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The Antiviral Effects of the Homeopathic Cinchona officinalis on the COVID-19 Virus

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Abstract

Background One of the most dangerous coronavirus diseases that might strike in 2019 is the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Results from several in vitro experiments show that hydroxychloroquine (HCQ) significantly lowers SARS-CoV-2 infections. Objective Due to the similarity in phytoconstituents between HCQ and Cinchona officinalis (CO), this study set out to investigate the antiviral efficacy of several homeopathic formulations of CO. Approaches After analyzing CO's chemical composition using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry, a comprehensive docking study was conducted. After measuring the binding ability of phyto-components, we docked them with several SARS-CoV-2 targets and compared the results. If two ligands have identical Glide docking scores, then the one with the lowest value must have the strongest binding affinity. The cytotoxicity of various homeopathic formulations was also examined using VeroE6 cells. One of them was CO mother tincture (CO-MT). In order to determine how CO-MT affected SARS-CoV-2 infected VeroE6 cells, in vitro trials were performed. A positive control was performed using Remdesivir, an antiviral that has a long history of usage. Completed Good Molecular docking studies showed that CO components can attach to many SARS-CoV-2 targets, including as nucleocapsid, RdRp, PLpro, and Mpro.

Keywords: Homeopathy *Cinchona officinalis* COVID-19 antiviral toxicity docking

angiotensin-converting enzyme 2 (ACE2) in the host, spike protein, and protein. Quinoline, an element in carbon monoxide, has the potential to bind the spike protein of SARS-CoV-2. With respect to binding capabilities, quinic acid outperformed the other components with respect to Mpro, PLpro RdRp, nucleocapsid protein, and ACE2 (allosteric site). Quinidine has a stronger binding affinity for ACE2. In terms of binding potential to SARS-CoV-2 proteins, including spike protein, PLpro, Mpro, and RNA-dependent RNA polymerase, HCQ was on par with other CO phytoconstituents. No harmful effects were seen with homeopathic CO-MT, and in vitro experiments shown that remdesivir prevented SARS-CoV-2 infection in VeroE6 cells by 99%, whereas CO-MT only managed an 89% reduction.
To summarize Our computational and in vitro results provide credence to the hypothesis that CO-MT might be a useful antiviral drug for COVID-19 therapy. The therapeutic potential of CO-MT in COVID-19 has to be further understood via in vivo study.

Introduction

Emerging in late December 2019 in Wuhan, China, coronavirus disease 2019 (COVID-19) quickly expanded to become a pandemic, driven by the new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).one, two There is a wide spectrum of symptoms, from moderate, self-limiting respiratory illness to advanced pneumonia, organ failure, and death. Much research and ongoing clinical studies have failed to identify any specific therapeutic medications that have existed to alleviate or eliminate a coronavirus infection.3–5 The National Institutes of Health have released recommendations and therapy techniques for COVID-19. In severe and critical instances, corticosteroids such hydrocortisone, dexamethasone, and prednisolone are indicated. However, in mild instances, they lower the immune response's protective capabilities, which puts the patient at risk for bacterial and fungal infections, a slowed recovery, and hypokalemia.6 Among the many alternative and complementary medicine modalities, homoeopathy ranks high in popularity. A chemical that produces the same symptoms in healthy

people may be used to treat illness, according to the "like heals like" concept. Some people believe that homeopathic treatments might help the body's immune system function better.7 Their potential immune-modulatory function impacts the susceptibility of the body to infections.8 Some people believe that the homeopathic medicine cinchona officinalis (CO) may eliminate parasites and cure low-grade fevers (whether they be intermittent, malarial, or recurrent). Cinchonine, quinidine, quinine, and cinchonidine are the main ingredients in CO bark.nine, ten Natural quinine and its synthetic analogues, such as chloroquine (CQ, C18H26ClN3) and hydroxychloroquine (HCQ, C18H26ClN3O), are structurally and therapeutically comparable. Some have speculated that the anti-malarial medications CQ and HCQ might be effective against COVID-19.11 The antiviral efficacy of HCQ against SARS-CoV-2.12 was shown in many in vitro investigations. We predicted that CO formulations would also possess antiviral capabilities against SARS-CoV-2 due to the chemical similarity between CO's active components and CQ and HCQ. Aiming to determine if homeopathic CO formulations are safe and effective against SARS-CoV-2 in vitro, the current research set out to do just that. Procedures and Supplies Unless otherwise specified, all chemicals and reagents used in the research are of analytical quality and were purchased from Sigma. The mother tincture (MT) and potencies were sourced from the GMP-certified Hahnemann Publishing Co. Pvt. Ltd. in Kolkata.

Investigation using UHPLC/Q-TOF-MS, or Ultra-High Performance Liquid Chromatography Quantitative Time-of-Flight Mass Spectrometry Using an Agilent 1290 Infinity LC system linked to an Agilent 6545 Q-TOF mass spectrometer with Agilent Jet Stream Thermal Gradient Technology, the phytoconstituents of CO-MT and poten-cies 3c, 6c, and 12c were identified by UHPLC/Q-TOF-MS. A diode array detector and auto-sampler were included in the UHPLC system. Using Agilent Mass Hunter Qualitative Analysis (Mass Hunter Qual, Agilent Technologies, Santa Clara, CA, United States), the raw data was deconvoluted into individual chemical peaks.Agilent Mass Hunter collection software, which has verified profiles of several phytoconstituents, was used to accomplish data collection on the LC-Q-TOF. That is why the m/z values of the specific chemical peaks were used to identify the phytoconstituents of CO-MT. Chemical Interactions All of the three-dimensional structures of the following targets were obtained from the Protein Data Bank: ACE2 (6LZG), Mpro (6LU7), PLpro (3E9S), RdRp (7BV2), Nucleo-capsid Protein (6VYO), and Spike Protein (7DWY). If accessible, their co-crystallized ligands were also included. Before being finally created using a LigPrep module in Schrodinger at a pH of 7.4, all of the ligand structures were exposed to the production of various conformations. In targets lacking co-crystalized ligands, the appropriate binding site(s) were located using the SiteMap tool. For ACE2, nucleocapsid protein, and spike protein, respectively, SiteMap produced 5, 5, and 3 locations. The software generated potential binding locations by considering the volume, size, and scores for the cavities. potential as a therapeutic target. Based on their druggable ratings, ACE2 and nucleocapsid protein were picked for site 1, whereas spike protein was chosen for site 2. Furthermore, docking at site 5, which is an allosteric site, was also taken into account for ACE2. For the targets described earlier and additional proteins using co-crystallized ligands, glide grids were made using SiteMap output. To conduct the molecular docking, we used Glide and its ExtraPrecision (XP) mode to create binding postures. As a measure of binding affinity, the Glide docking score (DS; negative number) is the best-docked position that was recorded for each ligand14,15.16 Coronavirus 2 Cytotoxicity Assay on VeroE6 Cells for In Vitro Antiviral Studies in the Setting of Severe Acute Respiratory Syndrome The experiment was carried out on a 96-well plate with three wells for per sample. A humidified incubator with 5% CO2 was used to incubate VeroE6 cells (1 × 10^4 cells per well) overnight at 37°C in order to establish a monolayer. The same day, VeroE6 cells were exposed to the chemical under test at the specified concentration (4 µL of the original sample, a 1:10 or 1:20 ethanol dilution, in a 200 µL reaction).Seventeen cells served as controls, and they were all exposed to the CO-MT solvent (1.40% ethanol). Hoechst 33342, a cell-permeant nuclear counterstain that generates blue fluorescence when attached to dsDNA, and Sytox orange dye, which stains dead cells alone, were used to stain both living and dead cells after 30 hours of incubation. To cover 90% of each well's area, 16 photos were obtained per well using Image Xpress Microconfocal microscopy (Molecular Devices) at a 10X magnification. In the photos that are stained with Hoechst, the program counts the total number of cells. It also counts the amount of dead cells that are stained with Sytox. Diagnostic Test for Antivirals (Immunofluorescence Evaluation) Follow the steps outlined in the preceding section to cultivate and treat VeroE6 cells with the test chemicals. For the control group, we used VeroE6 cells that had been treated with ethanol. At the outset, SARS-CoV-2 was introduced into VeroE6 cells at a MOI of 0.1 virions/cell.18 There was a 30-hour incubation period followed by cell fixation in 4% paraformaldehyde and permeabilization with 0.3% Tween-20. As a further step, VeroE6 cells were stained sequentially with a primary antibody that specifically identifies SARS-CoV-2 infected cells (SARS-CoV-2 nucleocapsid mouse monoclonal antibody: Catalog Number: 40143-MM05) and a secondary anti-mouse antibody conjugated to

Alexa Fluor 568. For nuclear staining, the Hoechst 33342 dye was used. The SARS-CoV-2 nucleocapsid (Alexa Fluor-568) and total nuclei (Hoechst) were stained with different chemicals, and 16 photos were taken per well (at 10X magnification). We counted total nuclei and nucleocapsid positive cells using Meta Xpress software's multi-wavelength cell scoring module, and we compared the results to the control. All SARS-CoV-2-related research took place in a biosafety level-3 laboratory, and all people engaged had the necessary equipment to ensure safety.19
Analyzing Statistics The data was statistically evaluated using GraphPad Prism V5 and the Student's t-test. Mean ± standard error of the mean is used to express the values.

Results

Identification of Constituents of *Cinchona Officinalis* Formulations

The specific gravity (0.8590 ½ 0.8556 g/mL; pH: 5.86), alcohol strength (135o ½ 77.03% v/v), and solid content (0.201% w/v) of the CO (Batch 0752) used in this investigation were all within the parameters specified by the Homeopathic Pharmacopoeia of India (HPI). Batch 0752 was almost colorless and devoid of sediments. The existence of three predicted spots with various Rf values (Rf: 0.07, 0.13, 0.33, and lambda max [λ max]: 278.80 nm) was shown by thin layer chromatography, which confirmed the identification and purity of the CO-MT. LC-MS analysis: Figure 1A shows the CO-MT total ion chromatogram (TIC), and Figure 1B compares the TICs of CO-MT at potencies 3c, 6c, and 12c. In comparing the ion chromatograms of CO-MT with its magnified view (►Fig. 1A and 1A1), the phytoconstituents of MT are clearly seen as specific peaks. The phytoconstituents of CO-MT that have been identified are listed in Table 1. There were no phyto-constituents detected in any of the three concentrations (3c, 6c, and 12c). The 2019 Coronavirus Disease Target Proteins in an In silico Investigation of Cinchona Officinalis Constituents
According to many sources, antiviral medicines can target specific SARS-CoV-2 proteins. In order to find out whether any of the CO phytoconstituents exhibited significant binding to SARS-CoV-2 spike protein, RNA-dependent RNA polymerase (RdRp), nucleocapsid protein, ACE2 (site of entry), Mpro, or PLpro, we conducted an in silico investigation. The results may be seen in Fig. 1 and Table 1. As positive controls in docking tests, we used HCQ, CQ, and remedesvir monophosphate (RMP), which are recognized SARS-CoV-2 inhibitors. The findings of docking the chosen components with the COVID-19 target proteins are shown in Table 2. The findings are for the optimal conformation with the best DS, even though these phytoconstituents may take on numerous forms. Table 2 allows us to draw three important conclusions. After ACE2 (site 1) and PLpro, remdesivir (RMP) binds well to RdRp. Both mono- and dianionic forms of remdesivir are possible; the binding scores of the two are similar, and remdesivir-monophosphate is the active form. HCQ binds well to the allosteric and active sites of ACE2, and CQ's binding score at site 5 is quite similar to HCQ's. The majority of HCQ and CQ's cationic interactions with target Asp and Glu residues are formed by these centers. On the other hand, Phe392 in the hydrophobic cavity is involved in cation-pi interaction with these medications via their cationic center (tertiary amine group) in the spike protein.

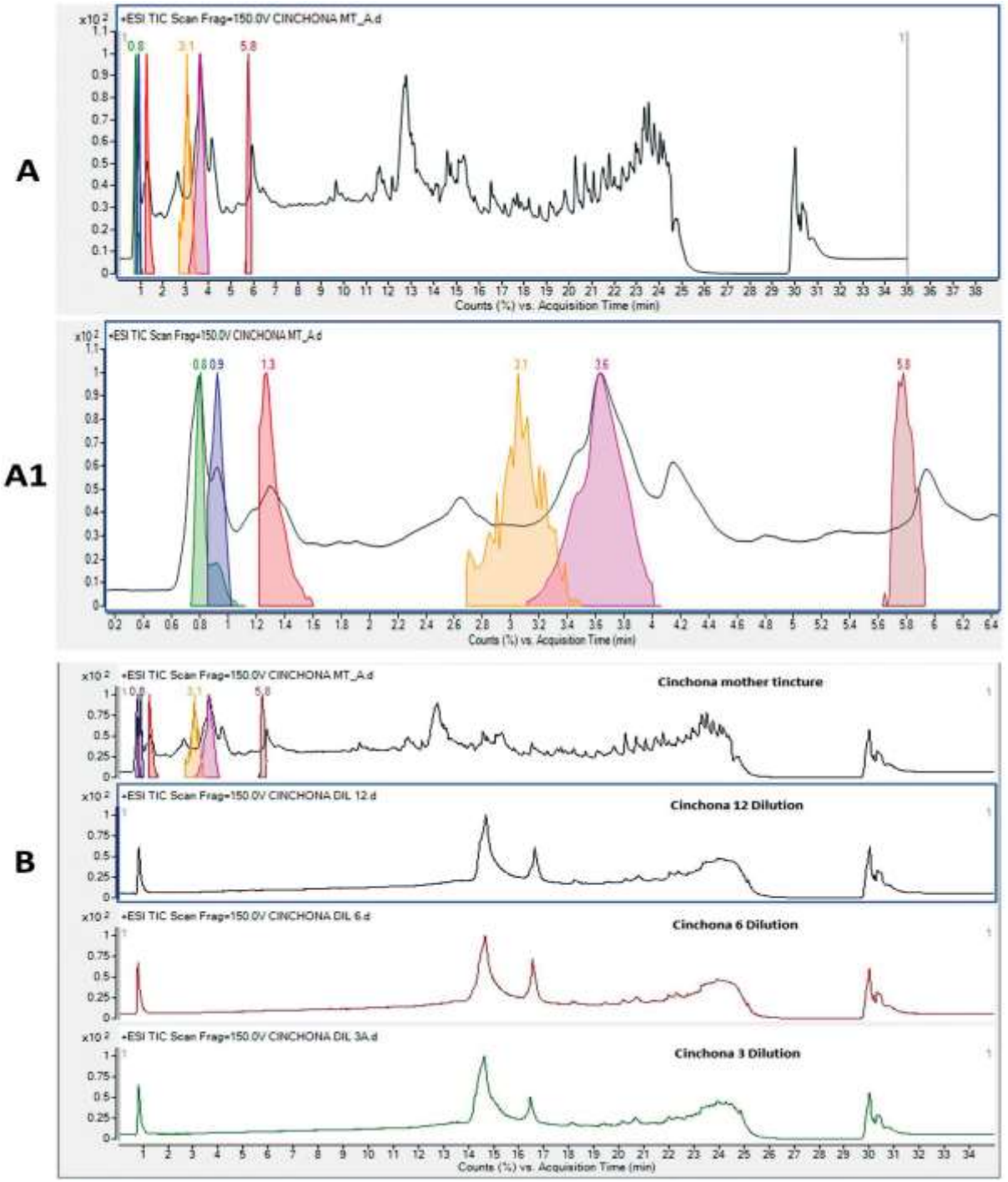


Fig. 1 LC-MS analysis of the *Cinchona officinalis* (CO) mother tincture and the potencies 3c, 6c and 12c. Identification of the components of CO-MT and potencies 3c, 6c and 12c were performed by UHPLC/Q-TOF-MS using the Agilent 1290 Infinity LC system coupled to an Agilent 6545 Q-TOF mass spectrometer with Agilent Jet Stream Thermal Gradient Technology. (A) Total ion chromatograms (TICs) of CO-MT.
(A1) Magnified view of A showing the peaks of identified molecules. (B) Comparison of TICs of CO-MT with potencies 3c, 6c and 12c. No constituents are found in 3c, 6c and 12c.

Table 1 List of compounds identified in *Cinchona officinalis* MT by molecular feature analysis

Formula	m/z	Mass	RT (Min)	Height	Compound Name
$C_7H_{12}O_6$	215.051 9	192.062 8	0.8	21430	Quinic acid
$C_{19}H_{24}N_2O_2$	313.190 3	312.183 0	0.9	236358	Dihydroquinidine
C_9H_7N	130.064 5	129.057 3	1.3	48299	Quinoline
$C_{19}H_{22}N_2O$	295.179 8	294.173 2	3.1	971894	Cinchonidine/ Cinchonine/ Epicinchonine/ Epicinchonidine
$C_{20}H_{24}N_2O_2$	325.191 5	324.184 6	3.6	601068 3	Quinine/Quinidine/ Epiquinidine/Epiquinine
$C_{20}H_{24}N_2O_2$	325.191 5	324.184 6	5.8	90594	Quinine/Quinidine/ Epiquinidine/Epiquinine

Abbreviation: MT, mother tincture.

Table 2 Docking results for the constituents against COVID-19 target proteins

Molecule	ACE2 (6LZG)		Mpro (6LU7)	PLpro (3E9S)	RdRp (7BV2)	Nucleocapsid (6VY0)	Spike (7DWY)
	S	S				Site1	S

	ite5	ite1					ite2
RMP_Monoanion	—5.35	—7.43	—5.45	—6.29	—5.72	—4.63	—5.38
RMP_Dianion	—5.78	—7.61	—5.36	—6.79	—7.11	—6.21	—4.56
Hydroxychloroquine	—8.78	—8.93	—4.98	—6.19	—5.46	—4.82	—5.93
Chloroquine	—8.50	—7.58	—4.04	—4.36	—3.66	—3.46	—7.00
Quinidine	—4.82	—6.11	—4.83	—2.53	—4.50	—4.50	—5.56
Cinchonine	—4.53	—3.46	—1.98	—5.69	—2.65	—3.39	—4.13
Dihydroquinidine	—5.37	—3.86	—3.89	—2.92	—2.37	—3.36	—3.94
Quinoline	—2.55	—3.26	—4.06	—3.57	—2.33	—2.36	—6.32
Quinic acid	—5.56	—5.59	—6.09	—6.00	—4.56	—6.24	—5.45

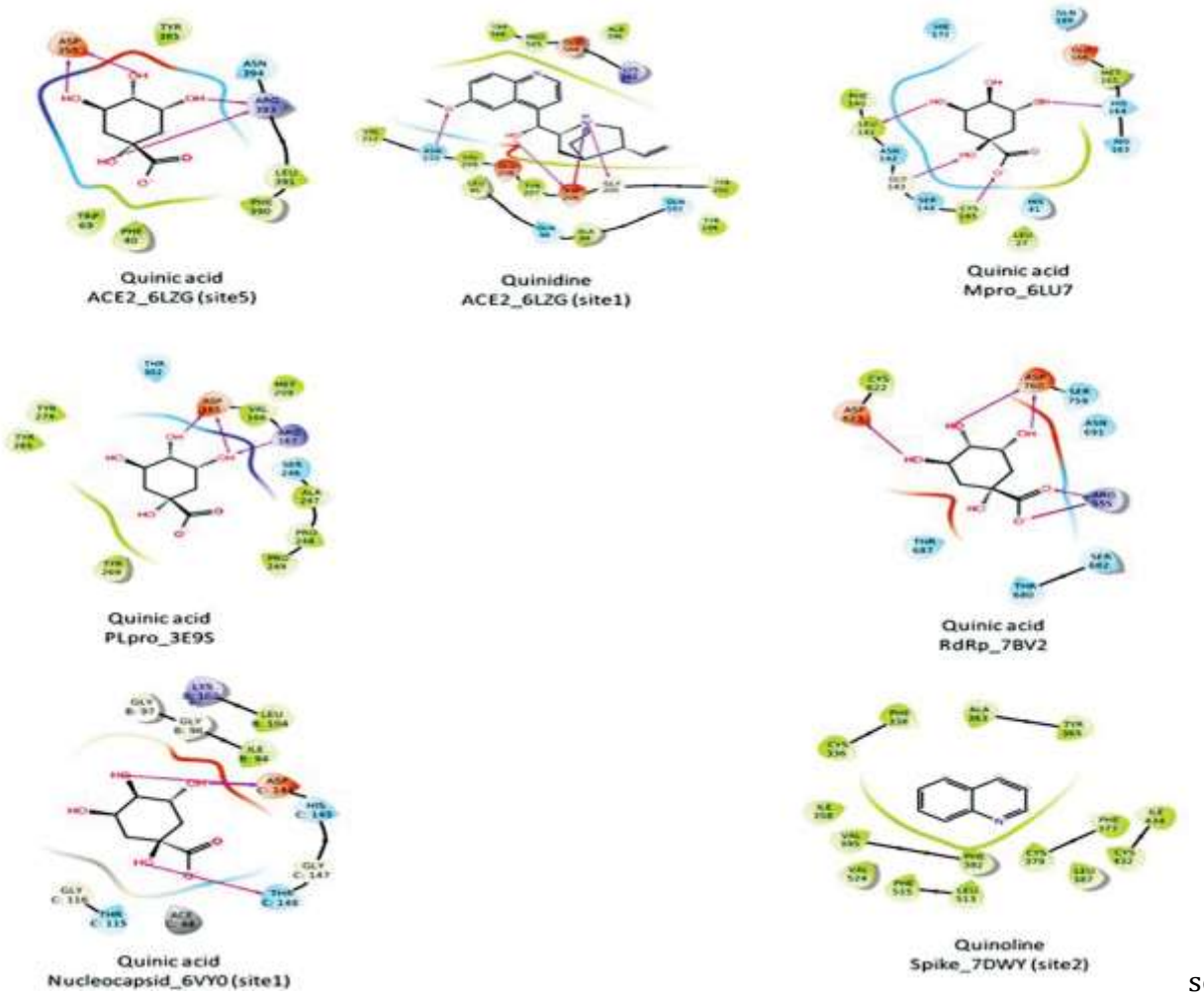
Abbreviations: COVID-19, coronavirus disease 2019; RMP, remdesivir monophosphate; PLpro, papain-like protease; RdRp, RNA-dependent RNA polymerase.

These results for HCQ and CQ are in agreement with the literature[20] and therefore validate our docking approach. Second, quinoline showed a DS of —6.32 against the spike protein, which is the best score among all phytoconstituents of CO and COVID-19 target proteins. It forms mainly hydro- phobic interactions in the active site of spike protein (►Fig. 2). Third, it is notable that RMP, HCQ, CQ, and three phytoconstituents of CO-MT (quinidine, quinoline and quinic acid) showed equivalent DSs, with a few exceptions. For example, quinic acid showed better binding capabilities with Mpro, PLpro RdRp, nucleocapsid protein and ACE2 (allosteric site) compared to other constituents. Quinic acid shows three to five hydrogen bonding interactions with different targets. Quinidine shows three hydrogen bonding interactions and one ionic interaction between the cationic center of ligand with ASP206 in site 1 of ACE2. In most targets, the dianion form of the phosphate group, the hydroxyl group of the sugar, and the amino group of RMP are involved in binding with residues of target proteins. Overall, these *in silico* data suggest that HCQ, CQ and specific phyto- constituents of CO-MT have equivalent potential to bind the spike and nucleocapsid proteins and could prevent SARS- CoV-2 entry into cells.

In vitro Antiviral Assay

In silico data suggested that HCQ, CQ and certain phytocon- stituents of MT have equivalent binding potential for the spike and nucleocapsid proteins of SARS-CoV-2 (►Table 2). Therefore, an antiviral assay against SARS-CoV-2 was performed in VeroE6 cells, which are commonly used for virus culture.[21] Initially, an immunofluorescence cytotoxicity as- say was performed to assess the safety of the CO formulations. The results confirmed that the CO-MT formulation and its potencies were not cytotoxic at the selected doses. Antiviral activity of CO-MT and potencies was tested in SARS-CoV-2 infected VeroE6 cells using the established antiviral drug remdesivir as a positive control. The total VeroE6 cell number was visualized with a nuclear stain (Hoechst blue color). Infected Vero cells were visualized with a dye conjugated to an antibody against nucleocapsid protein of SARS-CoV-2 (Alexa flour-568—orange color). Con- focal microscopy images clearly show the presence of Vero cells in all panels (►Fig 3C). As expected, uninfected VeroE6 cells show a Hoechst stain but no orange stain.

Similarly, SARS-CoV-2-infected Vero cells treated with remdesivir or CO-MT also show Hoechst stain and little or no orange stain, since both the



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drugs potently inhibited

Fig. 2 Binding mode of phytoconstituents of MT against different targets of SARS-Cov-2.

intracellular replication of the virus. Indeed, calculations revealed that remdesivir (10 µM) and CO-MT (concentration of 4 µL/200 µL) showed respectively a 99% and 89% inhibition of SARS-CoV-2 infection (►Fig 3B). However, SARS-CoV-2-
infected Vero cells treated with three potencies (CO 3c, CO 6c,

and CO 12c) showed only 5% to 15% inhibition of SARS-CoV-2 infection (►Fig 3B, –3C). This is consistent with our obser- vation that the active phytoconstituents of CO are absent in the three potencies (CO 3c, CO 6c, and CO 12c) (►Fig 1).

Thus, ►Fig 3B and –3C indicate that the antiviral activity of CO-MT is comparable to that of remdesivir, an established antiviral drug for COVID-19.

Discussion

This study was performed to identify the antiviral potential of CO against the SARS-CoV-2 virus, which causes COVID-19. First, we found that the homeopathic formulations of CO in

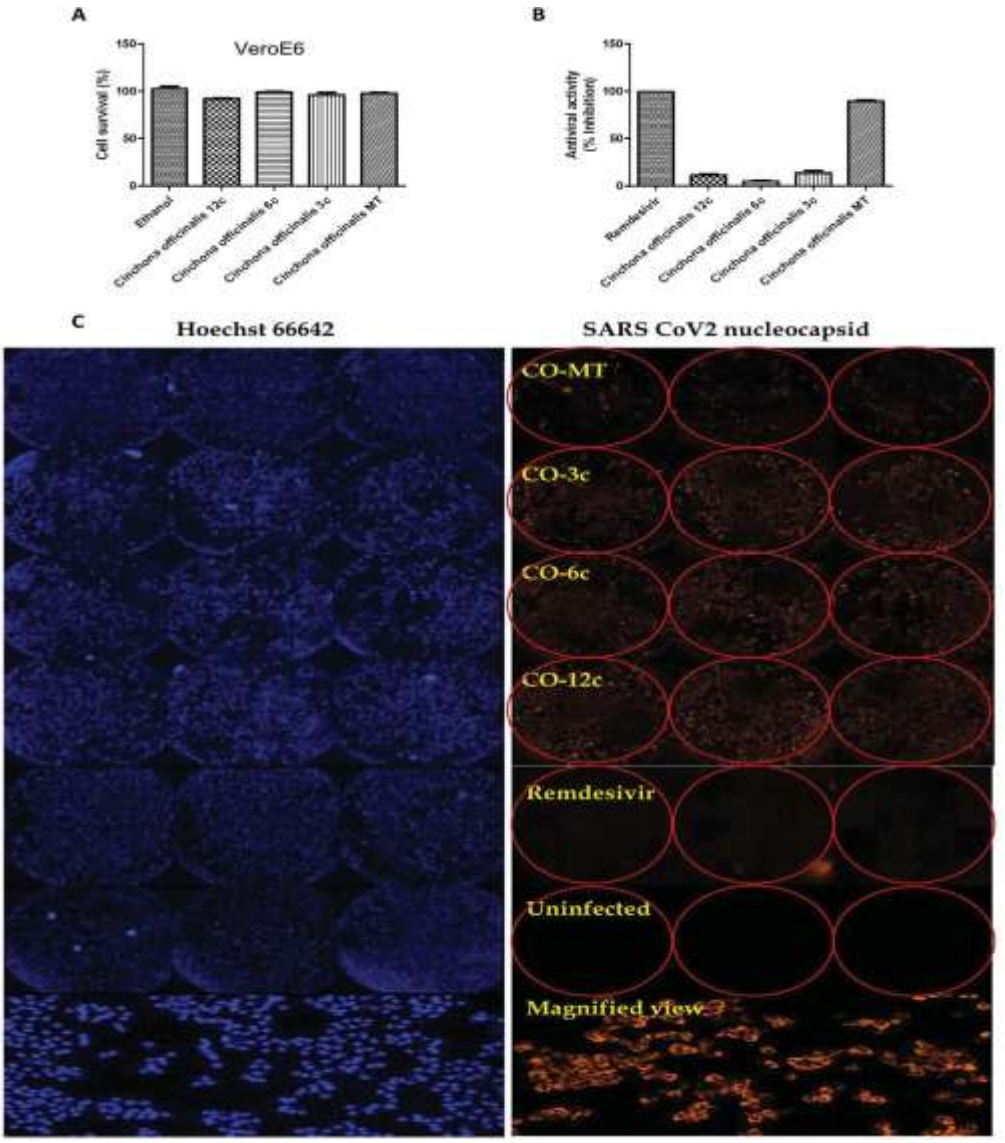

Fig. 3 (A) Effect of *Cinchona officinalis* (CO) on VeroE6 cells. Cells were incubated with the test substance at the indicated concentrations (4 µL of the sample [original, 1:10 or 1:20 dilution in ethanol] in 200 µl reaction). After 30 hours, cells were stained with Hoechst 33342 (live and dead cells) and sytox orange (dead cells). Cell viability was calculated by taking images (10X) of cells and total cell numbers from the software. (B) Antiviral activity of CO on SARS-CoV-2. The graph shows that SARS-CoV-2-infected cells treated with Remdesivir or CO-MT (4 µL/200 µL) show approximately 89% growth inhibition. Values are mean SEM. (C) Immunofluorescence images for the effect of CO on SARS-CoV-2 *in vitro*. Hoechst stained (*blue*) images (10X) indicate the total VeroE6 cell number. Alexa flour-568 stained cells (*orange*) indicate SARS-CoV-2-infected cells stained for viral nucleocapsid protein. Uninfected control cells lack an orange stain. SARS-CoV-2-infected cells treated with remdesivir or CO-MT have low orange stain since both drugs significantly inhibit viral replication. SARS-CoV-2-infected VeroE6 cells treated with CO-MT potencies show a bright orange stain because these potencies have weak antiviral activity. A magnified view of the cells stained with Hoechst and Alexa flour 568 is also provided for reference.

MT and three different potencies (3c, 6c and 12c) were not cytotoxic to VeroE6 cells (►Fig. 3A). Using state-of-the-art immunofluorescence methods to specifically identify SARS- CoV-2 infected cells and viable cells, we showed that CO-MT (dose of 4 μL/200 μL) and remdesivir caused >90% inhibition
of the viral replication in VeroE6 cells. Therefore, the *in vitro*
antiviral potential of CO-MT against SARS-CoV-2 is compa- rable to that of remdesivir. ►Table 2 shows quinoline had a good DS against the spike protein SARS-CoV-2. Quinic acid showed better binding potential with Mpro, PLpro RdRp,
nucleocapsid protein and ACE2 (allosteric site) compared to other constituents. Quinidine exhibited better binding to ACE2. Interestingly, docking scores of quinidine and quinic acid for the Mpro, PLpro, spike, nucleocapsid and RdRp proteins are very similar to those of HCQ and CQ for these proteins. Thus, compared to antivirals which exclusively target the spike protein, CO-MT could potentially be more effective against SARS-CoV-2 variants or mutants because five of its phytoconstituents can bind multiple SARS-CoV-2
target proteins. Therefore, the potent *in vitro* antiviral activ- ity of CO-MTobserved (►Fig. 3C) is strongly supported by the molecular docking data in ►Table 2.

A recent review examined the pathogenesis of systemic conditions of COVID-19 and compared them with the pathophysiological effects of various homeopathic medi- cines. The review concluded that the sphere of action of CO makes it a suitable medication for the relief of COVID-19 symptoms.[21] Our study supports this by establishing *in silico* and *in vitro* data, which provide clear scientific explanations and evidence for the antiviral potential of CO-MT against SARS-CoV-2.

Limitation of the Research

A key limitation of this research is the lack of a suitable experimental COVID-19 animal model at our laboratory to test the efficacy of the CO formulations *in vivo*.

Conclusion

The homeopathic remedy cinchona officinalis not only exhibits potent antiviral effects in VeroE6 cells, but it is also completely nontoxic. According to molecular docking experiments, HCQ, CQ, and certain CO phytoconstituents bind to SARS-CoV-2's primary protein targets (Mpro, PLpro, spike protein, nucleo-capsid protein, and RdRp) with a comparable affinity. The antiviral potential of CO has been proposed by others, and our work provides early evidence supporting this claim. Future in vivo research investigating the therapeutic potential of homeopathic CO for the treatment of COVID-19 infections may be designed using this useful foundation

Promising efficacy against SARS-CoV-2 have been shown by quinine derivatives Cinchona officinalis is a homeopathic preparation that showed promise in our in vitro and computational studies for treating COVID-19 infection.

Cinchona officinalis may have therapeutic promise in COVID-19, however further scientific data is needed to confirm this. Interest Conflic There is no financial or other conflict of interest among the writers. The National Institute of Homeopathy in Kolkata provided funding, and the Task Force of the Ministry of AYUSH in the Government of India gave its approval for the research hank You Notes

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