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COMBATING *XANTHOMONAS CAMPESTRIS*, A PLANT PATHOGEN WITH A UNIQUE FORMULATION OF BIOAVAILABLE PHYTOCHEMICALS

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Abstract: The prevalence of plant diseases continues to increase constantly. Traditional methods to combat plant diseases are harmful pesticides, chemical fertilizers, antibiotics, etc. These traditional methods have a negative impact on the environment as well as on humans. Owing to this fact, this research focuses on using a novel biocontrol strategy that is green, eco-friendly, and positively impacts the environment. Bacteria communicate with each other, also known as Quorum sensing, and infect plants. Quorum quenching is a novel biological method that is used to manage plant disease. Citrus canker is a dangerous plant disease that causes plant deaths after the pathogen causes it. The only way to manage it is to obliterate all susceptible plants. The incidence rate of citrus canker is increasing globally as the disease can be caused over a short distance. The incidence rate of citrus canker in orchards is 36.2% and shows a severity of up to 18%. Natural compounds have been identified as quorum-sensing inhibitors and one such example is phytochemicals, which are secondary metabolites and can be used as biocontrol agents against phytopathogens. In this project, three different essential oils; cinnamon leaf oil, clove bud oil, and Citral were used. All the essential oils were tested against *Xanthomonas campestris*, a plant pathogen, majorly affecting citrus fruits. Minimum inhibitory concentration (MIC) was done to check the minimal concentration at which these oils kill the pathogen. The Quorum Quenching (QQ) property of the above-mentioned oils was done using Anti-swarming or anti-gliding assay. Essential oils are hydrophobic and are solubilized using Sophorolipids (SLS). Using Sophorolipids, formulations were prepared which showed a synergistic effect in killing the pathogens.
Index Terms **- Quorum quenching, Quorum sensing, Sophorolipids, *Xanthomonas campestris*, Citrus canker, Phytochemicals**

INTRODUCTION-

Citrus canker is a disease caused by phytopathogen *Xanthomonas campestris*, and the disease was first identified in the United States in 1910. The disease is not harmful to humans but it affects the fruits and leads to dramatic losses. Production of citrus fruits is widespread in the world. India is one of the leading countries that harvest citrus fruits. Citrus fruits are prone to diseases caused by Bacteria and Fungi. Citrus canker is a devastating bacterial disease caused by *Xanthomonas sp.* A severe disease infection causes several effects, such as defoliation, dieback, badly discolored fruit, decreased fruit quality, and early fruit drop. [1] Traditional methods have been employed to treat the disease like chemical pesticides, but they are harmful to the environment. The residues of these pesticides reside in the soil contaminating the soil microflora and affecting the soil fertility, reducing the quality of the farm product. Along with their negative impact on the environment, they possess harmful effects on human beings like stinging, rashes, blisters, blindness, nausea, dizziness, diarrhea, and in the worst case death. Owing to all these problems novel strategies are being studied to prevent the diseases. Communication in Bacteria is achieved via Quorum sensing. Quorum sensing was first observed in *Vibrio fischeri* [2]. Quorum sensing (QS) is dependent on population density [3]. The cell-to-cell signaling is different in both Gram-positive bacteria and Gram-negative bacteria. The mechanism of signaling is different in both groups. In Gram-negative bacteria fatty acid derivatives, N- acyl-homoserine lactones (AHLs) act as signaling molecules. The AHL molecule contains a lactone ring and an acyl chain with varying numbers of carbon atoms (4-8). LuxI gene encodes for the enzyme AHL synthase which acts as a signaling molecule in *Vibrio fischeri.* Two substrate molecules that produce AHLs are acylated acyl carrier protein and S-adenosyl-L-methionine (SAM). The homoserine lactone ring is formed due to a lactonization reaction through a nucleophilic attack on the gamma carbon of SAM. Whereas in Gram-positive bacteria peptide derivatives act as signaling molecules. This system makes use of two proteins and therefore is known as a two-component system. The ABC transporter translocates the oligopeptides outside the bacteria. Once the concentration attains the threshold, it gets detected by sensor kinase. The receptor protein is autophosphorylated at histidine (H) and the phosphate group is translocated to aspartate residue (D) The phosphorylated regulator protein regulates the transcription of desired genes [4]. The signaling molecules of quorum sensing are responsible for the induction of virulence genes, pathogenicity, etc [5]. Quorum sensing controls various processes like bioluminescence, virulence gene expression, etc.

To avoid all these above-mentioned processes, quorum quenching is being used. Similarly, the phytopathogen has the quorum sensing signaling molecules through which they infect the plants. One strategy to protect plants from infection is disrupting the Quorum signaling mechanism. By degrading the AHLs, the signal is disrupted which leads to disruption of QS, and this phenomenon is called Quorum Quenching. [6]. Plants, algae, and bacteria can naturally inhibit the QS mechanism [7]. The extracts from *Cinnamomum verum* and *Syzygium aromaticum* can Quorum quench the *Xanthomonas campestris*. Extract from orange peel, also known as Citral can quorum quench the phytopathogen. *Xanthomonas campestris* affect a wide variety of plants that are cruciferous in nature for example, Cabbage, Broccoli, Cauliflower, etc.[8] Antibiotics are traditionally used to treat bacterial pathogens, but their usage has imposed multiple disadvantages, and severe most being AMR. AMR is generally inflicted by bacteria due to the formation of biofilms using the Quorum Sensing mechanism and pressure imposed by the overuse of antibiotics. In accordance with, that Quorum quenching has been used as an alternative. Unlike antibiotics which kill the bacteria, in Quorum Quenching the signaling mechanism is disrupted without killing the bacteria and thus bacteria enter the planktonic stage and are unable to show resistance towards Quorum Quenching. In Quorum Quenching, signaling AI molecules are inhibited and their detection by receptors is hampered or enzymes degrade them, and in turn, bacteria are unable to communicate, and processes like biofilm formation are inhibited. Thus, this novel strategy is gaining attraction worldwide over traditional methods. Quorum Quenching is non-toxic and bacteria cannot produce resistance against them and is a green, eco-friendly alternative to treat bacterial pathogens.

Aromatic plants have a natural ability to produce a wide range of volatile molecules, together which are called Essential oils. Essential oils are mostly secondary metabolites that possess biological activities like antimicrobial activity, antioxidant, anti-septic, anesthetic, and anti-inflammatory activity [9]. As essential oils are bio-products, they are more ecological and are used as alternative bio-control agents than chemical pesticides [6]. Trans-cinnamaldehyde, cinnamyl acetate, eugenol, L-borneol, caryophyllene oxide, b-caryophyllene, L-bornyl acetate, E-nerolidol, α-cubebene, α-terpineol, terpinolene, and α-thujene are some of the active components present in Cinnamon leaf oil. [7]. Similarly, the active compounds present in Clove bud essential oil are Eugenol (89%), eugenol acetate (5-15%), and β-cariofileno. [10]

Essential oils are hydrophobic in nature and need to be solubilized for their application. The solubility of essential oils was increased by adding emulsifiers. The phytochemicals used in this study are highly hydrophobic and emulsifiers were incorporated to convert them into their hydrophilic form. In this study, we used biosurfactant-sophorolipids (SL) as emulsifiers
as they can increase the hydrophilicity of essential oils. SLs are easy to synthesize as well as they are biodegradable, eco-friendly, biocompatible, and highly stable. C16-C18 fatty acids make sophorolipids unique from conventional SLs. In this study, for synthesizing sophorolipids, *Starmerella bombicola (*ATCC22214*)* is used.

RESEARCH METHODOLOGY

3.1 Production of Sophorolipid-

Materials used- Malt extract, Glucose, Yeast extract. Peptone, Fatty acid, Ethyl acetate, Erlenmeyer flask, Separatory funnel, Centrifuge, Incubator shaker, Rotary evaporator

MGYP- Media components (g/L)- Malt extract- 3, Glucose- 20, Yeast extract- 3, Peptone- 5,

Biosurfactants were synthesized from Yeast, *Starmerella bombicola* (ATCC22214). The inoculum was prepared in MGYP broth, inoculum buildup was done, and the flask was incubated at 30ºC at 180 rpm in a rotary incubator shaker.

The production medium used was carried out using the Resting cell method. In this method, *Starmerella bombicola* was grown in MGYP broth for 48 hours at 30ºC and was then transferred to 100ml of MGYP broth and incubated for 48 hours at 30ºC at 180 rpm. Later, the broth was centrifuged at 5000rpm for 10 minutes at 4º C. The supernatant was discarded and 100ml of 10% glucose solution was added along with 2% Fatty acid. The flasks were incubated at 30ºC at 180 rpm in a rotatory incubator shaker. Downstream processing was done using the Solvent extraction method. The solvent used for the extraction of Sophorolipid was Ethyl acetate. The sophorolipid is collected in the organic phase and is evaporated using a rotary evaporator and Crude sophorolipid was obtained.

3.2 Anti-microbial activities of phytochemicals-

3.2.1 Minimum inhibitory concentration (MIC)-

Materials used- Luria- Bertani agar (LBA), freshly inoculated culture, Essential oil (Cinnamon leaf oil, Clove bud oil), cork borer, Erlenmeyer flask, glass spreader, ethanol, petri dish.

Luria Bertani agar was sterilized in an Erlenmeyer flask at 121ºC at 15psig. The media was poured into sterile plates aseptically. 100µl of freshly inoculated culture was spread on LB agar.

The well diffusion method was employed Using a cork borer, wells were created onto the agar plates. Different concentrations of phytochemicals were tested and the plates were incubated at 30ºC for 24-48 hours. The zone of inhibition around the well was measured.

3.2.2 Quorum quenching through anti-swarming assay-

Materials used- 0.4%Luria Bertani agar, phytochemicals, dollar plates, pipette

0.4% Luria Bertani agar was autoclaved at 121ºC at 15psig . Media was poured into the plates and along with that 1µl/ml essential oil was added and mixed with the media using a sterile tip. Once it was solidified, the culture was spot inoculated at the center of the plate using a sterile tip. The plates were incubated upright at 30ºC for 24-48 hours. The assay plate containing culture without essential oil served as the positive control.

3.2.3 Anti-bacterial activity of blend: contact inhibition assay

Materials used- Luria Bertani agar, formulated blend, ethanol, petri plate, glass spreader

To check the anti-microbial activity of the blend, blends were formulated. In this study, SL and the test organism were allowed to come in contact with each other for a certain period, and after a definite time interval, the blend was plated using the spread plate technique. The plates were incubated at 30ºC for 48hrs. After the incubation period, the cell count was counted and compared with control.

3.2.4 Anti-biofilm assay-

Materials used- Dollar plates, cover slips, glass slide, 0.1% crystal violet, Saline, Nutrient broth, 30% acetic acid, distilled water, Incubator, Microscope

Freshly inoculated *Xanthomonas* was used.

The Optical density of the culture was adjusted to 0.1 at 540 nm. The coverslips were flame sterilized and placed in dollar plates aseptically. 2ml of culture was added, which served as Positive control (Fig 1 (a)). The unique formulation of bioavailable phytochemicals was added onto the coverslips placed in dollar plates marked as Test. The plates were incubated at 30ºC for 48 hours in an Incubator. After incubation, the coverslips were washed with Saline, and 0.1% of Crystal violet was added. Crystal violet was washed using saline and the coverslips were observed under a microscope at 40X. The procedure was followed as given by Desai, D., Hirlekar, S., Navale, G., Prabhune, A., Late, D., Dharne, M., & Walke, P. (2021). -2. [11] The quantitative analysis was carried out by calculating percent inhibition. The equation used was:

$$\text{Percent inhibition} = \frac{\text{OD of positive control} - \text{OD of test} \times 100}{\text{OD of positive control}} \quad \text{(Equation 1)}$$

3.2.5Anti-bioluminescence assay-

Materials used- Overnight cultures of E. coli pSB401 and E. coli pSB1142, AHL molecules 96 well plates, Formulated blend, Incubator shaker, Microplate reader

In this study, the bioluminescence assay was carried out by reporter strains *Escherichia coli* 401 and *Escherichia coli* 1142. Short-chain AHL molecules and long-chain AHL molecules were used. *Escherichia coli* 401 and *Escherichia coli* 1142 are lux-based biosensors that are activated by short-chain AHL molecules C4, C6, and C8 and long-chain AHL C10, C12, and C12 3-OXO. In this study, the QQ ability of the blend was checked through a bioluminescence assay and percent inhibition was calculated. The Optical density of the culture was adjusted to 0.1 at 600nm. The assay was done using a 96-well plate. An optical density-adjusted culture was added to the plate. The unique formulated blend was added to the respective plates. The Short chain and long-chain AHL molecules were added in respective wells in the desired volume. The plate was kept in an incubator shaker for 6 hours. the bioluminescence was measured using a microplate reader in luminescence mode.

$$\text{The percent inhibition was calculated as: } \% \text{ inhibition} = \frac{\text{Positive control-test}}{\text{Test}} \times 100 \quad \text{(Equation 2)}$$

IV. RESULTS AND DISCUSSION

4.1 Results-

Sophorolipids were produced using Starmerella bombicola. Different essential oils were tested for their anti-microbial activities. The Minimum inhibition concentration was studied for the essential oils against Xanthomonas campestris. In this study, essential oils were tested for their quorum quenching activity against Xanthomonas campestris. All three essential oils (Cinnamon leaf oil, Clove bud, and Citral) showing the best results in MIC were further tested for their ability to quench the test organism. The activity was checked by performing the anti-swarming assay. The plate containing essential oil had quenched the organism. The ani-microbial activities of all the obtained SLs were performed using the Contact inhibition method. The individual anti-microbial activity of all three SLs was also checked, whereas, blends were formed of Cinnamon leaf oil and Clove bud oil with all three SLs, and their anti-microbial activity was observed using the Contact inhibition method. Further testing of all the blends was carried out by performing an Anti-biofilm assay. Using the formula (equation 2) percent inhibition was calculated for all the blends containing SL and Cinnamon leaf oil and the highest percent of inhibition was observed for the Cinnamon leaf oil + Myristic Acid Sophorolipid (MASL) blend showing 75% of inhibition, followed by Oleic Acid Sophorolipid (OASL) showing 53% inhibition and showing least percent of inhibition Palmitic Acid Sophorolipid (PASL) with 30% inhibition. Anti-bioluminescence assay of the MASL blend was done using AHL molecules and the highest inhibition was observed against the C10 AHL signaling molecule.

Percent inhibition using Equation 1

Sr. No	Phytochemicals	Percent inhibition
1.	Cinnamon leaf oil	38%
2.	MASL + Cinnamon leaf oil	75%
3.	OASL + Cinnamon leaf oil	53%
4.	PASL + Cinnamon leaf oil	30%

Table 1 – Percent inhibition of biofilm of different Phytochemicals

Percent inhibition using Equation 2

Sr no	Formulated blend	Percent inhibition
1	MASL against C4	29.37%
2	MASL against C8	28.69%
3	MASL against C10	30.89%
4	MASL against C12	29.16%

Table 2 - percent inhibition of MASL blend against short-chain and long-chain AHL molecules

Discussion-

The incidence of Anti-microbial resistance is increasing extensively due to the overuse of antibiotics. To treat diseases caused by bacterial pathogens, novel technologies are being studied. Traditional methods have consequences and are harmful to the environment as well as to humans. In this study, Quorum sensing and Quorum Quenching are the approaches that were explored. The study focused on exploring natural bioactive compounds like natural, eco-friendly phytochemicals, which pose no harm to the environment. Even though these phytochemicals are advantageous, they are mostly hydrophobic, causing problems with their applicability. Owing to this problem, biosurfactants were used in this study as emulsifiers, increasing the bioavailability of these phytochemicals. Biosurfactants are eco-friendly, green and possess no harm to the environment, and are synthesized from GRAS yeast. 3 different SLs were synthesized and the solubility and efficacy of the phytochemical were studied. Oleic acid sophorolipid has 18 carbon chain monosaturated fatty acid backbone. Similarly, Palmitic acid sophorolipid has a 16-carbon chain length monosaturated fatty acid backbone. Whereas, Myristic acid sophorolipid has a backbone of 14-Carbon chain length fatty acid whose CMC value is less as compared to other SLs. All the 3 synthesized SLs were screened for their ability to disperse oil and MASL gave the best results as its zone of clearance measured around 32mm whereas OASL's zone of clearance measured about 16mm and PASL's measured 15mm. From this assay, we can say that MASL is a potent SL having the highest biosurfactant activity. The Minimum inhibition concentration was studied for the essential oils against *Xanthomonas campestris.* Cinnamon leaf oil showed maximum inhibition as compared to other EOs such as clove bud oil, citral, camphor, etc. QQ activity of the phytochemical was checked using an anti-swarming assay. The Positive control having broth and culture showed growth whereas the test having broth, test organism, and EO, showed no growth suggesting that the organism has been quenched by the essential oil. The contact inhibition method of all three SLs showed and 100% inhibition was observed when compared with control. The contact inhibition method was used for testing the activity of blends and the best results were obtained for Cinnamon leaf oil and MASL blend and Cinnamon leaf oil and OASL blend, However, Clove bud oil and OASL blend showed 95% inhibition. Biofilm is formed when the organisms communicate with each other and produce an exopolysaccharide layer which leads to film formation. The cell to cell communication takes place due to Quorum Sensing. At high cell concentration cells communicate through signaling molecules and film is formed. In QQ, antibiofilm is one of the confirmatory assays to check the ability of the component to QQ. In this study, the ability of the blend to Quorum quench the phytopathogen was checked. It was observed that MASL and cinnamon leaf oil blend gave the best results in quorum quenching *Xanthomonas campestris* (Fig 1). From the above assays, the MASL blend gave the best results. Anti-bioluminescence assay is one of the processes controlled by quorum sensing. The AHL signaling molecules are responsible for the luminescence as they interact with the receptor molecule and luminescence is observed. Whereas, when an anti-quorum sensing inhibitor is added, there is competition between the AHL molecule and the inhibitor and this leads to no luminescence or anti-bioluminescence. The reporter strains used in this assay were *Escherichia coli* pSB401 and *Escherichia coli* pSB1142. *Escherichia coli* pSB401 has short-chain AHL molecules as receptors and it was understood that for C4 AHL molecule and C8 AHL molecule, Palmitic acid sophorolipid gave high inhibition, and for C6 AHL molecule Myristic acid sophorolipid showed high inhibition. Similarly, *Escherichia coli* pSB1142 has long chain AHL receptor molecules and for the C10 AHL molecule, higher inhibition was shown by Palmitic acid sophorolipid whereas, for the C12 AHL molecule Oleic acid sophorolipid showed higher inhibition (Fig 2). In this study, sophorolipids are used as emulsifiers as they act as carrier molecules to increase the bioavailability of the hydrophobic phytochemicals. The AHL molecules used in this study are specific for Escherichia coli, whereas for *Xanthomonas campestris*, the Diffusible signal factor (DSF), acts as the signaling receptor and thus they can be specifically checked against these receptor molecules.

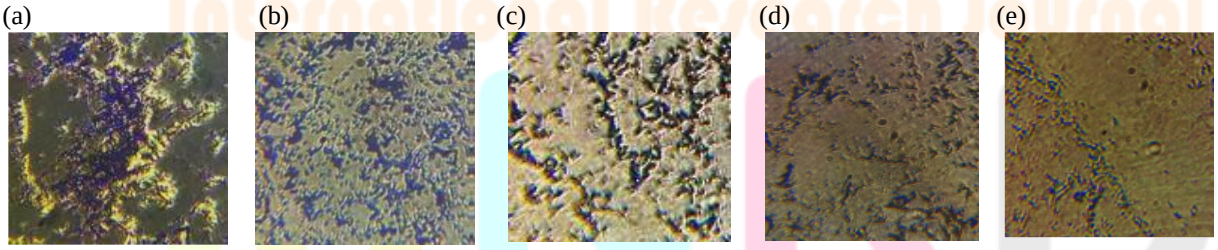
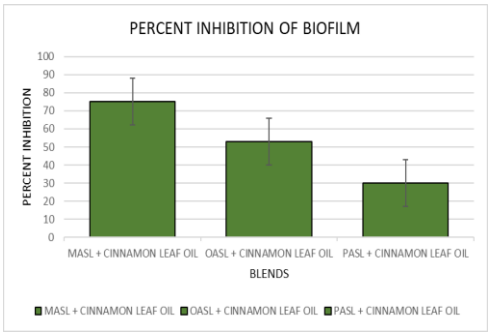
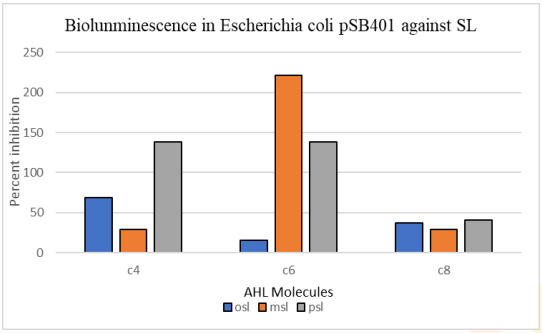


Fig 1- antibiofilm assay (a) positive control (b) cinnamon leaf oil (c) masl + cinnamon leaf oil (d) oasl + cinnamon leaf oil (e) pasl + cinnamon leaf oil



(b)



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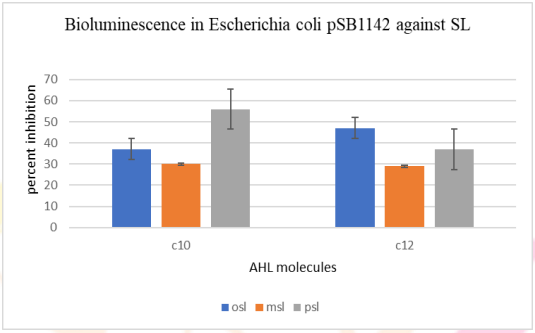

fig 2- (a) bar graph showing the comparison of inhibition of biofilm formation by cinnamon leaf oil blend (b) bar graph showing percent inhibition of the blends against short-chain ahl molecules (c)bar graph showing percent inhibition of blends against long-chain ahl molecule

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