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Anti-Viral Activity of Polyherbal Siddha Formulation *"Notchi kudineer"* Using Immuno-Fluorescence Assay and *In-Silico* Binding Studies of its Phytocompounds Against Various NSPs of SARS-CoV-2

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Abstract

Global health has suffered greatly because of the coronavirus disease 2019 (COVID-19) pandemic; as of late 2021, there had been over 230 million confirmed cases of illness and over 4.7 million deaths. Despite the existence of vaccines, COVID-19 is more aggressive in claiming millions of lives worldwide which is mainly due to the emergence of new variants of SARS-CoV-2 and rapid transmission rate. Thus, the world needs screening and developing new drugs with the potential of affecting multi-target through which the virulence of the virus can be largely restrained. '*Notchi Kudineer*' (NK) a polyherbal formulation from the Indian *Siddha* system of medicine, has proven therapeutic effect in various viral infections such as dengue and chikungunya and is used in the management of COVID-19. To further elucidate the efficacy and antiviral activity of *NK*, we carried out *in-vitro* and *in-silico* analyses. Initially, the cytotoxic assay was performed in the Vero E6 cell line to study the cytocompatibility nature of the formulation by SRB assay, and later antiviral activity was evaluated by immunofluorescence assay. Results of the SRB assay showed that NK has no cytotoxic effect. Indirect immunofluorescence assay shows a decrease in fluorescence intensity of SARS-CoV-2 infected cells which shows the reduced antigen of SARS-CoV-2. This reveals the significant inhibitory effect of NK extract on the virus at 200µg/ml concentration. An *in-silico* study was performed with the phytochemicals present in *Vitex negundo* against the Non-Structural Proteins (NSPs) of SARS-CoV-2 *viz*., nsp1, nsp3, nsp9, nsp15 and nsp10-16. A molecular docking study of the phytocomponents of *Vitex negundo* with the nsp sheds light on the inhibitory mechanisms over the different nsp. Thus, revealing the multiple actions of NK over the inhibition of the viral protein. These results showed the efficacy of NK and paved the way for further drug development for the treatment of COVID-19.

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Major Findings: NK extract is non-toxic to Vero E6 cells (SRB assay) and it inhibits viral replication by reducing antigen expression. NK phytochemicals strongly bind SARS-CoV-2 NSPs, among these Negundin-B shows the highest binding to nsp10-16 (-8.94 kcal/mol), suggesting antiviral potential.

Keywords: Antiviral Activity, *In-Silico, Notchi kudineer*, NSP, SARS-CoV-2

1. Introduction

The Coronavirus Disease-19 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) created a devastating crisis across the globe affecting the social, economic, political and quality of life. The incidence of the first case was identified in the Wuhan city, China, in December 2019[1]. According to World Health Organization (WHO), the COVID-19 pandemic has had a severe negative impact on global health; as of late 2021, there had been over 230 million confirmed cases of disease and over 4.7 million deaths.

Eventually, there was a rapid increase in the number of cases affected by this infection across the world and hence efforts were taken towards the development of antiviral agents, vaccines and prophylactic measures to combat the spread and virulence of SARS-CoV-2.

The pathogenicity of the virus begins with the entry of the host cell. SARS-CoV-2 makes use of its surface spike glycoprotein, to detect and bind specifically to the Angiotensin-Converting Enzyme 2 (ACE2) receptor which is found on the surface of the host cell[2,3]. Cleavage of the spike protein into two subunits namely S1 and S2 is essential for the entry of the virus into the cell and this cleavage process is mediated by the cathepsin and Transmembrane Protease Serine 2 (TMPRSS2)[4]. The S2 subunit is involved in the fusion of the virus with the cell membrane, thereby the entry of the virus into the cell *via* endocytosis is facilitated[5]. Once entered into the host cell, the RNA genome of the virus is released into the cytoplasm and replication occurs in the double-membrane vesicles which is derived from the endoplasmic reticulum. These double-membrane vesicles then join to form a complex membrane network.

Within the host cell, the RNA of SARS-CoV-2 undergoes translation to synthesize Non-Structural Proteins (NSPs) from the two Open Reading Frame (ORFs), ORF1a and ORF1b respectively. The SARS-CoV-2 genome produces 16 Non-Structural Proteins (NSPs) that are key players in viral replication, immune system evasion, and the development of disease.

Translation of ORF1a will produce pp1a (polypeptide 1a) of 440–500 kDa which splits into 11 NSPs. The -1 frameshift occurs in the ribosome upstream of the stop codon in ORF1a, thereby translation of ORF1b continues. Translation of ORF1b gives a polypeptide, pp1ab of 740–810 kDa that cleaves into 15 NSPs. The cleavage of the polypeptide is aided by the proteases nsp3 and nsp5, each exhibiting a domain that is identical to a papain-like protease and a 3C-like protease[6].

From the beginning of the COVID-19 pandemic, the sequence of SARS-CoV-2 is constantly varying throughout the years. These changes are due to the mutations that alter the transmissibility, drug resistance, virulence, and basic reproduction number that give rise to different SARS-CoV-2 variants. To assess the risk of SARS-CoV-2 variants, WHO (World Health Organization) in collaboration with the researchers has classified the variants as Variants of Concern (VOCs) and Variants of Interest (VOIs). Five variants are considered as VOCs - Omicron (B.1.529), Delta (B.1.617.2), Gamma (P.1), Beta (B.1.351) and Alpha (B.1.1.7), Gamma (P.1)[7].

The mutation is seen throughout the viral genome of VOCs, but these mutations cluster together on the structural proteins, particularly in the spike protein. The key reason is that mutations retained in the VOCs were found not to affect the virus amplification. Moreover, the NSPs of SARS-CoV-2 are more conserved compared to that of structural proteins and this indicates that any mutation in the NSPs will be lethal to the virus affecting its virulence and replication efficiency. Hence, the NSPs mutation were not retained during the evolution of the virus[8]. Thus, NSPs can be target. The Non-Structural Proteins (NSPs) of SARS-CoV-2 are considered ideal and vital for the virus's ability to infect and cause disease, thus making them key targets for the development of antiviral drugs against SARS-CoV-2.

In the absence of an antiviral agent against SARS-CoV-2 and its variants, repurposing the existing traditional medicine can be used as a prophylactic and therapeutic drug against this infection. Traditional medicines are easily available, cost-effective and free from

adverse events and it is worth considering as an alternative for the prevention and treatment of COVID-19.

A *Siddha* formulation named '*Notchi kudineer*' (NK) is used in the treatment of body pain, throat pain, fever, respiratory problems, *etc*. The key ingredients of this decoction include *Vitex negundo*, *Piper nigrum, Allium sativum* and *Piper betel*[9].

V. negundo Linn. (family - *Verbenaceae*) is widely used in modern as well as in traditional medicine for the treatment of different diseases[10]. Betulinic acid, oleanolic acid and ursolic acid that were found in the leaves of *V. negundo* were found to exhibit antibacterial, anticancer and anti-HIV properties. 3b-acetoxyolean-12-en-27-oic acid in seeds and isovitexin and vitexin in the roots are some of the active compounds in *V. negundo*. It is generally used in the treatment of fever, cold, headache, inflammation and pain[11].

In the current research work, the antiviral activity of the extract of *V. negundo* was tested against SARS-CoV-2 and its cyto compatibility were analysed using SRB assay. In addition, *in-silico* studies were performed against the key NSPs found in SARS-CoV-2 *viz*., nsp1, nsp3, nsp9, nsp15 and nsp10-16.

2. Materials and Methods

2.1 Cell Culture

The cells that are used in the current study are African green monkey kidney Vero E6 cells. The cells were cultured in a Minimum Essential Medium (MEM) which consists of L-glutamine and 10% Foetal Bovine Serum (FBS) and incubated at 37°C in a humidified CO_2 incubator. The media was replaced with fresh media once every two weeks. Once sufficient growth was attained, the cells were trypsinized and seeded on a 96-well microplate with a concentration of 1×10^4 cells or well. 100 µL of cell suspension is in each well and to attain monolayer culture, the plates were incubated at 37^0C with 5% CO_2 for 24 hours. After incubation, the existing media was removed from each well before performing the assay.

2.2 Preparation of NK

The exact composition of NK is as follows: *V. negundo* at 40%, *P. nigrum* at 20%, *A. sativum* at 20%, and *P. betel* at 20%. The NK powder of 20 mg was dissolvedin0.1 mL of DMSO, 20 µL of Tween 80, and 3.9 mL of MEM with L-glutamine (without FBS and antibiotics) to obtain a stock solution of 5 mg/mL. Serial dilution was done, and finally, NK extract with concentrations 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml was obtained and tested in two cell lines to analyse its cyto compatibility at minimum (3.125 µg/ml) and maximum concentration (100 µg/ml).

2.3 SRB Assay

The density of the cell is determined based on the measurement of protein content in the cell by Sulfo-Rhodamine B assay (SRB assay). This assay was performed for the screening of any compounds present in the NK that are toxic to the cells adhered in the 96-well plate. To the 96-well plate, 100 µl of the NK was added with a concentration ranging from 3.125–100 µg/mL. A cell without any NK serves as the negative control. The plates were incubated for 24 hours at 37^0 C under 5% CO_2 after which the cell viability is assessed. Post incubation, a monolayer of the cells was fixed with 10% Trichloroacetic Acid (TCA) and allowed to stand for 30 minutes at 4^0 C. After this, the plates were washed with distilled water and stained with 0.1 % SRB in 1 % acetic acid (100 µL) for 15 minutes. The excess dye was removed by washing the cells with 1% acetic acid four times and allowing them to air dry. The experiment was performed in triplicate. The optical density of the protein-stain bound complex was measured at 510 nm after dissolving the complex in 200 µL of 10 mM buffered Tris EDTA with the help of a microplate reader.

The intensity of the colour is directly proportional to the number of viable cells. The cell viability percentage is calculated as given below,

% of viability = Sample abs or Control abs x 100

The IC_{50} value of the NK extract was determined by using a dose-response curve plotted with the concentration of NK extract along the x-axis and the percentage of the growth inhibition on the y-axis.

2.4 Indirect Immunofluorescence Analysis

The monolayer of the Vero E6 cells was treated with two different concentrations of NK extract-100 and 200 µg/ml and then infected with SARS-CoV-2 at the MOI (Multiplicity of Infection) of 0.1. For positive control, the cells were treated with remdesivir (10 µM/ml). The cells were incubated at 37^0 C in a 5% CO_2 incubator for 30 hours. Upon the completion of the incubation, the cells were fixed with 4% paraformaldehyde for 2 hours and the cells were permeabilized by the addition of 0.3% tween-20 for 15 minutes and blocked with PBS. The cells were incubated

with the primary antibody, SARS-CoV2 Nucleocapsid mouse monoclonal antibody (Catalog Number: 40143-MM05) in PBS which has 1% BSA. After incubation, the cells were washed with PBS and then a secondary antibody, anti-mouse Alexa Fluor-568 was added to the cells. Hoechst 33342 was added to counter stain the nucleus and the fluorescence images were viewed under Image Xpress® Micro Confocal microscope (molecular devices). Using the multi-wavelength cell scoring module in Meta Xpress software, cells with nucleocapsid and the total nuclei of the cells were counted and compared to that of remdesivir-treated cells.

2.5 *In-Silico* Analysis

The phytochemicals that were found to be present in the *V. negundo* are obtained from the literature to perform molecular docking studies[11,12]. The 2D structure of phytochemicals was obtained from PubChem[13]. Dockage of the phytochemicals in *V. negundo* was done using Auto Dock to confirm the antiviral activity[14] and interaction with different SARS-CoV-2 non-structural proteins such as Crystal structure of nsp1 with N-(2,3-dihydro-1H-inden-5-yl)acetamide (PDB-8A4Y)[15], nsp9 with (PDB-7N3K)[16], nsp13 with Z2327226104 (PDB-7NNG)[17], nsp14 with Z71580604(PDB-5SMI), nsp15 with Tipiracil (PDB-6WXC)[18], nsp10-16 with tubercidin (PDB-8BSD). Docking was performed in Auto Dockand the phytochemicals were allowed to interact with the NSPs at the binding site[19]. After docking, the conformations of the protein-ligand complex were ranked and noted based on the binding affinity. The 3D-interaction between phytochemicals and NSPs proteins around 5Å was visualized using PyMOL[20,21].

3. Results

3.1 Cytotoxic Effect

Vitex Negundo is widely used for treating various diseases. Thereby, the cytocompatibility of the NK extract was assessed by SRB assay to measure the safety of the extract against Vero E6 cells. The results showed that after 24 hrs of treatment with varying concentrations of NK extract ranging from 3.125-100 µg/mL, the cell viability was found to be 81.58-98.98 % Table 1. The cell viability was found to be 81.58 % even when treated with 100 µg/mL of the NK extract. From the dose-response curve (data not shown), the IC_{50} of NK extract is found to be >100 µg. Thus, the *Siddha* drug NK extract is non-toxic to the cells and can be used to assess its antiviral activity against SARS-CoV-2.

3.2 Indirect Immuno-Fluorescence Test

An indirect immune-fluorescence assay was performed to confirm the antiviral activity of NK extract against SARS-CoV-2. The amount of nucleo capsid protein in Vero E6 cells infected with SARS-CoV-2 in the presence of NK extract and remdesivir was determined by indirect immuno fluorescence assay. Fluorescence intensity was directly proportional to the amount of SARS-CoV-2 nucleocapsid protein in the cells. A decrease in the fluorescence intensity is seen in cells treated with 100 and 200 µg/mL of NK extract and the percentage of virus inhibition is found to be 35.6% and 51.2% in Table 2. Remdesivir (positive control) was found to have inhibited the virus by 94.9%. From this, it is clear that the reduction in the count of infected cells as well as inhibition of bright fluorescence clearly explains that NK extract decreases the multiplication of the virus. The microscopy image of the SARS-CoV-2 nucleocapsid (Alexa flour-568) and Hoechst nuclei stain images was captured at 10X as shown in Figure 2.

The reduction in the fluorescence intensity may be ascribed due to the interruption of the synthesis of viral mRNA and hence the level of nucleocapsid protein decreases.

3.3 Molecular Docking

Due to the increase in the virulence and basic reproduction rate of SARS-CoV-2 variants, multi-targeting of the virus essential proteins is required. The key mutation occurs mainly in the structural proteins such as spike protein, nucleocapsid protein, *etc*., but the non-structural proteins are not affected by the mutation

Table 1. Concentration of NK extract and its corresponding cell viability percentage

Concentration (µg/ml) of NK Extract	3.125	6.25	12.5	25	50	100	control
% of Viability	98.98	96.76	94.47	91.55	88.19	81.58	100

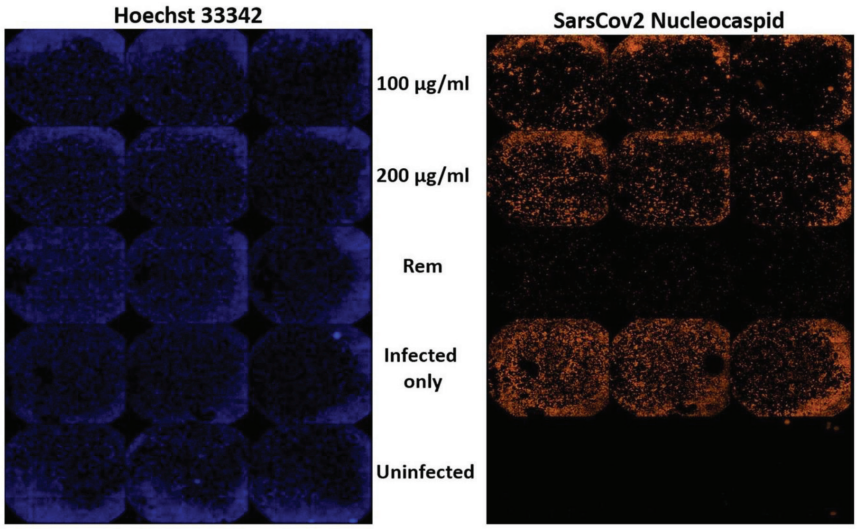


Figure 2. Dose response in IFA the antiviral activity of NK extract against SARS-CoV-2.

Table 2. Name of the compound with its concentration and the corresponding percentage of inhibition

Compound Name	Concentration (µg/ml)	Percentage of Inhibition
Remdesivir	10 µM	94.9
NK extract	100	35.6
NK extract	200	51.2

negundo is given in Table 3. A total of 48 phytochemicals in *V. negundo* were docked with the nsp1, nsp9, nsp13, nsp14, nsp15 and nsp10-16 using Autodock Tools. The binding energy provides the strength of the binding affinity of the ligands with the receptor. The lower the binding energy, the greater its affinity. It is noteworthy that all the phytochemicals exhibit good binding affinity with all the NSPs (except n-pentatriacontane which

Table 3. The docking results of various NSPs with various phytochemicals and its corresponding Binding energy, Hydrogen bonds and bond length

S.No.	NSPs	Phytochemicals	Binding Energy (kcal/mol)	H-bond	Bond Length (Å)
1	**nsp1**	**3b-acetoxyolean-12-en-27-oic acid**	**-8.69**	LYS125 (N-H…O)	2.4
2	**nsp9**	**Acetyl oleanolic acid**	**-7.05**	-	-
3	**nsp13**	**Acetyl oleanolic acid**	**-8.76**	ARG442 (N-H…O)	2.9
				ARG442 (N-H…O)	3.1
				ARG442 (N-H…O)	2.8
4	**nsp14**	**Sitosterol**	**-7.26**	-	-
5	**nsp15**	**Sitosterol**	**-7.72**	SER294 (O-H…O)	2.7
6	**nsp10-16**	**Negundin-B**	**-8.94**	ASP6897 (O-H…O)	2.9
				ASP6931 (O-H…O)	2.8

and hence targeting the non-structural proteins will help eliminate different variants of SARS-CoV-2. Molecular docking studies were performed targeting the Non-Structural Proteins (NSPs) namely nsp1, nsp9, nsp13, nsp14, nsp15 and nsp10-16 with the phytochemicals prevalent in *V. negundo*. The list of phytochemicals in *V.* does not bind with the nsp9) are studied as shown in supporting information.

Out of 48 phytochemicals, 3b-acetoxyolean-12-en-27-oic acid, Betulinic acid [3b- hydroxylup-20-(29)-en-28-oic acid], Ursolic acid [2b-hydroxyurs-12-en-28-oic acid], 60-p-hydroxybenzoyl mussaenosidic acid and Oleanolic

acid ranks the top five with good binding affinity with nsp1 having binding energy of -8.69 kcal/mol, -7.91 kcal/mol, -7.69 kcal/mol, -7.59 kcal/mol and -7.51 kcal/mol, respectively. The 3D interaction pattern of 3b-acetoxyolean-12-en-27-oic acid with nsp1 is shown in Figure 3.

The Nsp9 is a replicase protein and plays a key role in the replication of the virus. Docking with nsp9 shows that all the phytochemicals except n-pentatriacontane exhibit tight binding affinity. Acetyl oleanolic acid (-7.05 kcal/mol), Ursolic acid [2b-hydroxyurs-12-en-28-oic acid] (-6.68 kcal/mol), Oleanolic acid (-6.6 kcal/mol), 3b-acetoxyolean-12-en-27-oic acid (-6.38 kcal/mol) and Betulinic acid [3b-hydroxylup-20-(29)-en-28-oic acid] (-6.3 kcal/mol) were found to be the phytochemicals with high binding affinity among the other phytochemicals. Acetyl oleanolic acid has the least binding energy and its interaction pattern with nsp9 is shown in Figure 4.

Nsp13 exhibits ATPase and helicase activity which is involved in the unwinding of RNA helices. The phytochemicals were allowed to dock at the ATP-binding site of the nsp13 and it was seen that Acetyl oleanolic acid, Ursolic acid [2b-hydroxyurs-12-en-28-oic acid], 3b-acetoxyolean-12-en-27-oic acid, Oleanolic acid and Sitosterol has the high binding affinity compared to the rest of the phytocompounds present in the extract. The binding energy of these phytochemicals is -8.76 kcal/mol, -8.67 kcal/mol, -8.51 kcal/mol, -8.36 kcal/mol and -7.68 kcal/mol, respectively. Figure 5 shows the interaction between the Nsp13 and Acetyl oleanolic acid.

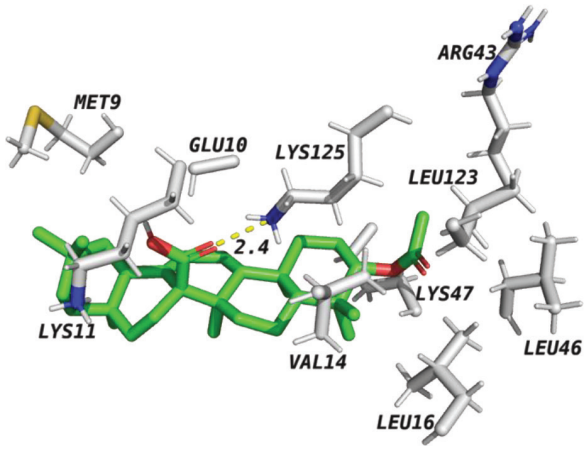


Figure 3. 3D interaction between nsp1 and 3-beta-Acetoxyolean-12-en-27-oic acid.

Nsp14 consist of guanine-methyl transferase domain at the C-terminal and an exonuclease domain in the N-terminal. This protein is found to be involved in the host immune escape and replication of SARS-CoV-2. Docking study of phytochemicals in *V. negundo* with nsp14 shows that out of 48 compounds Sitosterol, Betulinic acid [3b-hydroxylup-20-(29)-en-28-oic acid], Acetyl oleanolic acid, a-selinene and Oleanolic acid exhibits least binding energy of -7.26 kcal/mol, -6.92 kcal/mol, -6.86 kcal/mol, -6.72 kcal/mol and -6.54 kcal/mol, respectively. Sitosterol exhibits the strongest binding affinity with the nsp14 protein and its interaction as displayed in Figure 6.

Sitosterol, Ursolic acid [2b-hydroxyurs-12-en-28-oic acid], Vitrofolal-E, Acetyl oleanolic acid and Betulinic acid [3b-hydroxylup-20-(29)-en-28-oic acid] with binding energy -7.72 kcal/mol, -7.07 kcal/mol, -6.92 kcal/mol, -6.66 kcal/mol and -6.63 kcal/mol, respectively

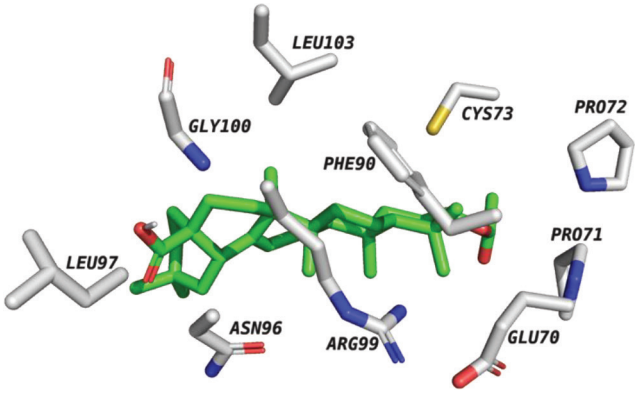


Figure 4. 3D interaction between nsp9 and Acetyl oleanolic acid.

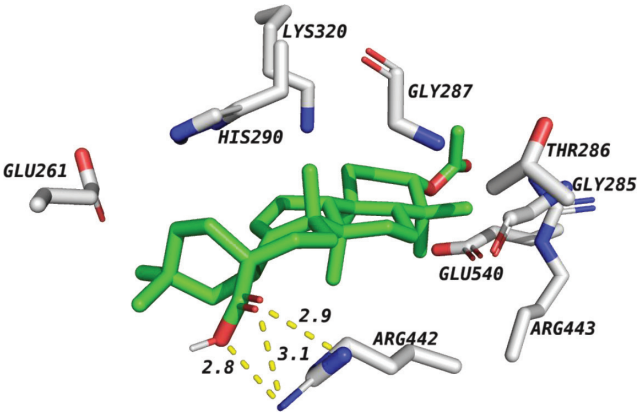


Figure 5. 3D interaction between nsp13 and Acetyl oleanolic acid.

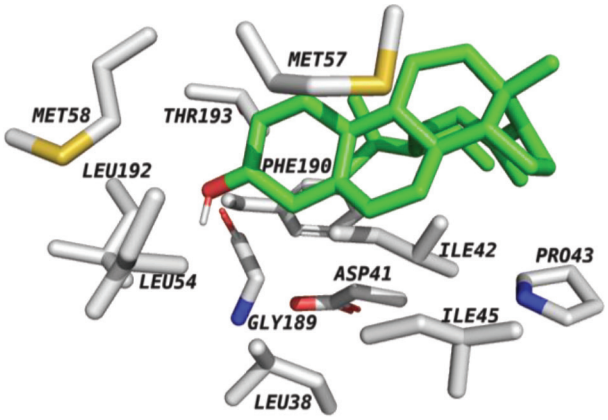


Figure 6. 3D interaction between nsp14 and sitosterol.

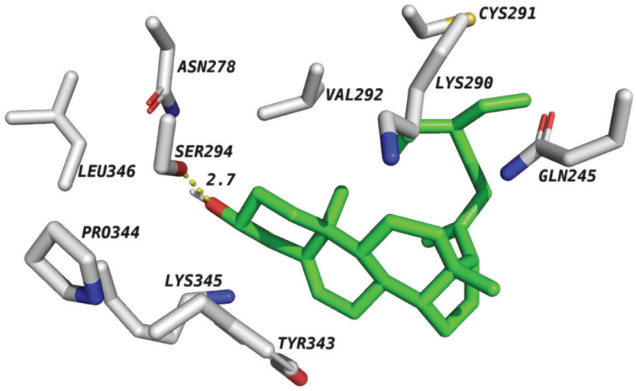


Figure 7. 3D interaction between nsp15 and sitosterol.

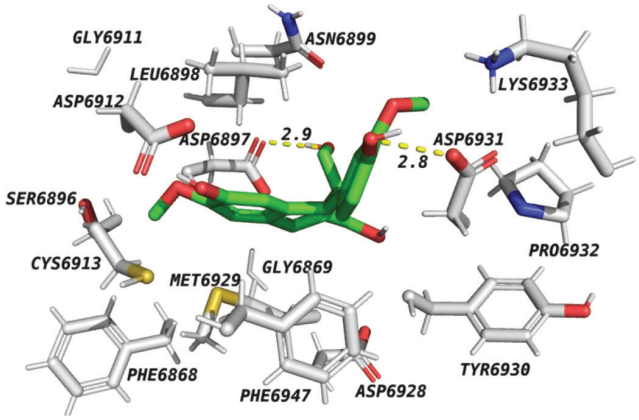


Figure 8. 3D interaction between nsp10-16 and negundin-B.

are the top five compounds with better binding affinity with the nsp15. This protein is found to be involved in the processing of sub-genomic RNA and RNA replication of SARS-CoV-2. The strongest binding affinity is found between the Nsp15 protein and Sitosterol which is given in Figures 7.

Nsp10 acts as a cofactor for the activation of nsp16. Nsp16 is involved in the formation of a 5 cap in viral mRNA by its 2-O-ribose methyl transferase activity. The complex nsp10-nsp16 mediates the methylation of the virus RNA thereby hindering the host recognition and reducing the immune response. Phyto chemicals were allowed to dock into the active of the nsp10-16 complex and the inhibitory effect of the phytochemicals was ranked based on the binding energy. Negundin-B (-8.94 kcal/mol), Oleanolic acid (-8.86 kcal/mol), Acetyl oleanolic acid (-8.86 kcal/mol), Negundin-A (-8.43 kcal/mol) and Ursolic acid [2b-hydroxyurs- 12-en-28-oic acid] (-8.42 kcal/mol) possess the strong binding affinity with the nsp10-16 complex. Figure 8 reveals the interaction between the Negundin-B and nsp10-16 complex. Table 3 gives information on phytochemicals with good binding affinity with the NSPs.

4. Discussion

Before the emergence of COVID-19, Notchi kudineer (NK), a Siddha formulation, was used for treating body pain, throat pain, fever, and respiratory problems. Key ingredients include *V. negundo, P. nigrum, A. sativum,* and *P. betel*. *V. negundo*, from the *Verbenaceae* family, is employed in both modern and traditional medicine. Compounds in its leaves, such as betulinic acid, oleanolic acid, and ursolic acid, have antibacterial, anticancer, and anti-HIV properties. Active compounds like 3b-acetoxyolean-12-en-27-oic acid in the seeds, and isovitexin and vitexin in the roots, are used to treat fever, cold, headache, inflammation, and pain.

In the light of the current study, the inclusion of *V. negundo*'s active compounds in NK and their potential antiviral properties against SARS-CoV-2 underscores the traditional and modern medicinal value of this formulation. The findings from molecular docking studies further support the role of these phytochemicals in targeting non-structural proteins, providing a promising multi-targeting approach for antiviral drug development.

Three conclusions about the antiviral activity of NK can be drawn from the presented work. First, the extract was found to exhibit cytocompatibility with Vero E6 cells. Second, a decrease in fluorescence intensity was observed in an indirect immuno fluorescence assay when SARS-CoV-2-infected cells were treated with *Notchi kudineer*, suggesting inhibition of virus replication. Third, molecular docking studies showed that the phytochemicals in *V.*

negundo exhibit tight binding affinity to the non-structural proteins nsp1, nsp9, nsp13, nsp14, nsp15, and the nsp10-16 complex, indicating that inhibiting these proteins could serve as a multi-targeting approach against SARS-CoV-2.

An effective predictor of human toxicity is crucial for analysing the safety of any product, which is essential in drug development. The evaluation of cytocompatibility is increasingly recognized. In current work, the cytocompatibility of the NK extract was analysed using the SRB assay. This assay is based on the uptake of pink-coloured sulfo-rhodamine B dye by basic amino acids in the cell. The intensity of the color correlates with cell viability[22]. NK extract-treated cells exhibited a significant increase in cell viability compared to an untreated control group with 100% viability. These findings suggest the development of a non-toxic medicine that can potentially be used as a therapeutic agent against COVID-19.

The antiviral activity of NK extract was determined using an indirect immuno fluorescence assay, which detects specific antigens within cells using fluorescent-labelled antibodies. A decrease in fluorescence intensity was observed, indicating a significant decrease in viral antigen expression in cells treated with NK extract. This suggests that the compounds in the extract target and interfere with the SARS-CoV-2 life cycle post-entry into the cell, demonstrating a potential virostatic effect against SARS-CoV-2.

Due to mutations in structural proteins like the spike protein and nucleocapsid protein, SARS-CoV-2 variants with high virulence have emerged, and no specific drug for COVID-19 treatment exists. A multi-targeting approach against the non-structural proteins of SARS-CoV-2 was therefore considered. These proteins are essential for viral protection and survival and remain consistent across SARS-CoV-2 variants.

Vitex negundo, the major herb in NK extract, was used for molecular docking studies. An in-silico study revealed significant binding of *V. negundo* phytochemicals to Papain-like protease (PLpro) of SARS-CoV-2, inhibiting the protease and thereby the viral life cycle[23]. For a multi-targeting approach, these phytochemicals were docked against SARS-CoV-2 non-structural proteins.

Nsp1 prevents host mRNA translation by triggering cleavage and blocking nuclear export to the cytosol, down regulating the host immune response[24]. The top compound, 3beta-acetoxyolean-12-en-27-oic acid, showed a binding free energy of -8.69 kcal/mol and a predicted IC50 of 26.48 nM. A single hydrogen bond was formed between its oxygen group and Lys125, with a bond length of 2.4 Å, and the complex was stabilized by hydrophobic and other weak interactions with Met9, Glu10, Lys11, Val14, Val16, Arg43, Leu46, Lys47, and Leu123.

Nsp9, encoded by ORF1a, is a replicase protein essential for viral replication through complex formation with nsp8[25]. Acetyl oleanolic acid showed a binding energy of -7.05 kcal/mol and a predicted IC50 of 6.82 µM. The stability of the nsp9-acetyl oleanolic acid complex was increased by hydrophobic and electrostatic interactions with Glu70, Pro71, Pro72, Cys73, Phe90, Asn96, Leu97, Gly100, and Leu103.

Nsp13, a helicase protein, functions with the NSP7/NSP8/NSP12 replication-transcription complex[26]. Acetyl oleanolic acid showed the lowest binding energy of -8.76 kcal/mol with nsp13. The complex was stabilized by three hydrogen bonds and other weak interactions with Glu261, Gly285, Thr286, Gly287, His290, Lys320, Arg442, Arg443, and Glu540.

Nsp14 has exonuclease and guanine-N7 methyl transferase domains for viral mRNA capping[27]. Sitosterol showed the highest binding affinity to nsp14 with a binding energy of -7.26 kcal/mol, stabilized by Leu38, Asp41, Ile42, Pro43, Ile45, Leu54, Met57, Met58, Phe190, Leu192, and Thr193.

Nsp15, an endonuclease necessary for viral RNA replication[28], showed good binding affinity with sitosterol, with a binding energy of -7.26 kcal/mol. The complex was stabilized by a hydrogen bond and other interactions with Gln245, Asn278, Lys290, Cys291, Val292, Ser294, Tyr343, Pro344, Lys345, and Leu346.

For efficient mRNA translation and immune response evasion[29], the nsp10-16 complex is required. Negundin-B showed the lowest binding energy of -8.94 kcal/mol with this complex, stabilized by two hydrogen bonds and other weak interactions with Phe6868, Gly6869, Ser6896, Asp6897, Leu6898, Asn6899, Gly6911, Asp6912, Cys6913, Asp6928, Met6929, Tyr6930, Asp6931, Pro6932, Lys6933, and Phe6947.

These findings indicate that NK extract could potentially be developed as a therapeutic agent against SARS-CoV-2 due to its cytocompatibility and antiviral activity targeting multiple non-structural proteins.

5. Conclusion

The current work evaluated the antiviral activity of the *Siddha* polyherbal formulation '*Notchi kudineer*' against COVID-19 infection. The results showed that NK is non-

toxic to the cells and has reduced the virus antigen in the infected cells. Further *in-silico* analysis showed that the phytochemicals in *V. negundo* exhibit binding affinity with an inhibitory nature against multiple targets of the non-structural proteins, namely nsp1, nsp9, nsp13, nsp14, nsp15, and the nsp10-16 complex. From these results, it is evident that NK can be used to treat infections caused by different variants of SARS-CoV-2 through multiple inhibitory mechanisms. Among the compounds analysed, the most effective antiviral compound was found to be Negundin-B, which showed the lowest binding energy of -8.94 kcal/mol with the nsp10-16 complex. This compound demonstrated strong binding affinity and inhibitory potential, making it a promising candidate for further development as an antiviral agent against SARS-CoV-2.

6. References

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