



Research Article

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Bioefficacy of *Terminalia chebula* extract against biofilm formation of common pathogens

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ABSTRACT

In the present study bioefficacy and biocidal activity of *Terminalia chebula* fruit water extract against common infectious bacteria; *Staphylococcus aureus*, *Bacillus subtilis* and mixed culture of *S.aureus* and *B.subtilis* (1:2) was assessed. The characterization and detection of biofilm formation by these bacteria was analyzed using common methods: Tissue culture plate method (TCP), Congo red agar method (CRA). The extract in three different concentrations 1%, 2% and 5% were used for optimization of biocide concentration using TCP method. The results from both detection methods indicated the effective biocidal activity of the extract on biofilm formation. The optimum extract concentration for reducing or inhibiting the biofilm formation in both the species was found to be 2%. The results suggests the use of the fruit extract as an biological alternative for eradication of biofilm formation in medical devices in place of commonly used hospital chemical biocides.

Key words: Mixed culture, *Terminalia chebula*, biocide, biofilm, *Staphylococcus aureus*, *Bacillus subtilis*.

INTRODUCTION

Microorganisms irreversibly attach on surfaces or to each other forming a matrix of extracellular polysaccharides called biofilm. Notable feature of these biofilm-forming organisms is their increased resistance to antimicrobial agents and their potential to cause infections through indwelling medical devices in patients [1, 2]. Higher antimicrobial concentrations will be required to eradicate the intrinsic biofilm forming organisms as the resistance can increase up to 1000 fold [3]. Many medical conditions are caused by biofilms; dental plaque, upper respiratory tract infections, peritonitis, urogenital infections and blood stream infections associated with indwelling medical devices [4, 2].

Both Gram-positive and Gram- negative bacteria form biofilm. Staphylococcal infections are the major source of nosocomial infections associated with implant device failure. The infections are associated with surgical sites, wound infections, catheters and prosthetic implants [5, 6].

Biocides used currently in hospitals are not efficient in controlling biofilm formation [7, 8] as the organisms have gradually become less susceptible to most biocides over long exposure [9]. The increasing resistance to existing biocides initiates the necessity to identify alternative compounds with antimicrobial activity. Identification of bioactive compounds from natural products (plants) has been reviewed extensively in recent studies [10]. The advantage of using natural antimicrobial agents is that they do not enhance the development of resistance as encountered with antibiotics [11].

Terminalia chebula is called as the 'King of medicine' in Tibet confined to its power of healing. The plant is used to treat diseases like constipation, ulcer, diarrhea, gastroenteritis, asthma, cough, tumor, anoxeria, hepatomegally, dyspepsia [12]. It has been reported to show antibacterial, antifungal, antiviral, antioxidant, hepatoprotective, cardioprotective and antidiabetic activity [13].

Considering the challenges of development of new antimicrobial compounds from natural products to overcome the existing resistance associated with antibiotics the potential of water extract of *Terminalia chebula* fruit against biofilm formation of the pathogens; *Staphylococcus aureus* and *Bacillus subtilis* was analyzed in this study. The effective concentration to inhibit biofilm formation was optimized.

EXPERIMENTAL SECTION

The fruit of *Terminalia chebula* was purchased from local natural drug store. All chemicals of analytical grade procured from Hi-Media, India were used for analysis. The microorganisms were procured from Marina Labs, Chennai. The organisms were maintained on *Leuria bertani* broth.

Preparation of extract

The fruit was washed with sterile water and dried. The dried fruit was crushed and powdered using a mortar and pestle. 1, 2 and 5% concentrates of the fruit powder in distilled water was prepared. The extract was collected by filtering the concentrates after 24 hours of incubation in agitated conditions using No.1 Whatmann filter paper. The filtrate was used for the biocide efficacy studies [14].

Preparation of inoculum

The organisms were subcultured in *Leuria Bertani* broth incubated at 37°C overnight. The mixed culture in LB broth was prepared in 1:2 ratios of *Staphylococcus aureus* and *Bacillus subtilis*. Overnight cultures of each organism were used for the analysis.

Efficacy against biofilm formation

The efficacy of the extract against the biofilm formation was tested by the commonly employed biofilm detection methods: Tissue culture plate method (TCP) and Congo red agar method (CRA).

Tissue culture plate method: The most commonly used method for detection of biofilm formation proposed by Christensen et al., 1985 [15] is considered the standard. Organisms were sub cultured in *Leuria Bertani* broth was incubated at 37°C for 24hours. The cultures were then diluted in 1:100 dilution in fresh broth. 200µl aliquots of each diluted culture were filled in sterile 96 well flat bottomed polystyrene tissue culture plates incubated for 24hr, 48hr and 72hr at 37°C. After incubation the contents of each was discarded by gently tapping followed by washing with Phosphate buffered saline (pH 7.2) thrice. The wells were then stained with 0.1% crystal violet solution, washed with deionized water and 90% ethanol to remove excess stain and kept for drying. Optical density of the stained adherent biofilm was read at 590nm using ELISA reader (Rayto ELISA Reader 2100). Fresh broth was used as control [16, 17, 18]. For analyzing the bioefficacy of the extract after 24hrs of incubation for biofilm formation, 50µl of the extracts in all three concentrations (1, 2 & 5%) were added to the respective marked wells and incubated for another 24hrs at 37°C. The absorbance was recorded by the same procedure mentioned above. The experiments were done in triplicate and repeated thrice and the mean OD value was considered. These OD values were considered as an index of attachment to surface and forming biofilms.

Congo red agar Method: Alternative method for screening biofilm formation was suggested by Freeman et al., 1989 [19] on a solid medium of brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was prepared by dissolving 3.7g of BHI, 5g of sucrose and 1g of agar in 100 of distilled water. The medium was supplemented with congo red which was prepared and autoclaved separately and added to the autoclaved medium on cooling (55°C) to make up a final concentration of 8g/l. The plates were inoculated with test organisms and incubated at 37°C for 24hrs. The formation of intense black colonies with a dry crystalline consistency on the plates indicates the biofilm formation [4, 17, 18]. For biocide action analysis, 5ml of the extract was added to 100ml of autoclaved medium after cooling to about 55°C in 2% concentration. The formation of black colonies with crystalline consistency represents intermediate biofilm forming organisms and those remained pink indicates weak biofilm formation.

RESULTS AND DISCUSSION

The results obtained from TCP method were compared with the positive control containing organisms in the form of mean OD values. Table 1 represents the mean OD values of biofilm formation of test organisms incubated for 24hr, 48hr and 72hr. Table 2 represents the OD values corresponding to biofilm formation in presence of extract in three different concentrations for S.aureus, B.subtilis and Mixed culture by TCP method. The significant difference in OD values in extract added wells indicates the efficacy of the extract in controlling biofilm formation. The biofilm formation was controlled in presence of extract in case of planktonic floating bacteria in each case and no significant difference in adherent biofilm eradication was noticed. The extraction of specific compounds from the extract can provide explanation for the eradication of formed adherent biofilms. The results obtained can be supported by the reports indicating the efficacy of *Terminalia chebula* extracts showing antibacterial activity against S.aureus and B.subtilis. Methanolic extracts of leaf, bark, stem bark and fruit of *Terminalia chebula* was reported to produce higher MIC for both S.aureus and B.subtilis [20]. Similarly the antibacterial activity of ethanol extract *Terminalia chebula* Retz.fruit against S.aureus [21] and B.subtilis [22] were also reported. In all three test organisms considered, 2% concentration of the extract showed higher rate of inhibition of biofilm formation. The result can be supported with the explanation that in case of increased extract concentration deposition of extract components in the walls of the wells forms a matrix aiding biofilm formation instead of inhibition and in contrast 1% concentration is insufficient for inhibiting the biofilm formation.

Table 1 Mean OD values for biofilm formation in test organisms

Hr	Control (OD)	Staphylococcus aureus (OD)	Bacillus subtilis (OD)	Mixed culture (OD)
24	0.161	0.171	0.164	0.258
48	0.154	0.262	0.241	0.303
72	0.148	0.414	0.366	0.370

Table 2 Results of TCP method for bioefficacy analysis of *Terminalia chebula* fruit extract on S.aureus, B.subtilis and mixed culture in triplicates

Hr	S.aureus (OD)	B.subtilis (OD)	Mixed culture (OD)						
	1%	2%	5%	1%	2%	5%	1%	2%	5%
24 hr	0.202	0.166	0.199	0.225	0.204	0.213	0.262	0.223	0.241

The CRA method results were well correlated with that of TCP method. Black colonies with no crystalline consistency were observed in S.aureus, B.subtilis and in mixed culture indicating formation of intermediate biofilm formation in each case. The plates incubated with amended extract showed reduction in biofilm formation in the order of S.aureus > B.subtilis > mixed culture. Figure 1, 2 & 3 represents the biofilm formation in control and in presence of extract for S.aureus, B.subtilis and mixed culture respectively. The efficacy of inhibition of biofilm formation in the mixed culture was less compared to individual organisms in both TCP and CRA methods, which can be explained by the inhibition of biocide action due to the predominance of B.subtilis which enhances the biofilm formation of S.aureus in medium.

Figure 1 Biofilm formations in control and in presence of extract in S.aureus



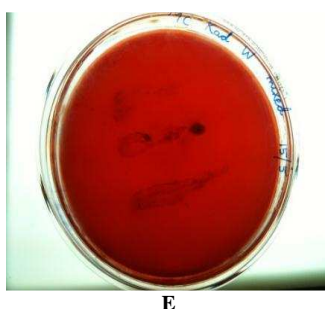
C
C- Control; T- agar inoculated with *Staphylococcus aureus*;
E- Agar amended with extract inoculated with *Staphylococcus aureus*

Figure 2 Biofilm formations in control and in presence of extract in *B.subtilis*



C
C- Control; T- agar inoculated with *Bacillus subtilis*;
E- Agar amended with extract inoculated with *Bacillus subtilis*

Figure 3 Biofilm formations in presence of extract in mixed culture



E
E- Agar amended with extract inoculated with mixed culture

From the results obtained in the present study it can be concluded that the water extract of *Terminalia chebula* fruit has the potential of inhibiting biofilm formation and can be subjected to isolation of active compounds that can be substituted for existing biocides and development of alternatives to antibiotics.

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