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RESEARCH ARTICLE

IN VITRO EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF ALUM AND IT'S *BHASMA* (*SPHATIKA BHASMA*)

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Abstract

To overcome the burden of antimicrobial resistance, greater attention has been paid to antimicrobial activity screening from natural products. One such natural products used for therapeutic purposes is Alum. Besides, Ayurvedic "*Bhasma*" formulations have been used for treatment of various ailments for thousands of years all over the world and widely in India. Hence, in the present *in vitro* study, antimicrobial activity of Alum as well as its *Bhasma- Sphatika Bhasma* was evaluated against total 10 organisms, viz., 4 gram negative [*Escherichia coli* (ATCC-10148), *Klebsiella pneumoniae* (ATCC-700603), *Pseudomonas aeruginosa* (Fisher's Immuno Type IV), *Salmonella typhi* (NCTC-786)], 4 gram positive [*Bacillus subtilis* (ATCC-9372), *Enterococcus faecalis* (ATCC-29212), Methicillin Resistant *Staphylococcus aureus* (*MRSA* ATCC-25923), *Sarcina lutea* (NCTC-2470)] and 2 fungi [*Aspergillus niger* (ATCC-16404) and *Candida albicans* (ATCC-10231)] using various assays such as MIC, MBC/MFC and Agar Well Diffusion method. In general, MIC values of *Sphatika Bhasma* were lower than that of Alum. Alum and *Sphatika Bhasma* revealed broad spectrum antibacterial activities inhibiting gram positive as well as gram negative organisms and moderate antifungal activities. Furthermore, different combinations prepared from Alum (AL), *Sphatika Bhasma* (SB) and standard antibiotics were subjected to determine their combinatorial effect against 10 organisms using Agar Well Diffusion Assay. The combinations (AL + CP), (SB + CP) and (AL + SB + CP) displayed synergistic effect against *P. aeruginosa*. Antimicrobial and Antioxidant potential of Alum and *Sphatika Bhasma* could be attributed to their Phenolic content. Overall, *Sphatika Bhasma* exhibited better Antimicrobial and Antioxidant activities than Alum.

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Introduction:-

Due to increase in irrelevant use of antibiotics and increasing susceptibility of microorganisms to antimicrobial agents, there are increasing numbers of drug resistance cases in most developing countries (Dongre and Bhagat, 2015). Since ancient times, the materials made by nature have been applied to treat human diseases (Shahriari *et al*., 2017). 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 (*Sphatika*) is an efficient, safe and eco-friendly inorganic compound, commercially available and is cost effective. Alum has various applications and is used as preservative, vaccine adjuvant, acid catalyst, antimicrobial, for water treatment to name a few (Amadi, 2020). Besides, Ayurvedic "*Bhasma*" formulations have been used for treatment of various ailments for thousands of years all over the world and widely in India (Verma and Yadav, 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). 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). It will be interesting to see whether *bhasma* of Alum (*Sphatika Bhasma*) possess any Antimicrobial activity and whether it's activity is better or similar to that of Alum and/or Antibiotics.

Antioxidants are chemical compounds which bind to free oxygen radicals and prevent these radicals from damaging healthy cells and can cause hindrance in pathogenic activity of microbes (Dontha, 2016). Many natural products have shown Antimicrobial as well as Antioxidant potential in the past (Angiolella *et al*., 2018; Kusuma *et al*., 2014). Hence, in the present *in vitro* study, antimicrobial activity of Alum as well as its *Bhasma- Sphatika Bhasma* was evaluated against total 10 organisms, viz., 4 gram negative [*Escherichia coli* (ATCC-10148), *Klebsiella pneumoniae* (ATCC-700603), *Pseudomonas aeruginosa* (Fisher's Immuno Type IV), *Salmonella typhi* (NCTC-786)], 4 gram positive [*Bacillus subtilis* (ATCC-9372), *Enterococcus faecalis* (ATCC-29212), Methicillin Resistant *Staphylococcus aureus* (*MRSA* ATCC-25923), *Sarcina lutea* (NCTC-2470)] and 2 fungi [*Aspergillus niger* (ATCC-16404) and *Candida albicans* (ATCC-10231)] using various assays. Furthermore, Alum and *Sphatika Bhasma* were also evaluated for their Antioxidant potential using DPPH assay.

Materials and Methods:-

1) Procurement of Material

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

2) Preparation of Test Solutions

The test solutions were prepared by dissolving Alum and *Sphatika Bhasma* in sterile distilled water independently (Stock solution= 100 mg/ml each). The test solutions were solubilized by dipping the tubes in boiling water bath.

3) Test Organisms used for Antimicrobial Assays

Antimicrobial 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), *Sarcina lutea* (NCTC-2470), *Aspergillus niger* (ATCC-16404) and *Candida albicans* (ATCC-10231).

Bacterial cultures were grown on Nutrient agar and suspended in Mueller Hinton Broth (MHB) for the assays, whereas, fungal cultures were grown on Sabouraud Dextrose Agar and were suspended in Sabouraud Dextrose Broth for the assays.

4) Assessment of Minimum Inhibitory Concentration (MIC)

Assessment of Minimum Inhibitory Concentration (MIC) was carried out according to Sánchez *et al*. (Sánchez *et al*., 2016) with slight modification in Eppendorf tubes. Briefly, 500 μl of different concentrations of extracts were prepared and transferred to each Eppendorf tube. Then, 100 μl of a fresh culture (approximately 10^6 CFU/ml) of test organisms was added. The tubes were incubated at 37^0C for 24 h for bacteria and at RT for 48 h for Fungi. MIC was defined as the lowest concentration of the test solution that restricted the visible growth

of microorganism tested. Sterile distilled water was used as negative control, whereas, standard antibiotics such as Ciprofloxacin, Amphotericin-B and Fluconazole were used as positive control.

5) Assessment of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

Assessment of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) was carried out according to Sánchez *et al*. (Sánchez *et al*., 2016) with slight modification. Briefly, to determine MBC/MFC, 12 μl from each concentration from MIC tubes was spotted on Mueller Hinton Agar (MHA) plates for Bacteria and on Sabouraud Dextrose Agar for Fungi and then the plates were incubated at 37^0C for 24 h for bacteria and at RT for 48 h for Fungi. MBC/MFC was defined as the lowest concentration of the test solution showing no bacterial/fungal growth. Sterile distilled water was used as negative control, whereas, standard antibiotics such as Ciprofloxacin, Amphotericin-B and Fluconazole were used as positive control.

6) Evaluation of Antimicrobial Activity

Antimicrobial activity of Alum and *Sphatika Bhasma* was performed using the Agar-well diffusion assay according to Sánchez *et al*. (Sánchez *et al*., 2016) with slight modification. Briefly, fresh culture (approximately 10^6 CFU/ml) was uniformly spread onto Mueller-Hinton agar (MHA) and Sabouraud Dextrose agar plates by sterile loop for Bacteria and Fungi respectively. Then, inoculated plates were allowed to dry at room temperature for 20 min. After that, wells of 6mm in diameter were made in the agar using a sterilized cup-borer and 100 μl of each test solution in different concentrations (10, 50 & 100 mg/ml) was added in the wells. Sterile distilled water was used as Negative control. Plates were incubated at 37^0C for 24 h for Bacteria, whereas, plates were incubated at RT for 48 h for Fungi. Ciprofloxacin, Amphotericin-B and Fluconazole were used as positive control. Antimicrobial activity was evidenced by the presence of clear inhibition zone around each well. The diameter of this zone was measured and recorded. Experimental results were expressed as Mean ± standard deviation (SD) for analysis performed in duplicate.

7) Combinatorial Effect on Bacteria

Combinatorial effect of Alum (10 mg/ml), *Sphatika Bhasma* (10 mg/ml) and Ciprofloxacin- standard Antibiotic (2 mg/ml) was evaluated using the Agar-well diffusion assay as stated earlier. Following different Combinations were prepared in 1:1 ratio and their effect was tested against 8 bacteria as stated earlier.

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)

8) Combinatorial Effect on Fungi

Combinatorial effect of Alum (10 mg/ml), *Sphatika Bhasma* (10 mg/ml) and standard Antibiotics such as Amphotericin-B (5 mg/ml) & Fluconazole (2 mg/ml) was evaluated using the Agar-well diffusion assay. Following different Combinations were prepared in 1:1 ratio and their effect was tested against 2 fungi as stated earlier.

1. Alum + *Sphatika Bhasma* (AL + SB)
2. Alum + Amphotericin-B (AL + AM)
3. *Sphatika Bhasma* + Amphotericin-B (SB + AM)
4. Alum + *Sphatika Bhasma* + Amphotericin-B (AL + SB + AM)
5. Alum + Fluconazole (AL + F)
6. *Sphatika Bhasma* + Fluconazole (SB + F)
7. Alum + *Sphatika Bhasma* + Fluconazole (AL + SB + F)
8. Alum + *Sphatika Bhasma* + Amphotericin-B + Fluconazole (AL + SB + AM + F)
9. Amphotericin-B + Fluconazole (AM + F)

9) DPPH radical-scavenging assay

The free radical scavenging activities of Alum and *Sphatika Bhasma* were measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay with slight modification (Nikhat *et al*., 2009). For this, 0.1 ml of DPPH solution (0.1mM) in methanol was added to 0.1 ml of different concentrations of Alum and *Sphatika Bhasma* in a microplate. After incubating for 30 minutes in dark, the absorbance was measured at 517nm using Multimode Reader (Synergy HT, BioTek). Ascorbic acid at various concentrations (0.4 to 4 µg/ml) was included as a standard. Negative control without Alum and *Sphatika Bhasma* was set up in parallel. The percent DPPH-

scavenging activity was calculated as, DPPH scavenged (%)= $A_{Negative\ control} - A_{Test}/A_{Negative\ control}$ X 100. Where, A is absorbance. The antioxidant activities were also expressed as IC_{50} values. Experimental results were expressed as Mean ± standard deviation (SD) for analysis performed in triplicate. Means, standard deviations and IC_{50} values were calculated using a Microsoft Excel program.

10) Phenolic Content Estimation

The total phenolic content of Alum and *Sphatika Bhasma* was determined using Folin-Ciocalteu reagent according to the method of Pandima Devi *et al*. (Pandima Devi *et al*., 2008) with slight modification. Gallic acid at various concentrations (2-20 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 phenolic content was calculated from the equation ($y= 0.0062x – 0.0014$, $R^2 = 0.9828$) obtained from the Gallic acid standard curve. The total phenolic content was expressed as Gallic acid equivalent (GAE) in mg/g of dry sample. Experimental results were expressed as Mean for analysis performed in triplicate.

Results:-

Table 1:- Minimum Inhibitory Concentration (MIC).

Sr. No.	Organisms	MIC (mg/ml)	
		Alum	***Sphatika Bhasma***
1	*E. coli* (ATCC-10148)	100	80
2	*K. pneumoniae* (ATCC-700603)	2	1
3	*P. aeruginosa* (Fisher's Immuno Type IV)	2	1
4	*S. typhi* (NCTC-786)	5	5
5	*B. subtilis* (ATCC-9372)	>100	>100
6	*E. faecalis* (ATCC-29212)	4	4
7	*MRSA* (ATCC-25923)	60	40
8	*S. lutea* (NCTC-2470)	2	1
9	*C. albicans* (ATCC-10231)	>100	>100
10	*A. niger* (ATCC-16404)	>100	>100

Table 2:- Antibacterial Activity against *E. coli* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	9 ± 0
50	14.5 ± 0.7	15 ± 0
100	18 ± 0	20 ± 0

Table 3:- Antibacterial Activity against *K. pneumoniae* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	NIL
50	NIL	NIL
100	17.5 ± 0.7	20.5 ± 0.7

Table 4:- Antibacterial Activity against *P. aeruginosa* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	12 ± 0
50	17 ± 0	20 ± 0
100	21.5 ± 0.7	25 ± 0

Table 5:- Antibacterial Activity against *S. typhi* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	NIL
50	11 ± 0	14 ± 0
100	15.5 ± 0.7	20 ± 0

Table 6:- Antibacterial Activity against *B. subtilis* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	9 ± 0
50	12 ± 1.4	15 ± 0
100	16.5 ± 0.7	19 ± 0

Table 7:- Antibacterial Activity against *E. faecalis* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	10 ± 0
50	15 ± 0	16 ± 1.4
100	18.5 ± 0.7	21 ± 1.4

Table 8:- Antibacterial Activity against *MRSA* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	NIL
50	14 ± 0	15.5 ± 0.7
100	17 ± 0	19.5 ± 0.7

Table 9:- Antibacterial Activity against *S. lutea* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	10 ± 0	10 ± 0
50	16 ± 0	18.5 ± 2.1
100	19 ± 1.4	25 ± 0

Table 10:- Antifungal Activity against *A. niger* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	NIL
50	NIL	NIL
100	NIL	NIL

Table 11:- Antifungal Activity against *C. albicans* by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	Alum	***Sphatika Bhasma***
10	NIL	NIL
50	NIL	15 ± 0

100	15.1 ± 0.1	20 ± 0

Table 12:- Antibacterial Activity of Ciprofloxacin (Standard) by Agar Well Diffusion Method.

No.	Organism	Zone of Inhibition (mm) [Mean ± SD]
1	*E. coli* (ATCC-10148)	13 ± 1.4
2	*K. pneumoniae* (ATCC-700603)	20 ± 0
3	*P. aeruginosa* (Fisher's Immuno Type IV)	15.5 ± 0.7
4	*S. typhi* (NCTC-786)	18.5 ± 4.9
5	*B. subtilis* (ATCC-9372)	45 ± 0
6	*E. faecalis* (ATCC-29212)	45 ± 0
7	*MRSA* (ATCC-25923)	29 ± 5.7
8	*S. lutea* (NCTC-2470)	58 ± 0

Table 13:- Antifungal Activity of Standards by Agar Well Diffusion Method.

No.	Organism	Zone of Inhibition (mm) [Mean ± SD]	
		Amphotericin-B	Fluconazole
1	*A. niger* (ATCC-16404)	13 ± 0	NIL
2	*C. albicans* (ATCC-10231)	16 ± 0	56 ± 1.4

Table 14:- Combinatorial Effect on Bacteria.

Organisms	Zone of Inhibition (mm)			
	AL + SB	AL + CP	SB + CP	AL + SB + CP
E. coli (ATCC-10148)	Nil	Nil	Nil	Nil
K. pneumoniae (ATCC-700603)	Nil	Nil	Nil	Nil
P. aeruginosa (Fisher's Immuno Type IV)	10 ± 0	48 ± 0	49 ± 0	47 ± 0
S. typhi (NCTC-786)	Nil	Nil	Nil	Nil
B. subtilis (ATCC-9372)	4.5 ± 6.3	40 ± 0	39.5 ± 0.7	37.5 ± 2.1
E. faecalis (ATCC-29212)	4.5 ± 6.3	35.5 ± 0.7	34.5 ± 0.7	33.5 ± 0.7
MRSA (ATCC-25923)	4.5 ± 6.3	20.5 ± 0.7	18 ± 1.4	15.5 ± 0.7
S. lutea (NCTC-2470)	10 ± 0	50 ± 0	49 ± 0	45 ± 0

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

Table 15:- Combinatorial Effect on Fungi.

No.	Combinations	Zone of Inhibition (mm)	
		A. niger (ATCC-16404)	*C. albicans* (ATCC-10231)
1	AL + SB	NIL	20 ± 0
2	AL + AM	11 ± 0	12.5 ± 0.7
3	SB + AM	11 ± 0	15 ± 0
4	AL + F	NIL	32 ± 1.4
5	SB + F	NIL	34 ± 0

6	AM + F	10 ± 0	NIL
7	AL + SB + AM	12 ± 0	15.5 ± 0.7
8	AL + SB + F	NIL	29.5 ± 0.7
9	AL + SB + AM + F	11 ± 0	NIL

Note: AL- Alum
SB- *Sphatika Bhasma*
AM- Amphotericin B (Standard Antibiotic)
F- Fluconazole (Standard Antibiotic)

Table 16:- DPPH Scavenging Activity.

Test	Concentration	% DPPH Scavenged	IC_{50}
Alum	10 mg/ml	3.95 ± 2.3	143.15 mg/ml
	20 mg/ml	9.58 ± 0.9	
	40 mg/ml	17.08 ± 1.3	
	60 mg/ml	19.99 ± 1.0	
	80 mg/ml	29.15 ± 2.9	
	100 mg/ml	35.61 ± 2.1	
Sphatika Bhasma	1 mg/ml	6.79 ± 2.7	9.82 mg/ml
	2 mg/ml	10.31 ± 1.9	
	4 mg/ml	23.46 ± 1.9	
	6 mg/ml	31.35 ± 2.6	
	8 mg/ml	41.01 ± 1.5	
	10 mg/ml	50.43 ± 2.4	
Ascorbic Acid (Standard)	0.4 µg/ml	1.93 ± 1.3	2.89 µg/ml
	0.6 µg/ml	8.62 ± 0.9	
	0.8 µg/ml	11.64 ± 0.7	
	1 µg/ml	17.87 ± 2.0	
	2 µg/ml	38.99 ± 2.1	
	4 µg/ml	67.22 ± 1.8	

Table 17:- Phenolic Content Estimation.

Test	Gallic Acid Equivalent (mg/gm)
Alum	74.8
Sphatika Bhasma	150.6

Note: Mean of triplicate determinations

Discussion:-

Antimicrobial resistance is one of the major public health problems, especially in developing countries like India. To overcome the burden of antimicrobial resistance, greater attention has been paid to antimicrobial activity screening from natural products (Satardekar *et al*., 2022). 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, Traditional knowledge will serve as a powerful search engine and most importantly will greatly facilitate intentional, focused and safe natural products research to rediscover the drug discovery process (Patwardhan *et al*., 2004). 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).

In the present *in vitro* study, antimicrobial activity of Alum as well as its *Bhasma- Sphatika Bhasma* was evaluated against total 10 organisms, viz., 4 gram negative, 4 gram positive and 2 fungi using various assays such as MIC, MBC/MFC and Agar Well Diffusion Method.

Dilution methods are the most appropriate one for determination of Minimum Inhibitory Concentration (MIC) values. Either broth or agar dilution method may be used to quantitatively measure the *in vitro* antimicrobial activity against bacteria and fungi. Furthermore, MIC is defined as the lowest concentration of assayed antimicrobial agent that inhibits the visible growth of the microorganisms tested (Balouiri *et al*., 2016).

In the present *in vitro* study, MIC assay revealed broad spectrum activity of Alum as well as *Sphatika Bhasma* by inhibiting Gram Positive as well as Gram Negative organisms. Broad spectrum antibacterial activity of Alum as well as *Sphatika Bhasma* corroborates earlier findings (Amadi and Ngerebara, 2017; Sahoo *et al*., 2019).

However, MIC value of *B. subtilis* and Fungi (*A. niger* and *C. albicans*) were found to be higher than 100 mg/ml. Thus, Alum as well as *Sphatika Bhasma* exhibited moderate Antifungal activity. In general, MIC values of *Sphatika Bhasma* were lower than that of Alum [Table-1].

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) can be determined after broth dilution by using agar plates to confirm the antimicrobial potential of tested natural products. (Balouiri *et al*., 2016).

Accordingly, the observations of MIC were confirmed by Minimum Bactericidal Concentration (MBC) assay and Minimum Fungicidal Concentration (MFC) assay using Muller Hinton Agar (MHA) and Sabouraud Dextrose Agar plates respectively [photoes not included]. It is clear from the result that the inhibitory activity was found to be concentration dependent (as the concentration increased, growth decreased) especially against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, *E. faecalis*, *MRSA* and *S. lutea*.

Besides, Agar well diffusion method is widely used to evaluate the antimicrobial activity of natural products, wherein the agar plate surface is inoculated by spreading a volume of microbial culture over the entire agar surface. Then a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer and a volume of antimicrobial agent at desired concentration is introduced into the well. Then, the ager plates are incubated under suitable conditions depending upon the test microorganisms. The antimicrobial agent diffuses in the agar medium and inhibits the growth of microbial strain tested (Balouiri *et al*., 2016). It should be further noted that different components have different diffusion abilities through the selected medium. These differences may contribute to the strength of antimicrobial action of a particular natural product/extract (Wijenayake *et al*., 2016).

In the present study, three different concentrations (10, 50 and 100 mg/ml) of Alum as well as *Sphatika Bhasma* were evaluated for their antimicrobial effect using Agar Well Diffusion method against 10 organisms. *Sphatika Bhasma* revealed higher Zone of Inhibition than Alum against *E. coli* at the concentration of 100 mg/ml. *Sphatika Bhasma* also showed Zone of Inhibition at the concentration of 10 mg/ml, whereas, Alum did not show any inhibitory zone at the same concentration against *E. coli* [Table- 2].

Similarly, higher Zone of Inhibition was noted with *Sphatika Bhasma* against *K. pneumoniae* than Alum. However, both *Sphatika Bhasma* as well as Alum did not show any inhibitory zones at the concentrations of 10 and 50 mg/ml respectively [Table-3].

Likewise, *Sphatika Bhasma* exhibited higher Zone of Inhibition at the concentration of 100 mg/ml against *P. aeruginosa*, *S. typhi*, *B. subtilis*, *E. faecalis*, *MRSA*, *S. lutea* and *C. albicans* in comparison to Alum [Table-4 to Table-9 and Table-11]. *Sphatika Bhasma* even displayed inhibitory zone at the concentration of 10 mg/ml against *P. aeruginosa*, *B. subtilis* and *E. faecalis*, wherein Alum was ineffective against the same organisms at 10 mg/ml. Furthermore, *Sphatika Bhasma* exhibited highest zone of inhibition against *P. aeruginosa* as well as *S. lutea* (25 mm) at the concentration of 100 mg/ml. However, Alum as well as *Sphatika Bhasma* did not show any Zone of Inhibition at any given concentration against *A. niger* [Table-10]. Thus, Alum and *Sphatika Bhasma* again revealed strong antibacterial and moderate antifungal activities through Agar Well Diffusion assay as well. The bactericidal effect of alum could be due to reduction in pH or deleterious effects on bacterial cell wall (Amadi, 2020). Besides, usually fast-growing microbial species are said to have more susceptibility to antimicrobial agents than slow

growing microorganisms. For example, a fungus requires more time to proliferate and hence, killing requires additional time than the bacterial species. At the same time drug permeability through the fungal membrane are much slower than the bacterial membranes (Wijenayake *et al*., 2016). This could be the explanation for the moderate or no activity noticed against the fungal strains included in the present study.

The standard antibiotic Ciprofloxacin which was included as a positive control in the present study displayed highest zone of inhibition against *S. lutea* and the inhibitory activity was further noted in the order of *S. lutea* > *B. subtilis* = *E. faecalis* > *MRSA* > *K. pneumoniae* > *S. typhi* > *P. aeruginosa* > *E. coli* at the concentration of 2 mg/ml [Table-12].

Fluconazole, a standard antifungal showed no zone of inhibition against *A. niger*. However, it showed the highest zone of inhibition against *C. albicans* at the concentration of 2 mg/ml [Table-13]. Another standard antifungal Amphotericin-B displayed the highest zone of inhibition (16 mm) against *C. albicans* at the concentration of 5 mg/ml. However, it also showed zone of inhibition (13 mm) against *A. niger* at the same concentration [Table-13].

Natural antimicrobial agents can act alone or in combination with standard antibiotics to enhance antimicrobial activity against a wide range of microbes. Some of these active compounds show both intrinsic antibacterial activity and antibiotic resistance-modifying activities and some of them while not effective as antimicrobials by themselves, when combined with antibiotics, can help overcome antibiotic resistance (Vaou *et al*., 2021).

Hence, in the present *in vitro* study, four different combinations prepared from Alum (AL), *Sphatika Bhasma* (SB) and Ciprofloxacin (CP)- standard antibiotic were subjected to determine their combinatorial effect against 8 bacterial species using Agar Well Diffusion Assay. The combinations were labelled as (AL + SB), (AL + CP), (SB + CP) and (AL + SB + CP). Among these 4 combinations, none of the combinations displayed Zone of Inhibition against *E. coli*, *K. pneumoniae* and *S. typhi* [Table-14]. However, Ciprofloxacin showed Zone of Inhibition at the concentration of 2 mg/ml against *E. coli*, *K. pneumoniae* and *S. typhi* when tested individually. Whereas, *Sphatika Bhasma* revealed moderate zone of inhibition at the concentration of 10 mg/ml when tested individually only against *E. coli*. However, Alum was not effective at the concentration of 10 mg/ml against *E. coli*, *K. pneumoniae* and *S. typhi*, as it did not show zone of inhibition against them when tested individually. Hence, it could be concluded that antagonistic effect of combinations was noted against *E. coli*, *K. pneumoniae* and *S. typhi*.

With combination (AL + CP), the highest zone of inhibition was noted against *S. lutea*. (SB + CP) combination showed the highest zone of inhibition against *P. aeruginosa* as well as *S. lutea*, whereas, the combination (AL + SB + CP) exhibited the highest zone of inhibition against *P. aeruginosa*. In general, the combination (AL + SB) displayed smaller zones of inhibition against *P. aeruginosa*, *B. subtilis*, *E. faecalis*, *MRSA* and *S. lutea*.

In case of *P. aeruginosa*, combinations (AL + CP), (SB + CP) and (AL + SB + CP) showed almost four times bigger zone of inhibition in comparison to that shown by *Sphatika Bhasma* (SB) alone [Table-4] at the concentration of 10 mg/ml. Furthermore, they showed three times bigger zone of inhibition in comparison to Ciprofloxacin (CP) alone at the concentration of 2 mg/ml. However, Alum did not show zone of inhibition against *P. aeruginosa* when tested at the concentration of 10 mg/ml individually. Thus, the combinations (AL + CP), (SB + CP) and (AL + SB + CP) displayed synergistic effect against *P. aeruginosa*.

In case of *B. subtilis*, zones of inhibition observed with combinations (AL + CP), (SB + CP) and (AL + SB + CP) were four times bigger than that observed with *Sphatika Bhasma* (SB) independently [Table-6]. Furthermore, they displayed little smaller zones of inhibition in comparison to Ciprofloxacin alone. However, Alum did not show zone of inhibition against *B. subtilis* when tested at the concentration of 10 mg/ml individually. Hence, the activity of these combinations against *B. subtilis* could be mainly due to Ciprofloxacin.

Similar effects were noted against *E. faecalis*. The zones of inhibition observed with combinations (AL + CP), (SB + CP) and (AL + SB + CP) were three times bigger than that observed with *Sphatika Bhasma* (SB) independently [Table-7]. However, they displayed little smaller zones of inhibition in comparison to Ciprofloxacin alone. Thus, the activity of these combinations against *E. faecalis* could be mainly due to Ciprofloxacin.

In case of *MRSA*, Alum and *Sphatika Bhasma* did not show any zone of inhibition individually at the concentration of 10 mg/ml. However, combinations (AL + CP), (SB + CP) and (AL + SB + CP) showed moderate zones of inhibition. However, these combinations displayed smaller zones of inhibition in comparison to Ciprofloxacin alone.

In case of *S. lutea*, the zones of inhibition observed with combinations (AL + CP), (SB + CP) and (AL + SB + CP) were almost five times bigger than that shown by Alum (AL) and *Sphatika Bhasma* (SB) independently at the concentration of 10 mg/ml [Table-9]. However, they displayed little smaller zone of inhibition in comparison to Ciprofloxacin alone. Hence, activity could be mainly due to Ciprofloxacin.

Overall, synergistic effects of combinations (AL + CP), (SB + CP) and (AL + SB + CP) were noted mainly against *P. aeruginosa*.

Moreover, in the present *in vitro* study, nine different combinations prepared from Alum (AL), *Sphatika Bhasma* (SB), standard antifungals- Amphotericin-B (AM) and Fluconazole (F) were evaluated for their combinatorial effect against 2 fungal species using Agar Well Diffusion Assay [Table-15]. More or less similar inhibitory activity was noted against *A. niger* with combinations (AL + AM), (SB + AM), (AM + F), (AL + SB + AM) and (AL + SB + AM + F) when compared to activity of Amphotericin-B alone. Hence, the inhibitory activity could be due to the standard antifungal Amphotericin-B, as Alum, *Sphatika Bhasma* and Fluconazole did not show any inhibitory activities against *A. niger* individually.

In case of *C. albicans*, the highest zone of inhibition was noted with (SB + F) combination. The zone of inhibition was found to be bigger than the Alum and *Sphatika Bhasma* alone; could be synergistic effect. However, it displayed smaller zone of inhibition in comparison to Fluconazole alone. Furthermore, combinations (AL + SB), (AL + F), (SB + F) and (AL + SB + F) did not show any zones of inhibition against *A. niger*. Likewise, combinations (AM + F) and (AL + SB + AM + F) did not show any zone of inhibition against *C. albicans*. Nevertheless, it is important to note here that Amphotericin-B and Fluconazole exhibited decent inhibitory activity against *C. albicans* when tested individually. Thus, combinations (AM + F) and (AL + SB + AM + F) showed antagonistic effect against *C. albicans*.

Overall, Alum and *Sphatika Bhasma* exhibited strong Antibacterial activity against gram positive as well as gram negative organisms and moderate Antifungal activity revealed through MIC, MBC/MFC and Agar well Diffusion Assays.

Antioxidants are becoming ever more interesting to scientists in food field and medicinal professionals due to their protective roles in food against oxidative deterioration and in the body against oxidative stress-mediated pathological processes (Munteanu and Apetrei, 2021). Antioxidants regulate various oxidative reactions naturally occurring in tissues. Furthermore, they terminate or retard the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as electron donors. In general, *in vitro* antioxidant tests using free radical traps are relatively straightforward to perform. Among free radical scavenging methods, DPPH method is furthermore rapid, simple (not involved with many steps and reagents) and inexpensive in comparison to other test models. DPPH method is based on the reduction of DPPH a stable free radical. The free radical DPPH gives a maximum absorption at 517nm (purple colour). When antioxidants react with DPPH, the stable free radical becomes paired off in presence of a hydrogen donor and is reduced to DPPH-H and as a consequence the absorbance decreases from the DPPH radical to the DPPH-H form, resulting in decolorization (yellow colour). More the decolorization more is the reducing ability. The test has been the most accepted model for evaluating free radical scavenging activity of any new drug. Furthermore, the conventional cuvette assay can be replaced by 96-well plate assay (Dontha, 2016).

In the present *in vitro* study, the antioxidant potential of Alum and *Sphatika Bhasma* was detected using DPPH assay. The antioxidant activity of *Sphatika Bhasma* was found to be far better than that of Alum. The IC_{50} value for *Sphatika Bhasma* was found to be 9.82 mg/ml, whereas, for Alum it was found to be 143.15 mg/ml [Table-16].

As evidenced, phenolic compounds contribute to antioxidative action through various mechanisms, viz., scavenging free radicals, by stabilizing lipid peroxidation, through redox properties and by chelating metals (Lakshmi *et al*., 2005; Banerjee *et al*., 2008). Hence, total phenolic content of Alum and *Sphatika Bhasma* was determined by Folin-Ciocalteu method. Phenolic content of *Sphatika Bhasma* was noted to be higher than that of Alum [Table-17].

Many natural products possessed Antimicrobial activity as well as Antioxidant potential *in vitro* (Angiolella *et al*., 2018; Kusuma *et al*., 2014). Phenolic compounds are a very important class of antioxidants as it directly affects bacterial growth and can cause hindrance in their pathogenic activity. The mechanism of action of antioxidants as antibacterial is still not fully understood but three basic mechanisms could be, outer membrane permeability, cytoplasm leakage and inhibition of nucleic acid formation. The antibacterial activity of polyphenols may also be due to the capacity of these compounds to chelate iron, vital for the survival of almost all bacteria. Polyphenols rupture the wall, increase the permeability of cytoplasm membrane and release lipopolysaccharides. Moreover, bacterial cells have negative surface charge because of the ionic groups. The exposure to phenolic compounds decreases this charge and the transport of solutes. Excess of phenolic compounds cause hyper acidification that makes the cytoplasm acidic and denatures the proteins present in the cytoplasm. So, the damage to membrane by acidification potentially explains the activity of phenolic compounds (Naqvi *et al*., 2019). Phenolic compounds are diverse group of bioactive metabolites that include flavonoids, flavones, flavanols, quinones and tannins. These compounds showed diverse mechanisms of action against different microbial strains as stated above.

In the present *in vitro* study, Antimicrobial activities and Antioxidant potential revealed by Alum and *Sphatika Bhasma* could be attributed to their Phenolic content.

Overall, *Sphatika Bhasma* exhibited better Antimicrobial and Antioxidant activities than Alum. It has been observed that these activities were proportional to phenolic content of *Sphatika Bhasma* as *Sphatika Bhasma* exhibited the highest phenolic content.

Thus, Alum and its *Bhasma*, *Sphatika Bhasma*, are the new hope to combat the dangerous threats posed by increasing evidence of antimicrobial resistance. However, their molecular mechanism underlying antibacterial actions need to be further investigated and identified.

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