Original article

Effect of Terminalia chebula, Trikatu formulation and their combination on Staphylococcal biofilm

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Abstract:

Introduction: The present study evaluates Terminalia chebula, Trikatu formulation and their combination for their inhibitory effect on biofilm formed by Staphylococci.

Methodology: Clinical isolates of Staphylococci were screened for their biofilm forming activity by Congo red agar, Tube and Tissue culture plate (TCP) method. Two strongly biofilm forming strains along with a standard biofilm forming strain (ATCC 35984- Staph. epidermidis) were selected to study the plant extracts by TCP method. Aqueous extracts of the Terminalia chebula, Trikatu and their combination with various concentrations were added to the inoculate during incubation and also after 24 hrs of incubation in separate assay systems. 24 hrs after incubation with the test drugs, the biofilm was quantified.

Results: None of the study drugs showed any effect on Staphylococci in biofilm state. TCh showed significant prevention of biofilm formation at the concentrations of 200, 400 and 800 µg/ml. The Combination showed significant prevention of biofilm formation at the concentrations of TCh and Tr: 100 + 50, 200 + 100 and 400 + 200 µg/ml. The combination showed significantly better biofilm prevention as compared to TCh.

Conclusion: Though the study drugs did not show any effect on Staphylococci in biofilm state, TCh and combination could prevent biofilm formation. Tr enhanced the biofilm formation preventing property of TCh, thus showing synergism. Further evaluation with the various constituents of these plant drugs is required.

Keyword: Staphylococci, Biofilms, Antibiotics

Introduction:

Biofilm-mediated infections in the hospital environment have a huge burden on the resources of the health services.[1,2] Centers for Disease Control and Prevention estimated that biofilms are associated with about 65% of nosocomial infections.[3] Staphylococcal infections are recognized as the most frequent biofilm-associated infections.[4] The effective control of such infections requires strategies that prevent the formation or promote the detachment of biofilms.[5] Many antimicrobial agents fail to act against biofilm forming organisms and result in poor outcome of an infection.[6,7] Numerous preclinical studies have been reported in the literature which evaluate antibiotics[8], disinfectants,[9] and plant drugs against biofilm forming organisms; however very few of them have shown promising effect.[10,11,12,13] Clinical studies with adequate sample size are hardly reported and no drug has been marketed as an antibiofilm drug.

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Ayurveda is a treasure mine of plants and many of these plant drugs have been evaluated to determine their antibacterial potential.[14,15,16] Hence the present study was undertaken to evaluate anti-biofilm potential of Ayurvedic plant drugs. Terminalia chebula (TCh) is reported to have antibacterial activity against pathogenic Gram positive and negative bacteria in vitro.[17,18] Hence it was felt worthwhile to select this plant to determine its ability to act against the microorganisms in biofilm states. Yogavahaktva is an Ayurvedic concept in which one drug facilitates passage of another drug to its site of action. Trikatu(Tr), a combination of Zinziber officinale, Piper longum and Piper nigrum in equal parts, has been mentioned to possess the yogavahaktva property in Ayurvedic text.[19] This combination is claimed to enhance the efficacy of the concurrently administered drugs.[20,21] It was of interest to find out whether it enhances effects of TCh when given concurrently with it.

Hence it was decided to study the effects of above-mentioned plant products against CONS and *Staphylococcus aureus* in the biofilm state and also their ability to prevent the formation of a biofilm. Thus two assay systems were planned - in one, it was decided to introduce the test drugs after formation of a biofilm by the organisms and in another before its formation.

Material and methods:

Prior to the initiation of study, an approval of the Committee for Academic Research Ethics was sought.

Test drugs-

Dried aqueous extracts of TCh and Tr formulation were used for the study. TCh was purchased from Natural Remedies Pvt. Ltd., Bangalore. The extractive value of extract of TCh was 20%.Tr formulation containing Zingiber officinalis, Piper longum and Piper nigrum in equal proportion was donated by Shri Dhootapapeshwar Auyvedic Research Foundation. The extractive value of Tr extract was 41.19%.

For each drug, 6 concentrations were selected based on the dose range (upper and lower) prescribed in Ayurveda assuming that the drug on oral administration gets completely absorbed from the GI tract, their concentrations in the total body water, extracellular fluid and plasma compartment were computed. To use TCh and Tr in combination, it was decided that the concentrations of both should be in the ratio of 2:1. Hence for this combination also 5 final concentrations were prepared (Table 1).

Table 1: Selected concentrations of the test drugs

Plant extract	**Concentrations** **(mcg expressed as crude powdered drug/ml)**
Terminalia chebula (TCh)	25, 50, 100, 200, 400 and 800
Trikatu formulation (Tr)	25, 50, 100, 200, 400 and 800
TCh + Tr (TCh + Tr)	TCh + Tr: 25 +12.5; TCh + Tr: 50 + 25; TCh + Tr: 100 + 50; TCh + Tr: 200 + 100; TCh + Tr: 400 + 200

Organisms

Biofilms producing ATCC 35984- *Staphylococcus epidermidis* was used as a positive control (HiMedia Laboratories Pvt. Ltd India) and nonbiofilm producing ATCC 12228- *Staphylococcus epidermidis* (HiMedia Laboratories Pvt. Ltd India) was used as a negative control for standardization of assay methods.

Staphylococci isolated from different clinical specimens i.e. pus, sputum, blood culture, endotracheal tip, central line tip, urine or other samples as a part of routine investigation and were screened for biofilm formation. Strains which demonstrate biofilms forming capacity were used in the study.

Congo red Agar method

A method described by Freeman, *et al*[22] was used for screening biofilm formation by Staphylococcus isolates. The isolates were cultured on the agar and incubated for 48 hours. Biofilm formation was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink (Fig 1). A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

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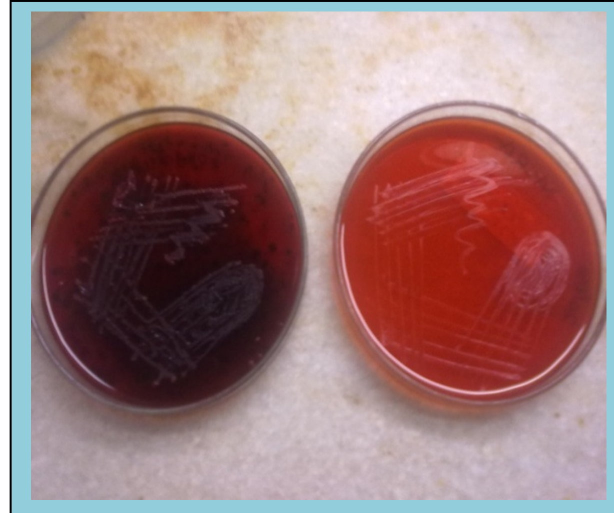

Fig. 1: Biofilm forming (left) and non biofilm forming Staphylococci on Congo red agar

Tube method-

A qualitative assessment of biofilm formation was done as described by Christensen, *et al*[23] Tryptic soya broth (TSB) with 1% glucose was used as media for growth of organisms. Ten ml of TSB glucose was inoculated with loopful of microorganisms and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS and stained with crystal violet (0.1%). Excess stain was removed immediately and tubes were washed with deionized water. Tubes were than dried in inverted position. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. (Fig. 2)

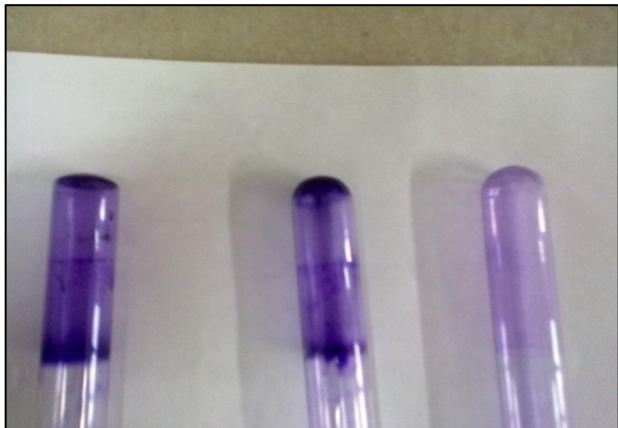

Fig. 2: Tube method showing biofilm formation indicated by staining with crystal violet (first 2 tubes)

Tissue culture plate method (TCP)

All the isolates were also screened for their ability to form biofilm by this method as described by Christensen, *et al*.[24] The method was modified by extending the duration of incubation to 24 hours.[25]

Loopful of organisms after incubation were added to 10 ml of TSB and the turbidity of inoculate was titrated to match with 0.5 Mcfarland standard (which corresponds to 10^8 organisms per ml). These matched inoculates were further diluted with TSB (1 in 100). Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates wells (Becton Dickinson, Falcon no. 3072, USA) were filled with 0.2 ml aliquots of the diluted cultures. Wells with only TSB served as control to check sterility of media. These tissue culture plates were incubated for 24 hours at 37°C. After incubation contents of each well was removed and washed four times with 0.2 mL of phosphate buffer saline (PBS) to remove free-floating 'planktonic' bacteria.

Biofilms formed in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off with deionized water and dried. Biofilm on all side wells get uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Adherence and the biofilm status were classified as shown by Christensen, *et al* [24] and Mathur, *et al*.[25] All the experiments were performed thrice in triplicate.

After standardizing these methods and confirming the biofilm formation of the clinical isolates, further experiments were done to evaluate the potential of the test drugs against the organisms in the biofilm state. TCP method was selected for the same. For this experiment along with standard strains, two of the clinical isolates were selected which were strongly adherent and thus indicative of high biofilm status. These included *Staphylococcus epidermidis* (coagulase negative) isolated from urine and *Staphylococcus aureus* isolated from blood.

The concentration of organisms (either clinical isolates like *Staphylococcus epidermidis* or *Staphylococcus aureus* or Standard strains) was adjusted to 10^6 organisms. For each well 0.2 ml aliquots of this diluted culture were added. No addition was made in TSB wells and they serve as media control. The plate was incubated at 37°C for 24 hours. After incubation either sterile PBS (negative control) or test drug concentrations were added (10µl each) and plates were then incubated for an additional 24 hours. After incubation, the same procedure as mentioned for TCP method was followed and OD reading obtained.

To evaluate ability of drugs to prevent biofilm formation by Staphylococci in the biofilm state, the same procedure was followed except that the drugs

were added during inoculation of the bacterial suspension and OD reading was taken after 24 hours. All experiments were performed in triplicate and repeated three times.

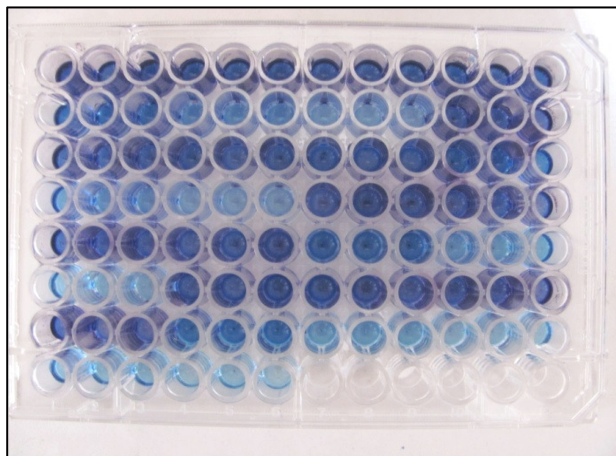

Fig. 3: Tissue culture plate method. Staphylococci incubated with various concentrations of Terminalia chebula extract

Analysis of data and statistical tests

The comparison between OD readings of drug concentrations with that of vehicle control was done by ANOVA followed by post hoc Tukey's test. OD of the most effective concentration of TCh with that of combination for a given organism was done using unpaired t test; $p < 0.05$ was considered significant.

Results:

Of the 100 isolates of Staphylococci screened for biofilm formation, TCP method detected 14 isolates (6 of *S. aureus* and 8 of CONS) as biofilm forming strains but 5 of them were undetected by Congo red agar method and 2 by the tube method. Only 9 of 100 strains of Staphylococci were found to be biofilm forming.

Table 2 and 3 shows the effects of TCh and Tr on the Staphylococci in biofilm state, respectively. There was no statistical difference amongst OD values observed after incubation of various concentrations of TCh and Tr with Staphylococci in biofilm state and the vehicle control. As shown in Table 4, the higher concentrations of TCh (200, 400 and 800 µg\ml) showed a reduction in OD values in a concentration dependent manner, 800µg/ml being the most effective concentration for all the three organisms.

Table 5 shows the effects of incubation of various concentrations of Tr on biofilm formation by Staphylococci. There was no statistically significant difference amongst OD values of different concentrations of Tr and those of vehicle control incubated with the organisms.

As seen from Table 6 none of the concentrations of combination showed a significantly different OD value than the control group for biofilm state. However, it showed a concentration dependent significant reduction in OD values, when tested for biofilm prevention (Table 7). The concentration of 400 µg/ml of TCh + 200 µg/ml of Tr showed the maximum lowering of OD values which was significantly lower than effect of 800 µg/ml of TCh (Table 8).

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Table 2 : OD Values obtained after incubation of TCh with stap. in biofilm state

Concentrations (µg\ml)	**OD values of ATCC 35984- *Staph. epidermidis* (n=18)**	**OD values of clinical isolate- *Staph. Epidermidis* (n=9)**	**OD values of clinical isolate- *Staph. Aureus* (n=9)**
PBS (Vehicle control)	1.22±0.03	1.24±0.05	1.06±0.01
TCh 25	1.32±0.03	1.30±0.04	0.99±0.05
TCh 50	1.31±0.04	1.23±0.04	1.01±0.09
TCh 100	1.26±0.02	1.37±0.02	0.97±0.02
TCh 200	1.29±0.02	1.31±0.04	1.02±0.03
TCh 400	1.35±0.03	1.34+0.02	1.03±0.01
TCh 800	1.33±0.08	1.21±0.01	1.03±0.02

OD= Optical density, PBS=Phosphate buffer saline, TCh= *Terminalia chebula*. Figures represent Mean ± SD. All the values are above 0.24.

One way ANOVA with post hoc Tukey's test: No significant intergroup difference (PBS *vs TCh*)

Table 3: OD values obtained after incubation of Tr with Staphylococci in biofilm state

Concentrations (µg\ml)	**OD values of ATCC 35984- *Staph. epidermidis* (n=18)**	**OD values of clinical isolate- *Staph. Epidermidis* (n=9)**	**OD values of clinical isolate- *Staph. Aureus* (n=9)**
PBS (Vehicle control)	1.38±0.03	1.33±0.08	1.07±0.02
Tr 25	1.34±0.02	1.31±0.07	1.02±0.05
Tr 50	1.31±0.03	1.26±0.04	1.03±0.09
Tr 100	1.27±0.02	1.29±0.03	1.02±0.02
Tr 200	1.33±0.01	1.31±0.05	1.02±0.05
Tr 400	1.35±0.03	1.32±0.02	1.03±0.01
Tr 800	1.33±0.07	1.31±0.01	1.03±0.06

OD= Optical density, PBS=Phosphate buffer saline, Tr= Trikatu. Figures represent Mean ± SD. All the values are above 0.24. One way ANOVA with post hoc Tukey's test: No significant intergroup difference (PBS *vs* Tr)

Table 4 :

Concentrations (µg\ml)	**OD values of ATCC 35984- *Staph. epidermidis* (n=18)**	**OD values of clinical isolate- *Staph. Epidermidis* (n=9)**	**OD values of clinical isolate- *Staph. Aureus* (n=9)**
PBS (Vehicle control)	1.29+0.06	1.42+0.18	1.05+0.06
TCh 25	1.34+0.03	1.34+1.14	1.02+0.07
TCh 50	1.29+0.03	1.33+0.15	1.03+0.03
TCh 100	1.25+0.1	1.28+0.14	1.02+0.03
TCh 200	0.86+0.36$^{\$\$}$	0.97+0.44$^{\$}$	0.41+0.02$^{\$\$}$
TCh 400	0.57+0.2$^{\$\$}$	0.48+0.16$^{\$\$}$	0.35+0.02$^{\$\$}$
TCh 800	0.30 ± 0.1$^{\$\$}$	0.23±0.008$^{\$\$,NS}$	0.32±0.09$^{\$\$,NS}$

OD= Optical density, PBS=Phosphate buffer saline, TCh= *Terminalia chebula*. Figures represent Mean ± SD. OD values for non-biofilm producing ATCC 12228 Staphylococci: 0.093±0.003

One way ANOVA with post hoc Tukey's test: \$$p<0.01$, \$\$$p<0.001$ (PBS *vs TCh*) , NS Not significant for ATCC strain incubated with 800 µg/ml of TCh vs clinical isolates incubated with 800 µg/ml of TCh

Table 5: OD values obtained after incubation of Tr with Staphylococci prior to biofilm formation

Concentrations (µg\ml)	**OD values of ATCC 35984- *Staph. epidermidis* (n=18)**	**OD values of clinical isolate- *Staph. Epidermidis* (n=9)**	**OD values of clinical isolate- *Staph. Aureus* (n=9)**
PBS (Vehicle control)	1.18±0.02	1.25±0.01	1.05±0.01
Tr 25	1.08±0.05	1.29±0.04	1.03±0.02
Tr 50	1.16±0.02	1.25±0.01	1.04±0.02
Tr 100	1.15±0.01	1.25±0.02	1.06±0.01
Tr 200	1.29±0.03	1.31±0.06	1.01±0.02
Tr 400	1.01±0.01	1.35±0.01	1.02±0.02
Tr 800	1.06±0.01	1.25±0.01	1.02±0.01

OD= Optical density, PBS=Phosphate buffer saline, Tr= Trikatu. Figures represent Mean ± SD. All the values are above 0.24. OD values for non-biofilm producing ATCC 12228 Staphylococci: 0.92±0.03.

One way ANOVA with post hoc Tukey's test: No significant intergroup difference (PBS *vs* Tr)

Table 6: OD values obtained after incubation of combinations of various concentrations of TCh and Tr with Staphylococci in biofilm state

Concentrations (µg\ml)	**OD values of ATCC 35984- *Staph. epidermidis***	**OD values of clinical isolate- *Staph. Epidermidis***	**OD values of clinical isolate- *Staph. aureus***
PBS (Vehicle control)	1.34±0.02	1.30±0.08	1.02±0.05
TCh+ Tr (25 +12.5)	1.41±0.04	1.26±0.04	1.01±0.09
TCh+ Tr (50 + 25)	1.46±0.02	1.27±0.03	1.07±0.02
TCh+ Tr (100 + 50)	1.39±0.015	1.31±0.07	1.02±0.04
TCh+ Tr (200 + 100)	1.35±0.03	1.33±0.02	1.03±0.01
TCh+ Tr (400 + 200)	1.33±0.08	1.31±0.01	1.06±0.02

N=9; OD= Optical density, PBS=Phosphate buffer saline, TCh= *Terminalia chebula,* Tr= Trikatu. Figures represent Mean ± SD. All the values are above 0.24.
One way ANOVA with post hoc Tukey's test: No significant intergroup difference (PBS *vs* TCh +Tr)

Table 7: OD values obtained after incubation of combinations of various concentrations of TCh and Tr with Staphylococci prior to biofilm formation

Concentrations (µg\ml)	**OD values of ATCC 35984- *Staph. epidermidis***	**OD values of clinical isolate- *Staph. Epidermidis***	**OD values of clinical isolate- *Staph. aureus***
PBS (Vehicle control)	1.32+0.07	1.33+0.03	1.07+0.03
TCh+ Tr (25 +12.5)	1.24+0.1.	1.244+0.03	1.06+0.05
TCh+ Tr (50 + 25)	1.33+0.08	1.34+0.09	0.53+0.03
TCh+ Tr (100 + 50)	0.53+0.11$^{\$}$	0.53+0.36$^{\$}$	0.39+0.01$^{\$}$
TCh+ Tr (200 + 100)	0.35+0.03$^{\$}$	0.36+0.19$^{\$}$	0.29+0.009$^{\$}$
TCh+ Tr (400 + 200)	0.17± 0.12$^{\$}$	0.23±0.007$^{\$}$	0.24±0.04$^{\$}$

N= 9; OD= Optical density, PBS=Phosphate buffer saline, TCh= *Terminalia chebula,* Tr= Trikatu. Figures represent Mean ± SD. All the values are above 0.24. One way ANOVA with post hoc Tukey's test: \$p<0.001 for PBS *vs* TCh +Tr; NS Not significant for ATCC strain incubated with TCh+ Tr (400 + 200 µg/ml) vs clinical isolates incubated with TCh+ Tr (400 + 200 µg/ml)

Table 8: Comparison of effects of 800 µg/ml of *TCh* and of combination of 400 µg/ml of TCh and 200 µg/ml of Tr on biofilm formation

Concentrations (µg\ml)	OD values of ATCC 35984- *Staph. Epidermidis* (n=18)	OD values of clinical isolate- *Staph. Epidermidis* (n=9)	OD values of clinical isolate- *Staph. Aureus* (n=9)
TCh (800 µg/ml)	0.30 ± 0.1	0.23±0.008	0.32±0.09
TCh + Tr (400+ 200µg/ml)	0.17± 0.12*	0.23±0.007NS	0.24±0.04$

OD= Optical density, PBS=Phosphate buffer saline, TCh= *Terminalia chebula,* Tr= Trikatu. Figures represent Mean ± SD.

Mann Whitney test; * $p<0.05$ vs TCh 800 µg/ml

Uunpaired 't' test; $ $p<0.05$, NS=Not significant vs TCh 800 µg/ml

Discussion:

The present study was undertaken to detect the anti-biofilm potential of Ayurvedic drugs. Since the organisms in biofilm state are less susceptible to killing, efforts are being directed towards approaches to prevent biofilm formation. Prevention of biofilm formation depends on ability of an antibacterial agent to decrease colonization.[26] It was therefore decided to undertake experiments to find out whether TCh and Tr (used individually) or in combination can prevent the formation of biofilm, along with activity on biofilm state.

Two clinical isolates tested positive by all the 3 methods along with the biofilm producing ATCC 35984 strain of *Staphylococcus epidermidis* were selected as test organisms. The TCP method was selected for the assay because this method is a gold standard. It is reported to be accurate, objective and reproducible having high sensitivity and specificity with no subjective errors.[25,7,27]

As seen from the Results, none of the selected concentrations of either TCh or Tr decreased the OD values of biofilms produced by all the 3 test organisms. This finding indicates that the selected plant drugs are not capable of disrupting the formed biofilms.

No study has been reported for TCh or Tr prior to this. Hence the results of the present study cannot be compared. However, it has been reported that xanthorrhizol isolated from *Curcuma xanthorrhiza*,[10,13] Salvipisone diterpenoids from *Salvia sclarea*,[11] Nidus vespae extract[12] have shown anti-biofilm properties. Many of these are active principles as against the aqueous extract used by us. Perhaps instead of using extract of TCh, use of the individual active principles in TCh like chebulic acid, gallic acid, Chebulagic acid, Ellagic acid, Chebulinic acid would have given positive results. Their concentrations in the extract are likely to be less and may be the reason for absence of effect.

TCh in concentrations of 200, 400 and 800 µg/ml was found to prevent biofilm formation when compared with vehicle control. The prevention of biofilm formation may be attributed to reduction in colonization, which may be secondary to reported antibacterial effects of TCh.[5] The finding that aqueous extract of TCh when used as a mouth rinse serves as an effective anti-caries agent corroborates findings of the present study as many of the organisms responsible for caries are biofilm forming.[28]

Tr, on the other hand, did not have any effect on biofilm formation by Staphylococci. In one study,[29] using disc diffusion technique various extracts of Tr have been shown to posses antibacterial activity. However, the concentration used was very high as compared to present study. At the same time the TCP method used by us is more sensitive than disc diffusion method. Kaveri, *et al* [30] have also reported that Tr does not exert any anti-bacterial effects per se.

As for the individual agent, none of the concentrations of the combination showed any effect on *Staphylococci* in biofilm state. This finding rejected our hypothesis that Tr being *yogavahi* can help in penetration of *TCh*, which can then act on the organisms. However, it is known that Tr facilitates passage of drugs from different groups across biological membranes, which differ from the exopolysaccharide exopolymer of biofilm with structural and functional properties.[2] The findings of the present study suggest that Tr is unable to penetrate the tough biofilm membrane. A second explanation would be that TCh may not be capable of eradicating bacterial load in biofilm as the organisms are slowly growing in biofilm. Hence another way to evaluate the effect of Tr is to combine it with an antibiotic which does not require bacteria in multiplying state (e.g. rifampicin, isoniazide, pyrazinmide) to exert its effect. However, this was beyond the scope of present study.

The combination against formation of biofilm formation showed a dose dependent inhibition in biofilm formation, the most effective concentrations being 400 µg/ml TCh and 200 µg/ml Tr.

The combination at the dose of 400 µg/ml of TCh and 200 µg/ml of Tr offered better protection against biofilm formation as compared to 800 µg/ml of TCh for *S.aureus* and ATCC strain of *S. epidermidis.* As the concentration of TCh in the combination was lower than when used alone, our study proves that the combination exerts synergism. This is a novel finding. This synergistic effect can be explained on the basis of additive antibacterial effect. However, as mentioned earlier the evidence for antibacterial effect of Tr is equivocal. Secondly in our study Tr has not reduced bacterial colonization as indicated by absence of its effect on biofilm formation. Therefore there is a possibility that it may be enhancing the antibacterial effect of TCh by increasing its diffusion across bacterial cells. This can be speculated on the basis of earlier studies wherein piperine (active principle in Tr) has enhanced the transcription inhibitory effects of rifampicin in mycobacteria.[31] There is also a possibility that it may be preventing its degradation by organisms or creating the adverse environment for production of biofilm. These possibilities need to be explored through future studies to understand the mechanism of this beneficial drug-drug interaction.

Antibiotics attain a higher concentration when applied to wounds[32] locally and serve as preventive agents against biofilm formation. In Ayurveda, TCh has been advocated for local use for conjunctivitis, for gargling in diseases of throat and mouth and as

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cleanser for wounds.[19] Also its effectiveness against caries have been demonstrated both, in *in vitro* and clinical studies.[28] The present study provides scientific rationale for these uses of TCh.

Further clinical applications of TCh to prevent growth of bacteria and consequently biofilm formation need to be explored through appropriately designed clinical studies having adequate sample size. It is also essential to explore further, the synergistic effect of combination which would reduce the dose of the principle drug. Though the present study did not prove the concept of *yogvahkatva* by Tr when used in combination with *TCh*, in the light of current evidences appropriate combinations with antibiotics need to be developed for evaluation of this important concept of our traditional medicine, and Ayurveda.

Conclusion:

Though the study drugs did not show any effect on Staphylococci in biofilm state, TCh and combination could prevent biofilm formation. Tr enhanced the biofilm formation preventing property of TCh, thus showing synergism. Further evaluation with the various constituents of these plant drugs is required.

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