

In vitro evaluation of antiviral efficacy of a Siddha Formulation against SARS-CoV-2

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Research Article

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Abstract

Introduction: SARS-CoV-2 virus caused COVID-19 pandemic with 218 million cases and 45 million deaths world over. It has challenged the already overburdened healthcare systems and created an urgent need to investigate solutions present in other healthcare systems. In this study Kabasura Kudineer is investigated as an intervention to influence the immune response which is beneficial for the host and stop the viral replication.

Methods: Kabasura Kudineer is a polyherbal formulation containing 15 herbal drugs mixed in equal quantity. It is an official Siddha formulation, used for phlegmatic fevers and flu-like symptoms. To conduct this study Vero E6 (CL1008), the African monkey kidney epithelial cell line was taken and infected with SARS-CoV-2 viral isolate. The Kabasura Kudineer was added in different concentrations; 0.5 mg/mL, 0.25 mg/mL, 0.12 mg/mL, 0.06 mg/mL and 0.03 mg/mL to the infected cells respectively. These cell plates were incubated for 3 days in 5% CO₂ incubator. Remdesivir was used as a positive control. The cells were fixed with formaldehyde, stained with crystal violet and plaques were visualised. Plaques were counted as PFU/ml.

Result: Kabasura Kudineer was found to exhibit good antiviral activity against SARS-CoV-2. The highest antiviral activity was 81.5% at a concentration of 0.5 mg/ml. The IC-50 value was found to be 0.2 mg/mL.

Conclusion: The antiviral efficacy of Kabasura Kudineer in our study showed reduction in the viral load which supports the results of clinical studies. Kabasura Kudineer can be used widely in a clinical setting as a treatment for COVID-19.

1.0 Introduction

SARS-CoV-2 virus is responsible for the ongoing pandemic known as COVID-19. More than 218 million cases have been confirmed worldwide and the disease has claimed around 45 million lives (3 September 2021 COVID Database, WHO) The wide spread of COVID-19 has highlighted the challenges that the already overburdened healthcare systems faces. Proper healthcare is required to care for the patients. COVID positive patients' world over have shown major symptoms like fever, cough and breathing difficulties (Vincent et al, 2020). In more severe cases, they can cause pneumonia, severe acute respiratory syndrome, kidney failure and even death (Pitchiah et al, 2020)

WHO initiated the "Solidarity Trial" in many countries to compare the medicines' effectiveness, such as Remdesivir, Lopinavir/ Ritonavir with interferon beta and Hydroxychloroquine against the Coronavirus infection (WHO- Guidelines 2019). Because of the side effects, antiviral therapy cannot be continued in the long run and relief from ailments is only symptomatic (Aminu Saleh Ahmad & Ruchi Sharma 2020). There is a need to look out for solutions present in other health care systems like Ayurveda, Siddha, Herbal medicine etc.

The concept of epidemics is very well defined and established in Ayurveda and Siddha. As per the siddha literature the contamination and vitiation of environmental factors such as- water, air, environment and season is responsible for the epidemics along with the disequilibrium of the doshas in the hosts that makes them more receptive to the disease (Jain et al, 2019)

In this study, Kabasura Kudineer is suggested as an intervention to influence the immune response in a way which is beneficial for the host and stop the viral replication. In case of Siddha medicine, many ingredients are immune-modulators and have the capacity to inhibit the virus by enhancing and restoring immunity. Kabasura Kudineer, an official Siddha formulation described in Siddha manuscript is used for phlegmatic fevers and is a dependable siddha prescription with flu-like symptom. (Kiran et al, 2020).

The Siddha & Ayurveda medicines are not usually tested in labs and there is lack of in vitro studies on these medicines. Kabasura Kudineer has shown great potential in RCTs and is required to explore its antiviral efficacy and mechanism of

action. Kabasura Kudineer is typically used as a concoction. To increase the ease of consumption, it was converted to a tablet form by Sri Sri Tattva (Sriveda Sattva Pvt. Ltd.)

Earlier research studies on few of the individual constituents of Kabasura Kudineer have shown several benefits; like *Piper longum* (one of the constituent of Kabasura Kudineer) has demonstrated significant anti-inflammatory activity (Kumar et al, 2009).

Ali et al, 2008, documented the pharmacological, phytochemical and toxicological properties of *Zingiber officinale* (Ginger). High concentration of fresh rhizome of ginger could stimulate mucosal linings to secrete the interferon, IFN- β , which helps in thwarting viral infection (Chang et al, 2013).

The hepatoprotective and immune-modulatory effects of *Tinospora cordifolia*, present in Kabasura Kudineer, were established by Bishayi et al in 2002. Extracts of *Tinospora cordifolia* possess antimicrobial activity against both gram-positive and gram-negative organisms (Tambekar, 2010). They also possess antioxidant properties (Subramanian et al, 2002). *Terminalia chebula*, also present in Kabasura Kudineer, has shown antiviral activity against the influenza A virus (Badmev and Nowakowski, 2000). Chebulagic acid and punicalagin isolated from *Terminalia chebula* inactivate the free respiratory viral particles and prevent early entry including attachment and penetration phases (Lin et al, 2013). The toxicity, anti-inflammatory, antipyretic, antibacterial and antioxidant activities of Kabasura Kudineer have been scientifically reported (Neethu Devasia, 2017; Koppala et al, 2020). A review article on the pharmacological activities of the ingredients of Kabasura Kudineer substantiates its use in swine flu (Thillaivanan et al, 2015)

This research study investigates the in-vitro efficacy of the well-known anti-viral Siddha Polyherbal Drug Kabasura Kudineer as a powdered tablet on COVID-19 infected Vero-E6 cell lines and discusses its potential in the treatment armamentarium for COVID-19.

2.0 Methodology

2.1 Kabasura Kudineer preparation: Kabasura Kudineer is a polyherbal formulation containing 15 herbal drugs mixed in equal quantity, these are; Chukku (*Zingiber officinale*), Thippali (*Piper longum*), Lavangam (*Syzygium aromaticum*), Cirukancoir ver (*Tragia involucrata*), Akkirakaram ver (*Anacyclus pyrethrum*), Muliver (*Hygrophila auriculata*), Kadukkaithol (*Terminalia chebula*), Adathodei elai (*Adhatoda vasica*), Karpooravalli (*Coleus amboinicus*), Kostam (*Saussurea lappa*), Seenthil thandu (*Tinospora cordifolia*), Siruthekkku (*Clerodendrum serratum*), Nilavembu (*Andrographis paniculata*), Vattathiruppi ver (*Cissampelos pareira*) and Korai kizhangu (*Cyprus rotundus*). The material was supplied in the powdered form by Sriveda Sattva Pvt Ltd, Bangalore (Sri Sri Tattva) and stored at 4°C until further use.

2.2 *Cell culture*: Vero E6 (CL1008), the African monkey kidney epithelial cell was obtained from Elabscience Biotechnology Inc. (Cat No. EP-CL-0491) and cultured in DMEM supplemented with 10% FBS and antibiotic antimycotic solutions at 37⁰ in humidified CO₂ (5%) incubator.

2.3 *Virus cells*: The SARS-CoV-2 viral isolate was obtained by BEI resources managed by ATCC. Isolate USA-WA1/2020 was isolated from an oropharyngeal swab from a patient with a respiratory illness who had recently returned from travel to the affected region of China and developed clinical disease (COVID-19) in January 2020 in Washington, USA. (Catalogue- BEI Resources)

2.4 *Test Material preparation*: A stock solution of the Kabasura Kudineer tablet powder was dissolved in DMSO and was made to 100X concentration. The Kabasura Kudineer 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, used from the assay. 100 μ l of the stock solution was added in all the wells. In positive cell control well, 100 μ l of PBS was added while for positive test control 100 μ l of remdesivir solution was added.

2.5 Plaque reduction assay: The assay plate was coated with approximately 30,000 Vero E6 cells in a solution of 200 μ L of DMEM containing 10 % FBS per well. A 96 well plate was used for the assay. The plates were incubated overnight (12–18 h) at 37° C. Three plates were used for controls as following: a) positive control (virus infected cells treated with remdesivir), b) virus only control (Vero E6 cells infected with virus and no test material), c) cell only control (Vero E6 cells without the infection or test materials). After overnight incubation, the excess of cell culture media was removed and 100 μ l of test material diluted to required concentrations was added. The following concentrations were used for Kabasura Kudineer: 0.5 mg/mL, 0.25 mg/mL, 0.12 mg/mL, 0.06 mg/mL and 0.03 mg/mL.

The test was performed in duplicates to nullify any error. After loading of the test material, the plates were incubated in 5% CO₂ incubator for an hour. After 1h of pre-incubation with the test material, the test material was removed and 30 μ L/well of a virus mix (prepared in infection medium) was added. The virus mix contained virus at multiplicity of Infection, (MOI) of 0.01. After virus was added, the plates were again incubated at 37° C in CO₂ incubator (5%) for 1 h with shaking at every 15 minutes. After 1 h, the medium containing the test material and the virus were removed from the wells. Thereafter, 200 μ L of DMEM: Carboxymethylcellulose (CMC) mixture again containing the test material at desired concentration was added to each well of the 96-well plates. The infected cell lines were incubated at the 37°C, 5% CO₂ for 3 days. After 3 days of incubation the CMC overlay was removed gently with a pipette and the cells were washed twice with PBS buffer. The cells were fixed with 200 μ L of 4% formaldehyde to each well and incubated for 30 minutes. Formaldehyde was removed and 100 μ L of 0.05% (w/v) crystal violet in 20 % methanol was added to each well and incubated for 20–30 minutes. After 30 minutes 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 IC-50 of the test material was determined using GraphPad Prism software (Version 9.0.1)

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.

3.0 Results

Plaque Assay Data table (Table 1) represents the various concentration of test material Kabasura Kudineer and positive control remdesivir and corresponding plaque forming units of different concentration.

3.1 Test Material

The test material (NF1) Kabasura Kudineer was found to exhibit good antiviral activity against SARS-CoV-2. The highest antiviral activity was 81.5% at a concentration of 0.5 mg/ml (Table 1). The IC-50 was calculated by considering the top value of 100 and baseline value of 0 and was found to be 0.2 mg/mL (Fig. 2)

3.2 Control

The IC-50 value of positive control (Remdesivir) was calculated by considering the top and baseline value 100 and 0, respectively (Table 1). The reported IC-50 was 1.3 μ M.

The test and negative control also ensured the efficacy of viral isolates, cell lines used and the test procedure. By providing respective controls, it was ensured that the antiviral effect in test material arm was caused only due to the antiviral efficacy of Kabasura Kudineer and not due to any toxicity.

Control (Remdesivir)

1: Virus only

2: Virus + Kabasura Kudineer

3: Virus + Remdesivir

4: Cells only

Table 1
Viral load reduction among different concentrations of Kabasura Kudineer

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	2	Average					
1	Virus only control		12	15	13.5	1000	45000	5.65	N/A	N/A
2	Cell only		0	0	0	N/A	N/A	N/A	N/A	N/A
3	Remdesivir	25 µM	1	0	0.5	1000	16667	4.22	1.43	96.30
	(Positive control)	12.5 µM	3	2	2.5	1000	50000	4.70	0.95	88.89
4	Kabasura Kudineer	0.5 mg/mL	2	3	2.5	1000	83333	4.92	0.73	81.48
		0.25 mg/mL	6	7	6.5	1000	216667	5.34	0.32	51.85
		0.12 mg/mL	8	11	9.5	1000	316667	5.50	0.15	29.63
		0.06 mg/mL	10	13	11.5	1000	383333	5.58	0.07	14.81
		0.03 mg/mL	15	11	13	1000	433333	5.64	0.02	3.70

4.0 Discussion

Herbal medicines have been used as therapeutic agents throughout the human civilization. Their use was challenged largely during 19th and 20th century by the advent of semi-synthetic and synthetic drugs. In recent times, though, the consumption of plant-based medicines has increased manifold because of increasing reports of adverse side effects and bacterial resistance to antibiotics (Mohammed, 2012; Capodice and Chubak 2021).

This research was aimed at investigating the antiviral properties of Kabasura Kudineer tablets in an *in vitro* experiment.

In vitro means that it is done outside of a living organism and usually involves isolated tissues, organs or cells. In our study Vero E6 cell line was used. *In vitro* tests are used to study the action mechanisms of active ingredients on biological systems and their release.

Efficacy can be defined as the performance of an intervention under ideal and controlled circumstances. In the present study we evaluated the antiviral efficacy of Kabasura Kudineer, a well-known Siddha polyherbal drug. The drug showed an 81.5% reduction in the viral load of SARS-CoV-2 at 0.5mg/ml in a DMSO extract. An earlier *in vitro* study by Devi MS et al, 2021, on Kabasura Kudineer (KSK) with water extract showed a 99.56% reduction in viral load after 48 hours; which supports the present study.

A review by Bchetnia M et al, 2020, on global SARS-CoV-2 described it as a communicable disease related to airway disorders that cause acute respiratory distress syndrome and severe pneumonia. The Siddha system of medicine has

several solutions for handling endemic/ epidemic diseases. It can be a powerful treatment modality COVID-19 management (Prakash et al, 2021)

Docking studies of bioactive compounds from Kabasura Kudineer has confirmed that it is having excellent binding efficiency with spike protein of SARS-CoV-2 (Naina Mohamed Pakkir Maideen, 2021) Chryseriol and Luteolin from Kabasura Kudineer inhibit ACE2 spike protein of SARS-CoV-2 (Kiran et al, 2020).

Pharmacological studies on Kabasura Kudineer have shown that it can be an effective drug in managing the symptoms of viral diseases affecting the respiratory system, due the promising anti-inflammatory, antiviral, immunoprotective and analgesic activities of its ingredients as well as several other medicinal benefits providing synergistic healing (Kumar et al, 2021).

Observing the importance of herbs in treating/ curing COVID patients; Ministry of AYUSH (Govt. of India) has recommended formulation of few plants, like, *Zingiber officinale*, *Piper longum*, *Syzygium aromaticum*, *Tragia involucrate*, *Anacyclus pyrethrum*, *Hygrophilla auriculata*, *Terminalia chebula*, *Adhatoda vasica*, *Plectranthus amboinicus*, *Tinospora cordifolia* etc (PIB, 2020). Many of them are constituents of Kabasura Kudineer. Several herbs have been studied earlier to establish their value in treating disease. A study by Vellingiri et al, 2020 also demonstrated the benefit of using herbs and medicinal plants, as well as their antiviral, anti-inflammatory and antioxidant properties.

Various clinical trials have been conducted on Kabasura Kudineer as a therapeutic option along with standard treatment for mild and moderate patients. The results demonstrate that clinical symptom resolution time was shortened by 3–6 days, cure rate was improved, disease progression was delayed, and it shortened the course of disease (Bala D, 2021; Natarajan et al, 2020). The Kabasura Kudineer increases the immunity and could act as immunomodulator, it can reinstate the respiratory health (Ramya et al, 2021). There was reduction in viral load of SARS-CoV-2 at the end of treatment (10 days) and time taken to convert patient for symptomatic to asymptomatic based on clinical symptoms in 10 days (Srivastava et al, 2021). Ministry of AYUSH has launched a massive nationwide campaign to distribute its proven poly herbal Siddha drug Kabasura Kudineer to strengthen its position in fight against COVID 19 pandemic (Jabaris SLS and Venkataraman K 2020)

5.0 Conclusion

This study supports the antiviral efficacy of Kabasura Kudineer by demonstrating an 81.5% reduction in the SARS-nCoV-2 viral load, which is in line with the symptomatic relief provided by Kabasura Kudineer in COVID patients in clinical studies. The results of our study support a wider usage of Kabasura Kudineer in clinical settings as a treatment for COVID-19.

Declarations

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Figures

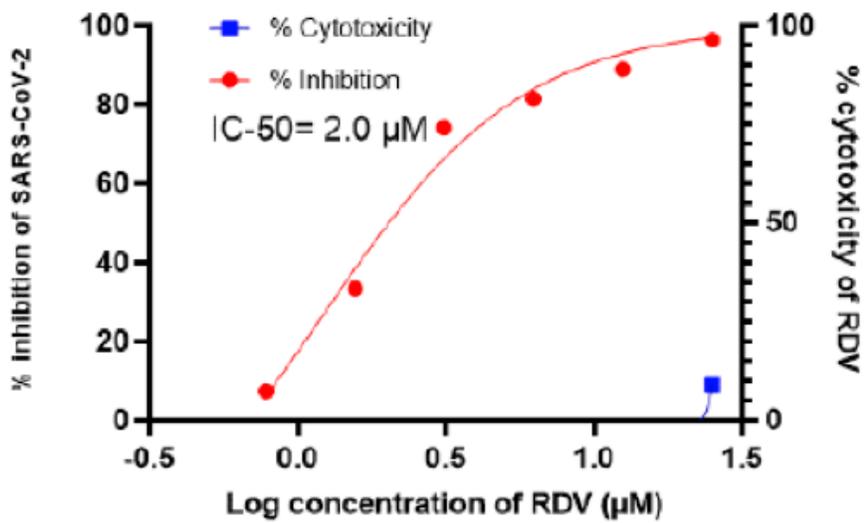


Figure 1

Antiviral Activity of Positvd. Control (Remdesivir)

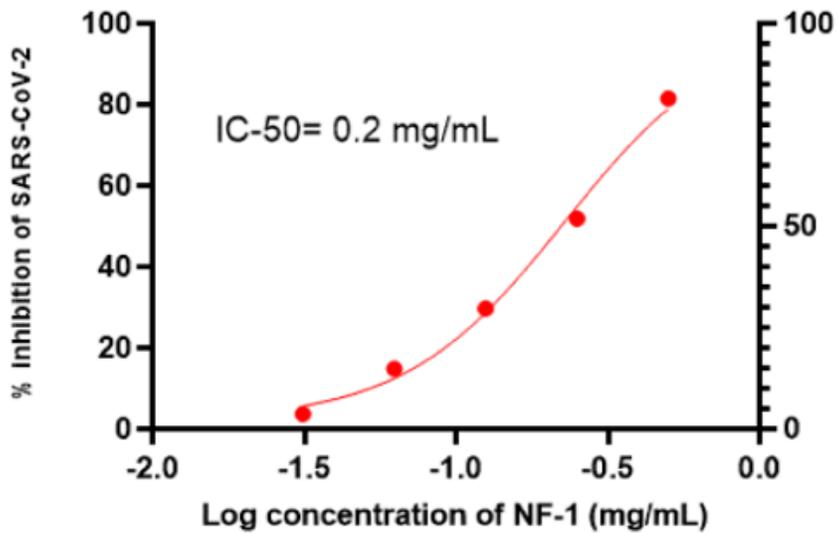


Figure 2

Antiviral Activity of Kabasura Kudineer

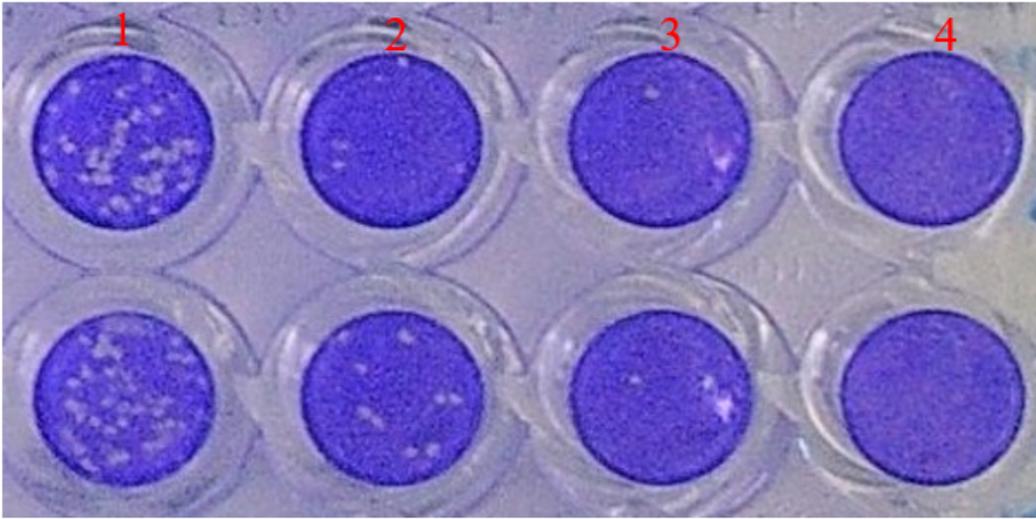


Figure 3

Plaque reduction assay of infected Vero E6 Cells