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Effect of Isoflavones Formononetin and Biochanin a to Tnhibit Biofilm Formation and Quorum sensing in Bacteria- Potential Application in the Prevention of Antibiotic Resistance in Bacteria

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Abstract: ***One of the greatest challenges faced by the human race today is the emergence of multidrug resistance by many of the dreadful disease causing pathogenic microorganisms. Injudicious and inappropriate use of antibiotics have paved the way for the emergence of such resistance, the problem which if not addressed may have the perilous consequence of claiming innumerable human lives in the future. Quorum sensing is a system of communication between bacteria based on population density. It is believed to play a very important role in contributing to increased virulence and emergence of bacterial resistance against antibiotics, disinfectants and detergents. Inhibition of quorum sensing signals and potentiating of quorum quenching is an important strategy to avoid emergence of drug resistance in bacteria. Plants have been the source of potent antimicrobials from the time immemorial. Biochanin A and Formononetin are is flavones - phytoestrogens reported to possess several beneficial effects including management of postmenopausal symptoms, prevention of bone loss and protection against osteoporosis etc, In the current study, an attempt has been made to understand whether phytoestrogens formononetin and biochanin A can interfere quorum sensing signals and inhibit biofilm formation thereby prevent the cross talk between the bacteria. This could be of immense therapeutic importance to manage the problem of multidrug resistance, antibiotic resistance in the future.***

Keywords: Formononetin, Biochanin A, Biofilms, Quorum Sensing, antibiotic resistance

I. INTRODUCTION

Quorum sensing (QS) is a cell communication in bacteria that helps it to monitor cell density in a population by sensing small signal molecules called auto inducers which are well recognised antimicrobial target. (Rutherford and Bassler., 2012). Bacteria employ QS systems to control a variety of physiological processes including exudation of virulence factors and biofilm formation (Ng, and Bassler, 2009;Rudrappa&Bais, 2008). The discovery of QS might have opened a new line of action against microorganisms. The control of bacterial virulence and biofilm formation could have many applications like (Xiong& Liu, 2010), reducing bacterial virulence against plants (Dong, Xu, Li, & Zhang, 2000) and as substitute or co-adjuvant therapy in bacterial infections (Lynch &Wiener Kronish, 2008). Plant survival involves the production and release of secondary metabolites into their immediate environment, not only as a means of defences against probable pathogens but also as an offense against competing species. They are geared towards the production of antimicrobial compounds that limit the ability of microbes to produce the factors required for virulence and successful colonization [Quaveet al, 2011]. Medicinal plants offer an attractive repertoire of phytochemicals with novel microbial disease-controlling potential, due to the spectrum of secondary metabolites present in extracts, which include phenolics, quinones, flavonoids, alkaloids, terpenoids and polyacetylenes [Packiavathy, et al., 2012]. Many phytochemicals are not highly effective as antimicrobial agents, instead they possess anti-pathogenic or anti-virulence properties, which are neither bactericidal nor bacteriostatic and therefore do not cause the development of resistant bacteria. Instead these compounds attenuate the expression of genes responsible for pathogenesis and virulence by interfering with quorum sensing (QS) and other related properties [Packiavathy, et al., 2012]. Phytoestrogens, a class of plant-derived phenolic compounds, have either estrogenic or/and antiestrogenic effects owing to their structural similarity to estrogens. Formononetin is a phytoestrogenicisoflavone found in soy-based foods. It displays potent antioxidant properties and acts as a selective inhibitor of ADH γ (the γ-isoform of alcohol dehydrogenase). Formononetin is a phytoestrogen from the root of Astragalusmembranaceus and an O-methylated isoflavone. It is effective against Giardia lamblia infection, at least partially by inducing detachment of trophozoites from intestinal mucosa

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(Medjakovic and Jungbauer, 2008). Biochanin A is an isoflavone phytoestrogen found in red clover (Trifoliumpratense) that is a selective agonist at ER-β estrogen receptors, and may have chemo preventive efficacy against breast cancer. In line with its low activity at ER-α estrogen receptors, it is essentially devoid of uterotrophic activity. Biochanin A is also a ligand for the aryl hydrocarbon receptor (AhR). It reduces arterial resistance and enhances microcirculation perhaps via effects on potassium and/or calcium ion channels. Induction of sulfotransferases for xenobiotic detoxification has been proposed as a mechanism of its cancer preventive effects (Medjakovic and Jungbauer, 2008).

In the current study, an attempt has been made to understand the inhibitory effects of few natural compounds of plant origin. Results of this preliminary study is expected to give leads as to whether further specialized studies could be conducted to examine the potential of these compounds to combat the problem of antibiotic resistance

II. MATERIAL AND METHODS

A. Procurement of Bacterial strains and culture conditions

Cultures of C.violaceum, E.coli, P.aeroginosaand P.fluorescencewere procured from Microbial Culture Collection, NCCS, Pune. The cultures were provided as stab cultures and were subcultured by inoculating C.violaceumon to LB agar and P.aeroginosa, P.fuoresenceand E.colion NA. The plates were incubated at 37°C for 24 hours.

B. Stock preparation

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

C. Antibacterial activity (Agar well diffusion method):

The antimicrobial activity was evaluated by the agar well diffusion method as described by (Hafizah, 2013). Briefly, LB agar was prepared (as per the requirement) and sterilized. The media was poured onto sterile petriplates and was allowed to solidify. To each plate 200µl of broth culture of the microorganism tested (C.violaceum, P.aeruginosa, P.fluorescens or E.coliasthe case may be) was spread on the media using sterile glass spreader. To each plate wells of 0.8mm was bored using cork borer. To the wells 20µl of different concentrations of the test compounds were added. The plates were incubated at 37 ^{0} 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 expressed in mm.

D. Biofilm inhibition assay

The biofilm inhibition assay was performed by microplate method as described by (Cady *et al.,* 2012). Briefly, the plate culture of the micoorganisms (*C.violaceum, P.aeruginosa, P.fluorescens*and *E.coli*as the case may be) was taken and a loopful of culture was inoculated into Luria Bertani broth. The broth was incubated at 37 ^{0} 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 microtiterplate. The plate was then incubated at 37 ^{0} C for 12 hours at 95% humidity and 5% CO2 in a CO2 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 on to a blotting paper. The plates were air dried in laminar air flow. 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.

E. 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 analysis. Comparisons were made with the control Vs treated groups.

III. RESULTS

A. Effect ofFormononetin and Biochanin A against some common pathogens - Antimicrobial activity testing by Agar diffusion method

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Table 1 shows the results of antimicrobial activity testing by agar diffusion method. Results implicate that none of the test substances used in the current study elicited antimicrobial actions against the bacterial species used in the study. Negative results were obtained with all the concentrations (25, 50, 75 and 100 µg/ml) of Formononetin and Biochanin A used in the study. Plates 1,2,3,4 and 5 illustrate the results of the antimicrobial assay of the test substances.

S.No	Groups	Microorganism against which tested and activity observed			
		E. coli	*P.aeruginosa*	*P.Fluorescens*	*C.violaceum*
1	Formononetin	*Negative*	*Negative*	*Negative*	*Negative*
2	Biochanin A	*Negative*	*Negative*	*Negative*	*Negative*

Plate 1 and 2: Effect of the Formononetin and Biochanin A against the growth of *P.aeruginosa and E.coli*

Plate 1

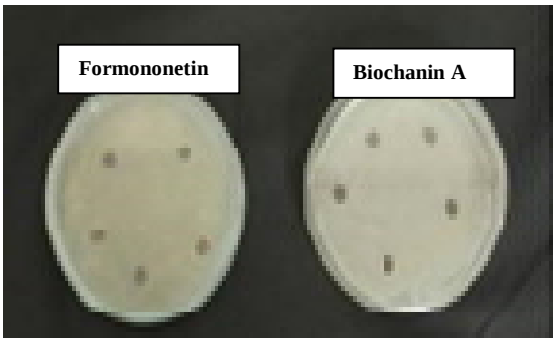


Plate2

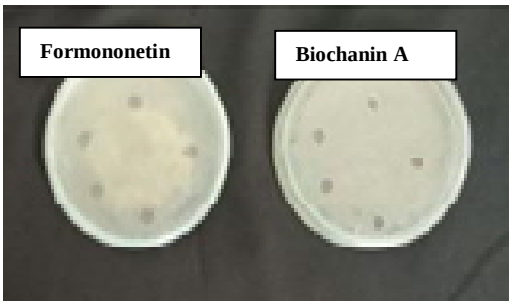


Plate 3 and 4: Effect of the test compounds against the growth of *P.fluorescens and C.violaceum*

Plate 3

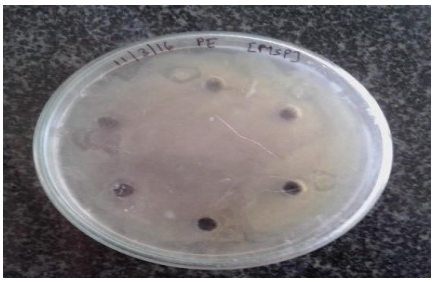


Plate 4



The results of the antimicrobial activity testing by agar diffusion assay were also illustrated in plates 1, 2, 3 and 4. No zones of inhibition was observed implicating that all the test substances tested were devoid of bactericidal actions against the bacterial species taken up for investigation in the study. This result indicated that the test substances were not cytotoxic and they did not induce bactericidal actions. This is of appreciable significance because the absence of cytotoxic or bactericidal effect will implicate that the compounds will not induce selection pressure on the bacterial species thereby necessitating the development of resistance by one or the other mechanisms.

B. Effect ofFormononetin and Biochanin A 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.

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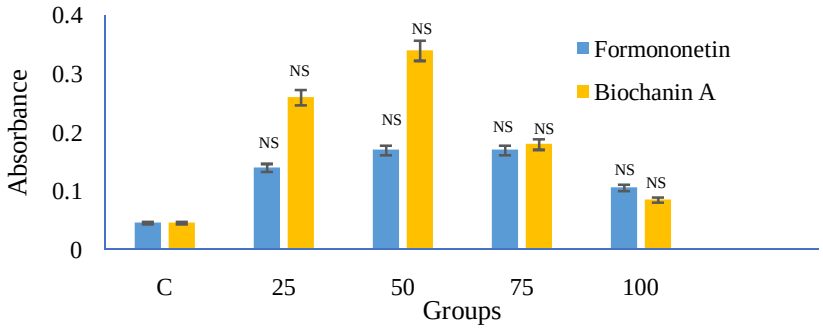


Figure 3.1: Effect of compounds Formononetin and Biochanin A on biofilm formation in *C. violaceum*.Values were expressed as mean ± SD. Students-t-test. Comparisons were made with the untreated control Vs treated groups. *** P<0.001, ** P < 0.01, * P< 0.05, NS- Non significant.

Figure 3.1shows the effect of compounds Formononetin and Biochanin A 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 lower doses. Interestingly, some proproliferative actions were observed with the compounds especially at 25, 50 and 75µg/ml which were found to be significant. The exact reason for this proproliferative actions induced by the compounds against this particular organism could not be understood at this stage of the study.

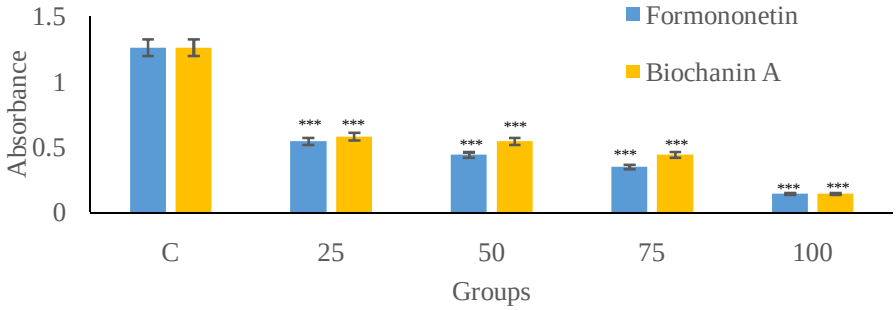


Figure 3.2: Effect of Formononetin and Biochanin A on biofilm formation in*E.coli*.Values were expressed as mean ± SD. Students-t-test. Comparisons were made with the untreated control Vs treated groups. *** P<0.001, ** P < 0.01, * P< 0.05, NS- Non significant.

Figure 3.**2**shows the effect of Formononetin and Biochanin A on biofilm formation in E. coli. Statistically significant dose dependent increase in biofilm inhibition was observed with maximum inhibition obtained at a concentration of 100µg/ml with both thetest compounds. The results implicate that both the test compounds were very effective in inhibiting biofilm formation in E. coli. The efficiency of biofilm inhibition against E.coli exerted by formononetin was found to be greater than that exerted by biochanin A (Formononetin>Biochanin).

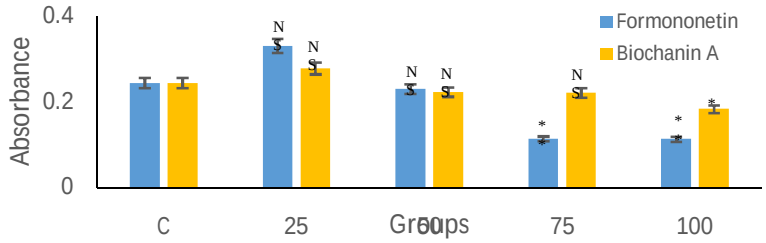


Figure 3.3 Effect of capsaicin, Formononetin and Biochanin A on biofilm formation in P. aeruginosa.Values were expressed as mean ± SD. Students-t-test. Comparisons were made with the untreated control Vs treated groups. *** P<0.001, ** P < 0.01, * P< 0.05, NS- Non significant.

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Figure 3.3shows the effect of Formononetin and Biochanin A on biofilm formation in P.aeruginosa. No significant inhibition in biofilm formation was observed at doses 25 and 50µg/ml with both the compounds. The high doses 75 and 100µg/ml showed a tendency for inhibition of biofilm formation with best results obtained at the highest dose. Biochanin A was found to be the less effective in inhibiting biofilm formation in P.aeruginosawhereas 100µg/ml of formononetin was found to be very effective in inhibiting the biofilms with the inhibition being statistically significant. Efficiency of biofilm inhibition against P.aeruginosawas in the order of Formononetin> Biochanin A**.**

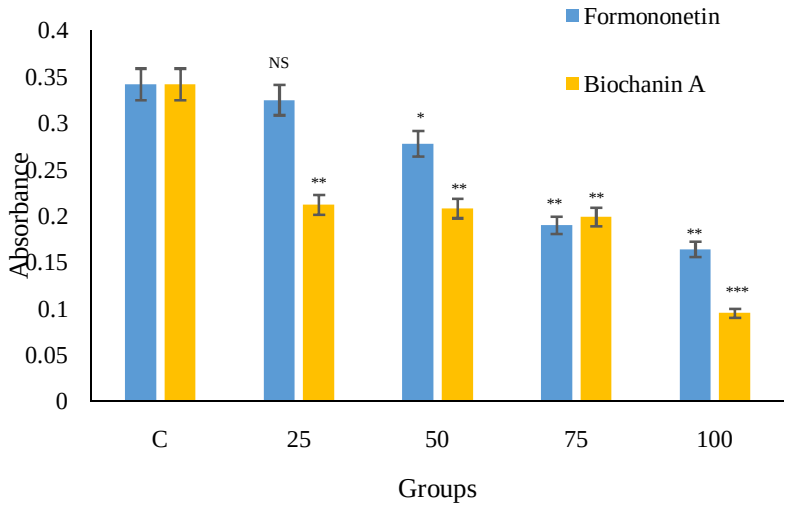


Figure 3.4. Effect of capsaicin, Formononetin and Biochanin A on biofilm formation in *P. fluorescens*Values were expressed as mean ± SD. Students-t-test. Comparisons were made with the untreated control Vs treated groups. *** P<0.001, ** P < 0.01, * P< 0.05, NS- Non significant.

Figure 3.4shows the effect of Formononetin and Biochanin A on biofilm formation in *P.fluorescens.* Interestingly, the compounds exhibited biofilm inhibition with maximum inhibition obtained at the highest dose. This inhibition in biofilm formation was found to be statistically significant at higher doses > 50µg/ml. Efficiency of biofilm inhibition against *P.fluorescens*exerted by Biochanin A was found to be superior to that exerted by formononetin
(Biochanin A >Formononetin).

IV. DISCUSSION

Quorum sensing is the regulation of gene expression in response to fluctuations in cell population density. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. These signals are produced while the bacterial population grows until a threshold concentration perceived by the bacteria is reached, resulting in the activation or repression of specific genes. Quorum sensing inhibition appears to be an attractive target in combating antibiotic resistance and multidrug resistance exhibited by microorganisms. Several quorum sensing inhibitors of synthetic and natural origin has been identified so far. Of natural quorum sensing inhibitors phytomolecules are found to be very effective against many bacterial species including E.coli, P.aeruginosa and P.fluorescens. Garlic, vanilla extract, phytoestrogens and several medicinal plant extracts were found to possess significant quorum sensing inhibitory properties.

In the current study, the quorum sensing inhibitory potential of two phytoestrogens were evaluated using the bioindicator organism C.violaceum(CV0 26). C.violaceumis a versatile Gram-negative ß proteobacterium that produces the violet non-diffusible antibiotic pigment violacein and additional antibiotics and enzymes affecting viruses, bacterial and eukaryotic cells. Since the production of violacein is quorum sensing (QS)-driven, it has become an important tool for bacterial QS signal bioassays, especially for the N-acylhomoserine lactone autoinducers (AHLs). A C.violaceummini-Tn5 mutant, CV026 (dependent on exogenous AHL for violacein production), was used as an indicator organism in the current study.

Both the test substances evaluated for antimicrobial activity (agar diffusion method) was found to be ineffective against all the bacterial species taken up for investigation in the study. No zones of inhibition were observed in the treated plates thereby indicating that the compounds used in the study did not have cytotoxic bactericidal actions.

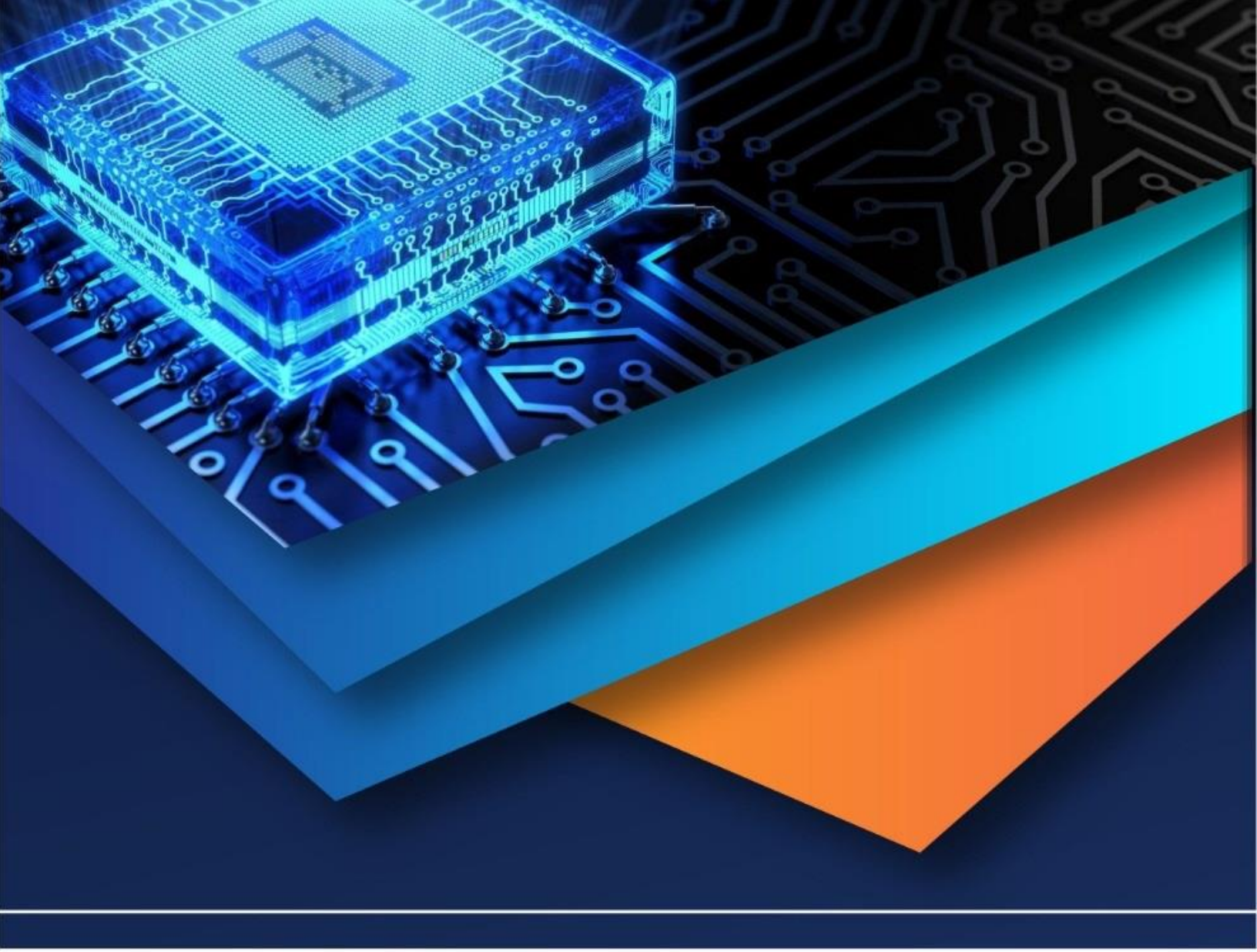
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With both the phytoestrogens used in the study being non-cytotoxic and non-bactericidal, the problems with selection pressure and consequent acquiring of antibiotic resistance which is the common problem faced with most of the currently available antimicrobials is overcome. This favourable fact along with significant inhibition in biofilm formation (which is crucial to the virulence of the bacterial species) the two phytoestrogens used in the current study have the potential as promising candidates for quorum sensing inhibition in bacteria.

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