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Bactericidal and Fungicidal Activities of Novel ProtecTeaV Formulations - Alcohol-Based Hand Hygiene and Surface Disinfectant Prototypes Containing Epigallocatechin-3-Gallate-Palmitate (EC16)

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ABSTRACT

Objective: *Currently used alcohol-based hand sanitizers and surgical hand rubs are not effective against alcohol-resistant microorganisms. We reported previously that nontoxic antioxidant food additive compounds derived from green tea polyphenols, particularly epigallocatechin-3-gallate-palmitate (EC16), are suitable for use in alcohol formulations to effectively inactivate nonenveloped viruses and bacterial spores. However, whether EC16 influences the bactericidal and fungicidal activity of alcohol is not clear. The objective of the current study was to determine the bactericidal and fungicidal activities of ProtecTeaV hand sanitizer and surface spray prototypes containing EC16.*

Methods: *The prototypes were tested according to the guidelines from the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA).*

Results: *As expected, EC16 did not reduce the bactericidal and fungicidal activities of ethanol. The hand sanitizer gel formulation was equally effective as 70% ethanol and met the tested standard, and the surface spray prototype met the EPA performance standard.*

Conclusions: *EC16 can be combined with ethanol without reducing antibacterial or antifungal activity, and the ProtecTeaV prototypes could be further developed into novel hand hygiene and surface disinfectant products with virucidal, bactericidal, fungicidal and sporicidal activities.*

Keywords

EGCG-palmitate, green tea, hand hygiene, surface disinfectant, bactericidal, fungicidal.

Introduction

A major problem for disease control and prevention is the lack of effective hand hygiene products that possess comprehensive activities against a wide spectrum of bacteria and bacterial spores, viruses, and fungi. Among pathogenic species and strains, a number of them are relatively resistant to the alcohol-based or non-alcohol-based hand hygiene products in current use, such as instant hand sanitizers or surgical rubs. For examples, there is no available hand sanitizer or hand rub effective against nonenveloped viruses (such as norovirus) or bacterial spores (such as *C. difficile* spores). Thus, guidelines from the Center for Disease Control and Prevention (CDC) recommend washing with soap and water to remove these pathogens from hands and skin. However, this method does not inactivate these viruses and spores, but rather

releases them into the waste water system, which is associated with potential contamination of the environment.

Diseases caused by the spread of new pathogens are on the rise. A deadly example is the SARS-CoV-2 virus responsible for the COVID-19 pandemic. Despite the fact that this enveloped virus is susceptible to proper preventive measures, including face covering, isolation, and hand hygiene methods, a large proportion of the world-wide population became infected, with a high number of associated deaths. Unfortunately, SARS-CoV-2 will not be the last lethal pathogen to infect the human race. The next new pathogens to evolve from the environment and spread (e.g., an Ebola-like virus) or to be introduced through biowarfare (such as anthrax) could be an alcohol-resistant nonenveloped virus or multi-resistant bacterial spores associated with a higher mortality rate. Human society is not prepared.

Chemical agents with virucidal and sporicidal activities against nonenveloped viruses and bacterial spores, such as bleaches, acids, bases, or strong oxidizing chemicals, are well known and are widely used in surface disinfectants. However, most are toxic and represent a degree of chemical hazard. Due to their toxicity and potential pollution of the environment, in the U.S. these agents are regulated by the Environmental Protection Agency (EPA), and in general they cannot be used in Food and Drug Administration (FDA)-regulated hand hygiene products. Regulations governing the sale of hand sanitizer products vary by country. In the US, over-the-counter (OTC) hand sanitizers with antibacterial (and fungicidal) activity claims are regulated by the FDA. According to their 24th April 2019 final rule, the only claims for active ingredients eligible for evaluation are for (denatured) alcohol (60-95% v/v), isopropyl alcohol (70-91.3% v/v) and benzalkonium chloride, although these ingredients had not been designated as "generally recognized as safe (GRAS)", requiring a special exemption to be provided during the COVID-19 pandemic [1].

We previously reported that lipid-soluble green tea polyphenols and derivatives possess antimicrobial activities. Specifically, tea polyphenol-palmitate, a GRAS food additive (FDA GRAS Notice 772), appears suitable for hand hygiene and surface disinfectant products [2]. The major content of tea polyphenol-palmitate is epigallocatechin-3-gallate-palmitate (EGCG-palmitate or EC16), the result of EGCG linked with one to three 16-carbon fatty acid - palmitic acid by ester bonds. EC16 has shown a strong antiviral activity against a broad spectrum of viruses [2]. When formulated with ethanol, it demonstrated virucidal activity against non-enveloped viruses such as norovirus, and sporicidal activity [3,4]. Therefore, alcohol-based formulations containing EC16 could become a new generation of hand hygiene and surface disinfectant products that simultaneously possess virucidal, sporicidal, bactericidal and fungicidal activities, without toxicity to humans or the environment. The current study focused on the bactericidal and fungicidal activities.

Within the required testing protocols for efficacy, neutralization controls are important to demonstrate that the effect of the antimicrobial agent is blocked immediately after sampling by dilution into an appropriate formulation (e.g., media containing appropriate chemical neutralizing agents). It is also important to demonstrate no toxic effects intrinsic to the test system or neutralizing agent [5,6].

In the present study, we tested the ProtecTeaV (PTV) alcohol-base hand sanitizer formulation containing EGCG-palmitate (EC16) against *S. aureus,* and *P aeruginosa* according to the EN 13727 Standard, and against *C. albicans* according to the EN 13634 Standard, whilst the ProtecTeaV PST70 alcohol-based disinfectant spray containing EC16 was testing against *S. aureus, P aeruginosa* and *S. enterica* using the EPA SOP MB 06-09. One potential source of variance for *P aeruginosa* test validity was identified, with a solution that did not require deviation from the EPA SOP.

Materials and Method

Formulations

ProtecTeaV (PTV) hand sanitizer was made with 70% v/v ethanol containing EC16, purified water, and standard gelling agents. ProtecTeaV PST70 surface disinfectant spray was made with 70% v/v ethanol containing EC16, purified water, and 0.3% citric acid. The precise EC16 contents of the formulations are proprietary information.

Bacterial and yeast stocks and culture

Stocks of the bacteria *Staphylococcus aureus* ATCC 15442, *Pseudomonas aeruginosa* ATCC 6538, and *Salmonella enterica* ATCC 10708, and the yeast *Candida albicans* ATCC 10231, were purchased from Microbiologics (Saint Cloud, MN). Cultures were initiated according to the supplier's directions and frozen stocks were prepared as directed in EPA SOP MB 06-09 and stored at -80°C.

For routine culture of *S. aureus* and *S. enterica* for spray testing and pilot experiments, 10ml of Synthetic Broth AOAC medium (Wright and Mundy Broth; HiMedia Laboratories, Mumbai, India) supplemented with 0.1% dextrose (SB) was used in 20 mm x 150 mm glass tubes with aluminum foil caps, while AOAC Nutrient Broth (NB; Aldon Corporation, Avon, NY), was used for spray testing with *P. aeruginosa*. A 24±2 h culture was initiated with 10µl of a frozen stock, and grown at 36°C without shaking. Ten microliters of this culture were then used to inoculate one or more tubes that were grown for 48±2 h. Titers were determined by serial dilution in AOAC PBS (prepared from monobasic and dibasic potassium phosphates according to AOAC 961.02), and plating on tryptic soy agar plates (TSA plates; Hardy Diagnostics, Santa Maria, CA), with growth at 36°C for up to two days, counting colonies on both days.

For sanitizer testing, 10 µl from a frozen stock of a bacterial species were streaked on TSA plates and the subculture grown for 18-24 h at 36°C. A Day 2 working culture was then prepared by spreading a loopful of subculture cells on a TSA plate and growing for 18-24 h. Subcultures of *Candida albicans* ATCC 10231 were prepared

from a frozen stock by growth on malt extract agar (MEA) plates (Seaweed Solutions Laboratories, at Amazon.com) at 30°C for 42-48 h. A working culture was prepared by spreading a loopful of cells on an MEA plate and growing for 42-48 h.

For bacteria, a factor of $1.2x10^9$ cfu/ml/OD_{600} was used to estimate cell density in liquid culture to prepare dilutions, and a factor of $0.75x10^9$ cfu/ml/OD_{600} was used for suspensions prepared from plates. For *C. albicans*, a factor of $1.6x10^7$ cfu/ml/OD_{620} was used.

Bacterial sanitizer testing

The PTV hand sanitizer gel was tested undiluted against *S. aureus* ATCC 15442 and *P. aeruginosa* ATCC 6538, according to the EN13727:2012 protocol. As a sanitizing control, 70% v/v ethanol/water was used. Tryptone solution (1g/l tryptone pancreatic digest of casein, 8.5g/l sodium chloride) was used as the diluent.

A 1 min exposure time with 0.30g/l bovine albumin interfering substance (designated as 'clean conditions' in the protocol) was used. For neutralization, tests were performed with a neutralizer comprised of Tryptone diluent containing 30g/l polysorbate 80 (Mystic Moments, Fordingbridge, Hampshire, UK), 3g/l saponin (Quillaja extract, Chemsavers, Bluefield, VA), and 3g/l soy lecithin (Velona Inc., Elk Grove Village, IL), as described in EN 13624 Table B.1, except the amount of saponin was reduced from 30g/l. To investigate the effects of the additives on neutralization versus simple dilution, tests were also performed with *S. aureus* using Tryptone diluent alone (i.e., without surfactants).

For this hand sanitizer test, as specified in the protocol for the 'modified method for direct use products' (with a final concentration of the product at 97% original), bacteria from a working culture plate were harvested with a loop and resuspended in diluent by shaking with glass beads (3 mm glass beads, Fisher Scientific, Suwanee, GA) to a working test suspension density (N) of 1.5-5 x 10^9 cfu/ml (based on optical density of a dilution). An aliquot of N (0.1 ml) was mixed with 0.2 ml 1.5% bovine albumin, and after 2 min incubation at 20°C, 9.7 ml of PTV hand sanitizer was added and mixed to form the test mixture (10^2 dilution; initial test concentration N_0). After 1 min incubation, a 1 ml sample was transferred to a mix of 8.0 ml neutralizer and 1.0 ml water (10^{-1} dilution). After 10s, 1 ml of this neutralized mix was plated in duplicate on TSA plates, and a 10-fold serial dilution prepared and plated. Plates were counted after 48 h growth at 36°C. The surviving cell count in the original test mixture (N_a) was calculated.

In parallel, validation suspensions for controls were prepared by diluting cell suspensions to give $3x10^3$ -$1.6x10^4$ cfu/ml (N_v) and $3x10^4$ -$1.6x10^5$ cfu/ml (N_{vb}) for the experimental and neutralizer controls respectively, based on OD_{600} values. The actual cfu/ml was determined by serial dilution and plating. Control A, validation of the experimental conditions (absence of toxic conditions in the experimental system), was performed by mixing 0.1 ml of N_v with 0.2 ml bovine albumin, and after 2 min, 9.7 ml of water was added (initial concentration N_{v0}). After 1 min, 1 ml was plated in duplicate. Control B, neutralizer control (verifying lack of neutralizer toxicity), was performed by mixing 1 ml of N_{vb} with 9 ml of neutralizer (10^{-1} N_{vb}), and then performing two serial dilutions of 0.5 ml to 4.5 ml of neutralizer (10^{-2}, 10^{-3}). After 10s, 1 ml of the last two dilutions was plated on TSA plates. Control C, method validation (testing for neutralization effectiveness), was performed by mixing 0.1 ml diluent with 0.2 ml bovine albumin, and adding 9.7 ml of sanitizer (or ethanol as control). After 1 min, 1.1 ml of this mixture was transferred to a mixture of 8.8 ml neutralizer, and after 10s, 0.1 ml of N_v suspension was added, followed by incubation for 30 min. One milliliter aliquots were then plated.

N_0, the cfu/ml in the test mixture at the beginning of the 1 min contact time, was calculated as 1/100 of the titer of N. A valid N value was between 1.5 and $5x10^9$ cfu/ml, and a valid N_0 value of 1.5 and $5x10^7$ cfu/ml. N_a, the number of surviving cfu/ml in the test mixture after the contact time, was calculated from the dilutions of the neutralized mixtures. The reduction in viable cells was expressed as the $\log_{10}$ of the ratio N_0/N_a. When no cells were detected after treatment, the reduction was calculated as $>\log (N_0/(N_a (=0)+1)) = \log(N_0)-\log(1) = \log(N_0)$.

Weighted averages for cfu values were calculated from dilutions and plates with between 14 and 330 colonies. Valid ranges for N_v and N_{vb} and were respectively $3.0x10^3$-$1.6x10^4$ and $3.0x10^4$-$1.6x10^5$ cfu/ml. For the controls, the cfu/ml at the beginning of each test (N_{vo}, N_{vbo}) was determined from the titers of N_v and N_{vb}. Valid controls had initial values between 30 and 160 cfu/ml. Valid control results were measured cfu/ml values $\geq$0.5x N_{vo} or N_{vbo}.

To confirm effective sanitization when no cells were detected, simplified testing was performed by mixing 50 µl of a 2-day culture (~$5x10^7$ cfu) with 450µl of either PTV hand sanitizer, 70% v/v ethanol or PBS (control), and after 1 min treatment the suspension was diluted with 9.5 ml PBS for neutralization by 20-fold dilution. One hundred microliters of this mixture, and of serial dilutions, were plated on TSA and cultured up to three days.

Fungal sanitizer testing

The PTV hand sanitizer was tested undiluted against *Candida albicans* ATCC 10231 according to the EN13624 protocol. A 1 min exposure time with 0.30g/l bovine albumin interfering substance (clean conditions) was used. Testing was performed with Tryptone diluent as the neutralizer (n=2), and with Tryptone diluent containing neutralizing agents as above (n=1).

The protocol was similar to that used for bacterial testing, with minor modifications specified in the protocol. The working test suspension (N) was 1.5-5 x 10^8 cfu/ml, while the validation and neutralizer control suspensions N_v and N_{vb} were still respectively $3x10^3$ -$1.6x10^4$ cfu/ml (N_v) and $3x10^4$ -$1.6x10^5$ cfu/ml (N_{vb}). A valid N value was between 1.5 and $5x10^8$ cfu/ml, and a valid N_0 value between 1.5 and $5x10^6$ cfu/ml. N_a, the number of surviving cfu/ml in the test mixture after the contact time, was calculated from the dilutions of the neutralized mixtures. The reduction in viable cells was expressed as the log of the ratio N_0/N_a, calculated as above.

Valid ranges for N_v and N_{vb} were the same as for bacterial testing. For the controls, the cfu/ml at the beginning of each test (N_{vo}) was determined from the titers of N_v and N_{vb}. Valid controls had initial N_{vo} values between 30 and 160 cfu/ml. A valid control result was a measured cfu/ml value $\geq 0.5x\ N_{vo}$.

Disinfectant spray testing against bacteria

The PST70 disinfectant spray was tested undiluted against *S. aureus* ATCC 15442, *P. aeruginosa* ATCC 6538, and *S. enterica* 10708 according to the AOAC Official Method 961.02, *Germicidal Spray Products as Disinfectants*, as implemented in the U.S. Environmental Protection Agency Protocol Standard Operating Procedure MB-06-09, Germicidal Spray Products as Disinfectants (GSPT): Testing of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica.* For each species at least three different batches of spray were prepared and tested.

Glass microscope slide carriers were cleaned by soaking overnight in 100% ethanol, followed by rinsing well with ultrapure water and then drying by evaporation. Slides were placed on Whatman #2 disks in glass petri dishes with lids, and sterilized by autoclaving.

In brief, 10 ml bacterial cultures of *S. aureus* and *S. enterica* were grown in Synthetic Broth AOAC media (Wright and Mundy Broth; HiMedia Laboratories, Mumbai, India), supplemented with 0.1% dextrose (SB media), in 20 mm x 150 mm glass tubes with aluminum foil caps. *P. aeruginosa* was similarly grown using Nutrient Broth (NB media, Aldon Corporation, Avon, NY). Cultures were grown at 36°C without shaking. A culture was started from a 10µl inoculum of a frozen stock, and after 24 h growth 10µl of this culture was used to inoculate 48-50 h cultures for use in experiments. In general, although permitted, consecutive 24 h cultures (up to four) were not used.

For *P. aeruginosa*, cells were aspirated from the middle of the culture tube to separate them from the pellicle. Aspirates contaminated with pellicle were discarded. The *P. aeruginosa* aspirate, or the two-day *S. aureus* and *S. enterica* cultures, were vortexed for about 4s and then allowed to stand for 10 min. The upper 6-7 ml was then transferred to a fresh tube. The OD_{650} (as specified in the SOP), as well as the OD_{600} (a commonly used wavelength that is also more comparable to the size of *S. aureus*) were used to determine a dilution of the culture in culture medium to a suspension concentration of about $1.6\text{-}1.8x10^8$ cfu/ml for *S. aureus* and *P. aeruginosa*, and about $1.2x10^8$ cfu/ml for *S. enterica* (confirmed by serial dilution and plating). This was determined to be sufficient to give an adequate recovery from the carrier in controls (see below). The load suspension (9.5 ml) was adjusted by addition of 0.5 ml FBS to 5% v/v fetal bovine serum (FBS) as organic soil.

Glass slides were loaded with 10 µl of load suspension, which was spread over a ca. 1.5 x 2cm area using a sterile 10 µl loop, and then dried at 36°C for 40 min at a relative humidity between 25 and 35%. This is the upper limit time allowed by the SOP, and under the conditions here was found to give drying of all slides within the time at up to 35% relative humidity.

The spray bottle containing PST70 was placed at a 45° angle, with the nozzle 6-8" from the carrier surface and set to jet spray. Three sprays were delivered (about 2.5-3ml), and a 1 min exposure time was used. After exposure the carriers were transferred with brief gravity draining to 20 ml of Letheen broth (LB) (Hardy Diagnostics, Santa Maria, CA) in a 50 ml polypropylene tube, mixed by shaking, and incubated at 36°C for 48 h. Tubes were also examined after an additional 24 h growth for confirmation.

Aside from routine sterility controls (media, diluents and carrier), untreated carrier growth, and culture purity testing, recovery was determined using six untreated carriers transferred directly to LB and vortexed for either 2 min (*S. aureus, S. enterica*) or 1 min (*P. aeruginosa*), followed by serial dilution in PBS and plating. Three of the recovery controls were placed in LB and vortexed before the start of carrier spray treatment (followed by storage at 4°C), and three were processed at the end of spray treatment (about 1 h later). Recoveries (total cfu) required by the SOP are $\log_{10}$ 5-6.5 for *S. aureus* and *P. aeruginosa* (geometric mean recovery of $1.0x10^5$-$3.2x10^6$ cfu), and $\log_{10}$ 4-5.5 for *S. enterica*, (corresponding to a geometric mean recovery of $1.0x10^4$-$3.2x10^5$ cfu).

As a separately conducted control, neutralization was tested according to EPA SOP MB-17-04, by spraying sterile (unloaded) carriers, and after 1 min treatment transferring to LB, followed by addition of 0.1 ml of a dilute bacterial suspension (a suspension at OD_{600} 0.1 was diluted 10^4, then four serial 10-fold dilutions were tested) to give a concentration in at least one dilution in the range of 5-100 cfu/ml. The final cell concentration was determined by plating on TSA. A parallel set of tubes with sterile, non-loaded, non-sprayed carriers was set up as a comparative growth control. Tubes were cultured for 48 h, with the appearance noted after 24 and 48 h.

For this study, the neutralization control was extended in two ways. First, the dilution series was continued to below an estimated 1 cfu per tube to establish a lower limit for detection. Second, an additional three unsprayed controls were included for each of the four lowest dilutions in the suspension series to allow determination of the lowest detectable cell concentration from a limit dilution using the Reed and Muench sampling method [7].

Routine microbiological techniques as designated in EPA SOP MB 06-09 were used to evaluate colonies and cultures for the growing species.

Results

Hand sanitizer bacteria testing

The PTV hand sanitizer formulation was tested undiluted (final concentration in test 97% original) against *S. aureus* and *P. aeruginosa* according to the EN13727:2012 protocol under "clean" conditions, with 0.3g/l bovine albumin interfering substance in the test mixture, and a 1 min exposure before neutralization. As a control for ethanol bactericidal activity in the system, 70% v/v

ethanol was tested in parallel (without a separate neutralization control for this material). Dilution of PTV or 70% ethanol to 7% by neutralization was expected to reduce the concentration below microbicidal levels. To test this dilution effect, pilot testing was performed with *S. aureus* using unsupplemented Tryptone diluent as the neutralizer. Although the neutralization controls showed effective recovery in the Method Validation Control C with recoveries >0.5xN_0, (consistent with blocking of the bactericidal effect by dilution), after 24 h of growth the majority of colonies from cells treated with PTV hand sanitizer were noticeably smaller than those on titer plates of cells exposed to either 70% ethanol or sanitizer formulation without EC-16, although they were of normal size after 48 h. This suggested an initial reduction in growth rate, consistent with a residual effect from EC16. When the surfactants were added to the Tryptone for neutralization, recovery remained efficient, but bacterial colony sizes in Control C were now indistinguishable from colonies on other plates. Therefore, Tryptone plus surfactants was used for neutralization in bacterial testing. No residual sanitizer effect was noted for *C. albicans.*

For both *S. aureus* and *P. aeruginosa*, all three replicate tests had initial and control suspension cell densities (cfu/ml) in the appropriate valid range (Table 1). Similarly, all three replicate controls gave recoveries >0.5 x the initial cell suspension density, indicating valid experimental conditions (Table 1). For *S. aureus* and *P. aeruginosa*, the mean percent recovery in Control A was 116 ± 21%(SD) and 80.3 ± 23.3% respectively for the two species, indicating no systemic toxicity under the experimental conditions.

For the Neutralizer Control B, all three tests for both *S. aureus* and *P. aeruginosa* gave an average recovery of 97.3 ± 26.3% and 61.0 ± 7.9% respectively, indicating no marked toxicity in the neutralizer formulation. Similarly, the Method Validation Control C gave average recoveries of respectively 97.6 ± 24.3 and 91.1 ± 26.7%, indicating effective neutralization. Therefore, all three sanitizer tests for each organism were conducted under valid conditions.

After treatment with either PTV hand sanitizer or 70% v/v ethanol, all three tests with both *S. aureus* and *P. aeruginosa* gave no colonies from plating neutralized cells, corresponding to a mean log reduction for each agent of respectively >7.49 (n=3) and >7.41 (n=3) with the two organisms. Since no cells were detected from plating 1 ml out of 10 ml total, a conservative estimate of fold reduction would be 10-fold lower; however, this would still give a >10^6-fold reduction in all tests.

Confirmation of bacterial sanitization

To confirm the elimination of viable bacteria, a simplified suspension test was performed. For *S. aureus*, the mean initial cell density in the test mix (determined from the PBS control) was 9.17x10^7 cfu/ml; again, no colonies were detected on the sanitizer or 70% ethanol treatment titers, for a mean log reduction of >7.95. Similar results were obtained for *P. aeruginosa*, with a mean initial cell density of 5.80 x 10^7, and no detected survivors after treatment, for a mean log_{10} reduction of >7.76.

In addition, a suspension time-kill test (ASTM E2315) against methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591) was conducted in a GLP laboratory. The result showed a log_{10} reduction of >5.52.

Table 1: PVT sanitizer testing according to EN13727 (bacteria) and EN13624 (yeast) protocols. Cells were treated with PTV sanitizer (9.7 ml/10 ml total) in the presence of 0.3g/l bovine albumin for 1 min before neutralization. The titers (cfu/ml) of the Test Suspension (N), the Validation Suspension (V_v) and the Neutralization Control Suspension (N_{vb}) were determined by serial dilution, and are shown as log_{10} values. N_o, the initial concentration of cells in the test mixture, is calculated as the 100-fold dilution of N. The log_{10} reduction was estimated as the log of the titer plate load of the test suspension assuming no killing (N_0x0.1x1). For the controls, the valid range for the initial cfu/ml of is N_vx 0.01 (A, C), or N_{vb} x 0.001 (B), with a range between 30 and 160 cfu/ml. The experimental values in the control columns show the measured control cfu/ml determined from the titer plates. A valid control result has a value ≥0.5 x the initial cfu/ml.

Organism	Test	Log_{10} Initial Suspension N cfu/ml	N_0 Log_{10} (cfu/ml)	N_v (cfu/ml)	N_{vb} (cfu/ml)	Control A (cfu/ml)	Control B (cfu/ml)	Control C (cfu/ml)	Mean colony #		Log_{10} Reduction	
									PTV	Ethanol	PTV	Ethanol
	Valid Range	9.17≤N≤9.70	7.17≤N_0≤7.70	3x10^3≤N_v≤1.6x10^4	3x10^4≤N_{vb}≤1.6x10^5	≥0.5x(30-160)	≥0.5x(30-160)	≥0.5x(30-160)				
S. aureus	1	9.28	7.28	4.43x10^3	5.73x10^4	62.0	57.5	25.5	0	0	>7.28	>7.28
	2	9.66	7.66	8.56x10^3	8.44x10^4	93.0	102.5	105.5	0	0	>7.66	>7.66
	3	9.52	7.52	6.34x10^3	6.39x10^4	63.8	44.5	47.5	0	0	>7.65	>7.65
	Mean	**9.49**	**7.49**								**>7.49**	**>7.49**
P. aeruginosa	1	9.49	7.49	6.45x10^3	6.08x10^4	42.5	36.5	78.5	0	0	>7.49	>7.49
	2	9.45	7.45	8.40x10^3	6.11x10^4	57.0	45	61	0	0	>7.45	>7.45
	3	9.29	7.29	5.64x10^3	5.77x10^4	56.4	40.0	44.5	0	0	>7.29	>7.29
	Mean	**9.41**	**7.41**								**>7.41**	**>7.41**
	Valid Range	8.17≤N≤8.70	6.17≤N_0≤6.70	3x10^3≤N_v≤1.6x10^4	3x10^4≤N_{vb}≤1.6x10^5	≥0.5x(30-160)	≥0.5x(30-160)	≥0.5x(30-160)				
C. albicans	1	8.20	6.20	3.97x10^3	3.98x10^4	28.8	50.6	52.0	0	0	>6.20	>6.20
	2	8.56	6.56	6.52x10^3	9.03x10^4	58.5	76.0	79.2	0	0	>6.56	>6.56
	3	8.40	6.40	7.69x10^3	5.89x10^4	71.9	40.6	68.6	0	0	>6.40	>6.40
	Mean	**8.39**	**6.39**								**>6.39**	**>6.39**

Hand sanitizer fungal (*Candida albicans*) testing

The PTV hand sanitizer was tested undiluted against *C. albicans* according to the EN13624 protocol. Two tests were performed using Tryptone diluent alone as the neutralizer, and one with Tryptone diluent containing additional neutralizers. No differences in cell growth were noted.

For all three tests, the density of cells (cfu/ml) in the initial suspension (N) and in the test suspension (N/100) were in the valid range. Similarly, all three tests had appropriate cfu/ml values for the validation suspension N_v and the control suspension N_{vB}. All three tests gave valid values for the three controls. For the Experimental Condition Control, A, the average recovery was 90.2±29.2 (sd)%, indicating no system toxicity. For the Neutralizer Control B, the average recovery was 100 ± 42%, indicating no marked toxicity in the neutralizer formulation. Similarly, the Method Validation Control C gave an average recovery of 104 ± 16%, indicating effective neutralization. Therefore, all three sanitizer tests for *C. albicans* were conducted under valid conditions.

For both agents (PTV, 70% ethanol), all three tests gave no colonies from plating neutralized treated cells, corresponding to a mean log reduction of >6.39 (n=3).

Evaluation of PST70 as a surface disinfectant spray

Independent batches of the PST70 spray (three per species) were tested against *S. aureus, P. aeruginosa* and *S. enterica* according to the EPA SOP MB-06-09 protocol, with 5% fetal bovine serum included in the cell suspension loaded on the slides as an organic soil.

Neutralization controls

For each species (*S.aureus, P. aeruginosa* and *S enterica*), all pairs of tubes (i.e., sprayed and unsprayed slides at each dilution) in the dilution range specified by EPA SOP MB 17 (5-100 cfu/ml) showed identical and substantial growth in unmodified Letheen broth at Day 1, relatively unchanged by Day 2.

S. aureus and *P. aeruginosa* both gave growth in tubes containing spray treated slides down through a test suspension estimated density of 34-35 cfu/ml (3-4 cfu in 0.1 ml added to the tube), and *S. enterica* with 11 cfu/ml (1-2 cfu added to tube). These values were within the 5-100 cfu/ml culture range for effective neutralization without toxicity as specified by the EPA protocol, and indicated that a positive tube Day 1 could arise from as little as one cfu.

To further evaluate the limit of detection of the test, further dilutions were performed in the same series, inoculated into three additional tubes (i.e., a total of five tubes at that dilution), and the Inoculated $Dose_{50}$ determined using the Reed and Muench algorithm. There was good agreement between the two methods for the estimated cell density, with 2/5 positive tubes for *S. aureus* at a cfu/tube of 1-2 (calculated as a dilution from plate titering results) and 1 cfu/tube ID_{50}. For *P. aeruginosa* there were 3/5 positive tubes, and for *S. enterica* 4/5 positive tubes, at an estimated cfu/tube of 1-2 (plating and ID_{50}). A further 10-fold dilution gave 1/5 positive tubes for each of the three species. Importantly, all tubes showing positive growth did so by Day 1, and no more tubes became positive Day 2 (or later).

Therefore, for each species, the lowest number of cells added to a tube required to give a positive result by Day 1 was about 1-2 cfu, consistent with an extremely sensitive test and normal growth giving a positive result by 24 h.

As a further test of neutralization, modified Letheen broth was used in the standard EPA protocol. This formulation has less surfactant, more nutrient, and sodium bisulfite as an additional neutralizing agent. Similar results were obtained as for unmodified LB, with positive tubes after 24 h of growth down to as low as 1-2 cfu added/tube.

Recovery controls

Within spray tests for bactericidal activity, *S. aureus* (grown in SB) gave excellent recovery from carriers (Table 2). Across three independent tests, the individual carrier recovery ranged from $1.05x10^6$ to $1.96x10^6$ cfu, with a mean $\log_{10}$ recovery for each test of 6.09, 6.20 and 6.24 (geometric mean 1.24×10^6, 1.57×10^6, and1.75×10^6 cfu), and an overall recovery of the original load of 95.8 ± 12.4 (sd)%.

Similarly, *S. enterica* gave recoveries in the valid range, although as expected from the protocol, substantially lower than for *S. aureus*. The individual carrier recovery ranged from $3.50x10^4$ to $6.30x10^5$ cfu, with a mean log recovery for each test of 4.81, 5.06 and 5.54 (geometric mean $6.44x10^4$, $1.15x10^5$, and $3.50x10^5$ cfu), and an overall recovery of the original load of 13.5 ± 7.6 (sd)%. The high recoveries in one test (above log 5.5) were acceptable, since the spray test gave passing results (see below).

Growth of *P. aeruginosa* in SB was found to result in highly inconsistent and generally poor recovery from control slides. After further testing of Nutrient broth (allowed by the protocol) and sampling technique, Nutrient broth (NB) was used for *P. eruginosa* testing, and only the middle region of the culture was sampled (about 2.6-6 ml), unless otherwise noted. Under these optimal conditions, the individual carrier recovery ranged from $1.16x10^5$ to $1.13x10^6$ cfu, with a mean $\log_{10}$ recovery for each passing test of 5.23, 5.54 and 5.63 (geometric mean $1.71x10^5$, $3.44x10^5$, and $4.21x10^5$ cfu), and an overall recovery of the original load of 18.3 ± 8.7 (SD)%.

Spray testing for bactericidal activity using optimized culture conditions

To further test the PST70 spray, samples were submitted to an independent laboratory for testing under GLP or non-GLP conditions according to EPA 06-09 (Table 3). Three different lots of the PST70 spray formulation were tested for bactericidal activity using three-four sprays at a jet setting (~2.5-3 ml delivered), and 1 min exposure before immersion in neutralizing media (unmodified LB or NB), under conditions compliant with EPA protocol MB 06-09. The PST70 spray passed the test, with no positive tubes with either organism.

Table 2: PST70 Spray testing according to EPA Protocol MB 06-09 under optimized culture conditions. The number of experimental tubes (60 carriers tested) showing growth Day 2 in each of three replicate tests with different batches of spray is shown. *P. aeruginosa* was cultured in Nutrient Broth. Bacterial suspension carrier loads contained 5% FBS as organic soil. No contaminants were detected in any tube. Positive tubes were confirmed as the test organism.

Organism	Test/ batch	Mean $\log_{10}$ carrier recovery cfu	Positive tubes/60	Pass/Fail
	Valid Range	5≤N≤6.5		
S. aureus	1	6.24	0	Pass
	2	6.20	0	Pass
	3	6.09	1	Pass
	Mean	**6.18**		
P. aeruginosa	1	5.23	1	Pass
	2	5.64	0	Pass
	3a*	5.43	0	Pass
	3b*	5.50	2	Fail
	Mean	**5.51**		
	Valid Range	4.0≤N≤5.5		
S. enterica	1	5.55†	0	Pass
	2	5.06	1	Pass
	3	4.81	0	Pass
	Mean	**5.14**		

* Tests were performed using the same batch

† The test was accepted as valid despite the carrier load being above the range limit, as no positive tubes were detected.

Table 3: PST70 Spray GLP testing in independent laboratories according to EPA Protocol MB 06-09 under non-GLP conditions. Carrier slides were exposed to the spray (3-4 sprays) for 1 min prior to neutralization. All tests gave appropriate control results for a valid GLP test.

Test Laboratory	Organism	Test	Growth Medium	Organic Soil	Average $\log_{10}$ carrier load	Positive tubes/60
MicroChem	***S. aureus***	1	SB	None		0
		2	SB	None		0
		3	SB	None	6.12 (three lots)	0
	P. aeruginosa	1	SB	None		0
		2	SB	None		0
		3	SB	None	6.12 (three lots)	0

Discussion

This study used the relevant European, EPA or FDA required tests to examine the bactericidal and fungicidal properties of two formulations, a hand sanitizer and a spray, both based on ethanol as the active agent, plus a lipophilic green tea polyphenol derivative (EC16).

Testing of the PTV hand sanitizer formulation was straightforward, and showed bactericidal and fungicidal activity comparable to 70% ethanol against *S. aureus, P. aeruginosa* and *C. albicans*, with a $>\log_{10}$ 7.4-fold reduction for bacteria, and $>\log_{10}$ 6.4 for yeast, satisfying *in vitro* regulatory requirements as a hand sanitizer for these organisms, and indicating no detrimental effect of the formulation on the antimicrobial activity of ethanol (Table 1). Interestingly, although using tryptone without neutralizing additives to dilute the ethanol to 7% (a concentration below standard antimicrobial levels) gave adequate recovery in neutralization tests with *S. aureus* to confirm elimination of overt cell killing by neutralization through dilution, we noticed the colonies from treatments grew more slowly than those from the controls, suggesting a persistent effect from another ingredient, likely EC16. This warrants further exploration. However, inclusion of the recommended neutralizing ingredients (Polysorbate 80, saponin and lecithin) removed this effect.

For the PST70 surface disinfectant spray, the EPA spray test is a stringent test of microbicidal activity. An extension of the neutralization control dilution series demonstrated that as little as 1-2 cfu added to a tube gave positive growth by Day 1. This means that at the limit, no more than 1 cfu can survive in a test to avoid a failing result (i.e., >1 positive carrier culture tube). Since about 10^6 cells are loaded on each of the 60 carriers in a test with *S. aureus* or *P. aeruginosa*, the overall reduction in survival must be about (60 x 10^6/(1 or 2)), or >log7.5, for a high probability of a passing result. For S. aureus, the spray test in our hands proved remarkably robust: we were unable to find any protocol-compliant conditions that in our hands could cause failing numbers of positive tubes. The orientation of the spray relative to the carrier, number of sprays (3 or 4), and jet versus mist spray all gave 0-1/60 positive tubes in tests.

We found that by paying attention to culture conditions (using NB for the culture of *P. aeruginosa*, as allowed by SOP 06-09) and culture sampling, the EPA spray test gave consistent results with all three species tested; using a one-minute exposure with three-four jet sprays was sufficient to give a passing test (0,1/60) in the large majority of tests performed with the inclusion of 5% FBS soil (Table 2). Therefore, the spray would meet the EPA regulatory

standard. Results from independent GLP-compliant laboratories are consistent with our results (Table 3).

In conclusion, under appropriate and test-compliant conditions, the ProtecTeaV sanitizer and spray formulations containing EC16 and ethanol proved to be highly effective in reducing the microbial load on contaminated surfaces and in suspension tests for hand sanitizer formulations.

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