Journal of Ayurveda & Holistic Medicine

https://jahm.co.in eISSN-2321-1563

ORA- Experimental Research

ANTIBIOFILM ACTIVITY OF *KASHAYA UPAKRAMA* (*SHASTI UPAKRAMA)*: AN EXPLORATORY STUDY ON COMMON WOUND ISOLATES.

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ABSTRACT :

Background: Wound healing management is a significant concern for uneventful healing preventing infection in surgery. *Vrana* and its *chikitsa* have received considerable attention from *Acharya Sushruta*. In *Shashti upakramas*, he described various formulations of *Vrana Shodhana* and *Vrana Ropana*. Complex microbial communities known as biofilms are encased in a self-produced biopolymer matrix that offers defence against host defence mechanisms and antimicrobial agents. **Objectives:** A major contributing factor to delayed wound healing is formation of biofilms, and in order to advance the evidence-based practice of *Shasti Upakrama* with reference to *Kashaya Upakrama*, biofilm experimental studies that are clinically relevant are required. **Material and Methods:** The current analysis employed strains of *Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus aureus* microorganisms which were collected by microbial type cultures then strains were maintained in laboratory using BHI **(**Brain heart infusion) media. *Pentabark Kashaya* was diluted in 10 serial concentrations to check the MIC & MBC concentrations. The acquired concentrations (MIC & MBC) were examined for exopolysaccharide and morphology of multi-species biofilm made up of *all 3 microbes* by fluorescence microscopy. **Results:** MIC values in this investigation were 6.25 µg/ml for *P.aerugenosa*, 12.5 µg/ml for *S. aureus*, and 12.5 µg/ml for *E. coli* and MBC values against the strains varied between 25 and 6.25 µg/ml. Both MIC and MBC concentrations showed time-dependent suppression of bacterial growth, with a sharp decline within 10 hours. According to the biofilm reduction experiment, *Pentabark Kashaya's* MIC and MBC concentrations considerably decreased the proportion of attached microbes to cells by 53.3% – 63.5%. This was further supported by fluorescence microscopy. **Conclusion:** *Pentabark Kashaya* – Ayurvedic Herbo-Mineral Formulations has shown anti-bacterial activities in both concentration inhibition by MIC and MBC, time inhibition by time kill assay. It has shown significant antibiofilm activity on commonly found wound microbes which were confirmed by fluorescence assay.

KEYWORDS: Antibiofilm activity, *Pentabark Kashaya*, Chronic Wound, *Shasti Upakrama* , *Kashaya Upakarma*

RECEIVED ON: 24-04-2025

REVISED ON: 04-05-2025,12-05-2025

ACCEPTED ON: 18-05-2025

Access This Article Online:

Quick Response Code:

Website Link: https://jahm.co.in

DOI Link: https://doi.org/10.70066/jahm.v13i4.1828

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CITE THIS ARTICLE AS

Srujana P, Pradeep. S Shindhe, Vijay M. Kumbar, Sayali Manjarekar. Antibiofilm Activity Of *Kashaya Upakrama* (*Shasti Upakrama*): An Exploratory Study On Common Wound Isolates. *J of Ayurveda and Hol Med (JAHM).* 2025;13(4):47-59.

1. INTRODUCTION–

Any interruption of the continuity of any bodily tissues, such as the skin, mucous membrane, or cornea, is referred to as a wound [1]. Since surgery necessitates uneventful healing, wound healing is a significant concern. Acute and chronic wound prevalence were 10.55 and 4.48, respectively, while the overall prevalence of wounds in the thousand populations was 15.03 [2].

Acharya Sushrutha, the father of surgery has mentioned wound management in the 1st chapter of *chikitsa Sthana* by explaining *Shasti upakarmas* [3]. They can be divided into first 12 *upakarmas* for *vranashopa,* next 8 *upakarma* as *pradhana shastra karmas*, and the rest can be divided into *paschat karma* like *Vranashodhana, Vranaropana* and *Vaikrutapaha* [4].Among these *Shasti upakarmas* few *upakarmas* like *kashaya, varti, kalka, gritha, taila, avachurnana, rasakriya and vranadhopana* has been told for *varna shodana* [5]. The drugs mentioned for *vrana shodana* are said to have *Krimighna* properties which may be correlated to antibacterial and antiseptic properties.

Previous studies have mentioned development of *Panchavalkaladi Kashaya* by mixing *Panchavalkal Kashaya* with definite proportions of *Shodhita Tutta, Spatika Kasisa* and by adding preservatives in appropriate proportions. The product was then manufactured by GMP certified KLE Ayurveda Pharmacy as *Pentabark Kashaya*. It is stable at room temperature for 12 months. In vitro antimicrobial study showed *Panchavalkaladi Kashaya* has the antibacterial activity against all the 3 test organisms' i.e. *S.aureus, Pseudomonas,* and *E coli*. Both in disc diffusion method and MIC. In vivo dermal toxicity was not observed so the drug is safe for application. *Panchavalkaladi Kashaya* has shown a very significant result in *Vranashodhana* and *vranaropana* effect in post-operative anorectal wounds. *Panchavalkaladi Kashaya* has Tannin, Alkaloids, Saponins which has shown significant result in various factors: *Vedana* (pain), Discharge reduction *(Srava*), Early promoting wound contraction rate (*vranaropana*), Antimicrobial activity (*vrana shodhana*)[6].

Sequential steps in the complex and dynamic process of wound healing are induction of the inflammatory process, regeneration, migration and proliferation of parenchymal tissue, extracellular matrix development, tissue remodeling, gaining wound strength, and clearly marked signs of healing within two to four weeks. Chronic wounds, on the other hand, do not heal in the same order and frequently become "stalled" in one phase. They also do not show signs of recovery beyond four weeks. Bacterial contamination and subsequent biofilm formation are characteristics of the majority of chronic wounds [7].

Complex microbial populations known as biofilms are shielded from antimicrobial agents and host defence mechanisms by a self-produced biopolymer matrix [8]. The development of the evidence-based practice of *Shasti Upakrama* with reference to *Kashaya Upakrama* requires clinically relevant biofilm experimental research, as biofilms are a significant contribution to delayed wound healing.

Hence this experimental study was conducted to investigate the antibacterial and antibiofilm activity of *Pentabark Kashaya* by bacterial adhesion, reduction assay and further confirmed by florescence microscopy.

2. MATERIALS AND METHODS

MATERIALS:

Pentabark Kashaya was procured from GMP certified KLE Ayurveda Pharmacy Belgaum.

In-vitro Study was carried out in Dr. Prabhakar Kore Basic Science Research Centre, KLE Academy of Higher Education and Research (KLE University), Belagavi 590010 after securing the ethical approval number BMK/21/PG/ST/5.

Staphylococcus aureus (MTCC No.96), *E Coli* - (MTCC No.443), *Pseudomonas aeruginosa* (MTCC No.741), BHI **(**Brain heart infusion) Hi media, Catalog number: 70192, Micro-centrifuge tubes (sterile), Incubators, Micropipette (10-1,000 µl of volume), Centrifuge tubes and machine were used.

METHODOLOGY:

Antimicrobial activity:

1) MIC Test

For MIC, each drug must be diluted nine times using BHI. In the first tube, 380 µl of BHI broth were mixed with 20 µl of trail drug. For dilutions, each of the next nine tubes were transferred with 200 µl of BHI broth. 200 µl of BHI broth was transferred from the original tube to the first tube. It was thought that the dilution was 10^{-1}. To create a 10^{-2} dilution, 200 µl from the 10^{-1} diluted tube was transferred to the second tube. Up to a 10^{-9} dilution, the serial dilution was performed. Two millilitres of brain heart infusion (BHI) broth were mixed with five microliters of the required organisms from maintained stock cultures. Each serially diluted tube received two hundred microliters of the previously mentioned culture suspension. Following a 24-hour incubation period, the tubes were checked for turbidity [9].

2) MBC Test

After plating each MIC dilution tube and incubating them for a full day, the colony count was calculated the next day. To ascertain whether the control drug has a bactericidal effect on the organism, MBC is utilized. In the absence of growth, it exerts a bactericidal effect [9].

3) Time kill assay

In accordance with CLSI recommendations, a time-kill assay investigation was carried out using the broth microdilution method. Bacterial suspension was incubated in microcentrifuge tubes containing the test material's control, MIC, and MBC values. Aliquots of the aforementioned mixture were serially diluted (down to 10^{-4}) and taken at various intervals (0, 6, 12, 24, and 48 hours). Coated on the plate of blood agar, colony counter was implemented to count the colonies after 48 hours of incubation, and the log10 CFU (colony forming units) vs. time was used to display the time-kill curve [10].

Antibiofilm activity:

1) Microtiter bacterial adhesion assay.

Microbes were anaerobically grown in BHI broth for 48 hours. MIC, MBC, 1.5 MIC, and 0.5 MIC concentrations of *Pentabark Kashaya* were added to a mixture of bacterial broth in 96-well fat-bottom microtiter plates. *Pentabark Kashaya* anaerobic incubation was carried out throughout the entire night at 37 °C. To get rid of unbound bacteria, the culture supernatant was taken out after a 12-hour incubation period and washed with the 7.2 pH phosphate-bufered saline (PBS) from Dulbecco. After 15 minutes of methanol fixation, the attached bacteria were allowed to air dry. After five minutes of staining the bound bacteria with crystal violet (0.1% w/v), the unbound dye was rinsed off with distilled water. Following a 95% (v/v) ethanol dissolution of the attached dye, the microtiter plate was left to air dry [11].

2) Biofilm reduction assay

In brief, each well was inoculated with bacteria that had been aerobically cultured for the entire night with 0.5 concentration of McFarland using BHI broth. 200 µl of sterile distilled water was added to the peripheral wells in order to minimize water loss. The plates were maintained at 37 °C for 72 hours in an aerobic atmosphere to give the bacteria time to grow and create the biofilms on the bottom of the well. The MIC and MBC concentrations of the test drug in culture media and culture medium alone (control) were then added to each well. The incubation was continued for a further twenty-four hours under the identical conditions. The supernatant was disposed of after 24 hours, and PBS was used to rinse the wells. After a fifteen-minute methanol fixation period, the samples were stained for five minutes with crystal violet (0.1% w/v) and then rinsed with distilled water. After the crystal violet was dissolved in ethanol, the samples' O.D. at 570 nm was measured using a microplate reader [11].

3) Fluorescence microscopy imaging

A bacterial cell suspension was cultivated overnight on sterile coverslips using BHI broth and then cultured in a 12-well microtiter plate (1200 µl per well). The plate was maintained at 37 °C in an aerobic condition for 72 hours in order to give the bacteria time to grow and create biofilms on the coverslips. The MIC and MBC concentrations of the test substance were added to each well following a 72-hour incubation period, and a control of material-deficient growth medium containing bacterial culture was used. The plate was maintained at 37 °C in the aerobic chamber for 24 hours. Following the careful removal of the culture supernatant, the coverslips were fixed with 4% paraformaldehyde for 30 minutes before being washed with PBS. The coverslips were then stained for 30 minutes in the dark using a solution of acridine orange (AO) and ethidium bromide (ETBR) (5 µg ml−each). The coverslips were cleansed with distilled water, and the biofilms were examined at 40 × magnification using a fluorescence microscope (Olympus BX41, New York, USA). ProRes ® Capture Pro software (Jena, Germany) was used to take the pictures, and Image J software was used to compare the fluorescence intensity of the treated and untreated groups [11].

3. RESULTS

Antimicrobial activity of *Pentabark Kashaya*:

The conventional broth dilution method was used to test *Pentabark Kashaya's* antibacterial properties against Staphylococcus aureus (MTCC No.96), *E. coli* (MTCC No.443), and Pseudomonas aeruginosa (MTCC No -741). According to Table No I, *Pentabark Kashaya* exhibited strong inhibitory action against Pseudomonas aeruginosa, *E. coli*, and Staphylococcus aureus. *Pentabark Kashaya*'s minimum inhibitory concentration (MIC) against the strains varied between **6.25 and 12.5 µg/m1**.

Table No. I-Antimicrobial activity of Test Samples

Organism	MIC(µl/ml)	MBC(µl/ml)
E. coli	12.5±0	25±0
S. aureus	12.5±0	25±0
P. aeruginosa	6.25±0	6.25±0

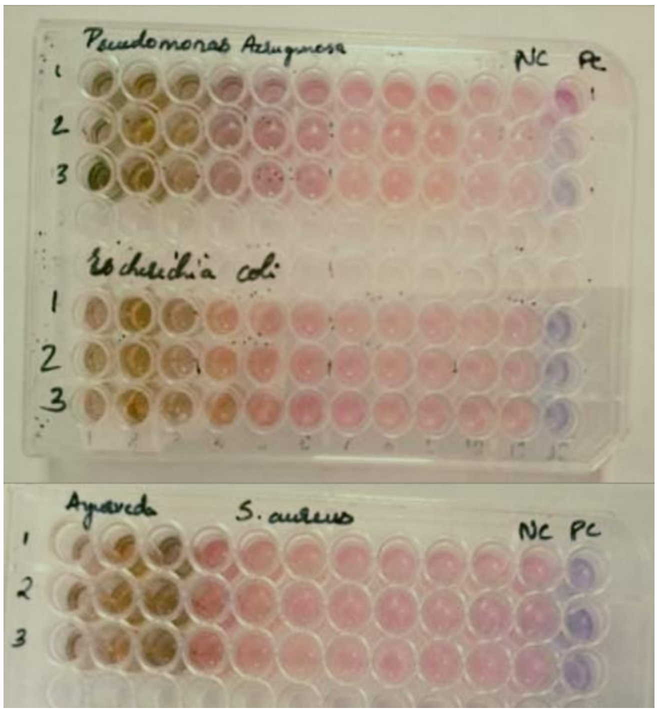


Image 1: Microtiter plate reading MIC.

Microtiter plate reading showing MIC of *Pentabark Kashaya* against *P aeruginosa, E Coli* and *S aureus* respectively.

In comparison to the initial inoculum, MBC is the lowest concentration at which 99.9% of the viable organisms are temporarily dead (Image 2). *Pentabark Kashaya*'s MBC values against the bacteria varied between **25 and 6.25 µg ml−1**.

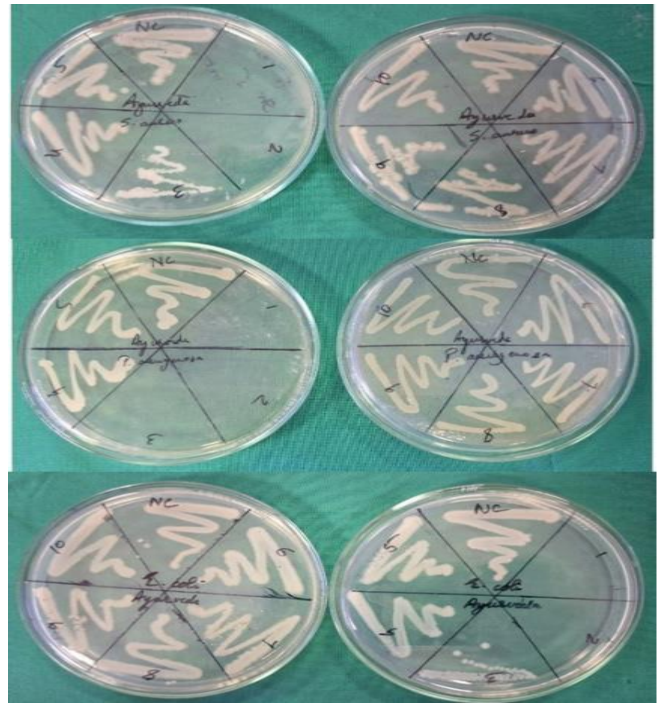
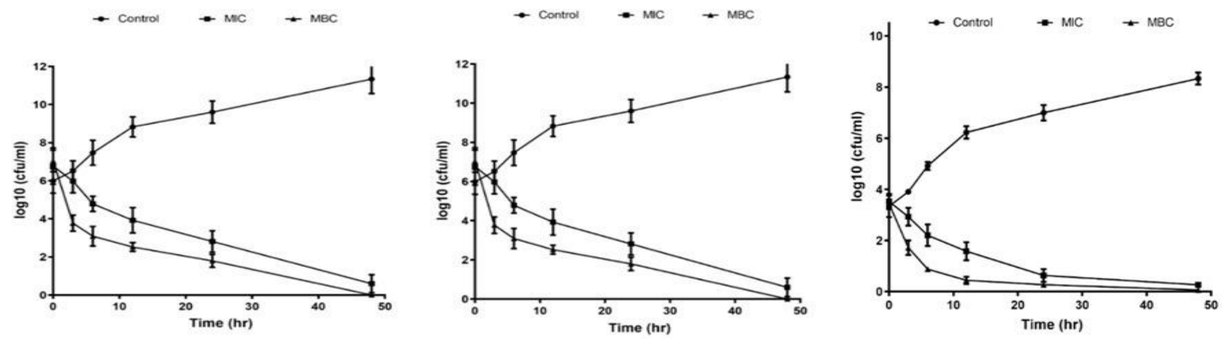


Image 2: Petri plates showing MBC values.

Petri plates showing MBC values of *Pentabark Kashaya* against *S. aureus, P.aeruginosa* and *E Coli* respectively.

Time kill assay:

Antibacterial effectiveness of *Pentabark Kashaya* against *Pseudomonas aeruginosa*, *E. coli*, and *Staphylococcus aureus* was further evaluated using the time-kill experiment. The bacteria were exposed to *Pentabark Kashaya*'s MIC and MBC concentrations for varying lengths of time prior to this test. At MIC concentration, bacterial growth was demonstrated to be time-dependently inhibited, whereas at MBC concentration, 100% suppression was attained in 48 hours [Graph No.1].



Graph 1:Time kill assay

Time kill assay of *E.coli, Staphylococcus aureus* and *P. aeruginosa* respectively.

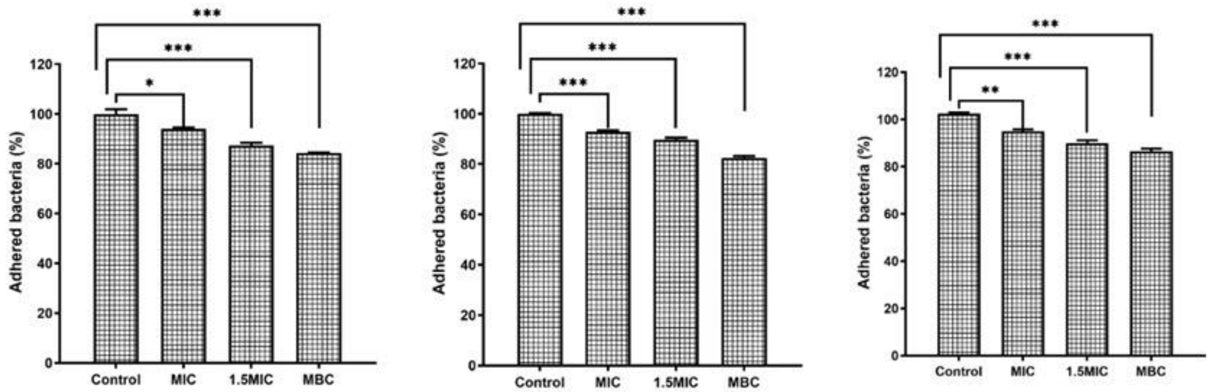
Anti-biofilm activities:

Microtiter bacterial adhesion assay:

The anti-adhesion activity of *Pentabark Kashaya* on the adhesion of *S. aureus, P. aeruginosa*, and *E. coli* was investigated [Table No II]. *Pentabark Kashaya's* MIC, 1.5 MIC, and MBC concentrations significantly reduced the adhesion of all the 3 microbes. [Graph No. 2].

Table No. II: Bacterial adhesion assay of *E. coli, S. aureus* and *P. aeruginosa.*

E. coli				
Concentration µl/ml	Adhered bacteria %			Mean ± SD of AB s %
NC	99.381	100.825	99.794	100.000 ± 0.734
MIC	92.165	97.629	95.258	95.017 ± 2.740
1.5 MIC	73.402	73.505	71.856	72.921 ± 0.924
MBC	52.371	53.299	54.330	53.333 ± 0.980
S. aureus				
Concentration µl/ml	Adhered bacteria %			Mean ± SD of AB s %
NC	106.55	95.38	98.07	100.00 ± 0.734
MIC	76.57	76.91	77.58	77.02 ± 0.51
1.5 MIC	70.61	68.85	68.60	69.35 ± 1.10
MBC	67.09	62.38	61.04	63.50 ± 3.17
P. aeruginosa				
Concentration µl/ml	Adhered bacteria %			Mean ± SD of AB s %
NC	106.549	95.382	98.069	100.00 ± 0.734
MIC	76.574	76.910	77.582	77.02 ± 0.51
1.5 MIC	70.613	68.850	68.598	69.35 ± 1.10
MBC	67.086	62.385	61.041	63.50 ± 3.17



Graph 2: Bacterial adhesion assay.

***Pentabark* Concentration (µl/ml)**

Bacterial adhesion assay of *E. Coli*, *S. aureus* and *P. aeruginosa*.

Biofilm reduction assay results:

Pentabark Kashaya's MIC, 1.5 MIC, and MBC concentrations significantly decreased the number of attached *S. aureus, P. aeruginosa*, and *E. coli* cells by up to 53.95% (p<0.001), 66.78% (p<0.001), and 66.78% (p<0.001), respectively, according to the biofilm reduction assay [Table No. III] [Graph No. 3].

Table No. 3: Biofilm reduction assay of *S. aureus, P. aeruginosa and E. coli.*

E. coli				
Concentration µl/ml	Surviving Cells %			Mean ± SD of SCs %
NC	99.381	100.825	99.794	100.000 ± 0.734
MIC	92.165	97.629	95.258	95.017 ± 2.740
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Concentration µl/ml	Surviving Cells %			Mean ± SD of SCs %
NC	106.55	95.38	98.07	100.000 ± 0.734
MIC	76.57	76.91	77.58	77.02 ± 0.51
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MBC	67.09	62.38	61.04	63.50 ± 3.17
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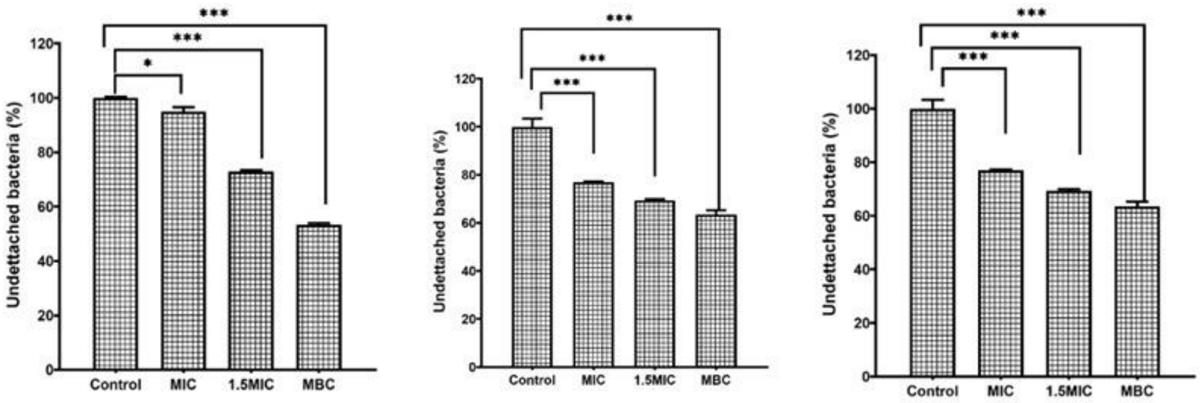
MBC	67.086	62.385	61.041	63.50 ± 3.17



Graph 3: Biofilm reduction assay.

Pentabark **Concentration (µl/ml)**

Biofilm reduction assay of *E. Coli*, *S. aureus* and *P. aeruginosa* respectively.

Fluorescence microscopy imaging:

The impact of *Pentabak Kashaya* on the produced biofilms was further validated by means of fluorescence microscopy and the live-dead staining technique. Image 3-5 shows the fluorescence microscopic images of the control samples (biofilms that were not treated) and the biofilm samples treated with *Pentabak kashaya* at both the MIC and MBC values. The biofilm treated with both MIC and MBC concentrations of *Pentabak kashaya* had red color, indicating considerable bacterial mortality, whereas the control samples were green. To determine the fluorescence intensity, ImageJ software was used to process the fluorescence microscopic images obtained during live dead staining. The fluorescence intensity of the control sample was measured at 100% in order to compare it to the fluorescence intensity of the other sample photographs. In comparison to the control, the percentage of live bacteria in the biofilms treated with *Pentabak kashaya's* MIC and MBC concentrations was 37.13% and 22.22% for ***E. coli***, 31.31% and 20.68% for ***S. aureus***, and 48.61% and 32.58% for ***P. aeruginosa***, respectively. [Table No. IV].

Table IV: Fluorescence assay of *S. aureus, P. aeruginosa and E. coli.*

E. coli				
Concentration µl/ml	Surviving Cells %			Mean ± SD of SCs %
NC	50.37	50.37	50.37	50.40133 ± 1.8372
MIC	36.465	36.465	36.465	37.13433 ± 1.995065
MBC	22.476	22.476	22.476	22.221 ± 1.645387
S. aureus				
Concentration µl/ml	Surviving Cells %			Mean ± SD of SCs %
NC	39.474	39.474	39.474	38.098 ± 1.588248
MIC	33.036	33.036	33.036	31.31867 ± 1.792185
MBC	22.159	22.159	22.159	20.685 ± 1.375373

P. aeruginosa				
Concentration µl/ml	Surviving Cells %			Mean ± SD of SCs %
NC	60.386	60.386	60.386	58.40533 ± 1.958394
MIC	50.081	50.081	50.081	48.61333 ± 1.408755
MBC	34.842	34.842	34.842	32.585 ± 2.19854

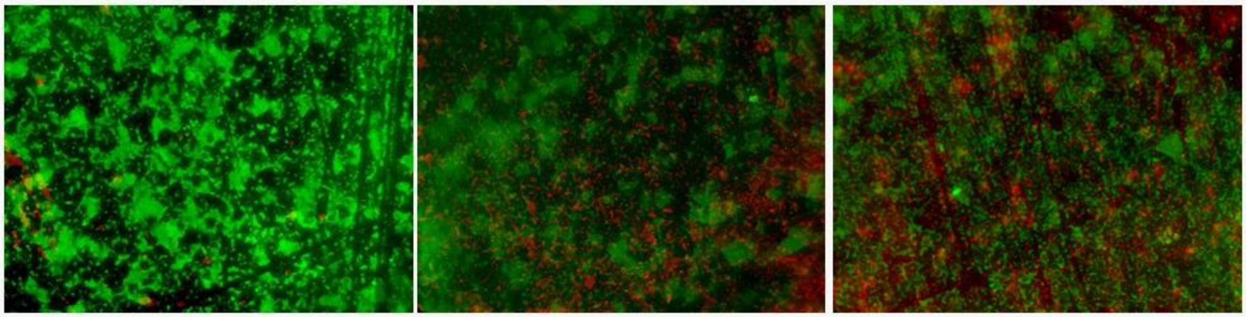


Image 3: Fluorescence E. coli biofilm.

The fluorescence after being incubated for 24 hours, a biofilm of *E. coli* developed on the glass slide surface, as seen in microscopic pictures. Biofilm that was left untreated (control), treated with *Pentabark* at a MIC concentration, and treated with *Pentabark* at an MBC concentration. ***p indicates a significant difference.

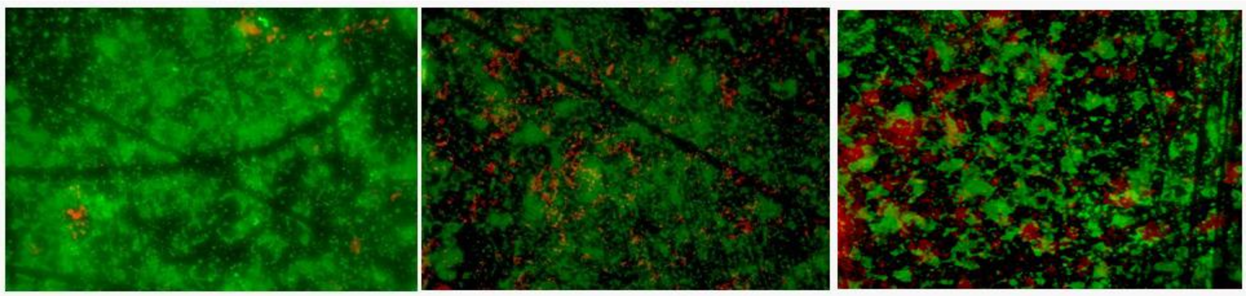


Image 4: Fluorescence S. aureus biofilm.

The fluorescence after being incubated for 24 hours, a biofilm of *S.aureus* developed on the glass slide surface, as seen in microscopic pictures. Biofilm that was left untreated (control), treated with *Pentabark* at a MIC concentration, and treated with *Pentabark* at an MBC concentration. ***p indicates a significant difference.

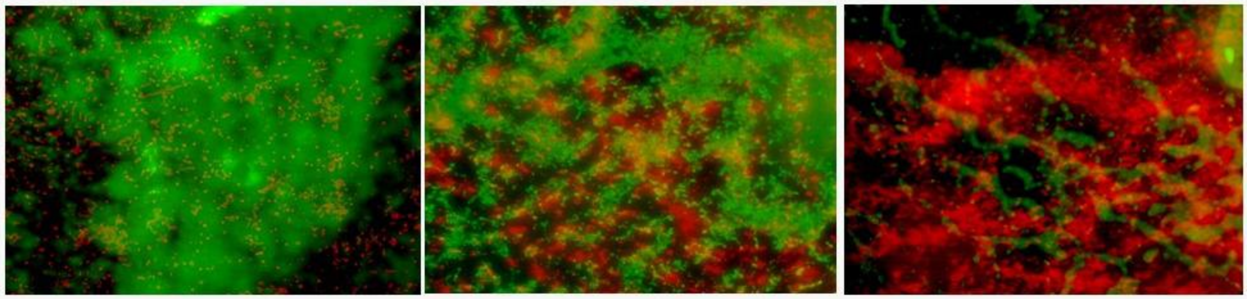


Image 5: Fluorescence P.aeruginosa biofilm.

The fluorescence after being incubated for 24 hours, a biofilm of ***P. aeruginosa*** developed on the glass slide surface, as seen in microscopic pictures. Biofilm that was left untreated (control), treated with *Pentabark* at a MIC concentration, and treated with *Pentabark* at an MBC concentration. ***p indicates a significant difference.

4. DISCUSSION

Wound healing is a complex process. Prevention of secondary infection and biofilm formation in the wounds will enhance wound healing process. In *shasti upakarma acharya sushrutha* have explained *upkarmas* like *kashaya, varti, taila* are explained immediately after *Asta vidha shastra kramas* which itself depicts for any *vrana upakrama* first line of management is *vrana shodhana* and the durgs mentioned for *these upakramas (kashaya, kalka, upnaha, utkarika* etc.) are having *kashaya, tikta, katu* rasa predominant drugs with *krimigna* and *vrana shodhana* propeties. There are several preparations available in *Ayurveda* classics which are exhibiting antibacterial, anti-fungal, antimicrobial and anti-inflammatory etc activities in compound preparation. Among such preparations *Panchavalkal Kashaya (Pentabark Kashaya)* is one of the preparatory medicines which is frequently used for the management of *Dusta Vrana* in the form of *Vrana Prakshalana.*

Previous series of studies have already established that *Pentabark Kashaya* is safe with no acute dermal toxicity, with stability of 12 months, has antibacterial activity and has phytochemicals like Tannin, Alkaloids, Saponins which has shown significant result in various factors like *Vedana* (pain), Discharge reduction (*Srava*), Early promoting wound contraction rate (*vranaropana*). *Pentabark Kashaya* has shown a very significant result in *Vranashodhana* and *vranaropana* effect in vivo study and as well as in clinical trial of post- operative anorectal wounds [12, 6].

To prove any drug or a compound has bactericidal / bacteriostatic or antibiofilm activity there are many tests to undergo like viable counting, after pegs are sonicated, vortexed, examination by confocal laser scanning microscopy among that most commonly used tests are bacterial adhesion assay, biofilm reduction assay, and further confirmation by florescence microscopy. Hence these 3 tests for antibiofilm activities were selected for this study.

Polymicrobial populations populate biofilm development in the majority of chronic wounds, causing widespread inflammation and delaying wound healing [13]. Gram-negative bacteria, which make up 61% of all microbial isolates, are typically the colonizing species in chronic wounds. In chronic infections, *S. aureus and P. aeruginosa* are the most often detected wound pathogens. The other harmful microbes are *Klebsiella pneumoniae, A. baumanni, E. coli, and Proteus mirabilis* [14]. Therefore, *Pentbark Kashaya's* antibiofilm properties were tested against the three most prevalent wound microbes: *S. aureus, P. aeruginosa, and E. coli*. To find out more about *Pentabark Kashaya*'s antibacterial properties in relation to the Time Kill assay, time-dependent suppression of bacterial growth utilizing the broth microdilution method and the MIC and MBC methods' concentration-dependent inhibition of bacterial growth.

The MIC and MBC values, which demonstrate concentration-dependent inhibition of *S. aureus, P. aeruginosa*, and *E. coli* bacterial growth, demonstrate *Pentabark Kashaya's* antibacterial activity. The MIC values of *Pentabark Kashaya* against *S. aureus, P. aeruginosa*, and *E. coli* are not the same as those from the previous investigation. MIC values of 50 µg/ml against *P. aerugenosa*, 0.8 µg/ml against *S. aureus*, and 3.12 µg/ml against *E. coli* were noted by Manjula et al. [12]. The MIC values for *P. aerugenosa*, *S. aureus*, and *E. coli* in this investigation were 6.25 µg/ml, 12.5 µg/ml, and 12.5 µg/ml, respectively [12]. Although the results of both studies show that *Pentabark Kashaya* has antibacterial activity, the variation in MIC values could be caused by variations in the drug's resources, purity, and relative percentage of phytochemical constituents, or by variations in the standard evaluation procedure used in various labs.

The perpetual relationship between antimicrobial drugs and bacteria strains is illustrated by the time-kill test [10]. The time-kill test indicates whether the antibacterial effect is concentration- or time-dependent. According to the results of this time-kill assay, *Pentabark Kashaya* has bactericidal qualities against *S. aureus, P. aeruginosa*, and *E. coli* at MIC and MBC concentrations. Because of *Panchavalkala, Kasisa, Tutta*, and *Spatika* have antibacterial qualities, the bacterial inhibition increases as the incubation period and *Pentabark Kashaya* concentration increased. As it shows presence of tannins, alkaloids, saponins as a phytochemical which are known to have anti-inflammatory, astringent, and antimicrobial activities[15]. *Shodhita Tutta* has antibacterial activity on *E. coli, S. aureus*[16]. *Spatika* (Potash alum) has bacteriostatic action with MIC of 2% conc [17].

Bacterial adhesion is one of the initial steps in biofilm formation. The interaction between the human matrix proteins with bacterial surface, by a specific protein to protein interaction helps in the attachment of bacteria [18,19]. The presence of tannins, alkaloids, and saponins, which have better antibacterial qualities of *Pentabark Kashaya*, may be the cause of the notable decrease in bacterial adhesion of *S. aureus, P. aeruginosa*, and *E. coli* at MIC and MBC concentrations.

Antibiotic penetration was hampered by the bacterial cells that were trapped in the biofilm's exopolymer matrix. *Pentabark Kashaya* significantly inhibited the pre-formed *S. aureus, P. aeruginosa,* and *E. coli* at MIC and MBC concentrations in a dose-dependent manner.

Fluorescence microscopy imaging using fluorescent dyes such ethidium bromide and acridine orange further validated the biofilm inhibition of *S. aureus, P. aeruginosa*, and *E. coli.* Acridine orange displays green fluorescence when it moves through healthy cells with intact cell membranes, but ethidium bromide only produces red fluorescence when it moves through dead cells [10]. Strong antibiofilm and bactericidal qualities are suggested by *Pentabark Kashaya's* ability to decrease the number of live bacteria at MIC and MBC concentrations. *Pentabark Kashaya* is therefore a useful tool for managing the biofilm of *S. aureus, P. aeruginosa*, and *E. coli.*

5. CONCLUSION

Pentabark Kashaya has shown antibacterial activities in both concentration inhibition by MIC and MBC, time inhibition by time kill assay. *Pentabark Kashaya* has shown significant antibiofilm activity on commonly found wound pathogens i.e *S. aureus, P. aeruginosa* and *E. coli* and confirmed by fluorescence assay an evidence-based *Kashya Upakarma* among *Shasti Upakarma.*

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Reviewing & editing- Dr. SP, Dr. PSS

Approval of final manuscript- All Authors

Conflict of interest: None

Source of support: None

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