

1 **Iota-carrageenan and Xylitol inhibit SARS-CoV-2 in cell culture**

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19

20 **Abstract**

21

22 COVID-19 (coronavirus disease 2019) is a pandemic caused by SARS-CoV-2 (severe acute
23 respiratory syndrome-coronavirus 2) infection affecting millions of persons around the world. There is an
24 urgent unmet need to provide an easy-to-produce, affordable medicine to prevent transmission and
25 provide early treatment for this disease. The nasal cavity and the rhinopharynx are the sites of initial
26 replication of SARS-CoV-2. Therefore, a nasal spray may be a suitable dosage form for this purpose. The
27 main objective of our study was to test the antiviral action of three candidate nasal spray formulations
28 against SARS-CoV-2. We have found that iota-carrageenan in concentrations as low as 6 µg/ mL inhibits
29 SARS-CoV-2 infection in Vero cell cultures. The concentrations found to be active in vitro against
30 SARS-CoV-2 may be easily achieved by the application of nasal sprays already marketed in several
31 countries. Xylitol at a concentration of 5 % m/V has proved to be viricidal on its own and the association
32 with iota-carrageenan may be beneficial, as well.

33

34 **Introduction**

35 SARS-CoV-2 is a single stranded positive sense RNA virus responsible for COVID-19. COVID-
36 19 has become one of the worst pandemics of our time counting more than 12,232,700 confirmed cases
37 and more than 554,000 deaths worldwide by July 10th, 2020 [1]. In most cases, COVID-19 manifests
38 itself clinically with flu-like symptoms as a mild or uncomplicated illness, eventually resolving
39 spontaneously. However, 15% of patients develop severe pneumonia that requires hospitalization and
40 oxygen support, and 5% of them need admission to an intensive care unit (ICU). More than half of this
41 patients may die [2]. Even children can be affected although with milder symptoms than adults and can be
42 transmitters of the disease. [3]. There are no adequate therapeutic or preventive medicines available, so
43 effective therapeutic approaches are urgently needed to reduce the spread of the virus and its death toll.

44 During the first days of disease the virus is localized mainly in the nasal cavity and the
45 nasopharynx [4,5]. Recent data show that high viral load and a long virus-shedding period was associated
46 with severe COVID-19 [6, 7]. Therefore, the use of antiviral nasal sprays would contribute to reduce
47 nasal and nasopharyngeal viral load, thus slowing down the disease progression in the treated patient and
48 the disease transmission to others in close contact with him or her.

49 Carrageenans are linear sulfated polysaccharides that are often extracted from red seaweeds.
50 Carrageenans are commercially available in the form of kappa (κ), iota (ι) or lambda (λ). They have been
51 used for years as thickening agents and stabilizers for food. At present they are extensively used in the
52 food (cold cuts, cheese, etc.) and in the cosmetic and pharmaceutical industry as suspension and emulsion
53 stabilizers. Their antiviral capacity has been described decades ago and has been experimentally
54 confirmed on herpes virus type 1 and 2, human papilloma virus, H1N1 influenza virus, dengue virus,
55 rhinovirus, hepatitis A virus, enteroviruses, and coronaviruses. Iota-carrageenan inhibits several viruses
56 based on its interaction with the surface of viral particles, thus preventing them from entering cells and
57 trapping the viral particles released from the infected cells. [8, 9, 10, 11, 12, 13].

58 Iota-carrageenan formulated into a nasal spray has proved to be safe and effective against virus
59 causing common cold [14,15,16]. In vitro studies in cell cultures (HeLa and Calu-3) and in primary
60 respiratory epithelial cells have shown inhibition of rhinovirus, Influenza virus and common-cold
61 coronavirus. Iota-carrageenan is most active against common-cold coronavirus, inhibiting the infection up
62 to 90 %. An iota-carrageenan spray reduced mortality by at least 50 % in mice infected with lethal doses
63 of H1N1 influenza virus [17]. In all cases the antiviral action of iota-carrageenan is more effective, when
64 administered preventively or in the early stages of disease and has shown synergy with other antiviral
65 agents. Studies performed on adults and children with common cold demonstrate effectivity of iota-
66 carrageenan nasal spray to alleviate the clinical symptoms and shorten their duration, as well as to
67 decrease the viral load of nasopharyngeal specimens and the relapses during the follow-up period [14, 15,
68 16, 18, 19, 20]. Iota-carrageenan-containing nasal sprays are already on the market in several countries in
69 the world.

70 Xylitol is a polyol that has been used as a sugar substitute in Finland since the 1960s. It is a
71 polyol, (formula $\text{CHOH})_3(\text{CH}_2\text{OH})_2$), which is obtained from xylan extracted from hardwood, which has
72 demonstrated multiple health benefits [21]. It has been extensively used in buccal health care to prevent
73 caries because of its antibacterial capacity. It is already being used in otorhinolaryngology as a nasal
74 spray and lavage for the treatment of rhinosinusitis and the prevention of otitis media. [22,23]. Studies “in
75 vitro” and in animal models has shown antiviral properties of Xylitol against human respiratory syncytial
76 virus (24).

77 Both iota-carrageenan and xylitol are safe for humans, being used in much larger amounts as food
78 additive and sweetener, respectively, than those that may be used for nasal delivery. Nasal safety of iota-
79 carrageenan by nasal and nebulization administration has been already confirmed empirically [25]. The
80 same holds for 5 % Xylitol water solution both applied as nasal spray and nasal irrigation [26], as well as
81 applied as a nebulization solution [27]. Both are included in nasal formulations already on the market for
82 use in children and adults.

83 Based on the above knowledge, an experiment was designed and carried out in a Biosafety Level
84 3 (BSL3) laboratory to investigate the SARS-CoV-2 inhibition capacity of three different candidate
85 preservative-free nasal formulations.

86

87 **Materials and methods**

88

89 **Cells and Virus**

90 Vero E6 cells were purchased from the American Type Culture Collection. Vero E6 cells were
91 grown in complete minimal essential media (c-MEM) (Corning, NY, USA) which included 5% fetal
92 bovine serum (FBS)(Gibco, Waltham, MA, USA), 5 mM penicillin/streptomycin (Gibco), and L-
93 glutamine (Gibco). Cells were incubated at 37°C with 5% CO₂. SARS-CoV-2 Isolate USA-WA1/2020,
94 was obtained from BEI Resources (catalogue number NR-52281, Manassas, VA, USA). Virus master

95 seed stock was prepared in T175 flasks of Vero E6 cells using a multiplicity of infection (MOI) of 0.1.
96 Each flask was harvested on day two post-infection and supernatant was centrifuged twice at 220 x g for
97 15 minutes to remove cellular debris. Titer of virus stock was determined by plaque assay on Vero E6
98 cells.

99

100 **Preparation of sample formulations**

101 All the formulations and placebos were prepared at Laboratorio Pablo Cassará S.R.L. (Argentina)
102 under aseptic conditions and provided by Amcyte (US) to the University of Tennessee Health Science
103 Center. Composition of different formulations is depicted in Tables 1 and 2. Samples 1, 2 and 3 were
104 diluted at stock concentrations of 1200 µg/mL, 120 µg/mL, 12 µg/mL and 1.2 µg/mL using samples P1,
105 P2 and P3 respectively as diluents. To determine antiviral efficacy of formulations by titer reduction
106 assay, sample formulations were used at a final iota-carrageenan concentration of 600 µg/mL; 60 µg/mL,
107 6 µg/mL and 0.6 µg/mL. Equivalent concentration of placebos (samples P1, P2 and P3) was used for titer
108 reduction assay as controls.

109

110 **Table 1. Composition of candidate nasal formulations (samples containing iota-carrageenan)**

Component	sample 1	sample 2	sample 3
Iota-carrageenan	1.7 mg/mL	1.2 mg/mL	1.2 mg/mL
Sodium Chloride	9 mg/mL	5 mg/mL	—
Xylitol	—	—	50 mg/mL ^a
pH adjusted to	6.00 – 7.00	6.00 – 7.00	6.00 – 7.00

111

112 ^a Equivalent to 5 % m/V

113

114

115 **Table 2. Composition of placebo samples used as diluents (samples without iota-carrageenan)**

Component	sample P1	sample P2	sample P3
Sodium Chloride	9 mg/mL	5 mg/mL	—
Xylitol	—	—	50 mg/mL ^a
pH adjusted to	6.00 – 7.00	6.00 – 7.00	6.0 – 7.00

116

117 ^a Equivalent to 5 % m/V

118

119 **Titer reduction assay**

120 Vero E6 cells were seeded in 12-well plates at density of 2.5×10^5 /well and grown overnight at
121 37°C under 5%CO₂. Next day, cells were washed with PBS (pH 7.2), followed by addition of equivalent
122 amount of c-MEM with reduced FBS (2%) and sample/placebo formulations. Formulations were
123 incubated with cells for 2 hr, after which the supernatant was removed. Cells were infected with 2.5×10^4
124 pfu (MOI=0.1) of virus for 1 hr at 37C, 5%CO₂ with rocking at 15 min intervals. After incubation wells
125 were washed with DPBS, and sample/placebo formulations were added at same concentrations. After
126 incubation for 2 days, well contents were collected. For titer reduction, wells with no treatment (only
127 virus) and cells only were included. Virus titer was determined by performing a TCID₅₀ assay using MTT
128 to measure cell viability. Virus endpoint titer was determined using the Reed-Muench formula and
129 expressed as log TCID₅₀/mL. Residual virus titer from sample/placebo formulation treated wells was
130 plotted against virus titer from untreated wells.

131

132

133 **Results**

134 To examine the antiviral effects of iota-carrageenan on SARS-CoV-2, three sample formulations
135 were developed and tested. Each of the three sample formulations were tested in a dose dependent manner
136 based on the concentration of iota-carrageenan and ranged from 600 µg/mL to 0 µg/mL. SARS-CoV-2
137 samples treated with 600 µg/mL and 60 µg/mL of sample formulation 1 were reduced > 3.75 Log when
138 compared to untreated control (Figure 1). The 6 µg/mL concentration of sample formulation 1 also
139 demonstrated an effect but to a lesser extent, with a 2.5 Log reduction in virus (Figure 1). No activity was
140 observed with 0.6 µg/mL of Iota-carrageenan (Figure 1). Lastly, there was no reduction in virus with P1,
141 suggesting that Iota-carrageenan and not the components of sample formulation 1 is inhibiting SARS-
142 CoV-2 (Figure 1)

143

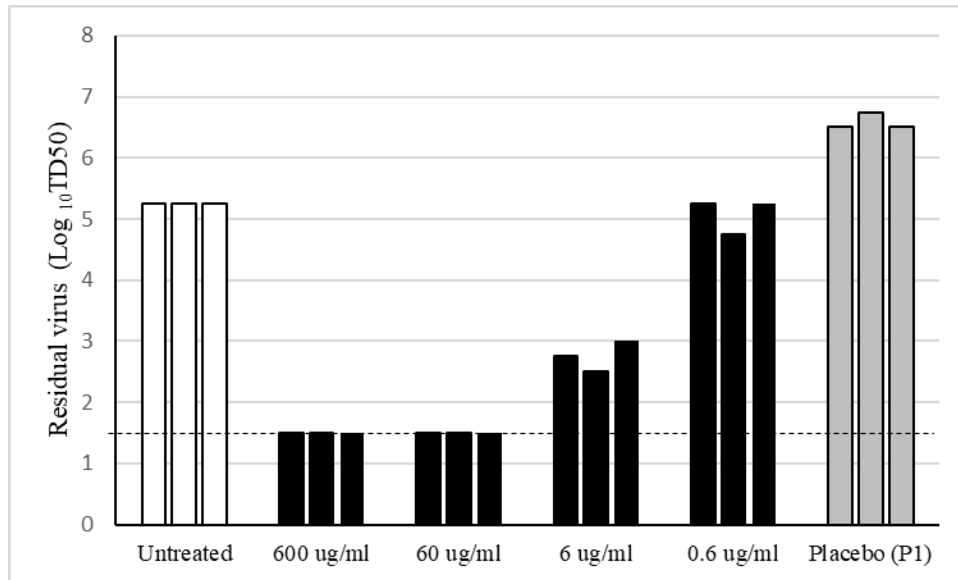
144 SARS-CoV-2 samples treated with dilutions of sample 2 at final iota-carrageenan concentrations
145 of 600 µg/mL, 60 µg/mL, and 6 µg/mL were reduced > 4.25 Log compared to untreated control (Figure
146 2). The 0.6 µg/mL concentration of iota-carrageenan was also not effective with sample 2 (Figure 2). No
147 reduction in virus with P2 was observed, suggesting that iota-carrageenan and not the components of
148 formulation 2 is inhibiting SARS-CoV-2 (Figure 2).

149

150 SARS-CoV-2 samples treated with dilutions of sample 2 at final iota-carrageenan concentrations
151 of 600 µg/mL, 60 µg/mL, and 6 µg/mL were reduced > 4.25 Log compared to untreated control (Figure
152 2). The 0.6 µg/mL concentration of iota-carrageenan was also not effective with sample 2 (Figure 2). No
153 reduction in virus with P2 was observed, suggesting that iota-carrageenan and not the components of
154 formulation 2 is inhibiting SARS-CoV-2 (Figure 2).

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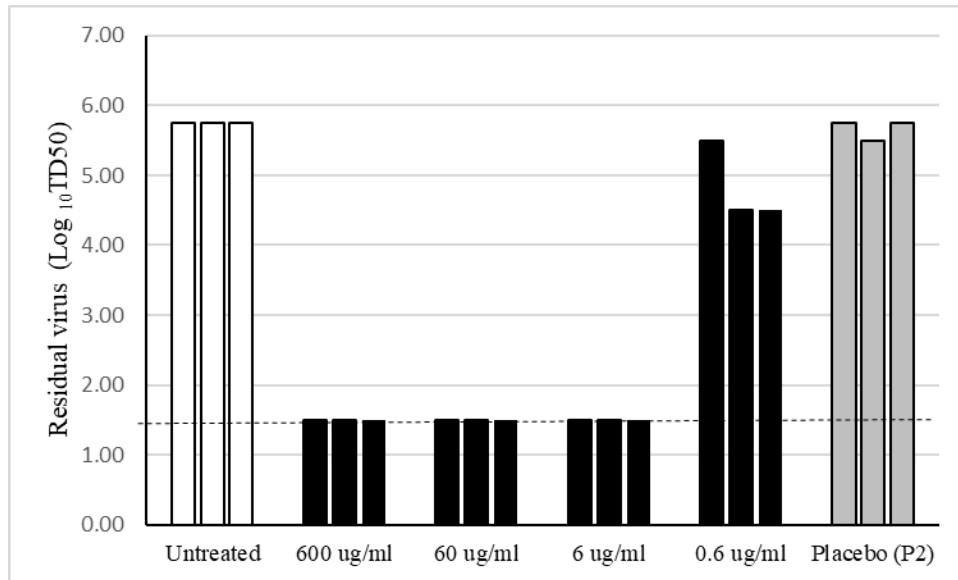
157

158 **Fig 1. SARS-CoV-2 viral titer after treatment with samples 1 and P1 (3 replicates per treatment).** Sample 1
159 composition: 1.7 mg/mL iota-carrageenan, 9 mg/mL sodium chloride, pH 6-7. Vero E6 were pre-treated with
160 dilutions of sample 1 with sample P1 (placebo without iota-carrageenan) to get 600 µg/mL, 60 µg/mL, 6 µg/mL, 0.6
161 µg/mL iota-carrageenan final concentration for 2 h. After a 2 h pretreatment, cells were infected with SARS-CoV-2
162 and incubated for 48h in the presence of the same dilutions of sample 1. Supernatants were harvested and virus yield
163 determined by an end point dilution assay (TCID₅₀). Controls consisted of untreated infected cells or infected cells
164 treated with P1 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed
165 as log TCID₅₀/mL. Dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate.
166 Underlying data reported in tables S3A and S3B as supporting information.

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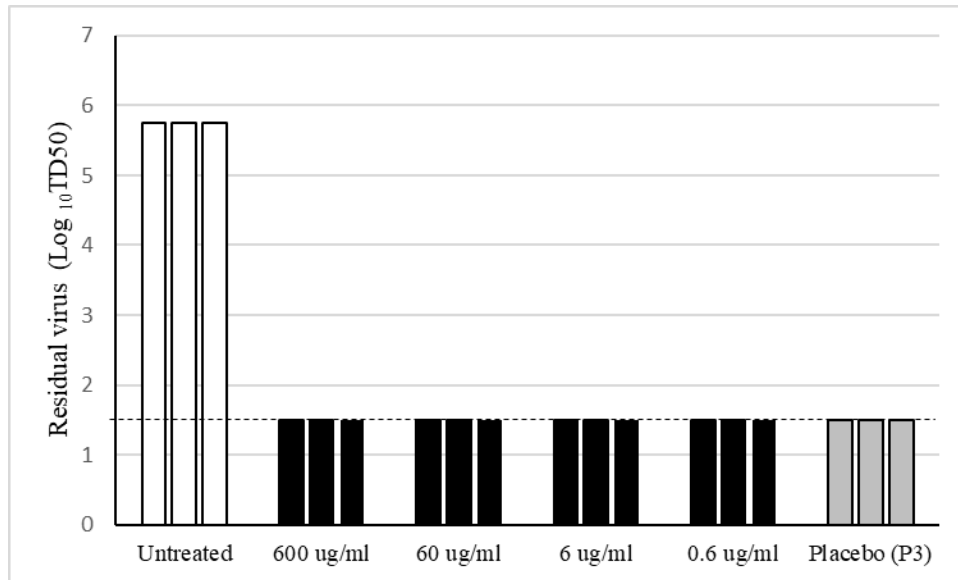


170

171 **Fig 2. SARS-CoV-2 viral titer after treatment with samples 2 and P2 (3 replicates per treatment)** Sample 2
172 composition: 1.2 mg/mL iota-carrageenan, 5 mg/mL sodium chloride, pH 6-7. Vero E6 were pre-treated with
173 dilutions of sample 2 with sample P2 (placebo without iota-carrageenan) to get 600 µg/mL, 60 µg/mL, 6 µg/mL, and
174 0.6 µg/mL final iota-carrageenan concentration for 2 h. After a 2 h pretreatment, cells were infected with SARS-
175 CoV-2 and incubated for 48h in the presence of the same dilutions of sample 2. Supernatants were harvested and
176 virus yield determined by an end point dilution assay (TCID₅₀). Controls consisted of untreated infected cells or
177 infected cells treated with P2 (no iota-carrageenan). Results were determined using the Reed and Muench formula
178 and expressed as log TCID₅₀/mL. Dotted line shows the limit of detection (LOD). Testing of samples was
179 performed in triplicate. Underlying data reported in tables S2A and S2B as supporting information.

180

181 All concentrations tested (600 - 0.6 µg/mL) with sample 3 demonstrated antiviral activity
182 including the P3 control that did not contain iota-carrageenan (Figure 3). Xylitol was present in this
183 sample formulation and not in sample 1 or 2. The result suggests this component might also exert an
184 antiviral effect.



185

186 **Fig 3. SARS-CoV-2 viral titer after treatment with samples 3 and P3 (3 replicates per treatment).** Sample 3
187 composition: 1.2 mg/mL iota-carrageenan, 50 mg/mL xylitol, pH 6-7. Vero E6 were pre-treated with dilutions of
188 sample 3 with sample P3 (placebo without iota-carrageenan) to get 600 µg/mL, 60 µg/mL, 6 µg/mL, and 0.6 µg/mL
189 final iota-carrageenan concentration for 2 h. After a 2 h pretreatment, cells were infected with SARS-CoV-2 and
190 incubated for 48h in the presence of the same dilutions of sample 3. Supernatants were harvested and virus yield
191 determined by an end point dilution assay (TCID₅₀). Controls consisted of untreated infected cells or infected cells
192 treated with P3 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed
193 as log TCID₅₀/mL. Dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate.
194 Underlying data reported in tables S3A and S3B as supporting information.

195

196 A comparison of all three samples tested indicate that iota-carrageenan (600 µg/mL, 60 µg/mL,
197 and 6 µg/mL) in samples 1 and 2 are effective at inhibiting SARS-CoV-2 (Table 3). Sample 3, which
198 contained xylitol, was the most effective and demonstrated an antiviral effect at all concentrations tested
199 (Table 3).

200

201

202

Table 3. Log reduction of TCID₅₀/mL found after 48 hs.

Iota-carrageenan	Sample 1	Sample 2	Sample 3
600 µg/mL	≥ 3.75	≥ 4.25	≥ 4.25
60 µg/mL	≥ 3.75	≥ 4.25	≥ 4.25
6 µg/mL	2.50	≥ 4.25	≥ 4.25
0.6 µg/mL	0.17	0.92	≥ 4.25
0 µg/mL ^a	None	None	≥ 4.25

203

204 The values reported are calculated as mean of untreated replicates minus mean of treated replicates. Mean
205 values used for this calculation are reported in Tables S1B, S2B and S3B as supporting information.

206 ^aThese are the placebo samples P1, P2 and P3 having the same components as samples 1, 2 and 3 except
207 for iota-carrageenan.

208

209 Discussion

210 Results from our study indicate that iota-carrageenan significantly inhibits SARS-CoV-2 in vitro.
211 Our results are encouraging for clinical use of iota-carrageenan nasal spray for the prevention and early
212 treatment of COVID-19. Clinical studies have demonstrated that iota-carrageenan nasal spray
213 formulations already effective in vitro against rhinovirus [11] proved to be clinically effective in
214 preventing and reducing symptoms and duration of common cold [14, 15, 16, 18]. Moreover, this study
215 was designed so that the concentrations tested in vitro resemble the immediate concentration in nasal
216 cavity and lower concentrations expected as iota-carrageenan is cleared from it. To do that we estimated
217 airway surface liquid volume to be in the range 50 – 375 µL in nasal cavity based on a surface area of the
218 nasal mucosa of 100 – 250 cm² [28, 29, 30, 31] and airway surface liquid height estimated as 5 – 15 µm

219 [32. 33]. If we take an average content of 200 μL of airway surface liquid in the nose plus 200 μL of
220 formulation after delivering one 100- μL of a 1.2 mg/mL iota-carrageenan solution in each nostril, the
221 immediate concentration of iota-carrageenan in the nasal cavity would be 600 $\mu\text{g}/\text{mL}$, coinciding with
222 the highest concentration tested in vitro and capable of reducing virus yield to the LOD in our assay.
223 Furthermore, considering that even 1/100 of this concentration is still active in vitro and that iota-
224 carrageenan may stay for 4 hours [19] in the nasal cavity, there is a reasonable chance that this nasal spray
225 may significantly help in the prevention and early treatment of COVID-19. Expected concentrations of
226 iota-carrageenan in the nasal cavity will be even higher, if we consider a nasal formulation containing 1.7
227 mg / mL (0.17 % m/V) as some marketed nasal sprays.

228

229 The other remarkably interesting result is that xylitol exhibits antiviral activity on SARS-CoV-2
230 based on the results obtained with sample P3. Xylitol has been demonstrated to reduce titers of Human
231 Respiratory Syncytial Virus in Hep-2 cells culture and in infected mice [24].

232 Despite the implementation of severe personal protection measures, pandemic continues to affect
233 a significant proportion of health care workers with severe consequences for them, their patients, and the
234 community. At the same time, most COVID-19 patients remain at home, thus increasing the likely
235 exposure of household members and caregivers. Providing them with simple interventions as nasal sprays
236 with either iota-carrageenan or xylitol in the same or different nasal devices may lower the risk of
237 infection progression and transmission.

238 An inhalation solution of the same composition may be effective in case of severe cases of
239 COVID 19. Even though there are studies showing safety of the use of both carrageenan and xylitol in
240 nebulization [25,27], clinical trials would be needed to fully confirm these hypotheses. Risk of spreading
241 the virus should be considered in this form of administration and due protection should be used to contain
242 it [34].

243 We are starting multicenter randomized controlled trials to evaluate the efficacy of iota-
244 carrageenan nasal sprays in health care staff assisting COVID-19 patients and in patients suffering from
245 COVID 19 and other persons in close contact with them. However, it must be stressed that this and other
246 similar nasal sprays are on the market and their safety profile is remarkable. The current COVID-19
247 emergency warrants the urgent development of potential strategies to protect people even if more robust
248 data on antiviral therapies is yet to come.

249

250 **Conclusions**

251 Iota-carrageenan inhibits SARS CoV-2 in vitro at concentrations easily achievable by nasal and
252 nebulization formulations. Furthermore, xylitol exhibits antiviral activity on SARS-CoV-2, as well. An
253 association with iota-carrageenan may be beneficial. There are already nasal sprays on the market having
254 similar formulations to some of those tested in this in vitro study with adequate safety profile. Clinical
255 trials are in progress to evaluate that nasal sprays based on the tested formulations are useful in the
256 prevention and treatment of COVID 19. The data presented here are certainly encouraging in this
257 direction.

258 **Acknowledgements**

259 The authors thank Augusto Pich Otero and his team at Laboratorio Pablo Cassará S.R.L.
260 (Argentina) for the supply of the sterile formulations for this in vitro study.

261

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