Antibiofilm effects of tannins from *Delonix elata* Linn.

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Abstract
Biofilm is a complex microbial population that is resistant to antimicrobials. The production of biofilms on biotic and abiotic surfaces causes high rates of morbidity and mortality in hospitalized patients. In the present study, the antibacterial and antibiofilm potential of crude tannins (ethanol and aqueous extracts) were employed against microbial pathogens. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined ranging from 0.125 – 8 µg/ml.

The specific biofilm formation index (SBF) was evaluated before and after the addition of plant extracts and tannins. Tannins caused a major reduction in biofilm formation by S. aureus (69.02%). The crude extracts and tannins may be potential candidates for the development of antibacterial drugs.

Keywords: Antibacterial activity, MIC, antibiofilm activity, tannins, aqueous extract, MBC.

Introduction

Biofilm is a complex existence of microorganisms found on the biotic or abiotic surfaces and is encased in an extracellular polymeric substances (EPS) matrix. As regards the response to antimicrobial treatments, biofilm-enclosed bacteria are 10-1000 times more resistant than that of planktonic bacteria[23]. Under challenging environmental conditions, biofilms are crucial for the survival of bacterial cells[9].

According to the National Institutes of Health, microbes associated with biofilms are responsible for 80% of microbial diseases in the human body[11]. There are a number of bacterial genera of human and veterinary importance such as *Escherichia*, *Staphylococcus*, *Pseudomonas*, *Pasteurella*, *Bacillus*, *Salmonella* etc. causing infections that are difficult to treat due to their ability to form biofilms[2,19].

This scenario urges an increasing search for potential drugs and drug scaffolds from natural resources, though there are a limited number of new antimicrobials in place. Research focused on natural products is a promising route of investigation because a considerable proportion of newly approved antibacterial drugs is either natural products themselves or derivatives of natural resources[17,21]. Compounds with special antibacterial characteristics that can be derived from natural sources are becoming more and more popular. Their use in dietary supplements or as raw materials i.e. for packaging or medical needs, has been considered.

Medicinal plants are non-toxic or less harmful, low-cost, conveniently accessible and safe resources for drug development. Herbs include phytochemical substances with antibacterial activity against planktonic and biofilm forms of bacteria. Polyphenols have also received a lot of interest recently and studies are being done to determine whether they have antiviral and antibacterial effects. Tannins are polyphenolic secondary metabolites which are located in the roots, wood, bark, leaves and fruits of many plants[3].

In plants, tannins play a role in protecting plants from predation. Tannins have potential anti-oxidants, anti-microbial[15], antiviral[14] and anticancer activities[25].

Delonix elata Linn. (family: Caesalpiniaceae) is commonly known as Vadanarayanan in Tamil. It is a deciduous tree and is widely distributed in the dry regions of India. Traditional healers in the Chitradurga district of Karnataka, India, used the leaves and stem bark extracts to treat jaundice, liver diseases, bronchial and rheumatic ailments[10]. The plant has also been used to treat joint pain and stiffness, particularly in the knees[26].

The leaves are used to treat newborn bronchitis, fever, malaria, flatulence and paralysis, as well as a carminative. Siddha practitioners and local people use the leaves of *D. elata* for treating arthritis and inflammation and are eaten as food. The aim of this study was to assess the biofilm inhibiting property of tannins extracted from the leaves of *D. elata*.

Material and Methods

Collection of plant sample: The fresh leaves of *Delonix elata* (Fig. 1a) were collected from Thoothukudi, Tamil Nadu and were authenticated by Dr. Soosai Raj, Assistant Professor, the Rabiant Herbarium, St. Joseph's College Trichy, Tamil Nadu. India. The collected leaves were washed several times with distilled water to remove the traces of impurities from the leaves. The washed leaves of *D. elata* were shade-dried for one week and ground into a powder using an electric mixer grinder (Fig. 1b).

Extract preparation: The powdered plant sample 5g was soaked in 25 ml of ethanol and distilled water for 24 hours and filtered with Whatmann no. 1 filter paper to obtain plant extract.

Figure 1a: *Delonix elata* - Habitat

Figure 1b: Dry sample of leaf

Phytochemical screening: The phytochemical screening of the crude extracts (ethanol and aqueous) such as alkaloids, terpenoids, flavonoids, phenols, tannins, saponins, steroids, phytosterol, anthroquinones, phlobatannins, gums and mucilage, fixed oil and fat was qualitatively performed accordingly to the methods described by Trease and Evans[24].

Extraction of tannin: The fine powder of the *D. elata* (12.5 g) was mixed with 62.5 ml of hot distilled water. The mixture was agitated for 3 hours in a mechanical shaker and filtered using Whatmann no. 1filter paper. After 3 days, it was concentrated in a water bath at 40^{o} C. After 72 hours, crude tannin was obtained[16]. The presence of crude tannin was confirmed by the ferric chloride test.

Microorganisms: Bacterial strains were obtained from the Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi, India. Amongst five bacterial strains investigated, two were Gram-positive strains viz., *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*) while the other three were Gram-negative strains viz. *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Salmonella typhi* (*S. typhi*). The collected bacterial strains were maintained at 4^{o}C on nutrient agar slants. From agar plates, approximately three to five grown colonies were isolated using an inoculation loop and inoculated in test tubes containing seven ml of nutrient broth and incubated over 24 hours at room temperature.

Antibacterial activity: Antibacterial activity of crude extracts and tannins was carried out by using broth dilution method[6].

Minimum inhibitory concentration (MIC): For determination of MIC, 1 ml of broth medium was taken into 10 test tubes for each bacterium. Different concentrations of plant extracts ranging from 0.125 to 8 µg/ml^{-1} concentration were incorporated into the broth and the tubes were then inoculated with 0.1 ml of inoculums of respective bacteria (10^{5}CFU ml^{-1}) and kept at 37°C for 24 h. The test tube containing the lowest concentration of extract which showed reduction in turbidity when compared with control, was regarded as MIC of that extract.

Minimum Bactericidal Concentration (MBC): The lowest concentrations of the extract in the post-incubation suspensions which did not harbor any bacterial growth upon spotting on MHA after overnight incubation at 37°C, were considered as the MBC values.

Crystal violet staining assay: The effect of crude extracts and tannins on biofilm formation was evaluated. Briefly, 300 µl of fresh nutrient broth was aliquoted into each test tube and inoculated with 100 µl inoculums of respective bacteria. Tubes containing medium with inoculums and those without extracts were used as control. Tubes were incubated at 37°C for 48h. After incubation, the supernatant was removed and each well was washed thoroughly with sterile distilled water to remove free-floating cells; thereafter, plates were air-dried for 30min and the biofilm formed was stained for 15 min at room temperature with 0.1% aqueous solution of crystal violet. Finally, the dye bound to the cells was solubilized by adding 250 µl of 95% ethanol to each well and after 15 minutes of incubation, absorbance was measured using spectroscopy at a wavelength of 570 nm.

Biofilm determination was made using the formula:

SBF = (AB - CW)/G

where SBF is the specific biofilm formation, AB is the OD 570 nm of the attached and stained bacteria, CW is the OD570 nm of the stained control and G is the OD630nm of cell growth in broth.

Results and Discussion

Medicinal herbs are augmented with vital phytoconstituents, which are a variety of primary and secondary plant metabolites accountable for anticancer, antiulcer, antidiabetic, antioxidant, anti-inflammatory, anti-microbial effects and other known biological activities. The percentage yield was 2.432 %. The qualitative phytochemical screening

of leaf extracts of *D.elata* revealed that alkaloids, terpenoids, phenols, flavonoids, tannins, saponins, steroids and phytosterol were present in ethanol extract and alkaloid, flavonoid, tannins and saponins in aqueous extract (Table 1). The ethanolic extract contained higher phytochemicals composition than aqueous extract. Alkaloids possess antimalarial[7], antimicrobial, anti-inflammatory, antimitotic, anticancer and antispasmodic antiparasitic[13] properties.

Flavonoids possess anti-inflammatory, anticancer, anti-aging, cardioprotective, neuroprotective, immunomodulatory, antidiabetic, antibacterial, antiparasitic and antiviral properties[4]. Tannins possess activities such as anti-oxidants[1], anti-microbial, antiviral and anticancer. Steroids possess activities such as antitumor, antimicrobial, antioxidant, anti-insecticidal, cardiovascular diseases and anti-inflammatory potential[22]. Saponins possess antioxidant, analgesic, immunostimulant, antimicrobial, antiviral, cytotoxic activities and anti-inflammatory and hemolytic effects[8].

Table 1
Qualitative phytochemical screening of crude extracts of *Delonix elata*

Name of the phytochemicals	Ethanol extract	Aqueous extract
Alkaloid	+	+
Terpenoids	+	-
Phenol	+	-
Flavonoid	+	+
Tannin	+	+
Saponins	+	+
Steroids	+	-
Phytosterol	+	-
Anthroquinone	-	-
Phlobotannin	-	-
Gums and mucilage	-	-
Oil and fat	-	-

'+' Present '-'Absent

Table 2
Antibacterial effect of ethanol extract of *Delonix elata*

Microorganisms	MIC (µg/ml)	MBC (µg/ml)	MIC_{index}
S. aureus	0.500	1	2
B. cereus	0.500	1	2
E. coli	2	4	2
K. pneumoniae	4	8	2
S. typhi	8	-	-

Table 3
Antibacterial effect of aqueous extract of *Delonix elata*

Microorganisms	MIC (µg/ml)	MBC (µg/ml)	MIC_{index}
S. aureus	1	2	1
B. cereus	2	4	2
E. coli	2	4	2
K. pneumoniae	-	-	-
S. typhi	-	-	-

Table 4
Antibacterial effect of tannins of *Delonix elata*

Microorganisms	MIC (µg/ml)	MBC (µg/ml)	MIC_{index}
S. aureus	0.125	0.250	2
B. cereus	0.250	0.500	2
E. coli	1	2	2
K. pneumoniae	1	2	2
S. typhi	2	4	2

The antibacterial activity of the crude extracts and tannins was tested by employing two methods such as Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC). The crude extracts (ethanol and aqueous) tannins were subjected to antibacterial activity against *B. cereus, S. aureus, E. coli, K. pneumoniae* and *S. typhi*. The MIC values for the extracts and tannins ranged between 0.125 – 8 µg/ml against the selected bacterial pathogens (Tables 2 - 4). The MIC results are comparable to those obtained in the broth dilution technique because the lowest MIC was obtained using the crude extracts and tannins showing the best antibacterial activity.

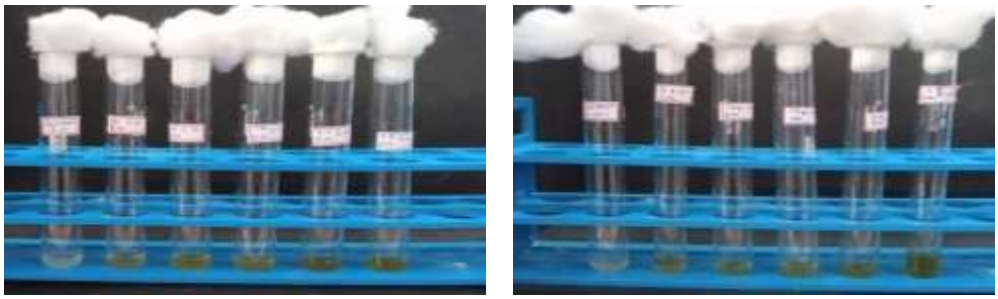
Ethanol extract inhibits the growth of *S. aureus* and *B. cereus* at 0.5 µg/ml, *E. coli* at 2 µg/ml and *K. pneumoniae* at 4 µg/ml (Table 2). Aqueous extract did not inhibit the growth of all tested bacteria significantly. The ethanol extract has more antibacterial activity than the water extract. Antibacterial activity of the *D. elata* leaf extracts against both Gram-positive and Gram-negative bacterial strains has been reported previously[18,20]. Alkaloids inhibit the bacterial growth of *Bacillus subtilis, S. aureus, E. coli, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger*. This may be attributed to the fact that these two groups differ by their cell wall component and the thickness of their cell wall[56]. The ability of tannin compounds to cause the bacterial colonies to disintegrate probably results from their interference with the bacterial cell wall, thereby inhibiting microbial growth.

Meanwhile, results of minimum bactericidal concentrations (MBC) are listed in tables 2-4, where tannins had the lowest MBC with a value of 0.250 µg/ml for *S. aureus*, 0.500 µg/ml for *B. cereus*, 2 µg/ml for *E. coli* and *K. pneumoniae* and 4 µg/ml for *S. typhi* (Table 4). The crude extracts revealed moderate activity against some microbial pathogens tested in this study. (Tables 2 and 3). It has been proposed that bioactive components could disrupt the permeability of bacteria's cytoplasmic membrane, hence reducing their growth. The anti-biofilm activity of ethanol and tannins of *D. elata* is shown in table 4. Tannins exhibited significant anti-biofilm activity against both Gram-positive and Gram-negative bacterial strains. The highest anti-biofilm activity was observed against *S. aureus* and *B. cereus* with 69% and 67 % respectively (Table 5).

Table 5
Antibiofilm activity of ethanol and crude tannins of *D. elata*

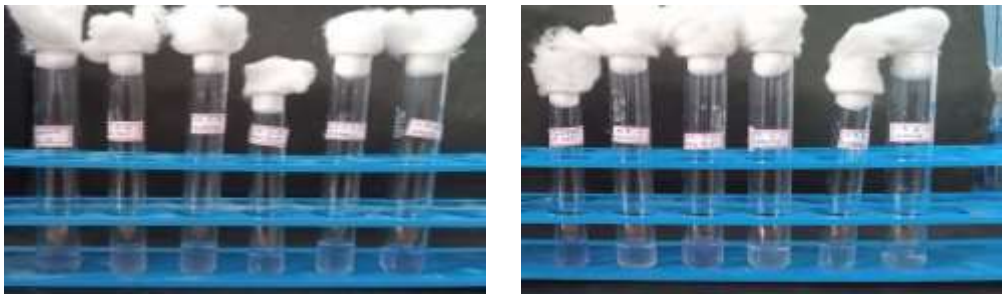
Microorganisms	Biofilm reduction (%)	
	Ethanol	Tannins
S. aureus	58.19 ± 0.21	69.02 ± 0.16
B. cereus	55.60 ± 0.14	67.48 ± 0.05
E. coli	56.01 ± 0.04	66.54 ± 0.24
K. pneumoniae	55.86 ± 0.01	65.33 ± 0.01
S. typhi	50.05 ± 0.17	62.33 ± 0.05

Mean ± Standard Deviation



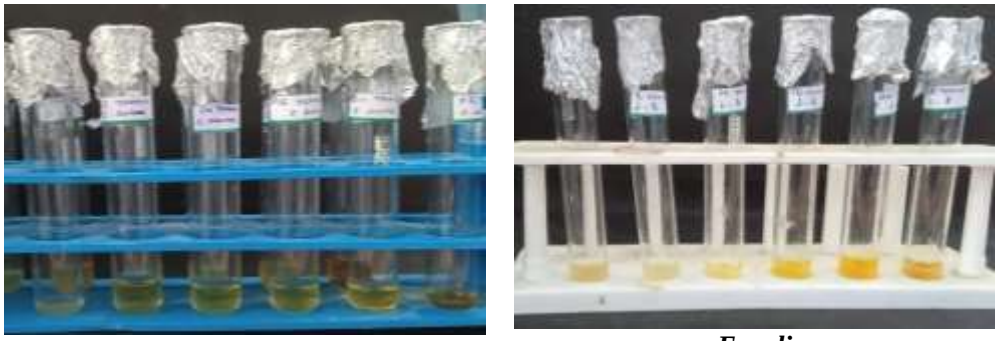
S. aureus ***E. coli***

Figure 2: Antibiofilm activity of ethanol leaf extract of *D. elata*



S. aureus ***E. coli***

Figure 3: Antibiofilm activity of aqueous leaf extract of *D. elata*



S .aureus ***E. coli***

Figure 4: Antibiofilm activity of tannins of *D. elata*

On the other hand, the highest antibiofilm activity was observed in the ethanol extract against *S. aureus* with 58% (Table 5, Figures 2, 3 and 4).

Conclusion

Biofilm-producing bacteria are naturally resistant to antibacterial drugs, which are the leading cause of illnesses in humans and animals. Numerous studies are currently being conducted to figure out the potential anti-biofilm agents from herbs. Because, unlike the conventional drugs, the plant bioactive compounds will ward off the growing threat drug resistance in microbes in the future. The findings of the present study highlight the potential of ethanol extract and tannins from the leaves of *D. elata* as antimicrobial agents. The results also confirmed the antibiofilm activity of the extracts and crude tannins against the selected bacterial pathogens.

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