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Research Article

EFFECT OF ISOFLAVONES AS ANTIBACTERIAL AND ANTI-QUORUM SENSING AGENTS AGAINST *CHROMOBACTERIUM VIOLACEUM*

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ABSTRACT

Multidrug resistance is one of the emerging challenges confronted by the human race by pathogenic microorganisms causing dreadful diseases. Usage of antibiotics inappropriately has caused resistance which could lead to extreme consequences on the lives of humans. Quorum sensing (QS) is communication between cells based on the bacterial population and density. QS plays a vital role in bacterial virulence and emerging bacterial resistance. *Chromobacterium violaceum* aids in detecting QS modulation which is measured by violacein pigment quantification. Genistein and daidzein are isoflavones-phytoestrogen found to be beneficial in managing post menopause systems and also antioxidant-rich. The present study was conducted to understand whether these phytoestrogens genistein and daidzein interfere to inhibit biofilm formation and quorum sensing by preventing cross talk between bacteria. Antimicrobial assay by agar well diffusion, biofilm and quorum sensing inhibition were evaluated using genistein and daidzein on *Chromobacterium violaceum*. Results indicated the cytotoxic effect of genistein wherein daidzein and genistein were found to have anti-quorum sensing and biofilm inhibition. This could be of therapeutic influence against antibiotic resistance in future.

Keywords: Genistein, Daidzein, Multidrug Resistance, Quorum Sensing, *Chromobacterium violaceum*.

1. INTRODUCTION

The prokaryotes are the unicellular organisms well known to lack the important functional unit; the nucleus, and any other kind of cell organelles. But the absence of the functional part and its cell organelles doesn't make the prokaryotes any less significant in nature. These organisms have undergone many evolutionary changes and have pulled many abilities to adapt themselves to nature.

One such ability is the "Quorum Sensing". Quorum sensing is simply known as the cell to cell communication that occurs in most of the prokaryotes, the bacteria. It is a term used to describe bacterial Cell to cell signalling involved in the determination of physiology [1]. It has been proven through studies that bacteria are capable of undergoing communications to coordinate several behavioural expressions [2].

Multiple resistance is considered to be a major threat to mankind and it is said to be just a rise of the resistances of this generation [3]. Many novel attempts, experiments are conducted to investigate and find a solution for this uprising problem. As the solutions are being identified, the same solutions are expected to restrain and work against the MDR and pathogenicity of the bacterial specimens. The bacteria always have been known to coordinate their communications or other behaviours through the Quorum Sensing mechanism, and this mechanism can include several pathways.

QS is the mechanism that is very much looked upon to be fought against as this solely leads most of the bacterial organism behaviour. This can be considered as the major site for drug attacks to improve the MDR situation of present times [4]. Many of the organisms are adapted to antimicrobial agents. They do this through spontaneous mutation or DNA transfer. Many of the bacteria including *streptococci, enterococci, salmonella* etc., now exhibit multiple drug resistance.

C. violaceum is known to cause sepsis and liver abscesses, but they are known to rarely infect humans, but when they do infect, the disease conditions are always fatal to the person affected. As these bacteria inhabit soil and water, this will be their mode of disease transmission.

They enter into the body through injured skin or cuts as skin makes contact with soil or water [5]. It proceeds from causing necrotizing metastatic lesions to multiple abscesses of the liver, lung, spleen, brain and lastly to fatal failure of multiple organs in the body. They are also known to cause urinary tract infections (UTIs). Antibiotics are usually used for treating the first proceeds of the disease. Pefloxacin, ciprofloxacin, amikacin etc. are some known antibiotics used in the treatment [6].

The azithromycin, a macrolide has been reported to have inhibition functions of the QS system and is also known to act on several virulence factors [7]. The analogue to AHL, the butyrolactones were known to be produced by *Streptomyces* species which is a naturally occurring intermediate of butanolides that is known to be effective on QS in *C. violaceum.*

Creating analogues to receptors whose main function is to transcript the signals have been found effective to suppress the QS signals. The compounds with a change in lactone moiety are introduced to give antagonistic reactions while binding and thus the signals are not produced [8].

Many of the natural sources have been proved to interfere in the QS mechanism. Several plants are known to mimic the AHLs signals that are produced by bacteria and this can affect the QS regulatory functions [9]. The QS system involves signal cascades, this can be directly inhibited or blocked at this stage by natural furanone compounds. The furanone that was isolated from red algae *Delisea pulchra* and the cinnamaldehyde from the cinnamon tree bark acts as blockades of signal transduction cascades of the QS system [10].

In the current study, we are attempting to evaluate the effect of isoflavones on antibacterial and anti quorum sensing activity.

2. MATERIAL AND METHODS

2.1. Procurement of Bacterial strains and culture conditions

The culture of *C.violaceum* (MCC2216) was procured from Microbial Culture Collection, NCCS, Pune. The cultures were provided as stab cultures and were subcultured by inoculating *C.Violaceum onto* LB agar. The plates were incubated at 37°C for 24 hours.

2.2. Stock preparation

Genistein and Daidzein were purchased from M/S Sigma Aldrich Co.(St Louis, USA). A stock solution of the test compounds was prepared by dissolving 1 mg of the Genistein and Daidzein in dimethyl sulfoxide (DMSO) and final concentration (0.1%) the test compound was prepared and frozen at -20°C in small aliquots. From the stock solution, appropriate dilutions were carried out to prepare various concentrations of the test compounds for different analyses.

2.3. Antibacterial activity (Agar well diffusion method)

The antimicrobial activity was evaluated by the agar well diffusion method as described by [11]. Briefly, LB agar was prepared (as per the requirement) and sterilized. The media was poured onto sterile Petri plates and was allowed to solidify. To each plate 200µl of a broth culture of the microorganism tested (*C.violaceum,* as the case may be) was spread on the media using a sterile glass spreader. To each plate, wells of 0.8mm were bored using a cork borer. To the wells, 20µl of different concentrations of the test compounds were added. The plates were incubated at 37°C for 24 hours. After the incubation period, the plates were observed for zones of inhibition around the test wells. The diameter of the zones (if any) was measured and the results were expressed in mm [12].

2.4. Biofilm inhibition assay

The biofilm inhibition assay was performed by the microplate method as described by [13]. Briefly, the plate culture of the microorganisms (*C.violaceum,* as the case may be) was taken and a loopful of culture was inoculated into Luria Bertani broth. The broth was incubated at 37°C till the absorbance was 0.1 at 600 nm. The broth was then diluted with fresh media in the ratio of 1:100. After dilution, 200µl of the diluted culture was added to the wells of 96 well microtiter plate. The plate was then incubated at 37°C for 12 hours at 95% humidity and 5% CO_2 in a CO_2 incubator (Forma Scientific, USA). After 12 hours of incubation, 100µl of different concentrations of the test compounds were added to the wells under sterile conditions. After this, the incubation was continued for another 6 hours. After incubation, the media was discarded by gently flipping the plate onto a blotting paper. The plates were air-dried in laminar airflow. After drying, the wells were stained by adding 50µl of 0.1% crystal violet. The excess stain was decanted and the wells were washed with distilled water. To each well, 200µl of 95% ethanol was added to dissolve the biofilm. The plates were read at 570nm in a microplate reader.

2.5. Quantitative assessment of quorum sensing inhibition-Quantitation of violacein pigment

The violacein pigment produced by the indicator organism *Chromobacterium violaceum* was quantified following the method prescribed by [14].

C. violaceum was cultured for 1 hour, till its OD reached 0.1 at 600 nm. The culture was inoculated into 5 conical flasks containing LB broth. Components according to the following table were added into the conical flasks. The flasks were incubated in a shaker incubator at 30°C for 24 hours. 5ml of the culture from each of the above flasks were taken in falcon tubes the next day. The contents were centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was retained. 1 ml of DMSO was added to falcon tubes to dissolve the pellet. The contents were vortexed vigorously for 30 seconds to solubilize the pigment violacein. The absorbance of the sample was read at 585nm using a colorimeter.

Table 1: Experimental setup for the quantification of violacein pigmen

Materials	Tubes				
	LB (ml)	LB+HHL (ml)	LB + HHL + Test Compound (50 µg/ml) (ml)	LB + HHL + Test Compound (100 µg/ml) (ml)	LB + HHL + Vanillin (ml)
LB broth	17.9	17.8	17.8	17.8	17.8
C. violaceum	1.0	1.0	1.0	1.0	1.0
HHL	-	0.2	0.2	0.2	0.2
Drug	-	-	1.0	1.0	1.0
DMSO	1.1	1.0	-		

2.6. Statistical analysis

All the experiments were done in triplicates on three different occasions. Values were expressed as mean ± SD. Statistical analysis was performed by students-t-test-test analysis. Comparisons were made with the control Vs treated groups.

3. RESULTS AND DISCUSSION

3.1. Effect of Genistein and Daidzein against some common pathogens

Fig 1 and Table 2 shows the results of antimicrobial activity testing by the agar diffusion method. Negative results were obtained with all the 20, 50 and 100µg/ml of Daidzein and Genistein of 100 and 50µg/ml of elicited antimicrobial action where 20µg/ml of daidzein showed negative results. Although different concentrations of the test substance have to be evaluated to know the exact dose at which antimicrobial action exists.

Table 2: Effect of test compounds on microbial action-agar well diffusion assay

Concentration (µg/ml)	Zone of inhibition
D1	Negative
D2	Negative
D3	Negative
G1	19.4 ± 0.84
G2	16.5 ± 1.57
G3	Negative

**Daidzein: D1-100 µg/ml, D2-50 µg/ml, G3-20 µg/ml; Genistein: G1-100 µg/ml , G2-50 µg/ml, G3-20 µg/ml*

The results indicate that daidzein at specified concentration showed no zone of inhibition implicating devoid of bactericidal action against *Chromobacterium violaceum* taken up for the study. Negative results implicated the absence of cytotoxic and bactericidal effect of test compound does not induce selection pressure on the bacterial species thereby necessitating the development of resistance by one or the other mechanisms [15].

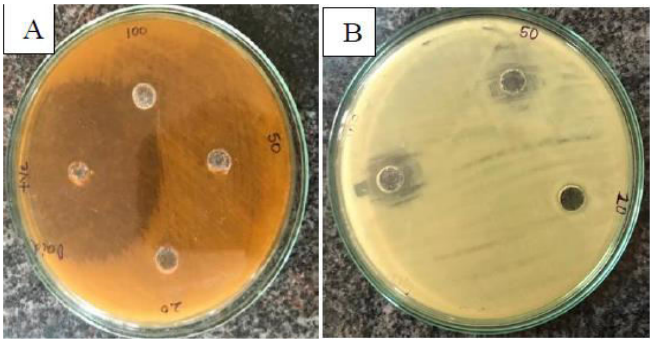

Fig. 1: Effect of the test compounds against the growth of *C.violaceum* A-Daidzein: B-Genistein

3.2. Effect of Genistein and Daidzein on biofilm inhibition- Microplate method

To understand whether the test substances used could interfere with biofilm formation and thereby act in interrupting the quorum-sensing signals in bacteria, the biofilm inhibition assay was performed.

Fig. 2 shows the effect of compounds Genistein and Daidzein on biofilm formation in *C. violaceum*. Results implicate that test compounds used did not show any significant inhibition of biofilm formation in *C. violaceum* at higher doses, whereas very low doses showed significant inhibition in biofilm formation.

The collection of microorganisms on the surface is termed biofilm formation which is a self-synthesized matrix. The inhibition of the biofilm so formed can be achieved by synthetic antibiotics but due to the development of antibiotic resistance, there is a need to shift to natural compounds that offer biofilm inhibition [04].

Fig. shows the effect Test compounds (100µg/ml, 50µg/ml)) on biofilm formation of *Chromobacterium Violaceum*. Data represent mean ± SD of 6 replicates. Student's t-test; ***P < 0.001, **P < 0.01, *P < 0.05 and NS- non- significant. Comparisons were made between treated group vs positive control.

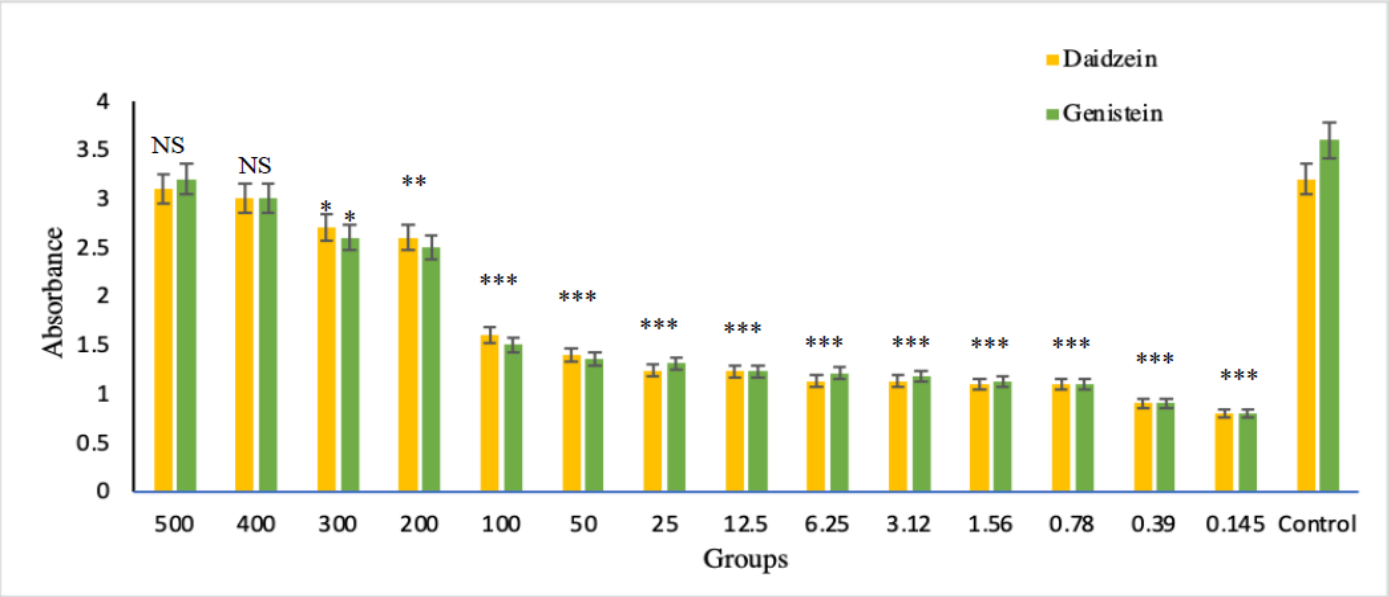

Fig. 2: Effect of test compounds on biofilm formation

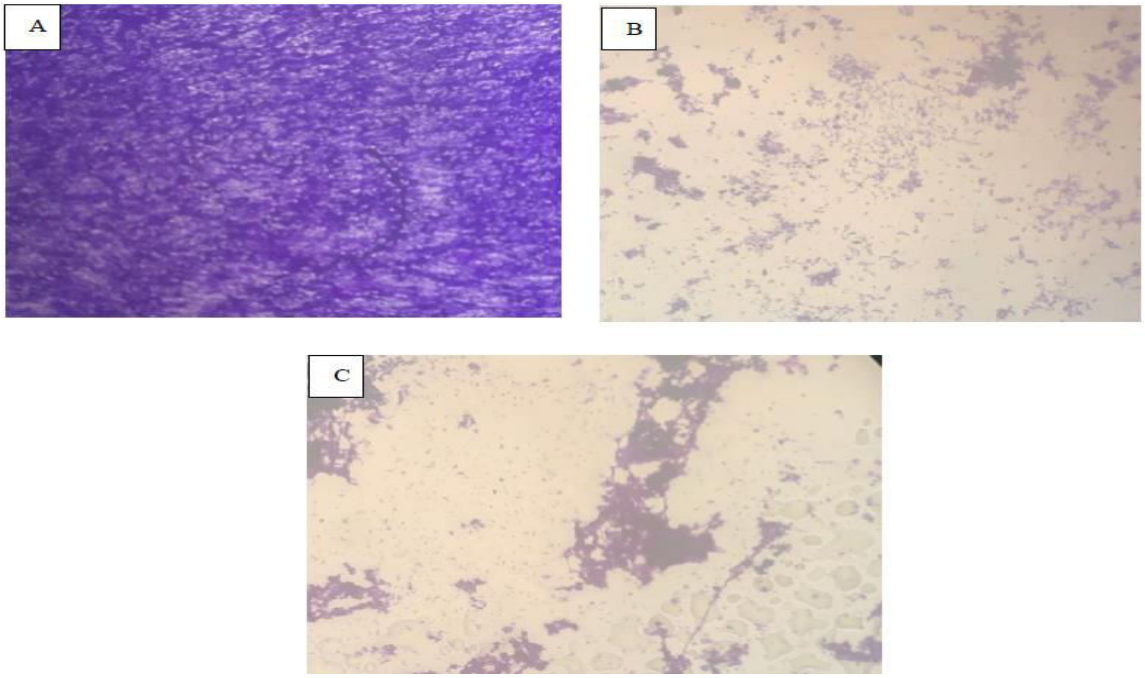

Fig. 3: Effect of the test compounds against the biofilm formation of *C. violaceum* A-Control; B-Daidzein; C-Genistein

3.3. Effect of test compounds on quorum sensing inhibition-quantification of violacein pigment

The effect of genistein and daidzein on inhibition of violacein pigment was assessed in *Chromobacterium violaceum*. The two concentrations (100µg/ml, 50 µg/ml) of test compounds showed a promising result in antimicrobial activity along with positive control (vanillin).

A statistically significant decrease in the formation of violacein pigment was observed at the dose 50µg/ml and 100µg/ml as compared to positive control. The results implicate that the results of test compounds could be potent anti-quorum sensing agents. Further investigations have to be performed to confirm the results.

The intercellular communication of the bacteria termed quorum sensing facilitates toxin production, motility, and biofilm formation in the bacterial species. Therefore, there is a need to inhibit the quorum sensing to disrupt the bacterial activities which can be done by blocking the AHL signalling pathway [16]. The violacein pigment formed by the *Chromobacterium* can be used to assess the inhibition of quorum sensing. Natural plant extracts should be used as a substitute for synthetic quorum sensing inhibitors to reduce bacterial pathogenicity and antibiotic resistance thereby reducing the severity of the infection.

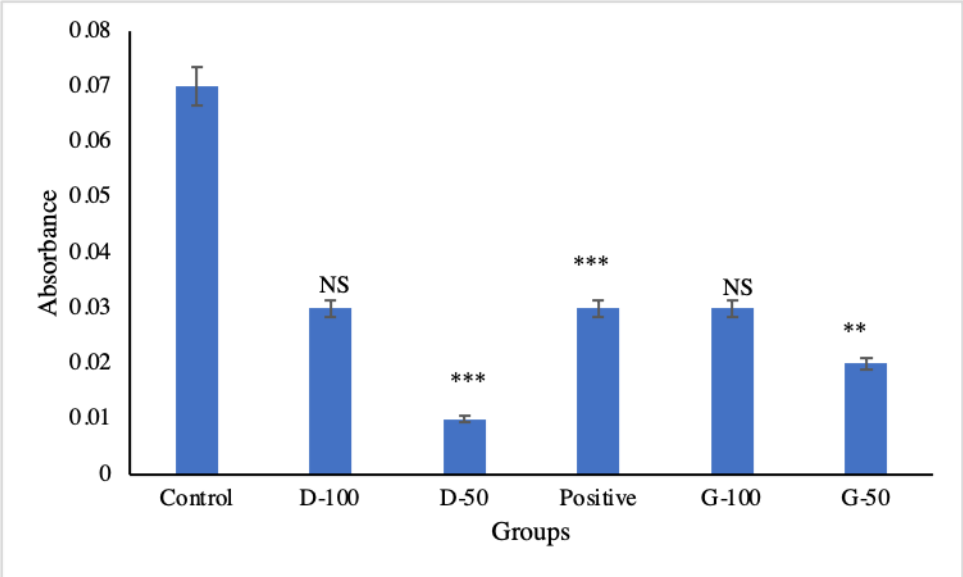

*Fig. shows the effect Test compounds (100 µg/ml, 50 µg/ml)) on biofilm formation of Chromobacterium Violaceum. Data represent mean ± SD of 6 replicates. Student's t-test; ***P < 0.001, **P < 0.01, *P < 0.05 and NS-non- significant. Comparisons were made between treated group vs positive control.*

Fig. 4: Effect of test compounds on quorum sensing inhibition- quantification of violacein pigment production of *Chromobacterium violaceum* - Quantification of violacein pigment

4. CONCLUSION

Phytoestrogens dietary supplements interfere with quorum sensing mechanism in bacteria. Dietary supplements hence could be potential source in development of anti-virulence therapies among various infections that have quorum sensing and biofilm forming capability. A plant based estrogen could act as dietary antioxidant and could be explored as quorum sensing inhibitors.

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Conflict of interest

None declared

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