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7 **A Nasal Spray Solution of Grapefruit Seed Extract plus Xylitol Displays**

8 **Virucidal Activity Against SARS-Cov-2 *In Vitro***

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30

31 **ABSTRACT**

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33 The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the

34 ongoing pandemic coronavirus disease 2019 (COVID-19) has triggered worldwide concerted

35 efforts in an attempt to identify effective therapies. In the present study, we have identified two

36 candidate agents with potential activity against SARS-CoV-2 which can be administered

37 intranasally, namely, xylitol and grape seed fruit extract (GSE). A commercially available nasal

38 spray (Xlear) combining xylitol and GSE has been available for years, but the antiviral effects of

39 this solution have not been documented. This *in vitro* study examined the virucidal effect of

40 Xlear against SARS-CoV-2. To this end, two independent sets of experiments were carried out to

41 test the hypothesis that Xlear is an effective (Experiment I) and replicable (Experiment II) means

42 to deactivate SARS-CoV-2. When tested against SARS-CoV-2, the test compound GSE 0.2%

43 was the only compound effective at reducing $>3 \log_{10}$ CCID₅₀ infectious virus from, $3.67 \log_{10}$

44 CCID₅₀/0.1 mL to an undetectable amount of infectious virus. The present results validated by

45 two independent sets of experiments, performed by different labs, on different viral strains,

46 provide early evidence to encourage further pilot and clinical studies aimed at investigating the

47 use of Xlear as a potential treatment for COVID-19

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49 **KEYWORDS:** *severe acute respiratory syndrome coronavirus, xylitol, grapefruit seed extract,*

50 *intranasal spray*

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54 **1 Introduction**

55 The initial global outbreak of the severe acute respiratory syndrome coronavirus 2
56 (SARS-CoV-2), responsible for the ongoing pandemic coronavirus disease 2019 (COVID-19),
57 was initially identified in Wuhan, China in December 2019. As of July 2020, there were more
58 than 13.3 million confirmed cases worldwide, with total deaths exceeding 573,000 (Dong et al.,
59 2020). Worldwide concerted efforts have been made in an attempt to characterize the disease and
60 identify effective therapies targeting SARS-CoV-2 including lines of studies focusing on the
61 route of infection, the potential routes of administration of therapeutic agents as well as the
62 potential efficacy of antiseptics (Meister et al., 2020). In this vein, a landmark study found that
63 the coronavirus infects the nasal cavity via the angiotensin-converting enzyme 2 (ACE2) protein
64 which appears to be the host-cell receptor for SARS-CoV-2 (Hoffmann et al., 2020). Since the
65 nasal epithelium cells have the highest percentage of ACE2 expressing ciliate cells in the
66 proximal airways, it is plausible to suggest that pharmacological agents such as sprays that are
67 used via the intranasal route of administration might be optimal candidates for providing
68 effective therapies against COVID-19 (Jia et al., 2005).

69 In a recent literature review conducted by Higgins et al. it is highlighted that intranasal
70 drug delivery represents an important area of research for viral diseases and COVID-19 (Higgins
71 et al., 2020). They concluded that the intranasal method of drug delivery has potential relevance
72 for future clinical trials in the setting of disease prevention and treatment of SARS-CoV-2 in
73 addition to other viral diseases (Higgins et al., 2020). Subsequently, Siddiqi et.al (2020), in a
74 diagram of COVID-19 disease progression, illustrated that the viral response phase is highest
75 during the early infection of the disease process, of which patients manifest mild constitutional
76 symptoms. Taken together the aforementioned studies support our rationale that therapeutic

77 strategies should be aimed at reducing the viral load in the nose by targeting this mild-moderate
78 phase of the disease process, and hence the use of a nasal spray might be an effective means to
79 accomplish this therapeutic strategy.

80 In the present study, we have identified two candidate agents with potential activity
81 against SARS-CoV-2 which can be administered intranasally, namely, xylitol and grape seed
82 fruit extract (GSE). Xylitol, a sweetener with antimicrobial and anti-inflammatory properties,
83 has been shown effective in decreasing the incidence of dental caries and improving chronic
84 rhinitis as well as important microbiota and immunological modulatory effects (Akgül et al.,
85 2020; Haukioja et al., 2008; Weissman et al., 2011; Xu et al., 2016). Xylitol has been reported to
86 have multiple health benefits as well as is generally safe and well-tolerated for most adults in
87 doses up to 35 grams per day and up to 20 grams per day in children (Salli et al., 2019; Storey et
88 al., 2007; Ur-Rehman et al., 2015). A derivative of grapefruit seeds, GSE, is associated with
89 abundant health benefits due to the presence of antioxidants and proanthocyanidin complexes
90 (Chacón et al., 2009). Also, GSE has been documented to have inhibitory effects against the
91 avian influenza virus, Newcastle disease virus, infections bursal disease virus, as well as other
92 pathogenic enteric viruses (Komura et al., 2019; Su and D'Souza, 2011). A commercially
93 available nasal spray combining xylitol and GSE, marketed as Xlear (American Fork, UT, USA),
94 has been widely used in the United States for several decades, but the antiviral effects of this
95 solution have not been documented. Accordingly, the aim of the present *in vitro* study was to
96 examine the virucidal effect of Xlear against SARS-CoV-2. To this end, two independent sets of
97 experiments were carried out to test the hypothesis that Xlear is an effective (Experiment I) and
98 replicable (Experiment II) means to deactivate SARS-CoV-2 the causative microorganism of
99 COVID-19.

100

101 **2 MATERIALS AND METHODS**

102 **2.1 Experiment I: Xlear Virucidal Activity Efficacy**

103 *2.1.1 Procedure*

104 SARS-CoV-2, USA-WA1/2020 strain, virus stock was prepared before testing by
105 growing 2 passages in Vero 76 cells. Culture media for prepared stock (test media) was
106 MEM with 2% fetal bovine serum (FBS) and 50 µg/mL gentamicin. Human rhinovirus 16,
107 strain 11757 purchased from ATCC (Gaithersburg, Maryland, USA), was grown in 3
108 passages of HeLa cells in MEM with 2% fetal bovine serum (FBS), 25 mM MgCl₂, and 50
109 µg/mL gentamicin. Test media is the growth media with 5% FBS.

110 *2.1.2 Virucidal Assay*

111 Test compounds including commercially available Xlear containing purified water,
112 11% Pure Xylitol (Shandon Lujian, Shandong, China), 0.6%NaCL (Saline), and 0.015%
113 GSE (Chemie Research & Manufacturing Co., Casselberry, FL, USA) were obtained from
114 the manufacturer in liquid form and stored at room temperature. The test compound 11%
115 xylitol in saline was diluted 1:2 with water before testing. Each solution was mixed directly
116 with virus stock so that the final concentration was 90% of each test compound and 10%
117 virus stock. A single concentration was tested in triplicate. Test media without virus was
118 added to duplicate tubes of the compounds to serve as toxicity and neutralization controls.
119 Ethanol (90%) was tested in parallel as a positive control and water only as a virus control.
120 The test solutions were incubated at room temperature (22 ± 2°C) for 15 minutes with
121 SARS- CoV-2 or Rhinovirus-16. The solutions were then neutralized by a 1/10 dilution in
122 the test media of each specific virus. The virucidal assays were performed in triplicate, then

123 after neutralization, the triplicate samples were pooled, serially diluted, and assayed for
124 infectious virus.

125 *2.1.3 Virus Quantification*

126 The surviving virus from each sample was quantified by standard end-point
127 dilution assay. Briefly, the neutralized samples were pooled and serially diluted using
128 eight log dilutions in test medium. Then 100 μ L of each dilution was plated into
129 quadruplicate wells of 96-well plates containing 80-90% confluent Vero 76 (SARS-CoV-
130 2) or HeLa cells (Rhino-16). The toxicity controls were added to an additional 4 wells of
131 Vero 76 or HeLa cells and 2 of those wells at each dilution were infected with virus to
132 serve as neutralization controls, ensuring that the residual sample in the titer assay plate
133 did not inhibit growth and detection of the surviving virus. Plates were incubated at $37 \pm$
134 2°C with 5% CO_2 for 5 days and at $33 \pm 2^{\circ}\text{C}$ with 5% CO_2 for 4 days for the SARS-CoV-2
135 assay and the Rhinovirus-16 assay, respectively. Each well was then scored for the
136 presence or absence of an infectious virus. The titers were measured using a standard
137 endpoint dilution 50% cell culture infectious dose (CCID₅₀) assay calculated using the
138 Reed-Muench (1948) equation and the log reduction value (LRV) of each compound
139 compared to the negative (water) control was calculated.

140 **2.2 Experiment II: Xlear Virucidal Activity Replication**

141 *2.2.1 Procedure*

142 SARS-CoV2/Switzerland/GE9586/2020 virus stock was amplified and titrated in
143 Vero E6 cells by plaque assay cultured in DMEM HG with 5% fetal bovine serum (FBS)
144 and 1% penicillin/streptomycin.

145 *Dose-response Assay*

146 Xlear nasal spray was serially diluted in DMEM HG and incubated with SARS-CoV2
147 (MOI 0.003 corresponding to 200 pfu/well) for 1 hour at 37°C and subsequently added on Vero
148 E6 cells for 1 hour at 37°C. The inoculum was then removed, cells were washed and overlaid
149 with DMEM HG with 5% FBS and Avicel 0.8%. 48hpi cells were fixed with PFA 4% and
150 stained with crystal violet. Plaques were counted and percent of infection calculated in
151 comparison with untreated wells. The experiments were performed twice independently, and
152 each was performed in duplicate.

153 *Virucidal Assay*

154 Xlear spray was mixed in different concentrations with SARS-CoV2 stock (10^5 pfu). The
155 compound was mixed directly with the virus solution with a final concentration of respectively
156 90%, 80%, 60%, or 20%. PBS was used as control. The solution and virus were incubated at 37
157 °C for 1 hour. The solution was then neutralized by a 1/10 dilution in test media. A 60%
158 condition was repeated in two independent experiments while the other dilutions were performed
159 in a single experiment in duplicate.

160 The infectious virus from each sample was quantified by standard end-point dilution
161 assay. 100 µL of each dilution were plated into quadruplicate wells of 96-well plates containing
162 80-90% confluent Vero 76 cells. Plates were incubated at 37°C with 5% CO₂ for three days.
163 Each well was then scored for the presence or absence of the virus. The end-point titers
164 (TCID₅₀) values were calculated using the Reed- Muench (1948) equation.

165 *2.2.2 Toxicity assay*

166 Vero-E6 (13000 cells per well) were seeded in 96-well plate. Xlear was serially diluted in
167 DMEM supplemented with 5% FBS and added on cells for 1h, followed by a washout, addition
168 of DMEM supplemented with 5% FBS for additional 48h hours. MTT reagent (Sigma Aldrich)

169 was added on cells for 3h at 37°C according to manufacturer instructions, subsequently cells
170 were lysed with pure DMSO and absorbance read at 570 nm. Percentages of viability were
171 calculated by comparing the absorbance in treated wells and untreated.

172 **3 RESULTS**

173 *3.1 Experiment I*

174 Virus titers and LRV of Rhinovirus-16 and SARS-CoV-2 when incubated with a
175 single concentration of the Xlear solutions are shown in Table 1. After a 15-minute contact
176 time, the Xlear nasal spray was not effective at reducing the infectious Rhino-16 virus.
177 When tested against SARS-CoV-2, the test compound GSE 0.2% was the only compound
178 effective at reducing $>3 \log_{10}$ CCID₅₀ infectious virus from, $3.67 \log_{10}$ CCID₅₀/0.1 mL
179 to an undetectable amount of infectious virus (Table 1). The Xlear nasal spray and the
180 GSE 0.2% had some toxicity in the top rows (1/10 dilution of the test sample) which may
181 have contributed to the virucidal effect of the GSE. The 11% xylitol and 11% erythritol
182 had no cytotoxicity. The positive control and neutralization control performed as expected.

183 *3.2 Experiment II*

184 SARS-Cov2 is inhibited in the dose-response assay (Figure 1) by different concentrations of
185 Xlear spray. However, the dilution 1:2 in medium evidenced damage to the cells with almost
186 complete loss of the cells, while with the dilution 1:6 a partial damage to the cell was evidenced,
187 while no morphologic changes in cells were visible from dilution 1:12 onwards. These results
188 were further confirmed with toxicity assays (Figure 1b).

189 In the virucidal assays (Figure 2), Xlear showed virucidal activity at the different
190 concentrations tested. Complete inhibition of viral infectivity was observed for the 90%, 80%,
191 60% condition, and a reduction of 2.17 log of viral titer in the 20% condition. In this assay, the

192 mixture of virus and Xlear was neutralized by a 1/10 dilution before addition on cells, therefore
193 diluting the compound below the toxic doses determined in the toxicity assay (Figure 1b).

194
195 **4 DISCUSSION**

196 The present study sought to evaluate the *in vitro* virucidal effects of a solution combining
197 xylitol and GSE in a nasal spray formulation known as Xlear. The novel results of this study
198 support our hypothesis that Xlear displays virucidal activity against SARS-CoV-2. The present
199 results validated by two independent sets of experiments, performed by different labs, on
200 different viral strains, provide early evidence to encourage further pilot and clinical studies
201 aimed at investigating the use of Xlear as a potential treatment for COVID-19.

202 Xlear is a solution of xylitol and GSE, in line with previous reports, the latter displayed
203 antiviral activity. Komura et al. demonstrated the efficacy of GSE as an antimicrobial agent on
204 avian pathogens including avian influenza virus, Newcastle disease virus, infectious bursal
205 disease virus, *Salmonella Infantis*, and *Escherichia coli* (Komura et al., 2019). Also, GSE has
206 shown similar antiviral activities against human enteric pathogens including Hepatitis A virus in
207 a dose-dependent manner (Su and D'Souza, 2011). Interestingly, GSE antiviral activity seems to
208 be particularly effective on enveloped viruses. Since SARS-CoV-2 is an enveloped virus the
209 GSE characteristics to induced or target the viral envelope should not be overlooked as candidate
210 therapies for COVID-19 emerge (Schoeman and Fielding, 2019). On the other hand, xylitol did
211 not show *in vitro* virucidal properties in the present study. However, it seems that the viral
212 protective effects of xylitol are evident *in vivo* a suggested by studies demonstrating ameliorating
213 effects against human respiratory syncytial virus and changes in the microbiota when consumed
214 orally (Uebanso et al., 2017; Xu et al., 2016).

215 The precise mechanism of action of GSE is poorly understood. However, according to
216 the present virucidal tests, the active component of the spray is the GSE, which is in line with
217 previous reports demonstrating that the extract was is effective to inactivate different enveloped
218 and non-enveloped viruses (Su and D'Souza, 2011).

219

220 Moreover, it seems that the mechanism of action of GSE targets the viral adsorption (or viral
221 binding) to a greater extent than viral replication. It is worth mentioning that studies of the
222 precise mechanism of action of GSE are beyond the scope of this work.

223 As with any research study, the present experimental design is not free from some
224 limitations. The minimum time required for the Xlear solution to exert the virucidal effect was
225 not investigated. Furthermore, to assess the relevance of the time-dependent effect of Xylitol
226 effect *in vivo*, it will be important to verify if the addition of the spray-on cells previously
227 infected at nontoxic doses would exert a reduction of the viral titer. Also, whether pre-treating
228 the cells with the spray and subsequently adding the virus would decrease the rate of infection
229 would be needed to assess the possible preventive use of the nasal spray.

230 **CONCLUSIONS**

231 This study demonstrates the strong virucidal effects against SARS-CoV-2 of the Xlear
232 nasal spray compound with xylitol and GSE. Using a virucidal nasal spray could become a
233 cutting-edge element in the prevention and treatment of COVID-19 disease. To further ascertain
234 the impact of this nasal spray in SARS-CoV-2, we propose to perform further a randomized
235 placebo-controlled study of intranasally delivered Xlear in patients with mild to moderate SARS-
236 CoV-2 and randomized placebo-controlled preventive trial in healthcare workers.

237

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- 297

298 **Table 1.** Virucidal efficacy of Xlear compounds against Rhinovirus-16 and SARS-CoV-2
299 after a 15-minute incubation with virus at $22 \pm 2^\circ\text{C}$.

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304 ^a Log₁₀ CCID₅₀ of virus per 0.1 mL. The assay lower limit of detection is 0.67 Log₁₀
305 CCID₅₀/0.1 mL.

306 ^b LRV (log reduction value) is the reduction of virus compared to the virus control

307

308 **Figure 1.** a) SARS-CoV-2 dose-response inhibition. Xlear was incubated at different dilutions
309 with SARS-CoV2 (200 pfu) for 1h at 37 C. At the end of the incubation, mixtures were serially
310 diluted and added for 1h at 37°C on Vero-E6 cells. Mixtures were then removed, and cells
311 overlaid with medium containing 0.8% avicel. Cells were fixed 48hpi and plaques were counted.
312 Results are mean and SEM of 2 independent experiments performed in duplicate. b) Xlear
313 toxicity evaluation. Different dilutions of the nasal spray were incubated for 1h (followed by
314 addition of medium for 47h) or for 48h on cells in DMEM 5% FBS. At the end of the incubation
315 MTT reagent was added on cells and percentages of viability were evaluated by comparing
316 treated and untreated wells.

317

318

319 **Figure 2.** SARS CoV-2 virucidal assay. Xlear was incubated with SARS-CoV2 (5×10^5 pfu) for
320 1h at 37 C. At the end of the incubation, mixtures were serially diluted and added on Vero-E6
321 cells. Cells were fixed 48hpi and scored for presence or absence of cytopathic effect and
322 TCID50/ml was determined. Results are mean and SD of two independent experiments.
323
324

	Tested Concentration	Virus Tested	Incubation Time	Virus Titer ^a	LRV ^b
Xlear	90%	Rhino-16	15-minute	5.0	0
Ethanol	90%	Rhino-16	15-minute	1.5	3.17
Virus Control	na	Rhino-16	15-minute	4.67	na
Xlear	90%	SARS-CoV-2	15-minute	3.0	0.67
GSE 0.2% in DI water	90%	SARS-CoV-2	15-minute	<0.67	3.0
Saline w/ 11% Xylitol	90%	SARS-CoV-2	15-minute	3.5	0.17
Saline w/ 11% Erythritol	90%	SARS-CoV-2	15-minute	4.3	0
Ethanol	90%	SARS-CoV-2	15-minute	<0.67	3.0
Virus Control	na	SARS-CoV-2	15-minute	3.67	na



