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Development of a Broad-Spectrum Biocide for Hospital Use on Surfaces Exposed to Pathogenic Microorganisms

Carlos Alvarado Almarza*, Sergio Ruiz Alvarez and Isandra Cartaya Mayora

Research and Extension Center in Environment, Biology, and Chemistry (AMBIOQUIM), Experimental Faculty of Science and Technology (FACYT), University of Carabobo (UC), Valencia, Venezuela.

***Correspondence:**
Carlos Alvarado Almarza, Research and Extension Center in Environment, Biology, and Chemistry (AMBIOQUIM), Experimental Faculty of Science and Technology (FACYT), University of Carabobo (UC), Valencia, Venezuela.

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ABSTRACT

Currently, nosocomial infections represent a major problem in health centers due to inadequate disinfection of their surfaces. Consequently, the objective of this research was to develop a broad-spectrum biocide for hospital use on surfaces. Firstly, various formulations were prepared using benzalkonium chloride, glutaraldehyde, isopropyl alcohol and citric acid as active ingredients. Then, their biocidal activity was determined by applying the neutralization dilution methods UNE-EN using Escherichia coli, Pseudomonas aeruginosa and Aspergillus niger as test strains. The results indicated that benzalconium chloride 0.15% w / v and the mixture of benzalkonium chloride 0.075% w / v with 50% v / v isopropyl alcohol turned out to be bactericidal and fungicidal during 5 min of contact and the mixture of benzalkonium chloride 0.075% w / v with 0.15% w / v glutaraldehyde for 15 min contact. Finally, two formulations were evaluated on hospital surfaces for 10 min of contact. The results showed that both benzalkonium chloride and the benzalkonium chloride-isopropyl alcohol mixture reduced 90% of mesophilic aerobes present but only benzalkonium chloride eliminated the pathogenic E. coli / fecal coliforms and Staphylococcus aureus microorganisms. On the other hand, none were efficient in the control of molds and yeasts.

Keywords

Broad-spectrum biocide, Pathogenic microorganisms, Hospital surfaces.

Introduction

Nosocomial infections (infections acquired within healthcare settings) remain a significant challenge in medical facilities. These institutions create an environment conducive to the proliferation of diverse microorganisms that threaten the health of patients and visitors. According to data from the World Health Organization (WHO), over one million individuals worldwide experience health complications due to infections contracted in hospital environments. Nosocomial infections account for approximately 30% of adverse events affecting patients (Llavina, 2012; Rutala & Weber, 2008).

Emerging bacterial pathogens currently observed in healthcare settings present new challenges for infection control; the principal species include Staphylococcus aureus, *Escherichia coli*, Pseudomonas aeruginosa, and Klebsiella pneumoniae (Llavina, 2012; Bassi, 2008). Likewise, certain molds and yeasts are increasingly implicated in infections, particularly among immunocompromised patients, with Candida spp. and Aspergillus spp. being the most prevalent (Nowrozi, 2013).

Cleaning and disinfection constitute the primary strategies to maintain environments free from microorganisms capable of adversely affecting patient and healthcare worker health. Disinfection protocols must ensure a reduction of the microbial load on equipment and surfaces to levels considered safe. Although disinfection does not guarantee complete microbial eradication, it allows for effective control of microbial populations, preventing them from posing a health risk. Consequently, disinfection enables the production of safe products intended for human use (Marín et al., 2008).

Biocidal products are substances designed to destroy, neutralize, inhibit, or otherwise control harmful organisms, particularly microorganisms, through chemical or biological mechanisms. Examples include disinfectants, preservatives, and pesticides (Scientific Committees, 2013).

Disinfectants play a critical role in controlling nosocomial infections. However, their antimicrobial efficacy is influenced by multiple factors, including formulation type, presence of organic load, temperature, dilution rate, pH, and microbial resistance. Additionally, the role of inanimate environmental surfaces in infection transmission has been reassessed. The U.S. Centers for Disease Control and Prevention (CDC) recognizes direct contact transmission via surfaces or body fluids, and indirect transmission via inanimate objects, as major pathways for microorganism spread (Argerich et al., 2005; Rutala & Weber, 2008; Villatoro, 2009).

Various microbiological methods have been developed to evaluate disinfectant efficacy, including suspension tests, surface evaluations, tube dilution, and agar diffusion methods. Suspension tests are the most widely employed for in vitro assessment of commercial disinfectants and antiseptics (Misirli & Aydin, 2011).

The objective of this study was to assess the antimicrobial efficacy of biocidal formulations based on benzalkonium chloride, glutaraldehyde, citric acid, and isopropyl alcohol, administered individually and in combination, against bacterial and fungal strains using the quantitative suspension method followed by surface tests under real-use conditions.

Materials and Methods

Selection of active ingredients, formulation adjuvants, and preparation of various biocidal formulations

Initially, a comprehensive literature review was conducted on hospital disinfectants to identify commonly used active ingredients, formulation adjuvants, and concentrations for surface disinfection in healthcare settings. Based on this investigation, comparative tables of active ingredients were developed, and employing a Moody selection matrix, the candidates for the new hospital biocide were chosen considering key parameters, including: 1) test microorganism and resistance profile, 2) recommended concentration, 3) exposure time, 4) efficacy, 5) health risk, and 6) compatibility with surfaces.

Following ingredient selection, concentration and exposure time were established to perform the preliminary microbiological analysis for each formulation containing a single active ingredient, guided by the aforementioned parameters and other critical factors. The selected active ingredients and exposure times are presented in Table 1.

Next, formulations were defined based on the active ingredient as either concentrated (requiring dilution in water) or ready-to-use, followed by the preparation of single active ingredient formulations using the manufacturing process employed in the disinfectant product industry. Upon obtaining the different formulations, quality control procedures specific to this product type were applied.

Table 1: Selected pre-experimental design for evaluating formulations prepared with a single active ingredient.

Compound	Selected concentration	Exposure time
Benzalkonium chloride	0.15% p/v	5 min
Citric acid	5.00% p/p	10 min
Glutaraldehyde	0.50% p/v	15 min
Isopropyl alcohol	70% v/v	5 min

To determine the biocidal activity of the formulations, a comprehensive review of international standards for in vitro evaluation of such products was conducted. For each methodology reviewed, critical application and validation parameters were considered, including: 1) type of microorganism used, 2) assay type: qualitative or quantitative, 3) simulation of realistic disinfectant use conditions—clean or dirty conditions (presence or absence of interfering substances) and the type of water used for product dilution (hard or distilled water), 4) exposure time to the chemical agent, and 5) evaluation temperature.

After analyzing all experimental conditions required for microbiological assays (bactericidal, fungicidal) and comparing methodologies of the same type according to the selected parameters, the following were chosen as the basis for studying the biocidal efficacy of the formulated products:

Dilution-neutralization method for chemical disinfectants based on the Spanish standard UNE-EN 1040:1997 and the French standard NF T 72-150:1995, for determining basic bactericidal activity (Álvarez et al., 2001).

Dilution-neutralization method for chemical disinfectants UNE-EN 1275:1996, for determining basic fungicidal activity (Galán, 2003).

After evaluating the single active ingredient formulations and considering their results in relation to the microbial resistance levels and active ingredient compatibility, formulations with two active ingredients were developed to assess potential synergistic effects. Binary combinations and concentrations of each active ingredient to be tested in these formulations were selected. Furthermore, evaluation times were established based on the results and in accordance with the exposure times proposed by the European UNE-EN standards, depending on the microorganism type (bacteria or mold). Consequently, an experimental design with three factors was constructed: binary formula at three levels, formula dose at two levels, and evaluation time at two levels, resulting in a randomized factorial design of 2x2x3, with 12 treatments per replicate and two replicates, totaling 24 treatments, as shown in Tables 2 and 3.

Considering the extensive scope of the investigation required to include all test microorganisms from the selected methodologies, it was decided to employ only those reported in the literature

as having the highest resistance to biocidal agents. Therefore, *Escherichia coli* and *Pseudomonas aeruginosa* were selected as challenge strains to evaluate the bactericidal activity of the prepared formulations, while *Aspergillus niger* was chosen to assess the fungicidal activity.

Table 2. Selected Factors and Levels.

Factor	*Symbol*	*Levels*	*Category*
Binary formula	F	F_1 F_2 F_3	F_1: Formula 1 F_2: Formula 2 F_3: Formula 3
Dose of the formulation	D	D_1 D_2	D_1: Maximum dose D_2: Maximum dose
Time evaluation	T	T_1 T_2	T_1: Minimum time T_2: Maximum time

Considering the extensive scope of the investigation required to include all test microorganisms from the selected methodologies, it was decided to employ only those reported in the literature as having the highest resistance to biocidal agents. Therefore, *Escherichia coli* and *Pseudomonas aeruginosa* were selected as challenge strains to evaluate the bactericidal activity of the prepared formulations, while *Aspergillus niger* was chosen to assess the fungicidal activity.

In vitro Evaluation

Preparation of Bacterial Strains

The test microorganisms *Escherichia coli* and Pseudomonas aeruginosa were obtained from the Microbiology Laboratory of Protinal®. The strains were revived on plates containing nutrient agar medium supplemented with glucose and incubated at 37°C for 18 to 24 hours. Subsequently, bacterial suspensions were adjusted to concentrations between (1–3) × 10^8 CFU/mL by measuring optical density at 620 nm until an absorbance range of 0.15 to 0.20 was achieved for Gram-negative bacteria. Serial 1:10 dilutions were prepared using 0.85% physiological saline solution up to 10^−6, from which 1 mL of the last dilution was plated in duplicate on nutrient agar with glucose and incubated at 37 °C for 18 to 24 hours for subsequent colony counting.

Preparation of the Fungal Strain

The test microorganism *Aspergillus niger* was obtained from the Biotechnology Laboratory at the Faculty of Engineering, University of Carabobo (LABIOT-UC). The strain was revived on Sabouraud agar plates and incubated at room temperature for 7 days. Subsequently, the fungal suspension was adjusted to concentrations between (1.5–5) × 10^7 CFU/mL by counting with a Neubauer chamber, using serial 1:10 dilutions prepared in 0.85% physiological saline solution supplemented with 0.10% Tween 80.

Neutralizer Validation

Prior to determining the biocidal activity of the disinfectants, it was necessary to validate the neutralizers. Various neutralizing agents were formulated based on literature reports indicating which compounds effectively halt the antimicrobial activity of the active ingredients under study. These neutralizers were evaluated following the experimental procedures outlined in UNE-EN standards. Toxicity testing involved exposing the test microorganism to the neutralizer for a defined period, followed by plate counting. Similarly, efficiency evaluation consisted of mixing the disinfectant with the neutralizer for a set duration before adding the microorganism, which was subsequently plated after an established contact time. A neutralizer was considered effective if it met both efficiency and toxicity criteria, specifically achieving at least 50% microbial survival relative to the initial inoculum. Neutralizers that satisfied the European normative requirements for neutralizing the tested disinfectants are presented in Table 4.

Bactericidal Evaluation

Tubes were prepared containing 2.25 mL of single-strength neutralizer and tubes with 0.90 mL of each disinfectant concentration. Then, 0.10 mL of the test suspension with bacterial concentration between (1-3) × 10^8 CFU/mL was added to each disinfectant tube, mixed, and incubated in a thermostatic bath at 20 °C for the selected exposure time(s) according to the experimental design to evaluate product activity. After this period, 0.25 mL from each tube was transferred to a tube containing neutralizer (2.25 mL at single strength), mixed, and held for 10 minutes in a thermostatic bath at 20°C. Subsequently, two 1 mL aliquots of the neutralized mixture were plated, incubated, and colony counts performed as previously described.

Table 3: Experimental design selected to evaluate the biocidal activity of binary formulations.

Formula	*Doe*		*Bactericidal activity*		*Fungal activity*	
			Time (min)		*Time (min)*	
F_1: Benzalkonium chloride + glutaraldehyde	D_1	Benzalkonium chloride 0,10% p/v Glutaraldehyde 0,1 % p/v	T_1=5	T_2=10	T_1=5	T_2=15
	D_2	Benzalkonium chloride 0,075% p/v Glutaraldehyde 0,15% p/v	T_1=5	T_2=10	T_1=5	T_2=15
F_2: benzalkonium chloride + citric acid	D_1	Benzalkonium chloride 0,10% p/v Citric acid 3% p/v	T_1=5	T_2=10	T_1=5	T_2=15
	D_2	Benzalkonium chloride 0,075% p/v Citric acid 3% p/v	T_1=5	T_2=10	T_1=5	T_2=15
F_3: benzalkonium chloride + Isopropyl alcohol	D_1	Benzalkonium chloride 0,10% p/v Isopropyl alcohol 50% v/v	T_1=5	T_2=10	T_1=5	T_2=15
	D_2	Benzalkonium chloride 0,075% p/v Isopropyl alcohol 50% v/v	T_1=5	T_2=10	T_1=5	T_2=15

Table 4: Appropriate neutralizing agents to inhibit the biocidal activity of formulations composed of one and two active ingredients.

Formulation	*Neutralizing*	*L (% p/v)*	*Tw (%p/v)*	*Glic (% p/v)*	*Ti (% p/v)*	*Bi (% p/v)*	*Hi (N)*	*El (% p/v)*	*Gluc (% p/v)*	*Cn (% p/v)*	*Ss (% p/v)*
Benzalkonium chloride	N2P3	0.50	0.70	---	---	---	---	---	---	---	0.85
Isopropyl alcohol	N3P5	---	---	---	0.50	---	---	---	---	---	0.85
Glutaraldehyde F_1 F_3	N2P7	0.70	0.50	1.00	0.60	0.25	---	0.25	1.00	0.80	0.85
Citric acid F_2	N1P9	0.70	0.50	1.00	0.60	0.25	0.10	0.25	1.00	0.80	0.85

Legend: L - soybean lecithin, Tw - Tween 80, Glic - glycine, Ti - sodium thiosulfate, Bi - sodium bisulfite, Hi - sodium hydroxide, El - yeast extract, Gluc - glucose, Cn - nutrient broth, Ss - saline solution.

A disinfectant concentration is considered bactericidal if it achieves a reduction of 10^5 in bacterial cell counts, i.e., the bactericidal efficacy (BE) must be BE ≥ 5 log, equivalent to a 99.999% bacterial reduction.

Fungicidal Evaluation

To 8 mL of disinfectant solution at the test concentration, 1 mL of sterile distilled water and 1 mL of *Aspergillus niger* fungal suspension at (1.5-5) × 10^7 CFU/mL were added, mixed, and incubated in a thermostatic bath at 20 °C for the selected exposure time(s) according to the experimental design for product activity assessment. Prior to completing the selected contact time, the mixture was stirred, and the reaction was neutralized by adding 1 mL of the test mixture to 8 mL of neutralizer plus 1 mL sterile distilled water. After 5 minutes, two 1 mL aliquots of the neutralized mixture were plated, incubated, and colony counts performed as previously described.

A disinfectant concentration is considered fungicidal if it achieves a 10^4 reduction in fungal cell numbers, i.e., the fungicidal efficacy (FE) must be FE ≥ 4 log, which corresponds to a 99.999% fungal reduction.

Statistical Analysis

Statistical analysis was conducted using a statistical software tool to identify the optimal formulations for evaluation timepoints and significant differences among them.

Field Evaluation

Following laboratory evaluation of formulations, field assessment was conducted. A review of studies on hospital disinfectant evaluations in clinical areas was performed to identify surfaces requiring broad-spectrum disinfection. Based on this, the surface for testing the best formulations obtained in the laboratory was selected.

The selected formulations were applied only in rooms of the Male Pneumology Service at a public hospital in Valencia, Venezuela, specifically on patient stretchers. For each surface, a 100 cm^2 area was defined, and sample collection was performed via swabbing into small screw-capped tubes containing 5 mL of 0.85% w/v physiological saline solution before and after spray application of each formulation. Serial dilutions were conducted, and 1 mL aliquots (in duplicate) were plated for mesophilic aerobes, coliforms, molds, and yeasts; 10 µL aliquots were plated on selective media for Staphylococcus aureus and *Escherichia coli*.

Formulations were evaluated using a 10-minute contact time, considering the in vitro results and CDC recommendations.

The selection of formulations with the best biocidal effect under field conditions was based on criteria established by the U.S. Food and Drug Administration (FDA) for critical areas in healthcare settings (Torrens et al., 2003) and on studies by Schmidt et al. (2012) regarding appropriate hospital surface hygiene, as detailed in Table 5.

Table 5: Standard values for the adequacy of cleaning and disinfection of critical surfaces in a facility de salud.

Disinfection level	*Mesophilic aerobes (UFC/cm²)*	*Molds and yeasts (UFC/cm²)*	*Pathogenic microorganisms (UFC/cm²)*
Optimus	< 2.5	< 2.5	0 (Absent)

Results and Discussion

Validation of Neutralizing Agents

The purpose of neutralization is to halt the microbicidal activity after the contact time between the disinfectant and the microorganism under study. This allows quantification of the product's efficacy by counting the surviving microorganisms. Without neutralization, if the disinfectant-microorganism mixture were directly plated for enumeration after the contact period, the assay would lack validity because the biocidal action would continue during plate incubation. In other words, the active ingredient(s) in the formulation would exert a residual effect, preventing accurate quantification of true microbial growth. Therefore, it is necessary to neutralize the active compounds of the formulation.

According to Russell (cited by Sutton et al. 2002), an effective neutralizer must meet three criteria: it should effectively inhibit the biocidal action of the solution; it should not exhibit high toxicity toward the test microorganisms; and neither the neutralizer nor the active ingredient should combine or react to form a toxic compound.

Each formulated neutralizer was evaluated against all microorganisms used to determine the antimicrobial activity of the developed disinfectants. However, only the results of the

evaluation against Pseudomonas aeruginosa and *Aspergillus niger* are presented in Tables 6 and 7.

In this study, upon defining an antimicrobial evaluation for hospital-use chemical products, it was demonstrated that each of the neutralizers employed in the assays effectively inhibited the chemical activity of the biocidal agents without causing toxicological effects on the test strains. This was evidenced by microbial recoveries exceeding 50% relative to the inoculum in both the toxicity and efficacy tests, in accordance with European standards.

Biocidal Evaluation *In Vitro*

Regarding the determination of the antimicrobial activity of the developed formulations, the results obtained from the pre-experimental evaluation against the studied strains are presented in Figures 1, 2, and 3.

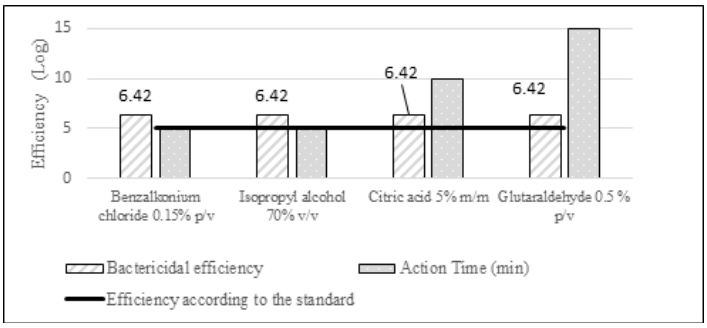

Figure 1: Bactericidal evaluation results of formulations containing a single active ingredient against *P. aeruginosa.*

The bactericidal and fungicidal efficiency achieved for each disinfectant exceeded the minimum thresholds established by the European standards employed, except for the formulation based on citric acid and glutaraldehyde against *A. niger*, which exhibited resistance under these conditions.

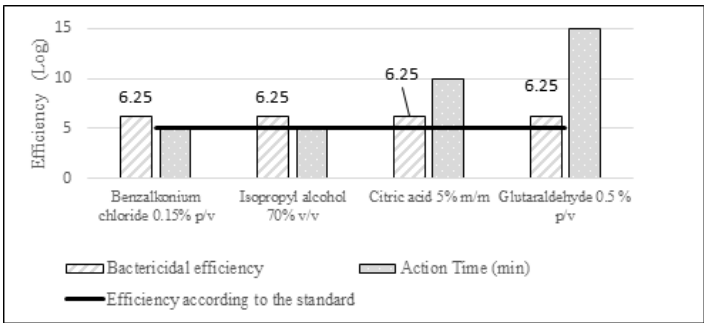

Figure 2: Bactericidal evaluation results of formulations containing a single active ingredient against *E. coli.*

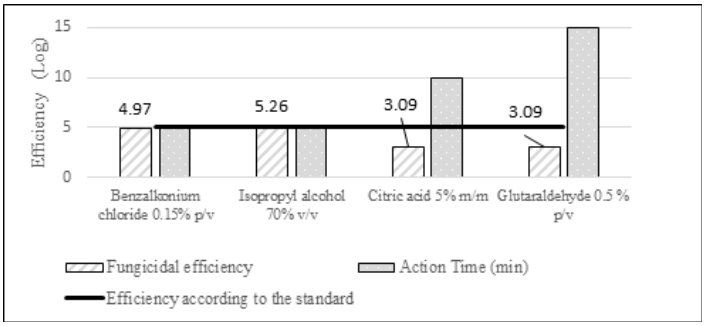

Figure 3: Results of the fungicidal evaluation of formulations containing a single active ingredient against *A. niger.*

The results obtained from formulations containing a single active ingredient varied depending on the microorganism type and the active compound used. Benzalkonium chloride exerts bactericidal action through three mechanisms: disruption of the cell membrane, protein denaturation, and enzymatic inactivation (Argerich et al., 2005). Both *E. coli* and P. aeruginosa were susceptible to the quaternary ammonium compound, consistent with Di Martino (2007), who reported minimum inhibitory concentrations (MICs) of 250 ppm and 62.5 ppm for P. aeruginosa and *E. coli*, respectively,

Table 6: Validation assay results of appropriate neutralizers for quenching the biocidal activity of formulations containing an active ingredient against *P. aeruginosa.*

Dilution 10^{-6}	***Compound***	***Neutralizing***	***Toxicity***		***Efficience***	
Average (UFC/mL)			***Average (UFC/mL)***	***Recuperation (%)***	***Average (UFC/mL)***	***Recuperation (%)***
290	Benzalkonium chloride	N2P3	290	100	274	94,48
	Isopropyl alcohol	N3P5	290	100	275	94,83
	Glutaraldehyde	N2P7	279	96,21	268	92,41
	Citric acid	N1P9	169	58,28	255	87,93

Table 7: Results of the validation assay for the appropriate neutralizers to halt the biocidal activity of formulations ase don an active ingredient against *A. niger.*

Dilution 1/10 de $1x10^{3}$ (UFC/mL)		***Compound***	***Neutralizing***	***Toxicity***		***Efficience***	
Theoretical value	***Average count***			***Average (UFC/mL)***	***Recuperation (%)***	***Average (UFC/mL)***	***Recuperation (%)***
100	80	Benzalkonium chloride	N2P3	76	95,00	74	92,50
		Isopropyl alcohol	N3P5	77	96,25	78	96,85
		Glutaraldehyde	N2P7	76	95,00	73	91,25
		Citric acid	N1P9	75	93,75	76	95,00

and with Osman et al. (2012), who achieved a 6-log reduction for both bacteria with 512 ppm after 1 minute of contact.

The results for *A. niger* are comparable to those reported for commercial products and previous studies, such as Tortorano et al. (2005), who demonstrated greater than 99.99% efficiency against A. fumigatus with 0.25% (w/v) for 5 minutes contact. Additionally, Korukluoglu et al. (2006) inhibited the growth of an *A. niger* strain using 0.50% (w/v) benzalkonium chloride for 5 minutes.

The efficacy observed for isopropyl alcohol was predictable, as literature affirms its rapid and effective activity against bacteria and viruses due to protein denaturation, which requires the presence of water. Kampf and Fröhner (2004) and Prince and Prince (2008) reported that contact times between 15 and 30 seconds are sufficient to achieve 5-log reductions across a broad spectrum of microorganisms, including the bacteria studied here.

Regarding *A. niger*, molds are generally more resistant to alcohols. Korukluoglu et al. (2006) showed that among tested microorganisms, molds were more resistant than yeasts; the fungicidal activity of 70% (v/v) isopropyl alcohol against *A. niger* depended on the isolate, requiring contact times exceeding 25 minutes for some strains. In contrast, the present study demonstrated a greater than 5-log reduction after 5 minutes contact, differing from previous findings, possibly due to strain-specific fungal resistance associated with insufficient disruption of spore walls and inhibition of sporulation and germination.

Glutaraldehyde acts as an alkylating agent targeting sulfhydryl, hydroxyl, carbonyl, and amino groups, thereby disrupting DNA, RNA, and protein synthesis. It also causes spore wall disruption and inhibits sporulation and germination (Argerich et al., 2005). This mode of action explains the high bactericidal activity observed, consistent with Osman et al. (2012), who achieved a 5-log reduction after 5 minutes exposure to 0.20% (w/v) glutaraldehyde against both bacterial strains. Namba et al. (1985) reported that a 0.50% (w/v) glutaraldehyde formulation applied for 10 minutes eradicates all bacteria, including resistant strains.

The fungicidal activity of glutaraldehyde was low, partially inhibiting *A. niger* growth. At the concentrations and contact times used, glutaraldehyde was unable to fully eliminate the mold. This was primarily attributed to two factors: acidic pH and low concentration. Rutala and Weber (2008) indicated that glutaraldehyde's biocidal activity is pH-dependent, with superior efficacy at alkaline pH values between 7.5 and 8.5 compared to acidic conditions. Mohamed (2004) corroborated this by showing that at 0.125% (w/v), glutaraldehyde exhibited a 3.4-log reduction at pH 8.4 after 60 minutes contact versus only 2.3-log at pH 4.2 under identical conditions, highlighting the significant impact of pH and concentration on efficacy.

Organic acids are postulated to exert antimicrobial effects by penetrating microorganisms and disrupting cell membranes (Medina & Valencia, 2008). Accordingly, citric acid demonstrated high bactericidal activity against both bacterial strains, consistent with Sumi et al. (2009), who reported a minimum inhibitory concentration (MIC) of 0.125% (w/v) for these Gram-negative bacteria—much lower than the concentrations evaluated herein. Conversely, the fungicidal activity of citric acid was very low and similar to that of glutaraldehyde. Shokri (2011) reported an MIC of 5% (w/v) citric acid against *A. niger*, a value inconsistent with our findings at the same concentration. Culver et al. (2005) noted that citric acid exhibits minimal fungicidal activity, being effective primarily against T. mentagrophytes (athlete's foot fungus), which is less resistant than *A. niger*.

Furthermore, experimental design results for mixtures containing two active ingredients indicated possible synergistic effects against the tested strains. For each formulation, bactericidal activity was similar across both bacterial strains regardless of dose or exposure time, meeting European standards (Figures 4 and 5).

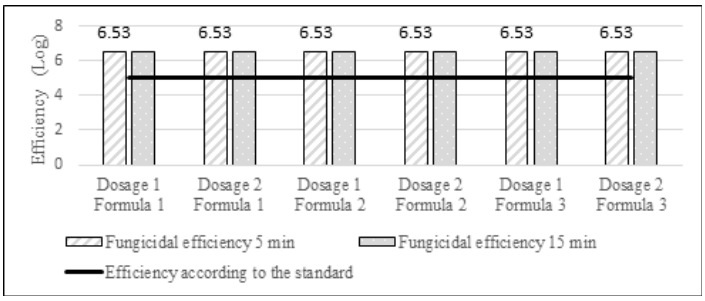

Figure 4: Bactericidal evaluation results of the binary formulations against *Pseudomonas aeruginosa.*

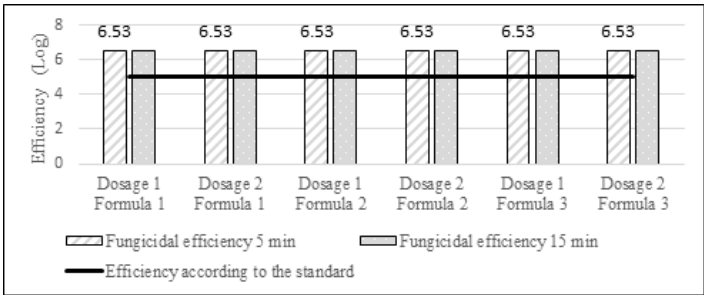

Figure 5. Bactericidal evaluation results of binary formulations against *E. coli.*

The experimental evaluation demonstrates that for the three formulations tested, at either dose and at both time points examined, the response variable (bactericidal efficiency) yielded consistent results for each of the two bacterial strains involved in the study, with counts below 1 CFU/mL and microbial reductions greater than 6 log. This clearly indicates that both strains were susceptible to all formulations tested.

In the case of *A. niger*, only formulation 1 at dose 1 and dose 2 at time point 2, as well as formulation 3 at any dose and time, achieved microbial reductions in accordance with European standards (Figure 6).

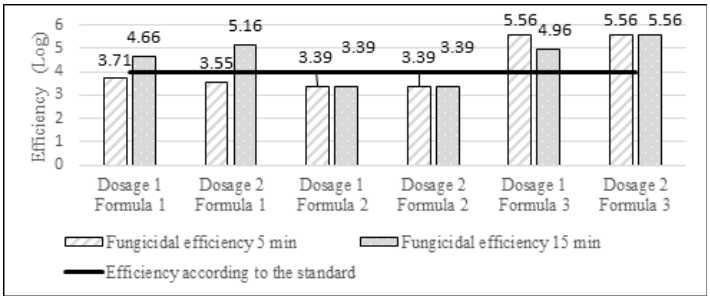

Figure 6: Results of the fungicidal evaluation of the binary formulations against *A. niger.*

Since the results obtained for the bacterial strains were similar, it was not possible to perform a statistical analysis to establish significant differences; however, for the fungal strain, it was feasible (Table 8). The results from the analysis of variance, conducted using a statistical tool on the data collected after evaluating the fungicidal activity of the binary formulations through a randomized experimental design, were reliable as they met the three assumptions: 1) the variance of the randomized error is not affected by the treatment applied, 2) uncontrolled variation is randomized, and 3) the samples are drawn from a normal population. The statistical results indicate significant differences among the variables.

Table 8. Analysis of Variance of the Factorial Design for Fungicidal Efficiency.

Fountain	*Sum of Squares*	*Gl*	*Middle Square*	*F-ratio*	*P-Value*
A:Formula	5,12597	1	5,12597	128,53	0,0000
B:Dosage	0,14127	1	0,14127	3,54	0,0794
C:Time	0,673086	1	0,673086	16,88	0,0009
AA	11,3617	1	11,3617	284,88	0,0000
AB	0,0200868	1	0,0200868	0,50	0,4888
AC	2,58201	1	2,58201	64,74	0,0000
BC	0,258614	1	0,258614	6,48	0,0224
blocks	0,0135452	1	0,0135452	0,34	0,5687
Total error	0,598227	15	0,0398818		
Total	20,7745	23			

The statistical design also enabled the identification of the optimal biocidal formulations based on two active ingredients: formula 1 at dose 2 and formula 3 at dose 1, with the latter corresponding to the optimization. In contrast, the best formulation based on a single active ingredient was benzalkonium chloride 0.15% w/v at a contact time of 5 minutes.

From the statistical analysis, the most influential factors on the response variable (fungicidal efficacy), in decreasing order, were: formulation type (A), formulation by time interaction (AC), time (C), and dose by time interaction (BC). Formula 1 at dose 2 and time 2 exhibited higher efficacy than dose 1 at the same time (Figure 6). This result demonstrates that dose 2 and time 2 (glutaraldehyde/ benzalkonium chloride ratio = 2:1) was the optimal dose-time interaction for formula 1, reflecting a synergistic effect between glutaraldehyde and benzalkonium chloride.

Furthermore, Osman et al. (2012) investigated the individual biocidal effects and the combined effect of glutaraldehyde with benzalkonium chloride on multidrug-resistant Gram-negative bacteria. Their results showed a clear reduction exceeding 6 log units when using a mixture of 2000 mg/L glutaraldehyde with benzalkonium chloride at concentrations of 8, 16, 32, and 64 mg/L, during exposure times of 15 seconds, 1 minute, and 5 minutes. The synergistic effect of these two active compounds was confirmed, as neither compound alone at these concentrations was able to eradicate the tested strains, which only occurred when acting together.

Similarly, Soliman et al. (2009) evaluated several commercial products, including TH4® and Microzal®, which are mixtures of quaternary ammonium compounds and glutaraldehyde, against isolated pathogenic bacteria (Pseudomonas aeruginosa, Staphylococcus aureus, *Escherichia coli*, and *Klebsiella oxytoca*) as well as molds and yeasts (*Aspergillus niger* and *Candida albicans*). The results demonstrated a synergistic effect, achieving 99.99% elimination of the strains after 5 minutes of contact under alkaline pH for bacteria and yeast, and 10 minutes for mold.

In the same vein, for formula 3, the dose-time interaction influenced fungicidal efficacy, indicating that dose 1 and time 2 (Figure 6) partially but less significantly reduced efficacy, suggesting this dose is not optimal for this formulation.

The study of the biocidal effect of two active ingredients acting simultaneously showed that formula 1 acts synergistically because benzalkonium chloride, as a surfactant (reducing surface tension), serves as a vehicle for glutaraldehyde acid to wet and penetrate the microbial cell, thereby increasing biocidal effect over a shorter time. Conversely, in formula 2, the active ingredients did not act synergistically to eradicate mold. This combined effect may be related to the reduction of benzalkonium chloride's efficacy when alone in solution (alkaline pH), since the binary mixture's pH is dominated by citric acid, and the microbicidal action of the quaternary ammonium was drastically reduced due to the acidic environment.

Finally, formula 3 exhibits a clear synergistic effect owing to the alcohol concentration used, which, in the presence of water and facilitated by the surfactant and wetting power of benzalkonium chloride, penetrates the cell, enhancing the biocidal effect of alcohol with both agents acting through their respective microbicidal mechanisms.

Considering the intrinsic resistance of *Aspergillus niger*, it is highly likely that for formula 2 to penetrate the cells and achieve fungicidal activity, the exposure time must be increased.

Field Evaluation

The field study allowed assessment of the real effect of the formulations under specific conditions. Prior to evaluating the performance of the benzalkonium chloride 0.15% w/v formulation, a high density of mesophilic aerobic bacteria (3.0 x 10^3 CFU/100 cm^2) and molds and yeasts (3.9 x 10^3 CFU/100 cm^2) was

detected, along with the presence of Gram-positive pathogenic bacteria such as Staphylococcus aureus and Gram-negativ*E coli*form bacteria (12 CFU/100 cm^2), including *Escherichia coli*.

Following application of the quaternary ammonium compound, results showed a significant reduction in aerobic mesophilic bacterial load, while total coliforms were not significantly reduced; however, fecal coliforms, including *E. coli*, were effectively eradicated (Figure 7). Similarly, S. aureus was undetectable post-application. These results demonstrate that benzalkonium chloride at 0.15% w/v was efficient in controlling the main pathogenic bacteria—S. aureus and *E. coli*—responsible for numerous nosocomial infections worldwide with high mortality rates.

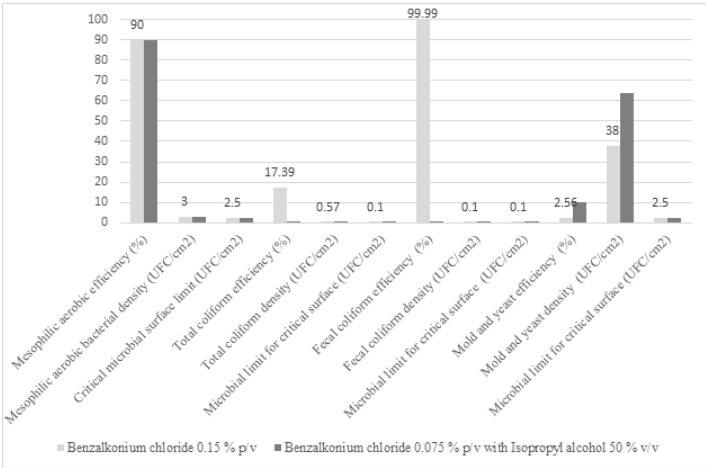

Figure 7: Field-level biocidal assay results for the two formulations evaluated after 10 minutes of contact.

The total coliform bacterial group, primarily composed of four genera—Enterobacter, Escherichia, Citrobacter, and Klebsiella (Camacho et al., 2009)—can be affirmed to have some surviving species after evaluation of the benzalkonium chloride-based formulation, except for *Escherichia coli*. This survival may be directly related to the resistance acquired by each strain due to the frequent use of the same antibiotics and disinfectants, which promotes resistance through biofilm formation (Rutala and Weber, 2008).

Benzalkonium chloride was ineffective at the tested concentration and exposure time in controlling molds and yeasts. Although the genera present in the evaluated area were not determined, it can be stated that they exhibited greater resistance than *Aspergillus niger* tested under laboratory conditions.

Prior to testing formulation 3 (benzalkonium chloride at 0.075% w/v combined with isopropyl alcohol 50% v/v), the sampled surface displayed a high density of mesophilic aerobic bacteria (3.0×10^3 CFU/100 cm²) and molds and yeasts (7.1×10^3 CFU/100 cm²). Following application of this formulation, a significant reduction in bacterial load was observed during the evaluation period; however, fungal load control was not achieved (Figure 7).

Conversely, no coliform bacteria, *E. coli*, or Staphylococcus aureus were detected, preventing assessment of the formulation's efficacy against these microorganisms under real-use conditions.

The efficacy of a hospital-grade product depends on multiple factors including temperature, concentration, contact time, presence of organic matter, target microorganism, and application method (Argerich et al., 2005; Medina and Valencia, 2008). Therefore, it can be inferred that products tested under actual usage conditions may exhibit different efficacies compared to those under controlled laboratory settings. Consequently, the hospital environment must be tightly controlled, since hands and air play a critical role in cross-contamination and nosocomial infections (Villatoro, 2009).

Based on this, it is presumed that airborne contamination played a significant role in inoculating surfaces in the pulmonology room where both products were evaluated, via spores of molds and yeasts. This was facilitated by the absence of air conditioning and open windows, allowing direct interaction between the room's interior and exterior environment. The molds and yeasts recovered from the evaluated surfaces differed between sampling sites (Figures 8.A and 8.B). Morphological characteristics suggest that the molds could belong to the genera Aspergillus and Penicillium, respectively, both known for high resistance to disinfectants and common airborne presence; however, confirmation is lacking as biochemical identification tests were not performed.

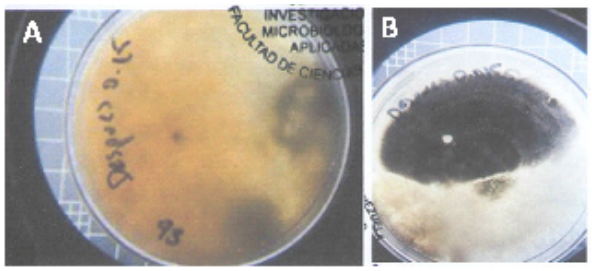

Figure 8: Molds present on clinical surfaces where hospital biocidal formulations were evaluated. A) Benzalkonium chloride 0.075% w/v with Isopropyl alcohol 50% v/v; B) Benzalkonium chloride 0.15% w/v.

It is also important to consider that both products were applied solely by spraying, and several researchers, including Argerich et al. (2005) and Rutala and Weber (2008), recommend performing a pre-cleaning of the surface to be disinfected. This step helps to remove part of the microbial load, thereby enhancing the disinfectant's efficacy. Additionally, friction or rubbing affects the microbial cell wall, facilitating the penetration of the disinfectant into the cell and causing cell death. Consequently, the application of friction during product evaluation could have increased the formulations' efficiency.

In summary, under the conditions in which both products were applied in clinical areas and in the comparative assessment between them, neither met all disinfection standards for critical hospital surfaces (Table 5).

Conclusion

Since the microbicidal activity of each hospital-grade product

depends on various factors, this study determined that although the evaluated formulations showed satisfactory results in in vitro assays, neither formulation significantly reduced the fungal load on hospital surfaces under field conditions. Therefore, reformulation of the active ingredient concentrations is recommended to achieve the effective elimination of the most resistant pathogenic microorganisms present on hospital surfaces through an appropriate disinfection protocol.

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