



Research Article

Virucidal Capacity of Novel ProtecTeaV Sanitizer Formulations Containing Lipophilic Epigallocatechin-3-Gallate (EGCG)

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Abstract

Current non-toxic or “Generally Recognized as Safe” (GRAS) countermeasures against viral entry into cells in the human body are often inadequate. The objective of this study was to evaluate ProtecTeaV, a novel alcohol-based instant hand sanitizer formulation containing lipophilic EGCG (derived from green tea extract), in comparison to commonly used hand sanitizers either with or without alcohol. A Standard 50% Tissue Culture Infective Dose (TCID₅₀) assay was used for determination of virucidal capacity against Poliovirus 1 (PV 1). Separately, neutralization by an ultra-filtration method was used to evaluate the mechanism of virucidal capacity. The results demonstrated that ProtecTeaV formulations (with and without gelling agent) reduced the TCID₅₀ by a factor of 6 (mean log₁₀-reduction of viral infectivity), 100-fold more than the reduction of viral infectivity (>4 log₁₀) mandated by internationally accepted standards. In addition, the virucidal effect of ProtecTeaV formulations was associated with direct and irreversible inactivation of PV 1, rather than a reversible inhibition mechanism. In contrast, two commonly used instant hand sanitizers failed to reduce PV 1 viral infectivity by >4 log₁₀. In conclusion, ProtecTeaV instant hand sanitizer formulations containing lipophilic EGCG possess effective virucidal capability with the potential for use in novel disinfectant and antiseptic approaches, pending additional research and development.

Keywords: Alcohol; EGCG; Hand sanitizer; Green tea; Viral infections

Introduction

Alcohol-based instant sanitizers gained popularity due to their ease-of-use and effectiveness against actively growing bacteria, as well

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as some viruses [1,2]. They are used routinely in clinics, schools, institutions, families, and on cruise ships for the purpose of prevention of microbial infections. However, current alcohol-based hand sanitizers have some weaknesses regarding the prevention of viral infections, and thus far, an alcohol-based hand sanitizer with persistent virucidal capacity has not been described. Alcohol evaporates in a matter of seconds after each application on the skin or other surfaces, leaving the skin surface unprotected until the next application. For example, although the concept “alcohol-based sanitizer is effective to inactivate influenza virus” has been well-accepted, a recent case-control human study indicates that alcohol-based hand sanitizer is not effective in school-aged children during outbreaks of influenza infection in community settings [3]. This result could be due to the short life of alcohol activity. More importantly, many non-enveloped pathogenic viruses such as norovirus, poliovirus, adenovirus and hepatitis A virus are resistant to alcohol [4-7]. Conliffe et al., reported that three of these-norovirus, rotavirus and adenovirus were responsible for the majority of healthcare-associated viral gastroenteritis cases among children in a large pediatric hospital setting [8]. Gastroenteritis caused by these viruses can be severe and costly to patients and healthcare providers [9]. In the US, norovirus alone is responsible for approximately 20 million acute gastroenteritis cases, 400,000 emergency visits, and 70,000 hospitalizations, with up to 800 deaths annually (US CDC, Norovirus: US Trends and Outbreaks). Another weakness of alcohol-based hand sanitizers is that alcohols do not inactivate bacterial endospores effectively [10,11]. Due to the weak virucidal capacity of alcohols against enteric viruses, and inability to inactivate bacterial spores and hydrophilic (non-enveloped) viruses, the US CDC 2008 Guidelines for Disinfection and Sterilization in Healthcare Facilities removed isopropyl alcohol and ethyl alcohol from the list of high-level disinfectants. In 2011, the CDC updated the guidelines for the Prevention and Control of Norovirus Gastroenteritis Outbreaks in Healthcare Settings. The new guideline for hand hygiene is: “during outbreaks, use soap and water for hand hygiene after providing care or having contact with patients suspected or confirmed with norovirus gastroenteritis”, because “very low-quality evidence was available to suggest that hand hygiene using alcohol-based hand sanitizers may reduce the likelihood of symptomatic norovirus infection”. In fact, the use of alcohol-based hand sanitizer was found to be a risk factor for norovirus spread vs soap and water wash in long-term care facilities [12]. In a human study, a 30-second incubation with alcohol (3% - 95%) was ineffective in reducing the infectivity of norovirus (genomic copy reduction <0.5 log₁₀); this was less effective than liquid soap or water rinse [13]. Although various chemicals inactivate non-enveloped viruses effectively, they are not feasible for use in hand sanitizers due to toxicity.

In recent years, plant-derived phytochemicals, such as extracts from grape seed, pomegranate, mulberry, cranberry, ginseng, persimmon, green tea and herbal essential oils, have been reported to exert antiviral properties against non-enveloped viruses, including enteric viruses [14]. Epigallocatechin-3-Gallate (EGCG) is the major polyphenol present in green tea leaves and its antiviral activities have been studied extensively. The most suitable derivatives of EGCG for topical applications are lipophilic EGCG compounds due to their global antiviral properties, high potency, persistency, and absence of

associated toxicity [15]. Results from a recent phase II clinical trial of the proprietary AverTeaX formula containing lipophilic EGCG for treating herpes labialis demonstrated that topical application of the AverTeaX formula provides high efficacy in treating herpes labialis [16]. Due to its unique antiviral activities, lipophilic EGCG could be used in a novel approach to a new generation of sanitizing products, either with or without alcohol. We hypothesized that lipophilic EGCG in surface-sanitizing formulations would be effective against human pathogenic viruses, with the potential for use as a global virucidal agent to prevent viral infections, particularly from alcohol-resistant non-enveloped viruses.

To test our hypothesis, the current study evaluated two proprietary alcohol-based instant hand sanitizer formulations, ProtecTeaV+cb and ProtecTeaV-cb both containing lipophilic EGCG (with (+) or without (-) gelling thickener), in comparison to two widely used alcohol-based hand sanitizers, one containing alcohol and the other a mixture of iodine and polyvinyl pyrrolidone. The internationally accepted method to test virucidal activity *in vitro* for disinfectants and cleansing agents was used [EN 14476-2013: Chemical and Antiseptics - Quantitative Suspension Test for the evaluation of virucidal activity in the medical area - test method and requirements (Phase 2/Step 1); Guidance Document Human-Use Antiseptic Drugs 2009, Minister of Health of Canada; and Technical Standard For Disinfection 2002 edition, Department of Health of People's Republic of China] to determine the efficacy (measured by TCID₅₀ assays) against Poliovirus 1 (PV 1, a non-enveloped virus resistant to alcohol) as a representative of virucidal activity. This virus is considered the most difficult to eradicate. To the best of our knowledge, this was the first attempt to test the virucidal capacity of lipophilic EGCG against a non-enveloped virus in alcohol-based hand sanitizer formulations.

Materials & Methods

African green monkey kidney VERO cells were obtained from the China Center for Type Culture Collection (CCTCC GDC029) and cultured in DMEM cell culture medium with 100U/ml penicillin, 100g/ml streptomycin and 10% fetal bovine serum. Lipophilic EGCG was provided by Camellix, LLC (Evans, GA, USA). PV1 was obtained from the CCTCC (GDV057). Carbomer (gelling thickener) was obtained from YuTai (Guangzhou, China). One-hundred percent ethanol analytical grade was from Hushi (Shanghai, China). Fetal Bovine Serum was from Gibco (Australia), DMEM cell culture medium from Gibco (China), trypsin from Gibco (Made in Canada), 96 well cell culture plates from LabServ (China). The 30K ultra filters used were Amicon Ultra-4 (Millipore, USA), 1.5 ml centrifuge tubes are from Axygen (Union, USA), and the ultra-pure water is from a Milli-Q (Millipore, USA). The centrifuge used was a Pico 17 (Thermo, Germany), and the microscope model was T1-SM TS100 (Nikon, Japan).

VERO cells were cultured in T75 flasks ($\geq 90\%$ confluent monolayer) and infected with PV1 at a Multiplicity of Infection (MOI) of 0.1-1 for 2-3 days until more than 80% cells showed a Cytopathic Effect (CPE). Stock virus suspensions were made by collecting and filtering the supernatant and the viral titer was measured and expressed as 10^8 TCID₅₀/0.1 ml by the TCID₅₀ protocol [17]. Aliquots were stored at -80°C until use.

Viral titration

Briefly, using 96 well plates, VERO cells were seeded at 10^4 cells per well and incubated for 24 h. Serial dilutions of PV1 in 10 fold

steps (10^{-1} to 10^{-10}) were prepared prior to incubation with the VERO cells in the 96-well plates. Medium from each well was removed and 100 μl of each dilution was added. Each dilution was tested with four duplicated wells. Unattached virus was aspirated off after incubation for two-hours at 37°C in a cell culture incubator with $5\% \text{CO}_2$, and fresh cell culture medium was placed in each well. The plates were then incubated for another 72 hours before being removed for CPE observation.

Testing sanitizer samples

Both ProtecTeaV hand sanitizer formulations contain 70% ethanol (v/v). ProtecTeaV-cb, hand sanitizer contains lipophilic EGCG, but without carbomer; ProtecTeaV+cb, hand sanitizer contains lipophilic EGCG and carbomer. Specifics of the proportions of lipophilic EGCG and carbomer are proprietary information. These formulations were tested against adf 0.1% PVP-I hand sanitizer (batch NO61040, expiration date 06/11/2016), containing a mixture of iodine and polyvinyl pyrrolidone (adf, Shenzhen, Guangdong, China), and Purell Instant Hand Sanitizer containing 62% ethanol (batch L62553, expiration date 03/2017, GOJO Industries, Inc., Akron, OH, USA).

Testing procedures

Testing solutions were prepared by mixing 50 μl PV1 suspensions (Average $10^{8.15}$ TCID₅₀/0.1 ml) with 450 μl of testing sanitizer samples. Mixtures were incubated at 20°C ($\pm 1^\circ\text{C}$) for 30, 60 and 90 seconds, followed by the virus infectivity assay [17]. For the positive control (all of the wells showing CPE), 50 μl of PV1 was mixed with 450 μl serum-free cell culture medium and incubated for 60 seconds. A series of 10-fold dilutions with serum-free cell culture medium were made prior to the virus infectivity assay. For the negative control (none of the wells showing CPE), cells were treated with culture medium only. The 96-well plates, with each well containing 0.1 ml cell culture media with (or without as negative control) sanitizer/PV1 mix, were incubated in a cell culture incubator at 37°C for 72 h prior to CPE observation by recording positive and negative wells for each testing sanitizer sample under the light microscope. TCID₅₀/0.1 ml (generated from 0.1 ml medium plus sanitizer/PV1 mix, expressed as TCID₅₀ hereafter) was calculated using the TCID₅₀ calculator (Marco Binder, Heidelberg University). The experiments were repeated independently three times on different days.

Physical neutralization by sanitizer assay

To determine if PV1 was inactivated by the ProtecTeaV+cb hand sanitizer formulation, instead of being inhibited temporarily, physical neutralization assays were performed using an ultra-filtration method according to the previously published standard [17]. Prior to performing the TCID₅₀ assay, the sanitizer formulation was mixed with PV1 suspension before ultra-filtration. Sanitizer (450 μl) was initially incubated in a 20°C ($\pm 1^\circ\text{C}$) water bath for 5 min, mixed with 50 μl PV 1 suspension, incubated for 1 min prior to a series of 10-fold dilutions, then transferred to 30 kDa ultra-filtration tubes. The tubes were centrifuged at 7500 g for 20 min the inner tubes were then inverted and placed in new sleeve tubes prior to centrifugation at 13,000 g for 5 min. The supernatants were collected and brought to 500 μl with cell culture medium. This treatment removed sanitizer and collected virus.

As a control to normalize the virus that might be lost during the ultra-filtration process (without sanitizer removal), sanitizer formulation was mixed with PV1 stock suspension prior to mock

Product	Active ingredients	Mean log ₁₀ -reduction of viral infectivity		
		30 s (SD)	60 s (SD)	90 s (SD)
ProtecTeaV- with L - EGCG	Ethanol (70%)	6.65 (±0)	6.65 (±0)	6.62 (±0.58)
ProtecTeaV+ with L - EGCG	Ethanol (70%)	5.98 (±0.49)	6.02 (±0.42)	6.53 (±0.40)
Purell	Ethanol (62%)	3.28 (±1.1)	2.85 (±0.61)	3.24 (±0.51)
adf	PVP-Iodine	2.88 (±1.07)	3.12 (±1.31)	2.75 (±0.98)

Table 1: Efficacy of four hand sanitizers either with alcohol (ProtecTeaV, Purell) or without alcohol (adf) against PV-1 using TCID50 assays. Data are from three independent experiments.

ProtecTeaV-: without thickener agent.

ProtecTeaV+: with thickener agent.

L-EGCG: Lipophilic Epigallocatechin-3-Gallate.

PV-1: Poliovirus 1.

SD: Standard Deviation of 3 independent experiments (n=3).

ultra-filtration. Sanitizer (450 µl) was initially incubated in a 20°C (±1°C) water bath for 5 min, mixed with 50 µl PV 1 suspension, incubated for 1 min before a series of 10-fold dilution, then transferred to 30 kDa ultra filter tubes (with an inner tube/filter and sleeve tube). The tubes were centrifuged at 7500 g for 20 min. Then the inner tubes were inverted and placed in the same sleeve tubes prior to centrifugation at 13,000 g for 5 min. The supernatants were collected and brought to 500 µl with cell culture medium. As a positive control without sanitizer incubation, PV1 suspension was ultra-filtered. PV1 suspension (50 µl) was mixed with 450 µl serum-free cell culture medium, and then transferred to 30 kDa ultra filter tubes. The tubes were centrifuged at 7500 g for 20 min. Then the inner tubes were inverted in new sleeve tubes prior to centrifugation at 13,000 g for 5 min. The supernatants were collected and brought to 500 µl with cell culture medium.

To normalize for the virus that might be lost during the ultra-filtration process (without sanitizer present), mock ultra-filtration was performed for PV1 without sanitizer. PV1 suspension (50 µl) was mixed with 450 µl serum-free cell culture medium prior to a series of 10-fold dilution and then transferred to 30 kDa ultra filter tubes. The tubes were centrifuged at 7500 g for 20 min. Then the inner tubes were inverted in the same sleeve tubes prior to centrifugation at 13,000 g for 5 min. The supernatants were collected and brought to 500 µl with cell culture medium.

The negative control was VERO cell culture without PV1 infection in duplicate wells. The supernatants from various treatments were used for the TCID50 assay according to the method described above. The experiments were repeated three times independently.

Statistical analysis

All experiments were reproducible and repeated three times with similar results. Results are expressed as mean log₁₀-reduction of viral infectivity (Table 1) and presented as bar graphs showing the actual PV1 infectivity from TCID50 assays and standard deviations (n=3) for each sample (Figures 1 and 2). Significance of differences between the four sanitizers was calculated using two-way ANOVA (Time one factor, three levels; Sanitizer second factor, four levels). Homogeneity of variance was assumed. ANOVA is somewhat robust to heterogeneity of variance with equal group sizes (as here), with a caution regarding an excess of false-positives. Sidak's multiple comparisons test was used for post-hoc testing. One-way ANOVA with Tukey's multiple comparisons test was used for the assessment of results from the physical neutralization by sanitizer assay. Significant differences were accepted for p<0.05 between samples.

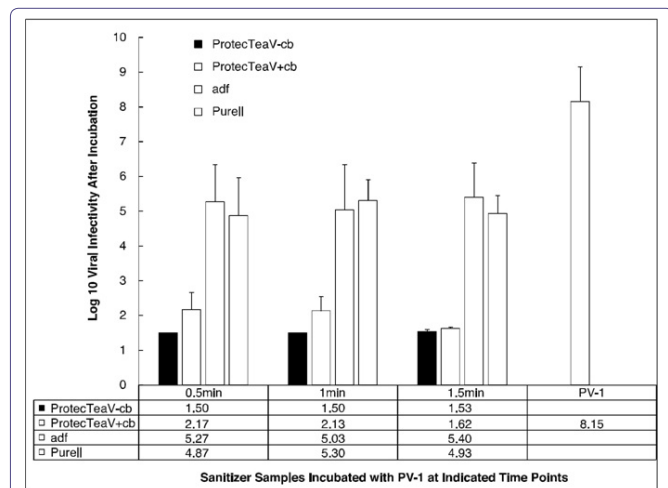


Figure 1: TCID50 assay results comparing virucidal capacity against PV-1 after incubation with different sanitizer samples for 30, 60 and 90 seconds. Data is from three independent experiments. TCID50 infectivity is presented as log₁₀ scales with standard deviations (n=3).

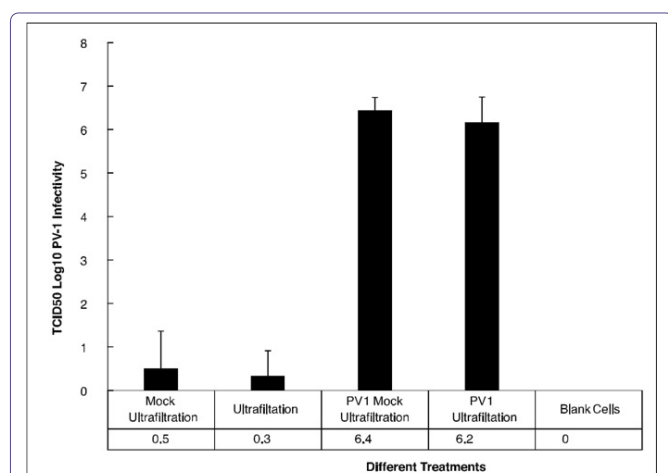


Figure 2: Mean log₁₀ of PV 1 infectivity (TCID50) after ultra-filtration of ProtecTeaV+ sanitizer formulation in comparison to mock ultra-filtration and untreated PV 1 with standard deviations. From left, lane 1: Mock ultra-filtration collected both virus and sanitizer after incubation and ultra-filtration; lane 2: Ultra-filtration only collected virus after incubation and ultra-filtration; lane 3: PV 1 mock ultra-filtration collected medium and virus after incubation with cell culture medium; lane 4: PV 1 ultra-filtration only collected virus after incubation with cell culture medium; lane 5: blank control using VERO cells only.

Results

Virucidal activities of different hand sanitizers

As shown in Figure 1, the positive control for PV 1 infection gave a mean TCID₅₀ 8.15 ± 1 (n=3) for the infectivity value. PV 1 incubated with ProtecTeaV-cb for 30 sec resulted in a reduction of 6 (\log_{10}) in the TCID₅₀ (1.5 ± 0), while ProtecTeaV+cb reduced the TCID₅₀ to 2.17 ± 0.49 . The adf sanitizer reduced the TCID₅₀ to 5.27 ± 1.07 after 30 sec incubation with PV 1, and Purell sanitizer decreased the TCID₅₀ to 4.87 ± 1.10 after 30 sec incubation with PV 1. Two-way ANOVA showed no main effect for time of incubation ($p=0.92$); that is, for any product, incubation times >30s had no detectable effect on viral TCID₅₀. There was no significant interaction ($p=0.91$). Sidak's multiple comparisons test for the main effect of product showed no significant difference between the two ProtecTeaV formulations ($p=0.70$), or between adf and Purell ($p=0.99$). However, all comparisons of ProtecTeaV formulations against adf and Purell showed highly significant reductions ($p<0.0001$) in the TCID₅₀.

In summary, both ProtecTeaV sanitizers reduced PV 1 infectivity by approximately 6 \log_{10} (from 8.15 to a range of 1.5 to 2.16). The adf and Purell sanitizers only reduced PV 1 infectivity by approximately 3 \log_{10} (from 8.15 to a range of 4.9 to 5.3). That is, the ProtecTeaV sanitizers possessed significant higher virucidal capacity than currently used hand sanitizers Purell and adf (Table 1).

Physical neutralization by sanitizer assay

To determine whether the virucidal activity of the ProtecTeaV+cb sanitizer was via inhibition or inactivation (destruction), sanitizer was removed after incubation by ultra-filtration prior to performing TCID₅₀ assays. As shown in figure 2, the virucidal activity of ProtecTeaV+cb was consistent with the TCID₅₀ results shown in Figure 1 and Table 1.

For the filtration control on untreated virus, PV 1 without sanitizer treatment after mock ultra-filtration showed a TCID₅₀ value of 6.4 ± 0.31 , while PV 1 without sanitizer treatment but underwent ultra-filtration had a TCID₅₀ value of 6.2 ± 0.58 . These values were not significantly different (one-way ANOVA, $p=0.949$). After PV 1 was incubated with the ProtecTeaV+ formulation for 60 second removal of the sanitizer by ultra-filtration, the mean TCID₅₀ value was reduced to 0.3 ± 0.58 , significantly lower than the untreated control ($p<0.0001$), but not significantly different from the treatment followed by mock ultra-filtration control (one-way ANOVA, $p=0.99$), which was also significantly lower than the untreated controls ($p<0.0001$).

Thus, after removal of sanitizer via ultra-filtration, ProtecTeaV treatment of PV 1 still resulted in a mean of 6.1 \log_{10} -reduction of viral infectivity in VERO cells.

Discussion

Due to the increasing evidence indicating a weak efficacy of alcohol-based hand sanitizer against non-enveloped viruses, especially norovirus and poliovirus [5], the 2011 CDC guidelines suggested using "proper hand washing with soap and running water for at least 20 seconds" as the most effective way to reduce norovirus contamination on the hands [18]. However, the implementation of new guidelines has not been associated with a reduction in norovirus outbreaks. Since 2011, rather than a decline in the number of norovirus outbreaks reported to the CDC, there was a spike during 2013-2014 (CDC NoroSTAT, 2009-2015). It is clear that in order

to reduce outbreaks and infections from norovirus and other non-enveloped or enveloped viruses, novel hand hygiene approaches that are more effective than hand washing with soap and water are in urgent need.

Results from the current study have demonstrated for the first time that alcohol-based sanitizer formulations containing lipophilic EGCG exert potent virucidal capacity. According to international standards (i.e. European standard for testing EN 14476 and Chinese CDC standard), virucidal disinfectants and antiseptics require a mandatory $\geq 4 \log_{10}$ virus infectivity reduction by 30s or 60s to be classified as such [19]. Our results from TCID₅₀ assays demonstrated that both ProtecTeaV hand sanitizer formulations, either with or without carbomer thickening agent, exceeded this mandatory requirement by 100-fold ($\geq 6 \log_{10}$) within the requisite time frame of 30-90 sec (Figure 1 and Table 1). In contrast, both the conventional alcohol-based hand sanitizer and non-alcohol-based hand sanitizer tested here failed to meet the internationally accepted standards. In addition, results from the neutralization assay indicated that the inactivation of PV 1 by ProtecTeaV formulations was irreversible, as virus incubated with ProtecTeaV formulations for 60s lost infectivity even after the sanitizer formulations were removed by microfiltration (Figure 2). The reduction of PV 1 infectivity in these neutralization assays was consistent with the TCID₅₀ assays (6.1 \log_{10} reduction, Figure 2).

The high potency of the ProtecTeaV hand sanitizer formulations demonstrated here was consistent with the antiviral properties of lipophilic EGCG, and previous studies showing antiviral activities of EGCG or its derivatives that did not use alcohol as the primary solvent. Although a synergistic effect might occur between alcohol and lipophilic EGCG, the contribution of alcohol is likely to be rather small, based on prior measures of EGCG effectiveness. Medications such as Veregen ointment against genital warts primarily induced by human papilloma virus and AverTeaX ointment for treating herpes labialis, either have EGCG (Veregen) or lipophilic EGCG (AverTeaX), but do not contain alcohol [16,20]. Previous studies using Dimethyl Sulfoxide (DMSO) as a solvent demonstrated that lipophilic EGCG is 24-fold more effective than native EGCG against influenza virus (H1N1) [21]. Another study using ethanol as a solvent showed lipophilic EGCG is 8.7 fold more effective against Herpes Simplex Virus 1 (HSV-1), in comparison to native hydrophilic EGCG [22]. In both studies, 50 μM of EGCG-mono-palmitate was sufficient to completely inhibit plaque formation by H1N1 and HSV-1 [21,22]. Although native green tea extract and EGCG exert significant antiviral activities against a wide spectrum of viral species, including Ebola virus, native EGCG is not able to effectively inactivate poliovirus within a concentration range (μM) that is active against other types of viruses [23,24]. The current study indicates that lipophilic EGCG in alcohol-based ProtecTeaV hand sanitizer formulations possesses high efficacy against poliovirus (Figures 1 and 2 & Table 1). It is interesting to note that Veregen ointment contains 15% of tea polyphenols (sinicatechins) with the majority being native EGCG (>55%). That is, the EGCG concentration in this prescription drug is >8.25%. In contrast, the lipophilic EGCG in AverTeaX ointment is significantly less than 1% (undisclosed proprietary formula). In summary, alcohol is not an important contributor to the virucidal capacity of lipophilic EGCG in the tested formulations, and the antiviral efficacy of lipophilic EGCG is significantly higher than native EGCG.

It is known that auto-oxidation is the major cause of instability of EGCG due to its antioxidant activity in aqueous preparations or

beverages [25,26]. Acylation of EGCG not only enhanced the antiviral activity but also increased stability by 10-fold [27]. Matsumoto et al., reported that EGCG-palmitate exhibits antibacterial and antifungal activities, with rapid bactericidal activity against MRSA ATCC43300 at $\geq 16 \mu\text{g/ml}$ [27]. Thus, lipophilic EGCG in an alcohol-based hand sanitizer (and other forms of surface disinfectants) is expected to have both strong virucidal and bactericidal activity. Future studies should include testing the persistency of the virucidal activity after application to determine the duration of protection by lipophilic EGCG on skin or other surfaces. In addition, lipophilic EGCG sanitizer formulations need to be tested against other non enveloped viruses, such as norovirus, adenovirus and enteric virus 71 (EV71), which work is underway in our laboratories (manuscript in preparation).

In conclusion, the ProtecTeaV alcohol-based hand sanitizer formulations containing lipophilic EGCG irreversibly inactivate non-enveloped polio virus 1 and exceeded the international standard for virucidal disinfectants and antiseptics. With superior antiviral activity and stability in comparison to native EGCG, lipophilic EGCG has the potential for use as a novel, effective and nontoxic component for the next generation of approaches to prevention of pathogenic viral infections.

Disclaimer

The views expressed in this manuscript are those of the author(s) and do not reflect official policy or position of the Department of the Army, Department of Defense or the US Government.

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