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3 ***Prunella vulgaris* extract and suramin block SARS-coronavirus 2 virus Spike**
4 **protein D614 and G614 variants mediated receptor association and virus entry**
5 **in cell culture system**

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35

1 **Abstract**

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3 Until now, no approved effective vaccine and antiviral therapeutic are available for
4 treatment or prevention of SARS-coronavirus 2 (SCoV-2) virus infection. In this study,
5 we established a SCoV-2 Spike glycoprotein (SP), including a SP mutant D614G,
6 pseudotyped HIV-1-based vector system and tested their ability to infect
7 ACE2-expressing cells. This study revealed that a C-terminal 17 amino acid deletion
8 in SCoV-2 SP significantly increases the incorporation of SP into the pseudotyped
9 viruses and enhanced its infectivity, which may be helpful in the design of
10 SCoV2-SP-based vaccine strategies. Moreover, based on this system, we have
11 demonstrated that an aqueous extract from the Chinese herb *Prunella vulgaris*
12 (CHPV) and a compound, suramin, displayed potent inhibitory effects on both wild
13 type and mutant (G614) SCoV-2 SP pseudotyped virus (SCoV-2-SP-PVs)-mediated
14 infection. The 50% inhibitory concentration (IC₅₀) for CHPV and suramin on
15 SCoV-2-SP-PVs are 30, and 40 µg/ml, respectively. To define the mechanisms of
16 their actions, we demonstrated that both CHPV and suramin are able to directly
17 interrupt SCoV-2–SP binding to its receptor ACE2 and block the viral entry step.
18 Importantly, our results also showed that CHPV or suramin can efficiently reduce
19 levels of cytopathic effect caused by SARS-CoV-2 virus
20 (hCoV-19/Canada/ON-VIDO-01/2020) infection in Vero cells. Furthermore, our
21 results demonstrated that the combination of CHPV/suramin with an
22 anti-SARS-CoV-2 neutralizing antibody mediated more potent blocking effect against
23 SCoV2-SP-PVs. Overall, this study provides evidence that CHPV and suramin has
24 anti-SARS-CoV-2 activity and may be developed as a novel antiviral approach
25 against SARS-CoV-2 infection.

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28 **Keywords:** SARS-CoV-2, spike glycoprotein (SP), pseudovirus, Chinese herb
29 *Prunella vulgaris*, Suramin.

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1 Introduction

2 The recent and ongoing outbreak of Coronavirus disease 2019 (COVID-19) has
3 called for serious and urgent global attention [1,2]. The COVID-19 disease is caused
4 by a newly emerged virus strain of Severe Acute Respiratory Syndrome (SARS)
5 known as SARS-CoV-2 [3]. Although the case fatality ratio (CFR) of COVID-19 can
6 only be detected at the end of the outbreak, an estimated global CFR was calculated
7 to be 5.5-5.7% in March 2020, which is shockingly more than seasonal influenza
8 outbreak [4]. While in August 2020, the infection fatality ratio was estimated by WHO
9 to be 0.5-1% [5]. Since the identification of the SARS-CoV-2 sequences [6], extensive
10 efforts worldwide have been focused on the development of vaccine and antiviral
11 drugs against SARS-CoV-2 with the hope to rapidly and efficiently control this new
12 human coronavirus (CoV) infection.

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14 SARS-CoV-2 belongs to a betacoronavirus subfamily that includes enveloped, large
15 and positive-stranded RNA viruses responsible for causing severe respiratory system,
16 gastrointestinal and neurological symptoms [7-10]. The human CoV was first
17 identified in 1960 and constituted about 30% of the causes of the common cold.
18 Among the identified human CoVs are NL63, 229E, OC43, HKU1, SARS-CoV, the
19 Middle East respiratory syndrome (MERS)-CoV, and SARS-CoV-2 [11,12]. A recent
20 study has revealed that SARS-CoV-2 was closely related (88% identity) to two
21 SARS-like CoVs that were isolated from bats in 2018 in China, but it was less related
22 to SARS-CoV (79%) and MERS-CoV (about 50%) [13]. The key determinant for the
23 infectivity of SARS-CoV-2 depends on the host specificity with the viral
24 surface-located trimeric spike (S) glycoprotein (SP), which is commonly cleaved by
25 host proteases into an N-terminal S1 subunit and a membrane-embedded C-terminal
26 S2 region [14]. Recent studies revealed that a SP mutation, Aspartic acid (D)
27 changed to Glycine (G) at amino acid position 614, in the S1 domain has been found
28 in high frequency (65% to 70%) in April to May of 2020, that was associated with an
29 increased viral load and significantly higher transmission rate in infected individuals,
30 but no significant change with disease severity [15]. Following studies also suggested
31 that G614 SP mutant pseudotyped retroviruses infected ACE2-expressing cells
32 markedly more efficiently than those with D614 SP [16].

33

34 Up till now, several compounds have been tested in numerous clinical trials, including
35 remdesivir, lopinavir, **umifenovir**, and **hydroxychloroquine** [17-21]. Moreover, some *in*
36 *vitro* research suggested that other drugs such as fusion peptide (EK1),
37 anti-inflammatory drugs (such as hormones and other molecules) could be potentially
38 used in the treatment of SARS-CoV-2 disease (reviewed in [22,23]). However, their
39 safety and efficacy have not been confirmed by clinical trials. Currently, specific
40 antiviral treatment drugs are still not available for SARS-CoV-2 infections [23].

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42 Traditional Chinese medicine holds a unique position among all conventional
43 medicines because of its usage over more than hundreds of years of history. Many
44 aqueous extracts of traditional Chinese medicinal herbs have been proven to have

1 antiviral activities [24], and most of these are generally of low toxicity, cheap and
2 readily accessible. As an easily accessible and low-cost natural source, they are
3 especially valuable as potential new sources for rapid responses against the ongoing
4 COVID-19 pandemic. *Prunella vulgaris*, widely distributed in China, Europe,
5 northwestern Africa and North America, is known as a self-heal herb and studies
6 have previously found that a water-soluble substance from Chinese Herb *Prunella*
7 *vulgaris* (CHPV) exhibit significant antiviral activity against HIV, HSV and Ebola virus
8 [25-28]. However, whether CHPV can block SARS-CoV-2 virus infection is unknown.
9 Another compound, Suramin, has also been previously shown to be a potent inhibitor
10 against HIV [29], while the subsequent studies revealed that its inhibitory effects on
11 HIV replication did not correlate with clinical or immunologic improvement [30,31]. A
12 previous study observed that suramin not only substantially reduced viral loads of
13 *chikungunya virus* (CHIKV) in infected mice, but it also ameliorated virus-induced foot
14 lesions in the mice [32]. Recently, Salgado-Benvindo C., *et al.*, reported that Suramin
15 is able to inhibit SARS-CoV-2 infection in cell culture by interfering with early steps of
16 the replication cycle [33].

17

18 In this study, we have established a highly sensitive SARS-CoV-2 SP-pseudotyped
19 virus (SCoV-2 SP-PVs) system and investigated the impact of the cytoplasmic tail
20 and a G614 mutant of SP on virus entry ability. We also examined two compounds,
21 CHPV and suramin, for their blocking activities in the SCoV-2 SP-PVs system and
22 SARS-CoV-2 infection, and the antiviral mechanism of their actions. Furthermore, we
23 investigated the synergistic effect of combining anti-SARS-CoV-2 neutralizing
24 antibody (nAb) with CHPV or suramin to enhance their anti-SARS-CoV-2 activity.
25 Overall, this study provides evidence for the first time that CHPV, an aqueous extract
26 from *Prunella vulgaris*, has potent anti- SARS-CoV-2 activity.

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28

1 **Materials and methods**

2 **Plasmid constructs**

3 The SARS-CoV-2 expressing plasmids (pCAGGS-nCoVSP, pCAGGS-nCoVSP Δ C
4 and pCAGGS-nCoVSP Δ C_{G614}) containing SARS-CoV-2 SP transgene (GenBank
5 accession No. MN908947) or corresponding mutated genes for SP Δ C and Δ C_{G614}.
6 The SP Δ C and Δ C_{G614}, were generated by mutagenic PCR technique. Primers are
7 following: SP Δ C-3'primer, 5_GCAGGTACCTAGAATTTGCAGCAGGATCCAC; D614G-5',
8 5_GCTGTTCTTTATCAGGGTGTTAACTGCACAG; D614G-3',
9 5_CTGTGCAGTTAACACCCTGATAAAGAACAGC. Mutated genes were cloned into the
10 pCAGGS plasmid and each mutation was confirmed by sequencing. The HIV vector
11 encoding for Gaussia luciferase gene HIV-1 RT/IN/Env tri-defective proviral plasmid
12 (Δ RI/E/Gluc) and the helper packaging plasmid pCMV Δ 8.2 encoding for the HIV
13 Gag-Pol plasmids are described previously [26,34].

14

15 **Cell culture, antibodies and chemicals**

16 The human embryonic kidney cells (HEK293T) and kidney epithelial cells (VeroE6
17 and Vero cells (ATCC, CCL-81)) from African green monkey were cultured in
18 Dulbecco's modified Eagle's medium (HEK293T, VeroE6) or Minimum Essential
19 Medium (MEM; Vero). HEK293T expressing ACE2 (293T_{ACE2}) was obtained from
20 GeneCopoeia Inc, Rockville, MD. All cell lines were supplemented with 10% fetal
21 bovine serum (FBS), 1X L-Glutamine and 1% penicillin and streptomycin. The rabbit
22 polyclonal antibody against SARS-CoV-2 SP (Cat# 40150-R007) and ACE2 protein
23 (Cat# 40592-T62) were obtained from Sino Biological and anti-HIVp24 monoclonal
24 antibody was described previously [35,36]. The HIV-1 p24 ELISA Kit was obtained
25 from the AIDS Vaccine Program of the Frederick Cancer Research and Development
26 Center. SARS-CoV-2 SP-ACE2 binding ELISA kit (Cat# COV-SACE2-1) was
27 purchased from RayBio. Anti-SARS-CoV-2 neutralizing Antibody (nAb) Human
28 IgG1(SAD-535) was purchased from ACRO Biosystems..

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30 **Preparation and purification of herb extracts of *P. vulgaris* L (CHPV)**

31 The dried fruitspikes of *P. vulgaris* L. (Labiatae) (Fig. 3A) were first soaked overnight
32 in deionized water at room temperature and then boiled for one hour. Then the cooled
33 supernatant was centrifuged (3000 g, 30 min), filtered through a 0.45 μ m cellulose
34 acetate membrane and finally lyophilized, as described previously [25]. The resulting
35 dark brown residue was dissolved in deionized water and stored at -20°C. A single
36 symmetrical peak corresponding to a molecular weight of polysaccharides
37 (approximately 10 kDa) in the aqueous extract from PV was detected by HPLC
38 analysis, as described previously [25]. Suramin (Cat# sc-200833) was purchased
39 from Santa Cruz BioTech and was dissolved in sterile H₂O and stored at -20°.

40

41 **Virus production, infection and inhibition experiments**

42 SARS-CoV-2 SP or SP Δ C pseudotyped viruses (SCoV-2-SP-PVs,
43 SCoV-2-SP Δ C-PVs, SCoV-2-SP Δ C_{G614}-PVs) were produced by transfecting
44 HEK293T cells with pCAGGS-SARS-CoV-2-SP, pCAGGS-SARS-CoV-2-SP Δ C, or

1 pCAGGS-SARS-CoV-2-SP Δ C_{G614}, pCMV Δ 8.2 and a Gluc expressing HIV vector
2 Δ RI/E/Gluc. After 48 hrs of transfection, cell culture supernatants were collected and
3 pseudotyped VLPs were purified from the supernatant by ultracentrifugation (32,000
4 rpm) for 2 hrs. The pelleted VPs were resuspended into RPMI medium and virus titers
5 were quantified using an HIV-1 p24 ELISA assay. The wild type SARS-CoV-2
6 (hCoV-19/Canada/ON-VIDO-01/2020, GISAID accession# EPI_ISL_425177) was
7 propagated and produced in Vero cells (ATCC, CCL-81).

8
9 To investigate the infection ability of SCoV-2-SP-VLPs, the same amount of each
10 SCoV-2-SP-PV stock (as adjusted by p24 levels) were used to infect different target
11 cells at 0.4×10^5 cells per well (24 well plate) for 3 hrs and washed. After 48 or 72 hrs,
12 the supernatants were collected and the viral infection rate was evaluated by
13 measuring Gaussia luciferase (Gluc) activity. Briefly, 50ul of Coelenterazine substrate
14 (Nanolight Technology) was added to 20ul of supernatant, mixed well and read in the
15 luminometer (Promega, USA).

16
17 To evaluate the anti-SARS-CoV-2 SP-mediated entry activity of CHPV or suramin,
18 various concentrations of herb extract or compound were directly added into target
19 cells at different time points before or after infection, as indicated. After 3hrs of
20 infection at 37°C, the cells were washed once to remove excessive residue
21 viruses/compound and cultured in fresh medium. The anti-SARS-CoV-2 effects of
22 CHPV or suramin were evaluated by measuring the Gluc activity or p24 levels in the
23 supernatant infected cultures.

24
25 Efficacy of CHPV or suramin against SARS-CoV-2 (hCoV-
26 19/Canada/ON-VIDO-01/2020, GISAID accession# EPI_ISL_425177) was evaluated
27 in Vero cells. The Vero cells were seeded into 96-well plates and reached a
28 confluency of 90% at the second day. Then each compound was diluted in assay
29 medium (MEM with 1X penicillin-streptomycin) and added to the wells (100 ul/well),
30 followed by adding 100 μ L of SARS-CoV-2 at a MOI of 0.01, resulting in a final 1X
31 drug concentrations. As positive controls, wells without drugs were infected with
32 SARS-CoV-2 at the same MOI. Cells were maintained for 72 hrs and then, virus
33 infection induced cytopathic effect (CPE) was monitored in each well.

34 35 **Binding Assay**

36 The inhibitory effect of CHPV or suramin on the interaction of SP-ACE2 was tested
37 with COVID-19 Spike-ACE2 binding assay kit. Briefly, 96-well plate was coated with
38 recombinant SARS-CoV-2 Spike protein. CHPV or suramin was then added to the
39 wells for 10 min followed by adding recombinant human ACE2 protein. After
40 incubation for 3 hours, wells were washed three times and a goat anti-ACE2 antibody
41 that binds to the Spike-ACE2 complex was added followed by applying the
42 HRP-conjugated anti-goat IgG and 3,3',5,5'-tetramethylbenzidine (TMB) substrate.
43 The intensity of the yellow color is then measured at 450 nm.

44

1 **Western blot (WB) analyses**

2 To detect cellular protein ACE2, SARS-CoV-2-SP, or SP Δ C in transfected cells or
3 SCoV-2-SP-VPs, transfected 293T_{ACE2} cells or VPs were lysed in RIPA buffer, and
4 directly loaded into the 10 % SDS-PAGE gel and the presence of each protein was
5 detected by WB with various corresponding antibodies.

6

1

2 **Results**

3 **Generation of a SARS-CoV-2 SP-pseudotyped HIV-1-based entry system**

4 We first established a sensitive SARS-CoV-2-SP-mediated virus entry system by
5 co-transfecting SARS-CoV-2 SP, a HIV-based vector (Δ RI/ Δ Env/Gluc) in which viral
6 reverse transcriptase/integrase deleted/envelope gene partially deleted and encoded
7 a Gaussia luciferase gene in the *nef* position [34], and a packaging plasmid
8 (pCMV Δ R8.2) in HEK293T cells (Fig. 1B). The Gaussia luciferase (Gluc) is a
9 bioluminescent enzyme that can be secreted into the media, enabling the analysis of
10 viral expression by direct measurement of Gluc activity in the supernatant. To do this,
11 we have constructed a full length SP (SARS-CoV-2-SP) and the C-terminal 17 amino
12 acid (aa) deletion SP (SARS-CoV-2-SP Δ C) expressing plasmids since previous
13 studies have reported that a carboxyl-terminal truncation of 17 amino acids of SARS
14 SP substantially increased SARS SP-mediated cell-to-cell fusion [37].

15

16 To examine the expression and incorporation of SARS-CoV-2 SPs and SP Δ C in the
17 cells and the SARS-COV-2-SP pseudotyped viruses (SP-PVs) and
18 SARS-COV-2-SP Δ C pseudotyped viruses (SP Δ C-PVs), lysates of both
19 virus-producing cells and pseudotyped viruses were analyzed by SDS-PAGE and WB
20 with a mouse anti-SP antibody, as indicated in Fig. 1C. As expected, the HIV capsid
21 Gagp24 protein was detected in all of the cell lysates and the pelleted SP-PVs and
22 SP Δ C-PVs pseudoviruses (PVs) by rabbit anti-p24 antibodies (Fig. 1C, lower panel).
23 The SARS-CoV-2 SP including S1/S2 were clearly detected in both
24 SARS-CoV-2-SPs and SARS-CoV-2-SP Δ C-transfected cells (Fig. 1C, lane 1).
25 Interestingly our results revealed that virus-incorporation level of SARS-CoV-2-SP Δ C
26 were significantly higher than that of SARS-CoV-2-SP (Fig. 1C, compare lane 4 to 3),

27

28 To test the infectivity of generated pseudoviruses, we infected 293T-ACE2 cells with
29 serial diluted amounts of pseudoviruses (25, 12.5, 6.25ng of p24) of SP-PVs or
30 SP Δ C-PVs for 3 hrs. The Gluc activities or Gagp24 of supernatants from infected
31 cells were measured at 24h, 48h or 72h post infection. The results showed that both
32 SP-PVs and SP Δ C-PVs can infect 293T-ACE2 cells and induce an increase of Gluc
33 activity in the supernatants in a dose dependent manner (Fig.1D, left panel). As
34 expected, the infectivity of SP Δ C-PVs was significantly higher than that of SP-PVs.
35 The infection of pseudoviruses in 293T_{ACE2} cells was further confirmed by detection of
36 the HIVp24 levels in the supernatants of infected cells through ELISA assay (Fig.1D,
37 right panel).

38

39 To test whether the infection is ACE2-dependent, we infected various cell lines,
40 including HEK293T, 293T_{ACE2} and VeroE6 with SP-PVs and SP Δ C-PVs, respectively.
41 The results showed that these pseudoviruses were only able to efficiently infect
42 293T_{ACE2} cells, and not HEK293T or VeroE6 cells (Fig. 2A). In parallel, we only
43 detected high level expression of the SARS-CoV-2 receptor ACE2 in 293T_{ACE2} cells,
44 but not in the 293T or Vero E6 cells (Fig. 2B).

1 In addition, we have generated a GFP⁺ SARS-CoV-2-SP-mediated virus entry system
2 by cotransfecting SARS-CoV-2 SPΔC_{G614}, a lentiviral vector that expressing GFP,
3 and the pCMVΔR8.2 in HEK293T cells and produced SPΔC_{G614}-PVs expressing GFP
4 (GFP⁺ SPΔC_{G614}-PVs). After 293T_{ACE2} cells were infected with GFP⁺ SPΔC_{G614}-PVs,
5 the GFP positive 293T_{ACE2} cells were clearly detected under fluorescent microscopy
6 (Fig. 2C)

7 8 **2. SARS-CoV-2 SP G614 variant exhibited stronger receptor association and** 9 **virus entry.**

10 Recent sequence analyses revealed a SP mutation, Aspartic acid (D) changed to
11 Glycine (G) at aa position 614, was found in high frequency (65% to 70%) in April to
12 May of 2020, indicating a transmission advantage to D614 [15]. In this study, we
13 have also generated constructs to express SCoV-2-SPΔC_{G614} (Fig. 1A,c) and
14 compared its virus entry ability with SCoV-2-SPΔC (SPΔC_{D614}). Our results showed
15 that SCoV-2-SPΔC_{G614} was incorporated into pseudotyped viruses similar to
16 SCoV-2-SPΔC_{D614} (Fig. 2D, compare lane 5 to lane 4). However, the
17 SARS-CoV2-SPΔC_{G614}-pseudotyped viral particles (SPΔC_{G614}-PVs) mediated
18 approximately 3-fold higher infection than that of SPΔC_{D614}-PVs (Fig. 2E), suggesting
19 that the SP_{G614} mutation increases SP-mediated viral entry.

20

21 **Evaluation of CHPV and Suramin for blocking SARS-CoV2-SP-mediated virus** 22 **entry**

23 Next we tested whether CHPV (Fig. 3A) and suramin could block SARS-CoV2
24 SP-mediated virus entry of 293T_{ACE2} cells. Briefly, 293T_{ACE2} cells were infected
25 with SPΔC-PVs in the presence of different concentrations (25, 50,75, 100 and
26 200ug/ml) of CHPV (Fig. 3B) or suramin (Fig. 3C), respectively. After 3 hour of
27 infection, the infected cells were washed to remove the viruses and compounds and
28 cultured with fresh medium. At 48 hrs post-infection, the supernatants were collected
29 and the virus-produced Gluc activities were measured for monitoring the infection
30 levels. Consistent with our previous observation [26], we did not detect any
31 CHPV-induced toxic effect on the cells for 3hs exposure, nor for Suramin.
32 Significantly, both CHPV and suramin were able to inhibit
33 SARS-CoV-2-SP-pseudotyped virus infection. The half maximal inhibitory
34 concentration (IC₅₀) of CHPV was 30 ug/ml (Fig.3. B, left panel, while IC₅₀ of
35 Suramin was about 40 ug/ml (Fig.3. B, right panel). The inhibitory effect of CHPV
36 and suramin on a SP mutant pseudotyped virus (SPΔC_{G614}-PVs) infection was also
37 tested, and results show that SPΔC_{G614}-PVs infection is also susceptible to CHPV
38 and suramin (Fig. 3C). Furthermore, the SARS-CoV-2-SPΔC_{G614} pseudotyped GFP⁺
39 virus infection was tested in the presence of the two compounds and results showed
40 that the pseudotyped GFP⁺ virus infection was efficiently inhibited by the presence of
41 CHPV and Suramin (Fig. 3D). All of these results demonstrate that both CHPV and
42 suramin exhibit strong inhibitory effect on both SPΔC_{D614}-PVs and SPΔC_{G614}-PVs
43 infection.

44

1 **Mechanistic analyses of actions of CHPV and Suramin against**
2 **SARS-CoV2-SP-mediated virus entry**

3 To gain more insight into the mechanism of how CHPV and Suramin are targeting
4 SARS-CoV-2 SP-PVs infection, each of the drugs (100 µg/ml) was added to 293T_{ACE2}
5 cells at various time points during the infection, as indicated in Figure 4. After 48 hrs
6 of infection, the supernatants were collected and measured for virus-expressed GLuc
7 activity. Results showed that a strong inhibitory effect was achieved when cells
8 were pretreated with CHPV or Suramin one hour before infection or when the
9 compounds were present simultaneously with SP-PVs (Fig. 4A and B). Interestingly,
10 even when drug was added at one hr post-infection, CHPV still exhibited nearly 70%
11 inhibition on SPΔC_{G614}-PVs infection (Fig. 4A), while for Suramin, a lower inhibitory
12 effect (about 30% inhibition) was also observed (Fig. 4B). When CHPV or Suramin
13 was added to culture after 3 hrs of infection, no inhibitory activity on viral infection was
14 observed (Fig. 4A and B). These results suggest that both CHPV and suramin act on
15 the entry step of SPΔC_{G614}-PVs infection.

16

17 To further determine whether CHPV or suramin is targeting the interaction of
18 SARS-CoV2-SP and its receptor, ACE2, , we used an *in vitro* SARS-CoV2-SP/ACE2
19 binding ELISA assay, as described in Materials and Methods. Additionally, an
20 anti-COVID-19 neutralizing antibody (SAD-S35) [38] was included in parallel.
21 Results revealed that the presence of either CHPV or suramin was able to specifically
22 target and significantly reduce the SARS-CoV2-SP-ACE2 interaction (Fig. 4C and D).
23 It should be noted that the neutralizing antibody (SAD-S35) also showed a strong
24 inhibition on SARS-CoV2-SP/ACE2 interaction (Fig. 4C and D).

25

26 **Combination of CHPV and anti-SARS-CoV-2 neutralizing antibody (SAD-S35)**
27 **mediated more potent blocking effect against SARS-CoV2-SP-PVs.**

28 As described above, both CHPV and Suramin can inhibit SARS-CoV2-SP/ACE2
29 interaction and SP-PVs infection. We also tested whether the combination of two
30 compounds could mediate a stronger anti-SARS-CoV-2 activity. Thus, we infected
31 293T_{ACE2} cells with SPΔC_{G614}-PVs in the presence of a cocktail of CHPV/Suramin (25
32 µg/mL per compound), or CHPV (50 µg/mL) or Suramin (50 µg/mL) alone. The results
33 showed that in the presence of a cocktail of CHPV/Suramin, SPΔC_{G614}-PVs was
34 inhibited to 78%, while in the presence of CHPV or suramin alone, inhibition rate was
35 65% or 40% (Fig. 5A). These results suggest that a combination of these two
36 compounds may be able to achieve more efficient inhibition against SARS-CoV-2
37 infection.

38

39 The anti-SARS-CoV-2 neutralizing antibody (SAD-S35) was also tested and showed
40 a dose-dependent neutralizing activity against SPΔC_{G614}-PVs with an IC₅₀ of 2.4
41 µg/mL (Fig. 5B). Next, we sought to determine whether the combination of CHPV or
42 Suramin with SAD-S35 could mediate a stronger anti-SARS-CoV-2 activity. Thus,
43 serially diluted SAD-S35 (0.625 to 2.5 µg/ml) was mixed with CHPV (25 µg/mL) or
44 Suramin (25 µg/mL) and added to the 293T_{ACE2} cells with SPΔC_{G614}-PVs

1 simultaneously. In parallel, same concentrations of SAD-S35 alone were used for
2 comparison. The results show that 1.25 µg/ml of SAD-S35 alone only resulted in an
3 approximately 25% decrease of infection. However, nearly 80% inhibitory effect was
4 achieved when the same concentration of SAD-S35 was combined with CHPV
5 (25µg/ml), or approximately 60% inhibitory effect was achieved when combined with
6 suramin (25µg/ml), while CHPV or suramin alone only mediated 50% or 38%
7 inhibition, respectively (Fig. 5C). All together, the results clearly indicate that a
8 combination of CHPV or suramin with SAD-S35 is able to more potently block
9 SARS-CoV2 infection. By including a low dose of nAb, the amounts of CHPV or
10 Suramin needed to achieve highly effective inhibition of SARS-CoV2 infection can be
11 reduced.

12

13 **Inhibitory effect of CHPV and Suramin on SARS-CoV-2 virus infection.**

14 Given that both CHPV and suramin are able to block the SARS-CoV2-SP
15 pseudovirus entry, we next tested whether these two drugs could block wild type
16 SARS-CoV2 virus infection and virus-induced cytopathic effect in Vero cells. The wild
17 type SARS-CoV-2 virus (hCoV- 19/Canada/ON-VIDO-01/2020) was used to infect
18 Vero cells in the presence of different concentrations of CHPV or Suramin. Briefly,
19 Vero cells were infected with SARS-CoV-2 (MOI of 0.01) in the presence of different
20 concentrations of CHPV or Suramin. After 72 hrs post-infection, as indicated (Fig. 6),
21 the SARS-CoV-2-induced cytopathic effects in Vero cells were monitored. Results
22 showed that SARS-CoV-2 infection causes dramatic cytopathic effect (CPE) in Vero
23 cells after 72 hrs post-infection, with cells displaying 100% CPE. Remarkably, in the
24 presence of CHPV or suramin (at 50 to 125 µg/ml), the SARS-CoV-2-induced
25 cytopathic effect (CPE) was significantly or completely inhibited in Vero cells. These
26 results provide strong evidence that the presence of CHPV or Suramin is able to
27 inhibit SARS-COV-2 infection.

28

29

1 Discussion

2 Because SARS-COV-2 is classified as an aerosol biosafety level 3 (BSL-3) pathogen,
3 the study of SARS-COV-2 infection and the investigation of different
4 anti-SARS-COV-2 compounds required highly restricted BSL-3 containment. This
5 condition has significantly limited the SARS-COV-2-related research activities in
6 microbiology laboratories. In this study, we established a highly sensitive
7 SARS-COV-2-SP pseudotyped HIV-based entry system, which encodes a Gaussia
8 luciferase (Gluc) gene as a reporter (Fig. 1B). Since Gluc can be secreted into the
9 supernatant after being expressed in the infected cells, it is very sensitive and
10 convenient for evaluating the level of SARS-CoV2-SP-mediated virus entry and may
11 be used for anti-SARS-CoV2-SP compound screening in a BSL-2 environment.

12
13 Previous studies have revealed that the cytoplasmic tail (CT) of SARS SP contains a
14 dibasic motif (KxHxx) that constitutes for an endoplasmic reticulum (ER) retrieval
15 signal which retains the full-length SARS-S protein in the lumen of the ER-Golgi
16 intermediate compartment (ERGIC) [39,40]. Deletion of 17 aa at the carboxyl-terminal
17 in the CT of SARS SP was able to increase SP transported to the surface of cells and
18 substantially increased SARS SP-mediated cell-to-cell fusion [37]. In the
19 SARS-CoV2-SP, there is also a dibasic motif (KxHxx) present in the CT (Fig 1A). In
20 order to increase SARS-CoV2-SP incorporation into pseudovirions, we have deleted
21 17 aa at the CT of SARS-CoV2 SP and generated a SARS-CoV2 SP Δ C expressor
22 plasmid (Fig. 1A, b). Indeed, our data showed that a significantly higher level of
23 SCoV2 SP Δ C protein was present in the pseudovirus (Fig. 1C), and induced
24 remarkably efficient infection in 293T_{ACE2} cells (Fig. 1D). This observation clearly
25 indicate that the dibasic motif in SARS-COV-2 SP is functional and a deletion of 17
26 amino acids substantially increased incorporation of SP into SARS-CoV2-SP-PVs
27 and enhance its infectivity. This information is also important for improving the design
28 of SARS-CoV2-SP-based vaccine strategies.

29
30 Recent sequencing analyses found a SARS-CoV2 SP mutation, Aspartic acid (D)
31 changed to Glycine (G) at aa position 614 in the S1 domain which was dominantly
32 detected in April to May of 2020 isolates, indicating a transmission advantage over
33 original SP D614 [15]. The following studies showed that SARS-CoV2_{G614} SP
34 mutant MLV pseudotyped viruses infected ACE2-expressing cells markedly more
35 efficiently than those with SARS-CoV2_{D614} [16,41]. Consistently, we also observed
36 the SARS-CoV2_{G614} Δ C-pseudotyped lentiviral particles enhanced the pseudotyped
37 virus entry compared to the SP_{D614} Δ C-PVs (Fig. 2E).

38
39 By using this SCoV2-SP- pseudovirus system, we have provided evidence for the first
40 time that the CHPV can efficiently prevent infections mediated by both
41 SARS-CoV2-SP_{D614} and -SP_{G614} pseudovirus infection in 293T_{ACE2} cells and
42 significantly block the infection of wildtype SARS-COV-2 in Vero cells. We also
43 revealed that CHPV blocks the entry of virus by directly interrupting the interaction of
44 SARS-CoV2-SP and ACE2 receptor by *in vitro* ELISA assay (Fig. 4). Interestingly,

1 the presence of CHPV at one hour post-infection is still able to efficiently inhibit
2 SARS-Cov2-SP pseudovirus infection (Fig. 4), suggesting that CHPV may not only
3 target SP/ACE2 binding, but may also act on the following fusion step(s). Overall,
4 our results provide convincing evidence for CHPV as a potential blocking agent
5 against SARS-COV-2 infection. In agreement with a recent study [33] that suramin
6 can inhibit SARS-COV-2 virus infection, we further provide evidence that suramin is
7 able to directly block SARS-CoV-2 SP-ACE2 interaction (Fig. 4D) and different
8 SARS-CoV-2 SP variants mediated virus entry (Fig 3, 4, and 5).

9

10 Another interesting observation in this study is that the combination of CHPV or
11 suramin with anti-SARS-COV-2 neutralizing antibody (nAb) could enhance their
12 anti-SARS-COV-2 activity. The nAb has great potential to be used as a preventing
13 agent in blocking SARS-COV-2 infection [42]. However, one disadvantage of using
14 nAb as an anti-SARS-COV-2 agent is its source limitation. Therefore, the finding of
15 the synergistic effect of a combination of nAb with other agents, such as CHPV or
16 Suramin is beneficial for (i) similar efficiencies would be achieved by using reduced
17 amounts of antibody and CHPV or Suramin, (ii) the combination of nAb and
18 CHPV/suramin will reduce the likelihood of viral resistance. Whether these enhanced
19 effects might be due to a combined effect through their different binding mechanisms
20 still needs to be investigated.

21

22 The effectiveness of CHPV and/or suramin against SARS-COV-2 infection *in vivo*
23 remains to be investigated. Our findings could be further validated in an appropriate
24 animal model and clinical trials for prevention of COVID-19. Since SARS-COV-2
25 infection initiates in the respiratory tract [43], the use of CHPV and/or Suramin as
26 nasopharynx agents (Nasal spray) to prevent initial SARS-COV-2 infection and
27 transmission in the respiratory tract will be a particularly attractive strategy, and will
28 require further efficacy studies. Overall, we demonstrated that CHPV and suramin
29 possess an anti-SARS-COV-2 entry inhibitor activity and functions at least partially by
30 interrupting SARS-COV-2 SP binding to its receptor. Additional *in vivo* safety and
31 protection studies will facilitate its application as an option to help control the ongoing
32 SARS-CoV-2 pandemic.

33

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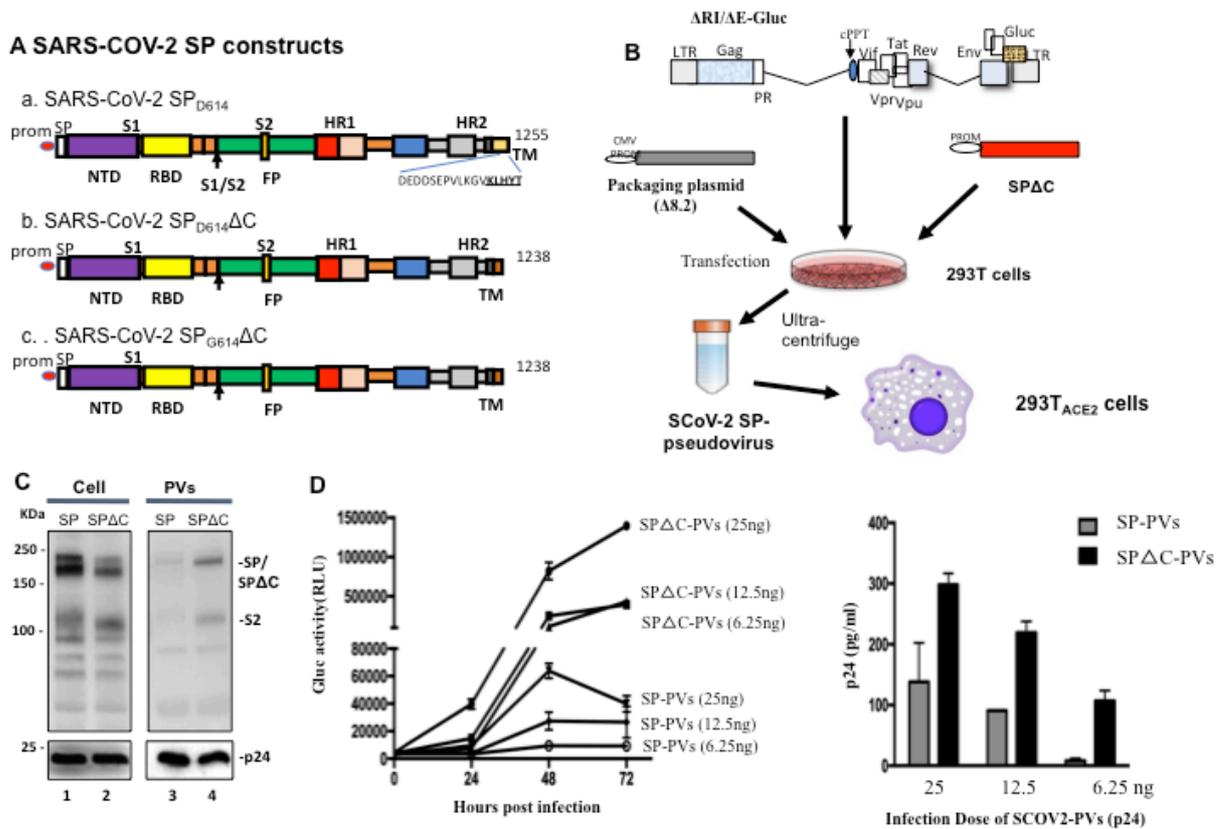
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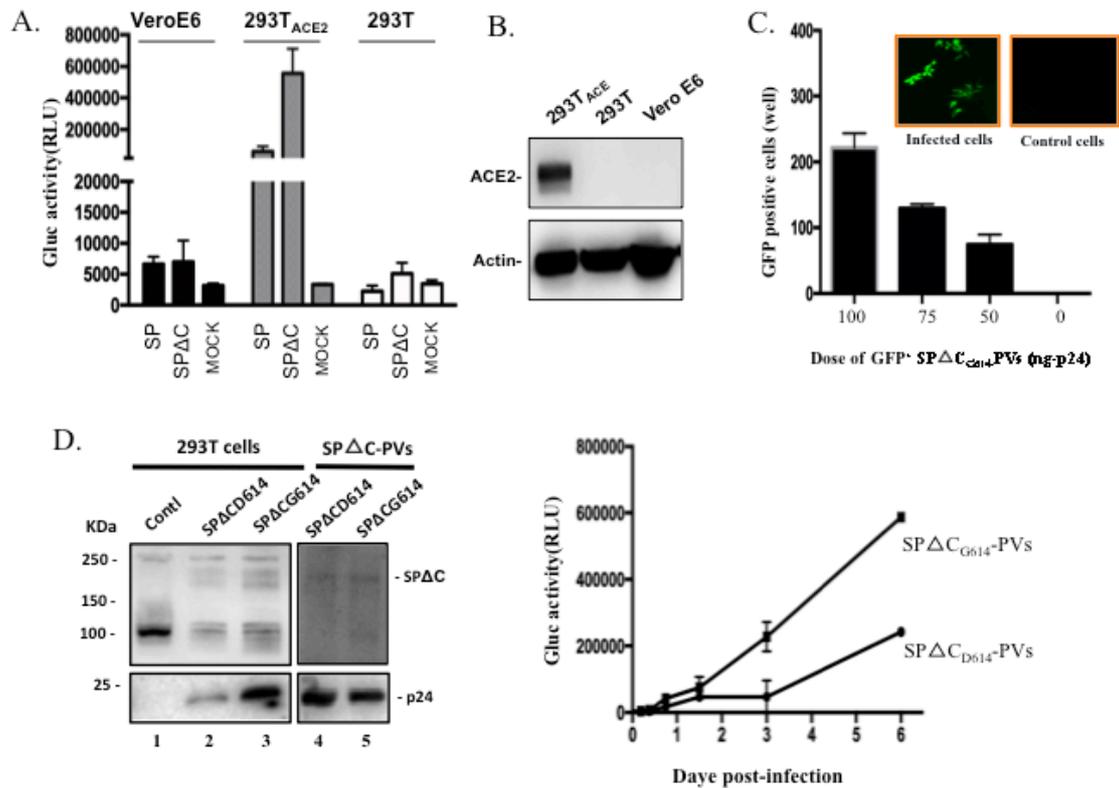
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2 **Figure 1. Generation of a SARS-COV-2-SP-pseudotyped lentiviruse particles**
 3 **(SCoV-2-SP-PVs).** A) Schematic representation of SARS-CoV-2SP,
 4 SARS-CoV-2SPΔC, and SARS-CoV-2SP_{G614}ΔC expressing plasmids. B) Schematic
 5 representation of plasmids and and procedures for production of
 6 SARS-COV2-SP-pseudotyped lentivirus particles (SCoV-2-SP-PVs). C) Detection of
 7 SARS-CoV-2 SPs and HIV p24 protein expression in transfected 293T cells and viral
 8 particles by Western blot (WB) with anti-SP or anti-p24 antibodies. D) Different
 9 amounts of SCoV-2-SP-PVs and SCoV-2-SPΔC-PVs virions (adjusted by p24) were
 10 used to infect 293T_{ACE2} cells. At different time intervells, the Gaussia Luciferase
 11 activity (Gluc) (left panel) and PVs-associated p24 (at 72 hrs) in supernatants was
 12 measured.

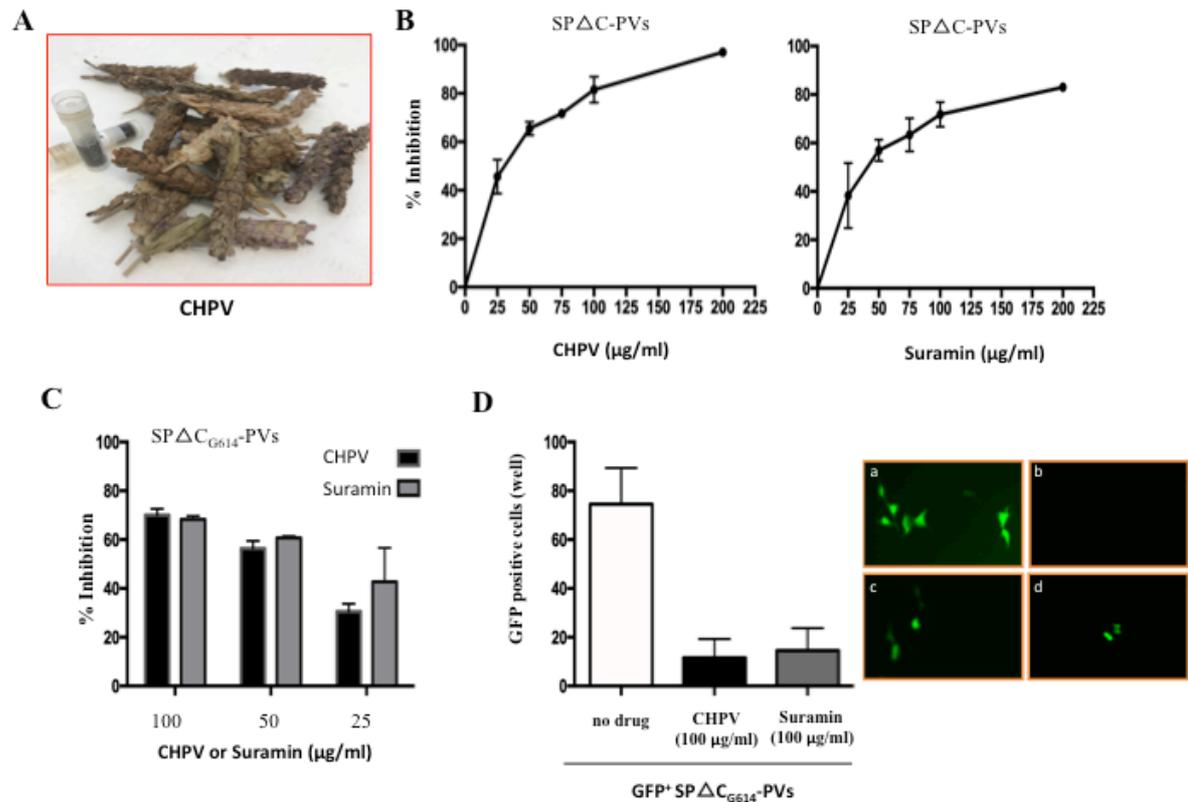
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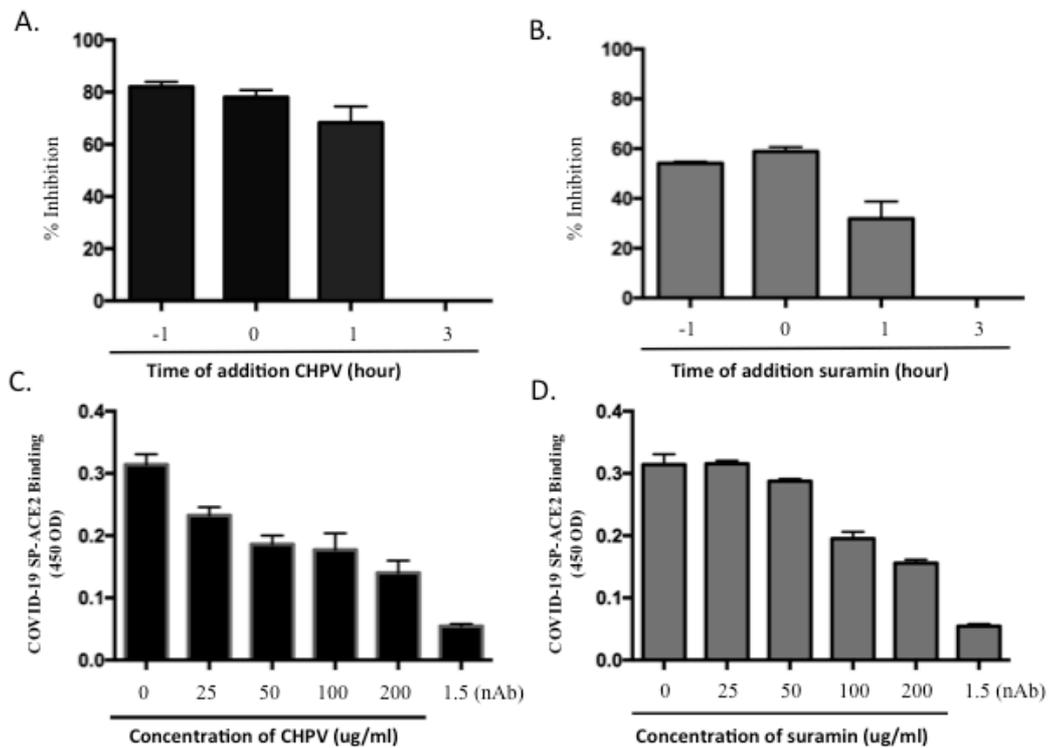
2 **Figure 2. SARS-CoV-2 SP-PVs's infection in different cell lines and**
 3 **SARS-CoV-2 SP_{G614} variant exhibited stronger virus entry.** A) 293T, 293T_{ACE2}
 4 and Vero-E6 cells were infected by equal amounts of SARS-CoV-2SP-,
 5 SARS-CoV-2SPΔC-pseudotyped viruses. At 48 hrs pi, the Gluc activity in
 6 supernatants was measured. B) the expression of SARS-CoV-2SP receptor, ACE2,
 7 in 293T, 293T_{ACE2} and Vero-E6 cells detected by WB with anti-ACE2 antibodies. C)
 8 The SPΔC_{G614}-GFP⁺PVs were produced with 293T cells and used to infect 293T_{ACE2}
 9 cells in 96-well plate After 48 hrs pi, GFP-positive cells (per well) were counted and
 10 photographed by fluorescence microscope (on the top of the panel). D) Detection of
 11 SARS-CoV-2 SPΔC, SPΔC_{G614} and HIV p24 protein expression in transfected 293T
 12 cells and viral particles by WB. E) Infectivity comparison of SPΔC-PVs and
 13 SPΔC_{G614}-PVs in 293T_{ACE2} cells. Equal amounts of SPΔC_{D614}-PVs and
 14 SPΔC_{G614}-PVs virions (adjusted by p24 level) were used to infect 293T_{ACE2} cells. At
 15 different days post-infection (pi), Gluc activity in supernatants was measured.

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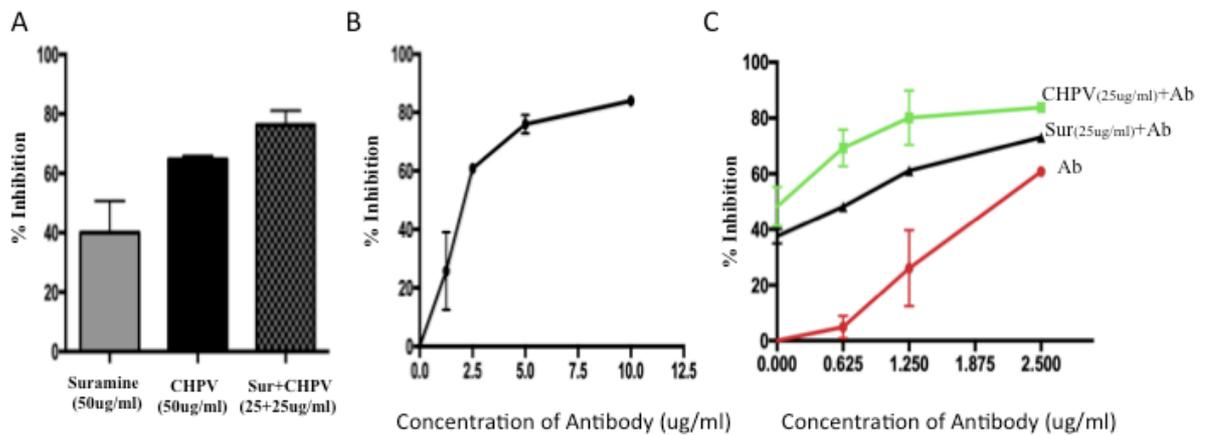
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Figure 3. SARS-CoV-2-SP-PV's infection was efficiently blocked by CHPV and suramin. A) Images of the dried *Prunella Vulgaris* flowers and its water extract (CHPV). B) Dose -response anti-SARS-CoV-2 analysis by Gluc activity for CHPV or suramin. 293T_{ACE2} cells were infected by equal amounts of SARS-CoV-2SPΔC-pseudotyped viruses in the presence of different dose of CHPV or suramin. At 48 hrs pi, the Gluc activity in supernatants was measured. (% inhibition = 100 x [1 - (Gluc value in presence of drug)/(Gluc value in absence of drug)]). C) Infection inhibition of CHPV or suramin on SARS-CoV-2-SPΔC_{G614}-PVs in 293T_{ACE2} cells. Equal amounts of SCoV-2-SPΔC_{G614}-PVs (adjusted by p24 level) were used to infect 293T_{ACE2} cells in presence of different concentrations of CHPV or suramin, in indicated at bottom of the panel. At 48 hrs pi, Gluc activity in supernatants was measured and present as % inhibition. Means ± SD were calculated from duplicate experiments. D) 293T_{ACE2} cells in 96-well plate were infected with SPΔC_{G614}-GFP⁺ PVs. After 48 hrs pi, GFP-positive cells (per well) were counted (left panel) and photographed by fluorescence microscope (right panel, a. Without drugs; b. Without infection; c. In the presence of CHPV (100 μg/ml); d. In the presence of suramin (100 μg/ml).



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Figure 4. Characterization of the mechanisms of CHPV and suramin for their anti-SARS-CoV-2-SP action. A) Time-dependent inhibition of SPΔC₆₆₁₄-PVs infection mediated by CHPV or suramin. CHPV (100 μg/mL) or suramin (100 μg/mL) was added at 1 hr prior to infection, during infection (0 hr), and at 1 hr, and 3 hr pi. The positive controls (PC) were 293T_{ACE2} cells infected with SPΔC₆₆₁₄-PVs in the absence of compounds. At 3 hrs pi, all of the cell cultures were replaced with fresh DMEM and cultured for 48 hrs. Then, the Gluc activity was monitored in the supernatant, and the data are shown as a percentage of inhibition (%). B) inhibitory effect of CHPV or suramin on SARS-CoV2-SP/ACE2 binding by ELISA as described in materials and methods. nAB: anti-COVID-19 neutralizing antibody (SAD-S35). The results are the mean ± SD of duplicate samples, and the data are representative of results obtained in two independent experiments.



1

2 **Figure 5. Enhanced inhibitory effects mediated by combination of CHPV and**

3 **suramin with neutralizing antibody (SAD-S35).** A) 293T_{ACE2} cells were infected

4 with SPΔC_{G614}-PVs in presence of CHPV (50μg/ml) or suramin (50μg/ml) alone or a

5 mix of CHPV and suramin (each with 25μg/ml). After 3 hrs of infection, cells were

6 washed and add fresh medium for 48 hrs. Then the supernatants were collected and

7 Gluc activity in the supernatant was measured and present as % inhibition. B)

8 Inhibitory effect of nAb SAD-S35 on SPΔC_{G614}-PVs infection. 293T_{ACE2} cells were

9 infected with SPΔC_{G614}-PVs in the presence of serially diluted SAD-S35 (1.25 to 10

10 μg/ml) for 3 hrs. Then infected cells were cultured in fresh medium. At 48 hrs pi., the

11 supernatants were collected and measured for Gluc activities and presented as %

12 inhibition. C) 293T_{ACE2} cells were infected with SPΔC_{G614}-PVs in the presence of

13 serially diluted SAD-S35 (0.625 to 2.5 μg/ml) alone or mixed with CHPV (25 μg/mL)

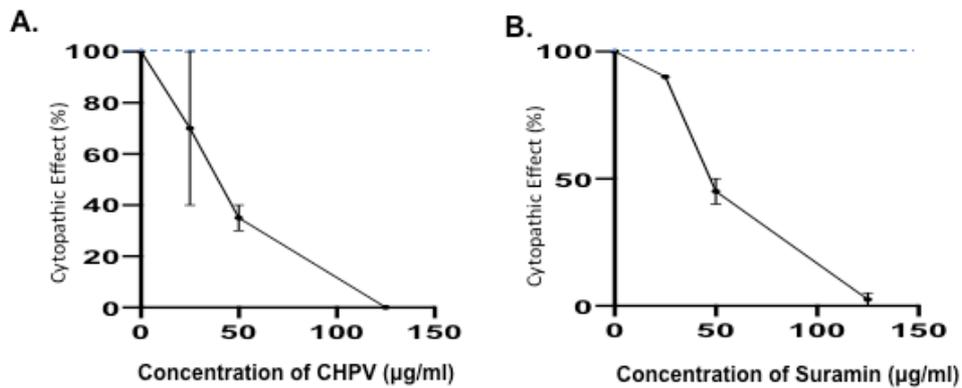
14 or Suramin (25 μg/mL) for 3 hrs and the infected cells were cultured in fresh medium.

15 At 48 hrs pi., the Gluc activities in the supernatants were measured and presented

16 as % inhibition. The results are the mean ± SD of duplicate samples, and the data are

17 representative of results obtained in two independent experiments.

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2 **Figure 6. Inhibitory effect of CHPV and Suramin on SARS-CoV-2**
3 **infection-induced cytopathic effects.** Vero cells were infected with a wild type
4 SARS-CoV-2 virus (hCoV-19/Canada/ON-VIDO-01/2020) in the presence or absence
5 of different concentrations of CHPV and Suramin. After 72 hrs pi., the SARS-CoV-2
6 infection-induced cytopathic effects in Vero cells were monitored. Error bars
7 represent variation between triplicate samples, and the data of (A) and (B) are
8 representative of results obtained in two independent experiments.

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