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AN INVITRO SCREENING OF ANTI MICROBIAL ACTIVITY OF SELECTED DISINFECTANTS AGAINST SELECTED MICROBIAL STRAINS

Vuchooru Gayathri*, Sruthi Prathapan, Raja Kotagiri, Ravi Kumar Yerram, Naresh Suserla

Department of Quality Control (Microbiology), Jodas Expoim Pvt. Ltd., Plot No. 55, Phase-3, Biotech Park, Karakapatla Village, Markook Mandal, Siddipet District, Telangana, India.

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***Corresponding Author**
Vuchooru Gayathri
Department of Quality Control (Microbiology), Jodas Expoim Pvt. Ltd., Plot No. 55, Phase-3, Biotech Park, Karakapatla Village, Markook Mandal, Siddipet District, Telangana, India.

ABSTRACT

The antimicrobial agents that are used to inhibit or prevent the growth of microbes on inanimate objects and floors: ideally killing of 99.999% are termed as disinfectants. The efficacy is a key attribute to a disinfectant for its successful application which depends on several factors. This disinfectant efficacy is demonstrated predominantly by means of use dilution method and hard surface carrier test in any pharmaceutical facility. Use dilution method can be employed to screen the disinfectants in terms of concentration and contact time, upon challenging them against certain selected microorganisms including environmental isolates from that pharmaceutical facility. Hereby, this paper emphasizes on performing the use dilution method to define the efficacy of commercially available disinfectants namely Protasan, Combatan and Novocide against targeted microorganisms listed in table-2. These three disinfectants displayed identical effective concentration of 2.5% and same contact time of 10 minutes on all the targeted microorganisms for a notable 3 log reduction in the microbial population. These outcomes paved a way for demonstrating the applicable concentration and contact time of a suitable disinfectant on a regular basis in a pharmaceutical facility which have to be further confirmed by the hard surface carrier test for satisfying its intended application on different types of surfaces in a pharmaceutical facility.

KEYWORDS: Antimicrobial agents classification, Mode of action of disinfectants, Disinfectant efficacy testing, Use dilution method.

INTRODUCTION

Antimicrobial agents are any substances of natural, semi-synthetic or synthetic origin that inhibits or kills the growth of microorganisms but causes little or no damage to the host cells. The term 'Antimicrobial' is originated from Greek words anti (against), micros (little i.e., not visible to naked eye) and bios (life). [1] The antimicrobial agents are classified as antibiotics and germicides. These antibiotics and germicides are further categorized as shown in the fig. 1.

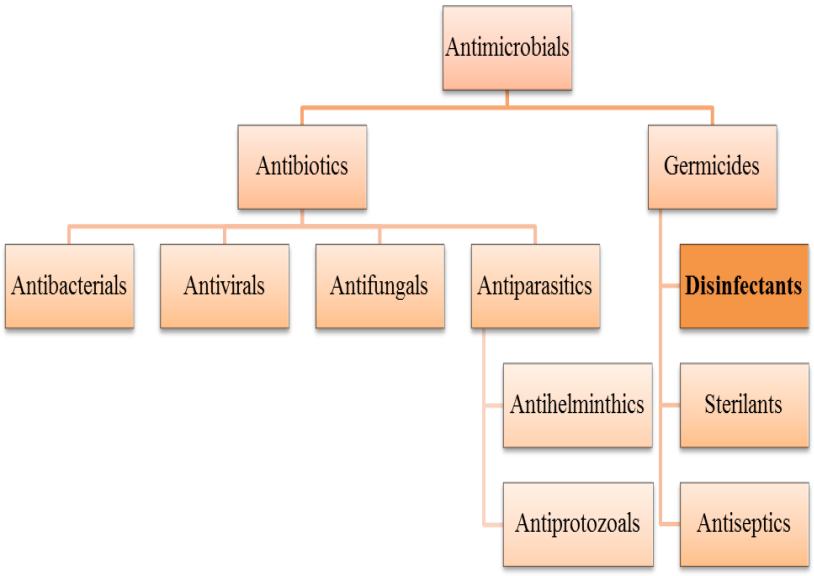

Fig. 1: Stratification of antimicrobial agents.

Definitions of different antimicrobial agents

The antimicrobial agents are defined depending upon their intended application. The disinfectants, sterilants, antiseptics have a prominent application in maintaining the aseptic environment in any manufacturing facility particularly in pharmaceutical and biotechnology industries.[2] The definitions and few examples of these three agents are clearly mentioned below.

Antibiotics[2]

- Substances produced by one microorganism that act against other organisms.
- **Examples**: Penicillin, Cephalosporin, Streptomycin.

Disinfectants[2]

- Chemicals used to inhibit or prevent the growth of microbes on inanimate objects and floors: ideally killing of 99.999%.

- **Examples**: 70% Isopropyl Alcohol (IPA), Combatan, Protasan, Novacide, Household bleach, Wescodyne.

Sterilants[2]

- They are chemical substances or physical processes that are applied to the inanimate objects to kill all microorganisms as well as spores including viruses.
- **Examples**: Physical agents: Moist heat sterilization Chemical agents: Sterilium, Ortho-Pthalaldehyde solution.

Antiseptics[2]

- Chemical agents applied directly to exposed body surfaces (skin and mucous membranes), wounds and surgical incisions to prevent vegetative pathogens.
- **Examples**: Dettol, Tincture of Iodine, Lemon

Disinfectants

Disinfectants are the chemicals used to inhibit or prevent the growth of microbes on inanimate objects and floors: ideally killing of 99.999%. There exist two well-known classifications of disinfectants. In other words, one classification is based on the type of backbone chemical compound present in the respective disinfectants and the other classification is based on their efficacy on different microorganisms in terms of medical criticality. The classification based on the backbone chemical compound is as follows: alcohols, aldehydes, halogens, peroxides, phenolics and quaternary ammonium compounds (Quats). On the other hand, the other categorization depending upon the disinfectant efficacy is as high-level, intermediate-level, and low-level disinfectants.[2]

Basically, the medical devices are categorized depending upon the criticality of their intended application as non-critical, semi-critical and critical devices. The non-critical devices are the items that contact intact skin, Eg: Stethoscopes; the semi-critical devices are the items that are associated with non-intact skin specifically mucous membrane, Eg: Endoscopes; the critical devices are the items that are in contact with sterile tissue or vascular system, Eg: Surgical instruments.[3] Based on this criticality, the disinfectants are defined as:

- **High-level disinfectants**[3]

The disinfectants destroy all micro-organisms except high numbers of bacterial spores particularly on heat sensitive semi-critical items are high level disinfectants.

Examples: >2% glutaraldehyde with a contact time of 20-45 minutes, 0.55% Ortho Pthalaldehyde with a contact time of 12 minutes, 1.12% glutaraldehyde with 1.93% phenol with a contact time of 20 minutes etc.

- **Intermediate-level disinfectants**[3]

The class of disinfectants destroys vegetative bacteria, mycobacteria, most viruses, most fungi but not bacterial spores which are meant for non-critical items are termed as intermediate-level disinfectants.

Examples: Chlorine-based products, phenolics, improved hydrogen peroxide of contact time of minimum 1 minute.

- **Low-level disinfectants**[3]

The class of disinfectants destroys vegetative bacteria, some fungi and viruses but not mycobacteria or spores specifically applied for non-critical items are the low-level disinfectants.

Examples: Chlorine-based products, phenolics, improved hydrogen peroxide, quaternary ammonium compounds (with 1 minute as minimum exposure time) or 70%-90% alcohol.

Mode of action of disinfectants

Disinfectants exhibit different mode of actions by means of their backbone chemical compound and the respective mode of actions are displayed in fig. 2.

Alcohols can cause damage to membranes followed by denaturing the proteins thereby resulting in cell lysis.[4] Aldehydes results in the cross linking of the macromolecules such as DNA, RNA and proteins present in the cell. Halogens inhibits the DNA and also results in oxidation of thiol groups to disulfides, sulfoxides, or disulfoxides. Peroxides manifest their activity resulting in free hydroxyl radicals formation which in turn oxidizing the thiol groups of enzymes and proteins. Hydrogen peroxide, the well-known common peroxide results in breakage of DNA strands. Phenolics result in leakage and uncoupling effects which can cause inactivation of enzyme systems. Quaternary ammonium compounds (Quats) disrupt phospholipid bilayers followed by damage of the cell membrane.[5]

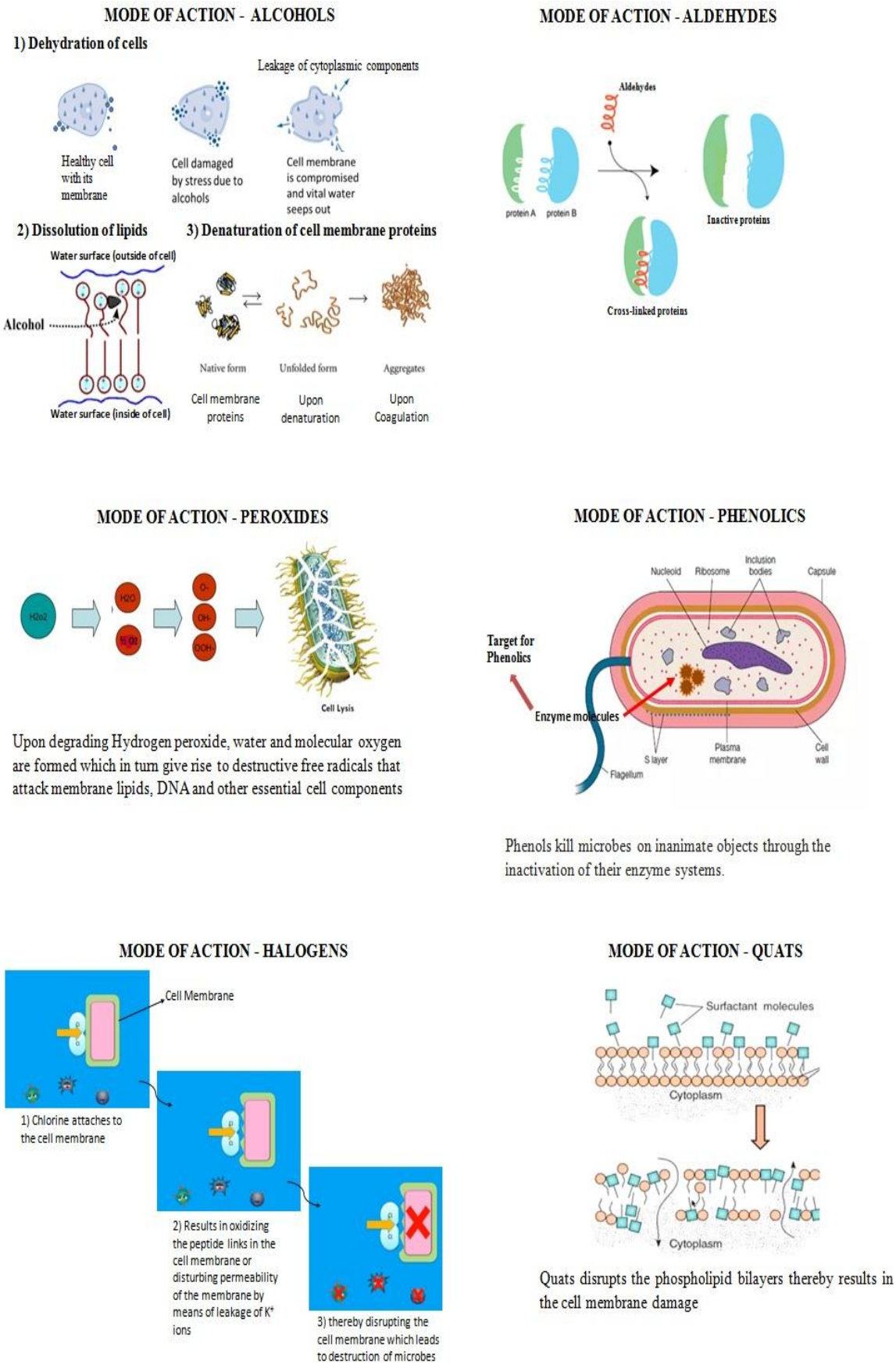

Fig. 2: Mode of actions of different chemical disinfectants.[5]

Disinfectant efficacy

Disinfectant efficacy is the crucial requisite for any disinfectant which is reported to be affected by particular factors such as 1) Types of contaminating microorganism, 2) Degree of contamination, 3) Type of the surface on which the disinfectant has to act, 4) Activity in organic matter and other compounds, 5) Type of chemical, 6) Concentration and quantity of chemical, 7) Contact time and temperature, 8) Residual activity and effects on fabric and metal, 9) Application temperature, pH and interactions with other compounds, 10) Toxicity to the environment and relative safety to humans that may be exposed, 11) Cost. In other words, a disinfectant should test for its efficacy to affix its degree of suitability for the targeted application without any adverse and unintended consequences.[2]

Hence this paper emphasizes on testing the efficacy of respective commercially available disinfectant formulations namely Protasan, Combatan and Novacide against selected microbial strains and environmental isolates from a pharmaceutical facility.

REVIEW OF LITERATURE

Phenol-coefficient is the relative strength of a disinfectant in killing specified microorganisms when compared to the phenol killing power under similar conditions. Strictly speaking the phenol coefficient of a particular disinfectant can be obtained by dividing the figure of the degree of dilution of that disinfectant at which it kills the microorganisms with the degree of dilution of phenol measured after providing same contact time and maintaining similar conditions.[6] As per United States Pharmacopeia, the standard methods to perform disinfectant testing are Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporicidal Carrier Test.[2] Phenol-Coefficient test is the AOAC adopted test developed in 1903 by Rideal and Walker in which the disinfectant is diluted and that degree of dilution is been compared with phenol to find the phenol-coefficient thereby depicting the efficacy of a disinfectant relative to phenol.[6,7] This method is applicable only to the disinfectants with similar chemical nature of coal tar.[6] The next method developed is Use-dilution method as a confirmatory method for the degree of dilution selected by means of Phenol-Coefficient test.[8] Any disinfectant efficacy testing comprises of two crucial steps. 1) Screening the disinfectants of different concentrations and contact times upon challenging recommended microorganisms and selected environmental isolates on various standard surfaces used in the manufacturing facility. 2) Statistical estimation of reduction and recovery

of microorganisms by comparing their number and isolation frequency before and after the application of disinfectant so as to determine its efficacy.[2]

Thereby, this paper focuses on the disinfectant efficacy testing of selected disinfectants i.e., Protasan, Combatan and Novacide by employing Use-Dilution method. The composition of Protasan, Combatan and Novacide is as displayed in the table-1 below.

Table 1: Composition of Protasan, Combatan, Novacide.

Sl. No	Name of the disinfectant	Composition of the disinfectant
1.[9]	Protasan	• Benzalkonium Chloride (50%) IP : 11.5% • Non-ionic Surfactants
2.[9]	Combatan	• Polymeric biguanide hydrochloride-12% • Benzalkonium chloride-10% • Formaldehyde-15% • Ethane dialdehyde-30%
3.[10]	Novacide	• 3% w/v Poly (hexamethylene biguanide) hydrochloride (PHMB) • 10% w/v Didecyl dimethyl ammonium chloride

MATERIALS AND METHODS

Test Microorganisms

ATCC equivalent cultures were procured from CSIR, PUNE, excluding environmental isolates. The test organisms that are targeted for testing the efficacy of disinfectants are as follows:

Table-2: Microorganisms targeted for disinfectant efficacy test.

Recommended Microorganisms as per USP	Environmental isolates
Aspergillus brasiliensis ATCC 16404 *Bacillus subtilis ATCC 6633* *Candida albicans ATCC 10231* *E.Coli ATCC 8739* *Pseudomonas aeruginosa ATCC 9027* *Staphylococcus aureus ATCC 6538*	*Alliococcus otitis* *Bacillus megatarium* *Kocuria rhizophila* *Kocuria rosea* *Kocuria varians* *Kytococcus sedentarius* *Leuconostoc mesenteroides ssp dextranum* *Micrococcus luteus* *Micrococcus lylae* *Staphylococcus arlette* *Staphylococcus caprae* *Staphylococcus cohnii* *Staphylococcus cohnii urealyticus* *Staphylococcus epidermidis* *Staphylococcus haemolyticus*

	Staphylococcus hominis *Staphylococcus warneri* *Staphylococcus xylosus*

Surface materials tested in hard surface carrier test

Table-3: Surface materials to be tested in hard surface carrier test.[2]

Material	Surface Characteristics	Locations of application in a Pharmaceutical facility
304 Stainless Steel	Grit polish	Various
Glass	Clear smooth	Walls (Windows)
Epoxy	Smooth	Floors
Delrin	Plastic smooth finish	Guides in filling LAF
Poly acetal	Semi smooth finish	In-feed conveyer belt
Poly urethane	Smooth finish	Covering tube for sensor cables inside the filling LAF
Silicone	Smooth finish	Gasket present on Bung vibratory bowl
MRC panels	Smooth finish	Filling room wall panels
MS (GI coated)	Smooth finish	Wall panels
Latex	Smooth finish	Gloves, Hypalon
PVC	Smooth finish	Curtains
Aluminum	Semi smooth finish	API canisters

Inoculum Preparation

Transfer 1 mL of the culture to 10 mL of sterile saline solution and perform serial dilution of the suspension up to 10^8 dilution. As the challenge microorganism dilution should be NLT 10^4 cells/mL, this selection been confirmed by implementing the spread plate method of the culture suspension upon maintaining appropriate negative control.

Disinfectant Preparation

The commercially available disinfectants named Protosan, Combatan and Novacide whose efficacy have to be tested were diluted to the concentrations of 2, 2.5 and 3%. Protosan is not been targeted on spore forming bacteria such as *Bacillus subtilis* and *Bacillus megatarium* as it is less sporicidal comparatively, whereas the other disinfectants efficacy has been tested by employing all afore mentioned microorganisms.

Use Dilution Method[2]

10 mL of prepared 2% concentrated disinfectant was taken in a test tube. NLT 10^4 cells/mL of any challenge microorganism was inoculated into each tube. The contents were allowed at three different contact times such as 5, 10 and 15 minutes and were filtered separately by means of employing membrane filtration method followed by the incubation of respective

petri plates. The same procedure was repeated at 2.5 and 3% concentrations of the respective disinfectant and for other mentioned disinfectants against each challenge organism. The positive and negative controls were maintained with 9 mL of saline solution and 1 mL culture of challenge microorganism as the contents in the positive control. After the incubation period, the number of CFU's were counted and respective log reduction values have been calculated.

$$\text{Log reduction (Log R)} = [log_{10}\ (\text{CFU Counts observed in positive control}) - log_{10}\ (\text{CFU Counts observed in test})]$$

Hard Surface Carrier Test[2]

The hard surface carrier test have to be carried out to find out the overall efficacy of a particular disinfectant on different types of surfaces as per table-3. This have to be performed based on the results of the use dilution method.

RESULTS AND DISCUSSION

Use Dilution Method

The use dilution method was performed for all the challenging organisms mentioned in table-2 and are represented in the tables below.

Table 4: Outcomes of use dilution method for *Staphylococcus aureus.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	48	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	12	> 3 log	07	> 4 log
	3		TNTC	< 3 log	10	> 3 log	NIL	> 3 log
Combatan	2	48	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	04	> 4 log	09	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	12	> 3 log
Novacide	2	48	TNTC	< 3 log	TNTC	< 3 log	NIL	> 3 log
	2.5		TNTC	< 3 log	02	> 4 log	05	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log

TNTC-Too Numerous to Count, Conc stands for Concentration

Table-5: Outcome of use dilution method for Pseudomonas aeruginosa.

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	43	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	02	> 4 log	01	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	43	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	05	> 4 log	09	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	11	> 3 log
Novacide	2	43	TNTC	< 3 log	TNTC	< 3 log	NIL	> 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	03	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log

Table 6: Outcome of use dilution method for Bacillus subtilis.

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	38	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	19	> 3 log	30	> 3 log
	3		TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
Combatan	2	38	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	05	> 4 log	10	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	11	> 3 log
Novacide	2	38	TNTC	< 3 log	TNTC	< 3 log	NIL	> 3 log
	2.5		TNTC	< 3 log	02	> 3 log	03	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log

Table 7: Outcome of use dilution method for Escherichia coli.

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	52	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	02	> 4 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	52	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	05	> 4 log	08	> 4 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	52	TNTC	< 3 log	TNTC	< 3 log	NIL	> 3 log
	2.5		TNTC	< 3 log	04	> 4 log	03	> 4 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table 8: Outcome of use dilution method for *Candida albicans.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	55	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	55	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	55	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	01	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-9: Outcome of use dilution method for Aspergillus brasiliensis.

Disinfectant		Initial CFU/ 0.1 mL (*10^4)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	35	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	35	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	35	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-10: Outcome of use dilution method for Staphylococcus arlettae.

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	40	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	40	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	40	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-11: Outcome of use dilution method for *Staphylococcus haemolyticus.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	45	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	45	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	45	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-12: Outcome of use dilution method for *Staphylococcus epidermidis.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	47	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	47	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	47	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-13: Outcome of use dilution method for *Staphylococcus hominis.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	52	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	52	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	02	> 4 log	05	> 4 log
	3		TNTC	< 3 log	01	> 4 log	NIL	> 3 log
Novacide	2	52	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-14: Outcome of use dilution method for *Kocuria rhizophila.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	50	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	03	> 4 log	02	> 4 log
	3		TNTC	< 3 log	04	> 4 log	NIL	> 3 log
Combatan	2	50	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	09	> 4 log	04	> 4 log
	3		TNTC	< 3 log	08	> 4 log	NIL	> 3 log
Novacide	2	50	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	01	> 4 log	01	> 4 log
	3		NIL	> 3 log	01	> 4 log	NIL	> 3 log

Table-15: Outcome of use dilution method for *Staphylococcus warneri.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	49	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	49	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	03	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	49	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	02	> 4 log	NIL	> 3 log
	3		NIL	> 3 log	04	> 4 log	NIL	> 3 log

Table-16: Outcome of use dilution method for *Kocuria varians.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	62	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	62	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	62	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	08	> 4 log	NIL	> 3 log
	3		NIL	> 3 log	18	> 3 log	NIL	> 3 log

Table-17: Outcome of use dilution method for *Micrococcus lylae.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	81	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	02	> 4 log	01	> 4 log
Combatan	2	81	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	81	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	04	> 4 log	NIL	> 3 log
	3		NIL	> 3 log	06	> 4 log	NIL	> 3 log

Table-18: Outcome of use dilution method for *Staphylococcus cohnii.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	47	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	02	> 4 log
Combatan	2	47	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	04	> 4 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	47	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	06	> 4 log

Table-19: Outcome of use dilution method for *Leuconostoc mesenteroides ssp cremoris*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	35	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	01	> 4 log	NIL	> 3 log
	3		TNTC	< 3 log	02	> 4 log	NIL	> 3 log
Combatan	2	35	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	04	> 4 log	NIL	> 3 log
	3		TNTC	< 3 log	05	> 4 log	NIL	> 3 log
Novacide	2	35	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	02	> 4 log	NIL	> 3 log
	3		TNTC	< 3 log	03	> 4 log	NIL	> 3 log

Table-20: Outcome of use dilution method for *Kocuria rosea.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	46	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	46	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	46	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log

Table 21: Outcome of use dilution method for *Alloiococcus otitis.*

Disinfectant		Initial CFU/ 0.1 mL (*10^4)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	51	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	51	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	51	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log

Table-22: Outcome of use dilution method for *Kytococcus sedentarius.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	57	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	57	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	57	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table-23: Outcome of use dilution method for *Staphylococcus caprae.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	48	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	48	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	48	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

Table 24: Outcome of use dilution method for *Staphylococcus xylosus.*

Disinfectant		Initial CFU/ 0.1 mL (*10^6)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	43	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	43	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	43	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log

Table-25: Outcome of use dilution method for *Staphylococcus cohnii ssp urealyticus.*

Disinfectant		Initial CFU/ 0.1 mL (*10^5)	Contact time					
			5 min		10 min		15 min	
Name	Conc (%)		CFU Final	Log R	CFU Final	Log R	CFU Final	Log R
Protasan	2	38	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Combatan	2	38	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log
Novacide	2	38	TNTC	< 3 log	TNTC	< 3 log	TNTC	< 3 log
	2.5		TNTC	< 3 log	NIL	> 3 log	NIL	> 3 log
	3		NIL	> 3 log	NIL	> 3 log	NIL	> 3 log

From the above results, it has been perceived that more than 3 log reduction was observed in all the targeted microorganisms with all the three disinfectants, after a contact period of 10 minutes. On the other hand, all the above outcomes depicted that the appropriate effective concentration on each and every challenged microorganism, in the case of disinfectants employed is 2.5%. Furthermore these results have to be the parameters to proceed onto the hard surface carrier test.

CONCLUSION

Disinfectant efficacy is vital in choosing a particular disinfectant for its intended application. Thereby in a pharmaceutical facility, they have to be challenged predominantly not only on few standard microorganisms but also on the environmental isolates from the facility till date. This was achieved by the use dilution method in which the efficacy in terms of concentration and contact time of the disinfectants Protasan, Combatan and Novocide on challenged microorganisms. The effective concentration and appropriate contact time found out by the use dilution method were 2.5% and 10 minutes respectively, on a common basis in the case of all three disinfectants and on all the challenged microorganisms. Further these results will form a basis to perform the hard surface carrier test on the coupons of 12 different types of surfaces as listed in table-3, with the three disinfectants of 2.5% concentration by means of maintaining a contact period of 10 minutes to establish the absolute efficacy of the particular disinfectant.

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