 5b) and isolate 4 (Fig 5c and 5d)

Biofilm Inhibition by plant sample extracts

Sample 4 was used for further testing. Percentage of inhibition was calculated using the formula stated below.

% of Inhibition = (OD in control -OD in treatment x 100) / OD in control

Biofilm formation can be inhibited by sample 4 and it is determined that 10% extract of sample 4 has a higher inhibitory effect than 5% of sample 4, against isolate 3 and isolate 4 as shown in Fig 6. It was noticed that 10% extract showed an inhibition of 73.82% and 5% extract showed an inhibition of 32.68% against isolate 3.

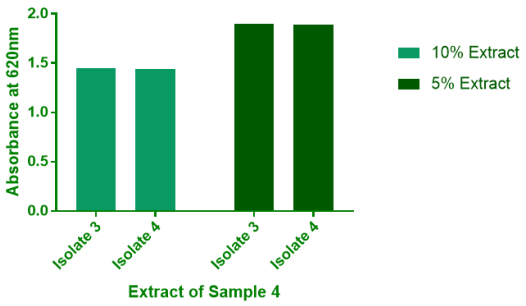


Figure 6: Biofilm inhibiting activity of sample 4

Against isolate 4, 10% extract exhibited a 51.35% inhibition and 5% extract showed 15.05% inhibition. The seed samples were found to have biofilm inhibition which could be due to presence of any photochemical group present in them. Individual compounds were not isolated nor further characterized to isolate the compound of interest that is able to confer the biofilm inhibition property. Hence, the specific compound(s) responsible for biofilm inhibition is unknown. Further isolation and detection of the phytochemical present that provide the anti-biofilm property is required.

CONCLUSION

Antibiofilm activity of *Tamarindus indica* seeds, that were subjected to various processing methods was performed. Zone of inhibition was noticed in sample 4, i.e., cooked seed extract, hence antibiofilm assay was performed on isolate 3 and isolate 4 using sample 4 extracts. Percentage inhibition for 5% and 10% extract of sample 4 against isolate 3 was found to be 32.65% and 73.82% respectively. Percentage inhibition for 5% and 10% extract of sample 3 against isolate 4 was found to be 15.05% and 51.35% respectively. Phytochemical group responsible for the antibiofilm activity was not isolated and hence requiring further work to be carried on to identify the compound. This will give an understanding of the mechanism of action providing antibiofilm effect.

Acknowledgement: The authors wish to acknowledge Department of Chemistry (PG Biochemistry) and the management of Mount Carmel College Autonomous, Bengaluru for funding this project and offering their facilities for the analysis.

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Source of Support: Nil, **Conflict of Interest:** None.