Original Research Article

An In-vitro evaluation of a polyherbal formulation, against SARS-Cov-2

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A B S T R A C T

Background: In the last two years, COVID-19 pandemic caused by SARS-CoV-2 has created a mass destruction among humanity causing a major health crisis around the world. With the emergence of new strains of the virus, lack of targeted drugs and antimicrobial resistance, there is a dire need to discover specific antiviral with minimum side effects targeted against COVID-19.

Objective: The present study evaluates the antiviral efficacy of a novel Ayurvedic polyherbal formulation, NOQ19, composed of a 13 well known herbs, in a cell-based setting.

Methodology: Vero E6 (CL1008), the African green monkey kidney epithelial cell, were infected with SARS-CoV-2 virus (isolate USA – WA1/2020) in a 96 well plate. NOQ19 test material was diluted at different concentration: 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml and 0.9 mg/ml. These different concentrations of NOQ19 were added to infected cells respectively and incubated for 3 days in 5% CO2 incubator. Remdesivir was used as a positive control. The cells were finally fixed with formaldehyde, stained with crystal violet and plaques were visualized. The number of plaques were counted to determine the PFU (plaque forming units)/ml.

Results: The results of the present study demonstrated an excellent antiviral efficacy of NOQ19 at 0.9 mg/ml concentration, eliminating 100% virus. The IC50 of the drug was found to be 0.2 mg/ml.

Conclusion: There is limited data around pre-clinical efficacy of polyherbal Ayurvedic drugs. Ayurvedic and herbal formations need to be tested in a preclinical setting to support the human data. The results of the present study demonstrated viral load reduction using NOQ19 in Vero E6 cell lines infected with SARS-CoV-2 virus. These result along with other preclinical and clinical trials could further evaluate the efficacy of NOQ19 as a potential therapeutic option in the fighting the COVID-19 challenge.
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1. Introduction

The Coronavirus disease (COVID-19) and associated pandemic is caused by the SARS-CoV-2 virus. Coronavirus are zoonotic in nature and their cross over to humans has led to multiple epidemics in the past as well. Betacoronavirus from the Coronaviridea family are a single stranded positive sense RNA virus with spike like projections which cause respiratory infections [1]. Before the current pandemic, NL63, OC43, 229E and HKU1 were among the common strains of coronavirus infecting humans [1]. Past decade

has seen the emergence of three new strains of coronavirus namely SARS CoV, MERS CoV and SARS-CoV-2 with increasing morbidity and mortality [2].

Wuhan, China reported cases of acute respiratory distress in late 2019. The subsequent outbreak of COVID-19 was declared as a pandemic by the World Health Organization (WHO) on March 11, 2020 [3]. The global crisis caused by the pandemics has effected 200 counties and emerged as one of the biggest health concerns of the decade. Pharmaceutical companies all across the globe have been in search of an effective antiviral treatment against this virus. Several drugs have been “repurposed” to manage the COVID-19 like chloroquine, hydroxychloroquine, favipiravir and other agents such as monoclonal antibodies, antisense RNA, corticosteroids and convalescent plasma. These drugs along with supportive therapy such as oxygen ventilation and fluid management act as the main
thertapeutics in COVID-19 management [4]. Among those; Remdesivir, a well-known antiviral drug against RNA viruses, has been extensively used in COVID-19 patients [5]. However, a major drawback of repurposing antivirals includes lack of therapeutic cure, side effects, insufficient studies on dosage and emergence of mutated strains [6]. However there is a need for an antiviral that can have a targeted approach against the virus.

The nature has provided humanity with a plethora of herbs containing phytochemicals which have been minimally explored in the context of COVID-19 treatment. Some of these compounds may exhibit good antiviral properties due to the presence of alkaloids, flavonoids, tannins and phenols [7]. The present study focuses on a novel Ayurvedic formulation, NOQ19 which contains 19 ingredients from 13 potent herbs. The drug includes herbs such as Ashwagandha (Withania somnifera), Bilwa (Aegle marmelos), Yashtimadhu (Glycyrrhiza glabra), Rasna (Pluchea lanceolata), Vasaka (Adhatoda vasica), Pippali (Piper longum), Haridra (Curcuma longa), Patha (Cissampelos pareira), Bhunimia (Phyllanthus fraternus), Bhumimia (Andrographis paniculata), Saptaparna (Alstonia scholaris), Tulasi (Ocimum sanctum) and Guduchi (Tinospora cordifolia) powder and extract. Each of these herbs have been used as fine powder or as both fine powder and extract. Some of these herbs are routinely consumed in India, the land of origin of Ayurveda, for their taste and health benefits. Due to their high antimicrobial activity, these herbs also act as a food preservative [8].

Ashwagandha (W. somnifera) is considered as a wonder drug and has been explored during the pandemic extensively. Through an in-silico study, four potential constituents of Ashwagandha (Withanoside II, Withanoside IV, Withanoside V and Sitosinioside I) have shown the highest docking ability with SARS-CoV-2 viral target protein Mpro, thus inhibiting its activity. Mpro protease enzyme is essential for viral propagation and replication of SARS-CoV-2 [9]. Another study demonstrated that Withanosides had the highest drug likeliness score and a positive human intestinal absorption in a molecular docking study [10]. Yashtimadhu (G. glabra), another component of NOQ19 also exhibits an excellent antiviral activity. A study from Frankfurt showed that, among the various components from Yashtimadhu, tested against SARS-CoV virus, glycyrrhizin, a component of Yashtimadhu, had the best viral replication inhibiting property [11]. Another animal study highlighted the role of glycyrrhizin in reducing the number of Angiotensin converting enzyme 2 (ACE2) receptors in the lung tissues. ACE2 is essential for SARS-CoV-2 binding and causing COVID-19. This activity is achieved by inhibiting 11 beta hydroxysteroid dehydrogenase type 2 enzyme (11btaHSD2) and activating mineralocorticoid receptor (MR). In intestinal cells, the co-expression of these three enzyme results in a reduction of ACE2 expression [12]. A molecular docking study revealed that tinosoroside, a component of Guduchi docked well with ACE2-RBD [13]. Another combined simulation study focused on the active phytochemicals from Ashwagandha, Bhunimia, Giloy (Guduchi) and Amla against a potential target Mpro of SARS-CoV-2. The study revealed that Amritoside and Apigenin-6-C-glucosyl7-O-glucoside from Giloy (Guduchi), and Pectolinarin and Astragalalin from Bhumimia showed the best affinity to COVID-19 Mpro [14]. Other ingredient of NOQ19 Bhumimia (A. paniculata), prevents blood clotting because of its antithrombotic property [15]. Haridra (C. longa) is a routinely used spice in India known for its antimicrobial properties. It can also modulate the cytokine release and therefore helps in clinical improvement of patients most viral infection [16]. The compounds used in the novel Ayurvedic formulation NOQ19 exhibit good antiviral properties against SARS-CoV-2 as noted in many simulated studies.

Objective: The present in-vitro study evaluates antiviral property of the formulation (NOQ19) against SARS-CoV-2 in a Vero E6 cell line based assay.

2. Methodology

2.1. NOQ19 preparation

NOQ19 contains a combination of Ashwagandha (W. somnifera), Bilwa (A. marmelos), Yashtimadhu (G. glabra), Rasna (P. lanceolata), Vasaka (A. vasica), Pippali (P. longum), Bhunimia (P. fraternus), Bhumimia (A. paniculata), Saptaparna (A. scholaris), Haridra (C. longa), Patha (C. pareira) herbs, Tulasi (O. sanctum) and Guduchi (T. cordifolia). The quantity of each herb varies and are as described in Table 1.

NOQ 19 was procured from Sriveda Sattva Pvt Ltd, Bangalore (Sri Sri Tattva). The drug was licensed by Ministry of AYUSH, Govt. of India with the license number- AUS782. It was supplied in the powdered form and stored at 4 °C until further use.

All the herbs and herbal extracts which constituted NOQ19 were subjected for quality control analysis and after approval process, ingredients were issued for production as fine powders. All the

| S.No | Name of the herb | Scientific Name | Part Used | Nature of herb | Quantity (mg) |
|------|------------------|----------------|-----------|---------------|--------------|
| 1    | Ashwagandha      | Withania somnifera | Root      | Fine Powder   | 30           |
| 2    | Bilwa            | Aegle marmelos   | Leaf      | Fine Powder   | 30           |
| 3    | Yashtimadhu      | Glycyrrhiza glabra | Root      | Fine Powder   | 20           |
| 4    | Rasna            | Pluchea lanceolata| Leaf      | Fine Powder   | 30           |
| 5    | Vasaka           | Adhatoda vasica  | Leaf      | Fine Powder   | 25           |
| 6    | Pippali          | Piper longum     | Fruit     | Fine Powder   | 30           |
| 7    | Bhumimia         | Phyllanthus fraternus | Plant     | Fine Powder   | 35           |
| 8    | Bhunimba         | Andrographis paniculata | Whole plant | Fine Powder | 30 |
| 9    | Saptaparna       | Alstonia scholaris | Stem bark | Fine Powder   | 30           |
| 10   | Haridra          | Curcuma longa    | Rhizome   | Fine Powder   | 25           |
| 11   | Patha            | Cissampelos pareira | Root      | Fine Powder   | 25           |
| 12   | Tulasi           | Ocimum sanctum   | Whole plant | Fine Powder | 20 |
| 13   | Guduchi          | Tinospora cordifolia | Stem     | Fine Powder   | 20           |
| 14   | Ashwagandha      | Withania somnifera | Root      | Aqueous Extract | 30 |
| 15   | Yashtimadhu      | Glycyrrhiza glabra | Root      | Aqueous Extract | 15 |
| 16   | Vasaka           | Adhatoda vasica  | Leaf      | Aqueous Extract | 25 |
| 17   | Bhumimia         | Phyllanthus fraternus | Plant     | Aqueous Extract | 30 |
| 18   | Bhunimba         | Andrographis paniculata | Whole plant | Aqueous Extract | 35 |
| 19   | Guduchi          | Tinospora cordifolia | Stem     | Aqueous Extract | 15 |
ingredients were blended with excipients followed by granulation and drying.

2.2. Cell line

Vero E6 (CL1008), the African green monkey kidney epithelial cell line was obtained from Elabscience Biotechnology Inc. (Cat no. EP-CL-0491) and cultured in DMEM supplemented with 10% FBS and antibiotic antimycotic solutions at 37 °C in a humidified CO2 (5%) incubator.

2.3. Propagation and quantification of SARS-CoV-2

The SARS-CoV-2 viral isolate (USA-WA1/2020) USA (Cat No: NR-52281) [17]. The virus was propagated in Vero E6 cells by following the standard protocol [18,19]. The virus inoculum was added on to preformed Vero E6 monolayer cells (T-75 flask) at multiplicity of infection (MOI) 0.01 and incubated at 37 °C for 1 hour in a humidified CO2 (5%) incubator with shaking at every 15 minutes. The flask was observed for virus induced cytopathic effect (CPE) at every 24 hours in comparison to the mock flask. With the clear CPE at 72-hours post infection (hpi), the supernatant was collected and centrifuged to clarify the supernatant and virus was harvested. The virus stock was stored at −80 °C in small aliquots until use.

The propagated virus was quantified by standard plaque assay [18], in brief, Vero E6 cells were plated (~2.5 × 105 cells/well) into 12 well plate in DMEM supplemented with FBS (10%) and incubated for 24 hours at 37 °C in a humidified CO2 (5%) incubator. Tenfold serially diluted virus stock (prepared in DMEM supplemented with 2% FBS) was inoculated in duplicate. The plate was then incubated for 1 hour in CO2 incubator for the adsorption of the virus. Infection medium was removed after the incubation period and overlayed with DMEM: Carboxy Methyl Cellulose (CMC). The SARS-CoV-2 infection study was carried out in high containment (BSL-3) facility.

2.4. Test material preparation

A stock solution of the NOQ19 powder was dissolved in DMSO at a concentration of 100 mg/ml. The solution was stirred on shaker for 6 h followed by centrifugation to clarify the solution. The NOQ19 was manufactured by Sriveda Sattva Pvt Ltd, Bangalore (Sri Sri Tattva). The stock solution was serially diluted at 1/20 with Phosphate Buffer saline (PBS) to obtain 5X solution, which was used for the assay. 100 μl of this solution was added in all the wells. 100 μl of PBS was added to the positive control well, while 100 μl of remdesivir solution was added to the positive test control.

2.5. Antiviral assay

The assay plate was coated with 200 μl of 30,000 (approx.) Vero E6 cells in a media of DMEM containing 10% FBS per well. A 96 well-plate was used for the assay. The plates were incubated overnight (12–18 hours) at 37 °C.

Three 96 well plates were used for controls: a) positive control (virus infected cells with remdesivir), b) virus only control (Vero E6 cells infected with virus without any drug), c) cell only control (Vero E6 cells without the infection or any drug). After overnight incubation, the excess of cell culture media was removed and 100 μl of test material diluted to required concentrations was added with SARS-CoV2 at MOI-0.01. The following concentrations were used for NOQ19: 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1 mg/ml.

The test was performed in duplicates to nullify any error. The plate was incubated for 1 hour at 37 °C in a CO2 incubator (5%). After 1 hour, the infection medium was removed and overlayed with DMEM:CMM containing NOQ19 at final indicated concentrations and returned the plate to CO2 incubator and incubated for 72 hours. After 72 hours, the cells were fixed with 200 μl of 4% formaldehyde added to each well and incubated for 30 minutes. Formaldehyde was removed and 100 μl of 0.05% (w/v) crystal violet in 20% methanol was added to each well and incubated for 20–30 minutes. After 30 min, the excess crystal violet was removed with distilled water and plaques were visualized. The number of plaques were counted to determine the PFU/ml, the log reduction and percentage viral load reduction in the presence of test material. The IC50 of the test material was determined using GraphPad Prism software (Version 9.0.1).

The experiment was conducted at Foundation for Neglected Disease Research (FNDR), Doddaballapur, Bangalore. FNDR's research and handling of SARS-CoV-2 has been endorsed by its Institutional Biosafety Committee. All SARS-CoV-2 studies were performed with approved standard operating procedures and conform to the safety requirements recommended by the Department of Biotechnology, Government of India. The study site (FNDR) was blinded to the components or any other details of the test material (NOQ19).

3. Results

Plaque Assay Data table (Table 2) represents the various concentrations of test material NOQ19, positive control remdesivir and

| Sl. No. | Sample        | Concentration | No. of Plaques | Dilution Factor | PFU/ml | Log PFU/ml | Log reductions of virus load (%) | Percentage Reduction of virus load (%) |
|--------|---------------|---------------|----------------|----------------|--------|------------|-------------------------------|--------------------------------------|
| 1      | Virus only control | 22 19 20.5   | 1000           | 6,83,333       | 5.83   |            |                               |                                        |
| 2      | Cell only     | 0 0 0 NA     | 1000           | NA            | NA     | 100.00     |                               |                                        |
| 3      | Remdesivir    | 25μM 0 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 4      | NOQ19         | 0.9 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 5      | NOQ19         | 0.8 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 6      | NOQ19         | 0.7 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 7      | NOQ19         | 0.6 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 8      | NOQ19         | 0.5 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 9      | NOQ19         | 0.4 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
| 10     | NOQ19        | 0.3 mg/ml 0 0 1000 | 0            | NA            | NA     | 100.00     |                               |                                        |
corresponding plaque forming units at different concentrations. The test was run in duplicates and the average plaque forming units were calculated.

3.1. Test material

The test material NOQ19 was found to exhibit antiviral activity against SARS-CoV-2. The highest antiviral activity noted was 100% viral load reduction at a concentration of 0.9 mg/ml (Table 2). At this concentration, NOQ19 was found to nullify the virus completely. The IC50 was calculated by considering the top value of 100 and baseline value of 0 and was found to be 0.2 mg/mL (Fig. 1).

3.2. Control

The IC50 value of positive control (Remdesivir) was calculated by considering the top value of 100 and baseline value 0 (Table 2). The reported IC-50 was 1.3 μM (Fig. 2). The test results correlated to internal report and various literature [20,21].

The virus only and cell only controls ensured the efficacy of viral isolates, cell lines used and the test procedure. By providing respective controls, it was ensured that the antiviral effects in test material arm were caused only due to the antiviral efficacy of NOQ19 and not due to any toxicity.

4. Discussion

Natural herbs and extracts have been used since centuries in India to cure different ailments. Although synthetic medicines are popular and widely used, health care professionals are once again appreciating the benefits of herbal medicine due to number of issues that use of synthetic medicines create. The side effects, emergence of antimicrobial resistance and cost are few disadvantages of synthetic medicines [22]. The current pandemic, emergence of ‘black fungus’ and lack of substantial cure for COVID-19 has led to questions regarding the efficiency of the pharmaceuticals in curing the population during such outbreaks. The present study evaluated the antiviral efficacy of a novel formulation NOQ19 composed of well-known medicinal herbs. This is the first study to test the in-vitro efficacy of the newly formulated drug NOQ19. The drug showed a 100% reduction in the viral load of SARS-CoV-2 at 0.9 mg/ml. A similar in-vitro study on one of the other popular herbal Siddha formulation, Kabasura Kudineer demonstrated a significant reduction, by 99.5%, in the viral replication of SARS-CoV-2 after 48 h, therefore inhibiting the further progression of the disease [23]. It is important to note that KSK has compounds similar to NOQ19 such as Guduchi, Pippali, Vasaka and Bhunimba. Also, another in-vitro study on Ashwagandha showed its potential benefit in reduction of HCV virus. The viral load reduction as measured by PCR was noted as 6.241 × 10^3 IU/mL at 25 mg/ml concentration [24]. Ashwagandha has a wide variety of pharmacological properties and has been routinely used for fever, respiratory disease like cough, asthma and for weight loss [25]. Glycyrrhizin, a major component of Yashtimadhu, showed that the expression of the viral antigens produced by SARS-CoV was much lower in the cultures treated with 1000 mg/L glycyrrhizin. Further, upon increasing the concentration to as high as 4000 mg/L, Glycyrrhizin completely blocked the replication. [26] Glycyrrhizin has been previously used to treat viral hepatitis and atopic dermatitis. The compound has antimicrobial, antiviral, anti-inflammatory and antioxidant properties [27]. Withanoside X and Glycyrrhizin are also responsible for preventing the viral replication among the SARS-CoV-2 virus [28,29]. In the present study we compared the polyherbal formulation with Remdesivir as a positive control. Remdesivir binds against target RNA dependent RNA polymerase (RdRp) enzyme of the SARS-CoV-2 and acts as a nucleotide analog, thereby inhibiting the synthesis [30–33]. Molecular docking studies revealed Tinosporin, cordifolide A, sitoindoside IX and curcumin from Guduchi (T. cordifolia) Ashwagandha (W. somnifera) and Haridra (C. longa) have demonstrated excellent binding affinities against RdRp protein [34,35]. Guduchi also contains several other phyto constituents such as tinocordifolioside, tinosporic acid, tinosporol, tinosporside, temberine etc that have antipyretic, anti-inflammatory and anti-diabetic properties [36]. Several of the other phytochemical constituents have also been studied for their molecular binding against various targets of SARS-CoV-2. Leaf of Vasaka was promoted by Ministry of AYUSH to aid with the respiratory symptoms noted in COVID-19 disease progression. The in-silico analysis of active alkaloid compound of Vasaka, Vasicine has demonstrated high docking affinities with ACE2 receptor thus blocking the binding sites of the virus [37]. Apart from Vasicine, the herb contains many alkaloids such as, Adhatodine, Adhatonine, Adhvasinone, Vasacinol, Anisotine and Hydroxypeganine which has antiviral, antibacterial, antioxidant, antitubercular properties [38]. Docking studies of Pippali against papain like protease of COVID-19 virus demonstrated I-Asarinin phyto component of Pippali as a potential component against COVID-19 [39]. In addition several other phytochemicals such as piperlongumine, piperlonguminine, piperidine, piperundecalidene contribute to its antibacterial, anti-inflammatory, immunostimulatory, anthelmintic, antispasmodic and cough suppressing properties [40].

Phytochemical compounds like sesline from Bilwa (A. marmelos) having anti-inflammatory properties and many other phytoconstituents from Guduchi (T. cordifolia) and Tulasi (O. sanctum) have...
shown good molecular binding activity against the Mpro protein of the virus [41–43]. Phytochemicals from Tulasi such as eugenol, urosolic acid, carvacrol, linalool contribute to its antimicrobial, antianaphylactic, larvicide properties [44]. Pure components of Patha (C. pareira) like pareirane, cisammine, magnoflorine having antiviral and antimalarial properties have demonstrated 40–80% inhibition of SARS-CoV-2 in infected Vero E6 cell culture [45]. The chemical constituents of Bhumina (A. paniculata) like andrographolide and dihydroxy dimethoxy flavone have demonstrated good binding sites with SARS-CoV-2 main protease enzyme and have antipyretic, anti-inflammatory, antihypertensive and anti-diarrheal properties [46]. The phytochemicals from Haridra like curcumin have extensive healing properties like anti-inflammatory, antimicrobial, antipatelet aggregation and gastro—protective activities [46]. Saptapartha (A. scholaris) has been widely used as a medicine in respiratory diseases like asthma, cough and COPD [47].

Previous studies have demonstrated an anti-inflammation action of various phytochemicals of Rasna (P. lanceolata) like Pluchine, Triterpenaeand Sorgumhol [48]. However, the current study is only an in-vitro evaluation of the drug in a Vero E6 cell line. Along with our results, further clinical trials have to be performed in order to test the safety and efficacy of NOQ19 in humans.

5. Conclusion

The present study explores a novel Ayurvedic formulation NOQ19 against COVID-19 in a Vero E6 cell based assay. The study results show complete elimination of the virus (100% antiviral efficacy) at a concentration of 0.9 mg/ml. The IC50 was found to be 0.2 mg/mL. The results highlights NOQ19’s potential efficacy against COVID-19. The present study along with preclinical studies clinical trial results could propose a beneficial therapeutic for the pandemic.

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Conflict of interest

The test resources were provided by Sriveda Sattva Pvt. Ltd (Sri tattva). Dr. Ravi Reddy is the chief scientific officer of Sriveda Sattva Pvt Ltd. In addition Dr Hari Venkatesh is the research head and development head at Sriveda Sattva Pvt. Ltd. Besides providing the NOQ19 intervention tablets, Sriveda Sattva Pvt. Ltd. was not involved in any aspect of this study. All the other authors have no conflicts of interest to declare.

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