Anti-SARS-CoV-2 in vitro potential of castor oil plant (Ricinus communis) leaf extract: in-silico virtual evidence

1 Introduction

SARS-CoV-2, the third challenging coronavirus to infect humans in the preceding 20 years, is the cause of coronavirus disease 2019 (COVID-19) [1]. The virus is a member of the family of zoonotic viruses known as Coronaviridae, which also includes other viruses that led to pandemic outbreaks in 2003 and 2012 as a result of the SARS-associated coronavirus (SARS-CoV-2) and the Middle East Respiratory Syndrome coronavirus (MERS-CoV), respectively [2, 3]. Both are highly contagious and can lead to high mortality [4, 5]. The greatest global catastrophe since the Second World War, however, has been the spread of COVID-19. Due to this virus’s quick spread and high level of contagiousness, a worldwide health disaster resulted. SARS-CoV-2 continues to be an issue for human health in terms of morbidity and mortality as well as financially [6]. Among the extra pulmonary organs and tissues that SARS-CoV-2 damages include the heart [7], kidney [8], liver [9] and brain [10]. The virus also has an effect on the ophthalmic system [11], the gastrointestinal system [12], the musculoskeletal system [13], the skin [14] and the cardiovascular system [15, 16]. Because of the virus’s quick spread, scientists and public health organizations are looking into investigate the possibility of repurposing existing drugs for the potential treatment of COVID-19 [17]. Effective treatment for COVID-19 and reduction in mortality rate are the primary aims of clinical treatment. So far, few specific and efficient pharmaceutical therapies for COVID-19 have been established. In October 2020, the FDA formally approved the use of intravenous remdesivir to delay the progression of sickness in hospitalised patients [18]. Recently, the FDA approved the oral antivirals Paxlovid (nirmatrelvir tablets and ritonavir) and molnupiravir for the treatment of COVID-19 in patients who are not hospitalised [19].

Nature provides a vast library of novel chemicals to explore and develop drugs to treat a variety of conditions, including viral infections [20]. Natural products (NPs) or their derivatives make up nearly one-third of all medications on the market [21]. Often, more efficient drugs can be designed based on the structure of natural compounds that display the desired property. NPs derived from plants and microorganisms are known for their antiviral activity via a
variety of mechanisms to prevent infection and boost host immunity [22]. NPs, or secondary metabolites, are a significant source of compounds required to create new antiviral agents. NPs include phenolic acids, terpenes, flavonoids, coumarins, lignans, alkaloids and proteins, all of which are crucial for inhibiting viruses and acting as complementary therapies against viral infection. The low toxicity of herbal medicine further supports screening for anti-COVID-19 agents [23, 24]. Numerous NPs have been tested to determine their effectiveness against coronaviruses and these tests revealed great promise for the treatment of coronavirus illness in terms of their activity against coronaviruses and have shown potential for the treatment of coronavirus infection. Bioactive compounds use a variety of mechanisms to inhibit coronaviruses; inhibition of angiotensin-converting enzyme 2 (ACE-2), 3-chymotrypsin-like protease (3CLpro) and papain-like protease (PLpro) are the three most attractive targets for drug development [25].

Ricinus communis L., commonly known as the castor oil plant, is a flowering plant in the Euphorbiaceae family. This soft-wooded shrub is found across the tropical world [26]. The plant has a global history in traditional medicine spanning thousands of years. Castor oil plant has been used as an anti-irritant, to treat liver infections, stomach-aches [27], flatulence, constipation [28, 29], inflammation, warts, colic, enteritis, fever and headaches. Numerous therapeutic benefits of castor oil phytochemicals include hepatoprotective [30], anti-nociceptive [31], antioxidant [32], anti-cancer [33], anti-inflammatory [34], antidiabetic [35], antifertility [36], antimicrobial [37] and wound healing activity [38]. From a phytochemical perspective, it has been well investigated; alkaloids, flavonoids, tannins, sterols and terpenes, including kaempferol, quercetin, gallic acid, ellagic acid, rutin and ricinin are distinctive constituents [28, 33, 39]. Numerous phytochemicals from R. communis are well-known antiviral agents [40–42] against HSV and Sindbis virus, supporting their traditional use in the treatment of fever and colds [43]. Additionally, four fractional extracts of castor oil leaves, including methanol, methylene chloride, ethyl acetate and butanol, have been shown to have antiviral effects against HAV, COXB4 and HSV [44].

An investigation of medications that target the viral protease revealed that the viral protease was recognized as an appealing target to inhibit COVID-19 replication [24]. 3CLpro, PLpro and spike (S) proteins are the primary targets for anti-SARS-CoV-2 agents [45]. 3CLpro is regarded as an alluring target among coronaviruses since it is essential in the processing of viral polyproteins translated from viral RNA. PLpro is an RNA-dependent RNA polymerase. Human angiotensin-converting enzyme 2 (ACE-2) and S-proteins intimately interact, enabling viral entry into cells. For anti-SARS-CoV-2 potential, certain medication candidates have previously undergone in-silico study. Molecular docking has been used to find phytoconstituents that potentially target the primary protease for the treatment of novel coronavirus (2019-nCoV) [46]. In-silico identification of targets for natural products (NPs) is an efficient, low-cost, time-effective and valuable virtual screening method. The two in silico approaches that are most extensively used are quantitative structure-activity relationship (QSAR) and molecular docking. The latter recognizes target bio macromolecules (DNA, RNA and proteins) for synthetic compounds and NPs [47, 48]. The technique also aids in addressing the processes by which NPs interact with their target proteins and could offer a plan for creating novel medications generated from NPs. Ligand-protein docking is used to develop several NP based drugs [49]. Understanding COVID-19 replication and infection processes using docking may advance the core idea of structure-based drug design. The latter may lead to the discovery of promising COVID-19 antiviral drugs [20].

Thus, the focus of this work is the isolation and identification of constituents of R. communis leaves using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS) and different chromatographic techniques, followed by evaluation of the potential cytotoxicity, anti-MERS-CoV and anti-SARS-CoV-2 activity. Moreover, we demonstrate in-silico anti-COVID-19 potential for inhibiting the main protease and spike protein using molecular docking tools for phytoconstituents and remdesivir.

2 Experimental

2.1 Plant material

R. communis L. leaves (castor oil plant) were collected in June 2019 from Bilbeis, Sharqiyyah Governorate, Egypt (31.1166° N and 30.6333° E). The plant material was collected in compliance with institutional, national and international (WHO) guidelines on good agriculture and collection practice (GACP) for medicinal plants. The planters approved the permit for the collection of leaves specimens and the samples were kindly authenticated by Mrs. Therisa Labib, consultant in plant taxonomy at the Orman Botanical Garden, Giza, Egypt. A voucher specimen (no. R. (03)2017) was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy (for Girls), Al-Azhar University, Cairo, Egypt. The plant name was verified with The Plant List (http://www.theplantlist.org/) on 15/07/2020.

2.2 Preparation of crude extracts

Powdered air-dried leaves of R. communis (1.5 kg) were exhaustively extracted three times at room temperature using 70 % methanol (3000 ml) for three days. The methanol extract was combined and
concentrated under vacuum at 45 °C to afford a brown crude extract (560 g). The concentrated extract was suspended in distilled H2O (700 ml) and filtered using filter paper. The filtrate and washings were combined and defatted with petroleum ether. The process was repeated (three times × 300 ml) and the obtained defatted extract (350 g) was concentrated using a rotary vacuum evaporator. The aqueous concentrated defatted crude extract was successively partitioned into methylene chloride and ethyl acetate fractions by extracting four times with 1500 ml of each solvent sequentially. The remaining aqueous layer was dried under a vacuum and then extracted with 1000 ml of methanol to provide the aqueous methanol fraction. Each fraction was pooled and concentrated under vacuum at a temperature not exceeding 40 °C to produce 70, 55 and 35 g of the methylene chloride, ethyl acetate and methanol fractions, respectively. Active extract constituents were identified using ultra-performance liquid chromatography coupled with hybrid triple time-of-flight mass spectrometry (UPLC-Triple TOF-MS/MS) analysis and column chromatography.

2.3 Isolation and purification using column chromatography

The methylene chloride fraction (30 g) was loaded onto a silica gel column, eluted with 100 % petroleum ether, followed by a gradual increase in polarity using dichloromethane (CH2Cl2). Similar fractions were combined based on thin-layer chromatography (TLC) pattern into four main fractions (A–D). Fraction B (12 g at 50 % Eth. Ether) was further subjected to a series of column chromatographic techniques including silica gel column and eluted with Eth. Ether: CH2Cl2 (95:5–10:90). Final purification used gel filtration (Sephadex LH-20) and elution with 100 % MeOH to produce compound RS [35 mg]. Fraction D (10 g at 100 % CH2Cl2) was loaded onto a silica gel column and eluted with 100 % CH2Cl2 followed by a gradual increase in polarity by adding MeOH until reaching 100 % MeOH. Similar fractions were combined based on TLC pattern into six main fractions (a–f). 100 % CH2Cl2 fraction (a, 4 g) was further subjected to a series of column chromographic techniques, including silica gel column chromatography elution with CH2Cl2: MeOH (95:5–10:90) and final purification by gel filtration (Sephadex LH-20) eluted with 100 % MeOH to produce compound RS1 [20 mg].

2.4 High-resolution UPLC-triple TOF-MS/MS analysis

The methylene chloride fraction was completely dried and then analyzed using a Triple TOF 5600+ System (AB SCIEX, Canada) [50]. LC columns were used for the separation process; IN-Liner filter disks column (0.5 μm × 3 mm; Phenomenex) as a pre-column and X select HSS T3 (2.5 μm, 2.1 × 150 mm; Waters, USA) as a main column. The LC column temperature was set to 40 °C. In 1 ml of mobile phase A working solution (5 mM ammonium formate buffer pH 3 containing 1 % methanol) was added to the plant sample (50 mg) to prepare 20 μl of stock solution (50/1000 μl), vortexed for 2 min followed by ultra-sonication for 10 min, centrifugation for 5 min at 10,000 rpm and then the stock sample was diluted with 1000 μl of reconstitution solvent. The final injected concentration was 10 μg/ml. The eluents were (A) 5 mM ammonium formate buffer pH 3 containing 1% methanol for the positive mode, (B) 5 mM ammonium formate buffer pH 8 containing 1 % methanol for the negative mode and (C) 100 % acetonitrile for the positive/negative mode. They were applied with multi-step linear gradient as follow: mobile phase A or B with gradient increase from 10 to 90 % of mobile phase C over 28 min at a flow rate of 0.3 ml/min to allow comprehensive elution of the different analytes. Analyst software TF 1.7.1 (SCIEX) for LC-QTOF control. Metabolite identification was performed by comparing regions of different classes using the retention time and mass spectrometry data (accurate mass, formula and fragmentation pattern) in both ion modes with those previously reported in the literature [32, 51–53].

UPLC-MS/MS is a sensitive and powerful universal analytical technique for qualitative and quantitative analysis and identification of different classes of secondary plant metabolites. Hence, both negative and positive ionization modes were used to identify R. communis leaf metabolites.

2.5 Preparation of stock solutions of tested plant samples

Stock solutions of the tested plant samples were prepared in 10 % DMSO in ddH2O and stored in stock concentrations of 100 and 5 mg/ml for crude extracts and pure compounds, respectively. Further dilutions for stock solutions were made with DMEM for use in in vitro experiments.

2.6 In vitro studies

2.6.1 Cell line and viruses: African green monkey kidney–derived Vero-E6 cells (ATCC No.CRL-1586) were purchased from the American Type Culture Collection, USA and sub-cultured in DMEM supplemented with fetal calf serum (FCS; 10 % v/v – Gibco), L-glutamine (2 mM) and 2 % antibiotics, antymycotic, penicillin (100 U/ml) and streptomycin (100 μg/ml). After subculture in growth media the cells were used for the antiviral assays with infection media 4 % bovine serum albumin and 2 % antibiotic antymycotic. Propagation of cells for up to four weeks; then, tissue culture flasks were incubated in a 5 % CO2 humidified atmosphere at 37 °C as a cell line. Middle East respiratory syndrome (MERS-CoV) related coronavirus isolate NRCE-HKU270 (Accession Number: KJ477103.2) and severe acute respiratory syndrome SARS-CoV-2 hCoV-19/Egypt/NRC-03/2020 (Accession Number on GISAID: EPI ISL 430820) were propagated on Vero E6 cells as previously reported [54] and harvested after the appearance of cytopathic effects (CPE). The viral stock was titrated using a plaque assay [55] and stored at −80 °C. All experimental work involving the SARS-CoV-2 virus was performed in a biosafety level 3 (BSL3) containment laboratory.

2.6.2 MTT cytotoxicity assay: This assay assesses concentrations of compounds that cause toxicity to 50 % of cells (CC50). Stock solutions of tested compounds were prepared in 10 % DMSO in ddH2O and diluted with Dulebeco’s Modified Eagles Medium (DMEM). Cytotoxic activity of the extracts was tested in VERO-E6 cells using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method with minor modification [56]. Briefly, Vero-E6 cells were cultivated in 96 well-plates (100 μl/well at a density of 3 × 103 cells/ml) and incubated for 24 h at 37 °C in 5 % CO2. After 24 h, cells were treated with various concentrations of the tested compounds diluted with DMEM in triplicates. Twenty four hours later, supernatants were discarded and cell monolayers were washed with sterile 1x phosphate buffer saline (PBS) three times. MTT solution (20 μl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 h followed by media aspiration. In each well, the formed formazan crystals were dissolved with 200 μl of acidified
isopropanol (0.04 M HCl in absolute isopropanol) = 0.073 ml HCl in 50 ml isopropanol). A multi-well plate reader measured the absorbance of formazan solutions at λ max 540 nm with 620 nm as a reference wavelength. Percent cytotoxicity compared to untreated cells was determined as:

\[
\% \text{ cytotoxicity} = \left(\frac{\text{Absorbance of cells without treatment} - \text{Absorbance of cells with treatment}}{\text{Absorbance of cells without treatment}}\right) \times 100
\]

A plot of percent cytotoxicity versus sample concentration was used to calculate the concentration, which produced 50 % cytotoxicity (CC50).

2.6.3 Screening for antiviral activity by plaque reduction assay:
Assays were performed as previously described (55) and modified as (57). Vero-E6 cells (10⁵ cells/ml) were cultured on six-well plates for 24 h at 37 °C. MERS-CoV and SARS-CoV-2 were diluted to give 10⁵ PFU/well and mixed with safe concentrations of tested compounds and subsequently fed to the cells after an hour at 37 °C of incubation. The cell culture plates’ growth media was removed and cells were inoculated with (100 µl/well) viral suspension containing the assessed compounds. After an hour for complete virus adsorption, the cell monolayers were overlaid with 3 ml of DMEM supplemented with 2 % agarose and the tested compounds, then agarose was allowed to solidify and the plates were incubated at 37 °C until the formation of viral plaques, which was observed daily for up to 3–6 days. Formalin (10 %) was added and incubation continued for 2 h, the plates were stained with 0.1 % crystal violet in distilled water. Control wells were included in each plate where untreated virus was incubated with Vero-E6 cells as a negative control, whereas remdesivir (Drug Control and Research, Cairo, Egypt) was used as a positive control for antiviral assays. All experiments were performed in triplicate. Finally, the cells were examined daily and plaques were scored. Percentage of reduction in plaque formation in comparison to control wells was recorded as

\[
\% \text{ inhibition} = \frac{\text{viral count (untreated) - viral count (treated)}}{\text{viral count (untreated)}} \times 100
\]

2.6.4 Inhibitory concentration 50 (IC₅₀) determination: In 96-well tissue culture plates, 2.4 × 10⁴ Vero-E6 cells evenly distributed in each well and incubated overnight at 37 °C in a humidified incubator with 5 % CO₂. Cell monolayers were then rinsed once with 1x PBS and subjected to virus adsorption for 1 h at room temperature (RT). Further, cell monolayers were overlaid with 100 µl of DMEM containing various concentrations of the test compounds and incubated at 37 °C with 5 % CO₂ for 72 h. Cells were fixed with 100 µl of 4 % paraformaldehyde for 20 min and stained with 0.1 % crystal violet in distilled water for 15 min at RT. The crystal violet dye was then dissolved using 100 µl absolute methanol per well and optical density measured at 570 nm using an Anthos Zenyth 200rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The IC₅₀ of the compound is the concentration required to reduce virus-induced cytopathic effects (CPE) by 50 % relative to virus controls (57).

2.6.5 Mode of action of virus inhibition: The possible mode of action of virus inhibition by the selected plant’s extracts was examined at three different stages of the virus propagation cycle and based on three main possible mechanisms of action: (i) Inhibition of budding and viral replication. (ii) The ability of each extract to inhibit the attachment of the virus to infected cells-membrane fusion known as blocking the viral entry (viral adsorption or protective activity) and (iii) the direct effect of each extract to inactivate the virus viability (virucidal activity) (57). For consistency, each mode was repeated three times independently of each other and the mean value of the three experiment of each mode was presented. Additionally, the above-mentioned mode of actions could account for the recorded antiviral activities either independently or in combinations. In this regard, the interaction between the selected extracts of the plant.

2.6.5.1 Post infection treatment (viral replication): In 6-well plate where VERO-E6 cells (10⁵ cells/ml) were incubated for 24 h at 37 °C. The virus was diluted to 10⁵ PFU/well and administered directly to the cells before being incubated for 1 h at 37 °C to ensure complete adsorption. Unabsorbed viral particles have been eliminated by washing cells three successive times with free-medium supplements. After 1 h of contact time with the extract different concentrations, 3 ml of 2X DMEM medium supplemented with 2 % agarose was overlaid to the cell monolayer. Plates were allowed to harden before being incubated at 37 °C until viral plaques appeared. Cell monolayers were fixed in 10 % formalin solution for 2 h and stained with crystal violet. In the control wells, Vero-E6 cells were incubated with the virus but not treated with the extract as (negative control), whereas remdesivir was used as a positive control. Finally, plaques were enumerated and the percentage reduction in plaques formation compared to control wells was recorded (57).

2.6.5.2 Cell pretreatment (protective activity) viral adsorption: This protocol was utilized to check for virus entry into the host cells by preventing adhesion to the cell surface. Vero-E6 cells were cultivated in six-well plates (10⁵ cells/ml) for 24 h at 37 °C for the viral adsorption assay. The plant extract was applied at different concentrations in a 200 µl medium without supplements and co-incubated with the cells (pretreatment) for 2 h at 4 °C. The unabsorbed excess extract was removed by washing cells three successive times with supplements free-medium then virus diluted was co-incubated with the pretreated cells for 1 h followed by adding 3 ml DMEM supplemented with 2 % agarose. Plates were left to harden and then incubated at 37 °C to allow the formation of viral plaques, fixed and stained as above mentioned to calculate percentage reduction in plaques formation in comparison to control wells where untreated Vero-E6 cells were directly infected with the virus (57).

2.6.5.3 Virus pretreatment (virucidal): The virus adsorption assay was carried out in six-well plate where the confluent monolayers of Vero-E6 cells were propagated (10⁵ cells/ml) for 24 h at 37 °C. A volume of 200 µl serum-free DMEM containing virus was added to the nonlethal concentration of the tested extract (1:1v/v). After 1 h of incubation for virus adsorption, the mixture was diluted using serum-free medium three times each 10-fold, which still allows the existence of viral particles to grow on Vero-E6 cells but leaves nearly no extract and 100 µl of each dilution was added to the Vero-E6 cell monolayer. After one-hour contact time,
DMEM over layer was added to the top of cell monolayer. Plates were left to solidify and then incubated at 37 °C to allow the formation of viral plaques, fixed and stained as above mentioned to calculate percentage reduction in plaques formation in comparison to control wells where cells were infected with the virus that was not pretreated with the tested extract [57].

2.7 Molecular docking

Molecular docking used was MOE-Dock 2014 software [58]. Chemical structures of compounds RS, RS1 and remdesivir were drawn with MOE builder, after that minimization was done by using the force field MMFF94x. Water molecules were removed and hydrogen atoms were added. Molecular docking was then accomplished using London dG and GB/VdW dG. Meanwhile, refinement was done by force field. 2D visualization of protein–ligand interactions used “Ligand Interactions” and the best pose was selected based on binding energy and binding interactions at the active site.

3 Results

3.1 Identification of isolated compounds

Two compounds were isolated and identified from R. communis: lupeol and ricinine (Figure S1, Table 1). The isolated compounds were identified using spectroscopic techniques, including ultraviolet-electrospray ionization mass spectrometry, 1H-nuclear magnetic resonance (NMR) and 13C NMR-DEPT 135 (distortionless enhancement by polarization transfer 135).

**Ricinine:** (4-methoxy-1-methyl-2-oxopyridine-3-carbonitrile) (RS1) was isolated as white powder. Positive ESI/MS showed m/z 165.068 [M + H]+ for molecular formula C8H8N2O2 and m/z 138 [M–C2H5]H. The 1H NMR (DMSO-d6, 500 MHz) spectrum (Table 1) showed δ ppm: 6.43 (1H, d, J = 8 Hz, H-5), 8.1 (1H, d, J = 8 Hz, H-6), 3.42 (3H, s, CH3), 3.97 (3H, s, OCH3). 13C NMR (125 MHz, DMSO-d6) spectrum (Table 1) showed δ ppm: 161.36 (s, C-2), 86.14 (s, C-3), 173.09 (s, C-4), 91.48 (d, C-5), 146.58 (d, C-6), 37.19 (q, C-7), 115.14 (s, C-8), 58.07 (q, C-9). The results are consistent with those reported in the literature [59].

**Lupeol:** (RS) it was isolated as white powder. Positive ESI/MS showed m/z 427.18[M + H]+ for C30H50O molecular formula and m/z 137 [M + H–C2O]H3O. The 1H NMR (DMSO-d6, 500 MHz) spectrum (Table 1) showed δ ppm 4.28 (1H, d, J = 4.25 Hz, H-3), 0.773 (3H, s, CH3), 0.876(3H, s, CH3), 0.766 (3H, s, CH3), 0.658 (3H, s, CH3). 13C NMR (125 MHz, DMSO-d6) spectrum (Table 1) showed δ ppm 38.72 (C-1), 27.62 (C-2), 77.25 (C-3), 38.97 (C-4), 55.31 (C-5), 18.42 (C-6), 34.30 (C-7), 40.81 (C-8), 50.29 (C-9), 37.14 (C-10), 20.87 (C-11), 25.14 (C-12), 38.03 (C-13), 42.83 (C-14), 27.45 (C-15), 35.53 (C-16), 43.02 (C-17), 48.24 (C-18), 47.87 (C-19), 150.68 (C-20), 29.65 (C-21), 39.94 (C-22), 28.57 (C-23), 16.16 (C-24), 16.40 (C-25), 16.28 (C-26), 14.80 (C-27), 18.24 (C-28), 110.16 (C-29), 19.42 (C-30). The results are consistent with those reported in the literature [60].

### Table 1: 1H and 13C NMR spectral data for identification of lupeol and ricinine (500/125 MHz, DMSO-d6) [59, 60].

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*Q = quaternary carbon, J = J-coupling value, s = singlet, d = doublet, t = tertiary.*

3.2 UPLC-triple TOF-MS/MS analysis

Retention times, molecular weight and fragment ions for each metabolite and their identities are presented in Table S2 a, b.
3.2.1 Mass spectral data for tentatively identified compounds in *R. communis*

UPLC-MS/MS was performed in positive and negative mode on methylene chloride fraction of *R. communis* leaves (Table S2 a, b). UPLC-MS/MS chromatogram shows the relationship between retention time of metabolites and intensity (Figure S2). Several classes of compounds were identified, including flavonoids, flavonoid glycosides, isoflavonoids, coumarins, alkaloids, phenolic acids, triterpenoids, amino acids and fatty acids. Five classes of flavonoids were identified in UPLC-MS/MS negative mode; flavanones, isoflavone, flavone, flavanol and dihydrochalcone. Flavanone (naringenin) was eluted at 1.39 min. Isoflavone (diadzein-8-C-glucoside) was eluted at 1.26 min. Flavone; luteolin, apigenin, acacetin (4'-methylated apigenin) and acacetin-7-O-rutinoside were eluted at 5.23, 12.01, 15.73 and 13.37 min, respectively. Flavonol; myricetin, queretin, kaempferol-3-O-α-L-rhamnoside, kaempferol-3-O-α-L-arabinoside and 3-methoxy-4,5,7-trihydroxyflavonol were eluted at 1.46, 5.16, 5.41, 7.18 and 13.37 min, respectively. Dihydrochalcone, phlorizin (glucoside of dihydrochalcone) and neohesperidin dihydrochalcone were eluted at 15.22 and 15.18 min, respectively. Most of these compounds were previously reported in *R. communis* [28, 33, 39, 44, 61]. Phlorizin (glucoside of dihydrochalcone), acacetin (4'-methylated apigenin) and acacetin-7-O-rutinoside were identified for the first time in *R. communis*.

In UPLC-MS/MS positive mode, five classes of flavonoids were tentatively identified; flavanone, flavanoneol, isoflavone, flavone and flavanol. Flavanone, 3’,4’,5, 7-tetrahydroxyflavonone and isosakuranetin-7-O-neohesperidoside were eluted at 5.91, 7.24 min, respectively. The flavanone; taxifolin was eluted at 1.39 min, isoflavone (4’-hydroxyisoflavone-7-O-glucoside) was eluted at 1.33 min, Flavone, 3-5 7-trihydroxy-4-methoxyflavone, acacetin (4’-methylated apigenin), acacetin-7-O-rutinoside and 3,4-dimethoxyflavone, acacetin were eluted at 1.4, 4.3, 17.89, 8.25 and 1.4 min, respectively. These latter compounds were among the most active identified compounds in the methylene chloride fraction from *R. communis* leaves via UPLC-MS/MS. Two alkaloids were identified in UPLC-MS/MS positive mode; trigonelline and ricinine which were eluted at 1.38 and 4.25 min, respectively. Ricinine was the major metabolite identified in methylene chloride fraction. Also, coumarins, scopoletin and daphnetin were identified in *R. communis*. Furthermore, lupeol, (a triterpene) was eluted at 5.63 min. The compounds were structurally identified by comparing retention times, high-resolution mass spectrometry data and fragment ions with reference substances and literature data.

### Table 2: Plaque reduction assay for extracts against MERS-CoV (NRCE-HKU270)

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration (µg/ml)</th>
<th>Viral count (PFU/ml) before treatment</th>
<th>Viral count (PFU/ml) after treatment</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>50</td>
<td>$27 \times 10^3$</td>
<td>$22 \times 10^3$</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$25 \times 10^3$</td>
<td>$25 \times 10^3$</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>$30 \times 10^3$</td>
<td>$30 \times 10^3$</td>
<td>0</td>
</tr>
<tr>
<td>Methylene</td>
<td>50</td>
<td>$27 \times 10^3$</td>
<td>$2 \times 10^3$</td>
<td>92.5</td>
</tr>
<tr>
<td>chloride</td>
<td>25</td>
<td>$10 \times 10^3$</td>
<td>$10 \times 10^3$</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>$22 \times 10^3$</td>
<td>$22 \times 10^3$</td>
<td>9</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>50</td>
<td>$27 \times 10^3$</td>
<td>$25 \times 10^3$</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$28 \times 10^3$</td>
<td>$28 \times 10^3$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>$29 \times 10^3$</td>
<td>$29 \times 10^3$</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3 Antiviral assay

3.3.1 Determination of cytotoxicity (CC$_{50}$) and antiviral activity for three fractions [MeOH, methylene chloride and ethyl acetate] against MERS-CoV2 by plaque reduction assay

CC$_{50}$ values for MeOH, CH$_2$Cl$_2$ and ethyl acetate fractions were 17.1, 0.4 and 8.2 mg/ml, respectively. Cytotoxicity tests used Vero-E6 cells and analysis using nonlinear regression analysis of plots of log concentration against percent cell viability with GraphPad Prism software (version 5.01).

Plaque reduction assay data were used to calculate percent inhibition against MERS-CoV for each extract (Table 2). Methylene chloride crude extract exhibited 92.5 % antiviral activity. So the same, this extract was also tested for antiviral activity against SARS-CoV-2 to determine IC$_{50}$.

3.3.2 Antiviral activity of methylene chloride extract against SARS-CoV-2 (NRC-03-nhCoV)

The methylene chloride extract showed promising antiviral activity against SARS-CoV-2, IC$_{50}$ = 1.76 µg/ml with high safety index, SI = 291.5 (Figure S3).

3.3.3 Antiviral activity of pure compounds [lupeol and ricinine] for MERS-CoV and SARS-CoV-2

Methylene chloride extract exhibited promising antiviral activity against MERS-CoV virus. Evaluation of pure compounds was performed against MERS and SARS-CoV-2 and calculated cytotoxicity concentration (CC$_{50}$), inhibitory concentration (IC$_{50}$) and safety index (SI) showed high antiviral activity of ricinine (RS1) against SARS-CoV2 and a significant safety
Percent inhibition for each mode of action is shown in Figure S3. Interestingly, the results showed that the best mechanism of action for antiviral activity was virucidal effect for ricinine and crude methylene chloride extract. Ricinine had a combination of viral inhibitory effect on SARS-CoV-2 at different viral stages. Ricinine showed >99% virucidal effect indicating that it possibly acts directly on the virion causing inactivation. Additionally, it showed 76% inhibitory effect during viral adsorption stage. Furthermore, the crude methylene chloride extract exhibited the virucidal effect with more than a 90% viral inhibitory effect. Lupeol showed almost nearly an approximately 63% inhibitory effect on virucidal as well as virus adsorption. This result confirmed the results of crystal violet assay which gave IC\textsubscript{50} equal 2.5, 1.7 and 19.5 µg/ml for ricinine, crude methylene chloride extract and lupeol, respectively.

### Table 3: Antiviral activity of ricinine and lupeol against MERS- (NRCE-HKU270) and SARS- (NRC-03-nhCoV).

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC\textsubscript{50} (Vero-E6) µg/ml</th>
<th>IC\textsubscript{50} SARS- (NRC-03-nhCoV) µg/ml</th>
<th>IC\textsubscript{50} MERS NRCE-HKU270 µg/ml</th>
<th>SI-SARS</th>
<th>SI MERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricinine</td>
<td>45.753</td>
<td>2.528</td>
<td>87.2</td>
<td>18,098</td>
<td>524.69</td>
</tr>
<tr>
<td>Lupeol</td>
<td>355.2</td>
<td>19.59</td>
<td>5.28</td>
<td>18.13</td>
<td>67.27</td>
</tr>
</tbody>
</table>

### 3.4 Molecular docking

#### 3.4.1 Docking into COVID-19 3CL\textsuperscript{pro}

The main protease (M\textsuperscript{pro}) is a critical enzyme encoded in SARS-CoV-2. The protein is also referred to as 3C-like protease (3CL\textsuperscript{pro}), an essential enzyme in viral RNA translation and maturation. Hence, the protease is crucial for viral infection and replication processes and is thus an attractive therapeutic target for developing anti-coronavirus drugs [62]. In this study we examined the antiviral activity of isolated pure compounds for SARS-CoV-2 via targeting 3CL\textsuperscript{pro} using molecular docking to evaluate molecular binding affinity of both compounds with 3CL\textsuperscript{pro} (PDB ID code: 6LU7). Lupeol and ricinine are anchored closely in the protease enzyme’s active site, with binding energies of −5.342 and −7.875 kcal/mol, respectively (Table 4). By comparison, **remedisvir** displayed a binding energy of −8.602 kcal/mol. Both tested compounds were able to form hydrogen bonds with a key amino acid Cys145. Further, additional amino acid residues; Met49, Gly143, His163 and Ser144, were involved in hydrogen bonding (Figures S6–S8). Lupeol docking indicated one hydrogen bond interaction of the OH group with the Cys145 residue (Figure S6). Ricinine was bound in the active site with four hydrogen bond interactions (Figure S7). Interestingly, the CN group participated in two hydrogen bonds with Gly143 and Cys145 residues, and C=O formed one hydrogen bond with the Ser144 residue. An additional hydrogen bond was formed by the interaction of the methyl group of OCH\textsubscript{3} with the Cys145 residue.

#### 3.4.2 Docking into COVID-19 (S) glycoprotein

SARS-CoV-2 attacks the host cell when the surface spike proteins (S-proteins) recognize and bind to ACE-2 receptors, leading to fusion between the viral envelope and host cell membrane, resulting in successful infection. Active S-protein inhibitors may reduce the severity of such virulent virus. Molecular docking can estimate the binding affinity of isolated compounds with the S-protein receptor-binding domain [63]. Molecular binding modes in the active site of S glycoprotein (PDB ID: 6VXX) identified docking energy scores and hydrogen bond interactions with essential amino acids residues (Figures S9–S11) (Table 5). **Remedisvir** exhibited the highest binding affinity with energy of −7.201 kcal/mol. This drug forms three hydrogen bonds with Lys304, Gln957, all essential amino acids residues. In contrast, **lupeol** showed the lowest binding affinity, −4.103 kcal/mol, via a single hydrogen bond with the Gln957 residue. **Ricinine**
Table 5: Molecular docking results of lupeol, ricinin and remedisvir in the active binding site of SARS-CoV-2 (S glycoprotein) (PDB ID: 6VX0).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Docking score (kcal/mol)</th>
<th>Interacting residues</th>
<th>Type of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupeol</td>
<td>−4.103</td>
<td>Glu309 (1H bond)</td>
<td></td>
</tr>
<tr>
<td>Ricinin</td>
<td>−6.622</td>
<td>Thr961, Lys964 (2H bonds)</td>
<td></td>
</tr>
<tr>
<td>Remedisvir</td>
<td>−7.201</td>
<td>Lys304, Glu309, Gln957 (3H bonds)</td>
<td></td>
</tr>
</tbody>
</table>

affinity, −6.622 kcal/mol, reflected two hydrogen bonds. The C=O group formed a hydrogen bond with the Lys964 residue and the nitrogen atom of the CN group formed a covalent hydrogen bond with the Thr961 residue. Additionally, an arene-H interaction was observed with the Gln957 residue.

4 Discussion

The frequent ineffectiveness of conventional medications in treating viral infections, along with the emergence of distinct viral resistance, has stimulated interest in plants as potential sources of substitute antiviral medicines. Natural compounds with high bioavailability, effectiveness and little cytotoxicity are successful constituents [64]. The efficacy of natural products against COVID-19 is known from previous in vitro studies of natural compounds, including phenolic acids, terpenes, flavonoids, coumarins, lignans and alkaloids. Such compounds may be important in complementary therapy against viral infections and might demonstrate bioactivity against coronaviruses [17, 65].

The current study elucidates the phytochemical profile of R. communis leaf constituents and evaluated the cytotoxicity, anti-MERS-CoV and anti-SARS-CoV-2 activities of three fractional extracts (methanol, methylene chloride and ethyl acetate) and two pure isolated compounds (lupeol and ricinine). The antiviral activity of the methylene chloride extract and two pure constituents was determined by examining the anti-replicative, protective and anti-infective effects of the extract in infected cells in culture. Each experiment was performed three times for consistency. Cytotoxic concentrations (CC_{50}) for each assay were calculated and compared with remdesivir as a positive control.

R. communis leaves are rich in phytochemicals; extracts revealed substantial content of phenolic compounds, flavonoids, terpenes, and alkaloids. Isolation of kaempferol, quercetin, their O-3-glycosides, naringenin, tannins and the alkaloid ricinine from R. communis is reported [27]. Numerous phytochemicals from R. communis are well-known to be antiviral agents [40–42, 44, 66]. In this study, UPLC-QTOF-ESI-MS/MS negative and positive modes were used to analyze the methylene chloride fraction of R. communis leaves; different classes of flavonoid and phenolic acid compounds were tentatively inferred. Most of these compounds were previously reported in R. communis [28, 33, 39, 44, 61], Phlorizin (glucoside of dihydrochalcone), acacetin (4′-methylated apigenin) and acacetin-7-O-rutinoside were identified for the first time in R. communis.

Interestingly, the results showed that the optimal mechanism of action for antiviral activity was the virucidal effect of ricinine and crude methylene chloride extract. Ricinine displayed a variety of viral inhibitory effects on SARS-CoV-2 at different viral phases. Since ricinine has a >99% virucidal effect, indicating that it possibly acts directly on the virus causing inactivation. Additionally, it demonstrated a 76% inhibitory effect during the viral adsorption stage. Furthermore, the crude methylene chloride extract exhibited the virucidal action with a viral inhibition value of more than 90%. Lupeol showed almost nearly a 65% inhibitory effect on virucidal activity as well as virus adsorption.

Among the three different extracts, the methylene chloride extract of R. communis leaves demonstrated the strongest in vitro activity against MERS-CoV. Viral replication was reduced by 92.5% (Table 3). Further, the anti-SARS-CoV-2 virucidal activity of this extract was over 90% (CC_{50} = 513.1 μg/ml and IC_{50} = 1.76 μM) (Figures S3 and S5). This activity might be attributed to the substantial flavonoid, phenolic acid, terpene and alkaloid content identified in this fraction. This interpretation is consistent with our results with molecular docking studies that suggest good affinity of two constituents of R. communis extract SARS-CoV-2. Substantial evidence supports the significant antiviral properties of pure flavonoids and flavonoid-containing plant extracts against MERS-CoV and SARS-CoV-2. According to molecular docking studies, the polyphenols from Curcuma (curcumin and its derivatives) and citrus sp. (hesperetin, hesperidin) exhibit a greater affinity for the S-protein than the reference compound, nafamostate [67]. Compared to remdesivir, naringenin demonstrated greater binding to viral spike proteins [68]. Flavonoids, such as kaempferol and iso-liquiritigenin, synergistically inhibited the SARS-CoV-2 M^{pro} and PL^{pro} in vitro [69]. In a computational analysis of a library of 80 flavonoids from natural phenolic compounds, certain flavonoids showed potential for binding to the active site of the SARS-CoV-2 major protease. The greatest binding affinity was demonstrated by hesperidin [25]. Also, rutin showed notable inhibitory activity against SARS-CoV-2 primary protease in silico. The flavonoids, myricitrin, baicalin and scutellarin, exhibited potent binding to SARS-CoV-2 M^{pro}. It’s interesting to note that these compounds also showed...
strong binding with other potential SARS-CoV-2 targets, such as viral receptor ACE-2 and block entry of the virus [70, 71].

Additionally, epigallocatechin gallate, gallocatechin gallate and quercetin were discovered to be powerful inhibitors of the SARS-CoV 3CLpro by in vitro and in silico analysis. Several phenolic acids, including caffeic acid, para-aminobenzoic acid, 4-methoxy cinnamic acid, 3,4-dimethoxycinnamic acid and chlorogenic acid were among the most efficient compounds identified in the methylene chloride extract and examined for their antiviral activity. Moreover, the antiviral effects of gallic acid, chlorogenic acid, quinic acid and caffeic acid were studied and they demonstrated a considerable efficacy against HSV-1, HSV-2 and influenza virus [40, 72]. Further analysis of the caffeic acid in elderberry stem extracts suggested that binding to S-protein may hinder HCoV adhesion to host cells [73].

UPLC-MS/MS analysis in the present study identified several alkaloids and terpenes in the methylene chloride extract of *Ricinus* leaves, including isolation and identification of two pure compounds, the triterpene lupeol and the alkaloid ricinine were eluted at 5.63 min and 4.25, respectively. Ricinine, a natural pyridine alkaloid, showed superior virucidal and adsorption activities against SARS-CoV-2 (IC₅₀ = 2.528 μg/ml, SI = 18,098) compared to MERS-CoV (IC₅₀ = 87.2 μg/ml) (Figure S4). These results fully agree with many previous studies of triterpene and alkaloid antiviral activity against SARS-CoV-2. The activity of pure terpenes has also been extensively studied [17, 20, 74, 75]. Glycyrrhizin, the main triterpenoid derived from licorice (*Glycyrrhiza glabra* L.) roots, is a powerful inhibitor of SARS-CoV replication in Vero cells, with a selectivity index of 67. Although it had a low selectivity index, it dramatically reduced the ability of all tested viruses to replicate while exhibiting little harmful effects [76]. Moreover, terpenoids from *Tripterygium regelii*, including celastrol, pristimerin, tingenone and iguesterin, inhibited SARS-CoV 3CL_pro [77]. Tanshinone IIA, tanshinone IIB, methyl tanshinonate, cryptotanshinone, tanshinone I, dihydrotanshinone I and rosmariquinone are examples of abietane-type diterpenoids isolated from ethanol extracts of *Salvia miltiorrhiza* that inhibited SARS-CoV 3CL_pro as well as PI_pro. It is interesting to note that this inhibition was selective because no inhibition of other proteases was observed [78]. To our knowledge, there are no prior reports of lupeol's antiviral activity against CoVs. However, EGVIR extract (a curcumin-piperine infusion) demonstrated immunomodulatory activity in *vitro* during the SARS-CoV-2 infection. Lupeol comprised 8.86% of the infusion constituents and is probably directly or indirectly responsible for some of its overall antiviral activity [79].

Numerous alkaloids have been investigated for their anti-coronavirus activity. Emetine can prevent MERS-CoV entrance into host cells and both lycorine and emetine alkaloids exhibit inhibitory effect against coronavirus replication in vitro [80]. Numerous alkaloids are specifically active against SARS-CoV [17, 20, 75]. Indigo is an alkaloid isolated from *Isatis indigotica* and can block the cleavage of 3CL_pro [81]. Most previously mentioned phytochemicals from different classes (flavonoids, phenolic acids, terpenes and alkaloids) were identified in *Ricinus* leaves; this literature reinforces our recent finding of significant antiviral activity of the methylene chloride extract and two leaf compounds. The likely culprits behind these outcomes are phenolics, terpene and alkaloid compounds.

Numerous studies have revealed that plant derived natural compounds might suppress SARS-CoV-2 replication by blocking 3CL_pro activity [21, 80]. Our findings show that ricinine binds to 3CL_pro with high affinity, −7.875 kcal/mol, due to the formation of four hydrogen bonds. Interestingly, the CN group participated in two such bonds with Gly143 and Cys145 residues and C=O formed a hydrogen bond with the Ser144 residue. The remaining hydrogen bond formed between the methyl group of OCH3 and the Cys145 residue. Cys145 is a critical amino acid in the catalytic dyad of SARS-CoV-2 3CL_pro. 3CL_pro is, thus, a promising anti-SARS-CoV-2 target. Lupeol showed a lower binding affinity for protease 3CL_pro, −5.342 kcal/mol. This terpene formed one hydrogen bond between the OH group and the Cys145 residue, suggesting anti-SARS-CoV-2 activity.

Several natural compounds block SARS-CoV entry into host cells. Lupeol in this study showed a binding affinity for −4.103 kcal/mol via a single hydrogen bond interaction with the Glu309 residue of the S-protein. Ricinine showed a higher relationship, −6.622 kcal/mol and two hydrogen bonds. The C=O group formed a bond with the Lys964 residue and the nitrogen atom of the CN group participated in two such bonds with Gly143, −5.342 kcal/mol. This terpene formed one hydrogen bond between the OH group and the Cys145 residue, suggesting anti-SARS-CoV-2 activity.

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5 Conclusions

This study is the first, to our knowledge, to show *in vitro* activity against SARS-CoV-2 and MERS-CoV by constituents in *R. communis* leaves, including two pure isolated compounds.
This beneficial activity might be due to various phytochemicals, particularly in the methylene chloride fraction of leaf extracts. This fraction showed the highest antiviral activity in MERS-CoV plaque assays. The most common compounds isolated and identified from the methylene chloride extract were ricinine and lupeol. The latter, a natural triterpene, showed the greatest activity against MERS-CoV. In addition to, almost nearly moderate an inhibitory effect on virucidal as well as virus adsorption. While ricinine, a natural alkaloid, showed superior virucidal antiviral activity against SARS-CoV-2. Ricinine exhibited the greatest affinity for two critical targets for anti-SARS-CoV-2 activity in silico and to be the most biologically active. These results might be a promising basis for developing these constituents or their derivatives as antiviral agents for the treatment of coronavirus infections. Additional research will be required to assess the effects of chemical modification of their structures, guided by the molecular docking tools and evaluate their in vivo activity to determine safe therapeutic levels before clinical trials in humans can be considered.

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Author contributions: Rawah H. Elkousy and Zeinab N.A. Said conceived and designed the research proposal. Mohamed Ali and Omnia Kutkat contributed to the design of the virology experiments, the latter performing the practical part. Rawah H. Elkousy and Salwa A. Abu El Wafa participated in the practical chromatographic section and contributed to data collection. Rawah H. Elkousy, Omnia kutkat and Salwa A. Abu El Wafa analyzed the data during the experiments and interpreted the results. Rawah H. Elkousy and Salwa A. Abu El Wafa wrote the manuscript with assistance, reviewing, editing and feedback from all. Zeinab N. Said and Mohammed Ali checked the theoretical results and approved the manuscript as the final version. All authors have read and approved the final manuscript.

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Ethics approval and consent to participate: The plant material was collected in compliance with institutional, national and international (WHO) guidelines on good agriculture and collection practice (GACP) for medicinal plants. The planters approved the permit for the collection of leaves specimens. Neither animals nor human used in this study (Not applicable).

Patent: This work has been submitted for patent application to patent office Academy of Scientific Research and Technology, Egypt under number 2022040600106.

Data availability: The manuscript includes all the information needed to support the study’s conclusions.

References


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