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Antibiofilm Activity of the Methanolic Extract of Nutmeg Oil against *Bacillus subtilis*

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Abstract

Biofilm is formed by one or different types of microbes that can grow on different surfaces. Bacteria, fungi, and many protists can form biofilms. Biofilm formation requires cell to cell signaling mechanism which is defined as quorum sensing. In this work, we have studied about the antibiofilm activity of methanolic extract of nutmeg against Gram-positive *Bacillus subtilis* bacteria. Our test extract inhibited biofilm production as well as eradication of pre-formed biofilm in *B. subtilis*. Extracellular polymeric substance (EPS) is the biopolymer of microbial origin in which biofilm producing microbes are embedded. When test extract was treated with organisms producing biofilms it can also reduce the production of EPS of biofilm. All the quantitative analysis of this work concludes that methanolic extract of *Myristica fragrans* seed extract inhibited biofilm formation by 47%, eradicated the pre-formed biofilm by 28.5%, and inhibited EPS formation by 60%. All these quantitative tests were confirmed by microscopic analysis. All Our study concludes that *M. fragrans* seed extract can inhibit biofilm formation and can destruct preformed biofilm in Gram-positive bacteria.

Keywords: *Bacillus subtilis*, Biofilm, Extracellular polymeric substance, Minimum biofilm eradication concentration, *Myristica fragrans*, Quorum sensing

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Introduction

The system of quorum sensing involves stimuli and response to signals released by bacterial cells that are present in the population. Through these responses bacteria can regulate genes that are responsible for the pathogenicity. Quorum sensing is a cell-cell communication through which bacteria converse with the same species or other species. Quorum sensing is mediated by specific signals which are different in Gram-negative and Gram-positive bacteria. These signals are called autoinducers which increase in their number when number of bacteria in that population was increased. When the concentration of these signals reached to a particular threshold level, expression of target genes responsible for particular phenotype occurs. Signals involved in quorum sensing are different in Gram-negative and positive bacteria. Acyl Homoserine Lactones are the signaling molecules in Gram-negative bacteria. AIPs are the signaling molecules in Gram-positive bacteria.

In this study, we tested inhibition of biofilm and eradication of biofilm by nutmeg oil against *Bacillus subtilis*.[1] Biofilm is defined as a collection of microorganisms that attached to surfaces and encapsulated in Extracellular Polymeric Substance (EPS). Many bacteria, fungi, and protists form biofilms on different surfaces. Dental plaque formation is the common example of biofilms that forms on the surface of teeth. In general, many pathogenic bacteria are resistant to many drugs. These multidrug-resistant bacteria communicate between same species and different species and forms biofilms. This mechanism of cell to cell communication of bacteria is called quorum sensing. Quorum sensing controls several phenotypes in bacteria such as biofilm formation, virulence factor producton, and motility. Quorum sensing inhibition leads to inhibition of biofilm formation. Prolonged treatment is required for biofilm forming bacteria which may lead to development of antibiotic resistant bacteria.[2] Biofilm formation in bacteria helps them to protect from many therapeutic treatments. Heavy usage of antibiotics to treat infections caused by biofilm forming bacteria results into evolution of antibiotic-resistant bacteria. Therefore, there is need to invent new treatment strategies which target pathogenicity rather than inhibiting bacterial growth.

Biofilm formation in *B. subtilis* has been primarily observed in NCIB3610 isolate. PS-216 is a naturally competent isolate of *B. subtilis* that forms highly structured biofilms which is used to study about biofilms.[3]

From ancient days India is well known for Ayurvedic medicines to treat many contagions. Nutmeg is a dietary phytochemical which contain antimicrobial properties, used to treat digestive problems. Nutmeg is used as a dietary phytochemical supplement which is used as a remedy for many digestive problems and also assorted contagions. This suggests that the Nutmeg may contain phytochemicals which can hinder the quorum sensing system. *Myristica fragrans* is commonly known for its aromatic, aphrodisiac and curative properties. Nutmeg is used to cure many digestive problems. It also helps to lower blood pressure, help to detoxify the body and to stimulate the brain. Nutritionally Nutmeg is rich in Vitamins A and C, energy, proteins, carbs, and fiber. Nutmeg has a lot of applications in cosmetic-related industries. By increasing the blood pressure nutmeg can cure heart problems. Nutmeg is used

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as an ingredient in cough syrups, because it can also help to cure respiratory related problems.[4] When test samples were treated with bacterial biofilms; first, the topology of biofilm was disrupted and finally bacteria growth will be inhibited.[2]

Materials and Methods

Bacterial Growth and Culture Conditions

Test extract was used to test against Gram-positive *B. subtilis* bacteria. This strain was maintained at 37°C in Nutrient media.

Antimicrobials Used

Penicillin, chloramphenicol, and tetracycline antibiotics were used to test antibacterial activity.[5] Quercetin was used as positive control to test antibiofilm activity.[6]

Test extract

Nutmeg (seed of *M. fragrans*) was made into fine powder and extracted with Soxhlet extractor by using 95% methanol at 50°C.[7] Extracted methanolic solvent was concentrated using rotary evaporator at 50°C. This concentrated extract was stored at 4°C until use.

Table 1: Broth dilution assay of *Bacillus subtilis* at 600 nm

S. No.	*Test sample*	*OD values at 600 nm*					*P-value summary*
		10 mg/ml	*30 mg/ml*	*50 mg/ml*	*70 mg/ml*	*90 mg/ml*	
1.	Negative control			1.538±0.008			
2.	DMSO			1.287±0.0080.821±0.011			**
3.	Penicillin	0.821±0.011	0.754±0.006	0.693±0.0080	0.598±0.010.821±0.011	0.475±0.01	****
4.	Chloramphenicol	0.797±0.008	0.685±0.01	0.5±0.05	0.486±0.006	0.382±0.007	
5.	Tetracycline	0.678±0.008	0.5±0.01	0.464±0.009	0.362±0.007	0.257±0.003	
6.	Quercetin	1.16±0.005	1.06±0.06	0.925±0.015	0.802±0.007	0.662±0.002	
7.	*Myristica fragrans*	0.823±0.002	0.695±0.004	0.613±0.008	0.521±0.011	0.42±0.07	

Values are means±SD of 3 values. **, **** are Significance at P=0.002 and P<0.0001, respectively

Table 2: Agar disk diffusion method of *Bacillus subtilis*

S. No.	*Test sample*	*Growth of Inhibition (cm)*					*P-value summary*
		10 mg/ml	*30 mg/ml*	*50 mg/ml*	*70 mg/ml*	*90 mg/ml*	
1.	DMSO			0.25±0.02			****
2.	Penicillin	0.7±0.03	0.8±0.03	0.85±0.01	0.9±0.02	1±0.2	
3.	Chloramphenicol	0.45±0.04	0.5±0.01	0.6±0.02	0.65±0.02	0.75±0.02	
4.	Tetracycline	0.9±0.1	1±0.2	1.1±0.1	1.2±0.06	1.5±0.1	
5.	Quercetin	0.3±0.01	0.35±0.02	0.4±0.03	0.45±0.01	0.5±0.03	
6.	*Myristica fragrans*	0.28±0.02	0.32±0.02	0.36±0.03	0.4±0.03	0.45±0.04	

Increase in growth of inhibition with increase in concentration of antibiotics and quercetin. Values are means±SD of 3 values. ****Significance at P<0.0001

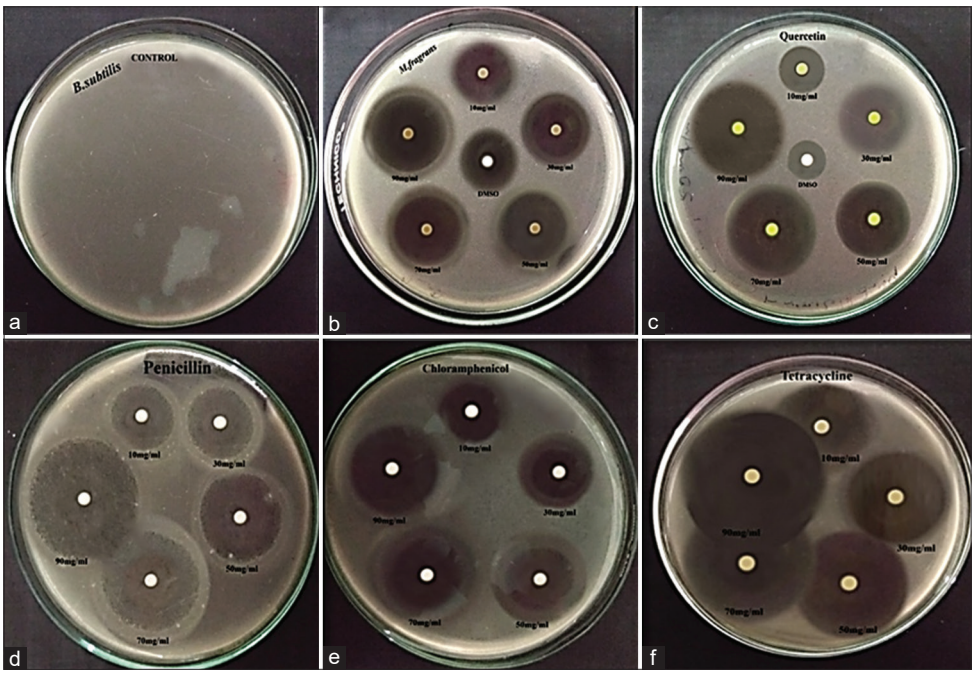

Figure 1: Agar disk diffusion method of *Bacillus subtilis*: (a) Control plate, (b) plate with *Myristica fragrans* discs, (c) plate with quercetin discs, (d) plate with penicillin discs, (e) plate with chloramphenicol discs, (f) plate with tetracycline discs

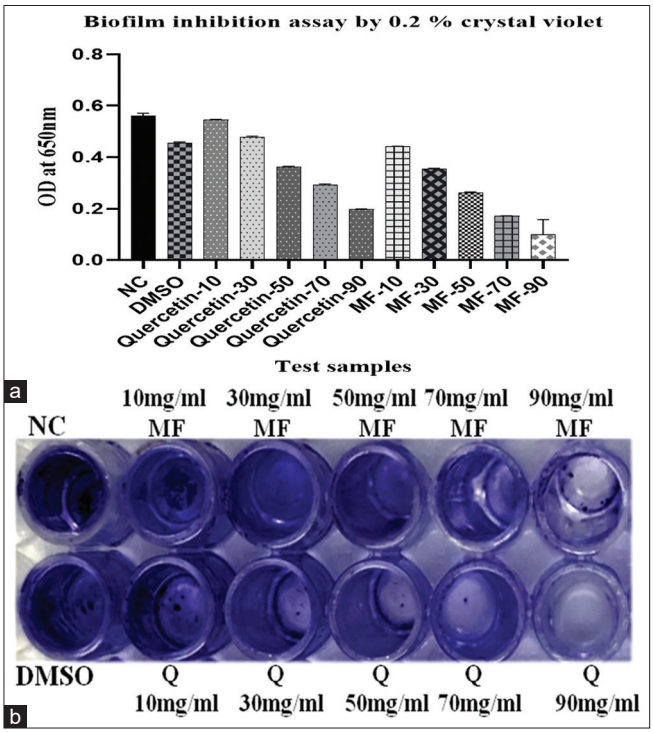

Figure 2: (a) Reduction of OD values with increase in the concentration of test extract. (b) Inhibition of *Bacillus subtilis* biofilm by *Myristica fragrans* extract: NC: Negative control, MF: *Myristica fragrans*, Q: Quercetin. Values are means ± SD of three values (*P*=0.0050)

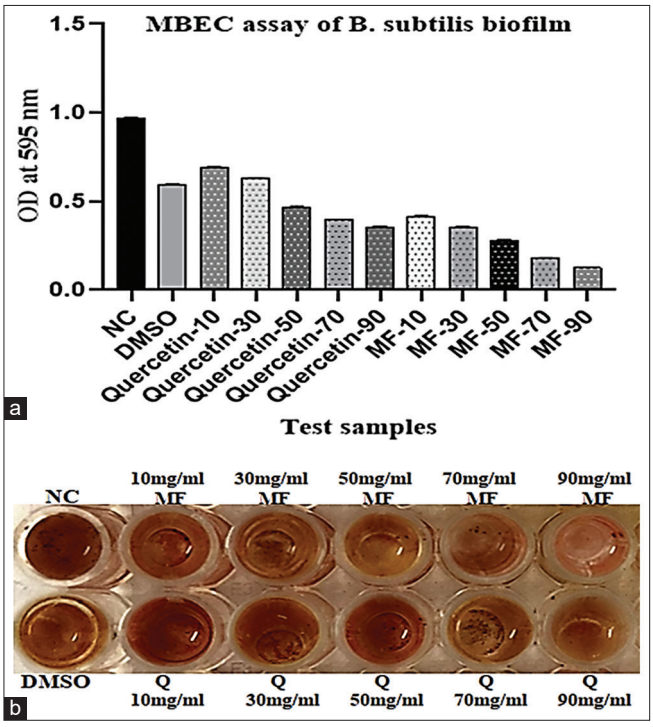

Figure 3: (a) Reduction in OD values by increase in the concentration of test extract. (b) MBEC assay of *Bacillus subtilis* biofilm: Inhibition of biofilm by *Myristica fragrans* and Quercetin extracts. NC: Negative control, MF: *Myristica fragrans*, Q: Quercetin. Values are means ± SD of three values (*P* < 0.0001)

Preparation of standards

Required concentrations of standards for antibiotics were prepared using distilled water. Standards of Quercetin and test extract were prepared by dissolving in DMSO. All stocks were stored at 4°C until use.

Minimum Inhibitory Concentration (MIC) Assay

Minimum concentration of test samples that inhibits the visible growth of bacteria is called MIC. Overnight culture of *B. subtilis* was diluted to 1 × 10^6 CFU/Ml.[8] 1–50 mg/mL concentrations of test extract were used for MIC assay. From 10 mg/mL concentration test extract showed visible growth of inhibition of bacteria. Therefore, 10–90 mg/mL concentrations of test samples were selected for all antibacterial and antibiofilm activity. Broth dilution assay and agar disc methods were used for MIC assay.

Broth Dilution Assay

Overnight culture of bacterial suspension (*B. subtilis*) was taken and OD was adjusted to 1 × 10^6 CFU/mL.MIC assay was done by taking 3 mL of broth medium, 300 µL of bacterial culture and 100 µL of test extract and antibiotics with 10 mg/mL, 30 mg/mL, 50 mg/mL, 70 mg/mL, and 90 mg/mL concentrations into separate test tubes and incubated at 37°C and 24 h. One tube with DMSO with 100 µL was added to test inhibition of bacterial growth by DMSO. Tube without test extract was treated as negative control and test tubes with antibiotics and quercetin were treated as positive controls. After overnight incubation antimicrobial activity of positive controls and test controls was observed by taking OD at 600 nm.[8]

Agar Disc Method

Agar test for MIC assay was done with *B. subtilis*. Overnight culture of bacterial culture was diluted to 1 × 10^6 CFU/mL and 100 mL of overnight culture was mixed with 1000 mL of molten agar media and poured into Petri plates (15 mL/plate). Sterile discs with test extracts of 10 to 90 mg/mL were inserted onto agar plates after solidification. Plates without test discs were treated as negative control and plates with antibiotics and quercetin were treated as positive control. Plates with *M. fragrans* extract discs were treated as test controls. All plates with positive, negative, and test control discs were incubated at 37°C for 24h.[9]

Effect of *M. fragrans* on Biofilm Formation

Biofilm formation assays were tested with *B. subtilis* organism using methanolic extract of *M. fragrans*.

Microtiter plate assay (MTT)

96-well microtiter plates were used for MTT assay of biofilm formation. Each well was filled with 200 µL broth, 30 µL overnight bacterial culture, and 20 µL test sample and incubated at 37°C for 24 h. Wells without test extract was treated as negative control; with test extract was treated as test control and wells with quercetin was treated as positive control. Wells with DMSO were also loaded for the comparison. After 24 h of incubation all wells were rinsed with distilled water to remove planktonic cells and incubated with 0.2% crystal violet (CV) for 15 min and again washed with distilled water

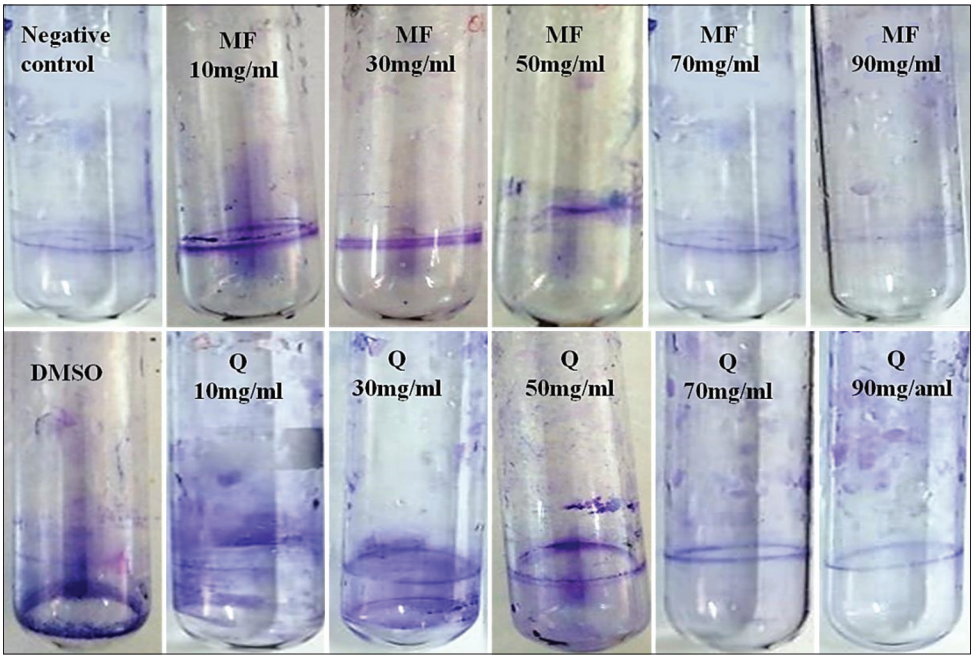

Figure 4: Tube test method: Inhibition of *Bacillus subtilis* biofilm by *Myristica fragrans*. MF: *Myristica fragrans*, Q-Quercetin, NC: Negative control

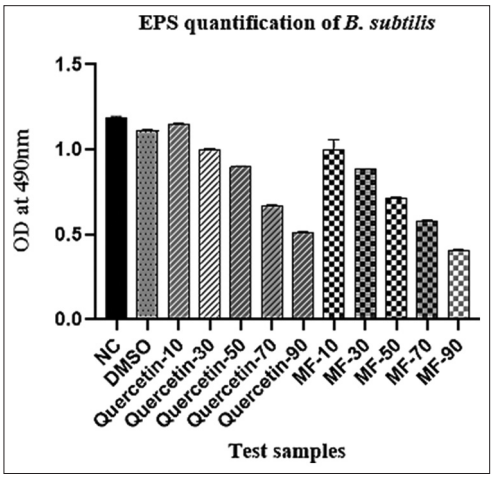

Figure 5: Extracellular polymeric substance extraction method of *Bacillus subtilis* biofilm. Values are means ± SD of 3 values ($P < 0.0001$)

until the unbound crystal violet was removed from the wells. All wells were filled with 95% ethanol to dissolve the biofilm-bound dye and dissolved biofilm was measured by taking OD at 650 nm in ELISA plate reader.[10]

Tube test

This test was done for the observation of inhibition of biofilm attaching to surfaces by *M. fragrans* extract. Boiling test tubes were used for the assay. Each tube was filled with 3 mL of broth, 300 µL of overnight bacterial culture (*B. subtilis*) and 100 µL of test samples. Controls are same as mentioned in the MTT assay. All tubes were incubated at 37°C and 24 h and washed with distilled water until planktonic cells were removed and stained with 0.2% CV for 15 min and washed with distilled water until unbound dye was removed.

Minimum biofilm eradication concentration (MBEC) assay

MBEC assay was done with respiratory dye 2-(-4-Idophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which was used for staining the biofilm. MBEC assay was done with MTT plates. Briefly each well of MTT plate was filled with 200 µL broth, 50 µL overnight culture of *B. subtilis* and incubated at 37°C for 24 h. After incubation period all wells were washed with PBS saline to remove unbound planktonic cells and incubated with test samples with increase in concentrations (10–90 mg/mL) for 16–18 h. All test, negative and positive controls are same as mentioned in MTT assay. Then, wells were washed again and incubated with 250 µL of 2 mg/mL INT dye for overnight at 37°C and OD at 595 nm was taken to observe the eradication of biofilm by test samples.[11]

EPS extraction method

In this assay, we can quantify the amount of EPS produced by *B. subtilis* after incubating the organisms with test samples. Briefly, an overnight culture of organism was incubated with broth medium and test samples as same as mentioned in the tube test method and incubated at 37°C and 24 h. After incubation period centrifuge the organism at 4°C for 30 min. Supernatant was filtered through 0.22 µm membrane filter and three volumes of chilled 100% ethanol was added to the filtered supernatant and incubate the mixture overnight at 4°C and allow to precipitate EPS. Precipitated EPS was quantified by phenol-sulfuric acid method. The amount of EPS was quantified by measuring the absorbance at 490 nm.[12]

Microscopic analysis

Inhibition of biofilm production by *M. fragrans* was confirmed by the microscopic analysis of biofilm. Light microscope and phase contrast microscopic analysis were used for the assay. For all techniques, *B. subtilis* were incubated in static position with broth

and test samples as mentioned in the tube test method. All tubes were incubated with 18 × 18 mm coverslips for the formation of biofilm on coverslips.

LCM

After incubation coverslips were washed with distilled water and stained with 0.2% crystal violet and incubated for 5 min and crystal violet was washed with distilled water to remove unbound dye. All coverslips incubated with and without test samples were observed under light microscope at ×40 magnification.[13]

PCM

Incubated coverslips were recovered and stained with 0.2% crystal violet for 2 min and washed with distilled water and allow drying. All stained coverslips were mounted on slides and observed under phase contrast microscope at ×40 magnification.[14]

Results and Discussion

MIC assay was confirmed both in broth and agar mediums. After incubation, all test tubes and agar plates were observed for growth of inhibition values. In broth, it was measured by taking OD at 600 nm and in agar growth of inhibition was measured in centimeters. A concentration dependent decrease in growth of bacteria was observed with all test samples [Tables 1 and 2]. A maximum decrease in the OD of bacteria was observed with the *M. fragrans* extract compared to Quercetin than antibiotics (penicillin, chloramphenicol, and tetracycline) [Figure 1].

There are few studies that *M. fragrans* have antimicrobial properties.[15] In this work first, we tested antimicrobial activity of Nutmeg oil against *B. subtilis* by both in broth and agar medium and MIC values were noted and 10–90 mg/mL concentrations were selected to test antibiofilm activity of Nutmeg. Penicillin, chloramphenicol, and tetracycline antibiotics were used as controls for antibacterial activity. Only quercetin was used as control for antibiofilm activity because quercetin is a pure flavonoid which can interact with quorum sensing signaling mechanism and reduce biofilm formation.[6]

Crystal violet bounded biofilm dissolved in 95% ethanol was measured by taking OD at 650 nm for all test, negative and positive controls. A concentration dependent decrease in the biofilm formation was observed with bacteria. Inhibition of biofilm formation with *M. fragrans* extract was much higher than that of Quercetin was observed [Figure 2].

The lowest concentration of the test extract at which biofilm was eradicated is called MBEC. The eradicated biofilm by the test samples was visually confirmed by INT dye and quantitatively measured by OD at 595 nm. After incubation with INT dye a concentration dependent increase in the biofilm eradication was observed. *M. fragrans* extract shown higher eradication of biofilm of bacteria than Quercetin [Figure 3]. This test confirms that extract can also help in eradicating pre-formed biofilms. MTP and MBEC assays determine the quantitative decrease in biofilm by adding test extracts.

After removal of the crystal violet dye from the tubes surface attached biofilm was examined for all tubes. A concentration dependent inhibition of biofilm formation on the walls of test tubes was observed. Tubes with *M. fragrans* extract showed less formation of biofilm on the walls of test tubes than tubes with Quercetin [Figure 4].

Tube test method confirms the direct visual identification of decrease in biofilm formation which is attached to the walls of test tubes by increasing the concentration of the test extract.

The amount of EPS produced by the bacteria after treatment with test extract was quantified by taking OD values at 490 nm. Amount of EPS produced by bacteria was decreased as increase in the concentration of test samples [Figure 5]. Values for *B. subtilis* were quantified. EPS production was decreased by the bacteria treated with *M. fragrans* than quercetin.

All coverslips that were incubated in *B. subtilis* bacterial culture with *M. fragrans* and quercetin samples were observed under light and phase contrast microscopes. A concentration dependent decrease in the biofilm formation was observed on all coverslips. Less formation of biofilm was observed on coverslips incubated

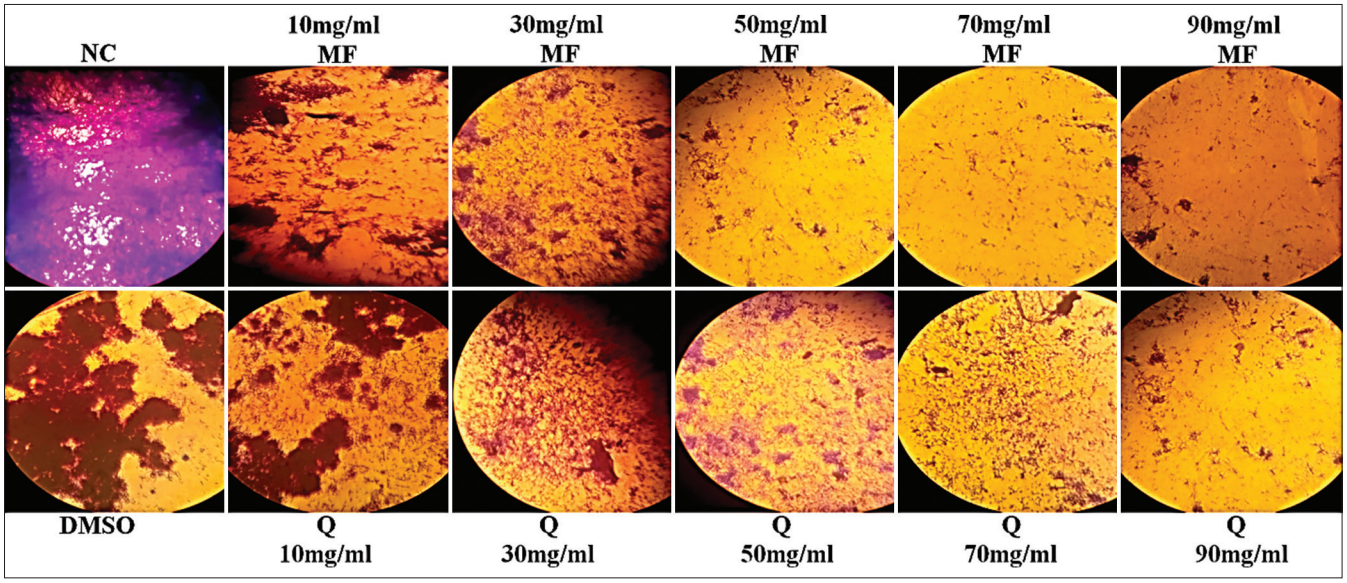

Figure 6: Light microscopic analysis of *Bacillus subtilis* biofilm. NC: Negative control, MF: *Myristica fragrans*, Q: Quercetin

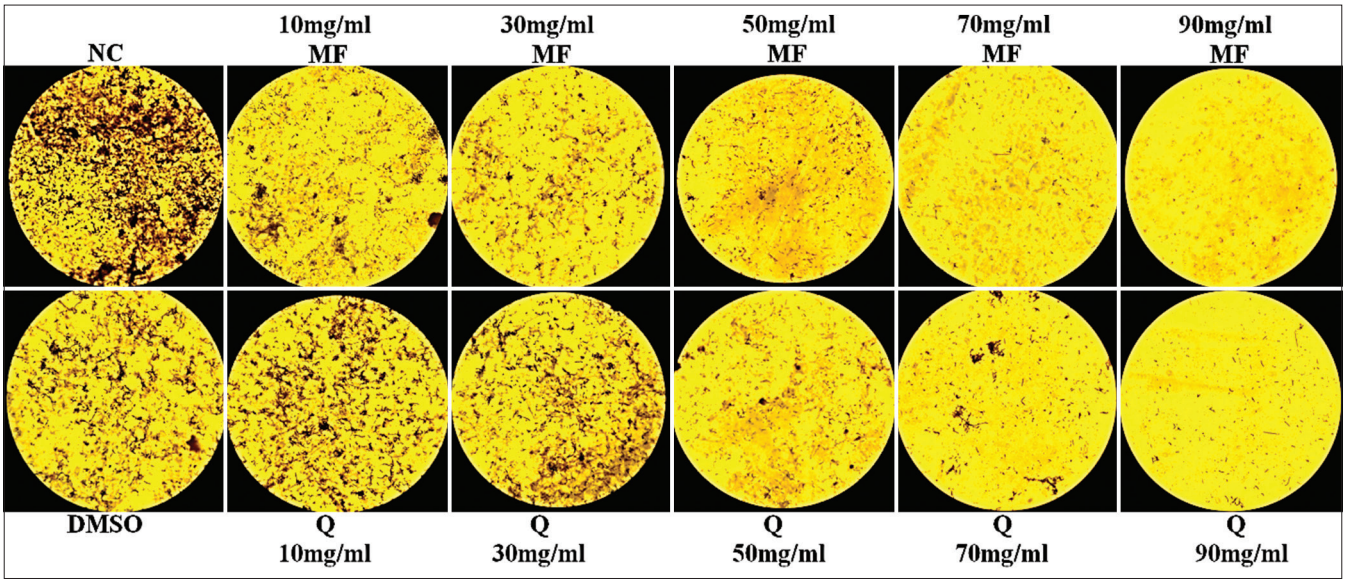

Figure 7: Phase contrast microscopic analysis of *Bacillus subtilis* biofilm. NC: Negative control; MF: *Myristica fragrans*; Q: Quercetin

with *M. fragrans* extract than quercetin [Figures 6 and 7]. All the quantitative test results were statistically significant with $P < 0.05$. These quantitative assays were supported by the microscopic analysis of biofilm.

These findings defines that nutmeg oil can inhibit planktonic and biofilm mode of growth in bacteria. Nutmeg is common well known house hold spice and used as flavoring agent in many food items. It can be used as a mood elevator because when a human eats nutmeg it opens heart, cleans mind, reduce bad fluids in body, reduce stress and anxiety, make strong, and put in a good state of mind.[16] Nutmeg has anticonvulsant activity because it has complex actions on the central nervous system.[17]

The activity of test extract in infected hosts helps to enhance the immunological defense against bacterial infectious diseases. The use of antibiotics to treat antimicrobial infections was banned in many countries because of the development of antibiotic resistance by microbes in humans. Therefore, the active test extract may potentially develop into herbal product which can be useful to cure microbial infections.[18]

Conclusion

Our results confirmed that the tested compound was targeted on biofilm formation of bacteria. Hence, this suggests that our test extract inhibited quorum sensing and it can be used as a natural therapeutic agent for the treatment of biofilm-caused infections. Detailed study of the test extract (pharmaceutical applications and chemical constituents of the nutmeg) needed to be investigate further.[18]

Bacterial biofilm formation is becoming highly global threat to health due to difficulty in treatment. Hence, searching for novel efficacious molecules to overcome this problem is a priority of this study. This appears to be the first study to determine the antibiofilm activity of Nutmeg. Our study confirms that our active test extract can reduce to colonize bacteria on surfaces of body thereby preventing infections. Spraying of natural compounds on vegetables may reduce contamination caused by many food pathogens.

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