Inhibition of Biofilm formation in plant pathogenic Xanthomonas *axonopodis* by *Cyanodon dactylon*

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Abstract*— Cyanodon dactylon is hardly perennial grass belongs to the family Poaceae and possess many pharmacological activities like antidiabetic, antioxidant, antidiarrheal, hepatoprotective, antiulcer, immunomodular, CNS (Central nervous system) depressant, antimicrobial and germicidal due to its phytochemicals. Cyanodon dactylon prevent bacterial pathogenicity without developing resistance. Cyanodon dactylon extract exhibited different degree of inhibition against selected gram positive and gram negative bacteria due to their varying polarity and solubility. Lemon (Citrus limon) is the third important species of citrus after Orange and Mandarin. Citrus canker causes severe infections such as defoliation, blemished, fruit, premature fruit drop, die - back of twigs and general debilitation of the tree. Organisms involved in the causes of citrus canker are Xanthomonas axonopodis pv. Citri, Xanthomonas campestris pv. Citri, Xanthomonas axonopodis pv. Aurantifolli. The bacterial genus (Xanthomonas axonopodis, Xanthomonas citri) causing citrus canker which lead to heavy yield losses. In the present study, Cyanodon dactylon showed effective inhibition against biofilm formation, protease, pectinase and elastase in test bacterial isolates.

Indexed Terms- Cyanodon dactylon, Xanthomonas sp., citrus canker, biofilm inhibition

I. INTRODUCTION

Cyanodon dactylon is hardly perennial grass belongs to the family Poaceae and possesses many pharmacological activities like antidiabetic, antioxidant, antidiarrheal, hepatoprotective, antiulcer, immunomodular, CNS (Central nervous system) depressant, antimicrobial and germicidal due to its phytochemicals. Cyanodon dactylon prevent bacterial pathogenicity without developing resistance. (Hamid Soraya et al., 2015). *Cyanodon dactylon* extract exhibited different degree of inhibition against selected gram positive and gram negative bacteria due to their varying polarity and solubility (B.K.Prashanth, 2017).

Fig.1 *Cyanodon dactylon*

Lemon (Citrus limon) is the third important species of citrus after Orange and Mandarin. The peel is a byproduct of lemon juice processing, with a high potential use. Two different tissues are found in what is colloquially called Lemon peel: Flavedo and Albedo.

Flavedo is the outer layer of peel, whose color varies from green to yellow. It is the source of essential oils. Albedo is the major component of lemon peel, and is a spongy and cellulosic layer laid under Flavedo. Albedo has high fibre content. Furthermore, the presence of associated bio active compounds (flavonoids and vitamin-c) with antioxidant properties in fresh lemon (Somayeh Sadat Fakoor Janati et al., 2012). Citrus canker causes severe infections such as defoliation, blemished, fruit, premature fruit drop, die - back of twigs and general debilitation of the tree (James. H. Graham et al., 2004). Organisms involved in the causes of citrus canker are *Xanthomonas axonopodis pv. Citri, Xanthomonas campestris pv.*

Citri, *Xanthomonas axonopodis pv. Aurantifolli* (Gottwald, 2005).

Citrus species are susceptible to a number of destructive diseases that are continuously emerging and which can severely limit production are totally decimate an industry of a country, the bacterial genus (*Xanthomonas axonopodis, Xanthomonas citri*) causing citrus canker which lead to heavy yield losses (Tennant et al., 2008). Citrus canker is a serious disease of most commercial citrus cultivars and some citrus relatives (K. Athira, 2017).

Xanthomonas axonopodis pv. *citri* (Xac) is a Gram negative obligate aerobic bacterium. The organism is also the phytopathogen responsible for citrus canker, a severe disease that affects most commercial citrus cultivars (Maria Laura Tondo et al., 2010). Many plant pathogens, including those belonging to the genus *Xanthomonas*, have been reported to secrete extracellular depolymerizing enzymes such as cellulases, xylanases, proteases and pectinases (Singh and Verma 1975; Chapon et al., 2001).

Pectinolytic organisms are a constant thread to spoilage of vegetables because of their extensive host range and widespread distribution. Between 10 to 30% of fresh vegetables are lost, mainly due to Microbial spoilage (Wakil et al., 2011). Most Pectin degrading microorganisms are associated with raw agricultural product and soil. Upto 10% of the bacterial genera in the soil have been shown to be pectinolytic, for instance *Achromobacter, Aeromonas, Arthrobacter, Enterobacter, Bacillus, Clostridium, Erwinia, and Flavobacter*.

Pectinolytic organisms belonging to the genera of *Agrobacterium* (causing Crown-gall disease), *Clavibacter* (potato ring rot, tomato wilt, fruit spot), *Erwinia* (blight, wilt and soft rot), *Pseudomonas* (leaf spots, galls, wilt, blight, soft rot and canker), *Clostridium, Bacillus, Xanthomonas* (leaf spot, cutting rot, canker and blight) *Streptomyces* (potato scab and soil rot, *Flavobacterium* and *Cytophaga* have been reported to be associated with post-harvest rots of various food crops. In order to cause plant infection bacteria need to enter into plant tissue. Most bacteria do this via stomates, wounds, or by the help of feeding insects. After entering the plant the aggressiveness of the plant pathogen varies. Biotrophs multiply and can stay within the host tissue for a long time before killing it, while necrotrophs multiply fast in the host tissue and are capable of destroying it rapidly (Alfano and Callmer, 1996). Bacteria-plant interactions are highly coevolved and dynamic processes at molecular, cellular and colony-tissue level. Understanding the different strategies and mechanisms the bacteria use for plant infection will aid in the development of better means to defend plants from bacterial diseases.

The most dangerous disease caused by the gram-negative bacterial pathogen *Xanthomonas axonopodis* pv. *Phaseoli* (*Xap*) and its fuscans, is a significant seed borne disease of common bean, Common bacterial blight (CBB).

CBB affects foliage, pods and seeds of common bean and is considered as the major problem in most common bean production areas of the world. During extended period of warm and humid weather, the disease can be highly destructive and causes losses in both yield and seed quality of bean in many production areas of Ethiopia. It is widespread throughout African's bean growing area and most prevalent at low to mid altitude under warm condition.CBB is seen wherever beans are produced and is an economically-important disease that can reduce yield from 10% to 45% depending on the environmental conditions and genotype (Belete, 2017)

Xanthomonas axonopodis pv. *Citri* (Xac) is an obligate aerobic phytopathogen constantly exposed to hydrogen peroxide produced by normal aerobic respiration and by the plant defense response during plant-pathogen interactions. (Maria Laura Tondo et al., 2010).This Organisms also possesses catalase which detoxifies the H_2O_2 converted into O_2 and water. The production of catalase from the organisms which catalyze H_2O_2 is difficult to killing the pathogens without affecting their growth (Liao and Wells, 1986).

One such attractive strategy is targeting the bacterial quorum sensing (QS) system, a cell density dependent mechanism mediated through small signaling molecules called auto-inducers to regulate the various gene expressions in bacterial organisms. The most extensively studied QS system is known to be present

in Gram negative bacteria, which utilizes N-acetyl homoserine lactones (AHLs) as signaling molecules. The growth and enzymatic activity of Gram-negative bacteria plays a vital role in food microbiology, where the spoilage of fresh foods such as fish, meat, milk and vegetables are mostly a consequence of the enzymatic degradative activity of Gram negative bacteria growing to high cell densities (10^8 to 10^9 CFU per gram) (Rivas et al., 2007). Interestingly, several enzymatic activities including saccharolytic, proteolytic, lipolytic, chitinolytic, cellulolytic and pectinolytic activities associated with the deterioration of foods have been reported to be regulated by QS system (Ammor et al., 2008).

Since, the enzymatic activities of several pectinolytic bacterial pathogens are regulated by signal mediated QS system, the blocking of such mechanisms will pave the way to prevent the severe economic losses in industrial sectors as well as to increase food safety. Although, QS has been extensively studied in bacterial pathogenicity and the reports on QS in bacterial food spoilage is very much scarce. Hence, the present investigation is intended with an aim to inhibit the QS regulated enzymatic activities of plant bacterial pathogens by the addition of *Cyanodon dactylon* as quorum sensing inhibitor (QSI) compounds.

II. BIOFILM FORMATION

Biofilm are complex aggregation of microbial cells in the self secreted exopolymeric matrix (Shigeta et al., 1997). A Biofilm can be formed by a single bacterial species, but more often biofilms consist of many species of bacteria as well as fungi, algae, protozoa, debris and corrosion products. Formation of a Biofilm begins with the attachment of free - floating micro organisms to the surface. These first colonists adhere to the surface initially through week reversible Van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion molecules such as pile. The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the Biofilm together and begins to excrete slimy material (Davies et a1., 1998).

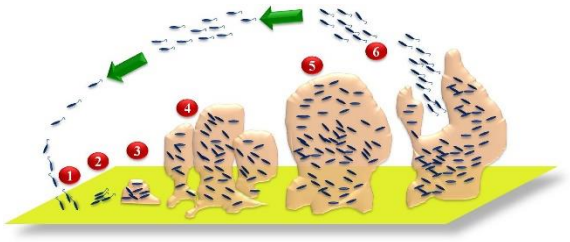


Fig.2 1.) Planktonic cells 2.) Swimming motility 3.) Swarming motility and EPS secretion 4.) Development of biofilm architecture 5.) Mature biofilm 6.) Detachment of bacterial cells from biofilm

Biofilm are usually found on substrates submerged in or exposed to some aqueous solutions. Bacteria living in the biofilm can have significantly different properties from free - floating bacteria , as the dense and protect environment of the film allows them to cooperate and interact in various ways. One benefit of this environment is increasing resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. This matrix protects the cell within it and facilitates communication among them through chemical and physiological signal. Some biofilm have been found to contain water channels that help distribute nutrients and signaling molecules. This matrix is strong enough that in some cases, biofilm can become fossilized (Mah and O 'Toole, 2001).

The bacterial cells on the surface of the biofilm are different from the cells within the biofilm matrix. The embedded cells behavior can change as the thickness of the biofilm changes. The surface cells, no matter how old the biofilm is, are likely to mimic surfaces cells of young biofilms which are metabolically active and large. These surface cells divide and increase the thickness of the biofilm. Little oxygen are available to embedded cells, therefore, they are smaller and grow slower. The bacteria exist in a somewhat dormant state, become active when cells in the outer layers are killed (Mah and O'Toole, 2001).

Some of the environmental signals that have currently been identified to regulate the structure of a mature biofilm are nutrient availability and quorum sensing and are not species specific. Nutrient availability regulates the depth of the biofilm in such a way that the maximal number of cells in a biofilm appears to

occur at suboptimal nutrient concentrations. At extreme nutrient-rich or very nutrient poor conditions, greater number of cells is in the planktonic phase where they have greater access to the local nutrients or can be distributed to a new environment. Similarly, QS control of the formation of channels and pillar like structure may ensure efficient nutrient delivery to cells in a biofilm (Stanley and Lazazzera, 2004).

Biofilms are important survival mechanism for bacterial cells. According to *in vitro* studies, they can avoid attack by host defense. Also, biofilms are much more resistance than planktonic cells to antimicrobial agents. For example, chlorinated of a biofilm is usually unsuccessful because the biocide only kills the bacteria in the outer layers of the biofilm. The bacteria within the biofilm remain healthy and the biofilm can grow again. Repeated use of antimicrobial agents on biofilms can cause bacteria within the biofilm to develop and increase resistance to biocides (Costerton et al., 1999).

One mechanism of Biofilm resistance to antimicrobial agents is the failure of an agent to penetrate the full depth of the biofilm. Polymeric substances like those that make up the matrix of a biofilm are known to retard the diffusion of antibiotics and solutes in general diffuse at slower rates within biofilms than they do in water. A second hypothesis to explain reduced biofilm susceptibility to antibiotics posits that at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow growing or starved state. Slow-growing are non growing cells are not very susceptible to many antimicrobial agents. A third mechanism of reduced biofilm susceptibility, which is more speculative than the preceding hypothesis, is that at least some of the cells in biofilm adapt a distinct and protected biofilm phenotype (Costerton et al., 1999; Mah and O'Toole, 2001). The expression of many phenotypic traits in microorganisms is governed by tight gene regulation and influenced by growth phase, nutrients, external stresses and multitude of other factors (Fuqua et al., 1994). Importantly, Biosurfactant production also becomes essential in maintaining biofilm architecture and allows flagellum-based propulsion over semisolid surfaces to form dense biofilm (Caiazza et al., 2005). Because factors such as EPS, biosurfactants, swimming and swarming motilities are mediated by QS and act as key players in the development of biofilm. So, targeting these factors through anti - QS strategies will lead to the prevention of biofilm.

Since, the QS plays a vital role in the virulence factor production and Biofilm formation, the inhibition of QS mechanism could be adopted as an alternative strategy to prevent the development of bacterial resistance and makes the pathogen become ineffective to establish successful infection without impose a selective pressure on bacterial growth (Hentzer and Givskov, 2003). This leads to the entry of quorum sensing inhibitor (QSI) compounds to treat bacterial infections.

III. QUORUM QUENCHING - AS AN ALTERNATIVE APPROACH

Quorum sensing systems are found in many bacteria that are pathogenic to plants, animals and humans (Williams, 2001). For most of the researchers explain a link between quorum sensing and virulence factor expression has been demonstrated. It has been reported that these pathogens probably increase their chances to infect their host successfully by delaying virulence factor production until the population density is large enough to overcome the host's immune system (Lamo Marin et al., 2007). It has been shown that inactivating the quorum sensing system of quorum sensing pathogens can conduce to a significant decrease in virulence factor expression (Swift et al., 1999) and inhibition of Biofilm formation.

As the importance of quorum sensing in virulence development of pathogenic bacteria became clear, disruption of quorum sensing was suggested as a new anti-infective strategy (Finch et al., 1998). Such targeting of quorum sensing systems is referred to as 'Quorum Quenching'. There is evidence that some plants and algae have evolved the ability to interfere with quorum sensing through the release of compounds which mimic the activity of AHL signals (Bauer and Robinson, 2002).

IV. QSI COMPOUND FROM NATURAL RESOURCES

The very first QSI compound furanone was identified

from red marine alga *D.pulchra (* Manefield et al., 1999).The south Florida medicinal plants extract such as Conocarpus erectus, Chamaecyce hypericifolia, Callistemon viminalis, Bucida burcera, Tetrazygia bicolor and Quercus virginiana were showed potential QSI activity against the various QS mediated phenomena in *P.aeruginosa* (Adonizio et al., 2008). Flavonoids like Naringenin, Kaempferol, Quercetin and Apigenin were effective antagonists against the Biofilm formation in *V.harveyi* (Vikram et al., 2010). The aqueous extract of edible fruits and plant namely *Ananas comosus, Musa paradiciaca, Manilkara zapota* and *Ocimum sanctum* exhibited QSI activity against the *C.violaceum* and *P.aeruginosa* PAOI (Musthafa et al., 2010). Cyanodon dactylon prevent bacterial pathogenicity without developing resistance. (Hamid Soraya et al., 2015).

The plant-derived dietary products have attracted widespread interest in the search of alternatives for microbial control. However, these compounds are widely considered due to their safety and have a long history of use in traditional medicine for the prevention and treatment of diseases and infections (Packiavathy et al., 2011; Musthafa et al., 2010). Based on these perspectives, the present study was initiated in order to inhibit the QS mediated virulence production and Biofilm formation in *Xanthomonas axonopodis* plant pathogens.

V. COLLECTION OF SAMPLES AND ISOLATION OF PATHOGEN

Infected lemons were collected from our college canteen, Kilakarai .The Collected lemons were washed thoroughly with sterile distilled water. After washing spoiled vegetables were surface sterilized with 95% ethanol. These punctures were made on decayed tissue using sterile inoculation needle and spread on the petriplates containing Nutrient agar. The plates were incubated at room temperature for 24h. After incubation, the suspected colonies were isolated dominantly on Nutrient agar plates.

VI. CULTURE CONDITION

Isolated colonies were maintained at room temperature. For experimental purpose, isolated bacterial pathogens were sub cultured in Nutrient agar medium.

VII. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after 24 h. of incubation. The most effective plant extracts which exhibiting a strong antibacterial activity at 10 mg/ml was manipulated to determine their MIC and evaluate their efficiency in controlling bacterial strains causing infections. The inhibition zones were measured by Vernier caliper and recorded against the concentrations of the effective plant extracts.

The MIC was recorded at the lowest concentration at which it showed complete inhibition of visible growth of the bacterial pathogens (Thenmozhi et al., 2009)

VIII. IN SITU LIGHT MICROSCOPIC OBSERVATION OF BACTERIAL BIOFILMS

Briefly 1% Overnight cultures of the test pathogen were added into MTP wells containing 1mL of fresh LB medium and cover glass of 1 cm^2 along with presence and absence of *Cyanodon dactylon* at various concentrations (300,350,400 µg/mL).After16 h of incubation at appropriate temperature, the cover glasses were rinsed three times with distilled water to remove the free planktonic cells and adhered biofilms in the cover stained with 0.2% crystal violet solution. Stained cover glasses were placed on glass slides with the Biofilm side pointing up and were visualized under light microscopy at magnification of ×40 (You et al., 2007)

IX. SWIMMING AND SWARMING ASSAYS

In swarming assays, Overnight cultures of the test bacterial pathogens were pointed at the centre of swarming plates consisting of 1% peptone, 0.5%NaCl, 0.5% of filter sterilized D-glucose with various concentrations of *Cyanodon dactylon* (300-400µg/mL) and the plate without Cyanodon dactylon as control. Plates were incubated at appropriate temperature in upright position for a 16-h period

(Deziel et al., 2001).The swarming migration was recorded by following swarm fronts of the bacterial cells. In Swimming motility assay, the overnight bacterial pathogens were point inoculated at the center of the swimming plates containing 1% tryptone, 0.5% NaCl and 0.3% agar and at respective concentrations of *Cyanodon dactylon* and incubated at appropriate temperature in upright position. The swimming migration was recorded by swim zones of the bacterial cells after 16h (Deziel et al., 2001).

X. DETERMINATION OF MIC

Based on the fact that the QSI compound not to have any adverse effect on bacterial growth, the MIC assay was performed to assess QS inhibitory concentrations of *Cyanodo dactylon.* The lowest concentration of *Cyanodo dactylon* that showed complete inhibition of visible growth was determined as MIC. The MIC of *Cyanodo dactylon* for each test bacterial pathogen was determined by doubling dilution method. The concentration well below the MIC was considered as sub-MICs which were not expected to inhibit the bacterial growth. Therefore, for QS inhibition studies , the sub-MICs were selected and used in subsequent assay. The selected sub-MICs were ranging from 100-400 µg/mL was subjected to Biofilm inhibition assay. In which, the sub-MIC concentrations of 100-250 µg/mL didn't show any reduction in Biofilm. The concentrations above 250µg/mL showed considerable reduction in Biofilm biomass quantification. So, the concentrations of 300, 350 and 400 µg/mL were fixed and move on to subsequent assays.

XI. IN SITU LIGHT MICROSCOPIC OBSERVATION OF BACTERIAL BIOFILM

The anti Biofilm property of *Cyanodo dactylon* in inhibiting Biofilm forming ability of test bacterial pathogens was further confirmed through light microscopic analysis. The results revealed that the untreated cover glasses displayed a well developed Biofilm growth of test bacterial pathogens such as *Xanthomonas axonopodis* (XA), while the *Cyanodo dactylon* treated cover glasses exhibited poorly developed thin Biofilm. Most notably, a reduction in number of micro colonies was observed when treated with higher concentration (400 µg/mL) of *Cyanodo dactylon* against all test bacterial pathogens.

XII. SWIMMING AND SWARMING INHIBITION ASSAY

As swimming and swarming migrations plays an important role in QS mediated bacterial Biofilm formation, an effort was made to examine the anti-QS potential of *Cyanodo dactylon* on QS depended swimming and swarming motility in *Xanthomonas axonopodis* .*Cyanodo dactylon* showed effective inhibition against swimming and swarming motility at higher concentration. It indicated that *Cyanodo dactylon* inhibited the swimming and swarming behavior and thereby prevents the subsequent Biofilm formation.

XIII. RESULT AND DISCUSSION

The Plant-bacteria interactions are long known and have three well-differentiated manifestations. The first is a direct relation between plants and pathogenic bacteria (for instance, *Agrobacterium* spp., *Erwinia* spp., *Ralstonia* spp., *Pseudomonas* spp., etc.) causing a state of disease. A second type is a direct interaction between plants and non-pathogenic bacteria (for example, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, etc.) leading to a beneficial association for both partners. The third type is a beneficial interaction between plants and microbes. However, the ultimate boundaries between a mutualistic and a pathogenic interaction can be fuzzy, and the recognition and signal-transduction processes leading to the plant response may be similar for both types of interactions (Baron and Zambryski 1995; Soto et al. 2006). The pathogenic interaction of plant-bacteria should be mediated via QS and the production of extracellular enzymes leads to the bacteria to cause disease in plants.

The QSI potential of *Cyanodon dactylon* was evaluated for their efficiency in inhibiting the Biofilm formation and its related characters. QS Mechanism plays a major role in the formation of Biofilm with complex architecture. The formation of biofilms by bacteria on plant surfaces is likely a survival strategy for the cells to withstand the harsh environment of the plant surface (wide temperature changes, desiccation, ultraviolet rays, and oxidative stress). Biofilm-

associated bacteria embedded in a matrix of extracellular polysaccharides (EPS) might be more difficult to remove from contaminated surfaces of produce or food processing areas than their solitary counterparts (Fett and Cooke 2003; Annous et al., 2004). In addition, flagella mediated swimming and the flagella and pili mediated swarming motility enhance the strength of Biofilm formation. The EPS production is likely shields bacterial cells within biofilms from desiccation and aids in resisting antimicrobial compounds. In the present study, the exposure of the respective concentration of inhibited the Biofilm biomass, EPS production, swimming and swarming motility significantly at their respective concentrations in test bacterial pathogens. Further, inhibition of Biofilm through light microscopic observation in *Cyanodon dactylon* treated and untreated bacterial pathogens showed visible reduction in their respective concentrations. Furthermore, molecular characterization of test bacterial pathogens will be done to confirm the isolated bacterial strains and studies requisite to evaluate the *Cyanodon dactylon* in in-vitro application of *Xanthomonas* sp. to reduce their pathogenicity in plants.

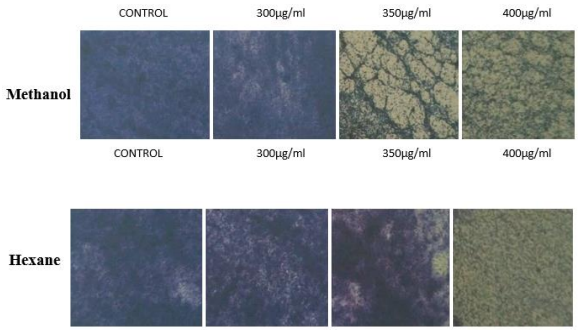
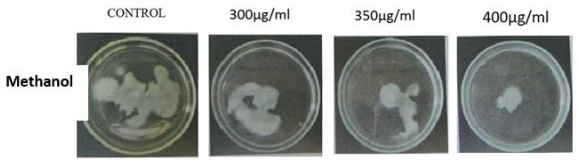


Fig.3 Representation of light microscopic images of compounds treated and untreated Biofilm against test bacterial pathogens.



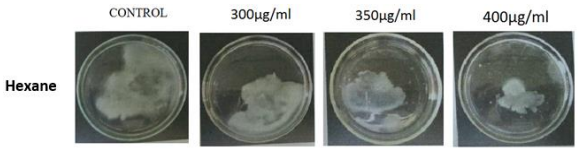


Fig.4 Inhibition of swimming motility in test bacterial pathogens Cyanodon dactylon at respective concentrations.

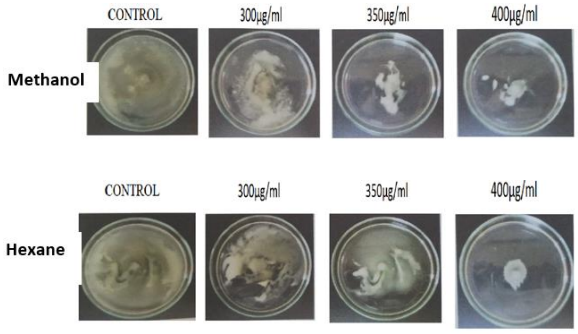


Fig 5. Inhibition of swarming motility in test bacterial pathogens by Cyanodon dactylon at respective concentrations.

CONCLUSION

In the present study, the QSI potential of *Cyanodon dactylon* was assessed against QS mediated virulence enzyme production and Biofilm formation in *Xanthomonas axonopodis*. In extracellular virulence enzyme quantification assay, the *Cyanodon dactylon* showed effective inhibition against protease, pectinase and elastase in test bacterial isolates. In light microscopic analysis revealed that the respective concentration of *Cyanodon dactylon* treated test bacterial pathogens showed reduced number of micro colonies compared with that of control. In swimming and swarming motility, the inhibition of motility was observed in the dose dependent manner. In conclusion, present study revealed the QSI potential of *Cyanodon dactylon* in reducing QS dependent phenomena in test bacterial pathogens, without any antibacterial activity. Therefore, from the obtained results predicted that *Cyanodon dactylon* showed potential anti-pathogenic and anti-Biofilm property might be used to reduce the severity of bacterial pathogens.

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