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Major Article

Virucidal activities of novel hand hygiene and surface disinfectant formulations containing EGCG-palmitates (EC16)

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A B S T R A C T

Background: Non-toxic hand hygiene and surface disinfectant products with virucidal activity against alcohol-resistant nonenveloped norovirus are in urgent need.

Method: Alcohol-based formulations were made with epigallocatechin-3-gallate-palmitate (EC16), an FDA accepted food additive. Based on in-house testing of formulations, 3 prototypes, PTV80 hand gel, PST70 surface disinfectant spray and PST70 surface disinfectant wipe, were selected from in-house tests for independent testing at GLP (good laboratory practice) laboratories according to EN 14476:2019 (hand gel), ASTM test method E1053-20 (spray), and ASTM E2362-15, E1053, and ASTM E2896-12 (wipe).

Results: The PTV80 hand gel prototype demonstrated a >99.999% reduction of murine norovirus S99 infectivity in 60 seconds. Carrier testing of the PST70 surface spray and surface wipe demonstrated reduction of feline calicivirus infectivity by >99.99% in 60 seconds. In addition, testing with human coronavirus and human herpes simplex virus demonstrated >99.99% efficacy in 60 seconds, consistent with broad spectrum virucidal activity.

Conclusions: The novel non-toxic prototypes containing EC16 were found to be suitable for use in future hand sanitizer gel, surface disinfectant spray and wipe products against norovirus. Products based on these formulations could be used safely to help prevent and control norovirus and other emerging virus outbreaks, pending future studies.

Many commercial surface disinfectants with activity against norovirus and/or bacterial spores contain toxic or corrosive chemicals such as chlorine-based compounds (e.g., hypochlorite/bleach, ammonium chloride), strong bases or acids, hydrogen/metal peroxides, and combinations of these chemicals,[1] some at high concentrations; as high as 50% for chlorine-based chemicals, up to 27.5% hydrogen peroxide, and 25% acids. These disinfectants are in use because nonenveloped viruses such as norovirus, and bacterial spores, are broadly resistant to alcohol-based products or products without the abovementioned chemicals. However, although many surface disinfectant products are not categorized as “toxic products”, certain products can represent a health hazard. For example, according to the American Association of Poison Control Centers, 47,632 bleach exposure cases were reported to the 55 US Poison Control Centers between Jan 1, 2020 and Nov 15, 2020, an increase of 31% compared to the same period in 2019. An EPA-registered surface disinfectant with sporicidal and virucidal claims (containing a combination of 27.5 hydrogen

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peroxide, 5.8% of peroxyacetic acid and 8% acetic acid) allegedly caused multiple symptoms in some healthcare-related workers.[2] Further, human error in use can lead to hazardous conditions. For example, misuse of 2 surface disinfectants (one contains 50% aluminum chloride hexahydrate and 50% citric acid monohydrate, and another contains 8.2% sodium hypochlorite) resulted in a fatal accident with a number of additional persons injured.[3] There is a need for surface disinfectants with activity against non-enveloped viruses and bacterial spores that have a lower risk to human health and the environment.

Due to the hazardous nature of the chemicals capable of eradicating alcohol-resistant microorganisms such as norovirus and bacterial spores, to the best of our knowledge, there is no hand hygiene product with the strength to effectively aid in the prevention of norovirus outbreaks or healthcare-associated infections due to bacterial spores (such as *Clostridium difficile* infection). Currently available alcohol-based hand sanitizers are not effective against norovirus,[4,5] and in fact, such sanitizers were found to be a risk factor for norovirus spread vs. soap and water wash in long-term care facilities.[6] Certain widely used alcohol-based hand sanitizer products have claimed effectiveness against norovirus, but the claims were removed after receiving a warning letter from the FDA (January 17, 2020, Case # 599132). Current CDC guidelines for hand hygiene against norovirus in healthcare settings remain with hand wash with soap and water, due to the lack of other effective methods.[7] However, hand washing with soap and water does not inactivate norovirus, but rather washes the virus off the hands into the water system.[8] It is known that norovirus can survive in water for 60 to 728 days, and it persists on various surfaces for prolonged time periods.[9] Therefore, an effective, environmentally friendly, non-toxic, and long-lasting strategy is in urgent need to combat alcohol-resistant microorganisms, and reduce the burden on healthcare and the associated social costs worldwide.

Epigallocatechin-3-gallate-palmitates (EGCG-palmitates or EC16), the major component of tea polyphenol-palmitate, is an FDA-approved antioxidant food additive and EPA-approved safe inert. We reported previously that EC16 possesses virucidal and sporicidal activities.[10-16] We have hypothesized that surface-applied formulations containing EC16, either used on human skin or other surfaces, are able to effectively eradicate alcohol-resistant microorganisms. Our long-term goal is to develop novel hand hygiene and surface disinfectant products with comprehensive virucidal, sporicidal, bactericidal and fungicidal activities, and we have sought to develop prototypes of an alcohol-based hand sanitizer gel, surface disinfectant spray and surface wipe, that have these activities. The bactericidal and fungicidal activities of these early prototypes was reported recently, showing the prototypes possess efficacy comparable to 70% v/v ethanol.[17] Our recent study showed that EC16, either in a preliminary hand sanitizer formulation or in a cell culture medium, was able to inactivate murine norovirus strain S99.[16] The current study was focused on the virucidal activities associated with the sanitizer prototypes we have since developed (PST70 surface disinfectant spray and wipe, and PTV80 hand sanitizer gel), measured in independent GLP tests according to regulatory standards set by the EPA for surface disinfectants, and Health Canada/EU for the hand sanitizer (the FDA does not allow a virucidal claim for hand hygiene products).

METHODS

Food grade EGCG-palmitates >90% (EC16) was purchased from Changxing Sanju Biotechnology Co., Ltd. Carbopol Ultrez 20 gelling agent was obtained from Voyageur Soap and Candle Co, and triethanolamine (TEA) from Carolina Biological Supplies. Fetal bovine serum (FBS) was from Atlas Biologicals, Inc. Dulbecco's Minimum Essential Media (DMEM) with high glucose, L-glutamine and sodium pyruvate was from GenDepot (Baker). Cell culture plasticware was purchased from Southern Labware Inc. Spunlace nonwoven polyester/rayon hybrid fabric was provided by Zhejiang Kingsafe Nonwoven Fabric Co., Ltd.

Virus stocks and tissue culture

For claims of anti-norovirus activity, the EPA requires GLP efficacy tests on Feline Calicivirus (FCV) as a surrogate for human norovirus, and Health Canada/EU require murine norovirus (MNV) S99 as a surrogate for human norovirus. MNV strain S99 passage 2 (Friedrich-Loeffler Institute Virusbank #RVB-651), and FCV strain F9 (ATCC #VR-782) were used. Host cells for MNV S99 were RAW 264.7, passage #15 mouse macrophage cells, and for FCV F9 the host cells were Crandell-Rees Feline Kidney Cells (CRFK) cells, passage #7 (ATCC#CCL-94). Human Coronavirus (betacoronavirus) strain OC43 (ZeptoMetrix Corporation, #0810024CF) was tested in the human colon epithelial tumor cell line HCT-8 (ATCC #CCL-244). Vero green monkey kidney cells (ATCC #CCL-81) were used as the host cells for herpes simplex virus type 1, strain HF (HSV-1, ATCC #VR-260). High titer virus stocks were prepared and stored as frozen aliquots at -80° C.

Host cells were maintained as monolayers according to routine tissue culture procedures. For Vero green monkey kidney, BHK-21 and LLC-MK-2 cells, the growth medium was Advanced Modified Eagle's Medium (MEM) with 10% FBS and 1% penicillin-streptomycin-amphotericin B. For RAW264.7 cells, Dulbecco's MEM (DMEM) was substituted for Advanced MEM. CRFK cells were grown in Advanced MEM with 10% horse serum, 1% antibiotic, and 1% GlutaMAX (Thermo Fisher Scientific), and HCT-8 cells were grown in RPMI-1640 with 10% FBS, 1% antibiotic and 1% GlutaMAX. All cultures were grown in a CO_2 incubator at 37°C except for the human coronavirus-infected HCT-8 cells, which were grown at 33°C.

For all tests, Maintenance Medium (MM) was the same as the growth medium except the serum concentration was reduced to 2%.

ProtecTeaV (PTV) hand sanitizer gel virucidal activity testing

In-house testing of hand sanitizer formulations against murine norovirus (MNV S99)

As a cost-effective approach prior to GLP testing, several formulations were selected to evaluate potential hand sanitizer gel prototypes in a simplified time-kill test screening against MNV S99 using standard sanitizer gel formulations (75%-78% ethanol) containing a range of EC16 concentrations (0.1%-1%) and 6%-8% sugar alcohol (the exact formulation remains proprietary). Formulations that met the Health Canada/EU required virucidal activity (>$\log_{10}$ 4 reduction within 120 sec) were designated as ProtecTeaV (PTV) formulations. The PTV formulations were tested against MNV S99 under soiled condition (virus stock containing 0.5% BSA).

Briefly, virus was mixed with 1.5% BSA at a 1:2 ratio before testing. For determination of viral titer, 150 μl the MNV virus/BSA mix was added to 350 μl MM, giving a 10^{-1} viral mix. A series of dilutions were made by adding 100 μl of this mix to 900 μl MM up to 10^{-8}. In a 48-well plate of cells, a 250 μl aliquot from each virus dilution (10^{-5}-10^{-8}) was added to designated wells in triplicate. For sanitizer tests, 0.3 ml of the virus/BSA mix was added to 9.7 ml sanitizer in a 50 ml centrifuge tube, mixed and incubated for 60 seconds at room temperature, after which a 100 μl aliquot was added to 0.9 ml MM (a 10^{-3} viral/sanitizer mix). A series of dilutions were made from this mix to 10^{-6}, and then 250 μl from each dilution was loaded into designated wells in triplicate for the $TCID_{50}$ assay. After a 1-hour incubation (viral absorption), the contents of each well were replaced with 250 μl MM. The plates were then placed in an incubator for at least 48 hours before observation for CPE.

Independent GLP laboratory tests

Based on the initial prototype screening, one of the PTV formulations, PTV80 hand sanitizer gel, was tested for virucidal activity against MNV and HSV-1 in an independent GLP (Good Laboratory Practice compliant) laboratory according to the European Standard EN 14476:2019, "Chemical Disinfectants and Antiseptics—Quantitative Suspension Test for the Evaluation of Virucidal Activity in the Medical Area—Test Method and Requirements (Phase 2/Step1), without modification. Testing was performed in accordance with Good Laboratory Practices (GLP), as specified in 21 CFR Part 58. Requisite testing of the product composition was performed by Nelson Laboratories Bozeman, LLC (formerly BioScience Laboratories).

For testing, host cells were seeded into multiwell tissue culture plates and used within 48 hours at a confluency of about 80%. The hand sanitizer gel was tested in the virucidal suspension test at an 80% v/v product dilution, with 0.3 g/l bovine albumin as the interfering substance (clean conditions). In brief, after equilibration to 20 °C, 1 ml of the test virus suspension was mixed with 1 ml of 3 g/l bovine albumin, and then 8 ml of hand sanitizer. After 1 minute incubation an aliquot of the treatment mixture was diluted with Dey-Engley Neutralizing Broth and then 10-fold dilutions were made in MM. Each dilution was plated onto 8 replicate wells of the host cells. Cytotoxic and cytopathic effects were monitored by inverted light microscopy after culture for 5-7 days at 37 °C.

Five controls were performed for the product test, each with 8 replicate wells. A Cell Culture control for normal growth (ie, absent virus or product treatment) was performed by replacing the growth medium with MM. A valid test required viable, attached cells at the end of the culture period. A virus control determined the initial titer of the untreated virus in the treatment mixture in the absence of treatment (by dilution with water not sanitizer), interfering substance and suppression/Dey-Engley Neutralizing Broth. A valid test required an absence of cytotoxic effect (such as changes in cell morphology or detachment) at a dilution at which a Virucidal Test could show a $\geq$4.0 $\log_{10}$ reduction.

$TCID_{50}$ determination

Viral and toxicity titers were estimated from the number of wells showing CPE using the Spearman-Kärger method, and expressed as the $-\log_{10}$ of the 50% titration end point for infectivity ($TCID_{50}$). The $\log_{10}$ reduction was calculated as ($\log_{10}$ $TCID_{50}$ virus control)- ($\log_{10}$ $TCID_{50}$ virus test).

PST70 surface disinfectant spray virucidal efficacy testing

In-house screening tests

A number of formulations were prepared using 70% v/v ethanol with selected concentrations of EC16 (0.1%-1% w/v) to make PST70 surface disinfectant spray prototypes for evaluation in a simplified time-kill test screening against FCV F9.

According to the results from these screening tests, various concentrations of citric acid was added to the formulations to adjust the pH. Formulations with a pH between 3 and 4 were designated as PST70 formula prototypes and tested against FCV F9 under clean condition (virus stock containing 0.5% BSA). Briefly, 0.1 ml of FCV F9 virus stock with 5% FBS was added to a sterile glass Petri dish, spread with a cell scraper and allowed to air dry. PST70 spray formulations with 70% ethanol were then applied directly onto the dried virus film by spraying 3-4 times from a spray bottle and incubated for 1 min. Then, 10 ml of MM was added to the petri dish to cover the film, and the film was mixed by scraping with a cell scraper. A series of 10-fold dilutions was made with MM to 10^{-5} by taking 100 μl of mix and adding to 900 μl MM in centrifuge tubes.

Virus control: the virus film was dried as described above, and 1 ml MM added. After a 1-minute incubation 10 ml MM was added to the film and mixed with a cell scraper. Dilutions were made as described above up to 10^7-fold. A 100 μl aliquot from each dilution was added to the designated wells (4 well repeat) in a 96-well plate for $TCID_{50}$ assay. After a 1-hour incubation, the contents of each well were replaced with MM. The plates were then placed in an incubator for 48 hours prior to observation for CPE.

Pre-GLP test: Based on the results from prototype tests, one PST70 prototype containing 0.1% EC16 was selected for a Pre-GLP test in 24 well plates. Briefly, for the Virus control, 0.2 ml of FCV F9 virus mix with 5% FBS was added to a sterile glass Petri dish, spread with a cell scraper and air-dried under a tissue culture hood. After drying, 2 ml of MM was added to the virus film, and after 1 minute 20 ml of MM was added to recover the virus. A series of 10-fold dilutions was made with MM to 10^{-7}. A 1 ml aliquot was loaded per well, 4 repeats per row for dilutions 10^{-3}-10^{-7}. The other row was used as an untreated cell control. After 1 hour virus absorption, the contents of the wells were replaced with MM and the plates incubated for 48 hours prior to CPE observation followed by $TCID_{50}$ calculation. For the PST70 spray test, 0.2 ml of FCV F9 virus stock with FBS 5% was added to a sterile glass Petri dish, spread with a cell scraper and air dried. The PST70 formulation was applied directly to the surface of the dried virus film using 3-4 sprays from a spray bottle at a 90° angle. After a 1 minute exposure 20 ml of MM was added to the petri dish, and mixed by scraping the film using a cell scraper. A series of 10-fold dilutions was made with MM to 10^5-fold by taking 500 μl of the mix and adding to 4.5 ml MM in 15 ml centrifuge tubes. One ml of the dilutions was then added to the designated wells of a 24-well plate, 4 repeats per row 10^{-2}-10^{-5}. The other 2 rows served as untreated cell control. After 1 hour virus absorption, the contents of the wells were replaced with MM and incubated for 48 hours prior to $TCID_{50}$ calculation.

Independent GLP laboratory tests

Disinfectant spray virucidal activity against FCV F9 and human betacoronavirus OC43 (selected because SARS-CoV-2 is also a betacoronavirus) were evaluated in an independent GLP laboratory according to Good Laboratory Practice Standards as specified in the requirement of EPA Office of Safety and Pollution Prevention OCSPP 810.2200, Disinfectants for Use on Hard Surfaces—Efficacy Data Recommendations (February 28, 2018). The tests were performed according to ASTM test method E1053-11 and E1053-20 (in 2 GLP laboratories on FCV F9, with consistent results) or E1053-20 (OC43). Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surface, and the Official Methods of Analysis, 961.02, AOAC Germicidal Spray Products as Disinfectants. Two independently made batches of disinfectant spray with identical formulation containing 0.1% EC16 were tested.

Briefly, sterile 100 mm diameter sterile glass Petri dishes were used as the carrier surface. High titer viral suspensions were adjusted to 5% FBS (for OC43 testing) or heat inactivated FBS (FCV testing), and 0.2 ml of suspension spread over the bottom inside dish surface using a cell scraper. Each virus inoculum was air dried at ambient temperature (about 22°C) until visibly dry.

The spray bottle was positioned with the nozzle 6-8 inches over the dish bottom surface, and 3-4 mist sprays applied to thoroughly wet the surface. After a 1-minute exposure at ambient temperature, an 18.0 ml aliquot of neutralizing medium was added, and a cell scraper used to recover remaining virus from the glass surface. A 10-fold dilution series was prepared in neutralizing medium, and each dilution plated in quadruplicate onto host cells grown in a 24 well plate. Cytotoxicity and viral cytopathic effect (CPE) were assessed by inverted light microscopy after 6 days growth for FCV, and 11 days for human coronavirus.

Acceptable virucidal activity required a ≥3 log_{10} reduction for both tested batches in the presence or absence of cytotoxicity, and at least a ≥3 log_{10} reduction beyond any observed cytotoxic level. A log_{10} reduction was calculated as for sanitizer testing. A % Reduction was calculated as 100 x (1-$TCID_{50}$ test/$TCID_{50}$ virus control).

PST70 surface disinfectant wipe virucidal efficacy testing

The surface disinfectant wipes were made using PST70 formulation-saturated nonwoven fabric rectangles (7 × 10 inches) sealed in plastic bags.

In-house Pre-GLP tests

A 1.0 ml aliquot of the virus suspension was transferred to a surface approximately 3.16" x 3.16" on a sterilized 150 mm x 20 mm sterile glass Petri dish carrier. A sterile cell scraper was used to spread the inoculum uniformly. The virus suspension was air-dried at room temperature until visibly dry. A saturated wipe was folded prior to passing over the contaminated surface. Each section of the carrier was wiped using 2 passes, where 1 pass equals a back-and-forth motion for a total of 4 motions per section. An unused area of the folded wipe was exposed for each section. The sections were treated in such a way that overlap was minimal. After the carrier was wiped, the dish was covered with a lid for 1 minute before adding 10 ml of MM to the Petri dish. The liquid was mixed with a cell scraper giving a 10^{-1} dilution of the original load. A series of 10-fold dilutions was made with MM to 10^5-fold by taking 500 μl of the mix and adding to 4.5 ml MM in 15 ml centrifuge tubes. One ml of the dilutions was then added to the designated wells of a 24-well plate, 4 repeats per row from 10^{-2} to 10^{-5}. The other 2 rows served as blank control. After 1 hour virus absorption, the contents of the wells were replaced with MM and incubated for 48 hours prior to $TCID_{50}$ calculation.

Virus control: The test viruses were applied and dried as described above. After addition of 10 ml MM to the contaminated surface and exposure for 1 min, the virus was scraped from the surface. Subsequent 10-fold dilutions were made in MM and plated in 4 replicates in 24-well plate from 10^{-4} to10^{-8}. After 1 hour virus absorption, the contents of the wells were replaced with MM and incubated for 48 hours prior to CPE observation and subsequent $TCID_{50}$ calculation.

Independent GLP test of PST70 wipe

Hard surface disinfection efficacy, when used on dry, non- porous, inanimate surfaces, was determined for of 2 independent batches of wipes (with a PST70 formulation identical to the spray) against FCV F9 as a surrogate for human Norovirus. EPA studies on surface disinfectants are required to follow OCSPP810.2200: Disinfectants for Use on Environmental Surfaces (February 2018), and OCSPP 81 0.2000: General Considerations for Testing Public Health Antimicrobial Pesticides (February 2018). In order to meet the EPA study requirement for the wipe test, the testing protocol was designed based on methods described in the American Society for Test Materials (ASTM) test method designated ASTM E2362-15, Standard Practice for Evaluation of Pre-saturated or Impregnated Towelettes for Hard Surface Disinfection and E1053- 11, Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surface, and ASTM E2896-12, Standard Test Method for Quantitative Petri Plate Method (QPM) for Determining the Effectiveness of Antimicrobial Towelettes (ASTM E2896-12 was withdrawn in 2020 after the tests were conducted).

Statistical analysis

For screening tests and pre-GLP tests, the primary statistical test for comparison of prototypes was either parametric one-way ANOVA (n >3 replicate experiments) or a t test, with appropriate adjunct tests for homogeneity of variance, etc., Alpha was 0.05.

The GLP tests do not have a statistical component; they require 3 repeated tests for 1 batch of the hand sanitizer, and with at least 2 independent batches for the surface disinfectant that all meet the required log_{10} reduction of specific organisms.

RESULTS

Initial formulations of hand sanitizers and surface sprays and wipes were tested in house using simplified versions of the requisite test, and modified as needed to achieve adequate reductions in viral yield. In some cases, formulations were then tested in-house according to protocols similar to those to be used for GLP testing. The results of in-house tests for the 3 prototypes are shown in Table 1.

GLP testing of virucidal activity

Formulations giving appropriate reductions in viral yield in in-house testing were designated as prototypes and submitted for independent testing at GLP laboratory (results summarized in Table 1). In addition to testing against the norovirus surrogates MNV S99 and FCV F9, the sanitizers were also tested against HSV1 and/or OC43 as an initial evaluation of the breadth of their antiviral activity.

Hand sanitizer

The PTV80 formulation at 75%-78% ethanol v/v concentration was tested according to European Standard protocol EN14476 in a GLP laboratory. The virucidal activity of the PTV80 hand gel prototype against both non-enveloped MNV S99 and enveloped virus HSV1 (selected as a human pathogenic virus and based on previous studies with EC16) were tested at the GLP laboratory.

In both nonenveloped MNV and enveloped HSV1 tests, a 10^3-fold dilution of the hand sanitizer was required to eliminate an observable cytotoxic effect at a dilution required to demonstrate a ≥4.0 log_{10} reduction. For both virus tests, all test acceptance criteria were met by the controls. The Virus and Suppression controls showed a difference of ≤0.5 log_{10}, indicating immediate neutralization of the product, and the Virus and Interference controls showed a difference of <1 log_{10}, indicating a lack of effect of the neutralized product on viral infection of cells.

Under the conditions of this evaluation, the Test Product, PTV80 Hand Gel, reduced the infectivity of MNV strain S99 (FLI Virusbank #RVB-651) by 5.500 log_{10} following a 60-second exposure to the active range concentration of 80%. Thus, in accordance with EN

Table 1
Virucidal activities of PTV80 hand sanitizer gel, PST70 spray, and PST70 wipe against 4 different viruses in GLP laboratories and Camellix research laboratories (In-house)

Sanitizer	MNV S99	HSV1	Surface disinfectant	FCV F9	OC43
PTV80 In-house	4.67-4.85	Not tested	PST70 Spray In-house	5.4-7.0	Not tested
PTV80 GLP	>5.50	>4.0	PST70 Spray GLP	4.13-4.63	4.25
			PST70 Wipe In-house	>4.50	Not tested
			PST70 Wipe GLP	>4.50	4.75

NOTE. GLP data was taken from the GLP laboratory final test reports (Tables 2,-5). All GLP tests were performed by Nelson Labs.

Table 2
GLP $TCID_{50}$ assay results of PTV80 hand sanitizer gel (80% concentration) against MNV S99 with 3 replicates according to EN 14476:2019 method

Parameter	Rep.	Dilutions, log10							TCID50, log10	*SE*	Log10 Reduction	K_R
		-2	-3	-4	-5	-6	-7	-8				
Test	1	CT	00000000	00000000	00000000	00000000	00000000	NT	≤2.500	0.000	**5.500**	0.378
	2	CT	00000000	00000000	00000000	00000000	00000000	NT	<2.500	0.000	**5.500**	0.378
	3	CT	00000000	00000000	00000000	00000000	00000000	NT	<2.500	0.000	**5.500**	0.378
VC	NA	NT	NT	44444444	44444444	44444440	44444444	44004400	8.000	0.189	NA	
NC	NA	NT	44444444	44444444	44444444	44444444	44444444	40000000	7.625	0.125		
CTC	NA	44444444	00000000	00000000	NT	NT	NT	NT	2.500	0.000		
IC	NA	NT	NT	44444444	44444444	44444444	44444444	00400004	7.750	0.164		
CC	NA					0000						

NOTE. Virus: MNV FLI Virusbank #RVB-651. Host Cell Line: RAW 264.7 (ATCC #TIB-71). Neutralizer: D/E Neutralizing Broth. Volume plated per well: 1.0 ml. 4-Monolayer completely destroyed by the virus or because of cytotoxicity. 0-No CPE present.
CTC, cytotoxicity control; IC, interference control; KR, standard error of log reduction; NA, not applicable; NC, neutralization control; NT, not tested; Rep, replicate; SE, standard error of logarithmic titer; VC, virus control.

Table 3
GLP $TCID_{50}$ assay results of PTV80 hand sanitizer gel (80% concentration) against HSV1 with 3 replicates according to EN 14476:2019 method

Parameter	Rep.	Dilutions, log10							TCID50, log10	*SE*	Log10 Reduction	K_R
		-2	-3	-4	-5	-6	-7	-8				
Test	1	CT	00000000	00000000	00000000	00000000	00000000	NT	2.500	0.000	**4.000**	0.354
	2	CT	00000000	00000000	00000000	00000000	00000000	NT	2.500	0.000	**4.000**	0.354
	3	CT	00000000	00000000	00000000	00000000	00000000	NT	2.500	0.000	**4.000**	0.354
VC	NA	NT	NT	44444444	44444444	44444440	00400000	00000000	6.500	0.177	NA	
NC	NA	NT	44444444	44444444	44444444	44404440	00000000	00000000	6.250	0.164		
CTC	NA	44444444	00000000	00000000	NT	NT	NT	NT	2.500	0.000		
IC	NA	NT	NT	44444444	44444444	40404444	00000000	00000000	6.250	0.164		
CC	NA					0000						

NOTE. Virus: Herpes Simplex Virus type 1 strain HF (ATCC #VR-260). Neutralizer: D/E Neutralizing Broth. Host Cell Line: Vero Host Cell Line (ATCC #CCL-81). Exposure Time: 1 minute. 4-Monolayer completely destroyed by the virus or because of cytotoxicity. 0-No CPE present.
CTC, cytotoxicity control; IC, interference control; KR, standard error of log reduction; NA, not applicable; NC, neutralization control; NT, not tested; Rep, replicate; SE, standard error of logarithmic titer; VC, virus control.

14476 the hygienic handrub Test Product, PTV80 Hand Gel, demonstrated virucidal activity via at least a decimal log reduction of 4 in MNV titer.

The result shown in Table 2 indicated PTV80 hand gel reduced MNV S99 infectivity (initial viral load (TCID50 8 log_{10}) by >99.999% (>log_{10} 5.5 average log_{10} reduction) with 0 CPE.

As shown in Tables 1 and 3 for HSV1, the PTV80 hand sanitizer prototype gave an average (n = 3) reduction in initial viral load ($TCID_{50}$ 6.5 log_{10}) of ≥4.0 log_{10} with 0 CPE.

Under the conditions of this evaluation, the Test Product, PTV80 Hand Gel, reduced the infectivity of Herpes Simplex Virus type 1 (ATCC #VR-260) by 4.000 log_{10} following a 60-second exposure to the active range concentration of 80%. That is, in accordance with EN 14476 the hygienic handrub Test Product, PTV80 Hand Gel, demonstrated virucidal activity via at least a decimal log reduction of 4 in Herpes Simplex Virus type 1 titer.

Therefore, for both MNV S99 and HSV1, the hand sanitizer formulation demonstrated effective virucidal activity (≥4.0 log_{10} reduction) and met the EN 14476 standard.

PST70 Surface disinfectant spray

Two batches of the PST70 surface spray were submitted to a GLP laboratory for full GLP tests against FCV and human coronavirus according to EPA guidelines current at the time. For FCV, the Initial Population Control $TCID_{50}$ was ≥7.5 log_{10}/ml. The Virus Control recovery $TCID_{50}$/ml (average) was 6.19 log_{10}. Effective neutralization was observed, with the Neutralization controls for each batch giving a $TCID_{50}$ within 1.0 log_{10} of the Virus Control (6.75 and 6.5 log_{10}). No cytotoxicity was observed at the least dilute mix tested (10^{-2}). Therefore, the test conditions were accepted as valid. Following a 1-minute exposure, Spray Batch 1 gave average reductions of FCV recoverable surface load of 4.63 log_{10}/carrier (>99.99%), and Spray Batch 2 gave average reductions of 4.13 log_{10}/carrier (≥99.999%) (Tables 1 and 4). Therefore, for FCV (a surrogate for human norovirus), the PST70 Spray Formulation exceeded the EPA standard of ≥3 log_{10} reduction in viral titer in the presence or absence of cytotoxicity (Table 4).

For human coronavirus OC43, the Initial Population Control $TCID_{50}$ was 6.5 log_{10}. The Virus Control recovery $TCID_{50}$/ml was 6.75 log_{10}. Effective neutralization was observed, with the Neutralization controls for each batch giving a $TCID_{50}$ within 1.0 log_{10} of the Virus Control (6.25 log_{10} each). The Virus Control recovery $TCID_{50}$/ml was 6.5 log_{10}. Cytotoxicity was observed at the least dilute mix tested (10^{-2}), but not at a 10^{-3} dilution. Therefore, the test conditions were accepted as valid. Following a 1-minute exposure, 2 PST70 Spray batches gave a reduction of coronavirus recoverable surface load of 4.25 log_{10} (>99.99%) with 0 CPE (Table 1). Therefore, for human coronavirus, the PST70 Spray Formulation exceeded the EPA standard of ≥3 log_{10} reduction in viral titer in the presence or absence of cytotoxicity (Table 5).

PST70 Surface disinfectant wipe (towelette)

The wipe prototype samples were submitted to the GLP laboratories for testing against both FVC F9 and human OC43 under full GLP conditions. The PST70 wipe samples were used in the Hard Surface Disinfection Test performed using 150 mm x 20 mm sterile glass Petri dish carriers inoculated with FCV (ATCC #VR-782) – a surrogate for human Norovirus. Batch # 1 of the Test Substance, PST70 Wipe, reduced the virus infectivity by >4.50 log_{10} (>99.99%) on 2 sets of test carriers, following a 1-minute exposure. Batch #2 of the Test Substance, PST70 Wipe, reduced the virus infectivity by >4.50 log10 (>99.99%) on 2 sets of test carriers, following a 1-minute exposure. The results of the PST70 wipe for FCV F9 efficacy were consistent

Table 4
GLP carrier test results of PST70 surface disinfectant spray against FCV with 2 batches

Dilution (- Log_{10})	Virus control		Test				Neutralization control		Cytotoxicity control		IP	CC
	Carrier 1	Carrier 2	Batch #1		Batch #2		Batch #1	Batch #2	Batch #1	Batch #2		
			Carrier 1	Carrier 2	Carrier 1	Carrier 2						
												0000
-2	NT	NT	00+0	++00	++++	++0+	++++	++++	0000	0000	NT	NA
-3	++++	++++	0000	0000	0000	0000	++++	++++	0000	0000	++++	
-4	++++	++++	0000	0000	0000	0000	++++	++++	0000	0000	++++	
-5	++++	++++	0000	0000	0000	0000	++++	++++	NT	NT	++++	
-6	++++	++++	0000	0000	0000	0000	++++	++++	NT	NT	++++	
-7	0000	0000	0000	0000	0000	0000	+000	0000	NT	NT	++++	
$TCID_{50}$ (log_{10})	6.50	6.50	1.75	2.00	2.50	2.25	6.75	6.50	1.50	1.50	≥7.50	
Average $TCID_{50}$ (log_{10})	6.50		NA								NA	
Log_{10} Reduction	NA		4.75	4.50	4.00	4.25	NA					
Percent Reduction			>99.99	>99.99	99.99	>99.99						

NOTE. The contact time is 60 sec after 3-4 sprays to the surface of each carrier according to ASTM test method E1053-20. Virus: FCV (ATCC #VR-782). Host Cell Line: CRFK Host Cell Line ATCC #CCL-94. Volume plated per well: 1.0 ml. Exposure Time: 1 minute. + -CPE (cytopathic/cytotoxic effect) present. 0-CPE (cytopathic/cytotoxic effect) not detected.
CC, cell control; IP, initial population; N/A, not applicable; NT, not tested.

with the in-house non-GLP test results (>log_{10} 4.5 reduction). Similarly, GLP carrier test results of PST70 surface disinfectant wipe samples were obtained against human coronavirus OC43 with 2 batches. (Virus: Coronavirus strain OC43 [ZeptoMetrix # 0810024CF]. Host Cell Line: HCT-8 Host Cell Line ATCC #CCL-244. Volume plated per well: 1.0 ml. Exposure Time: 1 minute). Testing of 2 different batches against human coronavirus OC43 showed log_{10} 5.25 reduction in viral titer. In fact, none of the wells showed CPE after 3 wipe motions and 60 sec contact time (data not shown due to journal guidelines on the number of tables).

DISCUSSION

The goal of the current study was to develop non-toxic hand hygiene and surface disinfectant (spray and wipe) prototypes that possess virucidal activity against non-enveloped viruses, and norovirus in particular, and that would meet regulatory standards in independent laboratory testing. For EPA-regulated hard non-porous surface disinfectants, the passing criterion for a norovirus claim is a >log_{10} 3 (99.9%) reduction in FCV titer, demonstrated beyond the cytotoxic level in carrier tests.[18] We reported previously that 70% ethanol alone or in a commercial hand sanitizer gel formulation failed to reach >log_{10} 3 reduction of FCV infectivity in 60 sec suspension time-kill tests or on a contaminated hard surface.[11] Similarly, another group reported that 70% ethanol only resulted in <log_{10} 2.15 reduction of the FCV titer.[19] These results confirm the resistance of this norovirus surrogate to ethanol. In contrast, the current study using the PST70 surface disinfectant spray prototype containing EC16 and citric acid under GLP conditions achieved >log_{10} 4 reduction (>99.99%) of FCV F9 titer after a 60 sec contact time (Table 1, Table 4), and exceeded the EPA required minimum efficacy (log_{10} 3) by 10-fold. Similarly, the PST70 surface disinfectant wipe prototype with the same formulation also reduced FCV F9 titer by > log_{10} 4 (99.99% reduction) in the requisite test (Table 1).

It is known that pH influences the efficacy of alcohol-based disinfectants. Certain human norovirus strains are relatively sensitive to alcohol-based disinfectants with either low (<3.0) or high pH (>11).[20] However, extreme levels of pH are corrosive. The EPA standard for liquid corrosive material is a pH greater than or equal to 12.5, or less than or equal to pH 2 (Introduction to United States Environmental Protection Agency Hazardous Waste Identification [40 CFR Parts 261]). Therefore, the formulation of the PST70 surface spray was made with only a small amount of citric acid, resulting in a pH of >3.0, to avoid a potential corrosive effect and/or waste disposal issues. In combination with 0.1% EC16 and 70% ethanol, the PST70 spray prototype evidenced a powerful virucidal activity against FCV F9 in 60 sec (>99.99% reduction) while maintaining a formulation that only contains ethanol, water, citric acid, and EC16, ingredients derived from plants and found in popular beverages (e.g., wine, lemon juice, green tea, etc.). Similarly, the PST70 spray prototype resulted in >log_{10} 4 reduction of human coronavirus OC43. This potent virucidal efficacy of the PST70 formulation was also observed for the wipe prototype using this formulation (Table 1).

It is possible that synergy played a significant role in the observed high virucidal efficacy. We reported recently that EC16 dissolved in cell culture medium with glycerol, without alcohol or citric acid, is able to reduce MNV S99 infectivity by >100 fold.[16] A potential mechanism for a synergistic effect with PST70 formulations could be that EC16 alters the viral coat, creating a structural change to allow alcohol to enter into the core of the virus, causing rapid and irreversible damage. Such a phenomenon of structural alteration by the combination of EC16 and alcohol has been observed for bacterial spores by electron microscopy.[14] It was reported that without alcohol, EGCG (hydrolyzed EC16) is able to cause a structure change of herpes simplex virus,[21] and EC16 is significantly more effective than EGCG.[22] The addition of citric acid lowers the pH in the formulation, in favor of viral destruction.

For the hand sanitizer gel prototype, the viricidal efficacy test was performed using a 60 sec time-kill suspension method against MNV S99 according to the method of EN 14476:2019 or ASTM E1052, with a passing criterion of >log_{10} 4 reduction of MNV titer, according to the recommendations from Health Canada and European Union (the US FDA does not allow virucidal claims for hand sanitizers). The PTV80 formulation does not contain citric acid and the pH is within a 6.0-6.5 range in consideration of frequent use on the skin. A previous report demonstrated that low pH in hand sanitizer formulations favors virucidal activity against FCV but not MNV, but a higher concentration of alcohol (ethanol) increased virucidal activity against MNV but not FCV. FCV is known to be less sensitive to ethanol than MNV. However, neither condition alone resulted in >log_{10} 4 reduction of viral titer.[23] Thus, the addition of EC16 in hand sanitizer formulations significantly increases the virucidal activity relative to formulations without EC16.

The virucidal activity of the earlier ProtecTeaV (PTV) formulations demonstrated they possessed virucidal activity against nonenveloped viruses such as FCV, polio virus-1, and MNV.[10,11,16] Consistent with our previous reports and the PST70 results described here, the GLP final test report of PTV80 hand sanitizer gel virucidal activity against

Table 5
GLP carrier test results of PST70 surface disinfectant spray against human coronavirus OC43 with 2 batches

Dilution (- Log_{10})	Plate recovery control	Test		Neutralization control		Cytotoxicity control		Virus sock titer	Virus control	NCC	CC
		Batch #1	Batch #2	Batch #1	Batch #2	Batch #1	Batch #2				
										0000	0000
-2	NT	CT	CT	NT	NT	++++	++++	NT	NT	NA	NA
-3	++++	0000	0000	++++	++++	0000	0000	++++	++++		
-4	++++	0000	0000	++++	++++	0000	0000	++++	++++		
-5	++++	0000	0000	++++	++++	NT	NT	++++	++++		
-6	++++	0000	0000	+++0	+00+	NT	NT	++++	++++		
-7	000+	NT	NT	0000	+000	NT	NT	0+00	0000		
$TCID_{50}$ (log_{10})/carrier	6.75	2.50	2.50	6.25	6.25	2.50	2.50	6.75	6.50		
Log_{10} Reduction	NA	4.25	4.25	NA							
Percent Reduction		>99.99	>99.99								

The contact time is 60 sec after 3-4 sprays to the surface of each carrier according to ASTM test method E1053-20. Virus: Coronavirus strain OC43 (ZeptoMetrix # 0810024CF) Host Cell Line: HCT-8 Host Cell Line ATCC #CCL-244. Volume plated per well: 1.0 ml. Exposure Time: 1 minute. + -CPE (cytopathic/cytotoxic effect) present. 0-CPE (cytopathic/cytotoxic effect) not detected.
CC, cell control; IP, initial population; N/A, not applicable; NT, not tested.

MNV S99 demonstrated an efficacy of >log_{10} 5.5 (>99.999%) reduction of viral titer, without any CPE (Tables 1 and 2). In contrast, an alcohol-based sanitizer with 79% ethanol was reported to reduce MNV infectivity by only >log_{10} 3.6.[23] That is, the PTV80 hand sanitizer with EC16 and 75-78% ethanol is approximately 100-fold more effective than the sanitizer with 79% ethanol, but without EC16. Thus, the PTV80 hand sanitizer gel prototype could enhance the efficacy of currently used healthcare hand antiseptics that are without markedly effective virucidal and/or sporicidal activity. Similarly, since the PST70 surface spray formulation is not toxic or corrosive, it could be developed into a surgical hand rub with virucidal activity against non-enveloped viruses.

In terms of the regulatory requirements for products, healthcare hand antiseptics, including healthcare personnel hand sanitizers and surgical hand rubs, are regulated by the FDA. It is important to note that EC16 is one of the green tea polyphenol-palmitates that is classified as GRAS by the FDA, and a safe inert approved by the EPA, but it is not an FDA-approved drug substance or active ingredient for labeling purposes. The limitation of the in vitro test on the PTV80 hand sanitizer gel prototype is that the evidence does not reflect in vivo safety and efficacy results required by the FDA/EU/Health Canada for healthcare personnel hand antiseptics. As FDA-regulated human OTC drugs, each healthcare topical antiseptic product must be tested for safety and efficacy in vitro and in vivo. For examples, clinical studies of healthcare hand antiseptics require safety tests, photo allergenicity test, human efficacy tests according to the ASTM E2755 protocol, plus in vitro time-kill tests according to the ASTM E2783 protocol against a list of microorganisms. However, bacterial spores and viruses are not on the FDA list of microorganisms. Indeed, the virucidal and sporicidal activities of the currently used healthcare hand antiseptic products are not known. Since these hand hygiene products are mainly alcohol-based hand gels or liquid rubs with alcohol concentration up to 90% (the alcohol concentration range allowed by the FDA is 60%-95%),[24] they are unlikely to have efficacy against alcohol-resistant microorganisms such as non-enveloped viruses and bacterial spores. Therefore, future preclinical and clinical studies on EC16 are warranted to evaluate its use as an active virucidal and sporicidal ingredient.

CONCLUSIONS

Here we report that alcohol-based hand hygiene and surface disinfectant prototypes containing EC16 demonstrate strong virucidal activities against norovirus (the surrogates MNV and FCV according to regulatory agency test requirements) and other pathogenic enveloped viruses such as HSV1 and human coronavirus OC43. The advantage of these prototypes resides in the safety of the ingredients, which can be found in consumable fruits or beverages (water, alcohol, green tea, and citrus), and the efficacy of the formulations. These alcohol-based prototypes also possess bactericidal and fungicidal activities, validated previously by GLP laboratories.[17] In combination with the sporicidal activities in similar EC16 formulations,[13,14] the non-toxic approach toward comprehensive disease prevention and disinfection solutions could lead to novel products with universal effects against circulating or emerging pathogenic bacteria, fungi, bacterial spores, and non-enveloped viruses, pending future studies and tests.

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