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INHIBITION OF SWARMING MOTILITY, BIOFILM FORMATION AND VIRULENCE FACTOR EXPRESSION OF URINARY PATHOGENS BY *EUPHORBIA TRIGONA* LATEX EXTRACTS

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Abbreviations:

EELE: *Euphorbia* Ethanolic Latex Extract;
DMSO: Dimethyl sulfoxide;
QS: quorum-sensing;
UTI: urinary tract infection

ABSTRACT

Euphorbia trigona latex has been used most effectively in the traditional Indian medicinal system of Ayurveda for the treatment of urinary tract infection (UTI). However, various organic and aqueous extracts of its latex were found not to possess any antimicrobial activity against several gram positive and gram negative organisms including *Proteus mirabilis* and *Pseudomonas aeruginosa* (selected for the present study) which are important urinary pathogens especially in patients with catheters over which they can form biofilms. The observation, that bacterial cell-cell communication or quorum-sensing (QS) is closely linked to swarming differentiation and virulence factor production in bacteria suggests that several bacterial pathogens may be prevented from establishing symptomatic disease *via* inhibition of their QS system by using anti-QS compounds. This is a viable alternative to the antibiotic-mediated growth inhibition or killing of pathogens, which invariably selects for multiple drug resistant cells. In order to investigate the effects of *Euphorbia* latex extracts on the pathogenicity of *Pr. mirabilis* and *Ps. aeruginosa,* their growth rate and some QS-controlled phenomena such as the swarming behavior, ability to form biofilm and virulence factor production, were monitored in the presence/ absence of the extracts. While growth rate was not affected adversely, a remarkable reduction in the swarm zone, virulence factor production and biofilm formation was observed which suggests that *Euphorbia* latex contains compounds which can potentially be developed as anti pathogenic drugs.

INTRODUCTION: The successful establishment of disease by several pathogens depends to a very large extent on their ability to perceive and respond rapidly to changes in cell density with the help of small signaling molecules. This phenomenon of quorum-sensing (QS) enables the cells to co-ordinate strategies which would not be successful if attempted individually or by a small number of bacteria [1]. Such density-dependent multi-cellular behavior in bacteria includes diverse processes viz bioluminescence, swarming, sporulation, antibiotic synthesis, etc [2].

Swarming, a process wherein short, oligoflagellated vegetative swimmer cells differentiate into aseptate multinucleate, filamentous, hyperflagellated swarm cells arranged in palisades or rafts enables bacteria to migrate rapidly and collectively over surfaces. *Pseudomonas aeruginosa* and *Proteus mirabilis* are opportunistic pathogens, especially among hospitalized and/or catheterized patients and menopausal women, the frequency of their involvement in UTI being next only to *E. coli* [3]. Both these organisms exhibit the swarming phenotype which plays a crucial role in the establishment of renal infections which begin with the colonization of the lower urinary tract followed by ascending migration of these urinary pathogens.

Pr. mirabilis and *Ps. aeruginosa* are also known to produce several virulence factors such as proteases, haemolysins, urease and rhamnolipids whose expression is considerably enhanced when the cells enter into the swarm mode [4]. Thus, swarming, and the associated expression of virulence factors enable them to successfully counter the host defense mechanisms and establish disease. Also, swarm cells of both these pathogens exhibit enhanced ability to invade epithelial cells, an important aspect in pathogenicity [5]. With their ability to grow in biofilms, especially over catheter surfaces they become highly resistant to most antibiotics leading to chronic or persistent UTIs [6]. With an increasing difficulty in treating such recalcitrant infections, there has been a growing interest in alternative therapies and the use of natural, especially plant based products.

Several species of the genus *Euphorbia* (*E. nerifolia, E. tirucalli E. trigona etc)* have been part of the Indian Ayurvedic pharmacopoeia for the treatment of infection and inflammation, including UTI. The latex of these plants is known to contain several di- and tri- terpenes and their esters which possess anti- infective properties [7] but the mode of action is not yet clear. Unlike a conventional antimicrobial agent which attempts to control disease by its microbiostatic or microbiocidal effect on cells, an anti-QS compound works by causing an interruption of the QS mechanism of pathogens.

This is known to attenuate virulence, without the selective pressure of developing drug resistance. Some plant and algal species are known to produce quorum-quenching compounds such as halogenated furanones [8]. A few aspects of the QS dependent phenomena in *Pr. mirabilis* and *Ps. aeruginosa* which determine their virulence and its attenuation by *Euphorbia* latex extracts have been investigated in this study.

MATERIALS AND METHODS:

Microorganisms, media and growth conditions:

Microorganisms: *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus mirabilis* and *Pseudomonas aeruginosa* were obtained from IMTech, Chandigarh, and routinely maintained on nutrient agar slants at 4˚C. An overnight liquid culture of the organisms in Luria Bertani (LB) broth was used in all the experiments.

Swarm agar:

Proteus: LB broth solidified with 1.5 g % agar [9]

Pseudomonas: LB broth supplemented with 0.5 g % casein hydrolysate, 0.5 g % glucose and solidified with 0.5 g % agar

All the swarm agar plates were dried overnight at 37 ˚C before use. Thereafter, the swarm agar plates were centrally inoculated with 5 µl of overnight cultures of the organisms and incubated at 37˚C for 24 hrs. Swarming *Proteus* cells undergo alternate cycles of differentiation and de-differentiation, producing a swarm zone with concentric growth rings on the agar surface [10] *Pseudomonas* swarms presents a dendritic fractal pattern on the agar surface [11].

Preparation of latex extracts: Latex extracts were prepared in various solvents such as petroleum ether, chloroform, and 70 % ethanol. 0.5 ml of latex was extracted twice with 5.0 ml of each solvent and the two aliquots were pooled, evaporated to dryness and the residue resuspended in 20 % di-methyl sulfoxide (DMSO) so as to obtain a five fold concentration. These preparations were used to study the anti-bacterial and anti-swarming properties.

Urea-LB broth: Filter sterilized urea solution was added aseptically to sterile LB broth so as to obtain a concentration of 0.5 mg ml^{-1}

Antibacterial activity: *Euphorbia* latex extracts in various solvents were tested for their antibacterial activity against gram positive, gram negative as well as drug resistant clinical isolates by standard disc diffusion and well methods using Mueller-Hinton agar.

Swarming motility assay: To study the effect of *Euphorbia* latex extracts on the swarming motility of *Pr. mirabilis* and *Ps. aeruginosa*, 5µl of an overnight culture of each microorganism was centrally inoculated on swarm agar plates containing various concentrations of the extracts. Two control plates were also set up for each study; one containing no additives and termed positive control and the other containing 20% DMSO and termed solvent control. All the plates were incubated at 37˚C for 20 hours. Thereafter, the diameter of the swarm zone was measured and compared to the control.

Bacterial growth assay: To study the effect of *Euphorbia* ethanolic latex extract (EELE) on growth of *Pr. mirabilis* and *Ps. aeruginosa*, growth was monitored turbidometrically for 24 hours in LB broth in the presence of EELE (50 µl ml^{-1}). A solvent control was also set up and both were compared to a positive control.

Urease assay (for *Proteus*): Urease activity in the presence and absence of EELE was assayed by estimating the unhydrolysed urea in urea-LB broth cultures [12] Briefly, urea-LB broth containing EELE (50 µl ml^{-1}) was inoculated with 100 µl of an overnight culture of *Pr. mirabilis*. At the end of an incubation period of 48 hours at 37˚C, the amount of urea hydrolyzed was compared to a positive control set-up, lacking EELE. An uninoculated urea-LB medium containing EELE was used to adjust blank and as a negative control, to rule out non-enzymic degradation of urea.

Rhamnolipid formation assay (for *Pseudomonas*): Rhamnolipid concentration was estimated by the orcinol method [13]. The concentration of rhamno lipids produced in the presence of EELE (50 µl ml^{-1}) in LB broth inoculated with 100µl of an overnight culture of *Ps. aeruginosa* was estimated after an incubation period of 72 hrs at 37˚C and compared to a similarly inoculated LB broth lacking EELE which served as the positive control.

Biofilm formation assay: The assay was performed using the microtiter plate method in the presence of EELE (50µlml^{-1}) for both *Pr. mirabilis* and *Ps. aeruginosa* [14]. Wells containing an equal volume of 20 % DMSO served as solvent control.

RESULTS AND DISCUSSION: It has been well demonstrated that swarming motility and virulence factor expression are coordinately regulated in several pathogens including *Pr. mirabilis* and *Ps. aeruginosa* which are often implicated in persistent UTI, especially in menopausal women and hospitalized &/or catheterized patients [15]. These organisms growing in a biofilm, also demonstrate greater resistance to antibiotics such as cephalosporins, aminoglycosides, and ciprofloxacin [16], necessitating the need for an alternative mode of treatment to treat such infections. Therefore, compounds which interfere with the QS mechanism of these pathogens will prove to be highly effective in controlling their pathogenicity.

Anti-microbial properties of *Euphorbia* latex extracts: The use of *Euphorbia* latex for UTI treatment prescribed in the practice of Ayurvedic medicine in India is proven to be highly effective even in recurrent UTI. However, an examination of the conventional antimicrobial activity of the aqueous and several organic solvent extracts of *Eu. trigona* latex revealed that these extracts possessed no antibacterial properties even against gram positive bacteria at concentrations of 20-50µl ml^{-1}. Meager antimicrobial activity was observed only when highly concentrated extracts were used. Hence we speculated that *Euphorbia* latex might act via its anti-QS properties against urinary pathogens and this investigation was initiated using ethanol (polar solvent), petroleum ether (non polar) and chloroform (intermediate polarity) extracts of the latex.

Effect of *Euphorbia* latex extracts on swarming motility: Swarming plays a crucial role in the pathogenesis of urinary pathogens; especially in catheter associated UTI [17]. The latex extracts in various solvents were found to differ in their potency, exhibiting a reduction of 20 %-60 % in the diameter of the swarm zone in case of *Proteus mirabilis* and 45 %-65 % in case of *Pseudomonas aeruginosa* (**fig. 1**).

Fig. 1: Effect of *E. trigona* extracts on swarm zone of *Pr. mirabilis* and *Ps. aeruginosa*

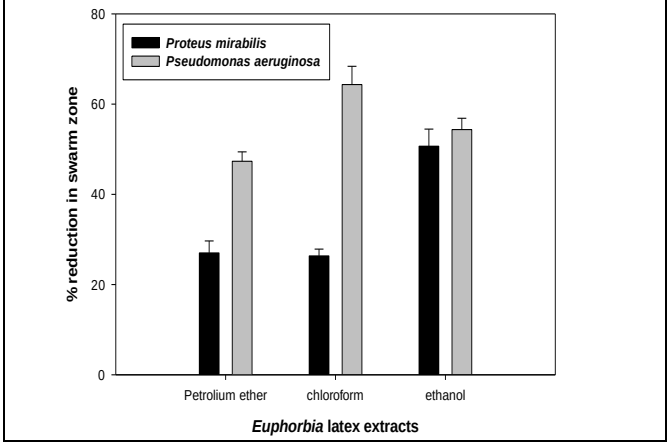

FIG. 1: EFFECT OF *P. TRIGONA* EXTRACTS OF *Pr. mirabilis* AND *Ps. aeruginosa*

Reduction in swarm zone diameter of *Ps. aeruginosa* in the presence of latex extracts is significantly greater as compared to *Pr. mirabilis*. ($p<0.01$); Extract concentration: 50µl/ml

Thus, in general, swarming motility of *Ps. aeruginosa* was found to be significantly more susceptible to the extracts ($p<0.01$). Apart from the obvious inhibition of swarming motility, both *Pr. mirabilis* and *Ps. aeruginosa* exhibited an alteration in their swarm pattern in the presence of the extracts. In case of *Pr. mirabilis,* the width of the individual swarm rings increased along with a concomitant decrease in the number of rings. Echeverrigaray *et al.,* have reported similar changes in the swarm pattern of *Pr. mirabilis* in the presence of pure terpenes [18].

In case of *Ps. aeruginosa* a marked change in the thickness and spacing of the radiating tendrils was observed. The dendrites were closely spaced and thin in the positive control and solvent control plates but broader and widely spaced in the presence of higher concentration of the extracts (>30µl ml^{-1}). Further, whereas maximum reduction in the swarm zone was observed with 70 % ethanolic latex extract for *Pr. mirabilis*, maximum

inhibition of swarming motility of *Ps. aeruginosa* was observed with chloroform extract. Petroleum ether latex extract was the least effective for both the organisms and a percent reduction of 20% for *Pr. mirabilis* and 42% for *Ps. aeruginosa* swarm zone was observed. *Euphorbia. spp* are known to possess anti-bacterial, anti-viral and anti-mycotic activity which is generally attributed to the di-and tri-terpenes in their latex [19]. These are essentially non-polar compounds. An assessment of the anti-swarming properties of several commercially available terpenes [18] reveals that these non-polar compounds do inhibit swarming at sub- inhibitory concentrations.

However, the present work demonstrates that latex extract in 70 % ethanol which contains predominantly polar compounds, is more potent than petroleum ether latex extract (which contains non-polar moieties) in restricting the swarming motility and causing a change in the swarm pattern of both *Pr. mirabilis* and *Ps. aeruginosa.* Further, it inhibits swarming in a dose-dependent manner, demonstrating >50 % inhibition of swarm zone diameter at relatively low concentrations (**fig. 2**).

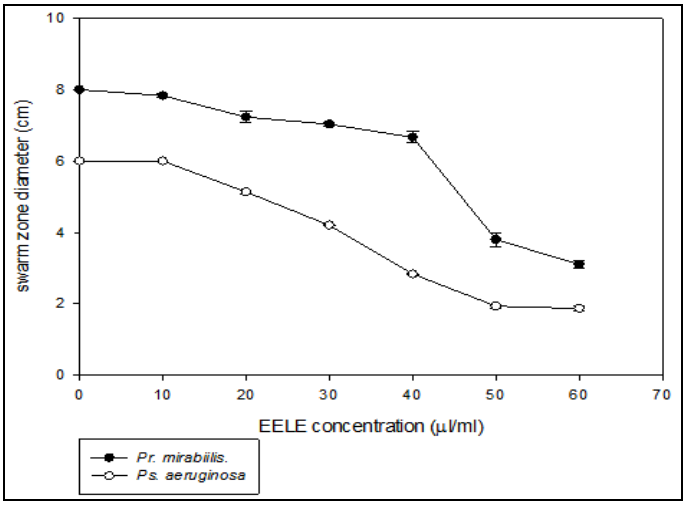

FIG. 2: EFFECT OF EELE ON SWARMING OF *Pr. mirabilis* AND *Ps. Aeruginosa*

Dose dependent reduction of swarm zone diameter: Each point represents mean of three independent observations ± S.D

Since 70 % ethanolic Euphorbia latex extract (EELE) was found to be almost equally effective against both the selected urinary pathogens, further studies, including inhibition of virulence factor expression and the ability to form biofilm were performed using EELE.

Inhibition of virulence factor expression by EELE: Swarming motility and expression of virulence factors are known to be closely associated and subject to QS control [20]. Hence the inhibitory effect of EELE on selected virulence factor production by *Pr. mirabilis* and *Ps. aeruginosa* was studied.

Inhibition of urease activity of *Pr. mirabilis*: Apart from swarming, urease-mediated urea hydrolysis by *Pr. mirabilis* is responsible for its ability to cause urolithiasis, blockage of catheters and extensive colonization of kidney and urinary tract endothelium and thereby its virulence [21]. This results in the creation of persistant foci of infection not easily removed by the flushing action of urine. A urease negative mutant, in fact, has been shown to colonize urinary tract endothelium at a significantly lower rate [22].

The activity of the extracellular urease produced in the presence and absence of EELE was assayed by estimating urea hydrolysis in urea-LB broth cultures inoculated with *Pr. mirabilis* after an incubation period of 48 hrs. The amount of urea hydrolyzed is indicative of the urease activity present. As expected, urease activity was found to be significantly ($p<0.01$) reduced in the presence of EELE at a concentration of 50 µl ml^{-1} (**fig. 3**) *in vitro.* Urease, an inducible enzyme causes an elevation of pH due to the release of ammonia, leading to the formation of bladder and kidney stones. Inhibition of urease activity in the presence of the bioactive compounds in the latex probably helps to maintain the pH of the urine *in vivo*, precluding the formation of struvites and facilitating the clearance of infection by the host defense mechanism.

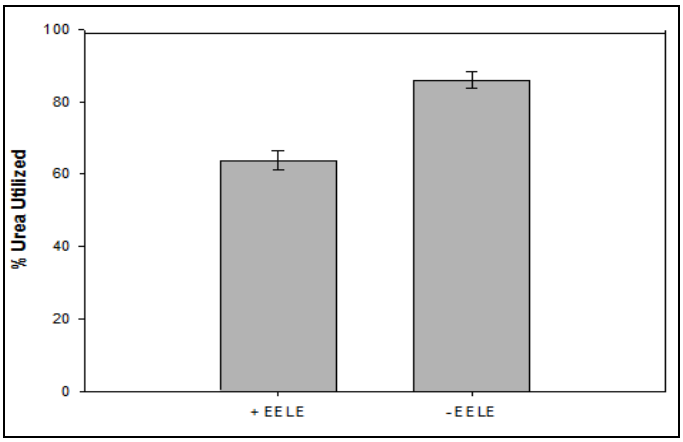

FIG. 3: UREASE ACTIVITY

Inhibition of Urease by EELE: A statistically significant (**p<0.001) inhibition of urease activity observed in the presence of EELE (50 µl/ml)

Inhibition of rhamnolipid production by *Ps. aeruginosa*: Rhamnolipids possess tensioactive properties, and are capable of modulating swarming in *Pseudomonas* [23]. They can inactivate tracheal cilia, indicating that they are virulence factors [24]. The concentration of rhamnolipids in the presence of EELE (50 µl ml^{-1}) was found to decrease significantly (p<0.005) as compared to the positive control (**fig. 4**). This further corroborates the notion that the mode of action of *Euphorbia* latex in UTI is by inhibiting the swarming and associated virulence factor production by urinary pathogens.

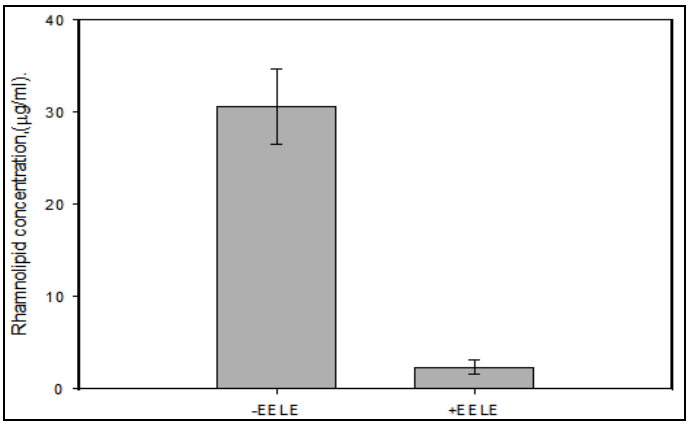

FIG. 4: EFFECT OF EELE ON RHAMNOLIPID PRODUCTION BY *Ps. Aeruginosa*

Reduction in rhamnolipid production: A statistically significant (p<0.001) reduction in rhamnolipid production observed in the presence of EELE (50 µl/ml)

Rhamnolipids also affect biofilm formation in *Ps. aeruginosa* and rhamnolipid defective mutants are reported to have a swarming pattern with broader tendrils as compared to the wild type strain[25]. A similar observation was made during our study of the inhibition of swarming motility in the presence of EELE. It has also been reported that the change in swarming phenotype could be due to the inability of the mutant to respond to or, alternatively, produce rhamnolipids and/or other swarm modulating factors. The ~10 fold decrease in rhamnolipid concentration in the presence of EELE observed in this study may thus be responsible for the change in swarm pattern as well as a reduction in the extent of biofilm formation.

Inhibition of biofilm formation: Biofilm formation plays a very important role in the pathogenesis of UTI pathogens [26]. It has been amply demonstrated that in the biofilm mode of growth, bacteria can cause chronic infections and the cells growing in such biofilms are resistant to phagocytes, antibodies and antibiotics. These properties are responsible for the characteristic failure of the host defense system and antimicrobial chemotherapy to clear biofilm pathogens, especially in device related infections [16]. Hence, compounds that inhibit biofilm formation will presumably be able to control or expedite clearance of such infections. Biofilm formation by both *Pr. mirabilis* and *Ps. aeruginosa* was studied in the presence/absence of EELE (50µl ml^{-1}) and the absorbance levels of the extracted crystal violet is indicative of the extent of biofilm formation by the selected UTI pathogens (**fig. 5**).

***Pr. mirabilis*:** The average absorbance levels of the extracted crystal violet was found to be 0.0108± 0.0065 in the presence of EELE and 0.0927±0.0387 in the solvent control. Thus the absorbance levels in wells containing EELE are >9 fold less as compared to the solvent control wells containing 20 % DMSO.

Ps. aeruginosa: The average absorbance levels of the extracted crystal violet were found to be 0.425±0.0.0693 in the presence of EELE and 0.633±0.0885 in the solvent control wells; the absorbance levels in the wells containing EELE being ~5 fold less as compared to solvent control wells containing 20 % DMSO.

Fig. 5: Effect of EELE on biofilm formation of *Pr. mirabilis* and *Ps. aeruginosa*

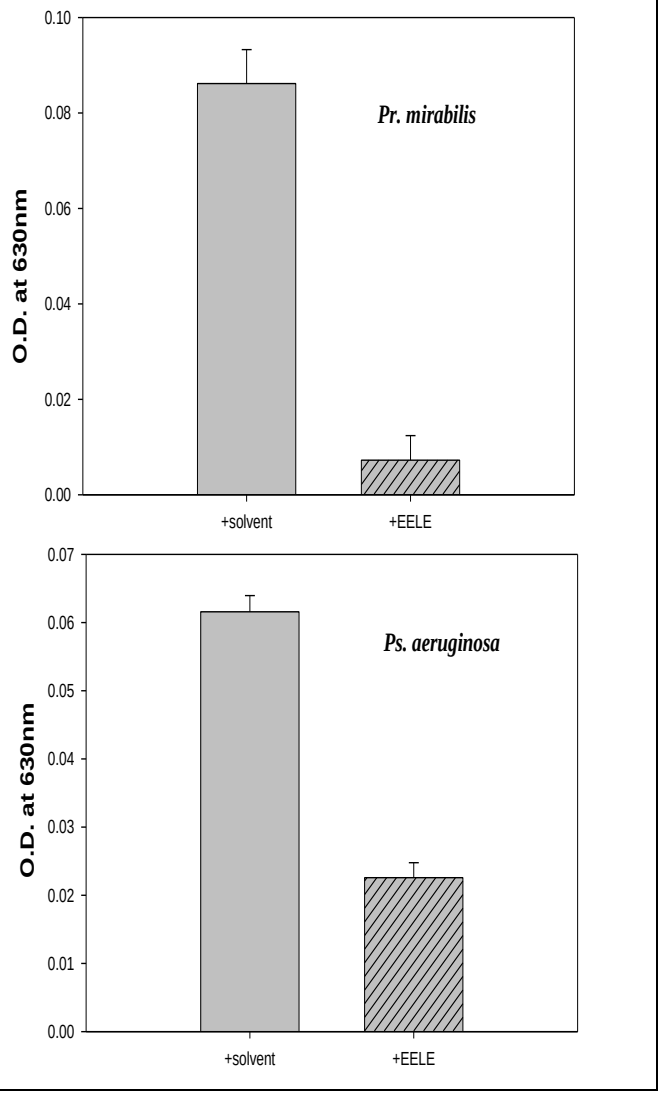

FIG. 5: EFFECT OF BIOFILM FORMATION BY *Pr. mirabilis* AND *Ps. Aeruginosa*

Biofilm formation *in vitro* is significantly reduced ($p<0.0005$) in the presence of EELE (50 µl/ml)

Both the selected urinary pathogens possess a QS-controlled ability to form biofilms adhering to bladder cells as well as indwelling catheters, where a crystalline biofilm by a mixed community of UTI pathogens leads to coating and blockage of catheters and formation of persistent foci of infection [17]. The statistically significant reduction in the extent of biofilm formation *in vitro* by both *Pr. mirabilis* ($p<0.01$) and *Ps. aeruginosa* ($p<0.03$) in the presence of EELE further substantiates the presence of anti-QS compounds in EELE.

Effect of EELE on the growth of *Pr. mirabilis* and *Ps. aeruginosa*: The swarming motility assay in the presence of EELE clearly demonstrates that the latex extract is not microbiocidal to either *Pr. mirabilis* or *Ps. aeruginosa*. In order to further confirm its non-toxic nature, the growth of both the organisms was monitored for 24 hrs in the presence of EELE at a concentration of 50 µl ml^{-1} in liquid culture at 37˚C and compared to a solvent control as well as a positive or normal control set up. It was observed, that in the initial stages of growth (upto 6 hrs), EELE seemed to have a very slight inhibitory effect on the growth rate of *Pr. mirabilis* (**fig. 6**) whereas in case of *Ps. aeruginosa* (**fig. 7**), the initial growth rate in the presence of EELE was at par with the positive control as well as the solvent control with a slight decrease in the growth rate in the mid-stages.

However, both the organisms grew to similar cell densities at 24 hrs .in the presence/absence of either solvent or EELE at concentrations at which swarming behavior is significantly inhibited. Hence, the inhibitory effect of EELE on all the QS-related attributes in this study is certainly not due to its toxic or growth inhibitory action.

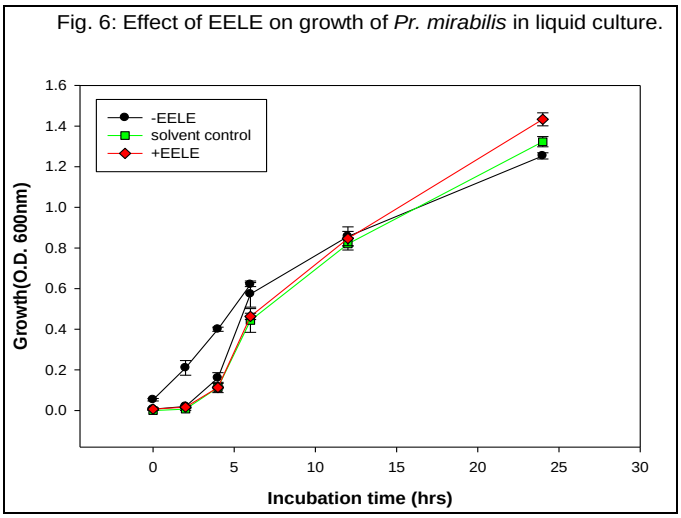

FIG. 6: EFFECT OF EELE ON THE GROWTH OF *Pr. mirabilis* AND *Ps. Aeruginosa*

Growth of *Pr. mirabilis* is not affected by EELE (50 µl/ml). Each point represents a mean of three independent observations ± S.D

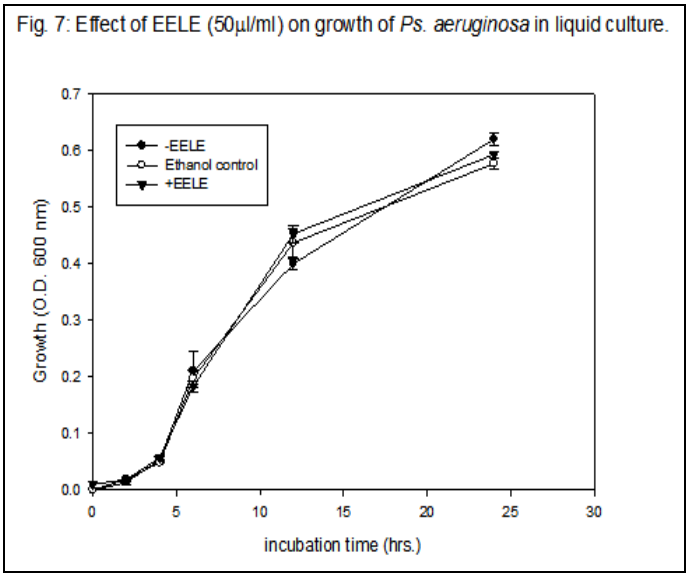

FIG. 7: EFFECT OF EELE 50µl/ml ON GROWTH OF *Ps. aeruginosa* IN LIQUID CULTURE

Growth of *Ps. aeruginosa* is not affected by EELE (50µl/ml). Each point represents a mean of three independent observations ± S.D

CONCLUSION: Thus the data generated in the present work on *Pr. mirabilis* and *Ps. aeruginosa* regarding inhibition of QS-related phenomena, *viz*, inhibition of swarming motility, alteration of swarming phenotype, reduction in the expression of virulence factors (formation of rhamnolipids & urease activity) and inhibition of biofilm formation in the presence of EELE suggests that the *Euphorbia* latex contains some compound(s) that act against these urinary pathogens by blocking / inactivating their QS molecules or interfering with their signaling system in some manner. Most UTI causing organisms are opportunistic pathogens and it seems quite likely that upon entry into the urinary tract, they produce disease only when their population becomes quorate, and they are able to express their virulence factors. Such anti-QS compounds present in *Eu. trigona* and other plants can hence attenuate the virulence of the pathogens without challenging their growth, thereby preventing the emergence of resistant strains and facilitating the elimination of pathogens by the host's immune system.

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