



Original Article

Evaluation of Anti-Quorum Sensing Activity and Anti-Biofilm Activity of *Prunus Avium* Fruit Extract

P Neeraja *, Ch Sravya, B Ramya bhanu, D Ravali

Geethanjali College of Pharmacy, Cheeryal (V), Keesara (M), Ranga Reddy, Telangana-501301, India.

ARTICLE INFO

A B S T R A C T

Received: 18 Apr 2018
Accepted: 02 May 2018

Bacteria inhabit dense, surface-bound communities, termed biofilms, within which they communicate and respond to local cell density through a process known as quorum sensing. Biofilms are estimated to be associated with 80% of microbial infections and the growth of micro-organisms in biofilms can enhance their resistance to antimicrobial agents. Targeting the bacterial communication system (quorum sensing, QS) can be chosen as one of the novel strategy to combat anti microbial resistance. In this work, *Prunus avium* fruit extract was prepared using sox let extraction. *Escherichia Coli* culture was maintained on nutrient agar medium and transferred on to L.B broth and bacterial extract is prepared using Orbital shaker incubator. *Escherichia Coli* Bio film was prepared on cover slips and slides. The intensity of bio film was measured by crystal violet assay method. The extra cellular polysaccharide was extracted. Anti quorum sensing activity of *Prunus avium* fruit extract was estimated using disk diffusion method. The anti biofilm activity was measured by crystal violet assay method. In this study *Prunus avium* fruit extract has shown promising anti quorum sensing activity against *Escherichia coli*. *Prunus avium* fruit extract was tested against urinary catheter infected with *Escherichia Coli* biofilm. There is a significant decrease in the intensity of the bio film of *Escherichia coli* after a period of 3 days.

Key words: Quorum sensing, biofilm, *Prunus avium*, anti biofilm agents and *Escherichia coli*

1. INTRODUCTION

Biofilm is a group of microorganisms in which microbial cells stick to each other irreversibly (not removed by gentle rinsing) and they often adhere to a surface. Bacteria exhibit this type of behavior by chemically signaling to one another by a process called quorum sensing¹. Quorum sensing bacteria releases a chemical molecule called auto inducers; these auto inducers modulate gene expression². Biofilms acts as a mechanism towards bacterial resistance³. Growth of micro-organisms in biofilms can enhance their resistance to antimicrobial agents. As a consequence antimicrobial therapy often fails to eradicate biofilms from the site of

Corresponding author *

P. Neeraja,
Associate Professor,
Department of Pharmaceutical biotechnology,
Geethanjali College of Pharmacy,
Cheeryal (V), Keesara (M), Ranga reddy, Telangana-501301,
India.
Email.id: neerajapodichety@gmail.com

infection. For this reason, innovative anti-biofilm agents with novel targets and modes of action are needed.

Biofilms have been found to be involved in variety of microbial infections⁴. An estimate was made that 80% of microbial infections in the body are caused due to biofilms. Infections in which biofilms infect include many common problems such as catheter infections, urinary tract infections, bacterial vaginosis, middle-ear infections, formation of dental plaque, gingivitis, endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves. Plaque is a biofilm formed on teeth⁵. The microorganisms accumulate on teeth and release high concentrations of bacterial metabolites leading to dental disease^{6,7}.

Prunus avium is commonly called as sweet cherry belonging to the family Rosaceae. Cherries are natural sources of antioxidants, Polyphenols, anthocyanins, sugars, Hydroxycinnamic acids, Anthocyanins and Flavan-3-ols. *Prunus avium* extracts has antimicrobial activity^{8,9}. Quorum sensing activity is shown by some plants¹⁰.

2. MATERIALS AND METHODS

Preparation of *Prunus avium* fruit extract:

Extraction procedure: Fresh cherry fruit of about 100g is taken and its seed and peel is removed. Then the fruit is soaked in methanol for about 24 hours. The soaked cherry fruit is soxhlet with methanol for about 6 hours at 60°C. Remove the mixture from soxhlet and evaporate the mixture to evaporate methanol. Centrifuge the mixture at 5000 rpm for 3 minutes and collect the supernatant liquid. The extract is temporarily stored in refrigerator.

Preparation of media:

Preparation of LB Broth: Triptone (10gm/ml), Yeast extract (5gm/ml), Sodium chloride (10gm/ml) is taken into a 1000ml conical flask. About 500ml of water is taken and slowly added in to the flask by shaking the flask until clear liquid is formed. The medium is sterilized in a autoclave.

Preparation of Agar Medium: Peptone (6gm), Beef extract (1.5gm), Yeast extract (3gm), Agar (15gm) are taken into a conical flask and water (1000ml) is added slowly in to the flask by stirring. A clear solution is formed and the medium is sterilized.

Organism and culture maintenance:

Escherichia coli culture was maintained on nutrient agar medium. The slants were incubated at 37°C for maximum growth. The fully grown slants were maintained at 4°C and were sub cultured for every 2 weeks.

Preparation of bacterial extract:

It is carried out in 250ml conical flasks containing 100ml of LB Broth. To the broth test microorganism i.e. *Escherichia coli* was added separately in two flasks. The inoculated broth is kept in orbital shaker incubator for 72 hours so that the microbes are evenly distributed in the broth. The bacterial extract is now used for preparation of biofilm.

Preparation of biofilm:

A clean groove slide is taken and immersed in a container of alcohol and sterilized by flaming. The slide is allowed to cool for a few seconds and then one or two drops of inoculated broth is added.

The groove slide is covered with an unbreakable cover slip which is also dipped in a container containing alcohol so as to sterilize. In the same manner five or six slides are prepared and placed in a incubator for 48 hrs under aseptic conditions.

After 48 hrs of incubation, using forceps which is sterilized by dipping in a container containing alcohol and by flame the cover slips are removed carefully without breaking and placed on a sterile filter paper.

The biofilm on the cover slips should appear as a slimy layer coating the under surface of the slide. Lifting rapidly may disturb the biofilm causing large sections to slough off the slide. In laminar air flow cabinet, agar medium is poured in petriplate and allow it to solidify. The biofilm formed cover slips are retained on agar medium and incubated again for 48 hours for more good results.

Alternative method:

Agar medium which is sterilized in autoclave is poured in petriplate and allow it to solidify in air flow cabinet. To the above medium sterilized cover slips are inserted, see that there is no air bubbles formation. Test organisms i.e. *Escherichia coli* was poured to different petriplate and incubated for 48 hours.

Detection of bio film:

The ability of biofilm producing microbes was tested by staining the heat fixed cells with crystal violet. The petriplate containing cover slips and slides were washed after incubation, three times with sterile distilled water to remove loosely associated cells. The plates were air dried and then dried in oven at 60°C for 45 min. Following drying, they were stained with 100 µL of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed 5 times with sterile distilled water to remove unabsorbed stain^{11,12}.

Anti quorum sensing activity of *Prunus avium* fruit extract:

Disk diffusion assay is selected for measurement of potential anti-quorum sensing activity.

Diffusion assay:

The Disk diffusion assay is an assay used to evaluate anti-QS activity by evaluating zones of inhibition around the disk. Each extract (50 µL) was loaded onto sterile disks (6 mm diameter), placed onto prepared LB plates containing biofilm formed cover slips of *Escherichia coli*. Plates were incubated in an incubator at 30°C overnight and anti-QS activity was detected by a ring of colorless, but viable cells around the disk. Measurements were made from the outer edge of the disks to the edge of the zones of anti-QS activity. Results were determined by observing the growth inhibition

Int J Pharma Res Health Sci. 2018; 6 (3): 2594-98
 colorless ring diameter, opaque circle around the extract loaded disk.

Anti bio film activity of *Prunus avium* fruit extract:

Anti bio film activity of *Prunus avium* fruit extract of *Escherichia coli* was qualitatively estimated by a method described by Xiao et al. 40 µL of exponentially growing cells were dispensed in wells prepared in cell culture plates. *Prunus avium* fruit extract was added to the wells and incubated for 24 h at 37°C. The concentrations of extracts were ranged from 10 to 100 µg/mL. The medium without extracts was used as the non-treated control. After incubation, media and unattached cells were decanted and washed with Phosphate Buffer Saline (PBS). Then the plate was air dried and it is stained with 0.1% (v/w) Crystal Violet (Sigma-Aldrich, Germany). 1000 µl of 95% (v/v) ethanol was added to each well for 1 h to release the stain. Cells were transferred to a new test tube for spectrophotometric analysis (OD570 nm)¹³. Inhibition mediated reduction of biofilm formation was calculated by the following formula
 % of inhibition = $\frac{OD \text{ in control} - OD \text{ in treatment}}{OD \text{ in control}} \times 100$

Effect of *Prunus avium* fruit extract on Bacterial (*Escherichia Coli*) Bio film in Urinary Catheter

About 5 mL of urine sample was injected into catheters to induce bio film formation on the internal surface of the catheter. *Escherichia coli* cultures were incubated on a freshly prepared sterile nutrient broth and injected into catheters. The catheters were stoppered with the catheter caps and incubated at 25°C for 120 h¹⁴.

Coating of catheter with the plant extracts: The catheters were immersed in warm water bath. This leads to tenderness of the catheter and allowed for easy coating of the inner surface layer of the catheters with the plant extracts. 20ml of the plant extract was injected into catheter. The catheters were placed such that the both end of the tubes were out of the warm water. This is then allowed for quick coating of the plant extract and evaporation of the solvent (ethanol) that was used to prepare the extract¹⁵.

3. RESULTS

Preparation and detection of bio film of *Escherichia coli*

Bio film of *Escherichia coli* was prepared in sterile cover slips. In laminar air flow cabinet, agar medium is poured in petriplate and allow it to solidify. The bio film formed cover slips are retained on agar medium and incubated for 48 hours for more good results. In another method, to agar medium containing cover slips and slides test organisms i.e. *E.coli* was added and incubated for 48 hours. The ability of biofilm producing microbes was tested by staining the heat fixed cells with crystal violet.

As *Escherichia coli* is a gram negative bacteria its cell wall consists of less amount of peptidoglycan. So it has increased permeability and increased pore size. When crystal violet was added to *E.coli* cell culture, blue color diffuses into the

cell. Blue colored stained cells were observed under projection microscope (Fig. 1).

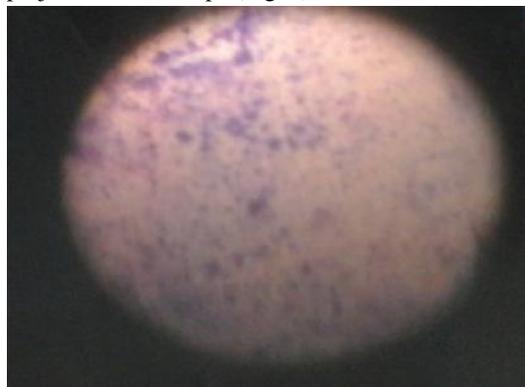


Fig1: Detection of bio film of *Escherichia coli*

Anti quorum sensing activity of *Prunus avium* fruit extract against *Escherichia coli* bio film was determined by disk diffusion assay (Fig.2). In this study *Prunus avium* fruit extract has shown promising anti quorum sensing activity against *Escherichia coli*.



Fig2: Anti quorum sensing activity of *Prunus avium* fruit extract

Anti bio film activity of *Prunus avium* fruit extract:

Bio film inhibition studies carried out using plant extracts have successfully inhibited bio film formation of *E.coli*. The plant extract inhibited bio film as dose dependent manner. The results were presented in Table 1.



Fig.3 Anti bio film activity of *Prunus avium* fruit extract in Crystal violet assay

Table 1: Measurement of Anti bio film activity of *Prunus avium* fruit extract:

S. No	Total bio film	% inhibition
Control	0.53 ± 0.02	0
10µg/ml	0.51 ± 0.01	7.57
25 µg /ml	0.43 ± 0.01	22.87
50 µg /ml	0.31 ± 0.02	47.51
70 µg /ml	0.29 ± 0.003	58.78

The effect *Prunus avium* fruit extract in Prevention of *Escherichia Coli* bio film in Urinary Catheter.

Prunus avium fruit extract was injected into the catheter coated with bio film of *Escherichia coli* and observed for a period of 15 days. There is a significant decrease in the intensity of the bio film of *Escherichia coli* after a period of 3 days (Fig 4, Fig 5).



Fig 4: *Escherichia Coli* bio film in Urinary Catheter.



Fig 5: Effect *Prunus avium* fruit extract in prevention of *Escherichia Coli* bio film in Urinary Catheter.

4. DISCUSSION

Microorganisms undergo various changes during their transition, these changes leads to formation of biofilm¹⁶. Biofilms are resistant to disinfectants when they are present on surfaces¹⁷. Many microbial species can form biofilms e.g. *Escherichia coli* strains^{18, 19}. *Escherichia coli* cause urinary catheter infections.

In this work, *Prunus avium* fruit extract was prepared using sox let extraction. *Escherichia coli* culture was maintained on nutrient agar medium and transferred on to L.B broth and bacterial extract is prepared using Orbital shaker incubator. *Escherichia Coli* bio film was prepared on cover slips and slides. The intensity of bio film was measured by crystal violet assay method. The extra cellular polysaccharide was extracted. Anti quorum sensing activity of *Prunus avium* fruit extract was estimated using disk diffusion method. The anti biofilm activity was measured by crystal violet assay method. In this study *Prunus avium* fruit extract has shown promising anti quorum sensing activity against *Escherichia coli*.

Prunus avium fruit extract was tested against urinary catheter infected with *Escherichia coli* biofilm. There is a significant decrease in the intensity of the bio film of *Escherichia coli* after a period of 3 days.

5. CONCLUSION

In summary, the selected *Prunus avium* fruit extract may have a potential, and serve as important as medicinal plants

in inhibiting quorum sensing of bacteria and further acts as a anti biofilm agent. Further study has to be done to identify the active compounds of the identified plant extract and mechanism of actions of quorum sensing by the active compound.

6. ACKNOWLEDGEMENT

The authors would like to thank Geethanjali College of Pharmacy, Hyderabad for providing infrastructure to carry out the work.

7. REFERENCES

1. Dufour D, Leung V, Levesque CM. Bacterial biofilm: structure, function, and antimicrobial resistance. *Endodontic topics*. 2010; 22(1):2-16.
2. Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. *Sensors*. 2012; 12(3): 2519–2538.
3. Jolivet Gougeon A, Bonnaure Mallet M. Biofilms as a mechanism of bacterial resistance. *Drug Discov Today Technol*. 2014; 11:49-56.
4. Bjarnsholt T. The role of bacterial biofilms in chronic infections. *APMIS Suppl*. 2013; (136):1-6
5. Parsek MR, Singh PK. Bacterial Biofilms: An Emerging Link to Disease Pathogenesis. *Annu Rev Microbiol*. 2003; 57: 677-701.
6. Donlan RM. Biofilms: Microbial Life on Surfaces. *Emerg Infect Dis*. 2002; 8(9):881-90.
7. Cosrerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science*. 1999; 284(5418):1318-1322.
8. Chong-Lek Koh , Choon-Kook Sam , Wai-Fong Yin , Li Ying Tan , Thiba Krishnan , Yee Meng Chong , Kok-Gan Chan . Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors*. 2013; 13(5):6217-6228.
9. Rovcanin BR, Cebovic T, Stesevic D, Kekic D, Ristic M. Antibacterial effect of *herniaria hirsuta*, *prunus avium*, *rubia tinctorum* and *sempervivum tectorum* plant extracts on multiple antibiotic resistant *escherichia coli*. *Bioscience journal*. 2015; 31(6) 1852-1861.
10. Rwaida A. Haidari A, Mona I. Shaaban, Sabrin RM. Ibrahim, Gamal A. Mohamed. Anti-quorum sensing activity of some medicinal plants. *Afr J Tradit Complement Altern Med*. 2016; 13(5):67-71.
11. Hassan A, Javaid Usman, Fatima Kaleem, Ali Khalid, Maria Omair, Muhammad Iqbal. Evaluation of different detection methods of biofilm formation in the clinical isolates. *Braz J Infect Dis*. 2011; 15(4):305-311.
12. Naves P, Del Prado G, Huelves L, Gracia M, Ruiz V, Blanco J, Rodríguez-Cerrato V, Ponte MC, Soriano F. Measurement of biofilm formation by clinical isolates of *escherichia coli* is method-dependent. *J Appl Microbiol*. 2008; 105(2):585-590.

13. Sanchez E, Rivas Morales C, Castillo S, Leos-Rivas C, García-Becerra L, Ortiz Martínez DM. Antibacterial and Antibiofilm Activity of Methanolic Plant Extracts against Nosocomial Microorganisms. *J. Evidence-Based Complementary Altern. Med.* 2016; 2016: 1-8.
14. Trautner BW, Darouiche RO. Role of biofilm in catheter-associated urinary tract infection. *Am J Infect Control.* 2004; 32(3): 177-183.
15. Adesina TD, Nwinyi OC, Olugbuyiro JA. Prevention of Bacterial Biofilms Formation on Urinary Catheter by Selected Plant Extracts. *Pak J Biol Sci.* 2015; 18(2):67-73.
16. Toole G, Kaplan HB, Kolter R. Biofilm Formation as Microbial Development. *Annu Rev Microbiol.* 2000; 54:49-79.
17. Jahid IK, Ha SD. A Review of Microbial Biofilms of Produce: Future Challenge to Food Safety. *Food Sci. Biotechnol.* 2011; 21(2): 299-316.
18. Ponnusamy P, Natarajan V, Sevanan M. In vitro biofilm formation by uropathogenic *Escherichia coli* and their antimicrobial susceptibility pattern. *Asian Pac J Trop Biomed* 2012; 5(3):210-213.
19. Saroj G, Vivek H, Sujatha K, Mallika Reddy K. Correlation between biofilm formation of uropathogenic *Escherichia coli* and its antibiotic resistance pattern. *J. Evol. Med. Dent. Sci.* 2012 ; 1 (3): 166-168.

Conflict of Interest: None

Source of Funding: Nil