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ANTIBIOFILM EFFECT OF ALUM (*SPHATIKA*) AND ITS *BHASMA* (*SPHATIKA BHASMA*) AGAINST *CANDIDA ALBICANS* AND ITS MIXED CULTURE WITH BACTERIA: AN *IN VITRO* STUDY

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ABSTRACT

Candida albicans biofilms are widely considered as major virulence factor and a key determinant to the high mortality rate. Furthermore, Infections that arise from mixed-species biofilms are considerably more difficult to treat than their single species counterparts. Therefore, prevention and eradication of Biofilms is crucial which can be achieved through natural products. Hence, in the present *in vitro* study, Antibiofilm potential of Alum (*Sphatika*) as well as its *Bhasma, Sphatika Bhasma* was evaluated against *C. albicans* and its Mixed Culture with bacteria (*C. albicans* + Mixed Bacteria) using crystal violet staining method at 37^0 C as well as at RT (25^0 C). Overall, present *in vitro* study has revealed mild antibiofilm activity of Alum as well as *Sphatika Bhasma* against *C. albicans*, however, strong antibiofilm potential against Mixed Culture (*C. albicans* + Mixed Bacteria). Moreover, the combination of Alum + *Sphatika Bhasma* exhibited the highest combinatorial effect against Mixed Culture at 37^0 C. In After-Treatment assay, overall good antibiofilm effect was noted with Alum, *Sphatika Bhasma* and the 11 combinations prepared with standard antibiotics at 37^0 C against Mixed Culture, wherein, *Sphatika Bhasma* displayed better inhibitory potential than Alum. Thus, the current *in vitro* study has exhibited predominant Antibiofilm potential of Alum as well as *Sphatika Bhasma* against Mixed Culture. The antibiofilm potential of Alum and *Sphatika Bhasma* against Mixed Culture revealed through present *in vitro* study could be attributed to their Antioxidant potential, Phenolic content, Flavonoid content and

presence of elements such as Aluminium, Calcium, Copper, Iron, Magnesium, Potassium, Sodium and Zinc.

KEYWORDS: Alum, *Sphatika Bhasma*, *Bhasmas*, Ayurveda, Antibiofilm, Natural Products, Mixed Culture.

INTRODUCTION

Candida albicans is a member of healthy microbiota, asymptomatically colonizing the gastrointestinal tract, reproductive tract, oral cavity and skin of most humans. However, alterations in the host microbiota, changes in host immune response or variations in the local environment can enable *C. albicans* to overgrow and cause infection. These infections range from superficial mucosal and dermal infection to haematogenously disseminated infection with sizable mortality rates.[1] A biofilm is a functional consortium of microorganisms attached to a surface and is embedded in extracellular polymeric substances produced by microorganisms. In natural communities, the microbial interactions observed are complex and often are mixed type. The mixed species biofilms are often thicker and more stable than mono-species biofilms.[2] Biofilm formation has been repeatedly demonstrated to protect the biofilm cells from many antimicrobial agents including antibiotics, biocides and host defence mechanism.[3] The ability of *Candida albicans* to form biofilms is a complex process. *Candida albicans* biofilms are widely considered as a major virulence factor and a key determinant to the high mortality rate attributed with Candidiasis.[4] Most manifestations of Candidiasis are in fact associated with the formation of *Candida* biofilms on surfaces which is associated with infection at both mucosal as well as systemic sites. Moreover, *Candida* biofilms share several properties with bacterial biofilms.[5] Mixed bacterial-fungal biofilms are associated with infections of catheters, orthopaedic prostheses, endotracheal tubes, biliary stents and acrylic dentures.[6] Infections that arise from mixed-species biofilms are considerably more difficult to treat than their single species counterparts. Hence, prevention of biofilm formation and the eradication of mature biofilms in the clinical settings present formidable challenges that must be overcome if the mortality associated with biofilm infection is to be addressed. Natural compounds have served as a source of novel alternatives to *C. albicans* biofilm treatment.[4] One such natural products used for therapeutic purposes for centuries is white Alum. Alum (*Sphatika*) is an efficient, safe and eco-friendly inorganic compound, commercially available and is cost effective. It is frequently used topically and internally in traditional systems of medicine including Ayurveda. Besides, Alum has various

applications and is used as preservative, vaccine adjuvant, acid catalyst, antimicrobial, for water treatment to name a few.[7] Besides, *Bhasmas* are the unique Ayurvedic metallic/mineral preparations, treated with herbal juices or decoction which are known in Indian subcontinent since 7th 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*.[8] *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.[9,10] 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).[11]

Alum and its *Bhasma* (*Sphatika Bhasma*) have revealed moderate antifungal activity mainly against *C. albicans* in our earlier study.[12] Furthermore, Alum and its *Bhasma* (*Sphatika Bhasma*) have also exhibited strong Antibacterial and Antibiofilm potential against the Clinical Isolates (Gram negative bacteria) as well as on their Mixed Culture in our previous study.[13] Based on these encouraging results, Antibiofilm activity of Alum and its *Bhasma*, *Sphatika Bhasma* were evaluated against *C. albicans* and the combination of *C. albicans* with Mixed bacterial Culture (Clinical Isolates) using various parameters in the present *in vitro* study.

MATERIALS AND METHODS

1. Procurement of Material

Alum was procured from local market, whereas, *Sphatika Bhasma* was procured from local Ayurvedic shop in Mumbai.

2. Preparation of Test Solution

The test solutions were prepared by dissolving Alum and *Sphatika Bhasma* in warm sterile distilled water individually (Stock solution= 100 mg/ml each). The prepared solutions were stored at 4^{0}C until further use.

3. Test Organisms used for Antibiofilm Assay

Antibiofilm activity of Alum and its *Bhasma* (*Sphatika Bhasma*) was evaluated against *Candida albicans* (ATCC-10231) and a Mixed Culture (*C. albicans* + Mixed Bacteria). Six

Clinical Isolates (Gram Negative Bacteria), namely, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris* and *Shigella flexneri* were included in the mixed bacterial culture. Bacterial cultures were grown on Nutrient agar and suspended in Mueller Hinton Broth (MHB) for the assays, whereas, fungal culture was grown on Sabouraud Dextrose Agar and was suspended in Sabouraud Dextrose Broth for the assays. Sub-culturing of above mentioned organisms and the media preparation was carried out by Mrs. Dipti Kolte, Senior Technical Assistant (STA), Bacteriology Department, Haffkine Institute, Mumbai.

4. Anti-Biofilm Assay

The effect of Alum and *Sphatika Bhasma* on microbial biofilm formation was evaluated in sterile 96-well polystyrene flat-bottom microplates according to Sánchez E *et al.*[14] with slight modification. Briefly, 200 μl of inoculated fresh Mueller Hinton Broth (10^6 CFU/ml) was aliquoted in triplicate to respective wells of sterile microplate. For *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), three concentrations such as 1, 10 & 100 mg/ml were used and one set of microplates was incubated at 37^0C for 48h, whereas, other set of microplates was incubated at RT (25^0 C) for 48h respectively. Wells containing microbial cultures with distilled water were used as controls. Ciprofloxacin and Fluconazole were included as a standard antibiotics. 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 microbial 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) or Fluconazole (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. Combinatorial Effect on Microbial Biofilms

a) For *C. albicans*, following combinations of Alum (100 mg/ml), *Sphatika Bhasma* (100 mg/ml) and Fluconazole (2 mg/ml) were prepared in 1:1 ratio and their combinatorial effect was tested against biofilm of *C. albicans*.

- Alum + *Sphatika Bhasma* (AL + SB)
- Alum + Fluconazole (AL + F)
- *Sphatika Bhasma* + Fluconazole (SB + F)
- Alum + *Sphatika Bhasma* + Fluconazole (AL + SB + F)

For the said study, 100 µl of fungal culture and 100 µl of combination solution were added to the microplates in triplicate. One set of microplates was incubated at 37^0C for 48h, whereas, other set of microplates was incubated at RT (25^0 C) for 48h. After incubation, supernatant was removed and the effect of combination solution on fungal biofilm was determined by Crystal Violet staining method as stated earlier.

b) For Mixed Culture (*C. albicans* + Mixed Bacteria), following Combinations of Alum (100 mg/ml), *Sphatika Bhasma* (100 mg/ml), Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were prepared in 1:1 ratio and their combinatorial effect was tested against biofilm of Mixed Culture.

- Ciprofloxacin + Fluconazole (CP + F)
- Alum + *Sphatika Bhasma* (AL + SB)
- Alum + Ciprofloxacin (AL + CP)
- *Sphatika Bhasma* + Ciprofloxacin (SB + CP)
- Alum + Fluconazole (AL + F)
- *Sphatika Bhasma* + Fluconazole (SB + F)
- Alum + *Sphatika Bhasma* + Ciprofloxacin (AL + SB + CP)
- Alum + *Sphatika Bhasma* + Fluconazole (AL + SB + F)
- Alum + Ciprofloxacin + Fluconazole (AL + CP + F)
- *Sphatika Bhasma* + Ciprofloxacin + Fluconazole (SB + CP + F)
- Alum + *Sphatika Bhasma* + Ciprofloxacin + Fluconazole (AL + SB + CP + F)

For the said study, 100 µl of microbial culture and 100 µl of combination solution were added to the microplates in triplicate. One set of microplates was incubated at 37^0C for 48h, whereas, other set of microplates was incubated at RT (25^0 C) for 48h. After incubation, supernatant was removed and the effect of combination solution on microbial biofilm 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.

6. Effect of Before-Treatment and After-Treatment on Microbial Biofilms

(a) For Before-treatment study against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), 100 µl of microbial culture (10^6 CFU/ml) was added to the microplates in triplicate and the plates were sealed and one set of plates was incubated at 37^0C for 48 h and other set was incubated at RT (25^0 C). After incubation, the cultures were aspirated carefully and Alum and *Sphatika Bhasma* (100 mg/ml each) were added to the plates and one set of plates was incubated further at 37^0C for 48 h and other set was further incubated at RT (25^0 C) for 48 h. After incubation, supernatant was removed and the effect of Alum & *Sphatika Bhasma* on microbial biofilms was determined by Crystal Violet staining method as stated earlier. For *C. albicans*, Fluconazole (2 mg/ml) was included as a standard and 4 combinations of Alum (100 mg/ml), *Sphatika Bhasma* (100 mg/ml) and Fluconazole (2 mg/ml) prepared in 1:1 ratio were also tested against biofilm of *C. albicans*. Likewise, for Mixed Culture (*C. albicans* + Mixed Bacteria) Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were included as standards and 11 combinations of Alum (100 mg/ml), *Sphatika Bhasma* (100 mg/ml), Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) prepared in 1:1 ratio were also tested against biofilm of Mixed Culture.

(b) For After-treatment study against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), 100 µl of Alum and *Sphatika Bhasma* (100 mg/ml each) was added to the sterile microplate in triplicate and the plates were sealed and one set of plates was then incubated at 37^0C for 48 h and other set was incubated at RT (25^0 C) for 48 h. After incubation, the test solutions were aspirated carefully and 100 µl of microbial cultures (10^6 CFU/ml) were added to the microplate and one set of sealed plates was further incubated at 37^0C for 48 h and the other set was further incubated at RT (25^0 C) for 48 h. After incubation, supernatant was removed and the effect of Alum & *Sphatika Bhasma* on microbial biofilms was determined

by Crystal Violet staining method as stated earlier. For *C. albicans*, Fluconazole (2 mg/ml) was included as a standard and 4 combinations of Alum (100 mg/ml), *Sphatika Bhasma* (100 mg/ml) and Fluconazole (2 mg/ml) prepared in 1:1 ratio were also tested against biofilm of *C. albicans*. Likewise, for Mixed Culture (*C. albicans* + Mixed Bacteria) Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were included as standards and 11 combinations of Alum (100 mg/ml), *Sphatika Bhasma* (100 mg/ml), Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) prepared in 1:1 ratio were also tested against biofilm of Mixed Culture.

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.

RESULTS

Table 1: Effect on Biofilm of *C. albicans*.

Test	Concentration (mg/ml)	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
Alum (AL)	1	13.45	15.56
	10	19.30	30.22
	100	14.62	30.22
Sphatika Bhasma (SB)	1	19.30	28
	10	24.56	33.33
	100	22.22	37.78
Fluconazole (F)	2	10.53	29.78

Note: Mean of triplicate determinations

Table 2: Effect on Biofilm of Mixed Culture (*C. albicans* + Mixed Bacteria).

Test	Concentration (mg/ml)	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
Alum (AL)	1	39.38	44.74
	10	95.08	92.02
	100	92.74	90.05
Sphatika Bhasma (SB)	1	51.88	44.80
	10	95.03	91.70
	100	93.66	90.68
Ciprofloxacin (CP)	2	89.51	83.46
Fluconazole (F)	2	20.92	Nil

Note: Mean of triplicate determinations

Table 3: Combinatorial Effect on Biofilm of *C. albicans.*

No.	Combination	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
1	AL + SB	22.22	34.67
2	AL + F	12.87	24
3	SB + F	26.32	32.89
4	AL + SB + F	21.64	28.44

Note: Mean of triplicate determinations

AL- Alum

SB- *Sphatika Bhasma*

F- Fluconazole

Table 4: Combinatorial Effect on Biofilm of Mixed Culture (*C. albicans* + Mixed Bacteria).

No.	Combination	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
1	CP + F	92.74	91.32
2	AL + SB	93.61	91.76
3	AL + CP	92.85	90.87
4	SB + CP	93.50	91.44
5	AL + F	92.24	89.67
6	SB + F	93.17	90.68
7	AL + SB + CP	93.12	91.13
8	AL + SB + F	93.06	91
9	AL + CP + F	92.57	90.43
10	SB + CP + F	93.17	91
11	AL + SB + CP + F	92.95	90.56

Note: Mean of triplicate determinations

AL- Alum

SB- *Sphatika Bhasma*

CP- Ciprofloxacin

F- Fluconazole

Table 5: Before Treatment Effect on Biofilm of *C. albicans.*

No.	Test	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
1	Alum (AL)	Nil	Nil
2	*Sphatika Bhasma* (SB)	Nil	Nil
3	Fluconazole (F)	Nil	Nil
4	AL + SB	Nil	Nil
5	AL + F	Nil	Nil
6	SB + F	Nil	Nil
7	AL + SB + F	Nil	Nil

Note: Mean of triplicate determinations

Table 6: After Treatment Effect on Biofilm of *C. albicans*.

No.	Test	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
1	Alum (AL)	Nil	Nil
2	*Sphatika Bhasma* (SB)	26.15	8.33
3	Fluconazole (F)	23.59	19.64
4	AL + SB	18.46	5.36
5	AL + F	25.64	Nil
6	SB + F	12.31	Nil
7	AL + SB + F	19.49	Nil

Note: Mean of triplicate determinations

Table 7: Before Treatment Effect on Biofilm of Mixed Culture (*C. albicans* + Mixed Bacteria).

No.	Test	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
1	Alum (AL)	Nil	Nil
2	*Sphatika Bhasma* (SB)	Nil	Nil
3	Ciprofloxacin (CP)	Nil	Nil
4	Fluconazole (F)	2.70	Nil
5	CP + F	Nil	Nil
6	AL + SB	Nil	Nil
7	AL + CP	Nil	Nil
8	SB + CP	Nil	Nil
9	AL + F	Nil	Nil
10	SB + F	Nil	Nil
11	AL + SB + CP	Nil	Nil
12	AL + SB + F	Nil	Nil
13	AL + CP + F	Nil	Nil
14	SB + CP + F	Nil	Nil
15	AL + SB + CP + F	Nil	Nil

Note: Mean of triplicate determinations

Table 8: After Treatment Effect on Biofilm of Mixed Culture (*C. albicans* + Mixed Bacteria).

No.	Test	Inhibition (%)	
		at 37^0 C	at RT (25^0 C)
1	Alum (AL)	69.05	56.65
2	*Sphatika Bhasma* (SB)	72.96	63.26
3	Ciprofloxacin (CP)	87.41	74.84
4	Fluconazole (F)	13.61	10.28
5	CP + F	86.96	73.76
6	AL + SB	65.48	55.79

7	AL + CP	84.98	72.83
8	SB + CP	82.48	67.79
9	AL + F	67.18	63.12
10	SB + F	71.94	66.07
11	AL + SB + CP	84.69	81.02
12	AL + SB + F	71.54	61.61
13	AL + CP + F	86.34	72.47
14	SB + CP + F	82.99	68.94
15	AL + SB + CP + F	82.88	68.51

Note: Mean of triplicate determinations

DISCUSSION

Candida species are the fourth most common cause of nosocomial bloodstream infections. These infections are associated with high mortality rate of approximately 50%. *Candida albicans* is the most frequent pathogen responsible for *Candida* infections. One specific feature of *Candida* species' pathogenicity is their ability to form biofilm which protects them from external factors such as host immune system defences and antifungal drugs. *C. albicans* is considered to be the biggest biofilm producer among the *Candida* species. Furthermore, the formation of mixed biofilms of *Candida* species has been described with different combinations between *Candida* species or with *Candida* and Bacteria.[15] Natural compounds have been proven to be active against fungal biofilms and are less likely to induce resistant phenotypes.[16] One such natural products used for therapeutic purposes is Alum.[17] Besides, many mineral and metallic *Bhasmas* are used for variety of health disorders. They are more powerful than herbs and have a faster healing action.[18] Moreover, based on previous encouraging results, Antibiofilm activity of Alum and its *Bhasma* (*Sphatika Bhasma*) was evaluated against *C. albicans* and combination of *C. albicans* with Mixed bacterial Culture (Clinical Isolates) by crystal violet staining method using various parameters in the present *in vitro* study.

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.[19]

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 microbial 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.[20] 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.[21] To reduce excessive content 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 microbial cultures were incubated in presence of test solutions. Three concentrations of Alum and *Sphatika Bhasma* mainly, 1 mg/ml, 10 mg/ml and 100 mg/ml were included against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) in the Anti-biofilm assay in the present study. Besides, the Antibiofilm assay was carried out at 37^0 C as well as at Room Temperature (RT= 25^0 C) against *C. albicans* and Mixed Culture.

In the present *in vitro* study, Alum displayed mild antibiofilm activity against *C. albicans* at both the temperatures (37^0 C as well as RT). However, the highest inhibitory activity was noted at RT at a concentration of 100 mg/ml. Likewise, *Sphatika Bhasma* exhibited the highest antibiofilm activity at RT at a concentration of 100 mg/ml against *C. albicans* [Table-1]. In case of Mixed Culture, the highest antibiofilm effect was noted at a concentration of 10 mg/ml at 37^0 C by Alum as well as *Sphatika Bhasma* [Table-2].

The standard antibiotic- Ciprofloxacin which was included as a standard in the present study, revealed the highest Antibiofilm effect against Mixed Culture at 37^0 C [Table-2]. Moreover, the standard antibiotic- Fluconazole revealed mild Antibiofilm activity against *C. albicans* as well as against Mixed Culture. However, it did not show any inhibitory effect at RT against Mixed culture [Table-1 & Table-2].

Combining antibiofilm agents with antibiotics is emerging as a promising strategy to eradicate biofilms.[22] Hence, the combinatorial Antibiofilm assay was carried out against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) at 37^0 C as well as at Room Temperature (RT) using crystal violet staining method [Table-3 & Table-4]. The combination of Alum + *Sphatika Bhasma* showed the highest combinatorial antibiofilm effect at RT against *C. albicans* [Table-3]. Furthermore, 11 different combinations prepared from Alum (AL), *Sphatika Bhasma* (SB) and standard antibiotics such as Ciprofloxacin (CP) & Fluconazole (F) were also evaluated to determine their combinatorial effect against Mixed Culture (*C. albicans* + Mixed Bacteria). The combination of Alum + *Sphatika Bhasma* exhibited the highest combinatorial effect against Mixed Culture at 37^0 C [Table-4].

In general, combination of SB + F revealed synergistic effect against Biofilms of *C. albicans* at 37^0C. However, combination of AL + F and AL + SB + F showed antagonistic effect at RT.

Furthermore, in case of Mixed Culture (*C. albicans* + Mixed Bacteria) combination of CP + F and AL + CP displayed synergistic effect at 37^0C, whereas, most of the combinations except AL + F, SB + F and AL + SB + CP + F showed synergistic effect against Biofilm of Mixed Culture at RT.

Antibiofilm assay was further divided into two separate and additional parts, viz., Before-Treatment and After-Treatment with Alum and *Sphatika Bhasma* individually. In Before-Treatment assay, the ability of test solutions to eradicate already established biofilms was evaluated, for which the microbial cultures were incubated first in the microplate wells for 48 h; One set of microplates was incubated at 37^0C and other set was incubated at RT (25^0 C). Then the cultures were replaced with test solutions and the plates were further incubated for 48 h at respective temperatures/conditions. Alum as well as *Sphatika Bhasma* did not show any antibiofilm effect in Before-Treatment assay against *C. albicans* as well as Mixed Culture. Even the combinations prepared with Alum, *Sphatika Bhasma* and the standard antibiotics did not display any inhibitory effect in Before-Treatment assay [Table- 5 & Table-7].

Besides, preconditioning of the surfaces with antimicrobial agents renders unfavourable conditions for the initial stage (attachment) of biofilm formation.[23] Hence, in After-Treatment assay, the microplate wells were first incubated with test solutions for 48 h; One

set of microplates was incubated at 37^0C and other set was incubated at RT (25^0 C). Then the test solutions were replaced with microbial cultures and the plates were further incubated for 48 h at respective temperatures/conditions. In After-Treatment assay, the highest antibiofilm activity was exhibited by *Sphatika Bhasma* against *C. albicans* at 37^0 C which was even higher than the 4 combinations included in the assay. However, the combinations such as AL + F, SB + F and AL + SB + F did not show any antibiofilm activity against *C. albicans* at RT. Furthermore, Alum did not display any inhibitory effect against *C. albicans* at 37^0 c as well as at RT in After-Treatment assay [Table-6]. In case of Mixed Culture (*C. albicans* + Mixed Bacteria), overall good antibiofilm effect was noted with Alum, *Sphatika Bhasma* and the 11 combinations at 37^0 C, wherein, *Sphatika Bhasma* displayed better inhibitory potential than Alum. Furthermore, the highest antibiofilm activity was exhibited by the combination of CP + F [Table-8].

The standard antibiotic Ciprofloxacin did not show any inhibitory effect against Mixed Culture (*C. albicans* + Mixed Bacteria) in Before-Treatment assay. However, it showed strong antibiofilm potential in After-Treatment assay at both the temperatures/conditions [Table-7 & Table-8]. Moreover, the standard antibiotic Fluconazole did not show any inhibitory effect in Before-Treatment assay at any of the temperatures/conditions against *C. albicans.* However, it showed mild antibiofilm activity against *C. albicans* in After-Treatment assay at both the temperatures/conditions [Table-5 & Table-6]. Additionally, Fluconazole displayed mild inhibitory effect against Mixed Culture in After-Treatment assay at both the temperatures/conditions, whereas, it revealed negligible antibiofilm activity against Mixed Culture only at 37^0 C in Before-Treatment assay [Table-7 & Table- 8].

Overall, present *in vitro* study has revealed mild antibiofilm activity of Alum as well as *Sphatika Bhasma* against *C. albicans*, however, strong antibiofilm potential against Mixed Culture (*C. albicans* + Mixed Bacteria). Likewise, our previous study has also revealed broad spectrum Antibiofilm effect of Alum as well as *Sphatika Bhasma* against mixed bacterial cultures (Mixture of Gram Negative Bacteria, Mixture of Gram Positive Bacteria and Mixture of all bacteria) of standard strains.[24]

Earlier studies have displayed phenolic content[12], flavonoid content[25] and presence of elements[26] such as Aluminium (Al), Calcium (Ca), Copper (Cu), Iron (Fe), Magnesium (Mg), Potassium (K), Sodium (Na) and Zinc (Zn) in Alum as well as *Sphatika Bhasma*. The antibiofilm potential of Alum and *Sphatika Bhasma* against Mixed Culture revealed through

present *in vitro* study could be attributed to their phenolic content, Flavonoid content and presence of above mentioned elements.

Phenolic compounds and flavonoids are a very important class of phytochemicals that affects microbial growth and can cause hindrance in their pathogenic activity.[27] Besides, Metals have shown strong efficacy against microbes growing in biofilms.[28] In Ayurvedic herbomineral formulations like *Bhasma*, metallic components are the essential part of the preparation with plant parts. The metallic components are not in free form, they are in complex form with organic components. This combination is achieved by selective Ayurvedic procedures like *Shodhana* and *Marana*. This procedure reduces the toxic effects of metallic components and enhances the potency of the preparation. Several such herbomineral formulations, viz., *Tambra Bhasma*, *Rasa Karpoor*, *Swasakuthara Rasa*, *Rajat Bhasma*, *Vyadhividhwansana Rasa*, *Tankanamruta Malahara*, *Rasa Sindoora*, *Mrityunjaya Rasa*, *Seetamshu Rasa*, *Udayabhaskar Rasa*, *Gandhak Taila*, *Gandhak Dhruti* and *Rasaka Bhasma* have displayed antimicrobial potential.[29] Presence of cations such as Al, Ca, Cu, Fe, Mg, K, Na and Zn in Alum as well as *Sphatika Bhasma* can be considered as available cations contributing to their overall Antimicrobial action. Such hypothesis, viz., presence of cations contributing to antimicrobial activity was postulated by Wijenayake *et al*.[30]

Additionally, earlier study has displayed antioxidant potential of Alum as well as *Sphatika Bhasma* which was detected using DPPH assay.[12] One of the main mechanisms that drives a microorganism to transit from a planktonic to a biofilm-sessile state is oxidative stress. Oxidative stress encountered by the microbial cells could be caused by abiotic stresses, presence of antimicrobials or the host immune system. Moreover, an elevation of reactive oxygen species (ROS) has been reported to upregulate certain microbial proteins which in turn leads to biofilm formation. Thus, oxidative stress in microorganisms does play an important role in the regulation of redox-defence mechanisms, the production of EPS and biofilm heterogeneity. Antioxidants were shown to possess potent anti-biofilm properties as they can reduce oxidative stress-mediated virulence in pathogenic microorganisms by scavenging free radicals.[31] Chemical compounds with antioxidant potential such as Alum and *Sphatika Bhasma* could therefore be used to treat biofilm-associated infections.

In general, following observations were noted in the present *in vitro* study,

1) Antibiofilm assay can be carried out at 37^0 C as well as at RT (25^0 C) against Mixed Culture (*C. albicans* + Mixed Bacteria).

2) The pattern of biofilm formation of Mixed Culture (*C. albicans* + Mixed Bacteria) was found to be different when the microplates were incubated at 37^0 C and at RT (25^0 C) respectively. The biofilm was formed on the wall/sides of the microplates (wells) that were incubated at 37^0 C, whereas, Biofilm was formed as a sharp ring in the microplates that were incubated at RT (25^0 C).
3) Antibiofilm assay against *C. albicans* can be conducted at 37^0 C as well as at RT (25^0 C).

Overall, the present *in vitro* study reveals prominent Antibiofilm activity of Alum as well as its *Bhasma*, *Sphatika Bhasma* against Mixed Culture of *C. albicans* with bacteria.

CONCLUSION

In the present *in vitro* study, Antibiofilm potential of Alum (*Sphatika*) as well as its *Bhasma, Sphatika Bhasma* was tested against *C. albicans* and its Mixed Culture (*C. albicans* + Mixed Bacteria) using crystal violet staining method. The current *in vitro* study has exhibited predominant Antibiofilm potential of Alum as well as *Sphatika Bhasma* against Mixed Culture. Moreover, the combination of Alum + *Sphatika Bhasma* exhibited the highest combinatorial effect against Mixed Culture at 37^0 C. In After-Treatment assay, overall good antibiofilm effect was noted with Alum, *Sphatika Bhasma* and the 11 combinations at 37^0 C against Mixed Culture, wherein, *Sphatika Bhasma* displayed better inhibitory potential than Alum. The antibiofilm potential of Alum and *Sphatika Bhasma* against Mixed Culture revealed through present *in vitro* study could be attributed to their Phenolic content, Flavonoid content, presence of metallic elements and their Antioxidant potential.

REFERENCES

1. Nobile CJ, Johnson AD. *Candida albicans* biofilms and human disease. Annu Rev Microbiol, 2015; 69: 71-92.
2. Ganeshkumar C, Anand SK. Significance of microbial biofilms in food industry: A review. International Journal of Food Microbiology, 1998; 42: 9-27.
3. Trachoo N. Biofilms and the food industry. Songklanakarin J Sci Technol, 2003; 25(6): 807-815.
4. Ponde NO, Lortal L, Ramage G, Naglik JR, Richardson JP. *Candida albicans* biofilms and polymicrobial interactions. Crit Rev Microbiol, 2021; 47(1): 91-111.
5. Jabra-Rizk MA, Falkler W, Meiller T. Fungal biofilms and drug resistance. Emerg Infect Dis, 2004; 10(1): 14-19.

6. Adam B, Baillie GS, Douglas LJ. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. J Med Microbiol, 2002; 51: 344-349.
7. Amadi LO. A review of antimicrobial properties of Alum and Sundry applications. ACTA Sci Microbiol, 2020; 3(4): 109-117.
8. Pal D, Sahu CK, Haldar A. The ancient Indian nanomedicine. J Adv Pharm Technol Res, 2014; 5(1): 4-12.
9. Tambekar DH, Dahikar SB. Screening antibacterial activity of some *Bhasma* (Metal-based Herbal Medicines) against enteric pathogens. Recent Res Sci Technol, 2010; 2(10): 59-62.
10. Sharma R, Prajapati PK. Nanotechnology in Medicine: Leads from Ayurveda. J Pharm Bioallied Sci, 2016; 8(1): 80-81.
11. Dongre S, Bhagat P. Antimicrobial Studies of *Rasaushadhies* (Herbomineral preparations): A Review. Int J Ayu Pharm Chem, 2015; 4(3): 228-237.
12. Rege AA, Kolte DB, Martin SS, *In vitro* Evaluation of Antimicrobial and Antioxidant activities of Alum and it's *Bhasma* (*Sphatika Bhasma*). Int J Adv Res, 2022; 10(8): 891-902.
13. Rege AA, Kolte DB, Ambre MS. Evaluation of Antimicrobial and Antibiofilm Potential of Alum *(Sphatika)* and It's *Bhasma* (*Sphatika Bhasma*) against Clinical Isolates- An *In vitro* Study. World J Pharm Pharm Sci, 2023; 12(8): 1331-1350.
14. Sánchez E, Morales CR, Castillo S, Leos-Rivas C, García-Becerra L, Martínez DO. Antibacterial and Antibiofilm Activity of Methanolic Plant Extracts against Nosocomial Microorganisms. Evid Based Complementary Altern Med, 2016; Article ID: 1572697.
15. Convalheiro M, Teixeira MC. Candida Biofilms: Threats, Challenges and promising strategies. Front Med, 2018; 5: 28.
16. Zara G, Budroni M, Mannazzu I, Fancello F, Zara S. Yeast biofilm in food realms: Occurrence and control. World J Microbiol Biotechnol, 2020; 36: 134.
17. Shahriari R, Salari S, Shahriari S. *In vitro* study of concentration-effect and Time course pattern of white alum on *Escherichia coli* O157:H7 growth. Afr J Tradit Complement Altern Med, 2017; 14(2): 311-318.
18. Swami Sada Shiva Thirtha. Minerals and Metallic *Bhasmas*, In: The Ayurveda Encyclopaedia- Natural secrets to Healing, Preservation and Longevity, Delhi, Sri Satguru Publications, 1998: 328.
19. Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, Desvaux M, Bonaventura GD, Hébraud M, Jaglic Z, Kačániová M, Knøchel S, Lourenço A,

Mergulhão F, Meyer RL, Nychas G, Simões M, Tresse O, Sternberg C. Critical review on biofilm method. Crit Rev Microbiol, 2017; 43(3): 313-351.

20. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp, 2011; JOVE 47 (doi: 10.3791/2437).
21. Shukla SK, Rao TS. An improved crystal violet assay for biofilm quantification in 96-well microtiter plate. 2017; bio Rxiv preprint doi: https//doi.org/10.1101/100214.
22. Sung K, Khan S, Ahn J. Foodborne pathogen biofilms: Development, detection, control and antimicrobial resistance. Pathogens, 2023: 12: 352.
23. Onsare JG, Arora DS. Antibiofilm potential of flavonoids extracted from *Moringa oleifera* seed coat against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. J Appl Microbiol, 2014; 118: 313-325.
24. Rege AA. *In vitro* Evaluation of Antibiofilm Potential of Alum (*Sphatika*) and it's *Bhasma* (*Sphatika Bhasma*) against Mixed Bacterial Biofilms. World J Pharm Pharm Sci, 2023; 12(2): 1211-1224.
25. Rege AA, Kolte DB, Martin SS. *In Vitro* Evaluation of Anti-Biofilm Potential of Alum And it's *Bhasma* (*Sphatika Bhasma*). Int J Adv Res., 2022; 10(9): 103-112.
26. Rege A, Kolte D, Martin S. Antimicrobial and Antibiofilm Effects of Alum (*Sphatika*) & Its *Bhasma* an *In vitro* Study. Germany; Lambert Academic Publishing, 2022.
27. Vaou N, Stavropoulou E, Voidarou C, Christina T, Bezirtzoglou E. Towards advances in Medicinal plant antimicrobial activity: A review study on challenges and future perspectives. Microorganisms, 2021; 9: 2041.
28. Teizel GM, Parsek M. Heavy metal resistance of Biofilm and planktonic *Pseudomonas aeruginosa*. Appl Environ Microbiol, 2003; 69(4): 2313-2320.
29. Ehsan M, Patil S, Nandi MK. An Appraisal on Antimicrobial Activity of Herbomineral Formulations. Int J Ayurvedic Med, 2022; 13(1): 15–21.
30. Wijenayake AU, Abayasekara CL, Pitawala HMTGA, Bandara BMR. Antimicrobial potential of two traditional herbometallic drugs against certain pathogenic microbial species. BMC Complement Altern Med, 2016; 16: 365.
31. Ong KS, Mawang CI, Daniel-Jambun D, Lim YY, Lee SM. Current antibiofilm strategies and potential of antioxidants in biofilm control. Expert Rev Anti Infect Ther, 2018; 16(11): 855-864.