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ORIGINAL ARTICLE **OPEN ACCESS**

Ficus racemose(Linn.) plant extract inhibits biofilm formation of *Pseudomonas aeruginosa* and *Proteus mirabilis* clinical isolates

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ABSTRACT

The aim of this study is to analyze the anti-biofilm activity of Ficus racemose plant against Pseudomonas aeruginosa and Proteus mirabilis clinical isolates. Among the total of fifteen isolates two biofilm-forming isolates, one from each species were isolated and subjected to different concentrations of the plant extract. It was observed that the F. racemose plant extract showed significant anti-biofilm activity at the tested biofilm inhibitory concentration of 0.5 mg/ml extract. Further, the microscopic analysis confirmed this result with a marked decrease in the biofilm architecture proportionately to the concentration of the plant extract. This is but a preliminary study, further studies are required to elucidate the molecular role of the phytochemicals of the Ficusracemose extract on interfering with the microbial biofilms.

Keywords: *Ficusracemose, anti-biofilm activity, Pseudomonas sp., Proteus sp.*

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INTRODUCTION

Biofilms are a collection of one or more types of microorganisms that adhere to a solid substratum and establish a strong colony growth embedded in an extracellular polymeric substance matrix. Biofilms may form on a myriad of surfaces, including living tissues, medical implants, water system piping in industries, natural aquatic systems. *Pseudomonas aeruginosa* and *Proteus mirabilis* are gram negative opportunistic pathogens responsible for acute and chronic infections in humans.The ability of these organisms to form complex biofilms has led to the development of the antibiotic resistance making it hard to be tackled by the host immune system and treatment with antibiotics. Majority of the infection of these organisms are localized to urinary tract and lead to urinary tract infection (UTI). These infections are also characterized with alternative reversible and irreversible attachments on the surface leading to dense biofilm formation with significant antibiotic resistance [1-8].

Indigenous medicines are an excellent source of alternative medicine to treat highly resistant microbial cells with multiple drug resistance patterns. *Ficusracemose Linn*. (Moraceae) is a prominent medicinal plant in India, which has been widely used in Ayurveda, the ancient system of Indian medicine, for various diseases/disorders including diabetes, liver disorders, diarrhoea, inflammatory conditions, hemorrhoids, respiratory, and urinary tract diseases. *F. racemose is* pharmacologically studied for various activities including antidiabetic, antipyretic, anti-inflammatory, antitussive, hepatoprotective, and antimicrobial activities. A wide range of phytochemical constituents have been identified and isolated from various parts of *F. racemose* . A comprehensive account of its traditional uses, phytochemical constituents, and pharmacological effects is presented in view of the many recent findings of importance on this plant.This study evalutes the antibiofilm activity of *Ficus racemose* against diabetes wound pathogens *Pseudomonas aeruginosa* and *Proteus mirabilis* [9-10].

MATERIAL AND METHODS

Fine fruits of *Ficus racemose* Linn. trees were collected in and around Coimbatore during the Dec 2020. The plant was identified and authenticate by Botanical Survey of India, Southern Circle, Tamil Nadu Agricultural University (TNAU), Coimbatore. The fruits were washed, shade dried, finely powdered, sieved

and stored in an air tight container until use. 10 g of fruit powdered were soaked in 50 ml of Methanol (Himedia, India) overnight. The extracts were filtered through Whatman No.1 filter paper to separate the filtrate. Removal of solvents from filtrate was done using rotary vacuum evaporator. Following vacuum evaporation, the dried extracts were collected and stored. Plant extract was dissolved in 100% Di Methyl Sulfoxide (DMSO) (Himedia, India) (v/v) and were diluted using sterile water prior to use [11-12]. The extracts were labelled as *F. racemose* Methanol extract (FR-M) for identification.

Bacterial strain and culture conditions

*Pseudomonas aeruginosa*and*Proteus mirabilis*bacterial culturewere obtained from Bioline laboratory, Coimbatore, Tamil Nadu. All the isolates were tested for their biofilm forming characteristics by observing the slime formation in routine media nutrient agar (Himedia, India). All isolates were cultivated and maintained in Todd Hewitt's broth at 37 °C. The biofilm forming isolates alone were used for further studies. Glycerol stock was maintained at -20 °C for further use.

Qualitative phytochemical analysis of plant extract

Test for Alkaloids -Plants methanol solvent extracts 2ml was treated with Dragendroff's reagent, the appearance of white colour indicates the presence of alkaloids.

Test for Flavonoids -Plants methanol solvent extracts 1ml was treated with magnesium and 1-2 drops of concentrated HCl. Formation of pink red colour shows the presence of flavonoids.

Test for Terpenoids -Plant methanol solvent extract 1ml add few drops of sulphuric acid and then shake well appearance of yellow colour indicates presence of terpenoids.

Test for Tannins -Plants four methanol solvent extracts 1ml was treated with 2ml of 5% neutral ferric chloride. A dark blue or bluish black colour product shows the presence of tannins.

Test for Phenols -Plants methanol solvent extracts 1ml was treated with 2ml lead tetra acetate solution. A precipitate production shows the presence of phenolic compounds.

Spectral Analysis

The FT-IR spectra of *F. racrmosa* fruit methanol extract was determined according to Sasidharan*et al.*, (2011). FT-IR spectrophotometer (Perkin-Elmer 1725x) at the range 4000-400 cm-1 and resolution of 4 cm^{-1}. The number of scan was 20.

Determination of the Minimal Inhibitory Concentration (MIC) and Antibacterial Assay

The MIC assay of the plant extracts followed by the antibacterial assay was performed according to the CLSI 2006 guidelines. The test bacterial cultures were subjected to the broth micro-dilution protocol. FR-M extract was prepared and incorporated to each well to obtain dilution of the active extract concentrations ranging from 8 mg/ml - 0.0625 mg/ml and incubated at 37 °C for 24h. The lowest concentration that resulted in inhibition of visible growth after overnight inhibition was recorded as MIC value.

Antibacterial activity was performed by agar well diffusion assay,against the *Pseudomonas aeruginosa* and *Proteus mirabilis*. Briefly, 100 µl of test bacterial suspensions culture were uniformly spread over the surface of nutrient agar plate. The agar plates were kept undisturbed for 10 min for the absorption of excess moisture. 50 µl of the methanol fruit extract was added in different concentrations to the wells. Then, the plates were incubated at 37 °C and the zone of inhibition was measured after 24 h. Sterile distilled water was used as negative control and Streptomycin (0.03 mg/ml) was used as antibiotic control [13-16].

Growth curve analysis

Growth curve analysis was done according to (Issac Abraham *et al*., 2012). Briefly, 1% of overnight test pathogens (0.5 OD at 600 nm) were inoculated in 50 ml of LB broth separately supplemented with 2 mg/ml of *Ficus racemose* solvent extracts. The flasks were incubated at 37 °C with 170 rpm agitation for 12 h in a rotator shaker. Cell density was measured using UV-visible spectrophotometer at every 1h interval at 623 nm [17-20].

Biofilm inhibition assays

Quantification of biomass inhibition

Quantification of biofilm biomass was performed using Micro titre plate assay (MTP) [21]. About, 1% overnight cultures (0.5 O.D at 600nm) of test pathogens were added in 1 ml of fresh LB medium in the presence and the absence of *Ficus racemose* solvent extracts (0.5-2mg/ml) separately. The samples were incubated at 37 °C for 16 h. After incubation, MTPs were emptied of free-floating planktonic cells and the wells were gently rinsed with sterile water. The biofilm were stained with 0.4% crystal violet (CV) (Himedia, Mumbai, India) solution. After 15 min, CV solution was discarded completely and wells were filled with 1 ml of 95% ethanol for de-staining. The biofilm biomass was then quantified by measuring the absorbance at OD 650 nm using multiplate ELISA reader (Biotek-ELX-800, India).

Microscopic observation of biofilm

Light Microscopic Analysis

For visualization of biofilm by light microscopy [21], The biofilms were allowed to grow on glass pieces (2x2 cm) placed in 24-well polystyrene plates supplemented with different solvent extracts of *Ficus racemose* (2 mg/ml) and incubated for 24 h at 37 ºC. The slides were stained using crystal violet and were placed on slides with biofilm pointing upwards. The slides were observed under light microscopy at magnification of ×40. Visible biofilms were documented with an attached digital camera[22-29].

RESULTS AND DISCUSSION

Phytochemical screening

The preliminary phytochemical screening of the FR-M extract was performed and the results were tabulatedin Table-1. The tested extract showed positive for most of the active plant biomolecules. According to Saha*et al.,* (2015) *Ficus racemose* fruit extract contained essential biomolecules like alkaloids, tannins, flavonoids, Terpenes and phenol.

Table1.Preliminary phytochemical screening of *Ficusracemose*

S.No	Bioactive compounds	FR-M
1.	Alkaloids	Positive
2.	Flavonoids	Positive
3.	Terpinoids	Positive
4.	Tannins	Negative
5.	Phenol	Positive

Spectral analysis

The FT-IR spectrum was performed to identify the functional group of the active components based on the peak value in the infra-red radiation (Table-2 and Fig-1). The FT-IR spectrum revealedseven functional groups between 500 to 4000 frequency wavelengths. The presence of intense bands at 817.28 cm^{-1}, 948.52 cm^{-1}, 1049.28 cm^{-1}, 1311.59 cm^{-1}, 1419.61 cm^{-1}, 1674.21cm^{-1},and 3641.60 cm^{-1}corresponding to C-H,C-H(S), H – C and C = C bond vibiration respectively indicate the presence of Alkyl groups, Aryl groups, Cyclohexane ring vibrations, Alcohol, Hydroxyl,Olefinic group, Alkene and Alcohol group compound.

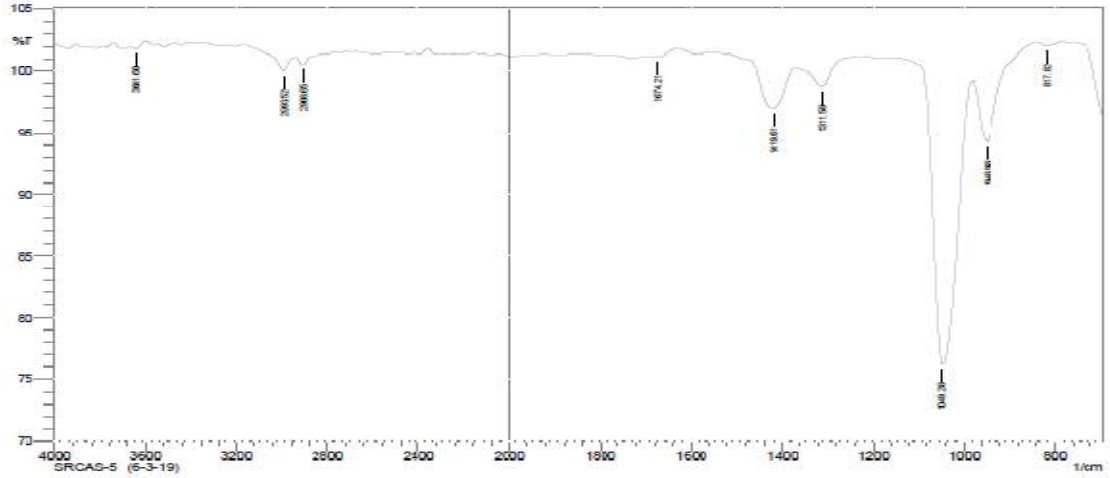

Fig 1. FTIR spectrum of *Ficusracemose* methanol (FR-M) extracts

Table 2. FTIR analysis of *Ficusracemose* methanol extracts

S. No	Frequency (cm^{-1})	Bond and type of Vibrations	Functional groups
1	817.28	C-H	Alkyl groups
2	948.98	C - H	Aryl groups
3	1049.28	C -H	Cyclohexane ring vibrations
4	1311.59	O - H	Alcohol and Hydroxyl compound
5	1419.61	C – H	Olefinic group
6	1674.21	C=C	Alkene
7	3641.60	O – H	Alcohol group

In our investigation of FTIR results correlated with functional groups present in the plant material that have fully potential medicinal property. A total of nine compounds with varying medicinal properties were observed. According to the previous reports compounds Alkyl groups, Aryl groups, Cyclohexane ring vibrations, Alcohol, Hydroxyl, Olefinic group, Alkene and Alcohol group compound consist of anti-cancer, anti-oxidant, anti-microbial activity were observed [29-31].

Antibacterial Activity Assay

Minimal Inhibitory Concentration (MIC) Assay

A total of 15 isolates were obtained from the clinical laboratory. Among the 15 isolates, six isolates were identified as *Pseudomonas aeruginosa* and nine were *Proteus mirabilis*, based on their characteristic's growth on the selective agar and beta haemolytic activity. It was observed and confirmed that two isolatesof *Pseudomonas aeruginosa* and *Proteus mirabilis* were biofilm formers as it produced slime colonies on the blood agar plates. Further, MIC was determined for the *Ficus racemose* against the test pathogens. The MIC of synergistic solvent extracts is represented in Fig 2.The MIC of *Pseudomonas aeruginosa* and *Proteus mirabilis* was recorded to be 1-4 mg/ml.

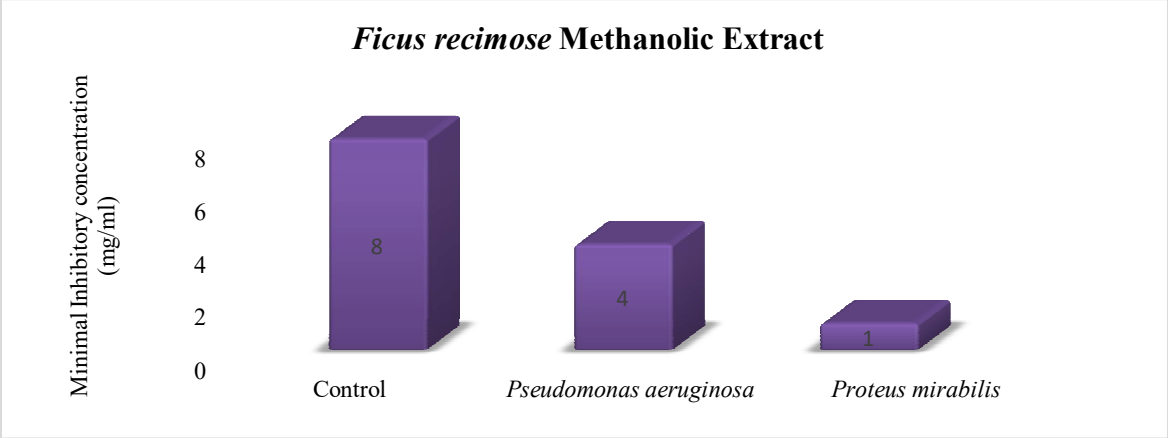

Fig 2. Minimal Inhibitory concentration of *Ficusracemose* methanol extracts

Agar well diffusion assay

Ficus racemose extracts were evaluated for their antibacterial activity at a concentration below MIC to assess the inhibitory nature at sub-MIC level by agar well diffusion assay. The methanol, extracts of *Ficus racemose* showed insignificant antibacterial activity against the tested biofilm forming pathogens as there was no zone of inhibition at 0.5 mg/ml concentration and also. The solvent extracts of *Ficus racemose* revealed varying degree of inhibition and are represented in Table 3.

Table3.Agar well diffusion activity of *Ficus racemose* methanol extracts

S.No	Test Organism	*Ficus racemose* (0.5 mg/ml)
1	*Pseudomonas aeruginosa* C1	-
2	*Proteus mirabilis* D1	-
3	Streptomycin (0.03mg/ml)(Positive control)	+++
4	DMSO (Negative control)	-

Growth curve analysis

The antibacterial activity of *Ficus racemose* solvent extracts were further tested by the growth curve analysis. The results indicate that all the extracts had no effect on the growth of the tested pathogens at 0.5 mg/ml concentration which was evident from absence in change of the cell densities between the treated and the untreated cultures. Results shown in (Fig 3 and Fig 4) revealed that the *Ficus racemose* extract did not possess any antibacterial activity at tested concentration against all test bacteria as compared to control. *Proteus mirabilis* and *Pseudomonas aeruginosa* belong to a diverse collection of Gram-Negative cocci that typically grow as rod and divide on single plane in chains of varying lengths in selection agar media and gram staining. The organisms are generally strong fermenters of carbohydrates, resulting in the production of lactic acid, a property used in the dairy industry. They are catalase, Oxidase and IMViC – negative [32-36].

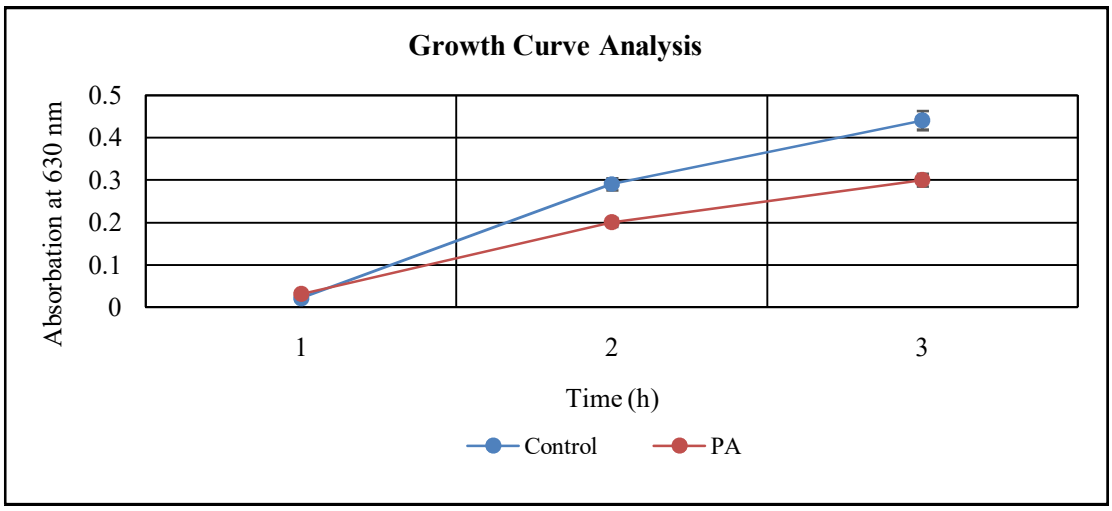

Fig 3. Growth Curve Analysis of *Ficus racemose* methanol extracts on *P. aeruginosa* C1.

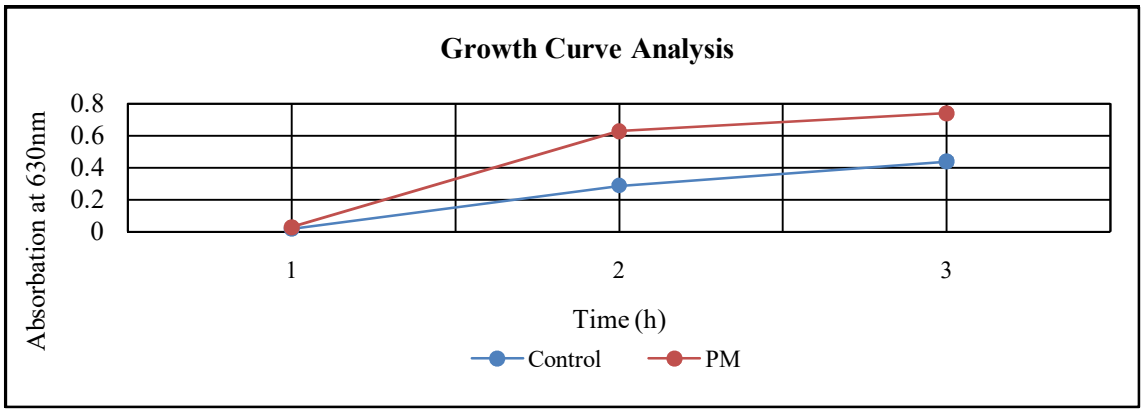

Fig 4. Growth Curve Analysis of *Ficusracemose* methanol extracts on *P. mirabilis* D1.

Biofilm inhibition assay

The effects of different concentrations (0.062-1mg/ml) of methanol extracts of *Ficus racemose* on biofilm-forming ability of test bacteria were determined. Evidently, a pronounced biofilm inhibition percentage was observed in methanol extracts of *Ficus racemose* against both test bacteria at a lowest concentration of 0.5 mg/ml. (Fig5) validated that methanol extracts of *Ficus racemose* have promising antibiofilm activity against the *Ficus aeruginosa* compared to control. Significant ($p < 0.05$) inhibition of biofilm was observed in methanol, extracts of *Ficus racemose*. The significant antibiofilm potential of methanol extracts against *Proteus mirabilis* and *Pseudomonas aeruginosa* were further elucidated via *in situ* microscopic analysis. Biofilm formation leads to increased resistance against antimicrobial treatments and host defenses which favor the growth of microorganisms in suboptimal environments. Generally, higher concentration of the antibiotics is required to kill bacteria in the biofilm. phase than their planktonic counterparts. Phytochemicals are used in the treatment of infections caused by bacterial biofilms as the compounds suppress the expression of genes responsible for pathogenesis by interfering with bacterial biofilm formation. Antibacterial activity assay elucidated insignificant inhibition of test pathogens at a concentration below MIC shown in Table-3. However, significant inhibition of biofilms was observed at the tested concentration which elucidated that the plant extract was interfering with the biofilm architecture without inhibiting the bacterial viability [36-59].

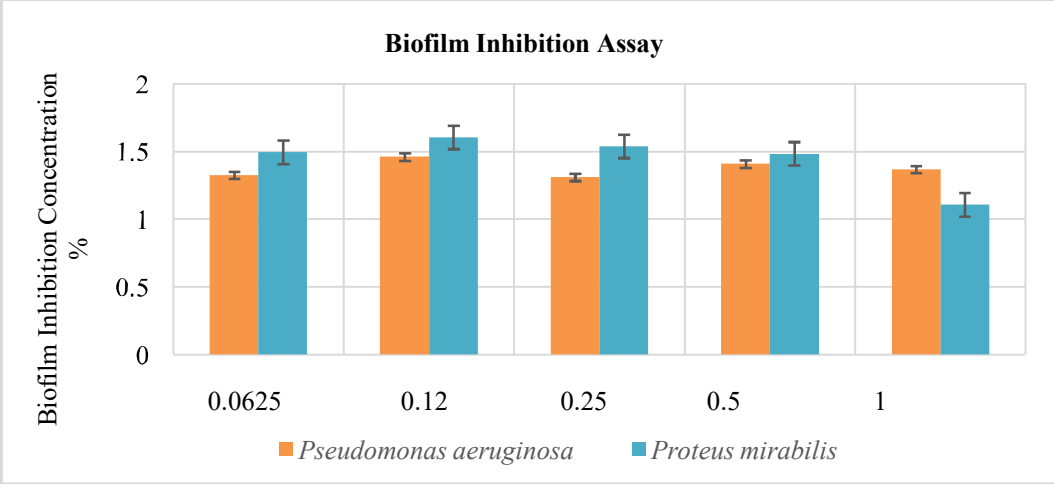

Fig 5. Biofilm inhibition assay at Sub Minimal Inhibition concentration.

Light microscopic analysis

In order to analyze the full potential of methanol extracts of *Ficus racemose* plants in biofilm inhibition, biofilm susceptibility analysis was carried out at biofilm inhibitory concentrations. The biofilm inhibitory potential of methanol extracts of both the plants at 1 to 0.0625 mg/ml concentration, on the test bacteria was also confirmed by microscopic visualization (Fig-6). Significant reduction in the biofilm formation was observed in all the test concentration when compared to their respective controls. This elucidated that the methanol *Ficusracemose* inhibited the biofilm formation at its initial stage.

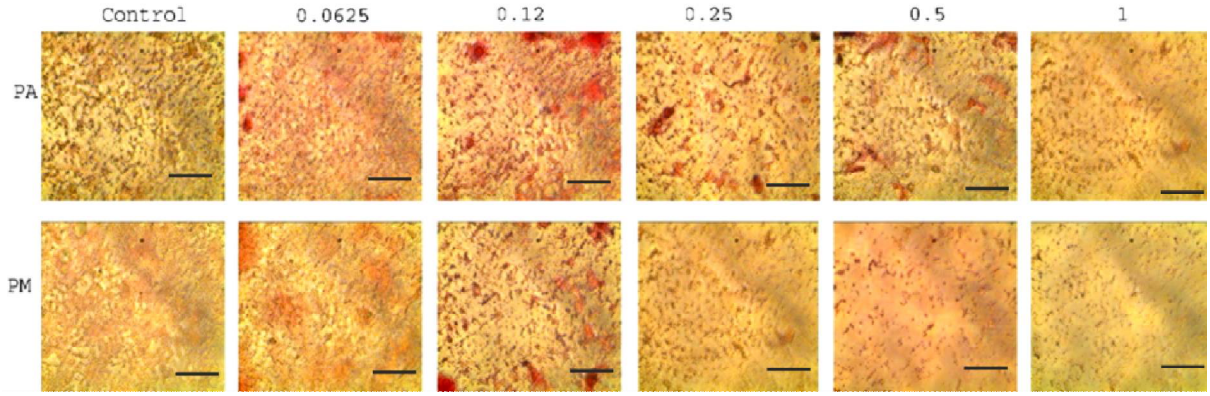

Fig 6. OlympusLight microscopic images of *S. pyogenes* biofilms grown in the absence and/or presence of *Ficusracemose* methanol extracts at test concentrations (Scale bar - 20µ at x200 magnification).

CONCLUSION

The plant holds great promise as a commonly available medicinal uses and it is indeed no surprise that the plant is referred to in the Indian traditional circles. The results of present investigation clearly indicated the *Ficus racemose* methaolic extracts contain the primary and secondary phytochemicals. The extracts contain an effective involve in different biological activity conformed by different in vitro studies. Furthermore studies are involved in understanding of action to exploit as potent of in vivo biofilm mechanism against pathogens.

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