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ISOLATION OF ANTIMICROBIAL AGENTS THAT DISRUPTS THE FORMATION OF BIOFILM IN STAPHYLOCOCCUS AUREUS

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Abstract

Biofilm formation is a multi-step process involves attachment of free floating bacteria to the solid matrix and secreting extracellular matrix substances finally succeeding in forming biofilm. Biofilm formation in bacteria helps in overhelming the environmental stressors, antibiotic resistance, offers enhanced protection against immunity and increased chances of genetic exchange. So, Phytochemical constituents that disrupt biofilm formation in microbes can help in designing new antibiotic agents. In this study we have selected five phytochemical constituents like *Vigna mungo*,*Vigna unguiculata*, *Magniferous indica*,*Vigna radiata* and *Lathyrus sylvestris* for the biofilm disruption. First we isolated S.aureus using nasal swab technique and confirmed the strain using Gram staining and catalase test. Futher to study the biofilm disruption we induced biofilm formation in *S.aureus* using solvents toulene and Glycerol. Biofilm confirmation was done by plating on EMB agar and Brain heart infusion agar. In the five phytochemical constituents *Vigna mungo* showed the positive response in disruption of toulene formed biofilm at concentrations 1.0% and 1.5%. Whereas *Magnifera indica* disrupted both toulene and glycerol formed biofilms at almost all concentrations (0.5%-2.5%) used.

Key words: Biofilm formation,*Vigna mungo*,*Vigna unguiculata*, *Magniferous indica*,*Vigna radiata* and *Lathyrus sylvestris*

Introduction:

Biofilm formation can contribute to enhanced antimicrobial resistance, offers protection against immune system causing highly potent infections. Disruption of biofilm can cause disruption of bacterial aggregates in to individual colonies with increased susceptibility to the antimicrobial agents. Understanding the mechanisms of biofilm formation and disruption, Defense mechanisms targeted by the immune system towards bacteria and extracellular matrix substances produced by bacteria can contribute significant advances in the microbial field leading to a pathway to prevent dreadful infections (Peng et al., (2022)).

S. aureus is one of the pathogen forms biofilms and studies by Liu et al., (2021) confirmed disruption of biofilms in *S.aureus is possible* by using antibiotic substilisin and calcium gluconate and oxytetracycline antibiotic alone is proven to be ineffective. Whereas Studies by Sudhir K Shukla , T Subba Rao (2017). proven to inhibit biofilm formation in bap-positive *S. aureus* V329 and as well as other *S. aureus* isolates (SA7, SA10, SA33, SA352) by proteinase K due to eDNA retention contributing finally to biofilm stability.

Studies so far on biofilm disruption in *S.aureus* includes targeting extracellular matrix proteins and use of antibiotics in combination but usage of phytochemicals for disruption is not reported till now. So, in this work we have selected five phytochemicals like *Vigna mungo*,*Vigna unguiculata*, *Magniferous indica*,*Vigna radiata* and *Lathyrus sylvestris* for study on biofilm disruption in *S.aureus.*

Materials and methods:

1. Isolation and Characterization of *S.aureus*:

For collecting the bacteria from source, Nasopharyngeal (NP) Swab technique is used. The swab is inserted in to the nose until it reaches the nasopharynx with out any puncture and the collected sample is transferred along with the swab and subjected to serial dilution. 10^{-7} or 10^{-8} dilution sample is preffered for culturing. 0.1ml of serially diluted sample of either 10^{-7} or 10^{-8} dilution is used for spread plate technique on selective media Mannitol salt agar.

1.1 Characterization of *S.aureus*:

Isolated *S.aureus* is characterized and confirmed using techniques like gram staining and catalase positive test.

Gram staining:

Gram staining is a principle technique used for differentiation of bacteria in to gram positive and gram negative. Crystal violet is the primary stain used to stain heat fixed smear for 1min followed by gram's iodine for 1min and acetone or ethanol for 15-30 sec. Finally the smear is counter-stained with saffranin for 1min and washed under running tap water after each staining procedure. The smear is allowed to air dry and examined under low power objective generally 10X.

Catalase Test:

Add a drop of distilled water on to the slide and transfer a loopful of culture to the water drop and mixed. Few drops of 30% H2O2 is added directly to the bacteria mixed water drop and observed for the result. Formation of bubbles indicate positive result.

2. Inducing Biofilm formation by using hydrophobic solvents:

Isolated *S.aureus* is sub-cultured in to nutrient broth and grown for overnight. 10ml of culture is aseptically transferred in to four different test tubes and to each test tube 1ml of toluene, hexane, acetone and glycerol is added and grown for overnight. Formation of white layer after 24 hrs in the tube surface confirms biofilm formation.

3. Testing of Biofilm disruption in *S.aureus* by Phytochemical constituents *Vigna mungo*,*Vigna unguiculata*, *Magniferous indica*,*Vigna radiata* and *Lathyrus sylvestris*:

5 test tubes are added with 10ml of cultured broth and 1ml of toulene to induce biofilm formation. After over night incubation to each test tube concentrations ranging from 0.5%-2.5% of phytochemical constituent is added and plated on nutrient agar under aseptic conditions and observed for the result.

Similar procedure is repeated with solvent Glycerol and all the five phytochemical constituents *Vigna mungo*, *Vigna unguiculata*, *Magniferous indica*, *Vigna radiata* and *Lathyrus sylvestris.*

Results:

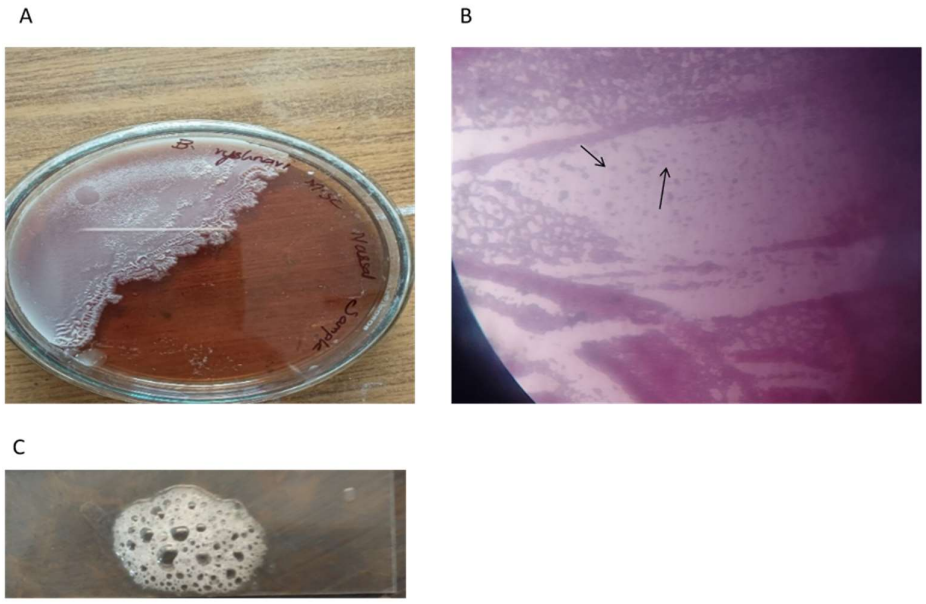

Figure:1 : Isolation and characterization of *S.aureus* (A) on Macconkey agar (B) using Gram staining (C) and catalase assay

The isolated bacteria can be confirmed as *Staphylococcus aureus* a gram positive cocci with catalase positive. Bacteria is collected using nasal swab and cultured using selective media Mannitol salt agar. Gram staining procedure was used to identify the isolated bacteria as gram

positive cocci, purple in color and from fig.1C the bacterium was confirmed to be catalase positive.

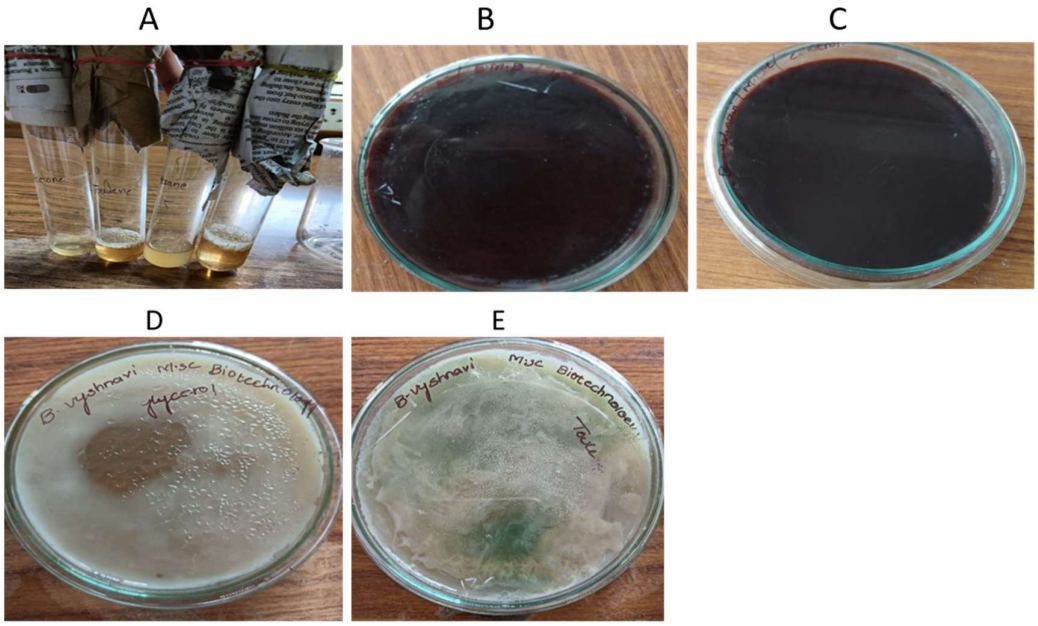

Figure:2 Inducing Biofilm formation and confirmation in *S.aureus* (A) using hydrophobic solvents Toulene,Hexane,Acetone and Glycerol (B) using EMB agar and (c) Brain heart Infusion agar + Malachite green

Addition of hydrophobic solvents induces biofilm formation in microbes and four different solvents like Toulene, Glycerol, Hexane and Acetone are used for the study. Biofilm formation was observed with only 2 solvents toulene and glycerol and the other two are not able to induce biofilms in *S.aureus*. Formed biofilms are subcultured on to surface of EMB agar and Brain Heart Infusion agar plates and used further to confirm the results and in fig 2E *Staphylococcus* stained green with the malachite green dye. From the fig 2D and 2E Toulene solvent can induce potent biofilm formation in *Staphylococcus aureus*.

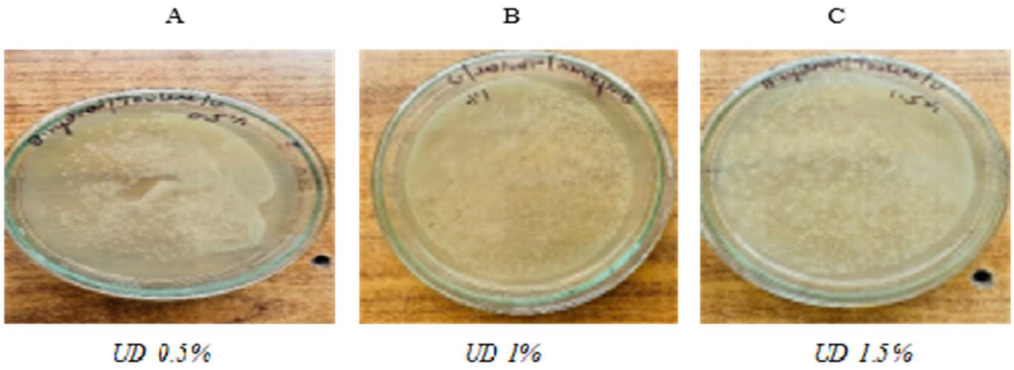

Figure:3 Biofilm disruption in *S.aureus* using 0.5%, 1% and 1.5% concentrations of *Vigna mungo* induced by toluene.

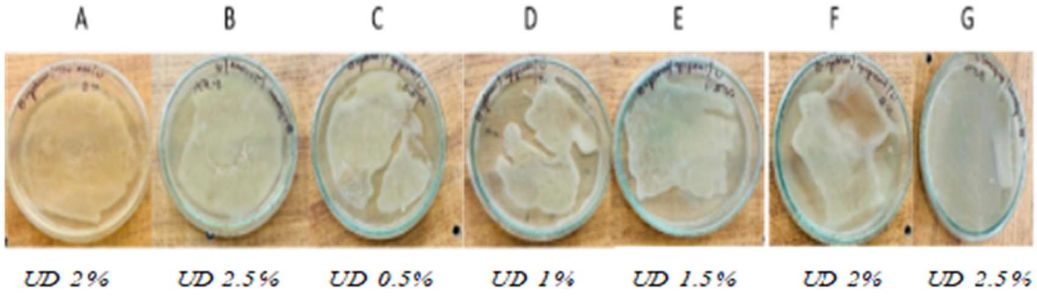

Figure : 4 Biofilm disruption in S.aureus Using 0.5% - 2.5% concentrations of *Vigna mungo* induced by toleune and glycerol. [(A-B) - Toulene and (C-G)- Glycerol)]

From the figure 3 and 4 *Vigna mungo* can disrupt biofilm formation in *Staphylococcus aureus* with 1.0 - 1.5% concentrations used induced by hydrophobic solvent toulene. With the concentration 0.5% the cells are dispersing from biofilm to colonize to new areas. From fig. 4C- 4G Glycerol induced biofilms showed reduced occupancy on the petri plate may be due to the less intense biofilm formation.

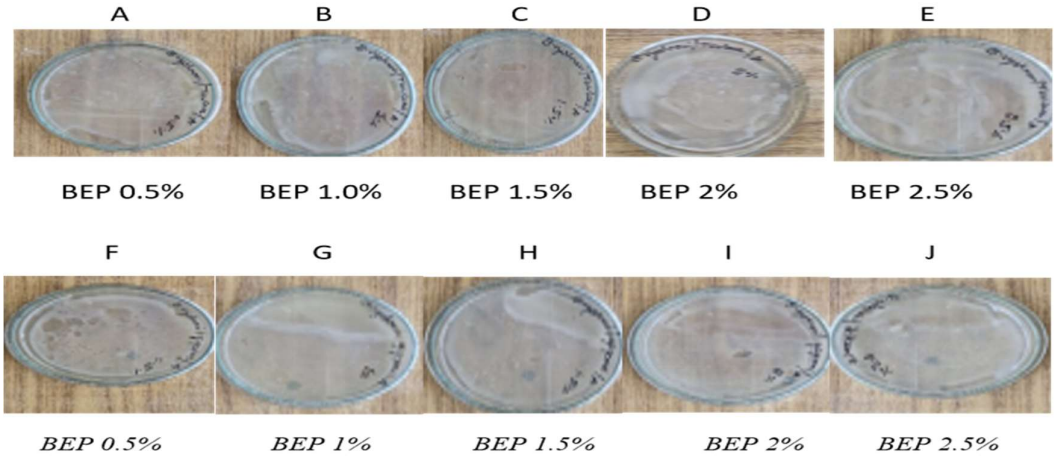

Figure:5: Biofilm disruption in S.aureus Using 0.5% - 2.5% concentrations of *Vigna unguiculata* (A-E) Biofilm induced using Toluene , (F-J) Biofilm induced using Glycerol

From the fig: 5 *Vigna unguiculata* phytochemical used is not able to disrupt the biofilm formation and where as *Magniferous indica* leaf constituents have the potential to disrupt the biofilm induced by both the solvents toulene and Glycerol with all the concentrations used for the study .

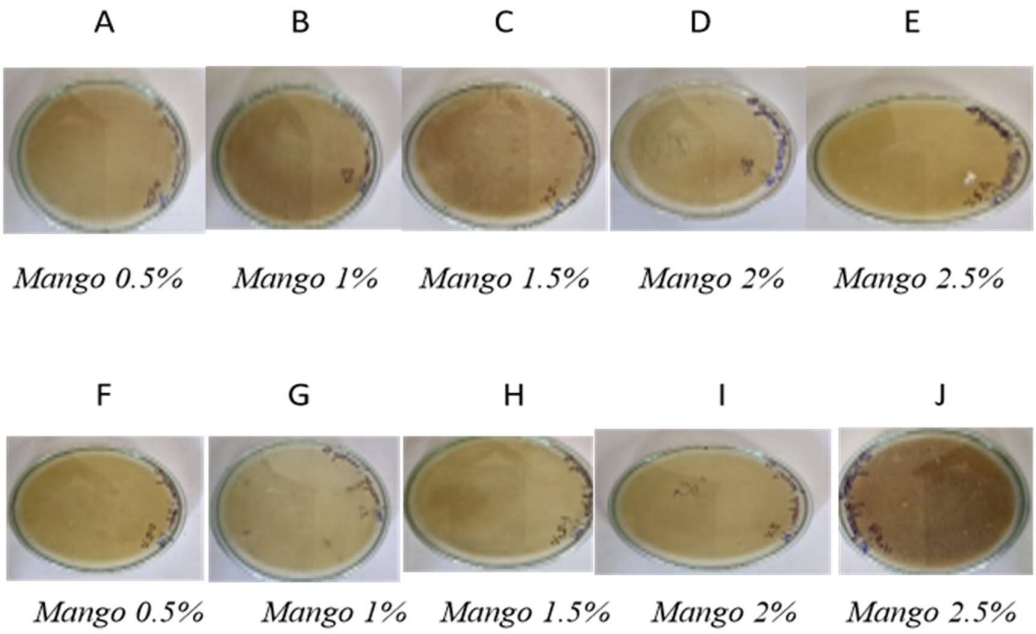

Figure: 6 : Biofilm disruption in S.aureus Using 0.5% - 2.5% concentrations of *Magniferous indica* (A-E) Biofilm induced using Toluene , (F-J) Biofilm induced using Glycerol.

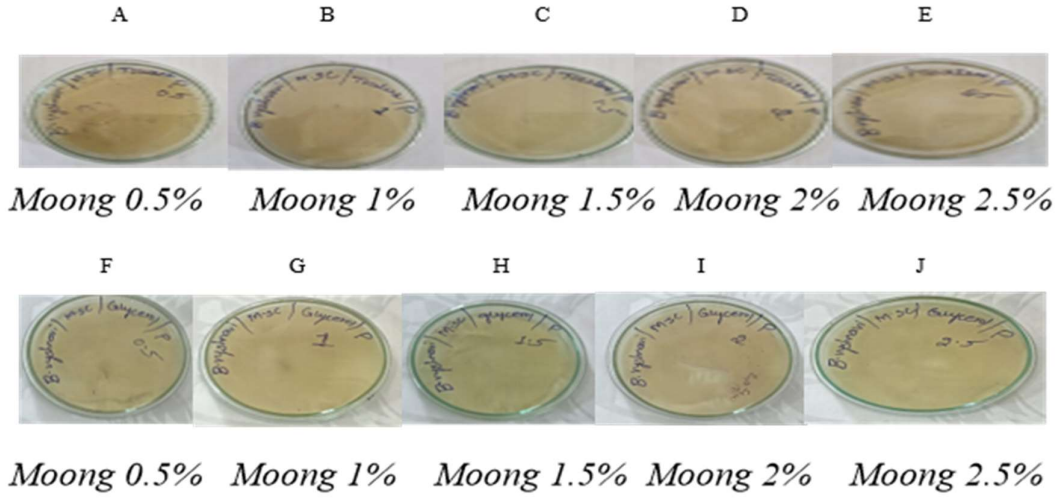

Figure: 7:Biofilm disruption in S.aureus Using 0.5% - 2.5% concentrations of *Vigna radiata* (A-E) Biofilm induced using Toluene , (F-J) Biofilm induced using Glycerol.

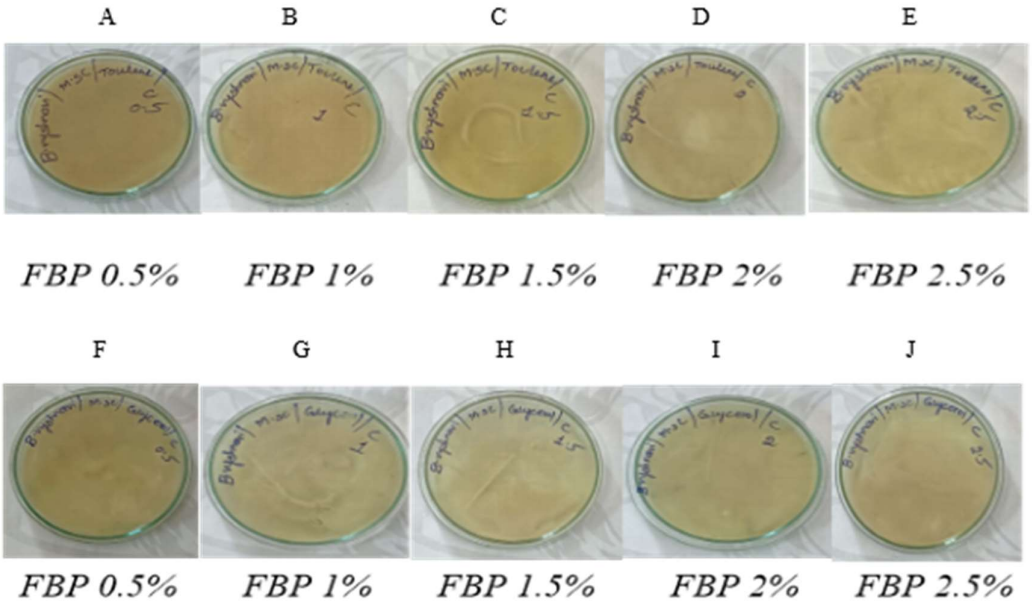

Figure: 8 : Biofilm disruption in S.aureus Using 0.5% - 2.5% concentrations of *Lathyrus sylvestris* (A-E) Biofilm induced using Toluene , (F-J) Biofilm induced using Glycerol.

Vigna radiata and Lathyrus sylvestris phytochemical constituents failed to disrupt biofilms formed by *Staphylococcus aureus* induced using toulene and glycerol at all the concentrations used (0.5% -2.5%) for the study.

Discussion and Conclusion:

Biofilm formation can contribute to enhanced antimicrobial resistance, protection against immune system causing highly potent infections. Disruption of biofilm can cause disruption of bacterial aggregates in to individual colonies with increased susceptibility to antimicrobial agents. *S.aureus* is one of the pathogen forms biofilms and current research involves targeting the biofilm disruption using anti microbial agents (Lin et al.,(2012)) and analyzing as well as targeting extracellular proteins present in *S.aureus* (Le KY et al., (2014)).

After initial attachment the *S.aureus* and *S.epidermis* secrete extracellular matrix proteins and polysaccharides and eDNA forming EPS. EPS enhances eDNA retention in biofilms and contributing to Biofilm stability (Sudhir K Shukla , T Subba Rao (2017)). The bacteria has array of exo enzymes that can target the bacteria for biofilm disruption to colonize to new surfaces. *S.aureus* and *S.epidermis* commonly form biofilms on the surgical devices there by acting as a means for the spread of nosocomial infections(Katrin Schilcher, Alexander R. Horswill (2020)).

Current research is to focus on the removal of biofilms to increase the susceptibility to antibiotics. Bacteriophage-encoded proteins, such as endolysins (Cheung AL, Ying P. (1994)), virion-associated peptidoglycan hydrolases, and exopolysaccharide depolymerases, have been

shown to be efficient against these structures (Diana G et al.,(2017)). However, the current screening techniques for identification of antibiofilm properties of phage derived proteins or enzymes associated with it has shortcomings.

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