Investigating the anti-SARS-CoV-2 activity of caffeic acid: A combined network pharmacology and laboratory study

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Abstract. The COVID-19 pandemic necessitated the urgent identification of effective antiviral agents, with natural compounds such as caffeic acid (CA) emerging as candidates based on broad-spectrum antiviral action. While prior computational studies suggested prospective therapeutic activity against SARS-CoV-2, a systematic, integrated study validating CA's multi-target mechanism and functional efficacy was lacking. This study was to explore and validate the molecular targets and antiviral efficacy of caffeic acid against SARS-CoV-2. Network Pharmacology (NP) was employed using GeneCards, DisGeNET, and OMIM to identify multiple inflammation and entry-related targets including ACE2, TNF-α, and NLRP3. PPI analysis revealed a highly connected network, predicting that CA targets the Renin-Angiotensin System (RAS) and key inflammatory mediators. Caffeic acid showed weak antiviral activity against SARS-CoV-2 in vitro, with an IC50 of 278 μM and SI > 1, which indicates weak activity according to antiviral screening standards and pharmacologically low potency. The predicted pathways may indeed be valid targets, but caffeic acid did not specifically affect the mechanisms contributing to the antiviral response. The weak activity measured may be due to non-specific interactions and not the pathways targeted by the predictions.

1 Introduction

The World Health Organization (WHO) declared COVID-19 as a global pandemic on March 11, 2020 [1]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel member of the genus Betacoronavirus capable of infecting humans and diverse type

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of animal. The virus possesses a positive-sense, single-stranded RNA genome and exhibits high pathogenicity, primarily targeting cells in the respiratory tract. This can result in respiratory illnesses that can escalate to severe and potentially life-threatening conditions [2].

According to WHO Dashboard data as of October 26, 2025, 136,000 new cases were reported, representing a substantial decrease of 29,716 cases compared to the preceding 28-day period [3].

The potential role of caffeic acid as an antiviral compound targeting SARS-CoV-2 was supported by studies using molecular docking, dynamic simulations, and ADMET predictions. Caffeic acid has been shown to interact with key viral proteins, suggesting the compound's therapeutic potential in medicating COVID-19.

Numerous phytochemical substances may have therapeutic benefits against SARS-CoV-2, possibly mitigating the onset and severity of COVID-19. Caffeic acid (CA), a natural substance present in coffee, exhibits several advantageous biological effects such as antiviral properties. Caffeic acid is a commonly occurring plant-derived polyphenol featuring two phenolic hydroxyl groups, and it is frequently found in coffee, fruits, and vegetables [4]. The compound demonstrates significant virucidal action toward the herpes simplex virus [5]. The COVID-19 outbreak has reinforced the urgent need to discover effective adjunct antiviral therapies, shifting research attention toward natural sources such as phytochemicals; within this context, caffeic acid has emerged as one of the strong candidates based on its broad-spectrum antiviral action. However, the state-of-the-art shows a gap in studies where no integrated study has systematically mapped and validated a multi-target mode of action of caffeic acid against SARS-CoV-2 through an integration of Network Pharmacology and functional in vitro assays. This research was intended to explore and validate the molecular targets and virus-inhibiting potency of caffeic acid against SARS-CoV-2 replication, to support the development of this compound as a prospective anti-SARS-CoV-2 agent.

2 Material and methods

2.1 Materials

Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) that was enhanced with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), 1% penicillin–streptomycin (GIBCO), and 2 mM L-glutamine (Sigma-Aldrich) at a temperature of 37 °C in an atmosphere containing 5% CO_2. The SARS-CoV-2 isolate, Caffeic acid with catalog number C0625 (Sigma-Aldrich), dimethyl sulfoxide (DMSO; Sigma, D-2650), MTT Cell Proliferation Assay Kit (Sigma), and semi-solid medium (7% carboxymethyl-cellulose in DMEM 1× with 1% Penicillin–Streptomycin) were used. Viral genomes were detected using the QIAmp Viral RNA Mini Kit (Qiagen) and One Step TaqMan real-time RT-PCR with the RT-PCR One kit Step (Thunderbird, Toyobo).

2.2 Network pharmacology

Network pharmacology entails the collection of target genes linked to caffeic acid compounds from the GeneCards database (https://www.genecards.org/) and the acquisition of genes associated with diseases, particularly coronavirus disease, from the DisGeNET database (https://www.disgenet.org/) and OMIM (https://www.omim.org/). Data were extracted from OMIM using the specific keyword "COVID-19", then genes that have strong phenotypic associations with severe acute respiratory syndrome, severe inflammatory response, or related organ dysfunction reported in the COVID-19 clinical literature were collected. A Venn diagram (https://bioinformatics.psb.ugent.be/webtools/Venn/) is employed to visualized the intersection of target genes between caffeic acid and coronavirus disease. Determination of overlapping genes between disease-related and compound-targeted genes using Cytoscape. Using these visualizations, a protein-protein interaction (PPI) network was constructed with STRING (https://STRING-db.org/). Confidence score cut off is determined with a score >0.700, with interaction sources namely experimental data, text mining, databases, and contextual predictions (such as co-expression), to build a comprehensive network. Following this, signaling pathway analysis is performed utilizing the KEGG Pathway (https://www.genome.jp/kegg/pathway.html) and Gene Ontology (GO) (http://geneontology.org/).

2.3 In vitro testing

2.3.1 Cytotoxicity testing

Vero cells were removed from the CO2 incubator and their conditions were observed using an inverted microscope. There were six treatment groups, each with three replicates. The treatment group consisted of cells treated with the compound, whereas the control group contained cells and media. Subsequently, 100 µl of culture medium was added to the control cell wells. A total of 100 µl of the prepared concentration series was dispensed into each well. The treated cells were kept at 37°C for a duration of 24 h. Subsequently, 100 µL MTT solution was poured to each well. The microplate underwent incubation for 4 h at 37°C. After formazan formation, 100 µL DMSO (stop solution) was dispensed to each well and absorbance was measured using a Glomax Microplate Multidetection Reader (Promega) at 595 and 750 nm.

2.3.2 Antivirus activity test

The study presented by Ohashi et al. [6] assessed the viral-inhibiting properties of caffeic acid through both pre- and post-infection treatment methods. Vero cells were placed in 6-well plates at a density of 2×10^5 cells per well, then left to incubate for 24 h at 37°C with 5% CO_2. Caffeic acid was applied in serial dilutions ranging from 1.25 to 10 µg/mL, with 50 µL added to each well for an hour. Following this, the caffeic acid-containing medium was removed, and the virus was introduced at a concentration of 400 PFU/ml or 200 TCID50 in 50 µL of DMEM with 2% FBS. After allowing the virus to absorb for an

hour at 37°C, the inoculum was removed and replaced with 150 µL per well of serial dilutions of caffeic acid in DMEM. The plates were subsequently incubated 48 h at 37°C with 5% CO_2.

2.4 Polymerase chain reaction (PCR)

Over a span of three days, the viral RNA was isolated from samples placed in 24-well microplates using the QIAmp Viral RNA Mini Kit from Qiagen, GermanyThis synthesized RNA was additionally used to generate a standard curve for one-step RT-PCR, enabling the conversion of cycle threshold (Ct) values into RdRP RNA copy numbers. The RT-PCR was performed using an Applied Biosystems 7500 Fast instrument and its dedicated software (Applied Biosystems, USA).

2.5 Quantification of antiviral activity by plaque assay

Vero cells, at a density of 2×10^5 cells per well, were plated in 6-well plates and incubated for 24 hours at 37°C with 5% CO_2. Following this, serial 10-fold dilutions of supernatants obtained from each antiviral assay (200 µL per well) applied to the cell monolayers and allowed to incubate for 1 hour at 37°C in 5% CO_2. After removing the virus inoculum, 1 mL of a semisolid medium (comprising 7% DMEM 1×, 2% FBS, and 1% penicillin-streptomycin) was addedAfter incubation at 37°C for 3 days, the cells were washed twice with PBS and stained using 4% formaldehyde/1% crystal violet, and counted. The percentage of inhibition was calculated by comparing the viral titers after caffeic acid treatment to those of the untreated control. Each treatment was performed in triplicate.

2.6 Statistical analysis

The cytotoxicity concentration 50% (CC50) was calculated through probit analysis. The PCR results provided the CT value, which was quantified as the viral load (copy number/µL) and analyzed with a regression curve to determine the IC50 value. The percentage of viral compound inhibition was assessed using plaque assay results. Statistical differences were examined using one-way ANOVA, following the Shapiro–Wilk normality test. A p-value of ≤ 0.05 was considered statistically significant. Experimental data were processed using Statistical Package for the Social Sciences (SPSS) version 24. The selectivity index (SI) was derived from the ratio of CC50 to IC50.

2.7 Ethical approval

The Ethics Committee for Health Research at the Faculty of Medicine at UIN Maulana Malik Ibrahim granted approval for this study (certificate number:105/40/EC/KEPK-FKIK/VI/2025).

3 Result and discussions

3.1 Network Pharmacology

The Venn diagram analysis in Figure 1(A) shows that of the 322 caffeic acid target genes linked to 91 COVID-19 target genes, nine potential target genes overlapped, demonstrating a relationship between the genes modulated by the compound and the disease-associated target genes. The nine potential target genes were ACE, TNF-α, NLRP3, ACE2, CCL3, AGT, CCL2, C3, and SELE.

STRING analysis yielded 9 nodes and 30 edges (Figure 1(B)), constituting a highly connected network, indicating that the proteins targeted by caffeic acid do not function independently but are part of an interdependent functional module crucial to the body's response to SARS-CoV-2 infection. Caffeic acid exhibits potential as an antiviral agent through its action on renin angiotensin system (RAS) proteins associated with viral infection. and organ injury. It also has potential as an anti-inflammatory agent that targets key inflammatory mediators. ACE, ACE2, and AGT are the core components of the RAS. The association of caffeic acid with these genes suggests the potential of the compound to modulate RAS, potentially disrupting viral entry or reducing ACE-mediated organ damage. TNF-α, NLRP3, CCL3, CCL2, and SELE proteins participate in the regulation of inflammatory processes and immune cell recruitment. The interaction of CA with these proteins suggests its potential as an anti-inflammatory agent. By inhibiting TNF-α or NLRP3, this compound may help mitigate potentially lethal cytokine storm in COVID-19 patients.

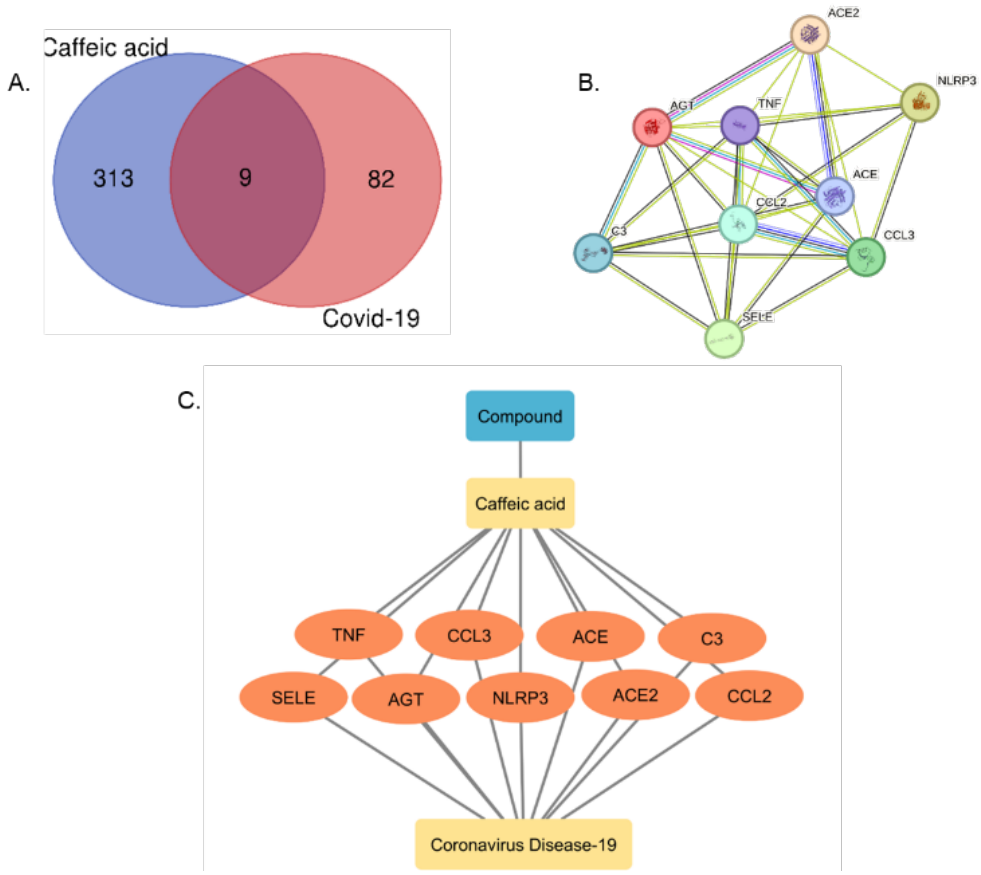

Fig. 1. A) Venn diagram illustrating intersecting target genes, B) PPI Interaction Analysis (9 nodes, 30 Edges, PPI enrichment p-value: 3.61e-13), C) Network visualization of caffeic acid with Covid-19 (Nodes 12, edges 19) (yellow: compound name, orange: target protein, dark yellow: Disease).

Target genes were visualized using the Cytoscape software. The Figure 1(C) displays the visualization results, which presents the relationships between the targets. There are nodes and edges, where nodes represent target genes, whereas edges (strings) indicate the interaction between target genes. Cytoscape results for caffeic acid in COVID-19 revealed 12 nodes and 19 edges.

Based on the identification results, the KEGG signaling pathway was identified as Coronavirus Disease 19 (COVID-19) (hsa05171) with a network count of 6 of 9. This pathway is associated with the caffeic acid target gene, with the highest number of protein interactions compared to other pathways, and is associated with COVID-19.

Based on the KEGG pathway analysis results shown in Figure 2, six related targets have significant potential for treating Covid-19. These six pathways included ACE, TNF, NLRP3, C3, ACE2, and CCL2. These target genes are associated with various pathways, including the renin-angiotensin system, downregulation of ACE2, TNF signaling, FcγR-mediated phagocytosis, signaling via Toll-like and NOD-like receptors, as well as the alternative, classical, and lectin pathways. Furthermore, Figures 3 shows gene ontology

bar graphs illustrating biological processes, cellular components, and molecular functions. Figure 4 shows bar diagram of the pathway analysis.

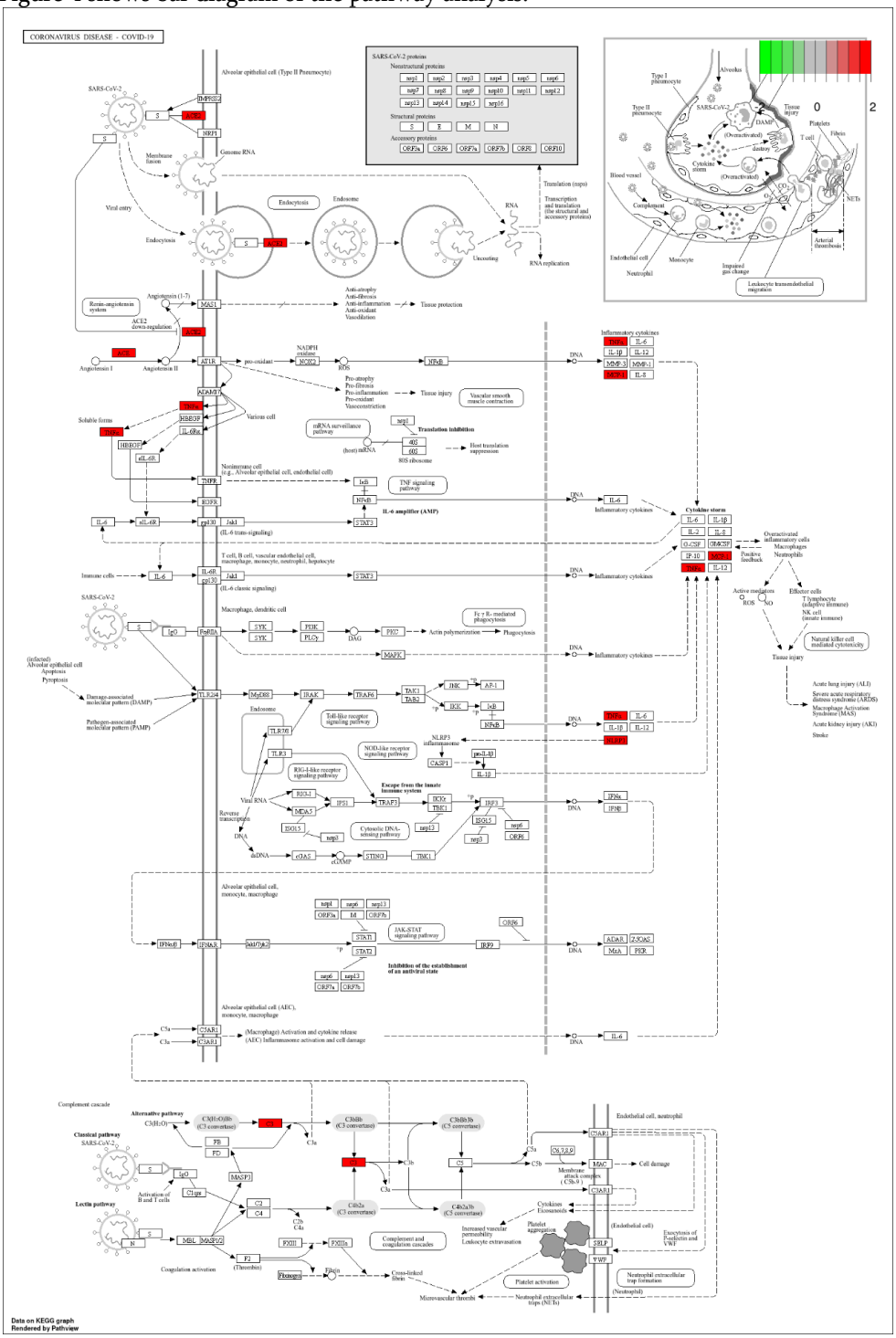

Fig. 2. Coronavirus diseases-19 pathway (hsa05171).

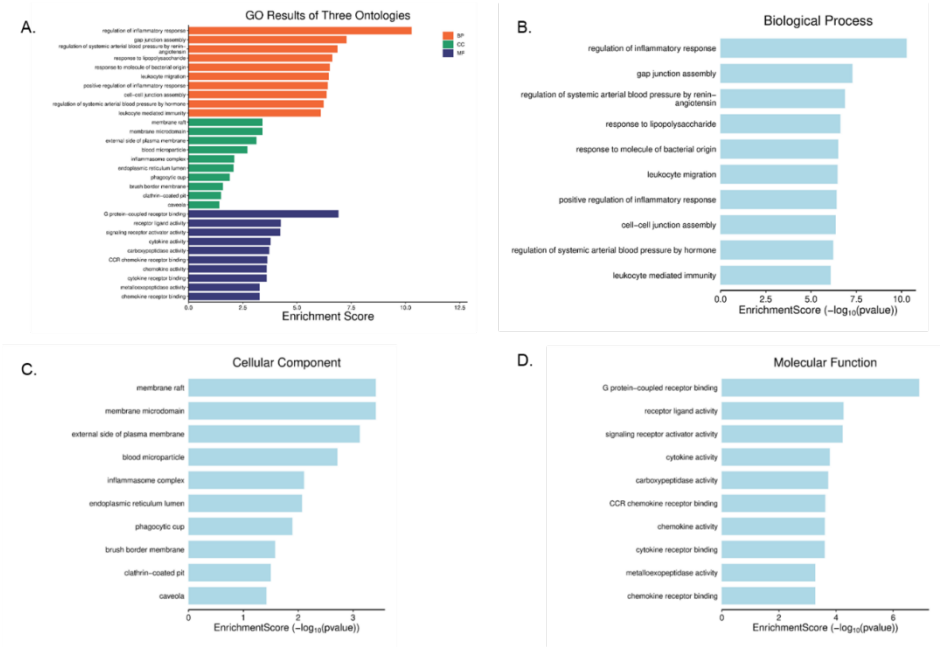

Fig. 3. A) Gene ontology (GO) bar graphs illustrating biological processes, cellular components, and molecular functions B) Bar diagram of the 10 biological processes with the highest potential; C) Bar diagram for the 10 cellular component with the greatest potential; D) Bar diagram for the 10 molecular function with the greatest potential.

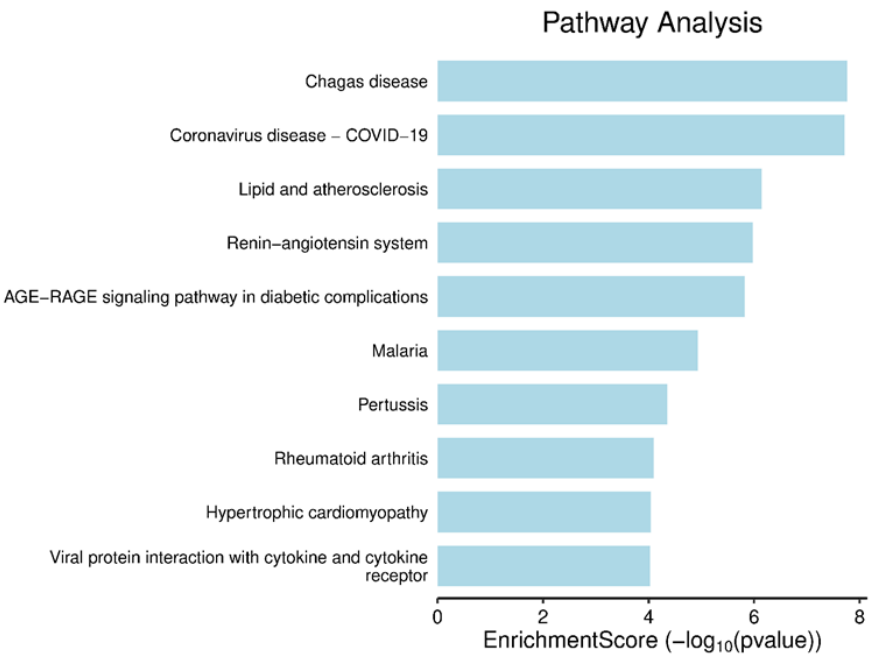

Fig. 4. Bar diagram of the pathway analysis.

The RAS is contributing in the process by which SARS-CoV-2 enters host cells, and the potential influence of active compounds on this pathway could be crucial in developing COVID-19 treatments. Angiotensin Converting Enzyme 2 (ACE2) acts as the receptor through which SARS-CoV-2 enters host cells and also functions as a component of the protective RAS pathway. This has led to investigations into RAS inhibitors and their possible impact on COVID-19 [7].

Caffeic acid shows promise with its therapeutic activities targeting viruses and inflammation, which may act against SARS-CoV-2 through interactions with the tumor necrosis factor (TNF) signaling pathway, an essential regulator of the inflammatory response and significantly heightened during COVID-19. This pathway plays a role in controlling inflammation as well as immune responses, thereby rendering it a target for caffeic acid's potential therapeutic effects against SARS-CoV-2. The infection of SARS-CoV-2 is known to disrupt several cytokine and signaling pathways, including TNF, leading to a severe inflammatory reaction often termed a "cytokine storm." Caffeic acid may exert its inhibitory effect on SARS-CoV-2 through modulating the TNF-dependent signaling pathway, thereby mitigating the excessive inflammatory response characteristic of critical cases COVID-19 [8].

By recognizing pathogen-associated molecular patterns (PAMPs), TLRs activate downstream pathways in the innate immune system, leading to the production of pro-inflammatory cytokines including interferons. This reaction is vital for initiating antiviral defenses. The activation of the TLR pathway, especially TLR2, TLR3, TLR4, and TLR7, has been implicated in SARS-CoV-2 infection. These receptors recognize viral elements, such as RNA, resulting in stimulation of signaling cascades responsible for producing IL-

1, IL-6, and TNF-α, which assist in managing viral infection by boosting antiviral activity. Caffeic acid might support a balanced immune response by reducing excessive inflammation, aiding viral clearance without causing significant inflammatory damage [9].

NOD-like receptors, especially the NLRP3 inflammasome, serve a key function in identifying PAMPs associated with viral infections such as SARS-CoV-2. When NLRP3 is activated, it results in the release of pro-inflammatory cytokines like IL-1β and IL-6. These cytokines are crucial for triggering the immune response, yet they may also drive the cytokine storm observed in patients with severe COVID-19 [10].

Regarding the inhibition of SARS-CoV-2 by caffeic acid, the classical, alternative, and lectin complement pathways signify different immune response mechanisms. The classical pathway is initiated with C1q attaching to the antigen-antibody binding complex, leading to the activation of a sequence of complement proteases that culminate in the creation of a membrane attack complex (MAC) to neutralize the pathogen [11]. During COVID-19, the excessive activation of this pathway may lead to tissue injury and exacerbate the clinical situation. Blocking the classical pathway can avert harm from complement components, while preserving other immune reactions.

The lectin pathway is initiated when mannose-binding lectin (MBL) attaches to carbohydrate structures on the surface of the pathogen, triggering a cascade of proteases, such as MASP-1 and MASP-2, which play crucial roles in the complement response. This pathway can be straightforwardly triggered by SARS-CoV-2, potentially resulting in excessive complement activation, particularly when the viral nucleocapsid interacts with MBL [12].

Caffeic acid may help regulate excessive activation of this complement pathway; however, understanding its mechanism related to SARS-CoV-2 requires further research. The classical, alternative, and lectin pathways contribute to the immune response that can be detrimental in COVID-19; hence, therapeutic agents such as caffeic acid might help alleviate complement-mediated harm by inhibiting or modulating these pathways.

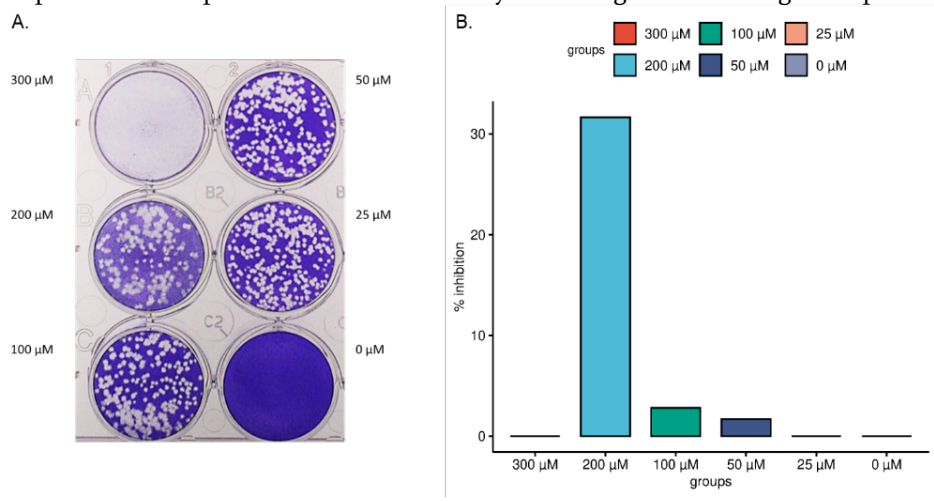

Fig. 5. Caffeic acid shows virus-inhibiting effects on properties against SARS-CoV-2 via treatments administered before and after infection. (A) Plaque assay on Vero E6 cells following pre- and post-infection SARS-CoV-2 exposure. (B) The image illustrates a reduction in the

inhibition percentage in Vero E6 supernatant following the pre-post-infection treatment with caffeic acid (n = 3). Inhibition percentages of 31.64%, 2.82%, 22.2%, and 1.69% were obtained at caffeic acid concentrations of 200, 100, and 50 µM, respectively.

The plaque assay demonstrated 31.64% inhibition of SARS-CoV-2 administered at 200 µM caffeic acid (Figure 5).

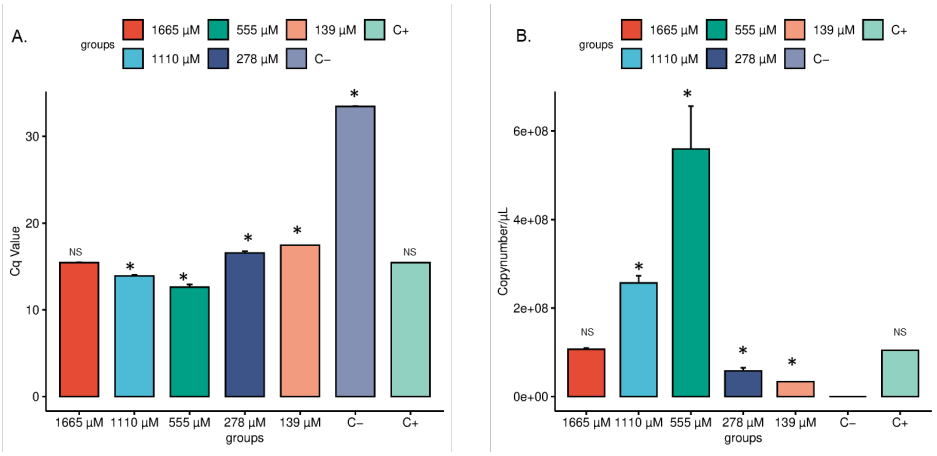

Fig. 6. Anti-SARS-CoV-2 activity of caffeic acid using the Polymerase Chain Reaction method. (A) Graph of Ct values based on the treatment groups. (B) Graph of viral load (copy number/µL) based on the treatment groups. The data were derived from three independent replicates. *P < 0.05; NS: not significant.

The results of the statistical analysis of antiviral efficacy using the PCR technique, presents as Figure 6, showing the Cq value along with the copy number/µL, with the RdRp gene as target. The results of the normality and homogeneity assessments for the Cq value variable indicated that the p-value was >0.05, indicating that the data exhibited a normal distribution and that the data variation was homogeneous. On all days of observation, the difference test revealed a significant variation ($p<0.05$). Post-hoc evaluation indicated a notable disparity between the negative control group and the concentrations of 1110, 555, 278, and 139 µM ($p<0.05$). Conversely, No significant difference was observed between the positive control group and the 1665 µM caffeic acid group ($p>0.05$). Furthermore, the Probit test results determined that the IC50 of Caffeic acid as an anti-SARS-CoV-2 agent was 278 µM.

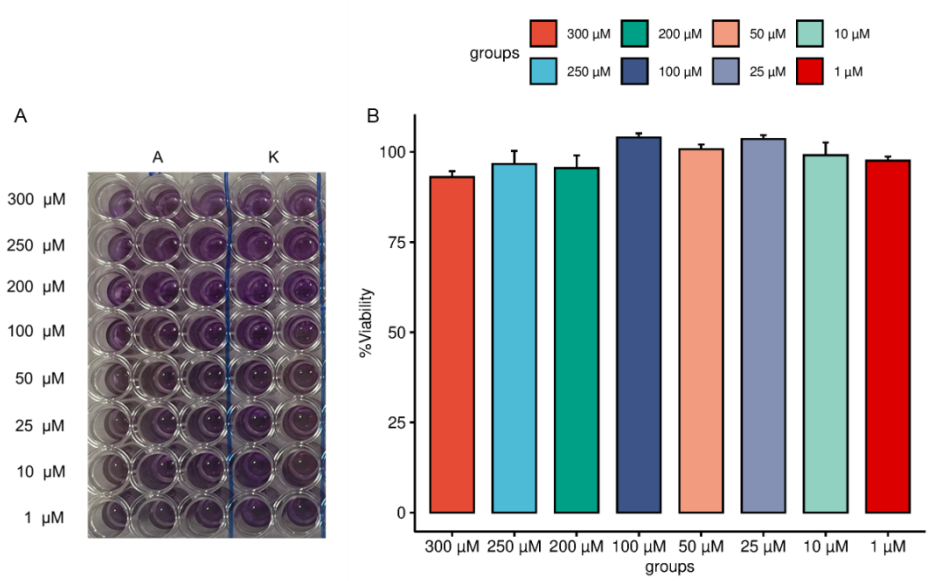

Fig. 7. (A) Results of the cytotoxicity test of caffeic acid on Vero cells. (B) Graph of % viability of Vero cells.

The cytotoxic test results (Figure 7) for the caffeic acid compound in Vero cells indicated that the IC50 was >300 µM. Thus, it can be inferred that the selectivity index of caffeic acid was greater than 1. Additionally, plaque assays revealed a 31.64% decrease in SARS-CoV-2 at 200 µM caffeic acid.

Caffeic acid exhibited an IC50 level of 278 µM against SARS-CoV-2, indicating that relatively high concentrations were required to inhibit the virus by 50%. This value indicates weak antiviral activity compared to other more potent compounds that typically achieve similar inhibition at much lower concentrations. Furthermore, the selectivity index (SI) of caffeic acid was reported to be greater than 1. While SI >1 generally indicates some degree of selectivity toward inhibiting viral activity rather than causing cytotoxicity, an SI slightly above 1 indicates a narrow therapeutic window. This means that the compound begins to exhibit toxic effects at concentrations not much higher than those required for antiviral activity. In the context of antiviral screening, both IC50 and SI values indicate that caffeic acid, while biologically active, may have low pharmacological potency as an antiviral agent for SARS-CoV-2. As a result of effective antiviral compounds typically exhibit lower IC50 values alongside high SI values, indicating efficacy and safety. Caffeic acid may still be valuable in combination therapy or as a lead compound for further structural optimization to increase potency [13].

Caffeic acid suppresses the replication of SARS-CoV-2 via various mechanisms. A crucial strategy focuses on blocking viruses from accessing host cells. Caffeic acid and related substances may inhibit the binding between the virus and ACE2 receptors on host cells, which is a crucial initial step in viral entry [14]. Additionally, caffeic acid is recognized for its immunostimulatory properties that can boost immune responsiveness, thereby limiting viral replication by fostering a more effective immune defense against SARS-CoV-2 [15].

This study integrated computational pharmacology analysis using protein-protein interaction (PPI) networks with experimental validation of the network through in vitro assays. However, significant disparities were found between the two components, limiting conclusions regarding the molecular mechanism by which caffeic acid inhibits SARS-CoV-2. Laboratory results indicated that caffeic acid had very weak antiviral activity, well above the pharmacologically feasible potency threshold. Network analysis predicted that caffeic acid targets key proteins in two important biological modules: the RAS and the inflammation-related pathway.

The weak antiviral activity experimentally demonstrated suggested that caffeic acid failed to interact effectively or efficiently with the key target proteins predicted by network modeling. The predicted pathways (RAS and inflammation) may indeed be valid targets, but caffeic acid did not specifically affect the mechanisms contributing to the IC50. The weak activity measured may be due to non-specific interactions and not the pathways targeted by the predictions. Chemical derivatization to improve binding affinity and cellular penetration is required to validate the biological relevance of these predicted pathways.

4 Conclusion

The ACE, TNF, NLRP3, C3, ACE2, CCL3, AGT, CCL2, and SELE genes were identified as targets of caffeic acid in COVID-19 using a network pharmacology approach. These genes participate in the disease through pathways including the renin-angiotensin system, TNF signaling, Toll-like receptor signaling, NOD-like receptor signaling, FcγR-mediated phagocytosis, ACE2 downregulation, as well as alternative, classical, and lectin pathways. In vitro testing showed that caffeic acid exhibited modest antiviral activity against SARS-CoV-2, with an IC50 of 278 μM and SI > 1, indicating weak activity according to standard antiviral screening criteria and pharmacologically low potency.

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