2,4-Dihydroxycinnamic acid as spike ACE2 inhibitor and apigenin as RdRp inhibitor in Nimbamritadi Panchatiktam Kashayam against COVID-19: an in silico and in vitro approach

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Abstract
Nimbamritadi Panchatiktam Kashayam (NPK) is an ayurvedic formulation composed of ingredients with potent anti-viral activities. We studied the interaction energy of 144 phytoconstituents present in NPK against spike receptor-binding domain (RBD) complexed with ACE2 protein (PDB ID: 6LZG) and RNA-dependent RNA polymerase protein (PDB ID: 7BTF) using Biovia Drug Discovery studio. The result indicated that 2,4-hydroxycinnamic acid exerts more significant binding affinities (28.43 kcal/mol) than Umifenovir (21.24 kcal/mol) against spike ACE2. Apigenin exhibited the highest binding affinities (54.63 kcal/mol) compared with Remdesivir (24.52 kcal/mol) against RdRp. An in vitro analysis showed a reduction in the number of lentiviral particles on transfected HEK293T-hACE2 cells as assessed by pseudovirus inhibition assay. At the same time, the tested compounds showed non-toxic up to 100 µg/ml in normal cells by MTT assay. The study highlights the plausible clinical utility of this traditional medicine against SARS CoV2.

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Introduction

COVID-19 is a global pandemic caused by a dreadful pathogen SARS-CoV2 (severe acute respiratory syndrome coronavirus-2), which resembles highly contagious fatal pneumonia with severe acute respiratory distress, which leads to a wide mortality ratio [1]. It is a highly enveloped, positive single-stranded RNA virus with four structural proteins: M protein, N protein, E protein, S protein, and sixteen non-structural proteins [2, 3]. SARS-CoV2 mediates endocytosis by adhering the spike protein homotrimers with the ACE2 receptors expressed on the surface of host cells, thereby aiding receptor recognition and membrane fusion [4, 5]. Following the viral entry into the host cell, another critical event involved in the viral lifecycle process is the Replication/Transcription complex (RTC). The formation of RTC helps in viral genome replication and subgenomic mRNA synthesis [6]. RNA-dependent RNA polymerase enhances a functional catalytic activity upon the formation of genome replication machinery; thus, it stimulates the frequent addition of hundreds to thousands of nucleotides and enhances elongation [7].

Traditional sources of Ayurveda have significantly contributed to averting the progression of almost all severe illnesses with potential effectiveness. Nimbamritadi Panchatiktam Kashayam (NPK) is an Ayurvedic herbal decoction mainly suggested during the infectious spread of Dengue and Malaria constituted with ingredients of superior anti-viral activities. It includes several herbal constituents such as Azadirachta indica (Neem), Tinospora cordifolia (Amrutha), Adhatoda zeylanica (Vasaka), Trichosanthes dioica (Pointed guard), Solanum Xanthocarpum (Kantakari,), etc. which are well known for its anti-viral activities. Among the former mentioned herbal components, Solanum Xanthocarpum (Yellow nightshade) constitutes 2,4-dihydroxycinnamic acid and apigenin. Also, apigenin is present in another herbal plant named Azadirachta indica (Neem) [8–13]. Targeting entry and replication of coronavirus with traditional formulation may be an ideal strategy, which can help further develop effective therapeutic interventions against SARS-CoV2 [14–17]. The present study identifies the most active phytochemicals within the formulation targeting the entry and replication pathway of the virus in silico and further confirms with in vitro studies.
Results and discussion

Evaluation of the effect of phytoconstituents against viral entry and viral replication using the in silico method of analysis

Molecular docking is a crucial platform for computer-aided drug design and structure-based biology that helps to predict drug–target interaction concerning binding geometry. Molecular docking favors the interpretation of the behavioral nature of drugs at the binding site and the characterization of drug–target complex [18]. We analyzed the ADMET properties of 90 phytoconstituents, among which 50 passed the ADMETox with critical properties. Finally, around 25 ligands were selected for further molecular docking, and the most active ligands were further subjected to in vitro studies (Figure S1 and Table S4). Lipinski’s rule of five indicates that 2,4-dihydroxycinnamic acid and apigenin possess drug-like properties, and there is no violation of the law (Supporting information Table S1) [19, 20]. In our present work, we determine the binding affinity of various phytoconstituents constituted within Nimbamritadi Panchatiktam Kashayam (NPK) toward spike RBD-ACE2 complex and RdRp compared with standard drugs. Several clinically available repurposed drugs like Chloroquine, Hydroxychloroquine, Umifenovir, and Remdesivir with existing literature were selected as standard drugs for comparison [21–24].

Results reveal that 2,4-dihydroxycinnamic acid interacts with nine amino acid residues: Asparagine 90 (ASN: 90), Lysine 26 (LYS: 26), Threonine 92 (THR: 92), Proline 389 (PRO: 389), Glutamine 96 (GLU A: 96), Asparagine 33 (ASN: 33), Leucine 29 (LEU: 29), Aspartate 30 (ASP: 30), and Valine 93 (VAL: 93). Reports indicate that the amino acids present at the spike RBD-ACE2 interface undergo glycosylation and favor the modifications for binding with the spike receptor-binding domain of SARS CoV2, enhancing the virion tropism [25, 26]. Additionally, 2,4-dihydroxycinnamic acid possesses a strong interaction bond with these amino acids due to two conventional hydrogen bonds. Also, the conventional hydrogen bond of 2,4-dihydroxycinnamic acid with spike ACE2 is at a short distance, making the bond more vital and stable [27]. Conventional hydrogen bonding is reported to be crucial in all types of biomolecular interactions. Thus, it is termed the “master key of molecular recognition.” Moreover, conventional hydrogen bonds are more specific and offer stereo chemical orientation. It is breakable under thermally activated reactions but offers long-term stability toward target–ligand interaction [28]. Similarly, apigenin possesses more amino acid interaction with RdRp and includes three conventional hydrogen bonding at the site of LYS: 545, ARG A: 553, and ARG A: 555. Hydrogen bonding of apigenin with these amino acid residues at the nucleoside triphosphate entry channel makes the bond more robust and can modulate the interaction. Thus, the binding of apigenin may enhance interaction with these amino acid residues and may help introduce the clinical activity of apigenin at the interaction site by inhibiting the activity of RdRp [29]. Physiochemical characterization and secondary structure analysis were done using online bioinformatics tools like ProtParam and SOPMA to identify the best PDB. The results revealed that 6LZG (spike RBD-ACE 2) and 7BTF (RdRp) are suitable PDB IDs for the selected active sites. Selected phytoconstituents docked with determined PDB IDs (6LZG and 7BTF), and the docking scores obtained compared with standard drugs Umifenovir and Remdesivir, respectively [30, 31]. Results indicate that 2,4-dihydroxycinnamic acid and apigenin are the best candidate molecules with PDB ID: 6LZG and 7BTF, respectively, based on docking score and number of amino acid interactions. Additionally, 2,4-dihydroxycinnamic acid and apigenin interact with identical hot spot amino acid residues as in the case of standard drugs like Umifenovir and Remdesivir, respectively. This indicates that they may be clinically promising in combating viral entry and replication of SARS CoV 2 by showing significant interactions with these hot spot amino acid residues present at spike RBD-ACE2 interface and at the RdRp site [25, 26, 29]. (Figs. 1, 2).

In vitro cell proliferation assay and Pseudovirion assay for SARS CoV2

MTT assay

The biological safety of the two most active compounds, apigenin, and 2,4-dihydroxycinnamic acid, was analyzed on Vero cells by MTT. Both the compounds exhibited a dose-dependent cytotoxic effect with IC50 of 87.55 µg/ml and 118.4 µg/ml for apigenin and 2,4-dihydroxycinnamic acid, respectively. However, the low-to-medium concentration range (1–50 µg/ml) did not exhibit a considerable cytotoxic effect. Apigenin and 2,4-dihydroxycinnamic acid exhibited cell viability of 76.92% and 81.16% on Vero cells after 48-h treatment at 50 µg/ml. Finally, based on the cell viability assay, we have selected a non-toxic concentration range of 1–50 µg/ml for both compounds for further in vitro Pseudovirion analysis Figs. 3 and 4 [32].

Pseudovirion assay

Pseudovirion assay offers a safe-effective protocol to study highly infectious and pathogenic viruses like SARS CoV2. SARS CoV2-spike-pseudotyped lentiviral particles
produced in transfected HEK293T-hACE2 cells have green fluorescence due to Zsgreen traceable marker. After 48 h treatment with NPK (1: 500 dilutions) upon Pseudovirion infected cell line, it exhibited a significant reduction in the fluorescent marker activity, which denotes its anti-SARS CoV2 effect. Importantly, this pseudovirion neutralization effect is almost similar to the standard virion entry inhibitor, Umifenovir, at 41.89 µM concentration. We evaluated the most active candidates within the NPK as evidenced by in silico. We observed a prominent inhibitory effect on the SARS CoV2 virus after apigenin and 2,4-dihydroxycinnamic acid treatment at 74.0083 µM and 111.01 µM, respectively, Fig. 5.

Several plant-based bioactive compounds exhibit pharmacological activities against various disease ailments [33]. Cinnamic acid derivatives and apigenin are associated with anti-viral effects. In a study by Zhang et al. and his team, apigenin blocked the EV71 infection by disrupting the viral RNA association with hnRNP (heterogeneous nuclear ribonucleoproteins) A1 and A2 proteins. Also, an estimated EC50 value of 10.3 µM and CC50 value of 79 µM for apigenin was found to block the EV71 infection. From the study, Zhang et al. and his team identified that apigenin selectively suppressed the GFP expression. Thus, apigenin was identified as an effective anti-viral agent against EV71 infection [34]. Several scientific reports state that cinnamic acid derivatives have been reported to elicit anti-viral activities. Amano et al. and his research team synthesized seventeen cinnamic acid derivatives and screened them to identify an effective anti-viral compound against the hepatitis C virus. Among the 17 selected compounds, compound 6 was found to suppress the viral replication of genotypes 1b, 2a, 3a, and 4a with an EC50 value of 1.5–8 µM and SI values of 16.2–94.2. Compound 6 is also phosphorylated f Tyr705 in signal transducer and activator of transcription 3 (STAT3). Compound 6 suppressed anti-viral activity but did not inhibit
JAK1/2-dependent phosphorylation of STAT3 Tyr705. Furthermore, results suggest that compound-6-dependent elevation of ROS is associated with the inhibition of HCV replication. The induction of oxidative stress by treatment with compound 6 may impair HCV replication by promoting lipid peroxidation. Thus, from the data obtained, the authors suggest that compound 6 significantly inhibits HCV replication via the induction of oxidative stress [35]. Based on the observation, we suppose that NPK and its active phytoconstituents such as apigenin and 2,4-dihydroxycinnamic acid can be developed as a significant anti-SARS CoV2 if further validated with detailed in vitro and in vivo [36].

Conclusion

The results showed that 2,4-dihydroxycinnamic acid from *Solanum Xanthocarpum* (Yellow nightshade) showed better binding affinities than Umifenovir in case of viral entry. Additionally, apigenin constituted within *Solanum Xanthocarpum* and *Azadirachta indica* (Neem) showed marked binding affinities more than Remdesivir toward blocking RNA-dependent RNA polymerase. In vitro SARS CoV2 assay confirmed that apigenin is more potent than 2,4-dihydroxycinnamic acid. Using phytoconstituents as an adjunct medication is another important question of herb–drug interactions requiring detailed experimental and clinical evidence. Another advantage with phytoconstituents is that the generation of adverse effects profiles is very low compared to other clinically available drugs.

Materials and methods

Database used for screening

The natural compound library of approximately 144 ligands constituted in Nimbamritadi Panchatiktam Kashayam
(NPK) was taken from ZINC (https://zinc.docking.org) and IMPAACT (Indian Medicinal Plants, Phytochemicals and Therapeutics) (https://cb.imsc.res.in/imppat/home) databases for the best ideal ligands against the reference targets. NPK is a marketed ayurvedic formulation that belongs to the expectorant category with a wide range of pharmacological activities. Additionally, Ayurvedic physicians advise the use of NPK for psoriasis and dermatitis. NPK is also known for its blood-purifying action. Anti-viral ingredients mainly constituted within NPK formulation include *Azadirachta indica* (neem), *Tinospora cordifolia* (amruta), *Adhatoda zeylanica* (vasaka), *Trichosanthes dioica* (pointed guard), *Solanum Xanthocarpum* (Kantakari), etc. (https://ayurvedapc.blog/2021/02/09). The literature-based survey was conducted to identify the constituents exhibiting the best anti-viral activity within Nimbamritadi Panchtatikam Kashayam. The 2D structures and chemical information of these phytoconstituents were obtained from PubChem, an open chemistry database at the National Institutes of Health (NIH) (https://pubchem.ncbi.nlm.nih.gov/) [37, 38]. These ligands’ drug-likeness properties were evaluated using the Lipinski rule to confirm molecular characteristics. The docking studies of selected phytoconstituents against SARS CoV2 with desired protein were performed using the BIOVIA Discovery studio (client version 17.2.0.1.16347). The method includes the identification of active proteins involved in the pathogenesis of SARS CoV2 from RCSB, ligand identification, ligand preparation, and molecular docking study [39].

**Drug likeness study**

Estimation of drug-likeness features with Lipinski’s rule states that the characteristics like electronic distribution, lipophilic nature, size of the molecule, and flexibility and hydrogen bond properties have a more significant impact on the mechanistic nature of the molecule within a living organism, such as protein affinity and transport, reactivity and toxicity, and metabolic stability. Following Lipinski’s rule, a molecule is considered to possess drug-like activity or is predicted to be membrane-permeable and easily absorbed.
**Fig. 4** Graphical representation of the in vitro toxicity of 2,4-dihydroxycinnamic acid at 570 nm. **a** Graphical representation of percentage cell viability of 2,4-dihydroxycinnamic acid. **c** Morphological evaluation of Vero cells upon treatment with 2,4-dihydroxycinnamic acid for 24 h.

**Fig. 5** The above figure demonstrates the lentiviral particle count on HEK293T-hACE2 cells upon 48 h treatment with Nimbamritadi Panchatiktam Kashayam (NPK), standard drug umifenovir (41.89 µM), apigenin (74.0083 µM), 2,4-dihydroxycinnamic acid (111.01 µM).
into the body; it should obey five criteria for drug-likeness mainly molecular weight, AlogP, hydrogen donor and acceptor, and several rotatable bonds [40].

**ADMET studies**

Lipinski’s rule analyzed ADMET properties such as absorption, distribution, metabolism, and excretion for the ligands that passed the drug-likeness properties. The AMET properties are generally predicted by deeply analyzing physico-chemical characteristics such as molecular weight (MW), polar surface area (PSA), lipophilicity (AlogP), Plasma protein binding (PPB), and aqueous solubility (logS) of selected compounds, closely related to certain features of drug molecules like absorption and bioavailability. ADMET studies are performed with ADMET Descriptors and Toxicity Prediction methods in Discovery Studio 2018. The information for establishing and validating these modules was obtained from various literature reports and experimental results [41].

**Protein identification structure retrieval**

Proteins employed for this study are mainly involved within the critical pathways possessing the mechanism of action of novel coronavirus SARS CoV 2. Experimentally illustrated 3D structures of the proteins like Spike RBD complexed with ACE2 and RNA-dependent polymerase (RdRp) were downloaded RCSB PDB (www.rcsb.org) [42, 43].

**Characterization of selected proteins**

PDB IDs representing spike RBD complexed with ACE2 and RdRp were set and subjected to ProtParam for computing physical and chemical parameters of proteins entered in Swiss-Prot or TrEMBL like pI value, instability index, half-life period, aliphatic index, and GRAVY. SOPMA is indicated for secondary structure prediction, which identifies the properties like the percentage of different forms of protein, such as alpha-helix, beta-turn, random coil, and so on. Finally, PDB ID: 6LZG representing spike – ACE2 and 7BTF stands for RdRp was selected [43].

**Protein preparation**

Proteins were prepared using the ‘prepare protein’ module implemented under the ‘Macromolecules’ module of the drug discovery studio. Water molecules and heteroatoms in the co-crystallized structure were removed, and the missing hydrogen atoms were added to the system. Protonation pH of 6.5 to 7.5 within the range of ionization was identified from the protein report. Possible tautomers and conformers for the proteins were predicted and the protein was thus prepared. Prediction of active sites in a protein is an essential strategy before docking. In this study, the accurate prediction of functional sites was done using Biovia Drug discovery studio visualizer 2020 [44].

**Ligand preparation**

Analysis of Drug likeness property by applying the Lipinski rule helped to filter all the enlisted phytoconstituents concerning factors similar to bioavailability by using a drug discovery studio. The selected ligands were subjected to evaluate ADMETox properties (Absorption, Distribution, Metabolism, Excretion, and Toxicity parameters) with Biovia drug discovery studio and SWISS ADME http://www.swissadme.ch. Around 25 ligands that have passed the ADMETox test were further estimated with molecular docking procedures. Filtered ligands and reference ligands, mainly chloroquine, hydroxychloroquine, and umifenovir were prepared by applying the designed ligands module from small molecule implemented in Biovia drug discovery studio [45].

**Molecular docking analysis**

The protein–ligand docking mechanism of the chosen protein–ligand complexes was performed using BIOVIA Discovery studio (client version 17.2.0.1.16347). We evaluated the interactions of crystal structures of spike RBD ACE2 complex and RdRp, respectively. The receptor structure was typed with the CHARMM force field before docking. The pH of the protein was adjusted to almost neutral 7.4 using a protein preparation module. Selected ligands were typed with a CHARMM force field and minimized by applying the smart minimizer minimization algorithm of Discovery Studio 2018, which constitutes about 2000 steps of steepest descent with an RMS gradient tolerance of 3 and conjugate gradient minimization. The active sites were determined from the PDB IDs 6LZG, and 7BTF, and the sphere radius was adjusted. CDOCKER module implemented under receptor–ligand interaction tool within DS is preferred for performing molecular docking. The prepared ligands were docked in the discovered pockets of the PDBs (6LZG and 7BTF) to evaluate the conformational flexibility during the refinement step and to predict CDOCKER ENERGY and CDOCKER INTERACTION ENERGY. In the study, each ligand was subjected to 10 runs in the docking software. Also, the active site of ligands was selected for the study, and in this site, we generated the grid box and further performed molecular docking analysis. The molecules with high docking scores were investigated to identify the molecules compared to
the standard drugs hydroxychloroquine, chloroquine, and umifenovir [30, 46–48].

**In vitro toxicity assay on Vero cells**

Chemicals: DMEM, FBS, and antibiotic solution (Gibco, USA), DMSO and MTX (Sigma, USA), 1X PBS (Himedia, India), 96-well tissue culture plates (Himedia, India).

Cell line: African Green Monkey Kidney cells and Vero cells were purchased from NCCS, Pune. The Vero cells were cultured in DMEM supplemented with 10% FBS, 100 mg/mL penicillin, and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO2 at 37 °C.

**Assay procedure**

The phytoconstituents selected from the molecular docking studies namely, 2,4-dihydroxycinnamic acid and apigenin were subjected to in vitro cell proliferation assay followed by trypsinization and pooling of cells into a 15-ml test tube. The cells were seeded [cell density: 1 × 10^5 cells/ml cells/well (200 µL)] into 96-well plates containing DMEM medium with 10% FBS and 1% antibiotic solution for 24–48 h at 37 °C. The wells were washed with sterile PBS and treated with various concentrations of the samples in a serum-free DMEM medium. Individually, the samples were replicated three times and the cells containing well plates were incubated at 37 °C using a 5% CO2 incubator (24 h). Followed by incubation, MTT (20 µL of 5 mg/ml) was added to the individual well plate. The cells containing well plates were again incubated for another 2 to 4 h until purple-colored precipitates were visible under an inverted microscope. At last, the medium along with MTT (220 µL) was detached from the wells and washed with 1X PBS (200 µL). In addition, the formazan crystals in the wash well plate were dissolved by adding DMSO (100 µL) and were agitated for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA), and the percentage cell viability and IC50 value were calculated using Graph Pad Prism 6.0 software (USA) [32].

**Anti-SARS CoV2 assay-pseudovirion assay for SARS CoV2**

The assay is based on the lentiviral backbone expressing ZsGreen as a traceable marker. We have utilized stable HEK293T expressing human ACE2 as the SARS permissive cells. The procedure involves transfection of HEK Lenti Cells (Invitrogen) with the expression vector encoding ZsGreen, a plasmid expressing spike, and plasmids expressing the minimal set of lentiviral proteins necessary to assemble viral particles (Gag/Pol, Rev). The cells were transfected with the expression vectors prepared via Quiaagen Midi prep using lipofectamine 2000 as per the manufacturer’s instruction. After 6 h, the cells were replaced with fresh medium containing serum. SARS CoV2- spike- pseudotype lentiviral particles from the transfected cells were collected at 48 h, filtered using a 0.45-micron filter, and infecting the HEK293T-hACE2 cells using polybrene as per the standard [36, 49].

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**Author contributions** LRN and ATP designed, conceptualised, and wrote the article. MM and VVR performed in silico analysis.

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**Data availability** All the necessary data available within the manuscript.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

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