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RESEARCH ARTICLE

IN VITRO EVALUATION OF ANTI-BIOFILM POTENTIAL OF ALUM AND ITS *BHASMA* (*SPHATIKA BHASMA*)

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Abstract

Microbial biofilms may form on wide variety of surfaces and are problematic in medical, industrial and environmental settings. Natural antibiofilm agents are promising candidates for treatment of biofilm-associated infections. One such natural products used for therapeutic purposes is Alum (*Sphatika*). Besides, Ayurvedic "*Bhasmas*" are used for treatment of various ailments in India. In the present *in vitro* study, anti-Biofilm potential of Alum as well as its *Bhasma- Sphatika Bhasma* was evaluated against total 8 bacteria, viz., 4 gram negative and 4 gram positive using crystal violet staining method. Antibiofilm activity of *Sphatika Bhasma* was noted in the order of *P. aeruginosa* > *S. lutea* > *E. coli* > *K. pneumoniae* > *S. typhi* > *MRSA* > *B. subtilis* > *E. faecalis*, whereas, Antibiofilm effect of Alum was found to be in the order of *S. lutea* > *P. aeruginosa* > *E. coli* > *MRSA* > *S. typhi* > *B. subtilis* > *K. pneumoniae* > *E. faecalis*. In general, *Sphatika Bhasma* exhibited better Anti-Biofilm activity than Alum. Furthermore, four different combinations prepared from Alum (AL), *Sphatika Bhasma* (SB) and Ciprofloxacin (CP)- standard antibiotic were evaluated for their combinatorial effect against 8 bacterial biofilms. In general, the combination (AL + SB) displayed the highest Anti-biofilm activity against all the 8 bacterial biofilms. Antibiofilm effects of Alum and *Sphatika Bhasma* could be attributed to their Flavonoid content. Moreover, to best of our knowledge, this is the first study revealing the anti-biofilm potential of *Sphatika Bhasma*.

Introduction:-

Biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping or natural aquatic systems. Characteristics of biofilm that can be important in infectious disease process include, (i) Detachment of cells or biofilm aggregates may result in bloodstream or urinary tract infections or in the production of emboli, (ii) Cells may exchange resistance plasmids

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with biofilms, (iii) Cells in biofilms have dramatically reduced susceptibility to antimicrobial agents, (iv) Biofilm-associated gram negative bacteria may produce endotoxins and (v) Biofilms are resistant to host immune system clearance (Donlan, 2002). Biofilm exists in more than 90% bacteria. Natural antibiofilm agents are promising candidates which could provide novel strategies for combating pathogenic bacteria and treatment of biofilm-associated infections (Lu *et al*., 2019). One such natural products used for therapeutic purposes for centuries is white Alum or Potash Alum (PA). Potassium Aluminium Sulphate (PAS) commonly referred to as "Potash Alum (PA)" or Alum is an efficient, safe, eco-friendly inorganic compound which is commercially available, cost effective and has various applications (Amadi, 2020). *Sphatika* or Alum comes from that category of drugs which is used internally as well as externally. *Sphatika* (Alum) is used from the ancient era as the best antiseptic and antimicrobial agent. Furthermore, pharmacological activities of Alum include anti-haemorrhagic, anti-cariogenic, anti-obesity, ulcer healing and larvicidal. In Ayurveda, it is used as an ingredient in various formulations and purification of water (Anand *et al*., 2019).

Besides, *Bhasmas* are the unique Ayurvedic metallic/mineral preparations, treated with herbal juices or decoction which are known in Indian subcontinent since 7^{th} century AD and are widely recommended for the treatment of a variety of chronic ailments. *Bhasmas* are claimed to be biologically produced nanoparticles and the concept of using nano-metal particles for treatment is prevailing since *Charaka Samhita* (Pal *et al*., 2014). *Bhasma* preparations involve conversion of the metal into its mixed oxides, during which the zero valent metal state is converted into a higher oxidation state. The significance of this "*Bhasmikarana*" is that the toxic nature of the resulting metal oxide is completely destroyed while introducing the medicinal properties into it (Tambekar and Dahikar, 2010; Sharma and Prajapati, 2016). Various *Bhasmas* have revealed antimicrobial potential in the past (Hebber *et al*., 2017; Mahapatra and Mahapatra, 2013; Prasanna *et al*., 2010; Shubha and Hiremath, 2010; Vaishagh *et al*., 2018).

Alum *bhasma* is called *Sphatika Bhasma* which is useful as *Kanthya* (for throat), as hair tonic, as *Vranashodhak* (cleanses wound), as *Vishaghna* (Anti-poisonous) and *Raktasthambak* (clots blood) (Dongre and Bhagat, 2015). However, antibiofilm activity of *Sphatika Bhasma* is not yet investigated. Likewise, reports regarding antibiofilm activity of Alum (*Sphatika*) are very scarce. Hence, in the present *in vitro* study, anti-biofilm activities of Alum as well as its *Bhasma- Sphatika Bhasma* were evaluated against total 8 bacteria, viz., 4 gram negative [*Escherichia coli* (ATCC-10148), *Klebsiella pneumoniae* (ATCC-700603), *Pseudomonas aeruginosa* (Fisher's Immuno Type IV), *Salmonella typhi* (NCTC-786)] and 4 gram positive [*Bacillus subtilis* (ATCC-9372), *Enterococcus faecalis* (ATCC-29212), Methicillin Resistant *Staphylococcus aureus* (*MRSA* ATCC-25923), *Sarcina lutea* (NCTC-2470)] using crystal violet staining assay.

Materials and Methods:-

1) **Procurement of Material**
   Alum was procured from the local market, whereas, *Sphatika Bhasma* was procured from local Ayurvedic shop in Mumbai.
2) **Preparation of Test Solutions**
   The test solutions were prepared by dissolving Alum and *Sphatika Bhasma* in sterile distilled water individually (Stock solution= 100 mg/ml each). The test solutions were solubilized by dipping the tubes in boiling water bath. The prepared solutions were stored at 4^0C until further use.
3) **Test Organisms used for Antibiofilm Assays**
   Anti-biofilm activity of Alum and *Sphatika Bhasma* was evaluated against *Escherichia coli* (ATCC-10148), *Klebsiella pneumoniae* (ATCC-700603), *Pseudomonas aeruginosa* (Fisher's Immuno Type IV), *Salmonella typhi* (NCTC-786), *Bacillus subtilis* (ATCC-9372), *Enterococcus faecalis* (ATCC-29212), Methicillin Resistant *Staphylococcus aureus* (*MRSA* ATCC-25923) and *Sarcina lutea* (NCTC-2470).
   Bacterial cultures were grown on Nutrient agar and suspended in Mueller Hinton Broth (MHB) for the assays.
4) **Anti-Biofilm Assay:**
   The effect of Alum and *Sphatika Bhasma* on bacterial biofilm formation was evaluated in sterile 96-well polystyrene flat-bottom microplates according to Sánchez *et al*. (Sánchez *et al*., 2016) with slight modification. Briefly, 200 μl of inoculated fresh Mueller Hinton Broth (final concentration 10^6 CFU/ml) was aliquoted in triplicate to respective wells of sterile microplate and cultured in presence of test concentrations (0.1, 1 & 10 mg/ml). Wells containing bacterial cultures with distilled water were used as controls. Ciprofloxacin was included as a standard antibiotic. Plates were sealed and incubated at 37^0C for 48h. After incubation, supernatant was removed and each well was washed thoroughly with sterile distilled water thrice to remove free-floating cells; thereafter plates were air-dried for 30 min and the biofilm formed was stained during 15

min at room temperature with 0.1% aqueous solution of crystal violet. Following incubation, the excess of stain was removed by washing the plate three times with sterile distilled water. Finally, the dye bound to the cells was solubilized by adding 250 μl of 95% ethanol to each well and after 15 min of incubation, absorbance was measured using Multimode Reader (Synergy HT, BioTek) at a wavelength of 570 nm. Effect on bacterial Biofilms was determined using the formula Percentage Inhibition = (Control − Test)/Control X 100, where Control is the OD_{570nm} of the stained Control wells containing distilled water and Test is the OD_{570nm} of the stained Test wells containing Alum or *Sphatika Bhasma* or Ciprofloxacin (standard) respectively. Experimental results were expressed as Mean for analysis performed in triplicate.

Note: The peripheral wells of the microplates were filled with sterile Distilled Water to avoid edge effect. Further, the sealed plates were placed in a tray and then kept in an incubator for the incubation to prevent loss of contents due to evaporation.

5) Effect of Before-treatment and After-Treatment on Bacterial Biofilms:

For before-treatment study, 100 µl of bacterial culture (10^6 CFU/ml) was added to the sterile microplate in triplicate and the plate was sealed and then incubated at 37^0C for 48 h. After incubation, the cultures were aspirated carefully and 100 µl of test solutions (10 mg/ml) were added to the plate and the sealed plate was incubated further at 37^0C for 24 h. After incubation, supernatant was removed and the effect of test solutions on bacterial biofilms was determined by Crystal Violet staining method as stated earlier. Ciprofloxacin (2 mg/ml) was included as a standard.

For after-treatment study, 100 µl of test solutions (10 mg/ml) were added to the sterile microplate in triplicate and the plate was sealed and then incubated at 37^0C for 24 h. After incubation, the test solutions were aspirated carefully and 100 µl of bacterial culture (10^6 CFU/ml) was added to the microplate and the sealed plate was further incubated at 37^0C for 48 h. After incubation, supernatant was removed and the effect of test solutions on bacterial biofilms was determined by Crystal Violet staining method as stated earlier. Ciprofloxacin (2 mg/ml) was included as a standard.

Note: The peripheral wells of the microplates were filled with sterile Distilled Water to avoid edge effect. Further, the sealed plates were placed in a tray and then kept in an incubator for the incubation to prevent loss of contents due to evaporation.

6) Combinatorial Effect on Bacterial Biofilms:

In the present study, Combinatorial effect of Alum (10 mg/ml), *Sphatika Bhasma* (10 mg/ml) and Ciprofloxacin- standard Antibiotic (2 mg/ml) was evaluated using Crystal Violet staining method as stated earlier. Following different Combinations were prepared in 1:1 ratio and their effects were tested against 8 bacterial biofilms.

1. Alum + *Sphatika Bhasma* (AL + SB)
2. Alum + Ciprofloxacin (AL + CP)
3. *Sphatika Bhasma* + Ciprofloxacin (SB + CP)
4. Alum + *Sphatika Bhasma* + Ciprofloxacin (AL + SB + CP)

For the said study, 100 µl of bacterial culture (10^6 CFU/ml) and 100 µl of different combination solutions were added to the sterile microplate in triplicate and the plate was sealed and then incubated at 37^0C for 48 h. After incubation, supernatant was removed and the effect of combination solutions on bacterial biofilms was determined by Crystal Violet staining method as stated earlier.

Note: The peripheral wells of the microplates were filled with sterile Distilled Water to avoid edge effect. Further, the sealed plates were placed in a tray and then kept in an incubator for the incubation to prevent loss of contents due to evaporation.

7) Flavonoids content estimation

The method of Oyedemi *et al*. (Oyedemi *et al*., 2011) with slight modification was used to estimate total flavonoids content of the Alum and *Sphatika Bhasma*. The method is based on formation of a complex flavonoids-aluminums. Briefly, a volume of 0.1 ml of 2% $AlCl_3$ ethanol solution was added to 0.1 ml of each test solution in a microplate. After one hour of incubation at room temperature, the absorbance was measured at 420 nm using Multimode Reader (Synergy HT, BioTek). Yellow colour indicated presence of flavonoids. Quercetin at various concentrations (10 to 100 µg/ml) was included as a standard. All the determinations were done in triplicate. Mean values of triplicate determinations were used to plot the graph. Total flavonoid content was calculated from the equation ($y= 0.0082x + 0.0512$, $R^2= 0.9883$) obtained from the Quercetin standard curve. Total flavonoids content was expressed as quercetin equivalent in milligrams per gram of dry sample. Experimental results were expressed as Mean for analysis performed in triplicate.

Results:-

Table 1:- Effect of Alum and *Sphatika Bhasma* on *E. coli* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	13.99	8.61
1	4.82	11.8
10	56.19	83.9

Note: Mean of triplicate determinations

Table 2:- Effect of Alum and *Sphatika Bhasma* on *K. pneumoniae* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	Nil	13.45
1	Nil	48.88
10	25.55	79.15

Note: Mean of triplicate determinations

Table 3:- Effect of Alum and *Sphatika Bhasma* on *P. aeruginosa* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	32.30	46.89
1	82.54	88.92
10	83.04	90.44

Note: Mean of triplicate determinations

Table 4:- Effect of Alum and *Sphatika Bhasma* on *S. typhi* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	Nil	11.9
1	Nil	29.59
10	40.97	73.64

Note: Mean of triplicate determinations

Table 5:- Effect of Alum and *Sphatika Bhasma* on *B. subtilis* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	Nil	2.78
1	Nil	0.69
10	37.60	34.72

Note: Mean of triplicate determinations

Table 6:- Effect of Alum and *Sphatika Bhasma* on *E. faecalis* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	7.04	23.89
1	2.82	15.56
10	21.83	32.22

Note: Mean of triplicate determinations

Table 7:- Effect of Alum and *Sphatika Bhasma* on *MRSA* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	Nil	Nil
1	Nil	Nil

10	55.61	60.38

Note: Mean of triplicate determinations

Table 8:- Effect of Alum and *Sphatika Bhasma* on *S. lutea* Biofilm.

Concentration (mg/ml)	Inhibition (%)	
	Alum	*Sphatika Bhasma*
0.1	7.89	Nil
1	9.70	Nil
10	85.12	88.56

Note: Mean of triplicate determinations

Table 9:- Effect of Ciprofloxacin on Bacterial Biofilms.

No.	Organisms	Inhibition (%)
1	*E. coli* (ATCC-10148)	62.57
2	*K. pneumoniae* (ATCC-700603)	42.75
3	*P. aeruginosa* (Fisher's Immuno Type IV)	84.13
4	*S. typhi* (NCTC-786)	61.75
5	*B. subtilis* (ATCC-9372)	Nil
6	*E. faecalis* (ATCC-29212)	Nil
7	*MRSA* (ATCC-25923)	Nil
8	*S. lutea* (NCTC-2470)	79.48

Note: Mean of triplicate determinations

Table 10:- Effect of Alum on Bacterial Biofilms (Before and After Treatment).

No.	Organisms	Inhibition (%)	
		Before Treatment	After Treatment
1	*E. coli* (ATCC-10148)	Nil	2.51
2	*K. pneumoniae* (ATCC-700603)	Nil	Nil
3	*P. aeruginosa* (Fisher's Immuno Type IV)	Nil	7.32
4	*S. typhi* (NCTC-786)	Nil	17.22
5	*B. subtilis* (ATCC-9372)	33.33	Nil
6	*E. faecalis* (ATCC-29212)	Nil	28.76
7	*MRSA* (ATCC-25923)	Nil	7.41
8	*S. lutea* (NCTC-2470)	39.71	18.84

Note: Mean of triplicate determinations

Table 11:- Effect of *Sphatika Bhasma* on Bacterial Biofilms (Before and After Treatment).

No.	Organisms	Inhibition (%)	
		Before Treatment	After Treatment
1	*E. coli* (ATCC-10148)	Nil	5.48
2	*K. pneumoniae* (ATCC-700603)	Nil	3.02
3	*P. aeruginosa* (Fisher's Immuno Type IV)	Nil	6.17
4	*S. typhi* (NCTC-786)	Nil	52.64
5	*B. subtilis* (ATCC-9372)	42.33	16.57
6	*E. faecalis* (ATCC-29212)	Nil	2.79
7	*MRSA* (ATCC-25923)	Nil	Nil
8	*S. lutea* (NCTC-2470)	Nil	Nil

Note: Mean of triplicate determinations

Table 12:- Effect of Ciprofloxacin on Bacterial Biofilm (Before and After Treatment).

No.	Organisms	Inhibition (%)	
		Before Treatment	After Treatment
1	*E. coli* (ATCC-10148)	29.68	41.56
2	*K. pneumoniae* (ATCC-700603)	Nil	49.71

3	*P. aeruginosa* (Fisher's Immuno Type IV)	Nil	51.23
4	*S. typhi* (NCTC-786)	Nil	54.37
5	*B. subtilis* (ATCC-9372)	Nil	Nil
6	*E. faecalis* (ATCC-29212)	Nil	1.55
7	*MRSA* (ATCC-25923)	Nil	20.86
8	*S. lutea* (NCTC-2470)	Nil	62.60

Note: Mean of triplicate determinations

Table 13:- Combinatorial Effect on Bacterial Biofilms.

Organisms	Inhibition (%)			
	AL + SB	**AL + CP**	**SB + CP**	**AL + SB + CP**
E. coli (ATCC-10148)	72.40	Nil	60.38	62.02
K. pneumoniae (ATCC-700603)	58.70	Nil	38.06	42.11
P. aeruginosa (Fisher's Immuno Type IV)	81.80	15.86	77.96	78.96
S. typhi (NCTC-786)	58.89	Nil	51.85	54.81
B. subtilis (ATCC-9372)	38.31	Nil	11.04	13.64
E. faecalis (ATCC-29212)	46.02	Nil	29.55	30.11
MRSA (ATCC-25923)	72.48	Nil	63.61	65.14
S. lutea (NCTC-2470)	64.74	Nil	51.16	50.58

Note: Mean of triplicate determinations

Note: AL- Alum
SB- *Sphatika Bhasma*
CP- Ciprofloxacin (Standard Antibiotic)

Table 14:- Flavonoid Content Estimation.

Test	Quercetin Equivalent (mg/gm)
Alum	67.90
Sphatika Bhasma	84

Note: Mean of triplicate determinations

Discussion:-

Microbial biofilms may form on wide variety of surfaces and are problematic in medical, industrial and environmental settings because these communities express biofilm specific properties such as increased resistance to antibiotics, UV light and chemical biocides, increased rates of genetic exchange, altered biodegradability and increased secondary metabolite production (Prakash *et al*., 2003). It has been found that Natural antibiofilm agents selectively kill persistent biofilms while allowing antimicrobials to diffuse into the biofilm matrix. These natural products work to degrade the biofilm matrix and destroy the released cells at various stages of biofilm cycle (Mishra *et al*., 2020; Raj and Thomas, 2021). Natural products have been used for a long time in treating human diseases and they contain many constituents of therapeutic value. One such natural products used for therapeutic purposes is Alum (Shahriari *et al*., 2017).

Besides, Ayurveda, the traditional medicinal system of India, extensively uses minerals and ashed metals (*Bhasmas*) as medicine because in contrast to herbal products the mineral products are long lasting and more efficacious and are prescribed in much smaller doses (Chaudhary and Singh, 2010). Furthermore, *Bhasma* is the oldest form of Nanotechnology and it is the scientific process in which metal is transformed into therapeutically active form using herbal ingredients (Dongre and Bhagat, 2015).

Based on encouraging results from our earlier work on antimicrobial potential of Alum and *Sphatika Bhasma*, we chose to further explore their antibiofilm effect. Our *in vitro* Antimicrobial study revealed broad spectrum antibacterial activities, inhibiting gram negative as well as gram positive organisms and moderate antifungal activities by Alum and *Sphatika Bhasma* [Rege *et al*., 2022]. Hence, in the present *in vitro* study, anti-Biofilm activity of Alum as well as its *Bhasma- Sphatika Bhasma* was evaluated against total 8 bacteria, viz., 4 gram negative and 4 gram positive organisms using crystal violet staining method. Crystal violet staining for biofilm quantification remains the most frequently used quantification technique in microtitre plate assays. These assays stain both live and dead cells as well as some components present in biofilm matrix, thereby being well suited to quantify total biofilm biomass. The method can be used with broad range of different bacterial species as well as yeasts or fungi. It also offers high throughput capability of the method, allowing testing of many different conditions simultaneously (Azeredo *et al*., 2017).

Likewise, the microtiter plate assay is an important tool for the study of the early stages in biofilm formation and has been applied primarily for the study of bacterial biofilms. This simple microtiter plate assay allows the formation of a biofilm on the wall and/or bottom of a microtiter plate. The biofilm formation is measured using the dye crystal violet (O'Toole, 2011). However, microtiter plate-based assays share issue of "Edge Effect". The "Edge Effect" poses serious concerns when antimicrobial efficacy of compounds is to be determined, as due to evaporation, concentration of "testing compound" increases which gives false crystal violet absorbance values (Shukla and Rao, 2017). To reduce excessive water loss and to maintain humidity, adding autoclaved water to peripheral wells and placing the sealed microplates in a tray significantly reduced the edge effect in the present study.

In order to the biofilms to form in the presence of test solutions, the planktonic cells would need to survive the test solution concentrations long enough to permit attachment. Therefore, this assay measures both cell attachment and biofilm proliferation in presence of test solutions. To assess the capability of test solutions to prevent the growth of biofilms, the bacterial cultures were incubated in presence of test solutions. Three concentrations of Alum as well as *Sphatika Bhasma* mainly, 0.1 mg/ml, 1 mg/ml and 10 mg/ml were included in the Anti-biofilm assay in the present study. In general, highest inhibition was noted at the concentration of 10 mg/ml. Furthermore, *Sphatika Bhasma* exhibited better Anti-Biofilm activity than Alum except against *B. subtilis*. *Sphatika Bhasma* displayed the highest inhibition against *P. aeruginosa* (90.44%) and the antibiofilm activity was further noted in the order of *P. aeruginosa* > *S. lutea* > *E. coli* > *K. pneumoniae* > *S. typhi* > *MRSA* > *B. subtilis* > *E. faecalis* [Table-1 to Table-8]. Besides, the lowest inhibition by *Sphatika Bhasma* was observed against *E. faecalis* with 32.22%. Additionally, in case of *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. typhi* the Anti-biofilm activity of *Sphatika Bhasma* was found to be concentration dependent, i.e., inhibition increased with the increasing concentration.

As far as Alum is concerned, the highest inhibition was noted against *S. lutea* (85.12%), followed by *P. aeruginosa* > *E. coli* > *MRSA* > *S. typhi* > *B. subtilis* > *K. pneumoniae* > *E. faecalis* [Table-1 to Table-8]. The lowest inhibitory activity of Alum was observed against *E. faecalis* with 21.83%.

The standard antibiotic- Ciprofloxacin which was included as a positive control in the present study, revealed the highest Anti-Biofilm activity against *P. aeruginosa* (84.13%) at the concentration of 2 mg/ml. However, it did not display any inhibitory effect against *B. subtilis*, *E. faecalis* and *MRSA* biofilms [Table-9]. It would be interesting to note here that Alum as well as *Sphatika Bhasma* exhibited Anti-Biofilm effect against *B. subtilis*, *E. faecalis* and *MRSA* biofilms.

Antibiofilm assay was further divided into two separate and additional parts, viz., Before-treatment and After-treatment with Alum as well as *Sphatika Bhasma* individually. In before-treatment assay, the ability of test solutions to eradicate already established biofilms was evaluated, for which the bacterial cultures were incubated first in the microplate wells for 48 h at 37^0C. Then the cultures were replaced with Alum and *Sphatika Bhasma* solutions respectively and the plates were further incubated for 24 h at 37^0C. In before-treatment sets, Alum showed the ability to eradicate biofilms of *B. subtilis* and *S. lutea*, whereas, *Sphatika Bhasma* revealed the ability to eradicate biofilm of *B. subtilis* only [Table-10 & Table-11].

Preconditioning of the surfaces with antimicrobial agents renders unfavourable conditions for the initial stage (attachment) of biofilm formation (Onsare and Arora, 2014). Hence, in after-treatment assay, the microplate wells were first incubated with Alum and *Sphatika Bhasma* solutions individually for 24 h at 37^0C. Then the test solutions were replaced with bacterial cultures and the plates were further incubated for 48 h at 37^0C. In after-treatment sets, Alum displayed the highest inhibitory effect against *E. faecalis* (28.76%). However, it did not show any inhibitory activity against *K. pneumoniae* and *B. subtilis* [Table-10]. Besides, the highest inhibitory activity of *Sphatika Bhasma* was noted against *S. typhi* (52.64%). However, it did not display any inhibitory activity against *MRSA* and *S. lutea* [Table-11].

The standard antibiotic Ciprofloxacin showed inhibitory effect against *E. coli* only in before-treatment assay. Besides, in after-treatment assay, the highest inhibitory activity by Ciprofloxacin was observed against *S. lutea* (62.60%). However, it did not show any inhibitory activity against *B. subtilis* in after-treatment assay [Table-12].

Probably it would be interesting to check the effect of Alum and *Sphatika Bhasma* after 48 h incubation (instead of 24 h) in before-treatment and after-treatment assays as the bacterial cultures were incubated for 48 h.

Furthermore, combination of antibiotic with natural antibiofilm agents was found to be more effective than the individual therapy with antibiotics (Mishra *et al*., 2020; Raj and Thomas, 2021). Hence, four different combinations prepared from Alum (AL), *Sphatika Bhasma* (SB) and Ciprofloxacin (CP)- standard antibiotic were evaluated to determine their combinatorial effect against 8 bacterial biofilms using crystal violet staining method [Table-13]. The combinations were labelled as (AL + SB), (AL + CP), (SB + CP) and (AL + SB + CP). In general, the combination (AL + SB) displayed the highest Anti-biofilm activity against all the bacterial biofilms. The combination (SB + CP) also showed good inhibitory effect especially against *E. coli*, *P. aeruginosa*, *S. typhi*, *MRSA* and *S. lutea* biofilms. However, the combination (AL + CP) showed moderate inhibitory activity that to against *P. aeruginosa* biofilm only. Among all the 4 combinations, the highest Antibiofilm activity was noted with the combination AL + SB against *P. aeruginosa* with 81.80%.

Overall, Antibiofilm effect of (AL + SB) combination observed in combinatorial study revealed almost average of the values that were noted with Alum and *Sphatika Bhasma* individually especially against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi* and *B. subtilis* biofilms. Besides, combinatorial effect of (AL + SB) combination was more than the average of the values that were noted with Alum and *Sphatika Bhasma* individually especially against *E. faecalis* and *MRSA*. Moreover, Antibiofilm effect of (AL + SB) combination exhibited less than the average of the values that were noted with Alum and *Sphatika Bhasma* individually especially against *S. lutea*.

Recently, Flavonoids were reported to inhibit biofilm formation of various organisms (Manner *et al*., 2013; Onsare and Arora, 2014; Raorane *et al*., 2019; Slobodníková *et al*., 2016). Hence, in the present *in vitro* study, flavonoid content of Alum and *Sphatika Bhasma* was estimated using $AlCl_3$ method, wherein, the flavonoid content of *Sphatika Bhasma* was found to be higher than that of Alum [Table-14]. Antibiofilm effects of Alum and *Sphatika Bhasma* could be attributed to their Flavonoid content.

Antimicrobial activity of flavonoids is thought to come from the power to form complexes with both extracellular proteins as well as with bacterial membranes. Therefore, their antimicrobial activity is through inhibition of bacterial virulence factors such as Quorum sensing signal receptors and enzymes, destabilization and permeabilization of the cytoplasmic membrane, inhibition of extracellular microbial enzymes and deprivation of the substrates required for microbial growth such as iron and zinc (Vaou *et al*., 2021). Thus, the antibiofilm effects of natural products are mainly relying on the following aspects, the inhibition of formation of polymer matrix, suppression of cell adhesion and attachment, interrupting extracellular matrix (ECM) generation and decreasing virulence factors production, thereby blocking quorum sensing network and biofilm development (Lu *et al*., 2019).

The biofilms are identified with increased resistance to antibiotics and antimicrobial agents, causing a troublesome burden on human healthcare. Treatment of biofilm associated infections is currently a complicated challenge for clinicians and microbiologists. Novel antimicrobial strategies are urgently to be developed to transcend problems with antibiotic resistance in microbial infectious diseases. Nanoparticles-derived natural compounds could be effectively used in Biofilm-based microbial infections (Lu *et al*., 2019). Silver nanoparticles are known to exhibit satisfactory antibacterial and antibiofilm activities against different pathogens (Siddique *et al*., 2020; Mohanta *et al*., 2020; Seo *et al*., 2021). Ayurvedic *Bhasmas* are the most ancient illustration of Nanotechnology which demonstrated nano-particle size in its finished form. This could be attributed to classical method of preparation of these *Bhasmas* (Chaudhary and Singh, 2010).

In present *in vitro* study, *Sphatika Bhasma* has displayed better Antibiofilm potential than Alum. To best of our knowledge the antibiofilm property of *Sphatika Bhasma* has not been reported before and therefore, result of present study can be considered as the first report about the Antibiofilm potential of *Sphatika Bhasma*.

Thus, Alum and *Sphatika Bhasma* can be used for combating the bacterial biofilms-related menaces. However, further research should be carried out for a better understanding of the exact mechanisms and also to determine pharmacodynamic and pharmacokinetic properties of the Alum as well as *Sphatika Bhasma*.

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