Indian Journal of Traditional Knowledge
Vol 20(3), July 2021, pp 679-684

Controlling progression of bacterial biofilm by herbal eye care formulation

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Received 13 August 2019; revised 06 April 2021

Pseudomonas aeruginosa and *Staphylococcus aureus* causing common eye-infection 'keratitis' develop resistance to antibiotics by forming biofilm. Present study evaluates anti-biofilm properties of three commercially available eye care herbal formulations against *P. aeruginosa* and *S. aureus.* Eye drop formulations were tested for total phenolic content and antimicrobial activity. Biofilm growth over glass in the presence of herbal formulation was examined under fluorescence microscope. The herbal formulations rich in phenolics, tested at 10% (v/v) concentration showed growth inhibitory effect on planktonic cells. During biofilm mode of growth, 10% of herbal formulation caused considerable decline in growth of biofilm, although did not inhibit it completely. The response to different herbal formulations varied markedly among the different bacterial strains tested. Herbal components can significantly affect the surface topology of biofilms, thereby restricting the attachment of the cell to the substratum.

Keywords: Antimicrobial, Biofilm, Herbal eye care, Keratitis, *Pseudomonas aeruginosa*, *Staphylococcus aureus*
IPC Code: Int Cl.[21]: A61K 9/00, A61K 9/08, A61K 38/00, A61K 38/48, A61K 45/06, C12R 1/385, C12R 1/445

Bacterial keratitis is a common eye disease caused by inflammation of clear tissue on the cornea. Ocular infection may lead to serious consequences including corneal ulcers, blurred vision, redness of eye and burning sensation of cornea[1]. Biofilm formation appears to be a critical factor for pathogenesis of keratitis. A healthy ocular surface is not an ideal environment for microbial colonization. However, bacterial colonization is feasible when the ocular surface comes in proximal contact with inert surfaces like contact lenses, intraocular lenses and punctual plugs[2,3]. Bacteria involved in a variety of ocular infections are associated with ophthalmic devices. Any artificial surface can accelerate the growth of biofilm and therefore promotes eye infection. Biofilm is a kind of bacterial colonization over a surface in which millions of cells adhere to each other and to the substratum via cementing biomolecules, generally an extracellular polymer of microbial origin[4]. The biofilm mode of growth aggravates microbial resistance to abiotic and biotic components. Hence, upon formation of biofilm, inherent pathogens are shielded against both host defense system and antibiotics[5].

Pseudomonas aeruginosa and *Staphylococcus aureus* commonly cause the inflammation of eyes by forming biofilm on the soft tissues of cornea[5]. Especially, contact lens users are more vulnerable to ocular infections. Contact lenses and lens case serve as reservoirs of various biofilm-forming pathogens[6]. A plethora of studies support the involvement of biofilm in ocular infection. *S. aureus* causes blepharitis, conjunctivitis and endophthalmitis[1], whereas *P. aeruginosa* causes ocular infections reported mainly among individuals with ocular implants[7].

Herbs are effective in the treatment of various eye ailments. The medicinal importance of herbs such as *Emblica officinalis, Glycyrrhiza glabra, Cassia fistula, Berberis asiatica, Butea monosperma,* etc. is recognized in Ayurveda[8]. Ancient ayurvedic scripts *Charak Samhita, Sushrut Samhita, Rasatarang* and *Astanghriday* explain the use of number of plants in eye disease as single or compound formulations[9]. Studies have supported the efficient use of ayurvedic medicines for managing eye infections[10]. Herbal formulations used for the management of eye infections largely focus on the antimicrobial aspects[11,12]. However, little and no attention has been given to incorporate anti-biofilm aspects in herbal

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formulation. The current study evaluates herbal formulations for potential to minimize biofilm formation by *P. aeruginosa* and *S. aureus*.

Materials and Methods

Microbial strains

Test cultures *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 29213) were received from Synergy Micropath Laboratory, Ahmedabad, Gujarat.

Herbal formulations

In present study, three commercially available herbal formulations (HF) for eye care having trade names Patanjali eye drop (PE), Netraprabha eye drop (NPE) and Itone herbal eye drop (IE) were used. The formulations contained different plant extracts along with a common ingredient honey. The details of formula herbs are printed on the commercial product and brief mention included in Supplementary file 1.

Total phenolic content

The total phenolic content of the herbal formulations was determined using the Folin-Ciocalteu reagent. Sample HF (0.5 mL), 1:10 diluted Folin-Ciocalteu reagent and 7.5% sodium carbonate (4.0 mL) were mixed. The content was incubated at 30°C for 30 min and A_{756} was measured. Total phenolic content was calculated using Gallic acid as the standard[13].

Antimicrobial effect

Antimicrobial activity of HF was evaluated using agar diffusion assay. Active (12 h) test cultures of *P. aeruginosa* and *S. aureus* were used to seed sterile Luria Bertani (LB) agar (Himedia, India) and poured into sterile Petri plates. After the content was solidified, test culture was swabbed over the surface using sterile cotton swabs. Using sterile cup-borer (8mm diam.), four wells were punched in the medium and were filled to the rim with test HF (100%) using sterile pipette. The plates were incubated in upright position at 37°C for 24 h. Zone of inhibition around the wells was measured to evaluate anti-microbial effect.

Effect on planktonic growth

Herbal formulations at the concentration of 10-50% in LB medium were studied for their effect on planktonic growth. Varying concentrations of eye drops were added to 18 h old active cultures (10^6 cells mL^{-1}) of *P. aeruginosa* and *S. aureus.* After incubation at 37°C for 24 h, growth was observed at A_{610}.

Effect on growth of biofilm

Glass microscopic slide was sterilized in a Petri dish by autoclaving and the slide was immerged in HF suitably diluted with LB broth. To this, 5 mL of 1:10 diluted active culture of *P. aeruginosa* or *S. aureus* was added along with 10% solution of test HF. The plates were incubated for 48 hours at 37°C. After incubation, slide was fluorescent stained using 0.02% Acridine orange[14]. The slides were observed under fluorescence microscope (under 10X objective) under green filter. The fluorescence microscopic images were analyzed using Image J software (National Institute of Health, USA) for the surface architecture of the biofilm. Mean biofilm growth was measured as raw integrated density. Biofilm surface topology was examined using integrated 3 D and surface plot tools of Image J[14,15].

Statistical analysis

Data were plotted along with Mean and Standard Deviation. Student's *t*-test was used to determine the differences in biofilm formation in the presence of herbal formulations tested. Value of $p<0.05$ was considered statistically significant.

Result and Discussion

Total phenolic content

The total phenolic content of the HF sample PE tested was noticeably high (27.5 mg mL^{-1}). The phenolic content of IE and NPE was 190 and 225 µg mL^{-1}, respectively. Sample PE contained *Citrus aurantifolia, Allium cepa* and *Zingiber officinale*, which are reportedly[16] rich in phenolics and flavonoids. Both IE and NPE are poly herbal formulations from several medicinal plants. Both herbal formulations contain *Emblica officinalis*, *Terminalia chebula*, and *Terminalia belerica* also rich in many phenolic compounds[17]. Phenolics have diverse therapeutic applications owing to potent anti-bacterial properties. Compounds such as ellagic acid, gallic acid, catechin, epigallocatechin, tannic acid and ferulic acid are essential phenolics with antibacterial properties[18–20].

Antimicrobial effect

The antimicrobial activity of the herbal eye drop was tested against *S. aureus* and *P. aeruginosa* using

agar well diffusion method. All the formulations showed inhibitory action against test cultures. However, the largest zone of inhibition was observed for PE (Fig. 1A). Effect of herbal formulations on planktonic cell growth was tested at the concentration between 10% and 50% in the broth cultures (Fig. 1B and C). At all tested concentrations, a significant decline in planktonic cell growth for both the test strains was observed. Both, *P. aeruginosa* and *S. aureus* were found to be more sensitive to all the herbal formulations tested (Fig. 1B and C). At 10% and 20% concentration of HF, 10-fold decrease in the growth of both strains was observed. At 50% concentration, complete loss of viability was observed. High phenolic level in the formulation is justified to maintain effective concentration after post-application dilution by tears. The findings from agar diffusion method correlates the level of antimicrobial activity of HF to its phenolic content ($p<0.05$).

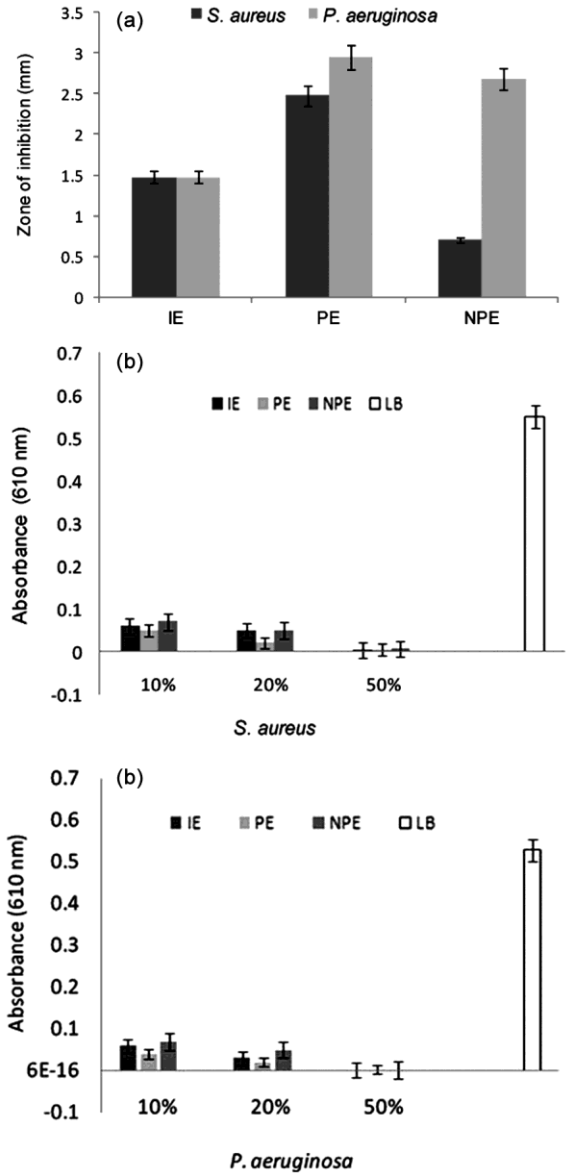

Fig. 1 — Effect of HE on growth (A) Agar diffusion method (B) Broth dilution method.

Herbs are a rich source of phytochemicals having antimicrobial potential. Phenolic compounds present in plants are not only rich in antioxidants but also exhibit marked antimicrobial properties[21]. Phenolic compounds show antimicrobial capability by inactivating enzymes or blocking cell wall components[22]. Gallic acid and ferulic acid are reported to inhibit *S. aureus* and *P. aeruginosa* growth by damaging their cell wall[19]. Formulation PE contains *Allium cepa*, a potential antibacterial herb which is also a rich source of gallic acid and ferulic acid[23]. Formulations IE and NPE contain *Emblica officinalis*, known to contain gallic acid[24]. Incorporation of such herbal components to conventional drug formulations can improve efficiency of antibiotics in treating bacterial infections[25].

Effect on growth of biofilm

The growth of biofilm by *S. aureus* and *P. aeruginosa* was monitored after staining with acridine orange in terms of raw integrated density using fluorescence microscopy. During planktonic mode of growth, both 10 and 20% HF caused 10-fold decrease in growth of biofilm. Thus, 10% HF was used to evaluate its efficiency during biofilm mode of growth. The decrease in growth varied considerably under both the conditions. Biofilm formed under unchallenged conditions covered the whole glass surface (Fig. 2A) within 48 h of growth. A significant decline in growth of biofilm was observed in the presence of eye drops ($p<0.05$). A 3-fold decrease in *P. aeruginosa* biofilm growth was observed in the presence of PE. Decline in biofilm growth was more eminent with *S. aureus*. It showed a 4-fold decrease in biofilm growth in the presence of IE. Decline in biofilm growth was 3-fold, with PE and NPE (Fig. 2B). The decrease in biofilm growth in response to HE differed in both strains. The response to herbal components also varied between two bacterial cultures. El-Ganiny *et al.*[6] also reported that the effect of herbal extracts on formation of biofilm by *P. aeruginosa* and *S. aureus* significantly differed. In their study, plant extracts were able to remove biofilm

formed by *S. aureus* on contact lenses surface, but the same extract could only weaken *P. aeruginosa* biofilm.

Effect on surface topology

The use of eye drop formulations in the present study explains construction of biofilm with different structural and surface topologies. The parameters estimated for these changes were 3 D surface plot and plot profile using Image J (Fig. 3). Both parameters varied significantly in the presence of different formulations. Culture of *P. aeruginosa* grows uniformly over the glass surface in the absence of any HF, whereas in the presence of IE, growth decreased,

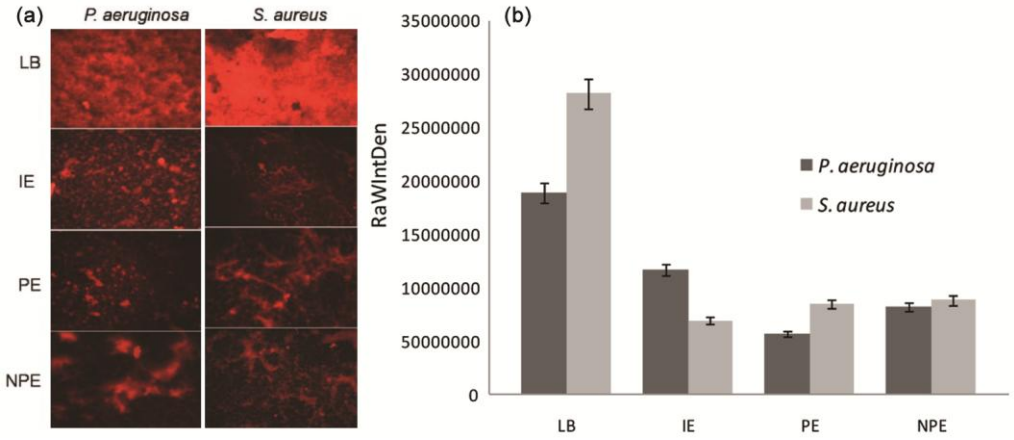

Fig. 2 — Microscopic changes in biofilm growth by HE (10X) (A) Fluorescence microscope images of biofilm (B) Biofilm growth expressed as raw integrated density of acridine orange bound to cells within biofilm.

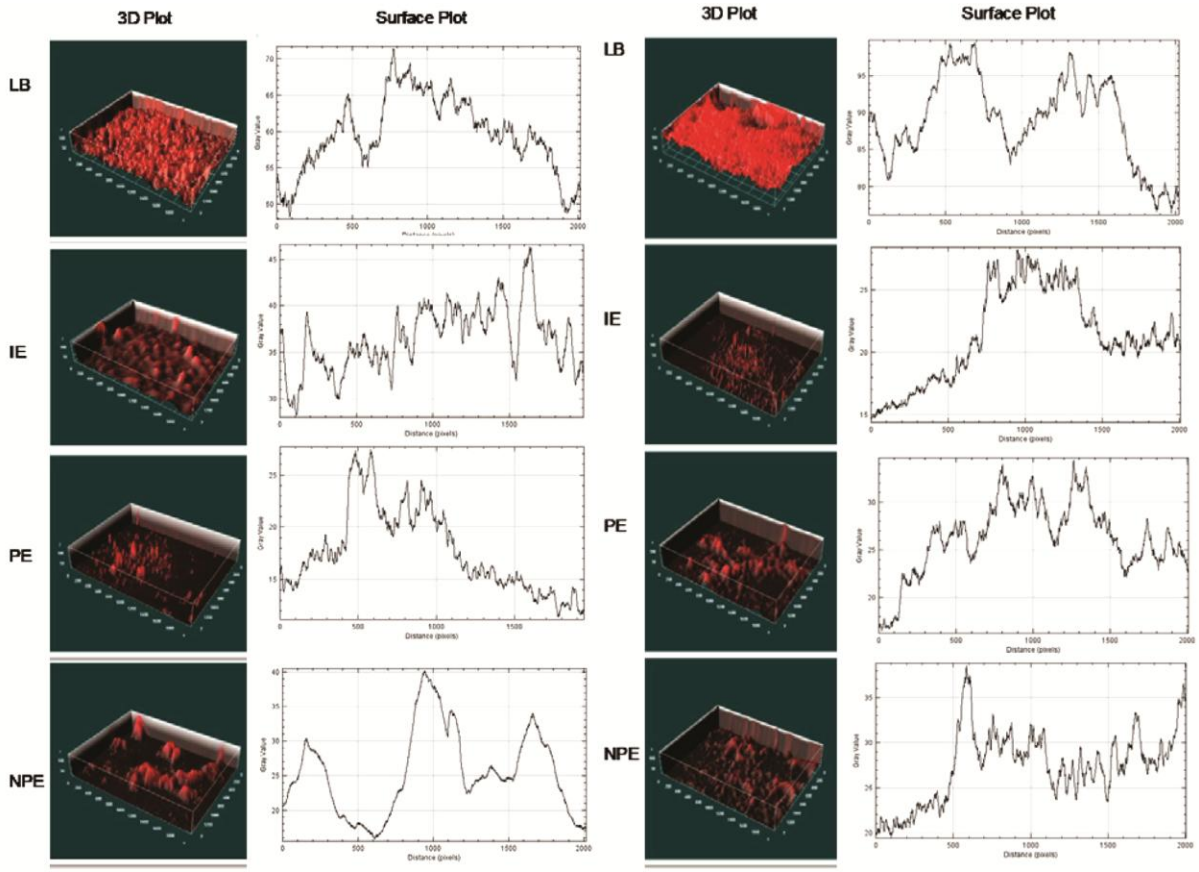

Fig. 3 — Surface architecture of biofilm in the presence of HE constructed using Image J (A) *P. aeruginosa* (B) *S. aureus*

but without significant effects on the surface topology of the biofilm. In the presence of both NPE and PE, coverage of biofilm decreased. The surface plot of the biofilm showed that it grows as a cluster confined to a small area, when NPE or PE were present (Fig. 3A).

The response to HF by *S. aureus* in biofilm mode of growth was significantly different from that by *P. aeruginosa*. The biofilm growth of *S. aureus* decreased in the presence of HF, but cell attachment to the surface and topological changes were consistent. It was also noted that test HF affected the thickness (Fig. 3B) of the film. Cluster growth was not observed for *S. aureus* biofilm in the presence of any HF. The findings indicate that the response to HF and resulting changes in biofilm structures differ considerably in both the strains studied.

The 3D surface plot of the biofilm was constructed to analyze the surface architecture. For both the strains, biofilms showed full surface coverage in the absence of any test eye drop. Mat-like growth with several microcolonies was normally observed, but in the presence of eye drop, changes in surface coverage and features of microcolony were evident. These findings indicate that at 10% concentration of HE, biofilm growth was not fully inhibited, but showed a significant decline in growth. The HE also restricted surface area covered by the biofilm.

Resistance to common antibiotics remains a major cause of treatment failure in the process of controlling infections and diseases. The herbal medicines contain a wide repertoire of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids; that dictate the therapeutic potency of the herbs as antimicrobial. Studies[26] supported antibacterial properties of phenolic compounds on *P. aeruginosa* by inhibiting *β*-lactamase activity and biofilm formation. Polyphenols from plants inhibit nucleic acid biosynthesis, enzymes and have anti-adhesive properties, which also prevents bacterial cell adhesion to the surfaces[27-29]. Plant extracts are effective in regulating virulence factors like swimming, swarming motility, synthesis of exopolysaccharides and formation of biofilm among pathogenic bacteria[30,31].

Honey is the common component in all eye drops tested. It is a natural compound effective in the treatment of corneal ulcer. Honey also exhibits antibacterial activity against ocular isolates and hence remained the most preferred natural product for ocular care[6]. Honey is also effective in inhibiting formation of biofilm by *P. aeruginosa* and *S. aureus*[32]. The component(s) commonly present in the most commercially available herbal eye drops are reported to inhibit bacterial growth as a single ingredient or in combination. Conversely, their quality of inhibiting biofilm is not well documented. The current study put forward an approach to evaluate the anti-biofilm properties of herbal formulations aiming at planning successful treatment regime.

Conclusions

The herbal formulations are effective in inhibiting growth of bacteria involved in eye infection. These herbs also retard bacterial attachment to the surface and alter biofilm topology. Phytochemicals present in plants can be a safe and preeminent alternative and additive to synthetic drugs. The response to antimicrobial agents differs in planktonic and biofilm mode of growth. The stage of growth should be the matter of concern while formulating and recommending the dosage. Thus, for planning successful treatment against any bacterial infection, anti-biofilm repertoire of the agent should also be taken into consideration.

Acknowledgement

Authors acknowledge Dr Pradeep Nawal, Synergy Micropath Lab- Ahmedabad for sharing the bacterial strains.

Conflict of interests

The authors declare that they have no competing interests. The study makes no relevance to demonstrate comparative therapeutic efficacy of any brand.

Author Contributions

N A K and K R designed the experiments. K R and N A K carried out experiments and analysis. B N Y, K R and N A K contributed towards interpretation of results and manuscript writing.

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