Synthesis and biological antimicrobial activity evaluation of furans from Lycopersicum against UPEC strains.

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ABSTRACT

Medicinal plants are being traced and screened for novel therapeutics which could address many of the ailments including ever rising antibiotic resistance. Urinary tract infections are also common and is affecting a vast ratio of the human population adversely affecting the economical and health status of individuals. This prompted us to screen for novel antimicrobials which could inhibit the growth and motility of these pathogens. Lycopersicum (tomato) is said to be anticancerous and antibacterial. For the first time, we focused on purifying compounds from the fruit, and studying the antibacterial activity. Qualitative and quantitative analysis of purified compounds were performed by disc diffusion, MIC, motility assay and gene expression studies. We concluded from our studies that, the compounds could inhibit the bacterial growth and its swimming and swarming ability. Even the gene members responsible for flagellar and adhesion movements were also found to be down regulated on treating with the compounds.

Keywords: UPEC, Tomato, Motility assay, MIC, Real time PCR.

Introduction: the infection of Urinary tract (UTIs) are most widely spread infection that affect huge number of population. Nearly hundred and fifty million people develops urinary tract infection annually which lead to high social costs (Flores *et al*., 2015). About 40% of women developed a urinary tract infection once during their life when about 11% women infected yearly with age above 18 years old and this may cost nearly 5$ billion to treat nearly eleven million cases in united states (Micali *et al*., 2014). Most predominant bacteria in the urinary tract is *Escherichia coli* which encompasses nearly in uncomplicated cases of UTI cases . strain of Uropathogenic *Escherichia coli* express a range of virulence factors, which enable the bacteria to establish UTI (Foxman *et al*., 2014). Urinary tract infection (UTI) account for most generally diagnosed disease specially for renal and urological diseases. Morbidity is mostly associated with hospitalized and outpatients. Also highly rates (50–90%) of uncomplicated cases mainly caused by uropathogenic *Escherichia coli* (UPEC) which is strain These bacteria reside in the urinary system after deviating from the bacteria in the digestive system naturally In the urinary system, these bacteria develop and grow, which develop new virulence factors to infect

this system , The diseases of this organ are linked to these bacteria (Shah *et al*., 2019). uropathogenic *Escherichia coli* may The use of a group of various virulence factors that play a role in causing urinary tract diseases (O'Brien *et al*., 2016). Most important surface structures that play a role in pathogenicity of UPEC is Several components of secreted substances and receptors such as flagella, capsule, substances secreted by bacteria, iron acceptors and others are present in these bacteria that help to make medicines or vaccines that eliminate these bacteria (Werneburg *et al*,).

There are a number of virulence factors associated with UPEC, e.g., fimbriae 1, hemolysis, serum resistance, hydrophobicity, but type 1 fimbriae accounts for more than 95% of the total *E. coli* virulence factor, causing UTI (Le Trong *et al*., 2014). Type 1 fimbriae has length approximately 0.5–1.5 μm. which represent the attachment structure of gram negative bacteria (Berne *et al*., 2015). Usually, bacteria use these structures to carry adhesions at their tip for addition to obtain nutrients and withstand shear forces (Tchesnokova *et al*., 2011). In *E. coli*, the 30-KDa subunit which called FimH represent a highly conserve ashesive structure of type 1 fimbriae which concerned its function for mannose specific adherence. (Le Trong *et al*., 2014). The FimH binding domain which abbreviated (LD) and mainly Consists of mannose which is the main cause of infection Which causes adhesion to the substrate, which in turn works to absorb nutrients (Tchesnokova *et al*., 2011).

Flagella are complex filamentous organelles that contribute to disease virulence, such as movement, chemical reaction, etc. (Kakkanat *et al*., 2015). The presence of the flagellum, which plays an important role in the movement of bacteria that helps it to rise to the urinary tract and infect more parts of the urinary system (Lane *et al*., 2007). There is research showing that flagella contribute to the adhesion and colonization of bladder epithelial cells in infected mice (Kakkanat *et al*., 2015).

One of the important topics that took place to know the organization, assembly and cellular synthesis of these bacteria flagella, one of the most important topics for several decades

In general, the flagellum consists of three regions of the basal body, the hook, and finally the extracellular filaments (Smith *et al*., 2009).

The researchers were interested in studying the components of the tomato fruit that contribute to reducing diseases, and the most important course of action of tomatoes is that it contains oxidizing substances that help reduce infection. (Iijima *et al*., 2008). In recent decades, the consumption of tomatoes and tomato products has been suggested to be able to reduce the risk of certain chronic diseases by preventing doxorubicin-induced cardiac myocyte oxidative DNA damage, reduce the levels of serum prostate specific antigen, and positively modulating other disease (Schummer *et al*., 2009). This component found in tomato antioxidants is very important in preventing infection with fungi, viruses and bacteria in plants or humans (García *et al*., 2009). (Jianguang *et al*., 2014). (Mourboul *et al*., 2012).

The study was this intended with an aim to evaluate the antibacterial activity of purified compounds (furans) against UPEC isolates and determine their effectiveness on the biofilm gene expression members.

Methods:

Bacterial Strains: *UPEC* strains procured from Silverline laboratories were revived on Luria bertani (LB) agar plates (tryptone 10gm, yeast extract 5gm, NaCl 0.5gm, agar 15gm) and incubated for 18-24hr at 37°C. Growth of the isolates was maintained optimum by screening the optical density at 580 nm (OD580).

Extraction of samples: Fresh tomatoes (50gm) were blended with about 200ml of extraction media (Ethanol and 5 % acetic acid; 95:5). The mixture was constantly stirred in dark for 72hr, and filtered using Whatman No.1 paper. The sediment obtained was then subjected to 20min of sonication using 50mL of same extraction media at 20°C. The contents were then filtered and second supernatant obtained was mixed with the first and evaporated using a rotatory evaporator. The

extracts were then stored at -20^{0}C for further analysis.

Compound purification: The compounds were purified by HPLC using a reverse phase column (250 × 4.6mm, 5µm, HiMedia). The temperature of the column was maintained at 25°C and gradient mobile phase (solvent A; water containing 46mg/L formic acid, solvent B: MeOH with formic acid 46mg/L). The flow rate was kept constant at 1.0ml/min throughout the assay with run time of 60min and 10min.

Experimental setup: In brief, about 0.4ml of LB broth was added to a 24 well plate accordingly. About 20µl of overnight culture was added to all the wells except the negative control (media alone). Imipenem (50µg/ml) was used as positive control (Mohammadzadeh *et al*., 2019) throughout the study. 5-Methyl furfural (5MF), 2-Pentyl furan (2PF) and Gamma-hydroxybutyric acid (GHB) purified from HPLC study was used as test compounds in the study. All of the compounds used were of 100µg/ml. The plates were then incubated at 37°C until optimum growth was observed.

Antibacterial activity: Antimicrobial activity of the purified compounds were tested against the UPEC strain by agar well diffusion method and broth dilution assay.

Antibacterial activity using the agar cup plate method: Overnight culture was pour plated onto LB agar plates and about 20µl of samples at varying concentrations (16, 32, 64µg/ml) were added into each well. Imipenem (20µg/ml) and Sterile distilled water were used as positive and negative control respectively. The antibacterial activity was confirmed first on the plate and later assayed using tube method.

Minimum inhibitory concentration (MIC): MIC values were determined by the broth dilution method (Luis *et al*., 2015). MIC was defined as the lowest concentration of compound that completely stops the growth of the microbes. Cell density about 5x 10^5CFU/ml were seeded into their respective wells. The assay was done in 96-well plate and two-fold serial dilution was used with varying concentrations of samples (0, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml). The plates following incubation were used for estimating the growth of the culture at 600nm. Imipenem was used as positive control with MIC of 20µg/ml.

Biofilm formation assay: Biofilm inhibition assay was done by ethanol acetone method as described by Hamzah Basil Mohammed *et al*, 2014. The 24hr incubated plate with treatments were washed thrice with PBS and stained with 2% crystal violet which was then solubilized with ethanol:acetone (80:20) after 30min of incubation and the absorbance was recorded at 590nm.

Cellular Hydrophobicity assay: Hydrophobicity assay of cell surface was estimated by measuring the microbial adhesion to solvents (Rodrigues *et al*, 2009). In brief, about 3.6ml of overnight culture (OD_{600}nm = 1.0) in 0.9% saline was added with 0.4mL xylene. The contents were mixed thoroughly for 1min and incubated at room temperature for 20min. Both the hydrocarbon and aqueous phases were allowed to separate and the OD600nm was recorded. The percentage of cell adhesion to the hydrocarbon was calculated using the formula: A (%) = $[(A_1 - A_2)/A_1] \times 100\%$; where A_1: Absorbance of the saline; A_2: Absorbance of culture and hydrocarbon. The degree of a strain's hydrophobicity was classified as strongly, moderately hydrophobic and hydrophilic basing on the percentage values of >50%, 20–50% and <20% respectively. All the experiments were done in triplicates.

Motility assay: Motility assay was studied by wet mount method as described by Ottemann, K. M, 2002. Motility was estimated using the soft agar plates (LB broth, 0.35% agar). In brief, a loopful of culture was stabbed into the agar medium about 3/4th of the way. The same was estimated with and without treatments (both positive and test compounds at 100µg/ml) on spate plates. Following inoculation, the plates were incubated at 37^{0}C and the diameter of the bacterial halo was measured after 48hr incubation. The more motile, more is the mobility which proves the ability of the cells to colonize effectively.

RNA purification: The same experimental set up was followed and following incubation for

48hr, the cells were centrifuged at 5000rpm for 10min and used for extracting the total RNA. The pellet obtained was resuspended in 5ml of lysis buffer (lysozyme (0.5mg/ml); Tris-HCl 10mM; EDTA 1mM, pH 8, 1% SDS) and incubated at 64°C for 2min in a water bath. After incubation, 5.5ml of 1M sodium acetate (pH 5.2) was added and extracted with an equal volume of water-saturated phenol (pH 5.5) and incubated at 64°C for 6min. The upper aqueous layer was then re-extracted with an equal volume of chloroform and ethanol,was used to precipitate the RNA. The RNA obtained was resuspended in RNase-free DNase treated water and used in RT PCR.

Real-time RT-PCR: cDNA synthesis was done using random primers and 2µg of total RNA was used with 1U of SuperScript II reverse transcriptase (Invitrogen). About 1.25µl of cDNA was used in the PCR amplification

Table 1: Table showing the primers used in the study. Gyrase gene (*gyrB*) was used as hosue keeping gene in the study.

Gene	Sequence	Size	Tm	GC %	Product
FliC	CAAACTGGGCGGAGATGATG	20	58.98	55	235
	GGAATCCAGACGGTTTTGCA	20	58.76	50	
fimh	CCAATGGTACCGCTATCCCT	20	58.94	55	249
	CGCTGGTGGTAGGAAATGGA	20	59.75	55	
hlyE	AGTATTCACAGGCAGCCTCC	20	59.46	55	214
	ATGCCGTCATCCAGTACCTT	20	58.8	50	
gyrB	TCGGCGACACGGATGACGGC	20	61.2	55	911
	ATCAGGCCTTCACGCGCATC	20	58.74	55	

The quantitative real-time PCR was performed as described Sudhakar Malla (2020). iQTM SYBR Green Supermix (HiMedia) was used along with primers (600nM) and 1µl of the RT products with a total volume of 12.5µl. The assay was done in duplicates along with its respective negative control, to confirm the positive amplification. Real-time assay was carried on in the Corbett Research cycler (Bio-Rad). The *fliC, fimh, hlyE* primers (both FW and RV) of about 600nM concentration was used along with 1.32µl of the RNA for about 40 cycles at 94°C for 30s, 60°C for 30s, and with an elongation at 72°C for 2min. initial dentauration at 94°C for 15min and final elongation at 72°C for 10min. Gyrase was used as house keeping gene [Souza GM , 2019] to compare the expression levels with respect to the treatment. Expression profiling was assayed using $\Delta\Delta^{Ct}$ method. The Ct values obtained for the gene of interest were all normalized to its housekeeping gene.

Statistical analysis: All the experiments were done in triplicates and correlation analysis was done with level of significance at 0.05. The data was analyzed with Prism software throughout the study.

Results and discussion:

Purified compunds: 12 compounds were collected from the column which might be responsible for antibacterial activity against UPEC. The compounds were identified and authenticated using their MS data by comparison with those of the NIST Mass Spectral Library.

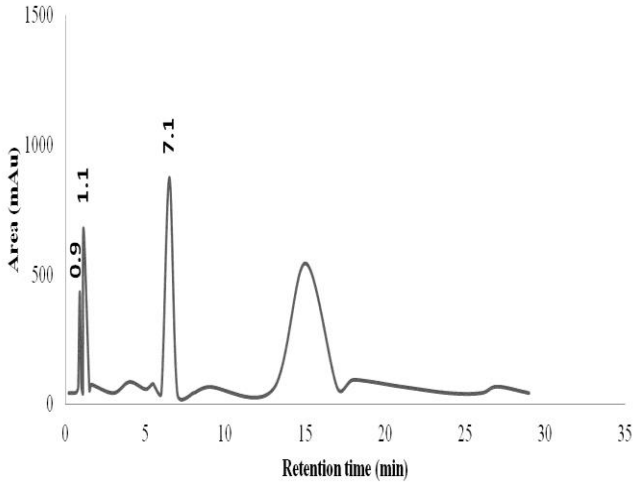

Figure1: HPLC chromatogram showing the purified fractions at 0.9, 1.1 and 7.1 retention times.

Disc diffusion assay: Among all the 12 sample compounds purified, only 3 (5MF, 2PF, GHB) of them showed significant positive inhibition

zones. The activity was found to be dose dependant when compared to the positive control. GHB was found to be equally competent to the positive control (17 ± 0.15) with inhibition zone of 16 ± 0.47 mm at 64µg/ml followed by 5MF (9.2 ± 0.15) and 2PF (8.6± 0.15). However, due to varying concentrations the sample effectiveness cannot be screened perfectly, as such minimum inhibitory concentration (MIC) was used. The correlation of inhibition zones between samples and varying concentrations were evaluated. There is a strong positive correlation of R2 of 0.99445 between the concentration and samples. A two way ANOVA was done to estimate the effectiveness of the samples on inhibition zones and varying concentrations. There was a significant effect observed between different concentrations of samples and zone of inhibition remembered at the $p<0.05$ level. All the samples showed significance to the treatment [$F(3,6) = 42.66972$, $p = 0.00019$] and was found to be dose dependant [$F(2,6) = 48.3858$, $p = 0.0002$]. Our results are in line with the reports of Ashraf A et al (2018) who confirmed similar activity of *Cuminum cyminum* on UPEC strains. Many of the medicinal plant extracts were tried before as antimicrobials and were successful.

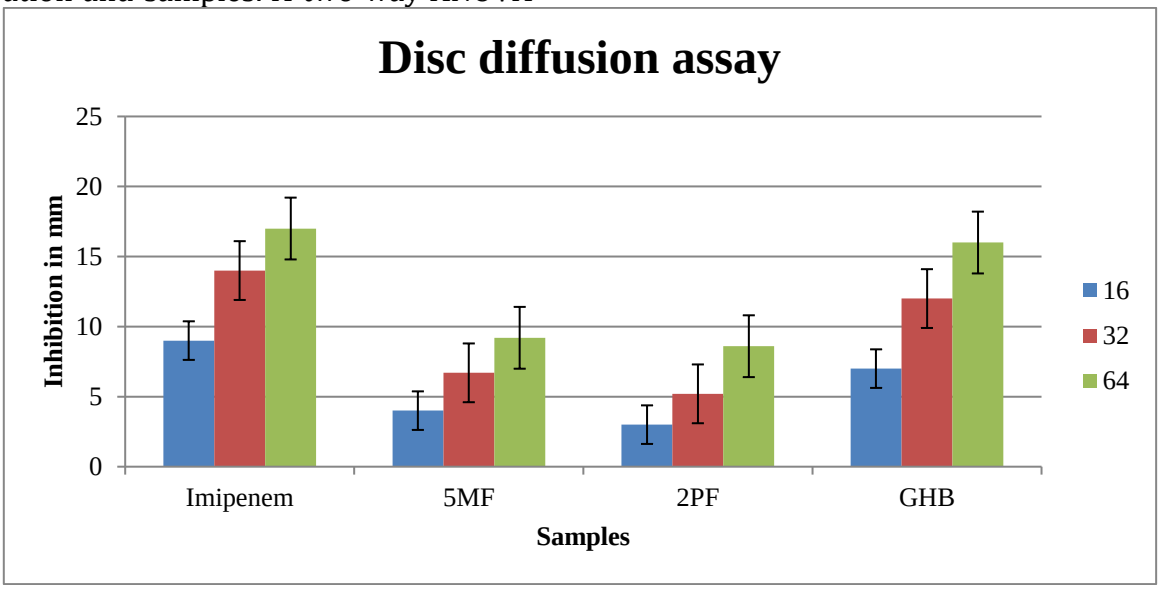

Figure 2: Graph showing the zone of inhibition against UPEC strains. All the values are the averages of triplicates. Incubation time was 48hr and the values are represented as value ±SD.

MIC values: The results of the MIC using broth dilution was shown in Figure 1. The results are in accordance with the disc diffusion method. GHB, 5MF and 2PF showed an inhibition of 91.23 ± 0.21, 72.34 ± 0.15 and 76.34 ± 0.54 respectively. All the samples showed dose dependant response when compared to positive control (not shown in graph). There is a strong positive correlation of R2 of 0.9928 between the samples and % inhibition samples. A two way ANOVA was done to estimate the effectiveness of the samples on % inhibition between samples at varying concentrations. There was a significant effect observed between different concentrations of samples and samples with zone of inhibition remembered at the $p<0.05$ level. All the samples showed significance to the treatment [$F(5,10) = 2858.419$, $p = 2.56E-07$] and was found to be dose dependant [$F(2,10) = 574.9127$, $p = 0.001566$]. The MIC values were found to be 16 ± 1.21µg/ml, 28 ± 0.45µg/ml and 44 ± 0.11µg/ml for GHB, 5MF and 2PF respectively when compared to positive control (20µg/ml). Saeidi S, 2015 also reported that various plant parts extracts of *Myrtus communis* L, *Amaranthus retraflexus* and *Cyminum cuminum* L were found to be effective against UPEC strains. They reported the low MIC values from these plant samples. Our results are again in accordance with these findings.

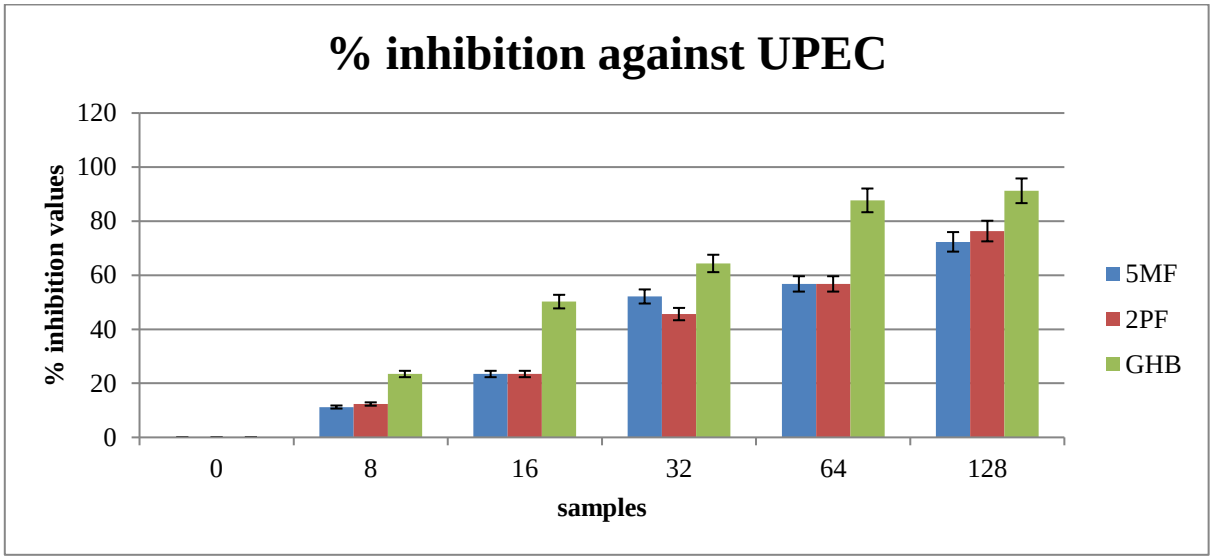

Figure 3: Graph showing the % inhibition against UPEC strains. All the values are the averages of triplicates. Incubation time was 48hr and the values are represented as value ±SD. Positive control with MIC (20µg/ml) was used in the study (not shown in graph).

Biofilm inhibition: The biofilm assay results are in accordance to the previous findings. The absorbance value of GHB, 5MF and 2PF at 128µg/ml was found to be 0.112± 0.06, 0.217± 0.54 and 0.435± 1.23 respectively when compared to positive control (0.092± 0.11). A two way ANOVA between the biofilm formation and sample treatment at varying concentrations was conducted to screen the effect of treatment on biofilm inhibition. There was a significant effect observed between different concentrations of samples and samples with biofilm inhibition remembered at the $p<0.05$ level. All the samples showed significance to the treatment [$F(5,10) =0.138583$, $p =2.08E-05$] and was found to be dose dependant [$F(2,10) =0.074242$, $p =0.001332$].

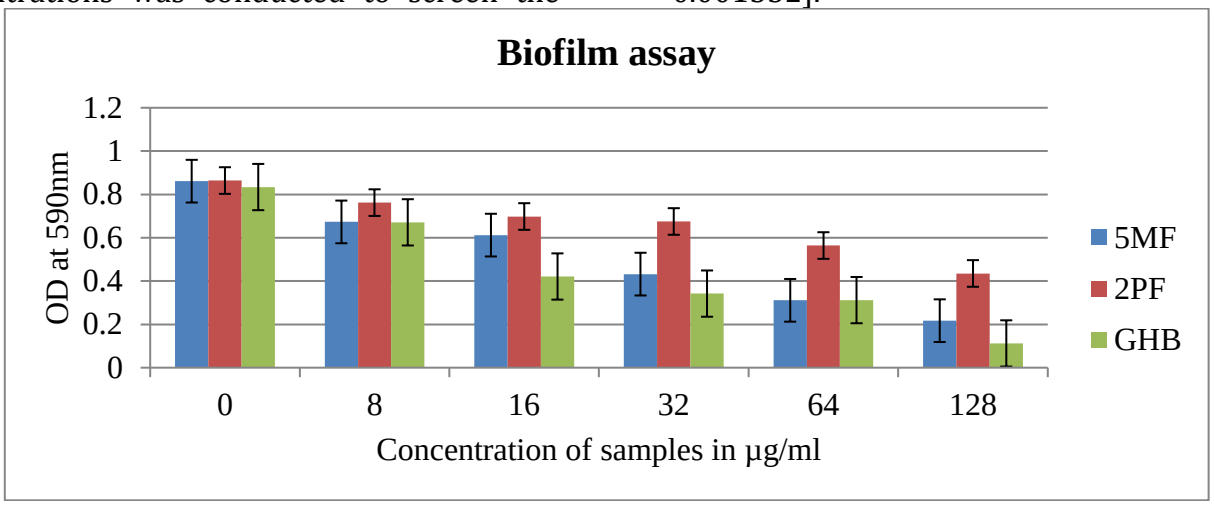

Figure 4: Graph showing the biofilm assay against UPEC strains. All the values are the averages of triplicates. Incubation time was 48hr and the values are represented as OD_{590} ±SD. Positive control with MIC (20µg/ml) was used in the study (not shown in graph). OD_{590} of negative control was 0.875± 0.24.

Cellular Hydrophobicity assay: *UPEC* strains showed susceptibility to form biofilms on substrates, in this case on the hdyroacrbon xylene. Hydrophobicity assay confirmed that the bacteria was able to form biofilm on hydrocarbon with 58.94%. The strains with

low hydrophobicity were found to be less effective in biofilm formation. Our results are in line with this statement, where the treatments showed positive effect in inhibiting the biofilms. The hydrophobicity seems to be decreased with increasing effectiveness of the extracts. The percent of cell adhesion to hydrocarbon was reported to be 13.12± 0.11, 21.11± 1.04 and 34.56± 0.32 for GHB, 5MF and 2PF respectively at 128µg/ml when compared to positive control (12.54± 0.21). There is a strong positive correlation of R2 of 0.9912 between the samples and % adhesion to hydrocarbon.

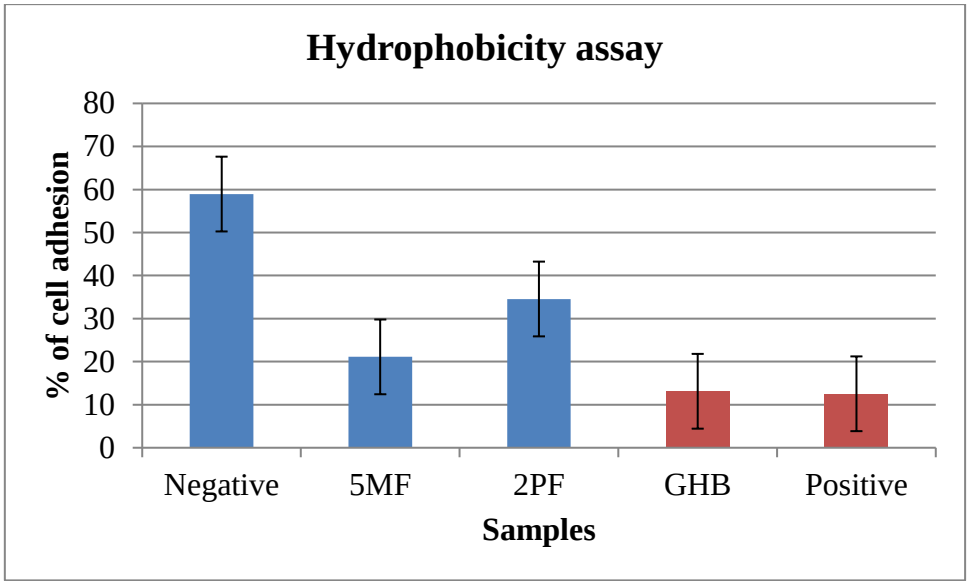

Figure 5: Graph showing the hydrophobicity assay on UPEC strains. All the values are the averages of triplicates. Incubation time was 48hr and the values are represented as % ±SD. Negative control was found to be 58.94± 0.44.

Motility assay: From our study, we could confirm the same findings as like in biofilm and MIC. In the plate with sample GHB, the colony growth was found to be inhibited when compared to negative control. The growth was profund in case of negative control with a diameter of about 13.45. The inhibition in the colony was seen in the plates with treatments GHB and positive control.

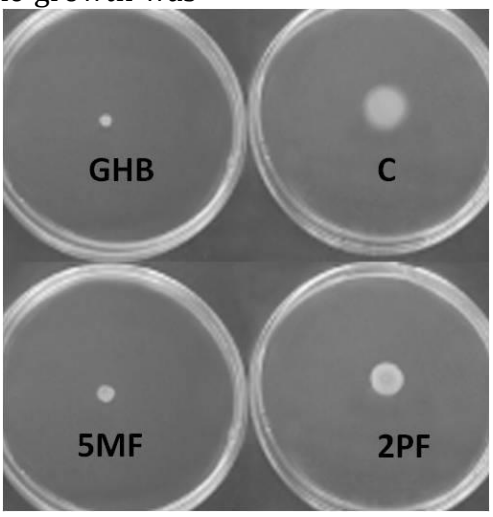

Figure 6: Images of LB agar plates showing swimming and swarming ability of UPEC under the treatment of purified compounds. GHB, 5MF, 2PF are the samples and C is the control without treatment

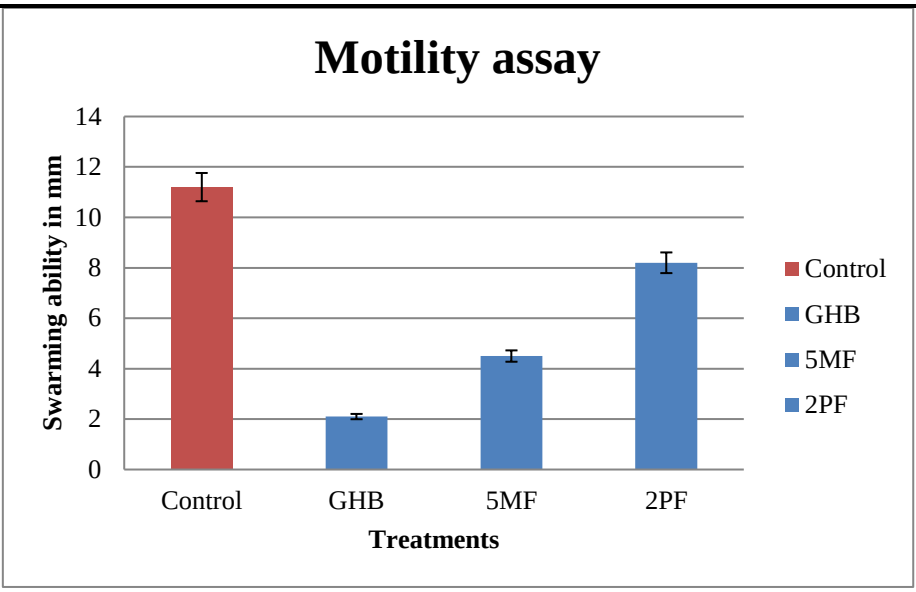

Figure 7: Graph showing the swarming ability of UPEC with and without treatment. GHB, 5MF, 2PF are the samples and C is the control without treatment. All the experiments are average of triplicates.

Real time PCR expression of gene members: Throughout the study, the results are normalized to the housekeeping gene (gyrase B) to reduce the noise in the background. The sample with the lower $\Delta\Delta^{Ct}$ values (11) was taken as calibrator.

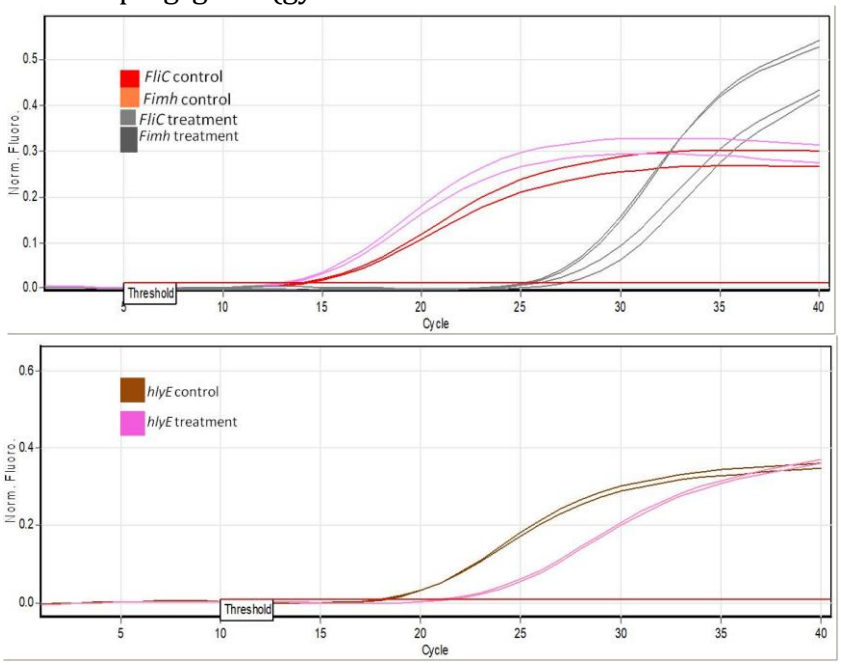

Figure 8: Images of quantification curves of the expression of FliC, FimH and HlyE for the bacterial samples under the treatment of purified extracts at 128µg/ml. Top: FliC and FimH; bottom: HlyE gene.

All the Ct values are average of duplicates. Ct value of the calibrator was 11 in the study. The ct values for the gene *FliC, FimH and hlyE* were found to be 13.4, 14 and 17 respectively. Housekeeping gene expression was considered to be 100% in the study. From the study and calculated $2^{-\Delta\Delta Ct}$ values, it was observed that all the 3 genes were underexpressed on treatment with GHB at 128µg/ml. The expression of the *FliC, and FimH* was found to be 64 and 32 times lowered than the control (without treatment). Even the expression of hlyE was lowered (4times), but the under expression of the member was less when compared to other 2 genes. There is a strong positive correlation of R2 of 0.8913 between the samples and treatment.

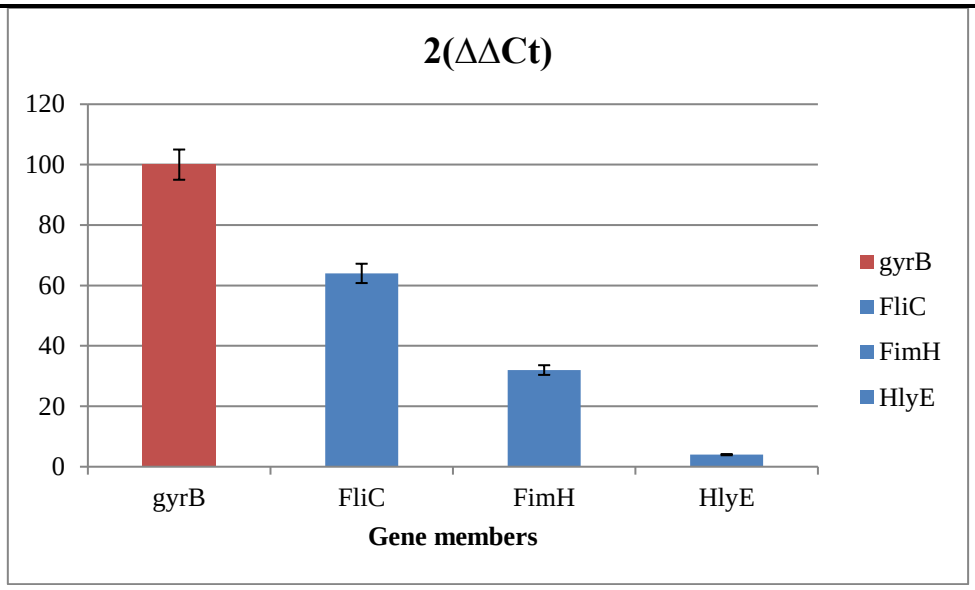

Figure 9: Graph showing the $2^{-\Delta\Delta Ct}$ values for the gene members on treatment with GHB at 128µg/ml. All the experiments were average of duplicates. Housekeeping gene expression was considered 100%. All the values are represented as value ±SD.

Previously studies done on UPEC strain with purified cranberry-derived proanthocyanidins cPACs, confirmed that they could inhibit the expression of the flagellin gene (fliC) which again slowed down the swimming and swarming motilities. Our motility assay results are very much similar to these findings, where the gene member FliC was repressed 64 times when compared to control (Hidalgo *et al*, 2011). Uropathogenic *E. coli* is said to form stronger biofilms than, the environmental samples without fimbriae gene (*FimH*). This confirms the role of FimH in motility and spreading. Our study confirmed the negative expression of FimH under the treatment of compounds, which could possible states the effect of the compound on the gene expression (Samah *et al*,, 2020).

Conclusion: In conclusion, biofilms play a critical role in pathogenesis and pose serious chronic threats for the public health. UPEC usually and commonly prevails and is considered nosocomial causing serious chronic ailments. Increasing antibiotic resistance to these strains is making biologists to look for more natural and novel compounds for treating these infections. In this study, we demonstrated that the purified compounds from Tomato (*Lycopersicum*) could inhibit the UPEC strains as is seen from our findings. It was found to decrease the swimming and swarming ability of the strain also. Even the gene expression studies on flagella and fimbria members also confirmed of the same activity. In summary, we can wind up by saying these plant compounds could act as novel antibacterial agents against UPEC strains.

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