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Evaluation of Antimicrobial Activity of ZITRITIDE, A Natural and Organic Antimicrobial Fogging Solution with Special Reference for Infection Prevention and Control in Hospital Environments and All Other Clean Room Facilities

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A B S T R A C T

The aim of the present study was to evaluate the antimicrobial efficacy of a natural and organic fogging solution, ZITRITIDE where *Citrus aurantium* Amara extract (Bioflavonoid Complex) acts as an active ingredient. The antimicrobial efficacy of the formulation at two different concentrations (0.2% and 0.5%) was assessed against eighteen bacteria, and four fungi prevalent in hospital and industrial environment. Both the concentration worked efficiently on gram positive, gram negative, spore forming anaerobic, and spore forming aerobic microorganisms. At 0.2% concentration *Mycobacterium tuberculosis* and *Methicillin Resistant Staphylococcus aureus* (MRSA) showed 2.43 log reduction and 2.05 log reduction respectively. Eleven bacteria of the group demonstrated 4 log reductions (99.99% killing efficiency) and 3 log reductions were achieved by four bacteria. At 0.5% product concentration 10 bacteria showed 99.99% killing efficiency with 4 log reduction value. The killing efficiency of the product for MRSA and *Mycobacterium tuberculosis* is 99% i.e. 2 log reduction at 0.5%. Antifungal activity at 0.2% and 0.5% concentration was highest for *Aspergillus flavus* with efficiency percent of 99.9999 i.e. 6 log reduction followed by *Aspergillus niger* (99.999), *Penicillium* species (99.99%) and *Candida albicans* (99.9%). Application of ZITRITIDE as a fumigant in clean room areas revealed 98%-100% reduction in bacterial count and 86%-100% reduction in fungal count in controlled areas and 80%-95% reduction in bacterial count and 89%-100% fungal count in uncontrolled areas. The ZITRITIDE was also fogged in hospital environment and found to be effective. The results demonstrated ZITRITIDE is quite effective in controlling hospital acquired infections (HAI). Being nontoxic and eco-friendly nature of the active ingredients, the advantage of ZITRITIDE over other chemical fumigant was that it can be fogged in the presence of personnel working in clean room areas and also in the presence of doctors, nurses, other clinical, non clinical professionals, patients, attendants, visitors and supporting staffs in hospitals.

Introduction

The present study focused on a 100% natural and organic antimicrobial solution for fogging/fumigating in all types of healthcare facilities and clean room environments.

The active ingredient in the product is derived from the peels of *Citrus aurantium* Amara (Bitter orange) extract (Bioflavonoid Complex). The potential for inadvertent

exposure of chemical fumigant to people and damage to surface or equipment is well known but due to lack of a suitable natural alternative, chemical fumigants are used worldwide. Thus the present study originated from the idea to minimize/eliminate the use of toxic chemical fumigants in all healthcare facilities and clean room environments. The product has been developed pro-actively to eliminate the incidences of microbial diseases which have become immune to chemical based alternatives in hospitals and many other public environments. The intended application is with special reference to infection prevention and control in hospital environments that consists of high risk to low risk areas and other healthcare facilities under various settings including Intensive care units (Neonatal ICU, Pediatric ICU, ICU's Cardio Thoracic Vascular Surgery, Respiratory infections (H1N1 units), Operation Theatres, Dialysis Unit, Burns Unit, Transfusion services unit, Central Sterile Services Department, Patient wards, Out Patient departments and so on.

The local name of the *Citrus aurantium* subsp. Amara is bitter orange and belongs to the family *Rutaceae.* It is a spiny evergreen tree and locally available in India. The common name in India is Narangi. Citrus plant is native to tropical Asia but it is also found in all tropical and subtropical countries. Phenethylamine alkaloids, octopamine, synephrine, tyramine, N-methyltyramine and hordenine are the most important bioactive constituents of *Citrus aurantium* fruits. In addition it is also rich in volatile oil, vitamin C, and flavonoids (hesperidine, naringin) and having beneficial effects on human health (Pellati *et al.,* 2002). Because of its increased use in various chronic and acute diseases, *Citrus aurantium* attains more research attention. Other uses include the uses of *Citrus aurantium* essential oil in foods, perfumes and also used in herbal medicines as a stimulant and appetite suppressant. In traditional Chinese medicine, it is used to treat nausea, indigestion, constipation, cancer, and cardiovascular effect.

C.aurantium essential oil contains linalool and limonenes (fragrant substance) that have antianxiety and sedative effects (Carvalho-Freitas *et al.,* 2002). Antidepressant-Synephrine-rich *Citrus aurantium* extracts have antidepressant effects (Song *et al.,* 1996). The whole *C. aurantium* peel contains citral, limonene, and several citrus bioflavonoid, including hesperidin, neohesperidin, naringin, and rutin. Evidence suggests that these substances also have antiviral effect (Song *et al.,* 1996).

The best method to kill or inactivate a micro organism, such as bacteria, fungi or a virus before it reaches a human cell is by using an effective antimicrobial agent, that is non-toxic to humans and animals, but toxic to any or all micro-organisms. Fogging is a sterilization technique that uses a special machine to create a mist which eliminates all pathogens, even ones that cannot be reached by conventional cleaning. Chemical based fogging solution generates many health related problems to the people/staff involved in fogging activity. Being so small, these particles remain suspended in the air as aerosols for long time and thus able to kill any airborne microbial contamination that they come to contact with. Gradually these particles settle onto all surfaces, even the areas never touched by conventional cleaning. Fogging solution continues to work and kill any surface contamination. Also there is a possibility that after few hours of fumigation, these aerosols remain in the air and inhaled by patients, staffs, doctors and all personnel involved in the room unknowingly. This may create a health problem in the long run if the solution is chemical based. Residual effect of chemical fogging solution is harmful. One of

the major concerns of the chemical based solution is the generation of resistant strains of microorganisms which are often fatal to the society. Examples include Methicillin Resistant *Staphylococcus aureus*, Vancomycin Resistant *Enterococus faecium*, and Gentamicin Resistant Gram Negative Bacteria that are always associated with Hospital Acquired Infection. But if the solution is in natural and organic form and at the same time it is good enough to destroy pathogenic microorganisms like chemical alternatives, then all the above concerns can be addressed with confidence.

Thus the main objective of this study is to analyze and report the antimicrobial efficacy of a natural and organic product where the active ingredient is derived from *Citrus aurantium* Amara extract (Bioflavonoid Complex) with the brand name of ZITRITIDE for application in hospital environments combating hospital acquired infections and other clean room facilities as well. Being nontoxic and eco-friendly nature of the active ingredients, the advantage of ZITRITIDE over other chemical fumigant is that it can be fogged in the presence of personnel working in clean room areas and also in the presence of doctors, nurses, other clinical, non clinical professionals, patients, attendants, visitors and supporting staffs in hospitals.

To our knowledge this is the first of its kind of natural and organic fogging solution where the antimicrobial efficacies against a broad spectrum of microorganisms are reported. In this study the antimicrobial efficacy of the ZITRITIDE (an antimicrobial fogging solution) has been evaluated against a broad spectrum of microorganisms that includes bacteria and fungi. In addition ZITRITIDE solution was evaluated practically in a clean room manufacturing environment and hospital during working hours.

Materials and Methods

Preparation of active ingredient for ZITRITIDE

ZITRITIDE is prepared from the superconcentraed solution that is derived from the extracts of Bitter orange (*Citrus aurantium*) (bioflavonoid complex).

The active ingredients are polymethoxy flavonoids (PMFs), (nobiletin and tangeretin) found in rich quantities in the peel of citrus fruits. PMFs were extracted from the peels of *Citrus aurantium* to make *Citrus aurantium* Amara extract (CAE) (Damián-Reyna *et al.*, 2015).

Preparation of dilution/concentration

The super concentrate is diluted to 1% with active ingredient concentration of 20% volume/volume (v/v). This is marked as stock solution. The stock solution is further diluted to 0.2% (4%v/v) and 0.5% (10%v/v) respectively with demineralized water.

Identification of microorganisms based on product application

The microorganisms involved were identified based on the application of the product and listed out. Then the antimicrobial activity of each product concentration against each microorganism was assessed as per section 2.4.

Assessment of antimicrobial activity using a time-kill procedure

The scope of this protocol is to measure the biocidal potential of a liquid antimicrobial formulation using a time-kill procedure (ASTM E2315).

Preparation of microbial culture

All the bacteria were grown on nutrient broth/or specific broth media up to 24h to 48h at 37 ^{O}C depending upon the test conditions (Table 1). For initial bacterial count, a saline control test tube (9mL) was spiked with 1mL of bacterial culture and enumerated by pour plate technique in nutrient agar and/or specific media wherever required. Fungi were cultivated in different media and cultivation conditions (Table 1a).

For testing the fungal culture, a spore preparation from a saline wash was used. For testing the test product 9 mL of product were inoculated with 1mL of each microbial culture separately, vortexed for 2 min. immediately. Each tube was kept for the specified contact time. After specified contact time, 1mL of sample mixture were taken and enumerated by pour plate technique. Further dilutions were made wherever necessary.

All the experiments were performed in duplicate. Log_{10} values of each count were calculated and the difference from the initial Log_{10} value was reported. Efficiency percent/percent difference was interpreted from the Table 2.

Application of zitritide for fumigation in clean room facility

ZITRITIDE was used at ten different locations of the clean room facility. With 5% ZITRITIDE the bacterial count and fungal count reduction was reported in the table (Table 9). In clean room area and controlled area the solution achieved 98%-100% reduction in bacterial count whereas reduction in fungal count was achieved in a range of 86%-100%. In uncontrolled area 80%-95% reduction in bacterial count was achieved whereas 89%-100% reduction was observed in fungal count.

Application of ZITRITIDE fogging solution in a clean room environment

For practical application 50mL of 1% ZITRITIDE was diluted with 950 mL of normal water to make it 5% solution of ZITRITIDE. This was used at ten different locations of the clean room facilities. For fumigation Biostar^{TM} ULV fogger machine was used as per manufacturer's instruction (Table 10).

Fumigation experiment protocol

Plates were prepared for settle plate exposure for fumigation requirement. Soyabean Casein Digest Agar for bacteria and Sabroaud Dextrose Agar media plates for fungi were prepared, marked and kept aside. Before fumigation (Pre-Fumigation) plates were exposed in different locations for 10 minutes. The fumigation was done at different locations as mentioned in the table (Table 9). After 20 minutes of fumigation (Post-fumigation) the petridishes were again exposed at different locations for 10 minutes. All the petridishes were collected and incubated at respective incubators (37 ^{o}C for 24 to 48 hour for bacteria and 25 ^{o}C for 5 to 7days for fungi). All the results were recorded.

Results and Discussion

Identification of microorganisms

All possible sites of applications for the product were identified and the microorganisms' presence in the particular site was listed in the table (Table 3 and Table 4). These microorganisms are generally prevalent in hospital and other environments. This includes high risk areas in hospitals under various settings including Respiratory infections (H1N1 units), Cardiothoracic surgery units, Intensive care units (Neonatal

ICU, Pediatric ICU, ICU's), Vascular Surgery, Operation Theatres, Dialysis Unit, Burns Unit, Transfusion services unit, Central Sterile Services Department, Patient wards, outpatient departments and so on.

Assessment of antimicrobial activity

At 0.2% concentration the product showed highest log reduction (5.03) for *Serratia marcescens*. The concentration works efficiently on gram positive, gram negative, spore forming anaerobic, and spore forming aerobic microorganisms.

Mycobacterium tuberculosis and MRSA showed 2.43 log reduction and 2.05 log reduction respectively. 4 log reductions (99.99% killing efficiency) were achieved by 11 bacteria of the group whereas 3 log reductions were achieved by four microorganisms (Table 5).

At 0.5% product concentration out of 18 bacteria, 10 bacteria showed 99.99% killing efficiency with 4 log reduction value.

Five bacteria of the group demonstrated 3 log reduction i.e 99.9% killing efficiency. The killing efficiency of the product for MRSA and *Mycobacterium tuberculosis* is 99% i.e. 2 log reduction (Table 7).

At 0.2% concentration the antifungal activity was highest for *Aspergillus flavus* with efficiency percent of 99.9999 i.e. 6 log reduction followed by *Aspergillus niger* (99.999), *Penicillium* species (99.99%) and *Candida albicans* (99.9%) (Table 6).

At 0.5% concentration, the antifungal activity of ZITRITIDE is 5.3222 log reduction followed by *Aspergillus niger* (5.3374), *Penicillium* species (99.99) and *Candida albicans* (99.9) (Table 8).

Zitritide combating Hospital Acquired Infection (HAI)

The prevalence of pathogens in hospitals which are usually involved in hospital based infections is taken into consideration in this study. 2 log reduction (99%) for MRSA in 10 min were observed whereas for Vancomycin Resistant *Enterococcus faecium*, *E.coli*, *S. aureus*, *P. aeruginosa* 4 log reduction (99.99%) were observed. The product's applications on HAI associated with fungi were also studied. *Aspergillus flavus* showed highest i.e. 6 log reduction in 5 minutes. *Aspergillus niger* is the next fungi in which the product application showed 5 log reductions. Upon *Candida albicans* 3 log reduction was observed with a contact time period over 15 mins (Figure1 and Figure 2). At both the product concentration, the effect on bacteria and fungi are same.

Application of zitritide for fumigation in hospital environments

Preliminary tests and trials have been conducted by the Dept. of Microbiology, Trivendrum Medical College, and ZITRITIDE's favorable report has been obtained after testing of the effectiveness of the pathogenic microorganisms (Sarala Devi, Personal Communication).

ZITRITIDE being a water-based broad spectrum anti-microbial product with 100% natural and organic ingredient can be fogged to control the levels of environmental microorganisms. It does not require heating and does not use any kind of chemical solvents or compounds or substances. The active ingredient in ZITRITIDE is derived from the peels of *Citrus aurantium* Amara extract (bioflavonoid complex).

Table.1 Media used for bacteria

Sl. No	Name of the Microorganisms	Media Used	Other Media/Solution Used for Analysis
1	*Acinetobacter* species	Leeds Acinetobacter Agar Base	Peptone Water, D/E Neutralizing Broth, Nutrient Agar, Chloramphenicol Yeast Dextrose agar, Blood Agar
2	*Bacillus cereus*	AK Agar No. 2 (Sporulating Agar)	
3	*Clostridium perfringenes*	Anaerobic egg agar Base	
4	*Clostridium sporogenes*	Anaerobic egg agar Base	
5	*Coagulase Negative Staphylococci*	Nutrient Agar	
6	*Enterococcus* species	Nutrient Agar	
7	*Escherichia coli*	EMB Agar	
8	*Klebsiella pneumoniae*	Mac Conkey Agar	
9	*Methicillin Resistant Staphylococcus aureus*	Nutrient Agar/Nutrient Broth	
10	*Mycobacterium tuberculosis*	Lowenstein-Jensen Medium	
11	*Proteus mirabilis*	Mac Conkey Agar	
12	*Proteus vulgaris*	Mac Conkey Agar	
13	*Pseudomonas aeruginosa*	Cetrimide Agar	
14	*Salmonella cholreasuis*	Bismuth sulphite Agar	
15	*Serratia marcescens*	Nutrient Agar	
16	*Staphylococcus aureus*	Baird Parker Agar	
17	*Streptococcus pyogenes*	Mannitol Salt Agar	
18	Vancomycin Resistant *Enterococcus faecium*	Vancomycin Resistant Enterococci (VRE) Agar	

Table.1a Media used for fungi

S. No.	Name of the Microorganisms	Media used for Analysis	Other Media/Solution Used for Analysis
1	*Aspergillus flavus*	Sabouraud Dextose Agar	Peptone Water, D/E Neutralizing Broth
2	*Aspergillus niger*	Sabouraud Dextose Agar	
3	*Candida albicans*	Corn Meal Agar/Potato Dextrose Agar	
4	*Penicillium sp.*	Sabouraud Dextose Agar	

Table.2 Efficiency percent for log reduction values

Log Difference	Efficiency Percent/Percent Difference
1 Log Reduction	90% Reduction
2 Log Reduction	99% Reduction
3 Log Reduction	99.9% Reduction
4 Log Reduction	99.99% Reduction
5 Log Reduction	99.999% Reduction
6 Log Reduction	99.9999% Reduction

Table.3 Zitritide - list of fungi

S. No.	Name of the Microorganisms	Applications
1	*Aspergillus flavus*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
2	*Aspergillus niger*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
3	*Candida albicans*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
4	*Penicillium sp.*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres

Table.4 Zitritide - list of bacteria

Sl. No	Name of the Microorganisms	Applications
1	*Acinetobacter* species	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
2	*Bacillus cereus*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
3	*Clostridium perfringenes*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
4	*Clostridium sporogenes*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
5	*Coagulase Negative Staphylococci*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
6	*Enterococcus* species	Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
7	*Escherichia coli*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
8	*Klebsiella pneumoniae*	Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
9	Methicillin Resistant Staphylococcus aureus	Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
10	*Mycobacterium tuberculosis*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
11	*Proteus mirabilis*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
12	*Proteus vulgaris*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
13	*Pseudomonas aeruginosa*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
14	*Salmonella cholreasuis*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
15	*Serratia marcescens*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
16	*Staphylococcus aureus*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
17	*Streptococcus pyogenes*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres
18	Vancomycin Resistant *Enterococcus faecium*	Fomites of Operating theatres, Patient rooms, Hospital Environments, Healthcare centres

Table.5 Assessment of antibacterial activity of ZITRITIDE @0.2% concentration

Sl. No	Name of the Bacteria	Initial Log_{10} Count	Contact Time	Final Log_{10} Count	Log_{10} Reduction Count	Efficiency percent (%)
1	*Acinetobacter* species NCIM 2886	6.415	5 Min	2.0414	4.3736	99.99
2	*Bacillus cereus* NCIM 2156	5.875	15 Min	2.857	3.018	99.9
3	*Clostridium perfringenes* NCIM *2677*	5.9868	10 Min	2.6232	3.3636	99.9
4	*Clostridium sporogenes* NCIM 2559	6.0414	10 Min	2.6128	3.4286	99.9
5	Coagulase negative Staphylococci MTCC 8924	6.813	10 Min	2.176	4.637	99.99
6	*Enterococcus species* NCIM 5253	6.531	30 Min	3.279	3.252	99.9
7	*Escherichia coli* NCIM 2065	6.8751	5 Min	2.6128	4.2623	99.99
8	*Klebsiella pneumoniae* NCIM 2957	7.447	5 Min	2.949	4.498	99.99
9	*Methicillin Resistant Staphylococcus aureus* MTCC 3610	5.978	10 Min	3.924	2.053	99
10	*Mycobacterium tuberculosis* MTCC300	5.732	5 Min	3.301	2.431	99
11	*Proteus mirabilis* NCIM 5296	6.7324	10 Min	2.3979	4.3345	99.99
12	*Proteus vulgaris* NCIM 2027	6.4914	10 Min	1.8451	4.6463	99.99
13	*Pseudomonas aeruginosa* NCIM 5029, ATCC 27853	7.643	10 Min	3.491	4.152	99.99
14	*Salmonella enterica* NCIM5256	6.5563	2 Min	1.6532	4.9031	99.99
15	*Serratia marcescens* NCIM 2919	6.7324	10 Min	1.699	5.0334	99.999
16	*Staphylococcus aureus* NCIM 5345, ATCC 6538	8.079	15 Min	3.477	4.602	99.99
17	*Streptococcus pyogenes* NCIM 2608	7.380	10 Min	2.881	4.499	99.99
18	*Vancomycin Resistant Enterococcus faecium* NCIM 5366	6.806	10 Min	2.788	4.028	99.99
Results are expressed as average values of two repeated experiments						

Table.6 Assessment of Antifungal Activity of ZITRITIDE @0.2% concentration

Sl. No	Name of the Fungi	Initial Log_{10} Count	Contact Time	Final Log_{10} Count	Log_{10} Reduction Count	Efficiency percent (%)
1	*Aspergillus flavus* NCIM 1316	6.7993	10 Min	1.0000	5.7993	99.9999
2	*Aspergillus niger* NCIM1317	6.9395	5 Min	1.6021	5.3374	99.999
3	*Penicillium species* NCIM 1108	6.1461	2 Min	1.6532	4.4929	99.99
4	*Candida albicans* NCIM 3100	5.5315	15 Min	2.3222	3.2093	99.9
Results are expressed as average values of two repeated experiments						

Table.7 Assessment of antimicrobial activity of ZITRITIDE @0.5% concentration

Sl. No	Name of the Bacteria	Initial Log_{10} Count	Contact Time	Final Log_{10} Count	Log_{10} Reduction Count	Efficiency percent (%)
1	Acinetobacter species NCIM 2886	6.415	10 Min	1.6532	4.7618	99.99
2	*Bacillus cereus* NCIM 2156	5.875	15 Min	2.756	3.119	99.9
3	*Clostridium perfringenes* NCIM 2677	5.9868	5 Min	2.3424	3.6444	99.9
4	*Clostridium sporogenes* NCIM 2559	6.0414	30 Min	2.6435	3.3979	99.9
5	Coagulase negative Staphylococci MTCC 8924	6.813	10 Min	2.041	4.772	99.99
6	*Enterococcus species* NCIM 5253	6.531	30 Min	3.362	3.169	99.9
7	*Escherichia coli* NCIM 2065	6.8751	5 Min	2.2553	4.6198	99.99
8	*Klebsiella pneumoniae* NCIM 2957	7.447	5 Min	2.699	4.748	99.99
9	Methicillin Resistant *Staphylococcus aureus* MTCC 3610	5.978	10 Min	3.785	2.192	99

10	*Mycobacterium tuberculosis* MTCC300	5.732	5 Min	2.820	2.913	99
11	*Proteus mirabilis* NCIM 5296	6.7324	10 Min	2.000	4.7324	99.99
12	*Proteus vulgaris* NCIM 2027	6.4914	5 Min	1.6628	4.8286	99.99
13	*Pseudomonas aeruginosa* NCIM 5029, ATCC 27853	7.643	10 Min	3.079	4.564	99.99
14	*Salmonella enterica* NCIM 5256	6.5563	2 Min	1.6532	4.931	99.99
15	*Serratia marcescens* NCIM 2919	6.7324	30 Min	1.6021	5.1303	99.999
16	*Staphylococcus aureus* NCIM 5345, ATCC 6538	8.079	10 Min	3.477	4.602	99.99
17	*Streptococcus pyogenes* NCIM 2608	7.380	10 Min	2.845	4.535	99.99
18	Vancomycin Resistant Enterococcus faecium NCIM 5366	6.806	10 Min	2.903	3.903	99.9
Results are expressed as average values of two repeated experiment.						

Table.8 Assessment of antifungal activity for ZITRITIDE @0.5% concentration

Sl. No	**Name of the Fungi**	**Initial Log_{10} Count**	**Contact Time**	**Final Log_{10} Count**	**Log_{10} Reduction Count**	**Efficiency percent (%)**
1	*Aspergillus flavus* NCIM 1316	6.7993	5 Min	1.4771	5.3222	99.9999
2	*Aspergillus niger* NCIM 1317	6.9395	5 Min	1.6021	5.3374	99.999
3	*Penicillium species* NCIM 1108	6.1461	2 Min	1.4771	4.6690	99.99
4	*Candida albicans* NCIM 3100	5.5315	15 Min	2.3222	3.2093	99.9
Results are expressed as average values of two repeated experiment.						

Table.9 Application of ZITRITIDE fogging solution in clean room facility

			Bacterial Colony Counts (In Cfu/Plate)			Fungal Count (In Cfu/Plate)		
Sl. No	**Area of Fumigation**	**Labelled as**	**Before Fumigation**	**After Fumigation**	**Percent Reduction (%)**	**Before Fumigation**	**After Fumigation**	**Percent Reduction (%)**
1	Biosafety Room (BS-MB)	(BS-MB)	6	<1*	**100**	3	<1*	**100**
2	Food Hall	FH	45	<1*	**100**	8	1	**88**
3	Primary Packing	PP	5	<1*	**100**	15	<1*	**100**
4	Quarantine Store, 43F	QS	13	<1*	**100**	1	<1*	**100**
5	Men's Washroom	MWR	207	11	**95**	7	1	**86**
6	Women's Washroom	WWR	55	10	**82**	TNTC (> 300)	3	**99**
7	Laser marking	LM	123	2	**98**	6	1	**100**
8	Production Area	PA	20	4	**80**	7	<1*	**100**
9	Pass Box, Production Area	PB1-PA	11	<1*	**100**	3	<1*	**100**
10	Humidity Chamber Room (Stability Room)	SR-MB	5	<1*	**100**	9	1	**89**

Results are expressed as average values of two repeated experiment
***No Microbial growth observed**
TNTC: Too Numerous to Count

Table.10 Manufacturer's instruction table for ULV fogger machine

Sl. No.	Room Sizes (m3/feet3)	Total Volume	Duration
1	30/1000	400 mL	5 mins
2	60/2000	800 mL	10 mins
3	90/3000	1200 mL	15 mins
4	120/4000	1600 mL	20 mins
5	150/5000	2000 mL	25 Mins
6	180/6000	2400 mL	30 mins

Figure I: Percent Efficiency of 0.2% ZITRITIDE for Microorganisms involved in Hospital Aquired Infections

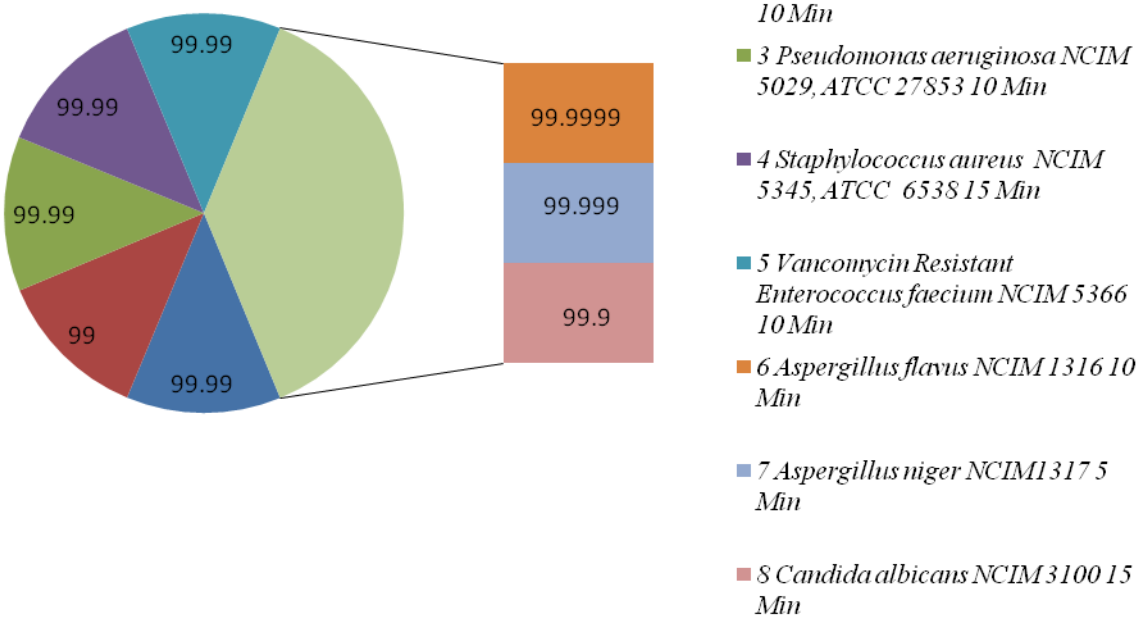
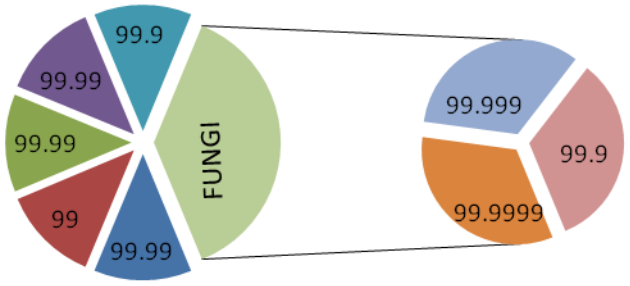


Figure II. Percent Efficiency of 0.5% ZITRITIDE for microorganisms involved in Hospital Acquired Infections



1 Escherichia coli NCIM 2065 5 Min

2 Methicillin Resistant Staphylococcus aureus MTCC 3610 10 Min

3 Pseudomonas aeruginosa NCIM 5029, ATCC 27853 10 Min

4 Staphylococcus aureus NCIM 5345, ATCC 6538 10 Min

5 Vancomycin Resistant Enterococcus faecium NCIM 5366 10 Min

6 Aspergillus flavus NCIM 1316 5 Min

7 Aspergillus niger NCIM1317 5 Min

8 Candida albicans NCIM 3100 15 Min

This study evaluated the Novel application of a natural and organic product (ZITRITIDE) derived from the above formulation as a fumigant/fogging solution in hospital environments. Infection control in any hospital or health care setting/facility is a very vital and important discipline concerned with preventing/controlling nosocomial and other healthcare associated infections/cross contaminations, to the patients, attendants, visitors, doctors, nurses and all other technical and non-technical support staff who practically come in contact with any or all such risks. The main advantage of ZITRITIDE over other chemical fumigants is that it was applied in the presence of doctors, nurses, other clinical, non clinical professionals, patients, attendants, visitors and support staffs and found to be effective (Sarala devi, Personal Communication).

As discussed in the results section the product acts efficiently with 18 bacteria and 4 fungi respectively prevalent in critical and high risk hospital departments. The product also works well for HAI pathogens at 0.2% and 0.5% concentration.

In a study *Citrus limonum* and *Citrus* aurantium essential oils (EOs) compared to 0.2% chlorohexidine (CHX) and 1% sodium hypochlorite (NaOCl) on multispecies

biofilms formed by *Candida albicans*, *Enterococcus faecalis* and *Escherichia coli*. *C. aurantium* EO and NaOCl inhibited the growth of all microorganisms in multi-species biofilms. 100% reduction of *C. albicans* and *E. coli*, and 49.3% reduction of *E. faecalis* were observed with *C. limonum* EO. A reduction of 68.8% of C. albicans and 86.7% of *E.coli* was observed in case of CHX and found to be less effective. Oliveria *et al* (2014) was observed the EOs was effective in controlling multi-species biofilms, and the antimicrobial activity of EOs was higher than those of CHX and NaOCl.

Usually the peels of the citrus fruits are considered to be the waste product of citrus processing industries. In the present study, the peel extracts of *Citrus aurantium* Amara were used as active ingredient in the product that exhibited inhibitory effect against eighteen (18) bacterial species and four fungi species. Many studies were reported regarding the antimicrobial efficacy of essential oils from peels and peel extracts of *Citrus* fruits. Madhuri *et al.,* (2014) evaluated antimicrobial efficacy of peel extracts of *Citrus aurantium* and *Citrus sinensis* against 3 bacteria by agar well diffusion assay. *K. pneumoniae* exhibited higher susceptibility to peel extracts whereas *B.cereus* was least affected. In our study we observed a 3 log reduction for *B.cereus* whereas 4 log reduction was shown in case of *K.pneumoniae. Bacillus cereus* being a gram-positive spore forming bacteria the efficacy might be less.

ZITRITIDE exhibited highest antifungal activity against *Aspergillus flavus* (6 Log Reduction), followed by *Aspergillus niger* (5 Log reduction). Upon penicillium species it showed 4 log reductions whereas on *C. albicans* it showed 3 Log reductions. It has been found that the peel extracts of *Citrus sinesis* significantly inhibited the growth of *Fusarium oxysporum* to higher extent when compared to leaf extract (OkWu 2007). *Citrus aurantium* peel extracts exhibited high antifungal activity (>50%) against *Candida capsici* as reported by Madhuri *et al.*, (2014).

ZITRITIDE also proved to be effective in combating nosocomial infections. In some critical areas the environment may be heavily contaminated with drug-resistant pathogens like MRSA, *Klebsiella pneumoniae*, *Acinetobacter* species and *Pseudomonas aeruginosa*. As reported by Taneja *et al.,* (2005), 27.3% of the environmental surfaces of various ICUs and emergency wards showed contamination of *Staphylococcus aureus*, and 30% of these were MRSA. In a MRSA outbreak study, Singh *et al.,* (2012) observed the contamination of MRSA in inanimate objects like medicine trolleys, the patient's cabinets, and railing of the beds, the nurses lockers, electric switches and door handles. It was emphasized in several studies that routine cleaning and hand washing alone were not sufficient to control the prolonged outbreaks of MRSA, but proper disinfection was required (Blythe *et al.,* 1998; Rampling *et al.,* 2001). Thus proper disinfection with an effective natural and organic solution is always better than chemical alternatives.

The significant killing efficiency of ZITRITIDE upon MRSA and other pathogens associated with Hospital Acquired Infections proved to be an effective fumigant and a safe alternative to chemical fumigants.

The application of ZITRITIDE in clean room manufacturing facility found to be quite effective as evidenced from the result (Table 9 and 10). The advantage of this fogging solution is that it can be applied in all areas in any time and in a working environment. In this study the fumigation was performed during working hours. People who are present at the time of spraying, fogging or fumigation, and inhale the micron particles of the

disinfectant can clear themselves of all the nasal and throat infections present in them and it will not cause Adina of the lungs. There is no need to shift or move anything or anyone at the time of spraying, fogging or fumigating and the area can be utilized immediately after disinfection. It is an eco-friendly solution and thus safe for people, animals, environment and even children. Thus the beauty of the solution is to apply it easily in all application areas of Hospitals and Industries in areas where chemical fumigants are restricted. It can solve many health problems that arise due to the use of chemical fumigants.

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