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Potential Therapeutic Use of EGCG-Palmitate Nanoparticles for Norovirus Infection

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ABSTRACT

Green tea catechins, especially epigallocatechin-3gallate (EGCG), have been widely studied for their antiviral activities against a long list of pathogenic viruses. However, the water soluble EGCG is unstable and undergoes auto-oxidation rapidly. To address this issue, lipid soluble EGCG was developed such as EGCG-palmitates (EC16) to be formulated in a variety of stable and effective products such as hand hygiene and surface disinfectant products. Recently, we developed an unconventional method to prepare EC16 nanoparticles and successfully formulated them in saline-based nasal applications. This novel nanotechnology allows EC16 nanoparticles to be used in aqueous nanosuspensions targeting viral gastroenteritis. The goal of the current study was to determine the stability and antiviral activity of water based EC16 nanosuspensions against murine norovirus S99 as a surrogate for human norovirus and as a future viral strain for animal studies.

Methods: EC16 nanoparticles were prepared using a facilitated self assembling method. The antiviral activity of the nanosuspensions of EC16 was assessed by TCID50 assays using RAW 264.7 cells. The stability of the nanosuspensions was determined by Zeta View evaluation following incubation in simulated gastric acid at pH 2. The morphology of the nanoparticles was captured by transmission electron microscopy.

Results: EC16 nanoparticles in water based and cell culture medium based suspensions reduced S99 viral infectivity by >99% either in direct contact with the virus or applied before or after viral challenges to the cells. The particle size and Zeta Potential of the nanosuspension remained consistent before and after HCl treatment for 60 min at 37 °C at pH 2. However, the number of particles was reduced by approximately 50%. The electron microscopy image of the nanoparticles was consistent with Zeta View data for the particle size range.

Conclusion: EC16 nanoparticles have the potential to be used as a novel approach to prevent and treat viral gastroenteritis, pending mechanism and *in vivo* studies.

Keywords: Norovirus; Green Tea Catechins; EGCG-Palmitate (EC16); Nanoparticles; Antiviral

Abbreviation: CDC: Center of Disease Control and Prevention; EGCG: Epigallocatechin-3-Gallate; MNV: Murine Norovirus; PBS: Phosphate Buffer Saline; EAOPs: EGCG Auto Oxidation Products; EPA: Environmental Protection Agency; ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; CPE: Cytopathic Effect; SD: Standard Deviation; NIDCD: National Institute on Deafness and Other Communication Disorders; NTA: Nanoparticle Tracking Analysis

Introduction

According to the Center of Disease Control and Prevention (CDC) of the United States, the leading pathogen that causes acute gastroenteritis is norovirus. Norovirus is also responsible for 58% of foodborne illness in the United States [1]. Globally, it is estimated that 685 million cases of norovirus associated illness were reported and 200 million cases are reported among children under five years of age each year, leading to 50,000 deaths from this age group, mainly in developing countries [2]. The total global norovirus associated per year is estimated as 200,000 [3]. The total global norovirus associated per year is estimated as 200,000 [3]. In addition to children, norovirus poses a life threatening risk to the immune population, causing chronic illnesses lasting weeks to years [4]. The current therapeutic approaches remain on symptomatic relief due to the lack of effective medication to rapidly mitigate viral replication in gastrointestinal systems [5]. A large number of compounds have been studied as candidates for new drugs against norovirus infection, including antivirals, monoclonal antibodies, and naturally occurring compounds such as vitamin A, curcumin, and citrate [6]. Among the candidates, nitazoxanide, a broad spectrum antimicrobial drug for treatment of parasite induced gastroenteritis, was one of the few opeful agents for immuno compromised patients. However, recently published ex vivo studies suggest that nitazoxanide exhibits poor antiviral activity against human norovirus [7].

Epigallocatechin 3 gallate (EGCG), a major green tea catechin, is another naturally occurring compound with a wide spectrum of antiviral property [8-15]. It is known that both human and murine norovirus (MNV) can be inhibited by green tea extract [16,17]. EGCG also demonstrated antiviral activity against norovirus. For example, Raw 264.7 cell pre treated with 100 μM EGCG reduced murine norovirus infectivity by >50% [18]. However, as a strong antioxidant, the water soluble EGCG is quickly self oxidized (auto-oxidation). In fact, EGCG dissolved in phosphate buffer saline (PBS) at 37°C completely converts to unstable EGCG auto oxidation products (EAOPs) within 4 hours [19]. Our group also confirmed that it is impossible to maintain EGCG's stability in a liquid formulation [20]. In addition to the instability, EGCG also forms insoluble aggregated precipitation under intestinal conditions, which could result in further reduction of antiviral efect *invivo* [21]. We reported previously that epigallocatechin-3-gallate-palmitate (EGCG-palmitate or EC16), a chemically stable lipid-soluble form of EGCG, is able to effectively inhibit influenza virus, norovirus, and herpes simplex virus. The antiviral activity of EC16 is significantly higher than that of EGCG [20,22-25]. Other advantages include that EC16 is an FDA categorized generally recognized as a safe (GRAS) compound (GRAS Notice 772) [26] and an Environmental Protection Agency (EPA) approved safe inert. Despite the potent antiviral activity of EC16 against a wide range of pathogenic viruses, the lipid soluble EC16 may not exert its antiviral activity in the hydrophilic environment in the gastrointestinal tract. This obstacle could be overcome by nanotechnology.

Indeed, EGCG nanoparticles have been developed through nanoparticle engineering. Previous studies demonstrated that EGCG in nanoparticle forms increased bioavailability and retention time in the small intestine environment [27,28]. However, the stability and antiviral activity of nanoparticle delivered EGCG in the gastrointestinal tract remain unknown. We hypothesize that EGCG-palmitate (EC16), in a unique nanoparticle form, has potential to become a new drug agent to treat and prevent viral gastroenteritis such as norovirus and rotavirus infections (both are nonenveloped viruses). Recently, our group developed a method, referred to as "Facilitated Self Assembling Method", to prepare nanoparticles of EC16 for human coronavirus inactivation [29-31]. The current study utilized this technology to prepare specific nanosuspensions to determine the stability and antiviral activity of EC16 nanoparticles against murine norovirus S99 to obtain initial data toward novel approaches to treat and prevent viral gastroenteritis.

Material and Methods

Virus and Cell Line

Murine norovirus S99 strain was obtained from Nelson Laboratories, Bozeman, MT. The RAW264.7 (ATCC# TIB-71) cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA).

Epigallocatechin-3-Gallate-Palmitates (EC16) and Other Supplies

EC16 was obtained from Camellix, LLC, Evans, Georgia, USA. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from ATCC (30-2002). Fetal bovine serum (FBS) was obtained from Neuromics (Edina, MN, USA). Penicillin, streptomycin, and amphotericin B solution (100×) was obtained from Corning (Glendale, AR, USA).

Preparation of EC16 Nanoparticles

The EC16 nanoparticles were prepared using the facilitated-self-assembling method (proprietary, patent pending). EC16 nanoparticle stocks were prepared at 1%, and 2% stabilized in pure glycerol for further dilution and use.

Determine the Antiviral Activities of EC16 Nanoparticles

1. Two types of EC16 formulations were prepared. A formulation containing 33% ethanol was made by dilution of EC16 nanoparticle stock with water and a food grade dispersing agent (proprietary) before addition of 100% ethanol to the final concentration of 33%. Formulations without ethanol were prepared by diluting the EC16 nanoparticle stock with water and the dispersing agent (for contact inhibition reaction only) or diluting the stock with plain DMEM, with or without the dispersing agent, for pre- and post infection incubation.
2. Contact inhibition assay using water based EC16 nanoparticles at 0.2%. RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin, streptomycin, and

amphotericin B. The viral infection assay and viral titer were performed in 96 well cell culture plates when the cells had reached 90% confluency. S99 virus was incubated with EC16 nanoparticles with 33% ethanol or without ethanol at 1:9 for 5 and 10 minutes prior to a series of 10 X dilutions using DMEM containing 2% FBS (MM) to 10^{-9}. The dilutions, as well as control (dilutions from water, or water and ethanol) were loaded into the designated wells in quadruplets. After a one-hour absorption, the viral dilutions were removed and 100 µl MM was added, followed by incubation at 37 °C with 5% CO2 for >4 days to allow a CPE (cytopathic effect) to become visible. Viral titer was calculated by a TCID50 protocol and software [32]. Three independent experiments were performed.

3. To test whether EC16 nanoparticles possess a post infection antiviral effect, RAW 264.7 cells were allowed to form a monolayer (90% confluent) in a 96-well cell culture plate prior to a 60 min infection of S99 virus in a series of 10 X dilutions to 10^{-9} before removal of free virus. Then, 100 µL of EC16 nanoparticles were applied to the designated wells for 10 or 30 min before being replaced by MM. The cytopathic effect (CPE) was captured after incubation for at least 4 days, followed by TCID50 calculations.

4. To test whether EC16 nanoparticles possess a pre infection antiviral effect, RAW 264.7 cells were allowed to form a monolayer (90% confluent) in a 96-well cell culture plate prior to a 20 min incubation with 0.02% EC16 nanosuspension or 0.05% EC16 nanosuspension, followed by infection of S99 virus in a series 10 X dilutions to 10^{-9} for 60 min and 5 hours, respectively. Then, the virus was removed and 100 µL of MM was added to each well. The cytopathic effect (CPE) was captured after incubation for at least 4 days, followed by TCID50 calculations.

Incubation of EC16 Nanoparticles in Acidic Conditions

To test the stability of EC16 nanoparticles in acidic conditions similar to stomach acid (pH 2), EC16 nanoparticles were prepared from the 2% stock by a 10 X dilution with phosphate buffer saline (PBS) as a 0.2% EC16 nanoparticle suspension. The 0.2% EC16 nanoparticle suspension was then added to a HCl/water solution with pH 1.93 at a 1:9 ratio, and incubated at 37 °C for 0, 30 and 60 min before neutralization with 10 X PBS. The resulting 0.002% EC16 nanosuspensions and untreated 0.002% EC16 nanoparticles in PBS were subjected to ZetaView evaluation.

Evaluation of Particle Size Distribution

Zeta View nanoparticle tracking analysis was performed according to a method described previously [12,43]. The particle size distribution and concentration were measured using the Zeta-View ×20 (Particle Metrix, Meerbusch, Germany) and corresponding software. The measuring range for particle diameter is 10-2000 nm. The four samples were diluted by the same volume of 1× PBS and then loaded into the cell. Particle information was collected from the instrument at 11 different positions across the cell, with two cycles of readings. Standard operating procedure was set to a temperature of 23 °C, a sensitivity of 70, a frame rate of 30 frames per second, and a shutter speed of 100. The post acquisition parameters were set to a minimum brightness of 20, a maximum area of 1000, a minimum area of 10, and a trace length of 15 [30].

Electron Microscopy Imaging of EC16 Nanoparticles

The 1% EC16 nanoparticle stock was diluted with PBS to 0.01% and fixed in 4% paraformaldehyde and 2% glutaraldehyde. 5 µl of the sample was removed and transfer to a Formvar/Copper 200 mesh grid and allowed to dry for 15 minutes. Excess solution was then removed using filter paper particles and was negatively stained by addition of 5 µl of 2% aqueous uranyl acetate. Multiple images were captured from each sample in a JEM 1400Flash Transmission Electron Microscope (JEOL, Peabody, MA) at 120kV, using a Gatan OneView Digital Camera (Gatan Inc., Pleasanton, CA).

Statistical Analysis

The primary statistical tests were parametric one-way ANOVA based on three or more repeated test points. Alpha was 0.05. GraphPad Prism version 6.0 software (www.graphpad.com) was used for most analyses. Reported errors are given as standard deviation (SD).

Results

Antiviral Activity of EC16 Nanoparticles.

Direct Contact Inhibition: As shown in Figure 1, at 0.2%, EC16 nanoparticles in water based suspension reduced S99 viral infectivity by more than >99% ($\log_{10}$ 2.31±0.31) after 5 min contact with the virus. After 30 min contact time, the infectivity of S99 virus was reduced by >99.9% ($\log_{10}$ 3.42±0.38), which was significantly higher than 5 min contact (One way ANOVA, p=0.008). The water/ethanol based suspension reduced S99 viral infectivity by $\log_{10}$ 2.63±0.14 (>99%) after 5 min contact, Which was not statistically different compared to water based suspension (p=0.12, n=4).

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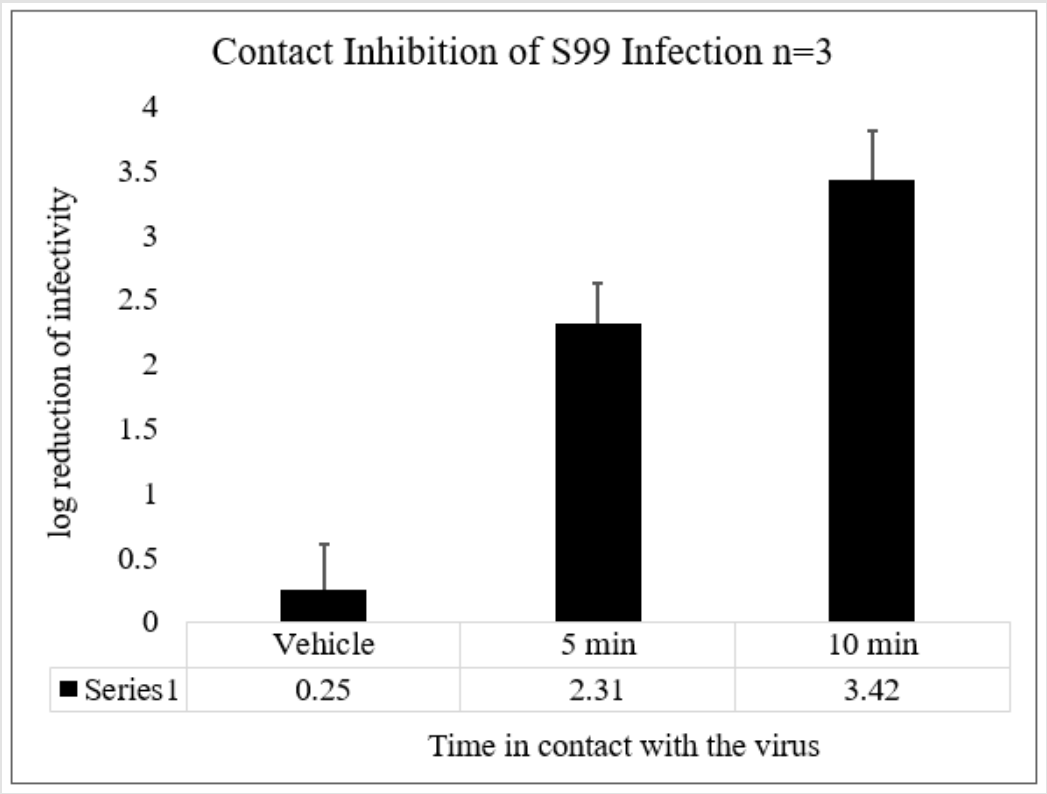

Figure 1: Time response of contact inhibition antiviral activity of water based 0.2% EC16 nanosuspension (n = 3). This nanosuspension was incubated with S99 virus at a 1:9 ratio (virus to formulation) for 5 and 15 min before 10x serial dilutions and TCID50 assay. The antiviral activity was calculated and expressed as $\log_{10}$ reduction ± standard deviation.

Post-Infection Inhibition of S99 Viral Replication: The EC16 concentrations in these experiments were 0.02% and 0.1%, as shown in Figure 2. The 0.02% nanosuspension was incubated with RAW264.7 cells for 10 and 30 min after the cells were infected by S99 virus. At this concentration and time points, EC16 nanosuspension reduced the viral replication by $\log_{10}$ 0.67 + 0.38 (approximately 66%, 10 min) and $\log_{10}$ 0.92 + 0.14 (close to 90%, 30 min). When the concentration was increased to 0.1%, 30 min incubation of EC16 nanosuspension reduced the viral replication by $\log_{10}$ 2.17 + 0.14 (>99%), which was a significant increase compared to 0.02% (One way ANOVA, p=0.0004).

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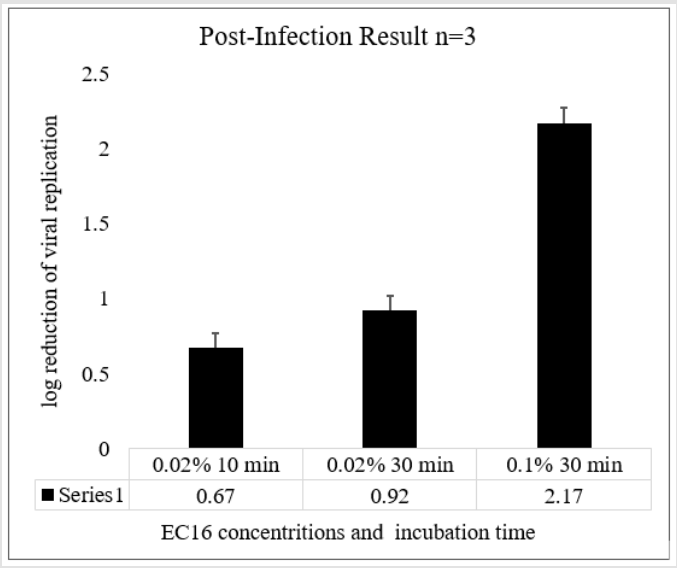

Figure 2: Post infection antiviral activity of water based 0.02% or 0.1% EC16 nanosuspensions (n = 3). The nanosuspensions were incubated RAW 264.7 cells in a 96-well plated challenged with S99 virus for 60 min. The antiviral activity was calculated and expressed as $\log_{10}$ reduction ± standard deviation.

Pre- Infection Inhibition of S99 Viral Replication: The RAW264.7 cells were incubated with EC16 for 20 min before infection. An EC16 concentration of 0.02% gave a log10 2.17 +/- 0.29 reduction at 1 hr post viral infection. An EC16 concentration of 0.05% gave a log10 2.42 +/- 0.?? reduction at 5 hr post viral infection.

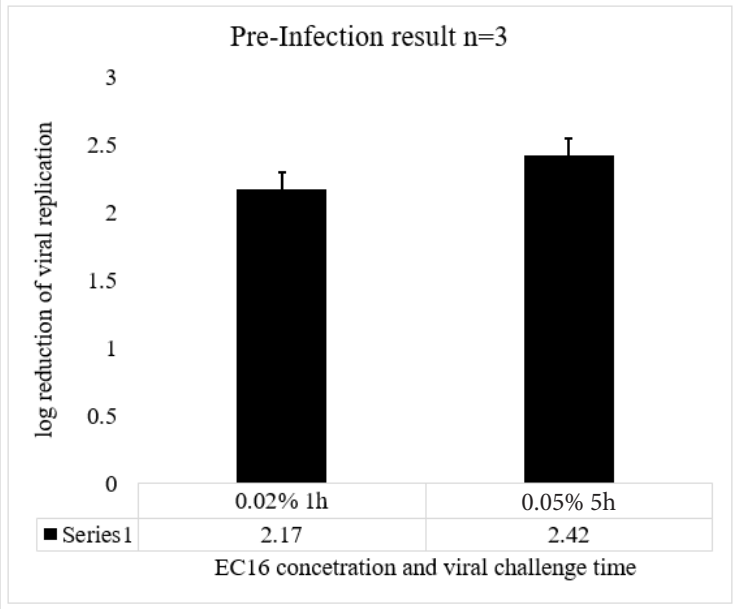

Figure 3: Pre infection antiviral activity of water based 0.02% or 0.05% EC16 nanosuspensions (n = 3). The nanosuspensions were incubated RAW 264.7 cells in a 96-well plated before a series of dilutions of S99 virus absorption for 60 min. The antiviral activity was calculated and expressed as $\log_{10}$ reduction ± standard deviation.

Nanoparticle Test in HCl Solution at pH 2 (Actual Reading1.93).

With consideration for oral administration, EC16 nanoparticle stock at 2% was directly suspended in PBS 1:9 to form a 0.2% suspension containing 10% glycerol. This suspension was incubated with HCl solution at pH 2 (1:9 ratio) at 37 °C. Samples collected at 0, 30 and 60 min neutralized by 10 X PBS, the resulting neutralized suspensions showed pH values of 6.09, 6.01 and 6.08, respectively, indicating the acidic incubation was terminated. The untreated EC16 nanoparticle suspension in PBS had a pH of 6.30. These samples were then subjected to ZetaView analysis.

Evaluation of Particle Size and Zeta Potential of HCl-Treated Samples

As shown in Figure 4A, the untreated EC16 nanoparticle size distribution was evaluated by nanoparticle tracking analysis (NTA) of 10-fold dilutions. The particles showed high polydispersity, with sizes ranging from 90.6 nm to 603.8 µm. The median size was 173.1 ± 182.3 nm (SD), and the cutoff size for 90% of the particles was <324.5 nm. The undiluted concentration was measured at $6x10^8$ particles/ml. Under acidic conditions but without incubation, the EC16 nanoparticle suspension also showed high polydispersity, with sizes ranging from 54.1 nm to 300.5 µm. The median size was 194.0 ± 129.6 nm (SD), and the cutoff size for 90% of the particles was <334.0 nm. The undiluted concentration was measured at $5x10^8$ particles/ml (Figure 4B). The Zeta Potential at 25°C was -42.01 ± 0.88 mV. After 30 min incubation in HCl solution at 37 °C, the EC16 nanoparticle suspension demonstrated sizes ranging from 100.5 nm to 410.3 µm. The median size was 195.5 ± 103.7 nm (SD), and the cutoff size for 90% of the particles was <330.8 nm. The undiluted concentration was measured at $4x10^8$ particles/ml (Figure 4C). The Zeta Potential at 25 °C was -41.80 ± 0.94 mV. After 60 min incubation in HCl solution at 37 °C, the EC16 nanoparticle suspension demonstrated sizes ranging from 125.0 nm to 354.7 µm. The median size was 194.9 ± 109.7 nm (SD), and the cutoff size for 90% of the particles was <362.8 nm. The undiluted concentration was measured at $3x10^8$ particles/ml (Figure 4D). The Zeta Potential at 25°C was -42.89 ± 0.62 mV.

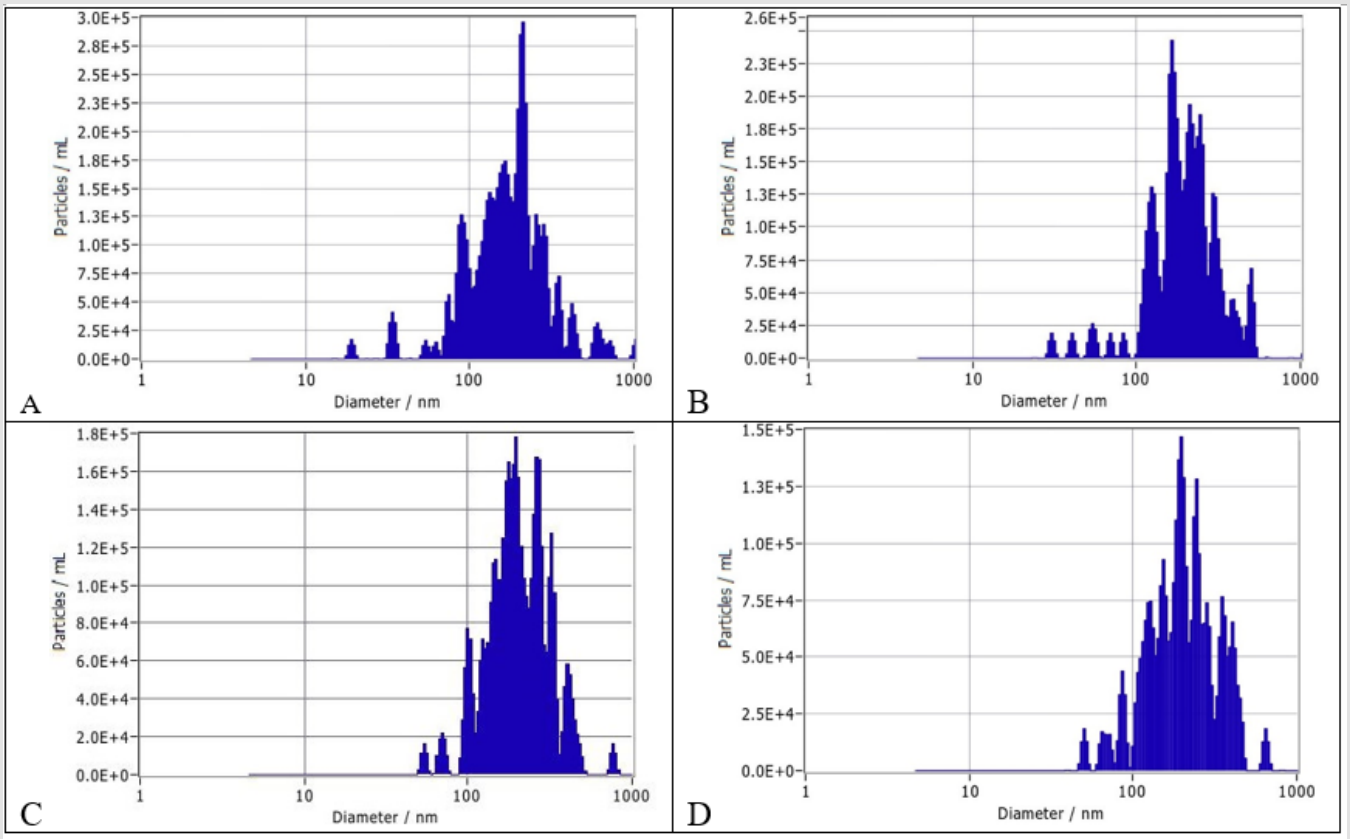

Figure 4: Zeta View size distributions of EC16 nanoparticles treated with HCl at pH 2.

A. Untreated EC16 nanosuspension in PBS.
B. EC16 nanosuspension in PBS with HCl at pH 2.
C. EC16 nanosuspension in PBS with HCl at pH 2 incubated for 30 minutes.
D. EC16 nanosuspension in PBS with 90% HCl at pH 2 incubated for 60 minutes.

Transmission Electron Microscopy

Figure 5 shows a representative TEM image of EC16 nanoparticle suspension fixed in 4% paraformaldehyde and 2% glutaraldehyde. The right panel shows a schematic chemical structure of EC16.

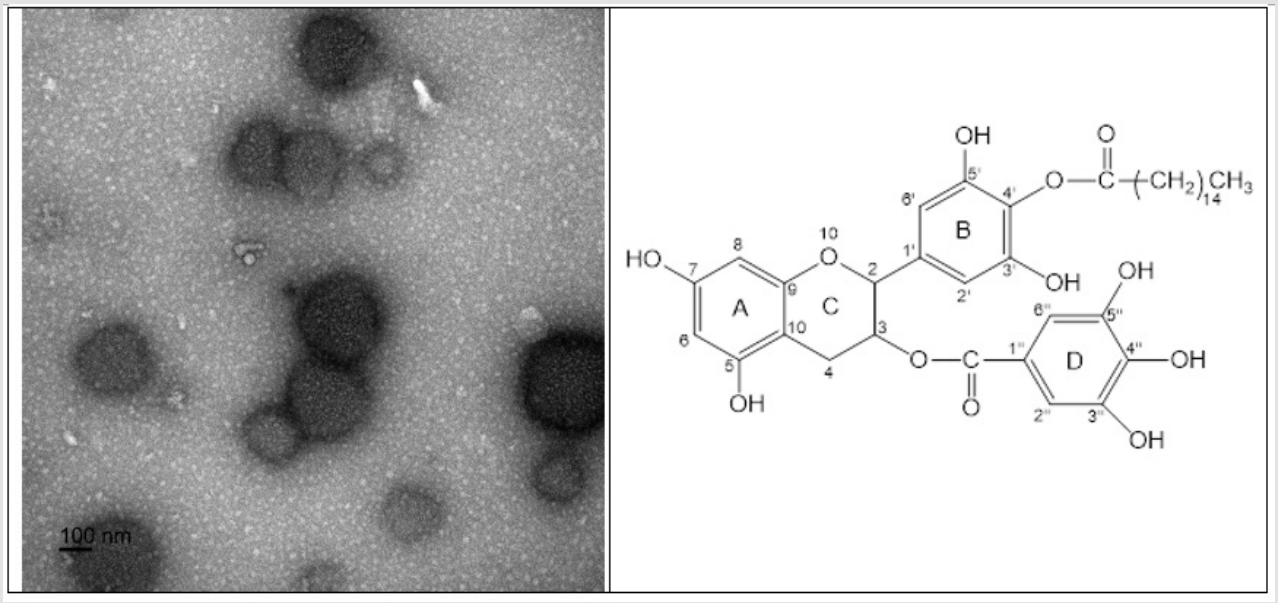

Figure 5: Representative TEM image of EC16 nanoparticles with a scale bar of 100 nm (Left) and a schematic structure of EC16 with a 16-carbon hydrophobic palmitoyl moiety linked with 4″ carbon of B ring (Right).

Discussion

The goal of the current study was to test our hypothesis that EC16 in nanoparticle form could be used as a novel approach to prevent and treat norovirus infection. We reported previously that EC16 in an alcohol-based hand sanitizer and surface disinfectant formulations is sufficient to rapidly inactivate norovirus (feline calicivirus and murine norovirus) [32]. However, elevated levels of alcohol content are not feasible for oral administration. The other obstacle to overcome is that EC16 is not water soluble, and therefore it may not be suitable for oral delivery to reach the intestinal lumen, an aqueous environment. Recently, we invented a facilitated self assembling method to prepare EC16 nanoparticles [29-31]. This nanotechnology was used to develop a nasal spray with strong antiviral activity against human coronavirus [29-31]. This novel approach enabled us to formulate either water based or alcohol based EC16 suspensions against non-enveloped murine norovirus S99 to collect initial efficacy and stability information. As shown in Figure 1, the 0.2% EC16 suspension demonstrated potent antiviral activity, with >99% and >99.9% inactivation of the virus in 5 and 10 min, respectively. The 10-min data was similar to the antiviral activity of the ethanol based (33%) EC16 nanosuspension on S99 virus (2.63±0.14 $\log_{10}$), but without statistical significance (p=0.12). Another water based EC16 nano formulation with 0.02% EC16 and 0.2% chlorohexidine gluconate also reduced S99 infectivity by 99% (data not shown).

This observation suggests that EC16 nanoparticles, even in aqueous suspensions without ethanol, are able to efficiently inactivate S99 virus. This result could be due to the large surface of the nanoparticles and the substantial number of the nanoparticles reacting with the viral surface proteins to alter the viral structure. Due to the wide spectrum of antiviral activity [8-15,20,22-25], the EC16 nanosuspension could be active against rotavirus, another major virus responsible for gastroenteritis and 200,000 deaths each year globally [33]. The second discovery from the current study is that EC16 nanoparticles are stable under an acidic environment (HCl at pH 2). The median particle size remains stable after 60 min incubation at 37 °C. There was no significant difference in particle size among the untreated and treated samples. However, the number of particles was reduced from untreated ($6x10^8$ particles/ml) to HCl-treated samples (5.0E, 4.3E and 3.0E + 8 particles/ml of 0, 30, and 60 min, respectively). This decline from $10^{8.8}$ (602,559,586) particles/ml to $10^{8.5}$ (316,227,766) particles/ml, represents an approximately 50% reduction in nanoparticle concentration after 60 min incubation in HCl (pH 2) at 37 °C. It is not clear what mechanism is associated with the reduction of particle numbers. There are multiple options to improve the durability of the particles. On the other hand, 50% loss of the nanoparticles through 60 min incubation with HCl at pH 2, a corrosive environment at 37 °C, is considered acceptable for a drug directly targeting the gastrointestinal system.

It is necessary to test the stability of EC16 nanoparticles under small intestinal fluid conditions in future investigations. It is crucial to consider the differences between in vitro studies and in vivo studies, which could be designed for multiple doses, or continued feeding in drinking water with EC16 nanoparticles, during the entire norovirus infection episode, rather than a single application in the current cell-based study. An important observation is that the Zeta Potential of all suspensions exposed to HCl remains consistent at approximately -42 mV, indicating the nanoparticles in the suspensions are potentially stable during the 60 min incubation period (A Zeta Potential value above +30 mV or below −30 mV is generally considered stable). It also confirmed that the EC16 nanoparticles are negatively charged, which is consistent with the EC16 nanoparticle structure, with the hydrophobic palmitoyl moiety in the center and the negatively charge EGCG moiety facing the aqueous phase [34]. It was reported that the antiviral activity of EGCG is partially due to the negative charge of the compound, which is in contact with the viral proteins, leading to protein structure alterations [35]. This information would help to evaluate the dosage range in future in vivo studies. Accordingly, the subsequent post-infection and pre-infection antiviral assays were based on reduced EC16 concentrations. For the post-infection assay, a single application EC16 nanoparticles at 0.02% or 0.1% in DMEM medium suspension were incubated with RAW 264.7 cells for 20 or 30 min after 60 min S99 viral infection of the cells.

Without direct contact with S99 virus, EC16 nanoparticles at 0.1% reduced the viral replication by $\log_{10}$ 2.17 + 0.14 (>99%) after a single 20 min post-infection incubation (Figure 2), suggesting a potential therapeutic effect at the 0.1% concentration during the 6-day observation period for CPE. When the dose and time were decreased, the viral inhibitory activity was less than 10% of that from 0.1% as shown in Figure 2. This result suggests that a sufficient number of EC16 nanoparticles is necessary for the efficacy against S99 virus. We chose a concentration between 0.02% and 0.05% to test the potential prevention effect of EC16 nanoparticles. As shown in Figure 3, DMEM suspension with 0.05% EC16 nanoparticles was incubated with RAW 264.7 cells for 20 min to allow the nanoparticles to attach the cells before aspiration. After the free nanoparticles were removed, the cells were infected with S99 virus for 5 hours prior to media change and observation. EC16 nanoparticles at 0.05% led to a 2.42 $\log_{10}$ 2.42 + 0.29 (>99%) reduction of S99 viral replication, while 0.02% EC16 nanoparticles resulted in $\log_{10}$ 2.17 + 0.29 (>99%) (Figure 3). That is, EC16 nanoparticles in 0.02% to 0.05% significantly reduced the infectivity of S99 virus (>99%). Interestingly, there is no statistical difference between the two concentrations (One-way ANOVA, p=0.35, n=3). One of the reasons to test 0.02% EC16 is that this concentration was tested extensively in our previous studies [29-31] and ongoing animal studies. This information will be useful for the future *in vivo* study design for treating viral gastroenteritis.

Regarding the potential toxicity of EC16 nanoparticles, cytotoxicity data from human primary epithelial cells an EC16 nanoparticle concentration at 0.1% is not associated with reduction of cell viability [30,31]. The TEM images demonstrated a unique feature of EC16 nanoparticles (Figure 5). The nanoparticles appear with a high polydispersity, consistent with ZetaView evaluation of the size distribution. It is noted that particles are not in round/sphere shape, and the contents appear packed in an organized manner, without an Apparant membrane like structure. This structure is consistent with the hydrophilic negatively charged surface from the EGCG moiety of the particles (data not shown, manuscript in preparation), which could enhance the antiviral activity by exposing a stronger negative charge in the solution than free EGCG molecules [35]. As shown in Figure 5, each EC16 nanoparticle (100 to >200 nm in diameter) presents a large surface of packed EGCG moiety with a powerful negative charge to a viral particle with 23-40 nm in diameter, leading to rapid destruction of the virus [35]. On the other hand, the mechanisms of forming stable nanoparticles without engineering warrant further studies. Based on the results, we report here for the first time that an aqueous nanosuspension of EC16, an FDA classified GRAS food additive, possesses strong antiviral activities against murine norovirus S99 either in direct contact, or applied before or after viral infection. The nanoparticles remain stable in size and charge after 60 min incubation with HCl solution at pH 2 in 37 °C, with nearly 50% loss of particle number. [36] The limitations of this study include only single applications of the nanosuspensions without repeated treatments in the design of the experiments; and simulated small intestinal fluid was not assessed. In conclusion, EC16 nanoparticles have the potential to be used as a new approach to prevent and treat viral gastroenteritis, pending mechanism and *in vivo* studies.

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