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1 **Green tea and Spirulina extracts inhibit SARS, MERS, and SARS-2 spike**
2 **pseudotyped virus entry in vitro**

3

4 Jeswin Joseph[1#], Karthika T[1#], Ariya Ajay[1], V.R. Akshay Das[1], V. Stalin Raj[1*]

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6 [1]Virology Scientific Research (VSR) Laboratory, School of Biology, Indian Institute of Science
7 Education and Research, Thiruvananthapuram, India.

8

9 [#]These authors contributed equally to this work

10 [*]Corresponding author: stalin@iisertvm.ac.in

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12 Keywords

13 SARS-CoV-2, SARS-CoV, MERS-CoV, antivirals, Spirulina, Green tea, EGCG, Sulphated
14 polysaccharides, medicinal plants

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20 **Abstract**

21 Coronaviruses (CoVs) infect a wide range of animals and birds. Their tropism is primarily
22 determined by the ability of the spike (S) protein to bind to a host cell surface receptor. The rapid
23 outbreak of emerging novel coronavirus, SARS-CoV 2 in China inculcates the need for the
24 development of hasty and effective intervention strategies. Medicinal plants and natural
25 compounds have been traditionally used to treat viral infections. Here, we generated VSV based
26 pseudotyped viruses (pvs) of SARS-, MERS-, and SARS-2 CoVs to screen entry inhibitors from
27 natural products. In the first series of experiments, we demonstrated that pseudotyped viruses
28 specifically bind on their receptors and enter into the cells. SARS and MERS polyclonal antibodies
29 neutralize SARSpv and SARS-2pv, and MERSpv respectively. Incubation of soluble ACE2
30 inhibited entry of SARS and SARS-2 pvs but not MERSpv. In addition, expression of ACE2 and
31 DPP4 in non-permissive BHK21 cells enabled infection by SARSpv, SARS-2pv, and MERSpv
32 respectively. Next, we showed the antiviral properties of known enveloped virus entry inhibitors,
33 Spirulina and Green tea extracts against CoVpvs. SARSpv, MERSpv, and SARS-2pv entry were
34 blocked with higher efficiency when preincubated with either green tea or spirulina extracts. Green
35 tea provided a better inhibitory effect than the spirulina extracts by binding to the S1 domain of
36 spike and blocking the interaction of spike with its receptor. Further studies are required to
37 understand the exact mechanism of viral inhibition. In summary, we demonstrate that pseudotyped
38 virus is an ideal tool for screening viral entry inhibitors. Moreover, spirulina and green tea could
39 be promising antiviral agents against emerging viruses.

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43 **Introduction**

44 The past three decades have witnessed a tremendous increase in the number of highly pathogenic
45 emerging viruses [1] which have caused serious global threats initiating diverse approaches for
46 understanding the biology of the virus and for robust vaccine and therapeutic development.
47 Coronaviruses are notable examples of zoonotic emerging viruses which can infect a diverse range
48 of mammals and birds including humans [2]. The major outbreak of human coronaviruses occurred
49 in 2003 wherein Severe Acute Respiratory Syndrome (SARS) CoV emerged from Chinese palm
50 civet and caused an epidemic in China with more than 8000 cases including 774 fatal cases [3].
51 With the help of public health authorities, the outbreak was effectively contained within six
52 months. Ten years later, another CoV termed Middle East respiratory syndrome (MERS) CoV
53 emerged from dromedary camel to human and is continuing to cause outbreaks in the Middle East
54 region [4]. To date, 2519 cases have been reported with mortality rates of 34% [5]. There is no
55 approved therapy or vaccine available for this virus yet. By the end of 2019, another pandemic
56 coronavirus named Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in
57 Wuhan, China [6] though unknown intermediate host. Till now, more than 8,000,000 cases and
58 450,000 deaths have been reported worldwide [7]. Likewise, novel or re-emerging viral outbreaks
59 may occur in the near future as well. Therefore, effective antiviral and therapeutic strategies are
60 required to control the ongoing or future outbreaks.

61 Coronavirus spike protein is a promising target for the development of antiviral compounds
62 because their cell tropism is primarily determined by the ability of the spike (S) protein to bind to
63 a host cell surface receptor and can block the virus entry at the early stage of infection. But drug
64 discovery for highly pathogenic viruses like SARS, MERS, Ebola, Lassa, or SARS-2 are
65 challenging due to the requirement for a BSL-3/BSL-4 laboratory containment facility. However,

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66 very limited facilities are available, especially in developing countries. Pseudotyped viruses
67 provide a substitute model in which the native envelope protein of a nonpathogenic BSL-2 virus
68 (vesicular stomatitis virus) replaced with an envelope glycoprotein of a highly pathogenic virus
69 like SARS, MERS, Ebola, or SARS-2 [8]. These viruses mimic a normal virus but are
70 noninfectious in nature. Moreover, they are replication-incompetent with a single round of
71 infection and hence can be used to do research in the routine BSL-2 laboratories. Pseudotyped
72 viruses have been used in diagnostics, vaccines, and high-throughput screening of entry inhibitors
73 for several BSL-3/BSL-4 level pathogens [8].
74
75 Natural products such as plants, algae, and seaweeds have always been implicated in multiple
76 fields of biology for their antibacterial, antiviral, and antifungal properties. Several medicinal
77 plants have been traditionally used to treat viral infections [9] and have been demonstrated with
78 their ability of their inhibitory effects (Table 1) on the replication or entry of several viruses like
79 herpes simplex virus (HSV) type 2, hepatitis B (HBV), influenza virus [10]–[13]and also other
80 emerging viral pathogens such as poxvirus and SARS [14]. Curcumin acts as an antiviral agent
81 against several viruses such as Parainfluenza virus type 3, vesicular stomatitis virus (VSV), HSV,
82 etc. [15]. Diammonium glycyrrhizin the main component of licorice root extract shown to have an
83 inhibitory effect on pseudorabies virus (PrV) [16]. Neem and Tulsi leaf extracts are potent antiviral
84 agents against influenza virus [17], [18]. Moreover, the extract of green tea, another natural
85 compound, inhibits human immunodeficiency virus (HIV), zika virus, influenza virus, and
86 hepatitis C virus (HCV) [19].

87 Spirulina is a commercially available dietary supplement which has been recorded for its diverse
88 properties. It is a free-floating cyanobacterium, which has 70% protein content and is rich in

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89 phenolic acids, essential fatty acids, sulfated polysaccharides, and vitamin B12 [20]. Extracts of
90 Spirulina have been shown to have antiviral activity against multiple enveloped viruses including
91 influenza virus, HSV, adenovirus, etc.[21] Apart from crude plant extracts, several specific
92 compounds from Green tea, catechins, such as epicatechin (EC), epigallocatechin (EGC),
93 epicatechin gallate (ECG), and epigallocatechin-3-gallate (EGCG) have been found to have
94 antiviral and anticarcinogenic properties [22] (Supplementary table 1). EGCG is a major
95 component and active catechin of green tea and has several bio modulatory effects such as anti-
96 allergic, anti-inflammatory, anti-tumor, antioxidative, and antiviral properties. EGCG, has been
97 reported to inhibit HCV, HIV, HSV type 1 and 2, enterovirus 71, influenza A, and other viruses
98 [10], [23]–[27]. For HIV, EGCG binds to the CD4 molecule at high affinity and inhibits HIV
99 gp120 binding to human CD4+ T cells [28]. Moreover, the EGCG-induced inhibition was observed
100 in a broad spectrum of HIV-1 subtypes as well. EGCG acts through direct inactivation of the virus
101 particle, inhibition of the protease adenain, and intracellular growth in vitro [29]. Sulfated
102 polysaccharides and Spirulan like compounds are the major components of spirulina extract, which
103 inhibits the entry of several viruses including HSV-1, hepatitis A, human cytomegalovirus
104 (HCMV), VSV, and HIV [13]. This clearly shows the importance of antiviral properties of natural
105 compounds, and hence could be promising therapeutic agents for emerging viral diseases including
106 SARS-CoV 2. In this study, we show the biological applications of pseudotyped coronaviruses
107 and the antiviral activity of Spirulina and green tea extracts using the developed pseudotyped
108 coronaviruses.

109 **Materials and Methods**

110 **Cells.** Vero E6 and HEK293T cells were grown in DMEM (Lonza, 12-604F) supplemented with
111 10% FBS (MP, 29101) and 1% penicillin/streptomycin. BHK21 cells were maintained in EMEM

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112 (Lonza, 12-611F) supplemented with 10% FBS and 1% penicillin/streptomycin (Lonza, 17-602E).
113 Huh7 cells were grown in RPMI 1640 medium (Lonza, 12-115F) with 10% FCS and 1%
114 penicillin/streptomycin. The cells were grown at 37°C in a CO_2 incubator.

115 **Plasmids.** Angiotensin converting enzyme 2 (ACE 2) gene (2.4kb) was PCR amplified from
116 cDNA of Vero E6 cells and the resulted amplicon was inserted between KpnI/XbaI restriction sites
117 in PCDNA3.1+ expression vector (pCDNA-ACE2). Dipeptidyl peptidase 4 (DPP4)(2.3kb) was
118 amplified from cDNA of Huh7 cells, as described previously [30], cloned between BamHI/NotI
119 in PCDNA3.1+ expression vector (pCDNA-DPP4). A synthetic construct of the full-length spike
120 of SARS-CoV-2 (aa residues 1-1273) was commercially synthesized from Genewiz, UK. The
121 coding sequence of spike S1 domain of SARS-2(aa residues 1-683), SARS (1-676), and MERS
122 (1-747) were amplified from synthetic spike construct and cDNA of SARS- and MERS-CoV
123 respectively. Amplicons were fused C-terminally with Fc domain of human immunoglobulin
124 (IgG) (aa residues 1-231) inserted via SacI /XhoI restriction sites in pCAGGS expression vector
125 (pCAGGS-SARS2-S1-Fc, pCAGGS-SARS-S1-Fc, pCAGGS-MERS-S1-Fc). For the production
126 of soluble ACE2, gene sequence encoding amino acid residues 1-635 was PCR amplified from
127 pcDNA-ACE2 plasmid introduced between N terminal CD5 signal sequence and C Terminal 6X
128 HIS- tag in pCAGGS-CD5 plasmid by Kpn1/Xho1 (pCAGGS-sACE2).

129 **Production of pseudotyped coronaviruses.** Full-length spike genes of SARS (aa residues, SARS
130 -2(1-1254) and MERS CoV lacking the endoplasmic retention signal sequence were cloned into
131 pCAGGS expression system (pCAGGS-SARS2-S, pCAGGS-SARS-S, and pCAGGS-MERS-S)
132 and were transfected using Polyethylenimine (PEI) (Polysciences, USA) at 1:3 ratio in HEK293T
133 cells. After 24 h post-transfection, cells were infected with VSVΔG/GFP pseudovirus, incubated
134 for 1 hour, and cells were washed thrice with PBS and added with infection medium DMEM with

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135 1%FCS. The cell supernatant was harvested after 24h post-infection (pi), cell debris was removed
136 by centrifugation at 3000rpm for 10 minutes and aliquots were stored at -80°C. For titration assay,
137 a day before the experiment Vero E6 and Huh7 cells were seeded in a 96 well plate at $2x10^4$ cells
138 per well and 100µl of serially diluted (1:10) pseudoviruses were added to the cells and incubated
139 for 1h. After incubation, cells were washed and replaced with fresh DMEM with 1% FBS and
140 were incubated at 37°C in a CO_2 incubator for 24h. GFP positive cells were counted and were
141 normalized to VSVΔG –GFP background values. The lowest dilution of the pseudovirus with GFP
142 positive cells was calculated and viral titer was calculated as infection units /ml as mentioned
143 previously [31], [32].

144 **Transmission Electron Microscopy (TEM).** Pseudoviruses were concentrated using Amicon
145 3kDa cut off columns (Merck, UFC800324). Tem micro liters of glutaraldehyde (2.5%) fixed
146 samples were drop casted on formvar carbon-coated copper grids and negatively stained with 1%
147 Uranyl acetate alternative solution for 1 minute (TED PELLA, Cat. No- 19485). The grids were
148 washed twice with distilled water to remove the excess stain and followed by imaging on Tecnai,
149 FEI Transmission electron microscope at 120 kV.

150 **Production of recombinant proteins.** Ten micrograms of plasmids encoding either spike S1-Fc
151 or sACE2 (pCAGGS-SARS2-S1-Fc, pCAGGS-SARS-S1-Fc, pCAGGS-MERS-S1-Fc, and
152 pCAGGS-sACE2) were independently transfected using PEI in HEK293T cells. After 12h post-
153 transfection, cells were replaced with fresh, freestyle expression media, and the cells were
154 incubated for 5 days at 37°C in a CO_2 incubator. Recombinant Fc fused S1 proteins containing
155 supernatants were harvested and cell debris was removed by centrifugation for 5 minutes at
156 1500rpm. S1 -Fc proteins were purified using Protein A-Sepharose beads (GE Healthcare Life
157 sciences) following the manufacturer's instructions. Proteins were eluted from the column using

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158 0.5M glacial acetic acid pH 3 and neutralized with 3M Tris-Hcl PH 8.8. Quality and quantity of
159 the purified proteins were analyzed by nanodrop, BCA, and SDS-PAGE. Further confirmed by
160 western blotting using Goat-anti human IgG conjugated with HRP (Bethyl, A80-119P). Soluble
161 ACE 2 was purified using Nickel -NTA agarose beads (Qiagen Cat.No-1018244), eluted with
162 200mM imidazole, and was analyzed by SDS PAGE and western blot using Goat anti-human ACE
163 2 polyclonal antibody (R&D, Cat.No-AF933).

164 **sACE2 blocking assay.** sACE2 (5µg/ml PBS) was preincubated with SARS2pv, SARSpv, and
165 MERSpv at 37°C for 1h and then the mixture was directly added on Vero E6 cells for 1h. After
166 incubation, the cells were replaced with fresh DMEM with 1% FCS and antibiotics and incubated
167 for 24h at 37°C in a CO_2 incubator. Next day, GFP positive cells were counted and calculated as
168 the relative percentage of infection.

169 **Pseudovirus neutralization assay.** SARS and MERS CoV polyclonal antibodies (gifted by Bart
170 Haagmans, EMC, The Netherlands). SARS polyclonal antibodies were preincubated with SARSpv
171 and SARS-2pv and MERS polyclonal with MERSpv (1:100 dilution) at 37°C for 1h. Then the
172 antibody virus mixture was directly added on Vero E6 cells. 1hpi, infection medium was replaced
173 with fresh DMEM with 1% FCS and was incubated at 37°C in a CO_2 incubator for 24h. GFP
174 positive cells were counted to calculate the antibody neutralization of the pseudoviruses.

175 **Surface expression of ACE2 and DPP4.** BHK21 or HEK293T cells were transfected with **either**
176 pCDNA-ACE2 or pCDNA-DPP4 using PEI at 1:3 ratio. After 6h post-transfection, the medium
177 was replaced with complete DMEM with 10% FCS and 1% antibiotics. Surface expression was
178 analyzed by incubation of either Goat anti-ACE2 or Goat anti-DPP4 (1:250) primary antibody and
179 secondary antibody with rabbit anti-goat conjugated with Alexa Fluor 594 (Immunotag-

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180 ITIF59418). The positive cells were visualized using Leica TCS SP5 II inverted confocal
181 microscope 63x oil objective.

182 **Cold water extraction of Spirulina (*Arthrospira platensis*) and Green Tea**. Spirulina and green
183 tea are available in routine organic/medical stores. Spirulina was purchased as capsules or powder
184 and green tea was purchased as dried leaves. Extracts were prepared as described elsewhere [33].
185 Briefly, Spirulina and green tea were powdered using mortar and pestle and the fine powder was
186 weighed at 20mg/ml concentration and were mixed in sterile distilled water and vortexed for 5
187 min. Next, the mixtures were freeze-thawed and the supernatants were collected by centrifugation.
188 Supernatants were filter sterilized using a 0.22µm membrane filter and stored at 4 °C until further
189 use.

190 **Screening of antivirals against pseudotyped coronaviruses**. In the first series of experiments
191 we incubated the cells with either Spirulina, green tea extracts or DMEM. After 30 minutes
192 incubation, cells were replaced with 100microlitre of all the four pseudotyped viruses. Parallelly,
193 we incubated all the fours pvs as well with the extracts or DMEM. After 30 minutes of incubation,
194 the mixtures were directly added to confluent Vero E6 cells and incubated for 1h. Then the cells
195 were washed and added with fresh DMEM 1% FCS and antibiotics. Next day, GFP positive cells
196 were counted and the percentage of infection was calculated.

197 **Spike and receptor interaction studies by S1-Fc binding analysis.** HEK293T cells were
198 transfected with either pCDNA-ACE2 or pCDNA-DPP4 plasmids. 24h post-transfection, spike
199 S1-Fc proteins (5µg/ml) of SARS, SARS-2 and MERS CoV and the cells were independently
200 treated with Spirulina and green tea extract for 90 min at 37°C. In the first set of experiments,
201 untreated spike S1-Fc proteins were added to pretreated cells followed by binding assay at 4°C. In
202 the next set of experiments, the preincubated extract - spike S1-Fc protein mixture was added to

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203 the untreated cells and were processed for binding assay. In both the experiments, following 1h of
204 spike S1-Fc incubation at 4°C, the cells were stained with Goat anti-human IgG conjugated with
205 FITC (1:300 dilution; A80-119F, Bethyl) subsequently counterstained with DAPI (Sigma Aldrich
206 Cat no D9542-10MG). Confocal images were acquired using Zeiss LSM 880 confocal laser-
207 scanning microscope with an objective at 63x oil. Imaging parameters were kept the same for
208 control versus treated samples. The images were processed using the Zeiss ZEN blue software.
209 **Statistical analysis**: Unpaired student's t-test was performed and only p values of 0.05 or lower
210 were considered statistically significant. For all statistical analyses, the GraphPad Prism 5 was
211 used.

212 **Results**

213 Emerging viruses are highly pathogenic and require sophisticated biocontainment facilities.
214 Research on these pathogenic viruses like SARS, MERS, or SARS-CoV-2 is challenging due to
215 the limited availability of BSL-3/BSL-4 laboratory facilities. Pseudotyped viruses are an
216 alternative that can mimic a live virus, but replication-deficient and can be used for research work
217 in the routine BSL-2 laboratory. For the generation of coronavirus pseudotypes, full-length spike
218 gene of SARS, MERS, and SARS-2 lacking the endoplasmic retention signal sequence were
219 amplified from cDNA (SARS and MERS) or the codon-optimized synthetic construct (SARS-
220 CoV-2) and amplicons were cloned into pCAGGS expression plasmid. The plasmid constructs
221 were confirmed by sequencing and restriction analysis and were transfected in HEK293T cells,
222 and protein expression was confirmed by immunofluorescent staining (data not shown). In order
223 to produce VSVΔG/GFP pseudotyped coronaviruses, cells were transiently expressed with the
224 respective plasmids encoding the spike glycoprotein of SARS, MERS, or SARS-CoV-2 in
225 HEK293T cells and VSVΔG/GFP pseudovirus was infected 24 hours post-transfection. Pseudo

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226 typed coronaviruses were harvested from the precleared cell supernatant and titrated in Vero E6
227 or HuH 7 cells, and the GFP positive cells (Fig 1a) were counted and calculated the infection units
228 per milliliter (IU/ml) The titer of the SARSpv was 1.2x105 IU/ml, MERSpv 1.4x104 IU/ml and
229 VSVpv 1x106.4 IU/ml whereas SARS CoV -2 yielded a less titer 5x103 IU/ml (Fig. 1b). The
230 presence of intact CoVpv particles in the supernatant was analyzed independently by Transmission
231 electron microscopy (TEM). Intact bullet-shaped virus particles of 200 nm size were observed in
232 each grid (Fig. 1c).

233 Proper maturation of the VSV pseudotyped coronavirus particles are critical for cellular entry and
234 infection. Next, we checked whether the polyclonal antibodies raised against MERS or SARS
235 CoVs binds and neutralizes the pseudotyped CoVs. SARS-CoV specific polyclonal antibodies
236 neutralizes SARSpv and also cross neutralizes SARS-2pv but not MERSpv (Fig 2a) whereas,
237 MERS-CoV polyclonal specifically neutralizes MERSpv but not SARSpv or SARS-2pv (Fig. 2b),
238 suggesting that antibodies specifically bind on the receptor binding S1 domain of the CoVpvs and
239 neutralize the virus infection. It is well known that SARS and SARS-CoV-2 use human ACE2 as
240 the entry receptor to initiate an infection cycle [33]–[35]. To confirm that the CoVpv entry is
241 mediated by its specific receptor, we produced a recombinant soluble form of Angiotensin-
242 converting enzyme 2 (sACE2) in HEK293T cells and purified using Ni-NTA affinity column. A
243 90 kDa band was observed in SDS PAGE and was confirmed by western blotting using ACE2
244 specific antibody (Fig. 2c). Incubation of sACE2 with CoVpvs blocked the entry of SARSpv and
245 SARS-2pv but did not affect MERSpv infection (Fig 2d).

246 Further, to confirm the receptor-mediated entry of the pseudotyped coronaviruses, we cloned the
247 complete ACE2 or MERS-CoV receptor dipeptidyl peptidase 4 (DPP4) and was transiently
248 expressed on the non-susceptible BHK21 cells. The surface expression of ACE2 and DPP4 was

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249 confirmed by immunostaining using ACE2 and DPP4 polyclonal antibodies (Fig 2e). Next, we
250 expressed the S1 domain of different spike proteins fused to the Fc domain of human IgG (S1-
251 hFc) in HEK293T cells, yielding recombinant proteins of approximately 140 kDa, which was
252 confirmed by western blotting (Fig 2c) and immunostaining (Supplementary Fig 1). Incubation of
253 recombinant SARS-, SARS-2 -S1-hFc fusion proteins enables binding on hACE2 whereas MERS-
254 S1-hFc bind on the hDPP4 transfected cells and no binding was observed in empty plasmid
255 transfected cells (Fig 2e). Subsequently, ACE2 transiently expressed on the surface of non-
256 susceptible BHK21 cells enable SARS- and SARS-2 pvs infection, whereas MERSpv or VSVpv
257 did not (Fig. 2f). Similarly, MERS CoVpv infection was observed in hDPP4 expressed BHK21
258 cells, whereas SARS-, SARS-2-, and VSV- pvs infections were similar to empty plasmid
259 transfected cells (Fig 2g). These data confirm that the developed pseudotyped CoVs use its specific
260 receptor to enter into the host cells.

261 Targeting viral entry through interaction with cell receptors is a preliminary goal for the
262 development of antivirals. Medicinal plants and natural compounds have been previously reported
263 to have antiviral activity against a diverse range of viruses [9], [36]. Among these, several natural
264 compounds are known to inhibit viral entry by binding to the viral glycoprotein or host cell surface
265 receptor and block the interaction of the virus and its receptor, thereby preventing the entry into
266 the host cells. Spirulina and Green tea extracts have been reported to have the ability to inhibit the
267 viral entry of enveloped viruses, including HCV, Influenza, HIV, VSV, etc. [21], [23], [26], [27],
268 [37]. Here, we screened the antiviral activity of Spirulina and Green tea extracts against
269 pseudotyped coronaviruses. First, to know whether crude extracts inhibit CoVpvs, we treated Vero
270 E6 cells as well as pseudotyped viruses (SARSpv, SARS -2pv, and MERSpv), including a VSVpv
271 positive control with 0.2mg/ml of cold water extracts (CWE) of Spirulina and green tea.

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272 Interestingly, a significant reduction in infection was observed in all pvs pretreated with the
273 extracts (Fig 3b, d) but not in any of the preincubated cells (Fig 3a, c). The results suggest that
274 CWE of Spirulina and Green tea inhibits pseudotyped coronavirus entry by attachment to
275 pseudovirus envelope protein rather than to the cell surface.
276 Next, to confirm that the observed inhibitory activity of the extracts was not due to proteolytic
277 cleavage of the spike glycoprotein, we incubated the spike S1-hFc protein with the extracts for 90
278 minutes and then the proteins were analyzed by western blotting. No protein degradation was
279 observed in any of the proteins in comparison to input (Supplementary 2). Next, we performed a
280 concentration-dependent inhibition assay for all pvs. We incubated pvs with varying
281 concentrations ranging from 0.1mg/ml -0.8mg/ml of Spirulina and green tea extracts. All three
282 CoVpvs were confirmed to be inhibited by CWE of Spirulina and Green tea with higher efficiency.
283 90% inhibition was observed in Spirulina treated SARSpv , SARS-2pv ,MERSpv and VSVpv
284 infection at 0.6mg/ml,0.5mg/ml,0.8mg/ml and 0.6 mg/ml respectively (Fig.4a)whereas green tea
285 treated pseudotyped viruses were inhibited at 0.1mgml, 0.12mg/ml, 0.1mg/ml and 0.25mg/ml
286 respectively (Fig.4b). Most importantly, Green tea CWE showed more inhibitory activity on
287 pseudotyped coronaviruses in comparison to Spirulina extract.

288 To understand whether the inhibitory mechanism of Spirulina and green tea extracts are mediated
289 through inhibition of the interaction of spike and its receptor, we performed an S1 binding assay
290 on HEK293T cells expressing either ACE2 or DPP4. Cells were preincubated with the extracts
291 followed by the addition of S1 spike proteins at 4ºC. Binding assay of spike (S1-hFc) proteins was
292 analyzed by immunostaining followed by confocal microscopy. Similarly, we incubated the spike
293 S1 proteins with the extracts and then the mixture was added on the ACE2 or DPP4 cells. Cells
294 treated with either of the extracts did not inhibit the interaction of S1 and its receptor ACE2 or

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295 DPP4 (Fig.5a). In contrast, all three S1 proteins incubated with the extracts of green tea is unable
296 to bind to its receptor whereas Spirulina treated S1 proteins did not inhibit S1 -ACE2 or DPP4
297 interaction (Fig.5b) suggesting that the Spirulina extract blocks the viral entry through an unknown
298 mechanism. However, green tea extract binds to the S1 domain of the spike and prevents the spike
299 receptor interaction. Further studies with live viruses are required to elucidate the exact
300 mechanism of antiviral action.
301
302 **Discussion**

303 The recent emergence of coronaviruses has caused serious global threats initiating diverse
304 approaches for biocontainment of these pathogens. Most of these emerging viruses are highly
305 pathogenic with limited therapeutic strategies and require BSL3/BSL4 facilities to handle these
306 pathogens. The ongoing pandemic of SARS CoV-2 which has spread across the world, is a notable
307 example that elucidates the need of diagnostics and robotic screening systems for identification of
308 antiviral compounds. However, the limited high-level biosafety equipped laboratories particularly
309 in developing countries inculcate the need for adapting to alternative approaches which can lead
310 to the handling of the viruses in a BSL2 facility. Here we report the production of VSV
311 pseudotyped virus particles for SARS CoV-2, SARS CoV, and MERS CoV. The developed
312 pseudoviruses were susceptible for infection in Vero E6 cells and other cells. We obtained a high
313 titer for SARS and MERS CoVpv whereas SARS CoV-2pv had a less titer which could be due to
314 less packaging efficiency in HEK293T cells which was consistent with the previous report [31].
315 Pseudovirus production in Vero E6 cells [31] and expression of TMPRSS2 might enhance
316 pseudovirus production and infection [33]. The developed pseudotyped viruses could be efficiently
317 neutralized by specific polyclonal antibodies. SARS specific polyclonal neutralizes SARSpv and

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318 SARS-2pv as shown elsewhere [35] whereas MERS polyclonal antibodies neutralized MERSpv
319 [36]. Therefore, pvs are useful tools for studying virus neutralization assay for highly pathogenic
320 viruses in BSL2 facility [9]. Next, we showed that pseudoviruses specifically bind its receptor and
321 enter into host cells. Previous studies report that pvs can be used to understand the entry of
322 pathogenic viruses including coronaviruses [33].
323

324 The inhibition of viral entry is a key target for the development of antivirals. Medicinal plants and
325 natural compounds have been reported to have a wide range of antiviral activities. Among these,
326 Spirulina and Green tea extracts have been reported to inhibit attachment of enveloped viruses
327 such as HCV, HIV, and Influenza virus [19], [21]. In our first study, we show that VSV is inhibited
328 by the cold-water extract of Spirulina and Green tea. In the case of green tea consistent with
329 previous studies, VSV entry is inhibited whereas cold water extracted Spirulina could inhibit the
330 entry of VSV. However, hot water extract of Spirulina could not inhibit VSV entry [38] which
331 might be due to the degradation of the major active compounds of Spirulina. but not hot water
332 extract. Next, we tested the antiviral activity of Spirulina and Green tea cold water extract on
333 pseudotyped coronaviruses. Treatment of cells with the extract did not inhibit viral entry whereas
334 preincubation of pseudoviruses with the extracts inhibited viral entry on Vero E6 cells suggesting
335 that the active compounds from Spirulina and green tea binds on the virus glycoprotein and block
336 the virus entry. To further understand the exact inhibitory mechanism, we performed an S1 binding
337 assay on DPP4 or ACE2 expressing cells using a mixture of S1 proteins preincubated with the
338 Spirulina and green tea extracts. Interestingly, all three S1 proteins treated with green tea abrogate
339 the interaction of spike with its receptor whereas Spirulina treated S1 proteins could bind to its
340 cellular receptor. The observed results suggest that green tea binds to the spike S1 domain and

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341 inhibits the viral entry. However, we speculate that EGCG, EGC, ECG, or EC, the most active
342 components in Green tea might be inhibiting the coronaviral entry not only binding exclusively to
343 S1 but also other domains of the viral envelope glycoprotein. These compounds are reported to
344 have antiviral entry inhibitory activity against different viruses [19]. On the other hand, the exact
345 mechanism of action of Spirulina extract is not understood. The major components in Spirulina
346 extract, sulfated polysaccharides and Spirulan like substances, might bind to enveloped
347 glycoproteins of CoVs and play a role in the inhibition of coronavirus entry. Further research
348 using the active compounds and live viruses is essential to understand the mechanism of action of
349 these extracts. In summary, we have successfully developed pseudotyped viruses for SARS-
350 SARS-2 and MERS CoV and demonstrated their applications including pseudovirus
351 neutralization, virus entry and most importantly, screening for identification of antiviral
352 compounds against viral entry. Moreover, natural products could be promising antiviral agents
353 for emerging viruses.

354 **Acknowledgments**

355 We thank Dr. Bart L. Haagmans and Mart M. Lamers, Erasmus MC, Netherlands for providing
356 SARS and MERS CoV polyclonal antibodies and cDNA. VSVΔG/GFP pseudovirus was received
357 from Yoshiharu Matsuura, Osaka University, Japan.We are grateful to the Central Instrumentation
358 Facility provided by IISER TVM and Mr Alex Andrews for TEM assistance.

359 This study was approved by institute biosafety committee (IBSC) No. BT/BS/17/447/2011-PID

360 **Fundings**

361 The study was supported by Indian Institute of Science Education and Research
362 Thiruvananthapuram (IISER TVM). J.J acknowledge financial support from Inspire PhD

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363 fellowship by Department of Science and Technology (DST). K.T acknowledges IISER TVM
364 IPhD fellowship financial support. A.A and V.R.AD acknowledge University Grants Commission
365 (UGC) and Council of Scientific and Industrial Research (CSIR) PhD fellowships respectively.

366 **Author's contributions**

367 V.S.R. designed and coordinated the study. J.J., K.T., A.A., and V.R.AD conducted the
368 experiments. All authors contributed to the interpretations and conclusions presented. J.J, K.T and
369 V.S.R. wrote the manuscript.

370

371 **Competing interests**

372 The authors declare that they have no competing interests.

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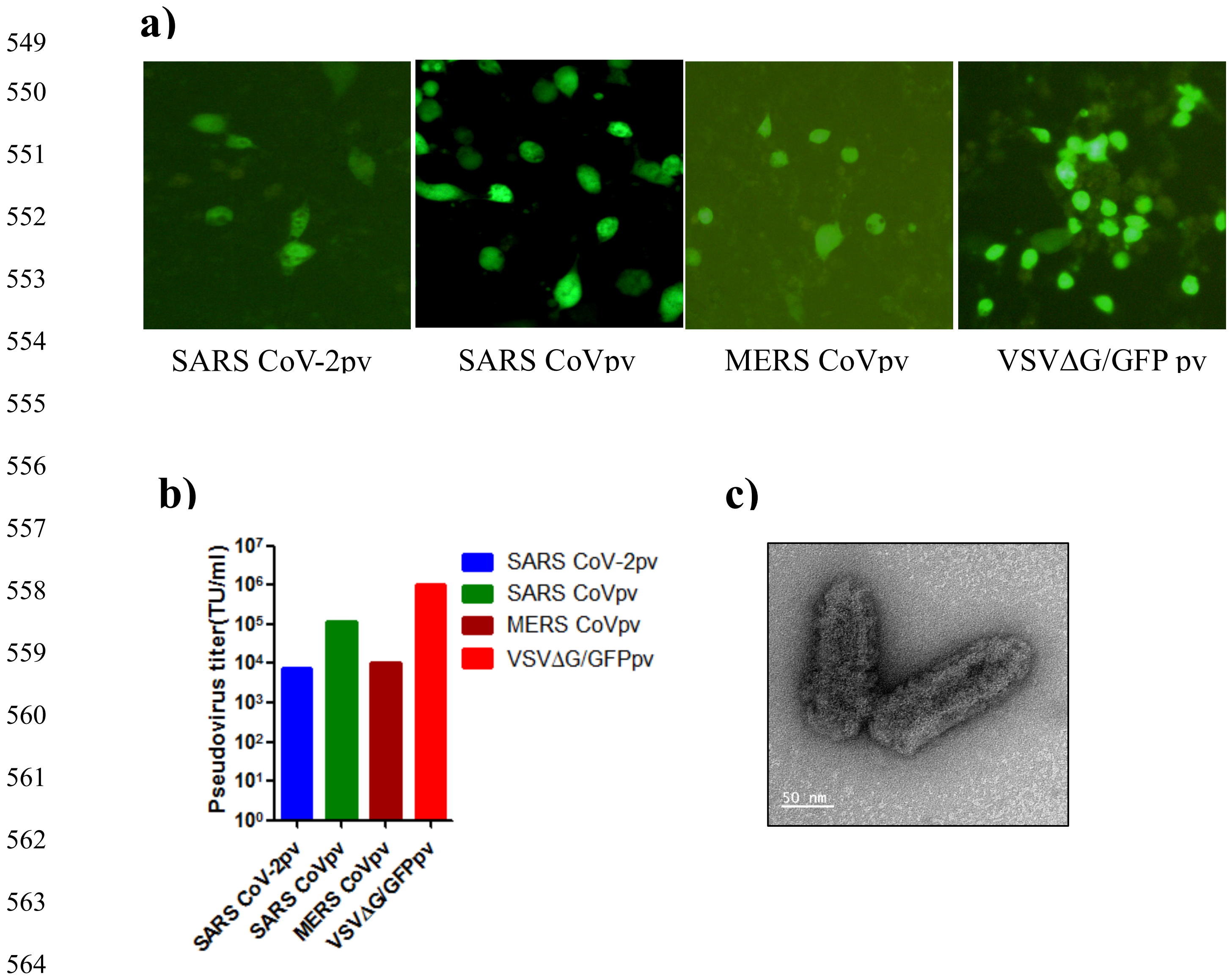

565 **Figure 1: Generation of VSV pseudotyped coronaviruses.**

566 **a)** Infection of SARSpv, SARS-2pv, MERSpv and VSVpv in Vero E6 cells. GFP expression shows
567 the infected cells. A single GFP positive cell corresponds to a single pv infection (200x zoomed
568 image). **b)** Serially diluted pseudotyped viruses were titrated on Vero E6 and Huh7 cells. The
569 lowest dilution showing GFP positive cells were calculated and viral titer is represented as
570 Infection units /ml (IU/ml). **c)**. Presence of intact pseudotyped viruses were aanalsyed by negative
571 staining and transmission electron microscopy which depicted pseudovirus particles of size
572 200nm. Representative image of pseudotyped SARS CoVpv.

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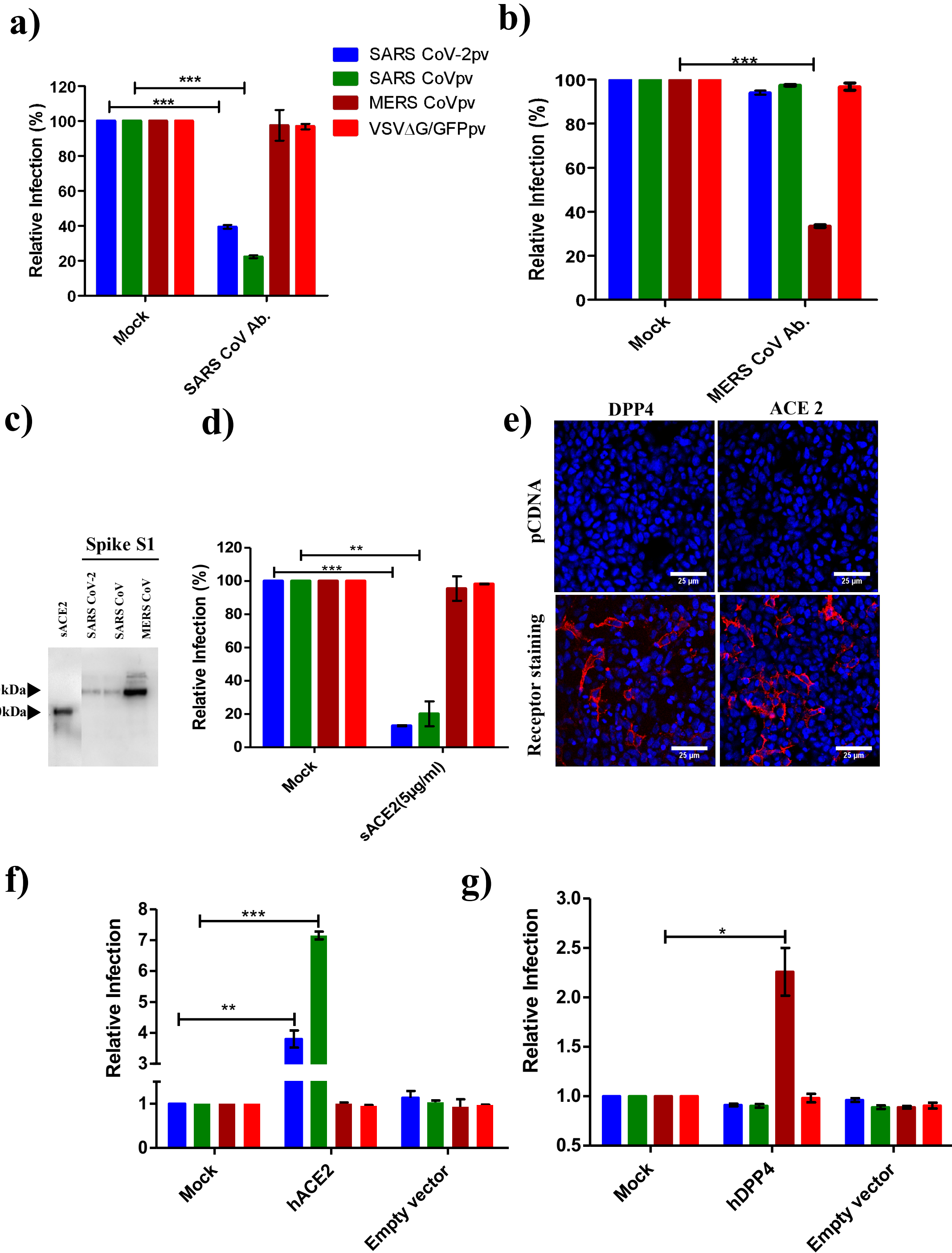

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587 **Figure 2: Antibody neutralization and receptor specificity of the developed pseudoviruses.**
588 Maturation of the developed pseudoviruses were assessed neutralization assay with **a**) SARS CoV
589 polyclonal antibody. Significant reduction in infection was observed in SARS and SARS -2pv **b)**
590 MERS CoV polyclonal antibodies and significant reduction was observed only in MERSpv but
591 not in others. **c)** 6X HIS- tagged recombinant sACE2 was expressed in Hek293T cells and was
592 purified by Ni-NTA based IMAC purification. The purified protein was confirmed by western
593 blotting using anti-ACE2 antibodies to detect a band of ≈90KDa. Similarly, SARS-, SARS-2- and
594 MERS -CoV spike S1 fused with Human Fc, the recombinant protein was produced and purified
595 with protein A sepharose beads. Approximately, 140kDa protein was purified and was confirmed
596 by western blotting using Goat anti human IgG HRP conjugated antibody. **d)** In order to validate
597 the specific interaction of the pseudotyped particles on mammalian cells, soluble ACE2 (sACE2)
598 was incubated with pvs followed by pv infection on Vero E6 cells**. e)** Surface expression of either
599 ACE2 or DPP4 on BHK21 cells were analyzed by immunostaining using Goat anti-ACE2 or goat
600 Anti-DPP4 antibody. Non permissive BHK21 cells were transiently expressed with **f)** ACE2 or **g)**
601 DPP4 followed by pvs infection. SARSpv and SARS-2pv were susceptible in ACE2 expressed
602 cells and MERSpv was susceptible in DPP4 expressed cells. Data shown is relative normalized
603 infection and error bar represents SD. Unpaired t test was performed for statistical analysis. P
604 values, $P<0.05$ =* $P<0.01$=** and $P<0.001$=***
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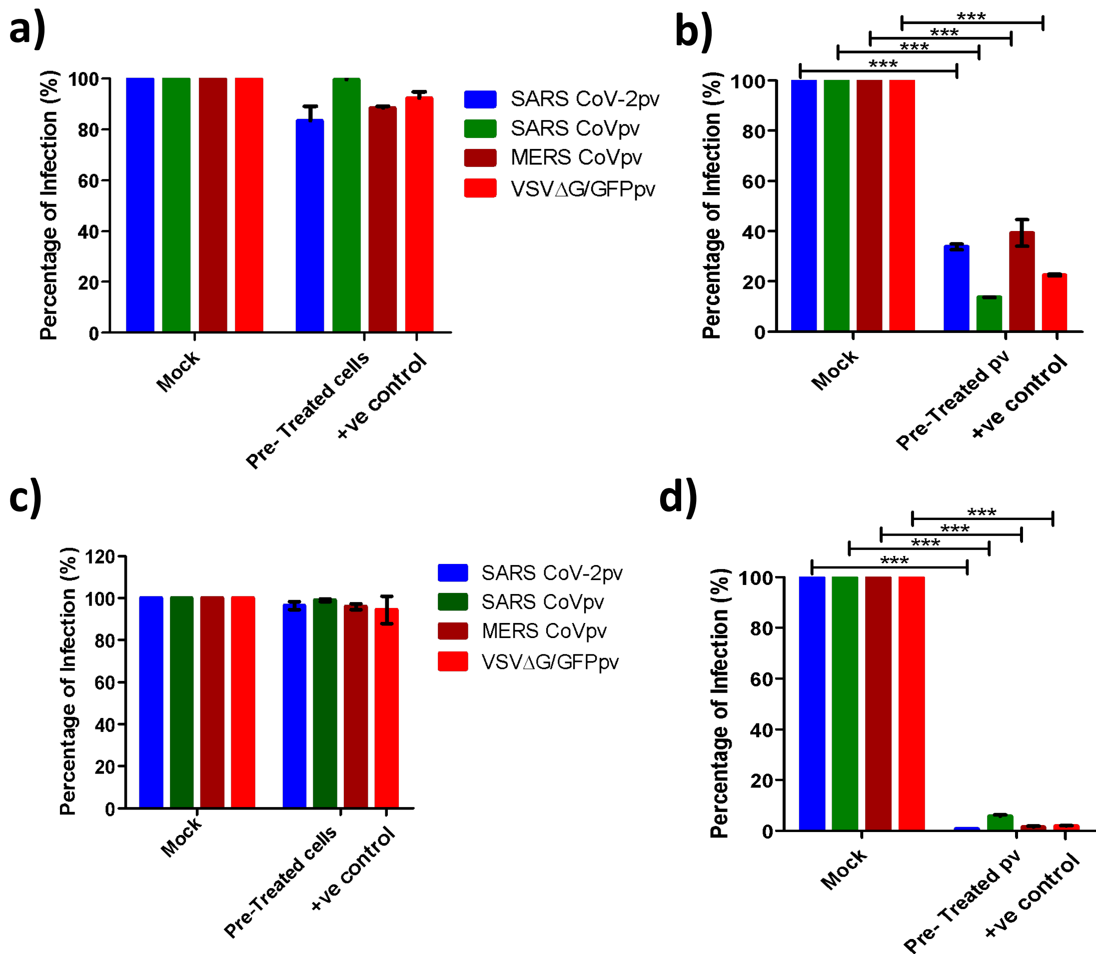

625 **Figure3: The cold water extract of Spirulina and Green Tea inhibits viral entry in a broad**
626 **range of coronaviruses *in vitro.* a and c) Monolayer** of Vero E6 cells were incubated either
627 Spirulina or green tea extract (0.2mg/ml) followed by pvs infection. **b** and **d)** pseudoviruses were
628 pre-incubated with Spirulina extract or green tea extract (0.2mg/ml) and then the mixture was
629 directly incubated on Vero E6 cells. Data represent the mean ± SD of an experiment with
630 duplicates. The experiment was repeated twice. Statistical significance was determined by
631 unpaired t test. P value, P<0.001 =***

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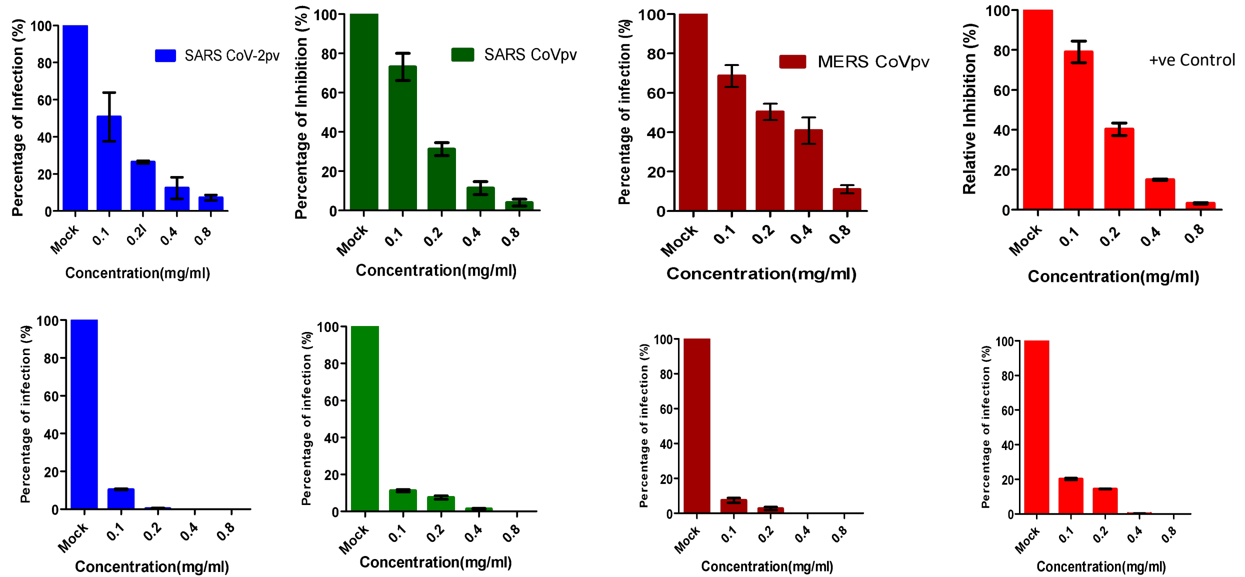

643 **Figure 4: Concentration dependent inhibition of peusotyped viruses by *Spirulina* and**
644 **green tea extracts.** Different concentrations (0.1mg/ml -0/8 mg/ml) of Spirulina and green tea
645 extracts were tested for the antiviral activity. **a)** represents Spirulina treated pseudotyped
646 coronaviruses and **b)** depicts green tea treatment followed by infection assay. Blue bar represents
647 - SARS-2pv, green-SARSpv, brown -MERSpv and red -VSVpv. Data represent the mean ±SD of
648 an experiment with duplicates. The experiment was repeated twice.

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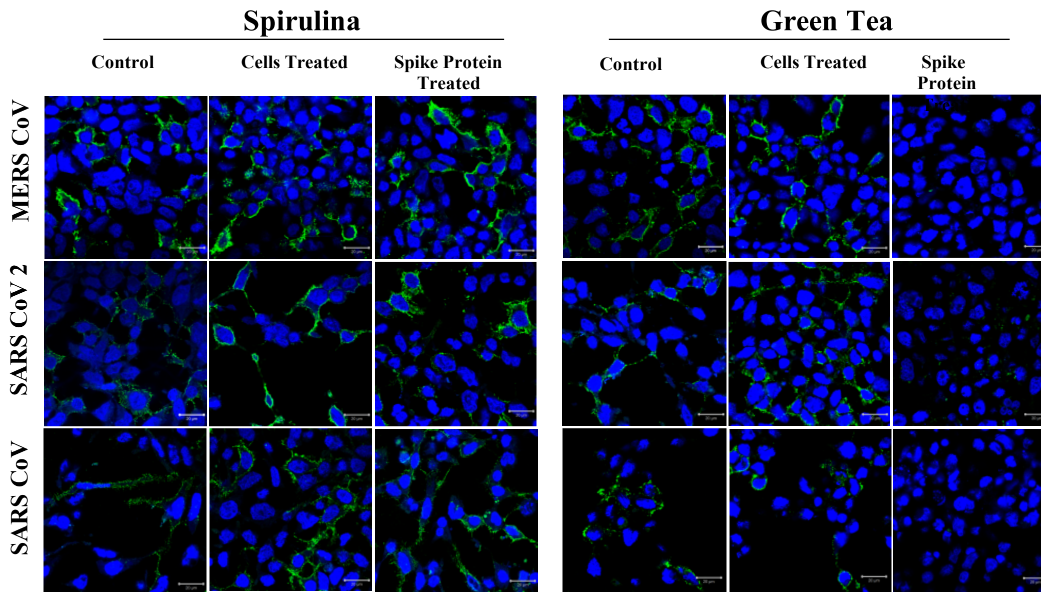

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661 **Figure 5: Pre-treatment of spike proteins with green tea extract inhibited S1-receptor**
662 **interaction. a, b**) DPP4 and ACE 2 plasmids were transiently transfected in HEK293T cells. 24
663 h post-transfection cells were independently incubated with PBS for 30 minutes followed by
664 spike S1-fc protein (both left panels), spirulina and green tea extracts (0.4mg/ml) followed by
665 S1-Fc protein (middle panels) and preincubated mixture of S1-Fc and Spirulina or green tea
666 extract (right panels). Binding of S1-Fc was immunostained by Goat anti-human IgG conjugated
667 with FITC and the binding of S1- Fc proteins were visualised by confocal microscopy. Green-
668 IgG -FITC and Blue- DAPI. Scale bar 20µm.

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677 **Table 1. List of antiviral activity of naturally available plant and seaweed extracts**
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Source	Target viruses	Mode of action	References
Olive leaves	Viral hemorrhagic septicemia rhabdovirus (VHSV), Hepatitis B virus (HBV) and Human Immunodeficiency Virus (HIV)	Cell to cell fusion, Secretion of HBV antigens, Fusion and integrase inhibition	[39]–[41]
Licorice Root	SARS-CoV, Human Respiratory Syncytial Virus (HRSV) HIV	Viral entry and replication	[42]–[44]
Gigartina Skottsbergii	Human Herpesvirus 1 and 2(HHV 1 and 2) Herpes simplex virus type 1&2(HSV-1 and 2)	Cell surface attachment and replication	[38]
Spirulina	HIV-1 Herpes simplex virus type 1 and 2 (HSV-1 and 2) Vesicular stomatitis virus (VSV) Influenza virus	Viral Entry, Replication, Cell membrane fusion Hemagglutination,	[21], [45], [46]
Green Tea	HIV-1 HSV-1,2 Influenza virus) Hepatitis B and C virus (HBV, HCV) Dengue virus (DENV) Zika virus (ZIKV)	Reverse transcriptase inhibition Cell surface attachment Neuraminidase activity inhibition Interference in replication and transcription Viral entry Viral particle destruction	[19], [26], [47], [48]

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