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Bioprospecting of *Terminalia arjuna* stem bark and its flavonoids for antimicrobial and anti-biofilm potential

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ABSTRACT

The formation of biofilms play crucial role in development of multiple drug resistance (MDR) in microorganisms and makes treatment more complex. Therefore, it is necessary to employ a strategy that effectively inhibits the formation of biofilm to treat MDR microorganisms. In the present studies, anti-biofilm activity of the methanolic extract of Terminalia arjuna stem bark (MeOH-TASB) and its purified flavonoids was evaluated against human pathogenic bacteria Staphylococcus aureus and Escherichia coli. The MeOH-TASB and its constituent flavonoids (baicalein and quercetin) showed potent antibacterial activity at relatively higher concentrations and inhibited biofilm formation at lower concentrations in both S. aureus and E. coli. Apart from inhibiting the formation of biofilm, MeOH-TASB, baicalein and quercetin disrupted pre-formed biofilms. These results merit further investigation of the potential of MeOH-TASB, baicalein and quercetin for the treatment of bacterial infections.

Keywords: Bioprospecting; *Terminalia arjuna*; Flavonoids; Antibacterial; Anti-biofilm

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INTRODUCTION

Infectious diseases caused by bacterial and fungal pathogens are among the many challenges to health and have a significant impact on the human being [1]. These are the major cause of morbidity and mortality in both developed as well as developing countries [2]. In earlies, antibiotics helped to treat infections effectively; however, inappropriate overuse/misuse of these antibiotics leads to development of multi-drug resistance (MDR) in the pathogens [3]. Development of MDR which is a major threat causes increase in the severity and complexity of the disease that can turn into life threatening one [4]. Several studies up till now indicated that the ability of bacteria to form surface adhered polymicrobial communities known as biofilms contribute largely to the development of MDR [5]. The biofilms formed by bacterial and fungal pathogens are of main concern because they impart up to 1000 times more resistant to antibiotics than planktonic cells [6]. Therapies that could target biofilm effectively are scanty due to inherent ability of biofilm of being resistant to antibiotics [7]. Thus, researchers are in continued search of novel agents that can combat MDR pathogens by inhibiting the formation of biofilm and further help in the reduction of development of drug resistance by lowering the selection pressure [8].

Several researchers have reported array of broad spectrum antibiofilm agents which are synthetic compounds, natural products or nanomaterials [9-11]. Among these, natural products are of more interest due to their safe nature and time tested traditional use. The traditional medicinal plants, those have been already in folkforine use to treat ailments/infections would be a good starting point to find natural products [12]. Among these, *Terminalia arjuna* (Family- Combretaceae) is most versatile medicinal plant commonly known as Arjuna having broad spectrum of biological activities. Many useful phytoconstituents present in the bark of *T. arjuna*; offer it antioxidant, anti-dysenteric, antipyretic, astringent, cardiotonic, lithotriptic, anticoagulant, hypolipidemic, antimicrobial and antiuremic properties [13]. In our previous work, we have reported that the *T. arjuna* stem bark methanolic extract is rich in polyphenols as well as flavonoids [14]. Many researchers have reported antipathogenic as well as antibiofilm

potential of polyphenols and flavonoids [1, 15-17). However, till date no report exist on antibiofilm activity of methanolic extract of *T. arjuna* stem bark and its constituent flavonoids. Therefore, the present study was undertaken to investigate, for the first time, the *in vitro* anti-biofilm potential of flavonoid rich *T. arjuna* stem bark extracts and its flavonoids (Baicalein and Quercetin) against human bacterial pathogens such as *E. coli* and *S. aureus*.

EXPERIMENTAL SECTION

Chemicals

Luria Bertani broth and Luria Bertani agar were purchased from Hi media Ltd. (India). Methanol, ethanol and DMSO used were of analytical grade and purchased from Merck Ltd (India). Crystal violet was purchased from Sigma Ltd (India).

Preparation of extract and purification of flavonoids

The methanolic extract of *T. arjuna* stem bark (MeOH-TASB) was prepared according to method described by Chaudhari and Mahajan [18]. Isolation of abundant flavonoids *viz.* baicalein (Bai) and quercetin (Que) was achieved (data communicated elsewhere).

Assessment of antibiofilm potential

Bacterial pathogens and their growth conditions

The equivalents of bacterial pathogens *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538) were procured from MTCC Chandigarh, India. These cultures were maintained on nutrient agar slants at 4 ºC.

Preparation of inoculum

Active cultures were prepared by inoculating a loopful of cells from the stock culture slants to Luria Bertani broth (LBB) tubes that were then incubated without agitation for 24 h at 37 ºC. The cultures were diluted with fresh LBB to achieve culture densities equivalent to 0.5 McFarland's standard (giving 10^5–10^6 CFU/mL).

Antibacterial activity

Antibacterial activity was determined by agar well-diffusion method [19]. Freshly prepared diluted bacterial pathogenic cultures were spreaded over LB agar plates by using sterile cotton-tipped swab. With the help of sterile cork borer; holes of 8 mm diameter were bored aseptically. The holes were inoculated separately with 100 µl filter (0.2 µM pore size) sterilized solutions of different concentrations MeOH-TASB, Bai and Que prepared in DMSO. Then plates were allowed to stand for 10 minute at 4 ºC in the refrigerator for proper diffusion of the test sample. Further, the plates were incubated at 37 ºC for 24 h. After incubation, the plates were observed for antibacterial activity, which was measured in terms of the inhibition zone (in mm). Deionized distilled water was used as a negative control. The antibiotic streptomycin (50 µg/mL) was used as standard antibiotic.

Determination of minimum inhibitory concentration (MIC)

The lowest concentration of the MeOH-TASB, Bai, Que and standard antibiotic streptomycin that inhibits the growth of the test cultures (human pathogenic bacteria) was defined as MIC and determined by the standard tube-dilution method by following CLSI guidelines [20]. The only modification done was use of LBB medium instead of Brain heart infusion medium. The MIC was taken as the lowest concentration of test samples that did not permit any visible growth. Each experiment was repeated thrice.

Crystal violet antibiofilm assay

The Antibiofilm activity of MeOH-TASB, Bai and Que was evaluated by using Crystal violet microtiter plate assay [1]. These pathogenic cultures were inoculated to polystyrene microtiter plates (96 well) containing fresh liquid medium having various concentrations of tests and subsequently incubated at 37 ºC. After 24 h of incubation, the content of the each well was discarded using micropipette to remove unattached cells, and the wells were washed three times with sterile distilled water. The attached cells were stained with 0.4 % crystal violet for 10 to 15 minute. Further, the crystal violet was removed and the wells were thoroughly washed three times with sterile distilled water. The cells associated with the crystal violet were destained with absolute ethanol for 30 minute and the absorbance was recorded at 595 nm to quantify antibiofilm activity of the MeOH-TASB, baicalein and quercetin using formula:

$$\text{Biofilm inhibition percentage} = [(\text{ACT}_{595\text{ nm}} - \text{AT}_{595\text{ nm}})/\text{ACT}_{595\text{ nm}}] \times 100$$

Where, $\text{ACT}_{595\text{ nm}}$- absorbance of control; $\text{AT}_{595\text{ nm}}$- absorbance of test

The lowest concentration that produced maximum (at least 90%) biofilm inhibition was considered to be the biofilm inhibitory concentration (BIC).

The disruption of biofilms

The pre-formed biofilms were treated with MeOH-TASB, Bai and Que in order to evaluate their potential to disrupt the already established biofilms [21]. Biofilms of *E. coli* and *S. aureus* were grown separately in the wells of a 96 well polystyrene plate. After incubation, the wells were washed with sterile distilled water. The MeOH-TASB, Bai and Que were then added to each well at their BIC containing fresh medium and further incubated for 24 h at 37 °C. Controls without MeOH-TASB, Bai and Que were run concurrently. After incubation, the wells were washed, stained with 0.4% crystal violet, destained, and absorbance was measured at 595 nm as above. The percentage of biofilm disruption was calculated using formula;

$$\text{Biofilm disruption percentage} = [(\text{ACT}_{595\ \text{nm}} - \text{AT}_{595\ \text{nm}})/\text{ACT}_{595\ \text{nm}}] \times 100$$

Where, $\text{ACT}_{595\ \text{nm}}$- absorbance of control; $\text{AT}_{595\ \text{nm}}$- absorbance of test

These experiments were performed three times, with replicates of six, and average values were calculated.

The effect on the architecture of the biofilm

The architecture of biofilm of *S. aureus* and *E. coli* were studied microscopically in the presence and absence of MeOH- TASB, Bai and Que at their BICs [1]. Biofilms were grown on 1 cm^2 glass slides placed in the wells of the 12 well tissue culture polystyrene plates. Following incubation, the slides were washed three times with distilled water and stained with crystal violet. After drying, the slides were examined under a microscope. Images were acquired, with externally attached Sony camera (13 mega pixels with 5X zoom).

Statistical analysis

All experiments were carried out in triplicates. The data was analyzed by One Way ANOVA followed by Fisher's LSD test for significant differences using Minitab-16.1.1.0 software. Graphs were plotted using Origin 8.1 software.

RESULTS AND DISCUSSION

Antibacterial activity

Antibacterial activity of MeOH-TASB, Bai and Que evaluated against *E. coli* and *S. aureus* by agar well diffusion assay is shown in Figure 1, 2 and 3 respectively. Also the antibacterial activity measurements in terms of zone of inhibition are summarized in Table 1. All three test samples showed inhibitory effect on both *E. coli* and *S. aureus* in agar well diffusion method, and their inhibitory effects increased in concentration dependent manner. The Que showed more inhibitory activity against *E. coli* and *S. aureus* both as compared to Bai. The *E. coli* was more sensitive to MeOH-TASB, Bai and Que as compared to *S. aureus* which could be due to the structural differences in their cell wall.

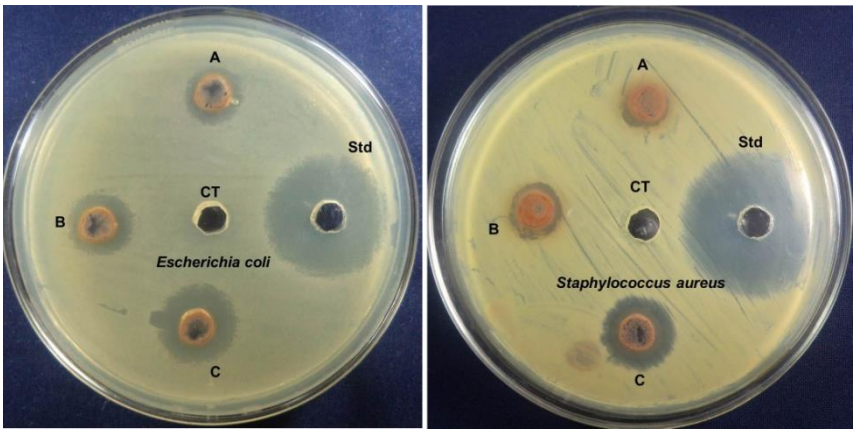

Figure 1: Antibacterial activity of methanolic extract of *T. arjuna* stem bark against *E. coli*and *S. aureus* (A-5, B-10, C-15 mg/ml, CT-DMSO and Std- streptomycin 0.05 mg/ml)

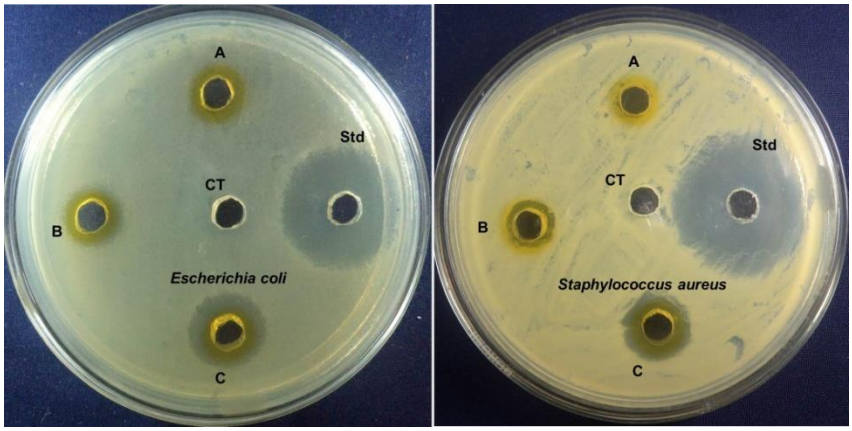

Figure 2: Antibacterial activity of baicalein against *E. coli* and *S. aureus* (A-0.5, B-1.0, C-1.5 mg/ml, CT- DMSO and Std- streptomycin 0.05 mg/ml

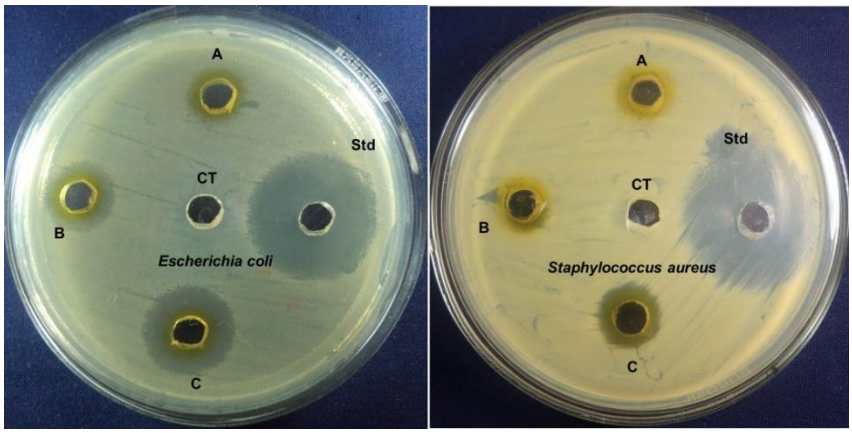

Figure 3: Antibacterial activity of quercetin against *E. coli*and *S. aureus* (A-0.5, B-1.0, C-1.5 mg/ml, CT- DMSO and Std- streptomycin 0.05 mg/ml

Table 1: Antibacterial activity ofMeOH-TASB, baicalein and quercetin against *Escherichia coli* and*Staphylococcus aureus* evaluated by agar well diffusion assay

Sr. No.	Test sample	Concentration used mg/ml	Zone of inhibition measured in mm*	
			Escherichia coli	*Staphylococcus aureus*
1	MeOH-TASB	5.00	12.33 ± 0.58	9.67 ± 0.58
		10.00	15.67 ± 0.58	14.33 ± 0.58
		15.00	18.67 ± 1.15	16.67 ± 1.15
2	Baicalein	0.50	ND	ND
		1.00	11.33 ± 0.58	10.00 ± 0.00
		1.50	19.00 ± 1.00	15.00 ± 1.00
3	Quercetin	0.50	10.33 ± 0.58	ND
		1.00	16.67 ± 0.58	12.33 ± 0.58
		1.50	22.33 ± 1.15	16.00 ± 1.00
4	Streptomycin	0.05	29.33 ± 1.53	34.67 ± 1.15

*ND- not detected, Streptomycin was used as a standard, * Zone of inhibition includes the diameter of well (8 mm). All values are expressed as mean ± standard deviation (n=3).*

Minimum inhibitory concentration (MIC)

The MIC of MeOH-TASB, Bai and Que determined by broth macro dilution assay against *E. coli* were 2.50, 0.25 and 0.25 mg/mL, respectively and that for *S. aureus* were 5.0, 0.50 and 0.25 mg/mL, respectively. The higher MIC values were observed for *S. aureus* as compared to *E. coli* which supported the results observed in agar well diffusion assay.

Antibiofilm assay

The anti-biofilm potential of MeOH-TASB, Bai and Que at different concentrations of these tests was measured against *E. coli* and *S. aureus* (Figure 4, 5 and 6). The antibiofilm activity of MeOH-TASB, Bai and Que was significantly ($p \leq 0.05$) increased with increase in their concentration up to certain limit, beyond this concentration there was no significant increase ($p \leq 0.05$) in their antibiofilm potential. This concentration limit of respective test

was considered as biofilm inhibitory concentration (BIC). The BICs of MeOH-TASB, Bai and Que for *E. coli* were observed to be 0.500, 0.050 and 0.050 mg/mL respectively and those for *S. aureus* were 0.250, 0.050, and 0.050 mg/mL, respectively. There was no significant difference ($p \leq 0.05$) in the antibiofilm potential of Bai and Que against both organisms for the concentration more than BIC of test sample.

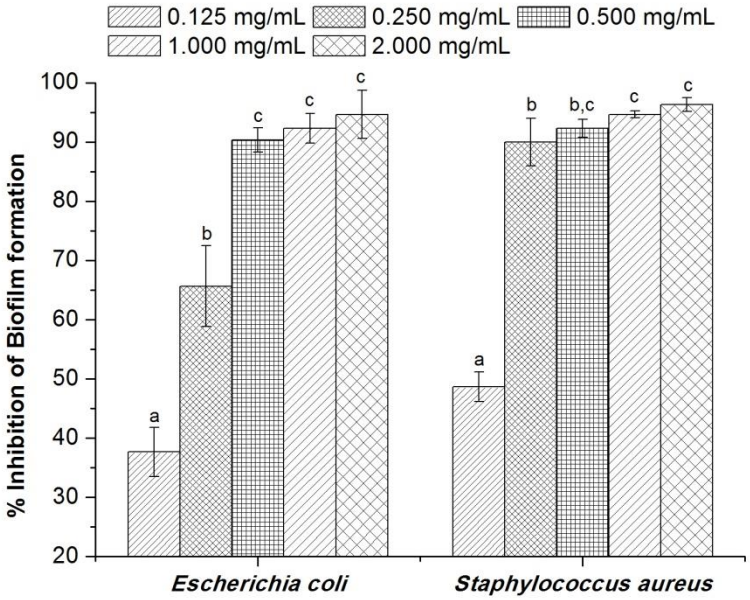

Figure 4: The effect of MeOH-TASB on the formation of bacterial biofilms (*E. coli* and *S. aureus*). In a group the bars which share common letter above it indicates, values (means) are not significantly different from each other at $p \leq 0.05$ by Fischer LSD test

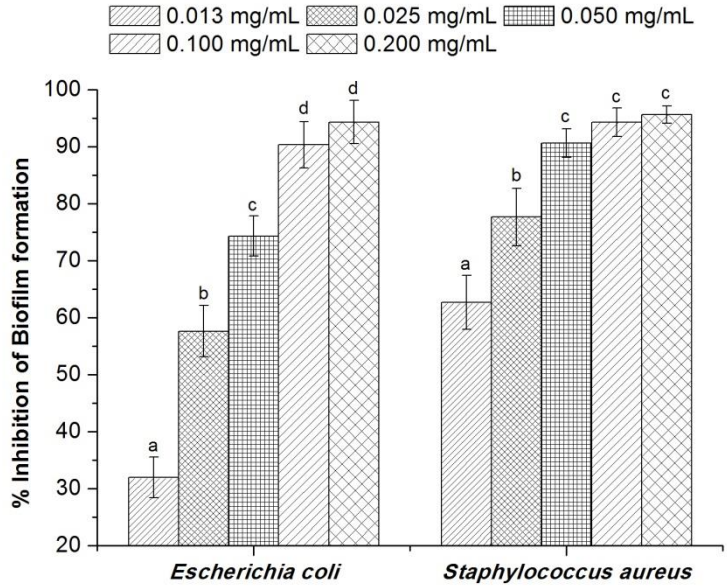

Figure 5: The effect of Baicalein on the formation of bacterial biofilms (*E. coli* and *S. aureus*). In a group the bars which share common letter above it indicates, values (means) are not significantly different from each other at $p \leq 0.05$ by Fischer LSD test

Namasivayam and Roy [22] reported antibiofilm potential of several medicinal plants such as *Azadirachta indica*, *Vitex negundu*, *Tridax procumbens and Ocimum tenuiflorumi* against *E. coli* and the results of the present investigation are also in good agreement. The mechanism of *E. coli* biofilm formation is complex however the inhibition of extracellular polymeric substances (EPS) and curli production are the well understood reasons for the inhibition biofilm formation in *E. coli* [23]. The curli are adhesive fimbrial structures of varying lengths with 4-7 nm width, appearing as extracellular fibers possessing the characteristics of amyloid fibers, produced by enterobacteriaceae members, including *E. coli* tends to form large aggregates [24]. The plant extracts and its constituent flavonoids are known to inhibit formation of curly and production of EPS in *E. coli* [25]. Therefore, in the present investigations, antibiofilm activity of MeOH-TASB, Bai and Que against *E. coli* could be attributed to their possible role in the inhibition of the EPS and curli production in *E. coli*.

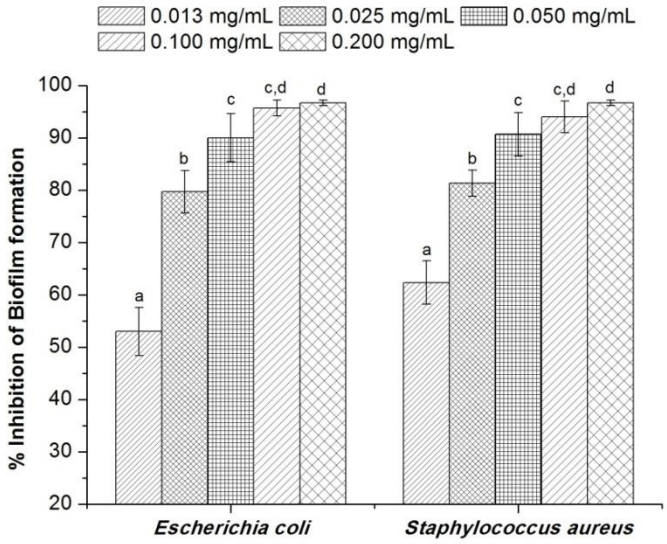

Figure 6: The effect of Quercetin on the formation of bacterial biofilms (*E. coli* and *S. aureus*). In a group the bars which share common letter above it indicates, values (means) are not significantly different from each other at $p \leq 0.05$ by Fischer LSD test

The mechanism governing the formation of biofilms by *S. aureus* cells is also complex process and down-regulation of the expression of the *ica* gene is the best understood mechanism in the inhibition of biofilm formation [26, 27]. According to Lee et al., natural compounds like flavonoids down regulate expression of *ica* gene [28]. Therefore, inhibition of biofilm formation in *S. aureus* by MeOH-TASB, Bai and Que can be attributed to the possible role of these tests in the repression of *ica* gene.

The disruption of biofilms

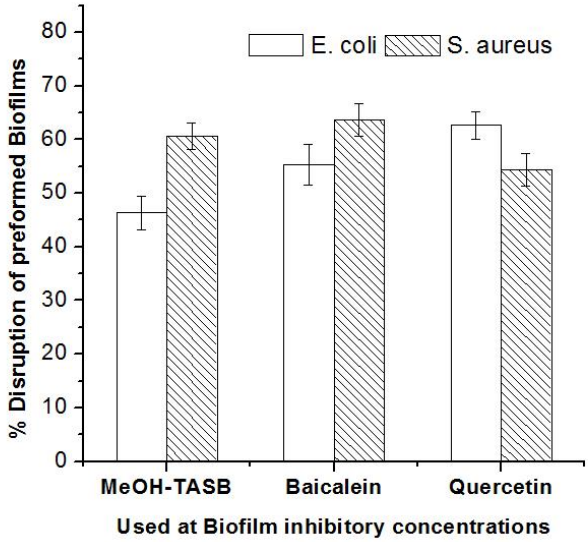

Figure 7: The potential of the MeOH-TASB, baicalein and quercetin to disrupt pre-formed biofilms of *E. coli* and *S. aureus*

The MeOH-TASB, Bai and Que showed potent ability of disruption of already established biofilms of *E. coli* and *S. aureus*. The MeOH-TASB and Baicalein showed higher percentage disruption of *S. aureus* biofilm than *E. coli* whereas, vice versa was observed in case of quercetin (Figure 7). The result of this experiment illustrate the remarkable ability to disrupt already established (mature) biofilms of *E. coli* and *S. aureus* by MeOH-TASB is mainly due to the presence of flavonoids, baicalein and quercetin. This is verifying with the result of antibiofilm activity of flavonoid rich fraction of *Moringa oleifera* seed coat [29]. Similarly, phenolic compounds of pomegranate extract were also reported to inhibit the formation of biofilms as well as disruption of preformed biofilms of *E. coli*, *S. aureus* and methicillin resistant *S. aureus* [1].

The architecture of the biofilm

The effects of MeOH-TASB, Bai and Que on the architecture of biofilms formed by *E. coli* and *S. aureus* were studied by light microscopic examination under 100X objective lens so as to get 1000X magnification.

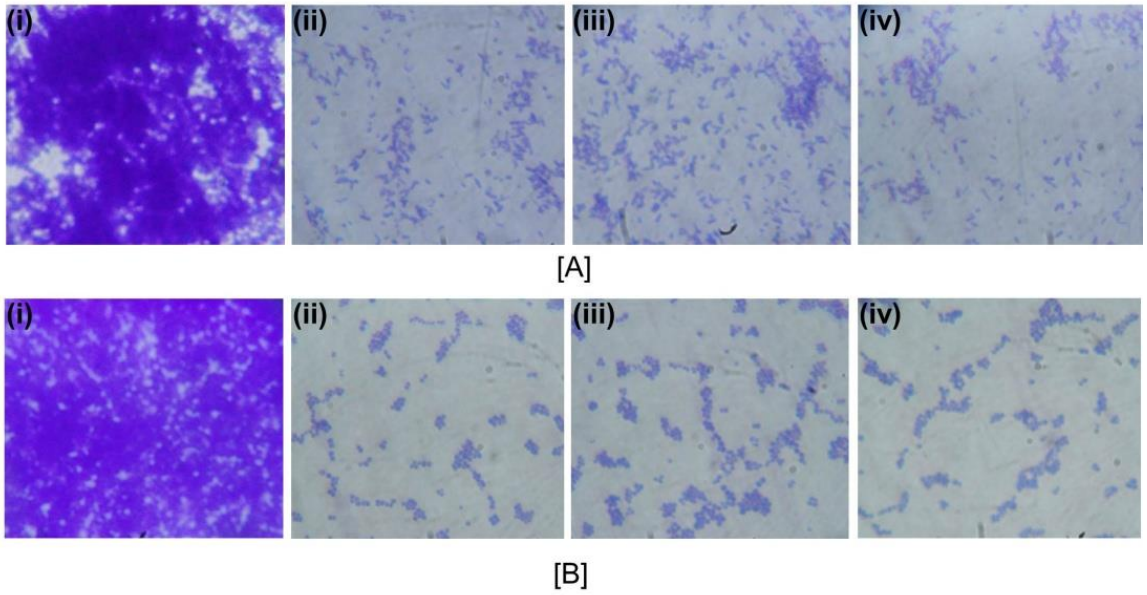

Figure 8: Architecture of (A) *E. coli* biofilms (B) *S. aureus* biofilms: (i) untreated control; (ii) treated with MeOH-TASB; (iii) Bai and (iv) Que

The untreated cells of *E. coli* and *S. aureus* formed highly dense and uniform biofilms on the surface of glass slide whereas, cells in presence of MeOH-TASB, Bai and Que at their BIC exhibited a notable reduction in the number of bacteria adhered to glass surface and didn't formed biofilms. The presence of MeOH-TASB, Bai and Que resulted in adhesion of comparatively very few bacterial cells to the glass surface that too are in very small sized aggregates, which were reduced to small clusters or even single cells at some places (Figure 8A and 8B). These observations supported the results obtained by crystal violet antibiofilm assay.

CONCLUSION

The present studies demonstrate potential antibacterial and antibiofilm activity of *T. arjuna* stem bark and its constituent flavonoids. The results of the study show potential antibiofilm activity MeOH-TASB and its extracted flavonoids baicalein and quercetin as they are able to inhibit biofilm formation and also responsible for disrupting the already established biofilm. These results strongly support the notion that plants are important resource of biofilm inhibitors and useful to control biofilm-associated infections caused by *E. coli* and *S. aureus*. Further investigation on these findings by mechanistic approach could be helpful in the development of herbal drugs to combat against serious bacterial infections involving resistant biofilm.

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