Clinical and non-clinical exploration of Thinqure20 (a herbal composition) in treatment and prophylaxis of novel coronavirus and in other pathogens.

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Research Article

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Abstract

Objective

Ongoing pandemic is being tackled by many in many ways. Simple yet effective age-old formulations might hold a key in better management of the disease. Objective was to run a series of studies to make and explore one such formulation through reverse-pharmacology. Thinqure 20 is a polyherbal formulation that contains *Piper longum, Piper nigrum, Zingiber officinale*, and rock salt. Thinqure20 was designed to work as an antiviral drug, especially against SARS CoV-2 virus. Hence the present Clinical and subsequent non-clinical studies were carried-out to evaluate efficacy, safety and tolerability of Thinqure 20 in Covid-19 infection.

Methods

Clinical study was conducted to evaluate safety, efficacy and tolerability of Thinqure 20 in Covid-19 patients (CTRI/2021/03/032471). The treatment duration was 5 days. Patients were assessed for efficacy, safety, and tolerability of Thinqure20. Subsequently, non-clinical in-vitro cell line study, antiviral and antifungal, COVID SPIKE ACE2 inhibition studies were done on Thinqure20 extract. ASTM guidelines were followed for the same.

Results

The clinical study showed the mean percentage reduction in the viral load from baseline to end of the study visit of Thinqure 20 to be 75.4%, where the minimum reduction was 59.3%, while the maximum reduction in viral load was 100%. There were 04 clinical adverse events reported which were mild in nature. Viral load testing was done by Quantitative RT-qPCR for SARS-CoV-2 (Covid-19) RNA method from NABL accredited lab. A total of 30 patients were enrolled in the study. 29 patients completed the study. In the invitro study on cell-line, the extract showed potential antiviral activity against MS2 bacteriophage, a non-enveloped virus, influenza virus, and human coronavirus with IC90 value at > 10mg/ml. The sample also presented antifungal ability against *Mucor racemosus* with EC50 value at concentration 1mg/ml. Thinqure 20 was tested for its ACE2 receptor inhibitory activity.). The kit-based SARS inhibitor showed 89.00% ACE 2 receptor inhibition when used as per KIT’s instructions.

Conclusion

The results showed that Thinqure 20 has potential against non-enveloped viruses and enveloped viruses including human coronavirus. It also showed antifungal activity against *mucor* strain. Evidences support that the use of Thinqure 20 has beneficial effects in the treatment of Covid19 which may be linked to a decrease in total viral load in patients. It provided better relief for the recovery of the COVID-19 disease.

1. Introduction

Coronaviruses (CoVs) are the single-stranded RNA virus that invades animals and humans causing diseases. The human coronaviruses (HCoV) were first identified in 1962 which was associated with respiratory tract infection[1]. Two highly pathogenic and contagious human coronaviruses: severe acute respiratory syndrome (SARS CoV) and the Middle East respiratory syndrome (MERS-CoV) were identified in 2002 and 2012 which were found to transmit from animals to humans and humans to humans [2]. In December 2019, a novel human coronavirus termed as SARS CoV-2 was identified which was considered responsible for the outbreak of Covid-19 by the World Health Organization [2]. This virus emerged in different regions of the world. The disease has since spread worldwide, leading to an ongoing pandemic.

Age and gender have been shown to affect the severity of complications of Covid-19. The rates of hospitalization and death are less than 0.1% in children but increase to 10% or more in older patients. Men are more likely to develop severe complications compared to women as a consequence of SARS-CoV-2 infection. Patients with cancer and solid organ transplant recipients are at increased risk of severe Covid-19 complications because of their immunosuppressed status [3].

Symptoms of Covid-19 are variable, ranging from mild symptoms to severe illness. Common symptoms include dizziness, headache, anosmia, ageusia, visual impairment, cough, muscle pain, sore throat, fever, diarrhoea, and breathing difficulties [4,5]. This virus possess genome and replicating RNA molecules for pathogenesis and replication. It starts replicating by binding its viral spike (S) glycoprotein to a complementary angiotensin-converting enzyme 2 (ACE2) receptor in the host cell [6]. After binding to ACE2 receptors, protease of host cells cleaves and allow virus to enter into host cells through endocytosis [1,7]. Virus replicates in host cell with the help of RNA dependent RNA polymerase by synthesizing new genomic RNA [8].

Herbal medicines have been used to treat various infectious and viral disease since ancient time. Remedies based on traditional herbs have been investigated in clinical trials and reviewed in a publication available [29]. Ayurveda is the ancient school of traditional medicine in India which has history of medicines derived from medicinal plants validated for thousands of years for several diseases and used for various viral infections.[30]

Below discussed Ayurvedic formulation influenced by traditional formulation which showed high virucidal efficacy providing better management to the Covid-19 patients within 3 to 4 days with administration of a mouth dissolving tablet four times a day. The herbal medicines imparts disease prevention or cure by building up immune system or by modulating inflammatory cascade [9]. Thinqure 20 is a polyherbal mouth dissolving tablet formulation which contains *Piper longum* Linn. (Pippali), *Piper nigrum* Linn. (Black pepper), *Zingiber officinale* Rosc. (Sunthi), and rock salt. The components of Thinqure 20 are well known medicinal remedies in Ayurveda: the traditional system of medicine. Pippali, black pepper, and sunthi have been proven for their antiviral activities [10–15]. The black pepper has been

found useful in the treatment of Covid-19 via inhibition of ACE-2 receptor inhibition [16,17]. *Piper nigrum* and *Piper longum* both possess potent antioxidant and anti-inflammatory activity [18]. These properties can provide the symptomatic relief to the Covid-19 patients. Zingiber officinale is a natural antioxidant, immune enhancer and has been found beneficial in the treatment of Covid-19 [19]. The rock salt contains trace minerals such as magnesium, zinc, sodium, chloride, and potassium which play a very important role in building up immune system. Additionally, the ionic salts can increase the absorption of other medicinal products [20]. Hence the study was carried to evaluate the efficacy and safety of Thinqure 20 in Covid-19 patients.

2. Materials And Methods

Materials

Thiqure 20 FDA approved polyherbal formulation – A standardized and ready to use formulation was obtained from Thinq pharma CRO, Mumbai.

Manufacturing and Standardization of Thinqure 20

Sr. No.	Ingredients	Part	Part
1.	*Zingiber officinale* Rosc.	Fruit powder	1
2.	*Piper longum* Linn	Fruit powder	1
3.	*Piper nigrum* Linn	Rhizome powder	1
4.	Rock salt	Not applicable	1

Method of preparation -All dried and pulverized ingredients numbered from 1 to 4. Mixing of the powders well in grinder and making the tablets of 500 mg active. The formulation Thinqure 20 showed presence of 0.55 ± 0.08% piperine, while 0.061 ± 0.01% of gingerol.

The Thinqure 20 was standardized by HPLC method to evaluate the piperine and gingerol content in the formulation.[31,32]

3. Clinical Experience Of Thinqure20

Study design and objectives

When the said formulation was given to few Covid19 patients, symptoms were reduced remarkably in two days. To explore the efficacy clinically, an EC approved, CTRI registered (Registration No.: CTRI/2021/03/032471) study was conducted between 12th Aug 2020 to 12th Nov 2020. The primary objective of the study was to evaluate efficacy of Thinqure 20 in Covid-19 patients. The secondary objectives were to assess safety, and tolerability of the formulation following this fixed dose combination

treatment in Covid patients. The study was performed in Yashwantrao Chavan Memorial Hospital, Pune, India and was performed in compliance with good clinical practice and applicable laws and regulations.

Thinqure20 is basically herbal composition for which we had acquired marketing license through FDA.This was distributed with local physicians govt Covid Care Centre . Feedback was expected to be received as an anecdotal research. Based on initial feedback we started receiving positive results and hence ,it was decided to conclude to retrospective CTRI registered study.

Patients

The study included male (21) and female (09) patients between age 18 to 75 years with known Covid positive status by RTPCR test in the last 72 hours. Eligible and consenting patients were taken nasal and oral swab for quantitative RTPCR (RTqPCR). This helped us get the virus count. Patients with history of intracranial bleeding, or suffering from haemoglobinopathies, active malignancies, pregnant or lactating women were not considered for the study. Tablet Thiqure20 was given for mouth dissolution. They were asked to take one tablet (to keep in mouth and to get it dissolved there, in 2 to 3 minutes) four times a day.

After screening with inclusion and exclusion criteria, total 30 patients were included in the study. At the time of screening, a written informed consent was taken and their demographics, medical history, including that of any other treatment was recorded. Additionally, vital signs including pulse, BP, temperature, body weight, and physical examination and viral load by RT-qPCR were also assessed.

Treatment

Patients were asked to put tablets in the mouth, slowly to be sucked till entire tablet is dissolved in the mouth and not to swallow or chew or crush. The first dose was 2 tablets of Thinqure 20 and from second dose it was reduced to 1 tablet at a time. One tablet was given every 4 hourly (08:00 AM, 12:00 noon, 04:00 PM and 08:00 PM) for 5 days. Out of 30 patients, 29 completed the treatment, one patient was excluded from the study due to non-compliance of the treatment.

Outcome parameters

The change in the viral load from the baseline to end of study was recorded with RT-qPCR test. Evaluation of vital signs including pulse, BP, temperature, body weight, and physical examination, recording of concomitant medication, dietary and lifestyle counselling were done to evaluate the safety of Thinqure 20. The safety and tolerability of the Thinqure 20 was to be determined by serious adverse events (SAE) and adverse events (AE) regardless of the causal relationship to the study drug.

Statistical analysis

The viral load after treatment was statistically compared with viral load before treatment by performing Wilcoxon rank sum test as it is performed when sample size is below 40. The data was represented as Mean ± SD (Standard Deviation). The $p< 0.05$ was considered as significant. The analysis was performed using statistical software GraphPad Prism Version 8.

RESULTS - Viral load

The treatment with Thinqure 20 for 5 days significantly ($p<0.001$) reduced the viral load in the Covid 19 Patients when compared with viral load before treatment (Figure 1).

Change in viral Count			
Screening No	Baseline visit (Day)0)	End of study visit (Day 5)	Reduction in %
1001	2990000	34400	99%
1002	9380000	128000	99%
1003	66600000	39200	100%
1004	635000000	77500	100%
1005	85200000	378000	100%
1006	59200	12500	79%
1007	6070	2470	59%
1008	1550000	337000	78%
1009	2700000	7020	100%
1010	17100000	70900	100%
1011	24800000	402000	98%
1012	7640000	477000	94%
1013	1150000	27900	98%
1014	33400	8720	74%
1015	126000	5210	96%
1016	8450000000	3E+09	64%
1017	27879043	1494286	95%
1018	48872	1321	97%
1019	3008331	9500	100%
1020	169055586	1.1E+07	94%
1021	441937736	2.6E+07	94%
1022	6765628	1550000	77%
1023	70800	279000	-294%
1024	89700	114000	-27%
1025	27879043	1494286	95%
1026	48872	1321	97%
1027	3008331	20866	99%
1028	169055586	549237	100%
1029	441937736	1457126	100%
1030	6765628	86088	99%

Table 1: % decline in Viral count od subjects exposed to Thinqure 20.

Mean percentage reduction in viral load for all patients was observed to be 75.4 %, where the minimum reduction was observed to be 59.3 %, while maximum reduction in viral load was observed to be 100 %. Thinqure 20 is observed to be safe and effective in controlling viral load in COVID-19 patients. P-value. Change from baseline to end of study visit is <.0001

- Decline in Viral Load was observed to be more than 90% for 22 patients
- Decline in Viral Load was observed to be more than 70% for 6 patients

Safety and tolerability

Total 4 adverse events were recorded throughout treatment duration. The adverse events were Flatulence, Diarrhoea, and Headache, which lasted for few hours, and did not require any intervention. No adverse event led to serious adverse event (Table 2).

Table 2: The assessment of safety and tolerability of Thinqure 20.

Adverse Events	Thinqure 20 (N=29)
	Number of Adverse Events n (%)
Headache	02 (6.66%)
Diarrhea	01 (3.33%)
Flatulence	01 (3.33%)

No patient reported advancement in the disease severity and everyone got relief from the symptoms and discharge from the hospital / covid care center without any complications. Clinical study thus confirmed safety, tolerability and efficacy of Thinqure20.

Sporadic experiences which prompted the investigators to take-up a clinical study confirmed Thinqure20 as a potential candidate in the management of Covid19. To further explore the product's efficacy, applying reverse-pharmacology[33,34] principle, investigators conducted non-clinical studies.

4. Non-clinical - Invitro Studies

Extraction of Phytochemicals from Thinqure 20

Sample 50 g was crushed in mortar and pestle and the crushed sample was placed into a thimble of a Soxhlet apparatus. The sample was refluxed in Soxhlet for 6 hours Two different solvents were used and carried out in two different Soxhlet apparatus viz., Ethanol and Methanol at ratio of 1:100 of the sample: solvent. The extracts were concentrated in rotary evaporator. The concentrate was dried in hot air oven. Both the extracts were diluted in 1:2 volume in distilled water in 0.1% Tween 20 to give combined concentration of 100 mg/ml. The samples were further diluted in respective media for specific tests at either 50mg/ml, 10mg/ml and 1mg/ml. The extracts were stored at 4^{o}C.

Invitro- Antiviral activity of Thinqure 20 extract against Human Coronavirus

Human Coronavirus HCoV-229E (Surrogate of SARS-CoV-2) belongs to the family Coronaviridae and subfamily Orthocoronavirinae. HCoV-229E is one of seven known coronaviruses (HCoV-OC43, HCoV-NL63, HCoVHKU1, MERS-CoV, SARS-CoV-1, SARS-CoV-2) to infect humans. The cell line used for viral infection was MRC-5 cell line (ATCC CCL-171) as host and was maintained in Eagle's minimum essential media supplemented with inactivated fetal bovine serum and 1% antibiotic suspension. The assay was performed as per the test guidelines mentioned in the test standard ASTM E 1052: 2020. Briefly one part of the virus suspension of the density 1.5 x 10^8 PFU/ml was mixed with 9 parts of 10mg/ml Thinqure 20 extract. The sample with the viral suspension was incubated for 4 hours at 37^{o}C. The viral-sample suspension was then neutralized with Soya Caesin Digest Lecithin Polysorbate Broth (SCDLP) and was serially diluted followed by plating in quadruplicate to host cell monolayer in a 24-well plate. After incubating the plate for 5-7 days, the log-reduction and percent reduction in coronavirus in the plate was detected by cytopathic test method. **Test Method to Assess the Activity of Microbicides against Viruses in Suspension. (2020).** ***ASTM*****. Published. https://doi.org/10.1520/e1052-20**

Thinqure 20 extract tested for anti-corona virus activity showed 97.76% reduction in the viral load at the end of 4 hours. The data was found to be statistically significant with p values less than 0.01. The extract showed antiviral and antifungal efficacies with high percent reduction ability and EC90 (concentration at which the percent reduction is 90%) at very low concentrations viz., 1mg/ml for anti-influenza and anti-mucor assay and >10 mg/ml for anti-corona virus activity. Molecular docking experiments have proven the Nsp15 target ability resulting into slowing down of virus replication and strong molecular interaction of curcumin, rosmarinic acid, piperine, gingerol, ursolic acid and alpha terpinyl acetate towards inhibition of SARS-CoV 2 replication (Kumar et al., 2021). Combination of Curcumin and Piperine have been extensively reviewed and the duo of drugs are constantly considered for the treatment strategy that could be adopted against COVID-19 spread (Miryan et al., 2021). However, the current study is evidential towards proving the anti-corona virus activity of piperine and gingerol extracts, and the authors have further studied its COVID-19 treatment efficacy on human volunteers in the same study.

Sample concentration	Contact Time	Set	Log Infectivity Titre Value at 0 min	Log Infectivity Titre value after contact time		Average Log	Percent Reduction
				Control	Test		
10 mg/ml	4 hours	SET 1	5.62	5.56	3.90	1.645	97.76*
10 mg/ml		SET 2	5.50	5.45	3.82		
10 mg/ml		SET 3	5.62	5.60	3.96		

Table 3: Average post treatment log reduction in human corona virus activity .

Indicates the data is statistically significant for Dependent T-test at p value 0.01

This data supports the statistically significant viral load decline noticed in clinical study. Thinqure20 extract showed around 98% reduction (anti corona virus activity).

Anti-viral activity of Thinqure 20 extract against the representative of non-enveloped viruses- MS2 bacteriophage

The assay was conducted as per the testing guideline ASTM E1052:2020. The strain of bacteria used as a permissive host for growing MS2 bacteriophage was *Escherichia coli* ATCC 15597. The samples were tested at a concentration of 10 mg/ml and 50 mg/ml. Plaque inhibition assay was used to determine the virucidal activity of Thinqure 20 extract against MS2 bacteriophage. Two contact times were tested viz., 1 hour and 4 hours. Briefly 1 part of viral suspension and 9 parts of sample was mixed for designated periods of contact time, aliquots were drawn, neutralize with Dey-Engley (DE) Broth and were serially diluted to compare virus count in the sample versus control. Neutralizer control was also maintained. Virus counting was performed via quantifying the Plaque forming units visible as area of clearance. Log reduction and percentage reduction was calculated. **Test Method to Assess the Activity of Microbicides against Viruses in Suspension. (2020). *ASTM*. Published. https://doi.org/10.1520/e1052-20**

Thinqure 20 sample was tested for anti-MS2 bacteriophage using *Escherichia coli* as the permissive host. There was no effect of neutralizer on the viral count as compared to control without sample. The experimental viral control showed 48 PFU/ml and neutralizer control showed 52 PFU/ml. However, Thinqure 20 sample showed significant effect at all the tested concentrations in comparison to the control without sample. There was 0.82 log reduction at 50mg/ml for 4 hours timepoint with a percent reduction of 85.00%. The sample showed significant anti-viral activity against non-enveloped MS2 virus when tested *in-vitro*.

Initial count	Log	Concentration	Time Points	PFU/ml	Log	Log Reduction	Percent Reduction
8.4×10^4 PFU/mL	4.92	10 mg/ml	1 hour	2.80×10^4	4.44	0.48	66.66*
			4hours	3.80×10^4	4.57	0.35	54.768
		50 mg/ml	1 hours	1.77×10^4	4.24	0.68	78.92*
			4 hours	1.26×10^4	4.10	0.82	85.00*
Control without sample				1.00×10^5	5.00	0.00	00.00

Table 4: Average post treatment log reduction in MS2 Bacteriophage .

Indicates the data is statistically significant for Dependent T-test at p value 0.01

Thinqure20 sample showed significant anti-viral activity against non-enveloped MS2 virus when tested in-vitro.

Anti-viral activity of Thinqure 20 extract against the representative of enveloped viruses- H3N2 Influenza Virus

The assay was conducted as per the testing guideline ASTM E1052:2020. The prepared test Virus Influenza A virus (H3N2): A/Hong Kong/8/68: ATCC VR-1679 inoculum 1.80 x 10^7 PFU/ ml was added to the test product and the virus recovery control media each at a ratio of 1:9. At the end of contact time (1 hour and 4 hours), the test and recovery suspensions were neutralized. Neutralised suspensions along with controls were plated in Quadruplicate to host cell monolayers of MDCK cell ATCC CCL-34 in 98 well plates. Plates were incubated for 5 - 7 days duration for Visible CPE by examined under microscope. The infective unit measured by $TCID_{50}$ method. The sample concentrations were checked before initiating the experiment for toxicity and non-toxic concentrations were chosen for conducting the test. Log of Antiviral efficacy, reduction and percentage antiviral efficacy was calculated. **Test Method to Assess the Activity of Microbicides against Viruses in Suspension. (2020). *ASTM*. Published. https://doi.org/10.1520/e1052-20**

The sample Thinqure 20 was tested on a representative of enveloped virus Influenza A virus (H3N2): A/Hong Kong/8/68: ATCC VR-1679. The sample showed time and dose dependent increase in anti-influenza virus activity with statistical significance ($p<0.01$). At 1 mg/ml the percent reduction was found to be 98.80 at 4 hours ($p<0.0001$). Whereas, a 99.9% reduction was achieved at 10 mg/ml of sample concentration upon incubating for 4 hours contact time with the virus. This concentration and contact time were thus chosen for anti-covid activity *invitro.* A recent paper on piperazine in combination with berberine and has shown to depict activity at very low concentration of 90.25µg/ml. Gingerol from ginger have been insinuated to have anti-avian influenza virus H9N2 (Enkhtaivan et al., 2018). Similar data was obtained from the current study where the combined effect of gingerol and piperine had significant anti-influenza activity when tested *invitro* (Rasool et al., 2017).

Sample concentration	Contact Time	Set	Log Infectivity Titre Value at 0 min	Log Infectivity Titre value after contact time		Average Log	Percent Reduction
				Control	Test		
1 mg/ml	1 hour	SET 1	5.80	5.70	4.90	0.82	84.95*
1 mg/ml		SET 2	5.90	5.80	4.80		
1 mg/ml		SET 3	5.60	5.60	5.00		
1 mg/ml	4 hours	SET 1	5.80	5.76	3.90	1.92	98.80*
1 mg/ml		SET 2	5.90	5.85	3.75		
1 mg/ml		SET 3	5.76	5.62	3.82		
10 mg/ml	1 hour	SET 1	5.80	5.75	3.80	1.79	98.37*
10 mg/ml		SET 2	5.90	5.72	3.90		
10 mg/ml		SET 3	5.68	5.60	4.10		
10 mg/ml	4 hours	SET 1	5.80	5.76	2.50	3.11	99.92*
10 mg/ml		SET 2	5.90	5.85	2.60		
10 mg/ml		SET 3	5.68	5.62	2.80		
50 mg/ml	1 hour	SET 1	5.80	5.75	3.26	2.33	99.52*
50 mg/ml		SET 2	5.90	5.82	3.42		
50 mg/ml		SET 3	5.68	5.60	3.50		
50 mg/ml	4 hours	SET 1	5.80	5.76	1.80	3.81	99.98*
50 mg/ml		SET 2	5.90	5.85	1.90		
50 mg/ml		SET	5.68	5.62	2.10		

		3					

Table 5: Average post treatment log reduction in influenza virus activity .

Initial Influenza Virus Suspension count 1.50 x 10^8 PFU/ml Indicates the data is statistically significant for Dependent T-test at p value 0.01

Thinqure20 sample showed time and dose dependent increase in anti-influenza virus activity with statistical significance ($p<0.01$).

Antifungal activity of Thinqure 20 extract against *Mucor racemosus* ATCC 42647

This assay was performed as per the test guidelines mentioned in the test standards ASTM E 2315– 16. Three concentrations of Thinqure 20 extract 1, 10 and 50 mg/ml were prepared and each concentration was inoculated with test fungi individually with an initial seeding density of 10^8 CFU/ ml. After the specified exposure time of 1 hour and 4 hours surviving microorganisms were recovered by drawing an aliquot, neutralizing and performing Standard Pour plate Technique. Culture count was ascertained by dilution Blank. Adequate Validation of Neutralizing agent was also carried. Test was carried out in duplicate and average count was taken as CFU/ ml. Note: The neutralizer showed no effect on the fungal count and hence was considered to be neutral with no positive or negative effect on fungal growth. Log reduction and Percent reduction in fungal count upon exposure to extract was calculated in comparison to control without fungus.

Thinqure 20 extract was analyzed for anti-fungal activity using *Mucor racemosus* ATCC 42647 as a representative of mucor genera. The sample showed antifungal activity above 90% at all the tested concentration. The samples at concentration 1 and 10 mg/ml showed a dose dependent increase in the activity, however it showed no activity increase with respect to time at the tested concentrations. Nevertheless, at 50 mg/ml a dose and time dependent increase in kill percentage was observed. Due to the anti-oxidant and anti-inflammatory ability of gingerol, curcumin, piperine and other phytoactives, it is thought that these drugs can prove beneficial in the management of inflammatory disorders (Prajapati et al., 2021; Timalsina et al., 2021). In the current study, the pharmacological activities of piperine and gingerol were evidently seen and realized when tested invitro on *Mucor racemosus*. To the best of our knowledge, this article is amongst the firsts to prove anti-mucor potential of both the drugs; gingerol and piperine, together in a formulation.

Concentration	Time Points	Initial Count CFU/ml	CFU/ml	Log	Log Reduction	Percent Reduction
1 mg/ml	1 hour	5.00×10^4	4.00×10^4	3.60	1.09	92.00*
	4hours	Log=4.69	4.00×10^4	3.60	1.09	92.00*
10 mg/ml	1 hours	4.80×10^4	3.00×10^4	3.47	1.21	93.75*
	4 hours	Log=4.68	3.00×10^4	3.47	1.21	93.75*
50 mg/ml	1 hours	5.20×10^4	3.00×10^4	3.47	1.24	94.23*
	4 hours	Log=4.71	2.00×10^4	3.30	1.41	96.15*

Indicates the data is statistically significant for Dependent T-test at p value 0.01

Thinqure 20 extract sample showed antifungal activity for Mucor racemosus ATCC 42647 above 90% at all the tested concentration.

5. Anti-ace2 Receptor Activity

The sample Thinqure 20 extract after its positive effects on coronavirus when checked invitro and on human volunteers was tested for its ACE2 receptor inhibitory activity. This receptor is known worldwide as an entry port for the infection caused by SARS-CoV-2 by its surface glycoproteins. Hence the interaction between SARS and ACE2 serves as an important target for effectively reducing the viral role. This was explored for Thinqure 20 to check its ability as a SARS-ACE2 interaction blocker. The assay was conducted using a kit procured from Cayman Chemicals (SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit, Item No 502050). The sample was prepared in ethanol at 100mg/ml and was further diluted to 1 mg/ml in the Immunoassay buffer C (1X). The samples were prepared at dilutions 1, 0.5, 0.25, 0.125, and 0.0625 mg/ml, and similar controls were maintained for ethanol. The test system contained 50µL of different dilutions of Thinqure 20 extract or 50µL of SARS-CoV-2 inhibitor control or solvent, 50µL of ACE2 Inhibitor Screening reagent, and 50µL of Spike Inhibitor Screening reagent. The solvent ethanol was used as an initial 100% SARS-ACE2 activity. The plate was covered and incubated at 37ºC for 60 mins on an orbital shaker. After I hour, the plate was washed four times with wash buffer and the plate was dried at RT. To the plate 150µL of Anti-His-HRP conjugate was added followed by incubation for 30 mins at room temperature. After washing and drying the plate, 175µL of TMB substrate was added,

incubated on an orbital shaker for 15 mins. Finally, 75µL of stop solution was added to the plate and was read at 450nm.

Thinqure 20 showed SARS-CoV-2 Spike-ACE2 inhibition

Thinqure 20 extract showed anti SARS-CoV-2 Spike-ACE2 activity when tested invitro. At 0.0625 mf/ml the reduction was seen to be by 2.31% which was not found to be statistically significant ($p > 0.05$). However, at higher concentrations of 0.125mg/ml the inhibitory percentage was found to be 22.69% till 1.0mg/ml where 78.46% of the reaction was inhibited ($p < 0.01$ using One-Way ANOVA). The kit-based SARS inhibitor showed 89.00% inhibition when used as per KIT's instructions.

6. Discussion

A novel coronavirus (Covid-19) has resulted in global mortality and morbidity. The plasma viral load is directly associated with progression of the disease severity [21,22]. Increase in plasma viral load leads to worsening of disease severity, marked reduction in lymphocytes count, and significant increase in plasma inflammatory markers such as interleukin 6 (IL-6), and C-reactive protein (CRP) leading to increased mortality [21].

Thinqure 20 treatment showed significant reduction in the viral load indicating its beneficial role in the inhibition of disease progression and mortality rate. In addition, the active components of the Thinqure 20 (piperine and gingerol, zingeberene) possess potent anti-inflammatory antiviral activities [23,24]. This property of thinqure 20 may also help in reducing the cytokine storm and further worsening of conditions.

The angiotensin converting enzyme – 2 (ACE-2) receptors play a very pivotal role in viral entry and its expression in the major vital organs such as heart kidney and lungs [1]. SARS-CoV-2 virus causes balance between ACE and ACE-2 and activate the renin angiotensin aldosterone system (RAAS) leading to entry of virus in host cell and its replication [25]. Inhibition of ACE-2 receptors and RAAS system can limit the viral load and disease progression by inhibiting viral entry and replication in the host cells [1]. The piperine, zingiberene and gingerol have proven ACE inhibitory activity [26,27]. This action of Thinqure 20 may be attributed to ACE-2 inhibition and inhibition of viral entry in the host cells. Founded results suggests that phytochemicals from Thinqure20 might be effective as a therapeutic as well as prophylaxis against COVID-19 by preventing viral attachment to the host cells through inhibition of spike glycoprotein.

Molecular docking results are indicated with excellent binding affinity of phytochemical piperine, zingiberene from Thinqure20 against SARS-CoV-2 spike glycoprotein and its cellular receptor along with MolDock score and other parameters [26,27].

Mole dock study shows phytochemicals from of *Zingiber officinale* as entry inhibitor of SARS-COVID 2 VIRUS. Gingerol and Zingiberene had effective binding activity with ACE2 receptors [26,27,28].

Thinqure 20 comprising of *Piper longum*, *Piper nigrum*, *Zingiber officinale* and rock salt, possess various active chemicals like piperine, zingiberene, gingerol which play its role for its effectiveness against SARS-Co2 virus. Thinqure 20 might be acting on single target or multiple targets for inhibiting SARS-CoV2 viral load.

Covid-19 can cause significant discomfort, continuous cough, fever/high temperature (37.8°C or greater), loss of, or change in, sense of smell or taste, respiratory failure, shock, or multi-organ dysfunction, reduce quality of life, and lead to a loss of productivity. In this study Thinqure20 have been seen in effectively managing the disease probably by viral load depletion. Treatment with Thinqure 20 was safe and was well tolerated. Adverse event experienced during clinical trial; all of them were mild in the nature and did not require special attention. In addition, there were no serious adverse events reported at any time during the study, and no patient deteriorated further anymore.

Adhering to the principles of reverse-pharmacology[33,34], Thinqure20 was taken for *in vitro* studies. Thinqure20 was found effective with statistical significance not only in anti-corona virus activity, but also for non-enveloped viruses- MS2 bacteriophage, the representative of enveloped viruses- H3N2 Influenza Virus and in antifungal activity against Mucor racemosus ATCC 42647.

Thinqure20 seems to have potential in enveloped, non-enveloped viruses and also in the fungus treatment.

7. Conclusion

Evidence supports that the use of Thinqure20 has beneficial effects in the treatment of Covid19. It provided better relief for recovery of the COVID-19 disease.. Overall Thinqure20 is well-tolerated, safe and is effective in COVID-19 patients, especially with reference to the reduction in viral load. *In vitro* studies showed efficacy in reduction in viral load in corona virus, enveloped and non-enveloped viruses and fungus as well. Overall, Thinqure20 could serve as a good ‘anti-pathogen’ in general. Invitro evidence also supports that use of Thinqure20 has beneficial effect as prophylaxis as shows ACE2 inhibition against SARS COVID SPYKE.

Declarations

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CONFLICT OF INTTEREST

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Milind Gharpure – GM Natural Products

Nkhil verma – GM Clinical research

Ravi Gulgule – Joint Managing director

Sanjay vekariya – Clinical research

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Figures

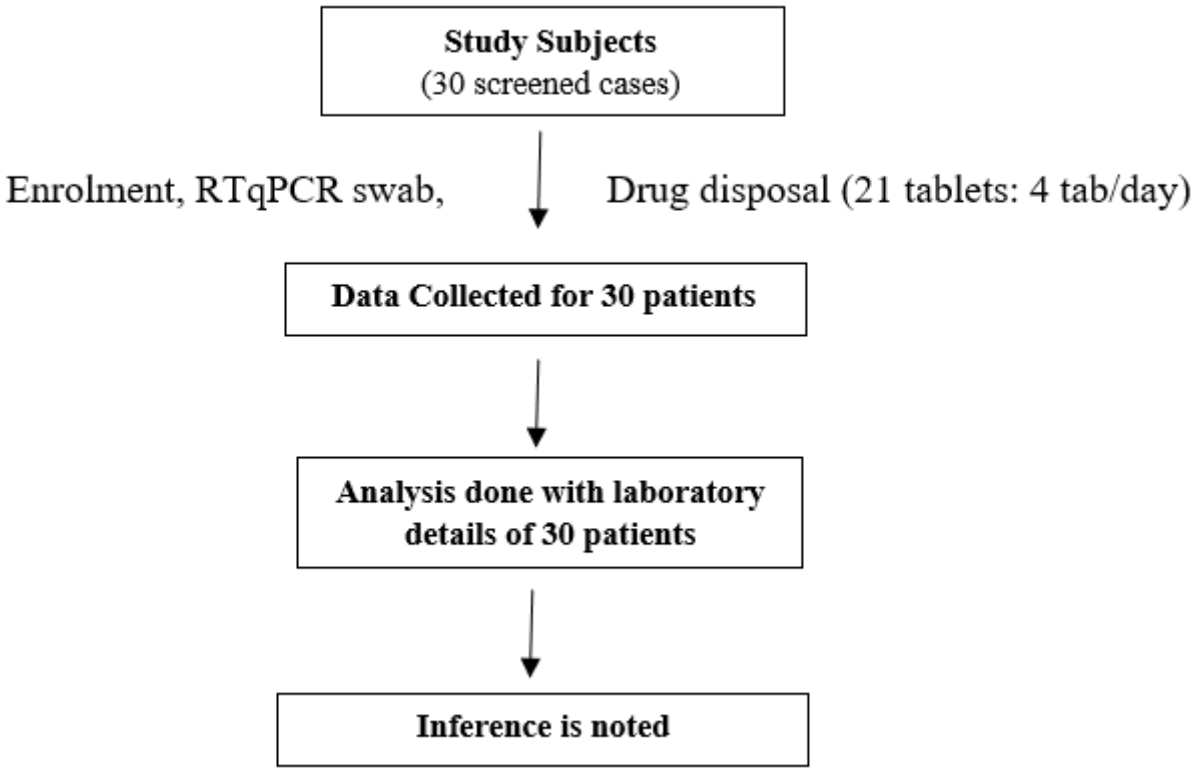

Figure 1

Flow Diagram of the Trial

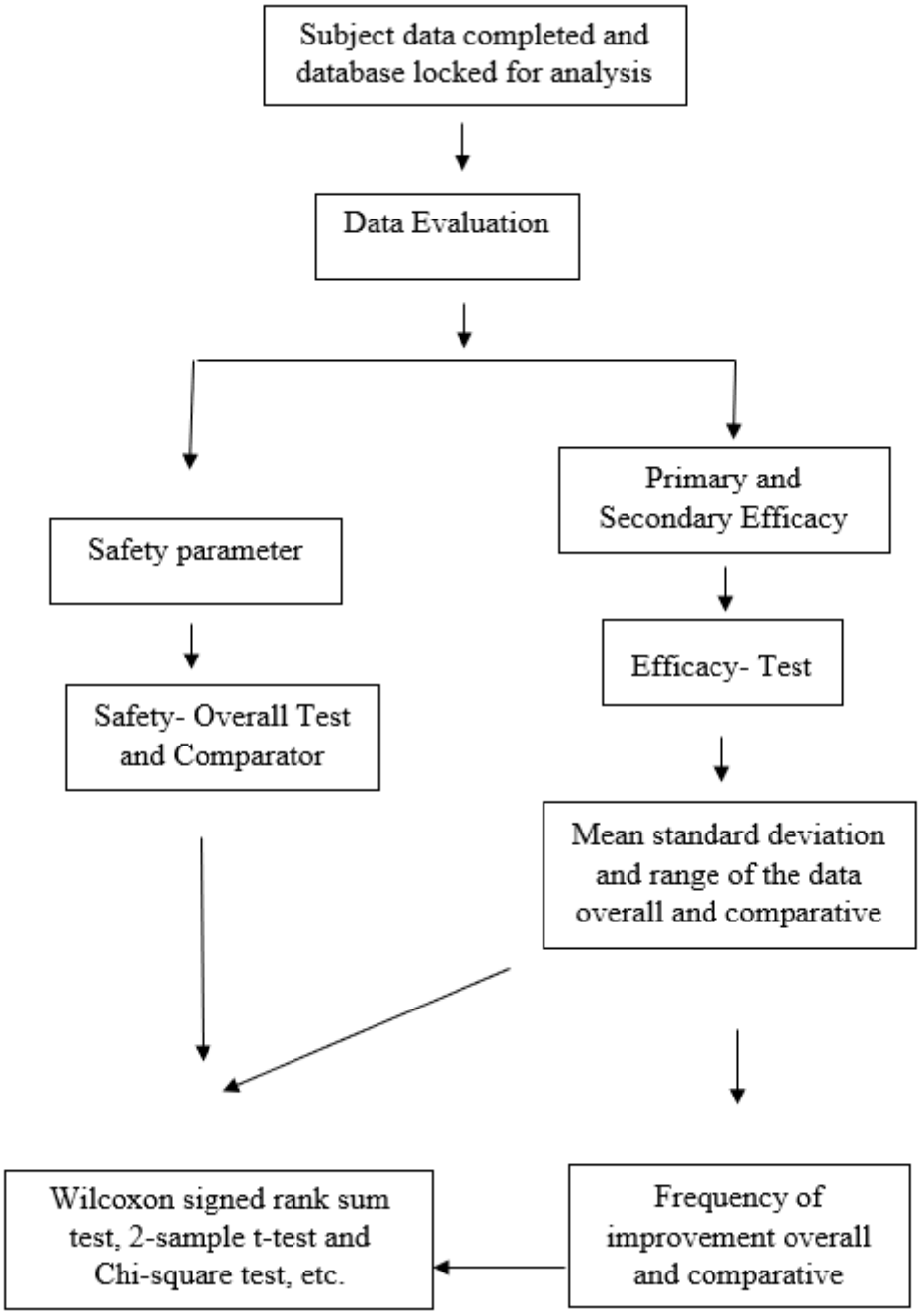

Figure 2

Flow Chart for Processing of Data

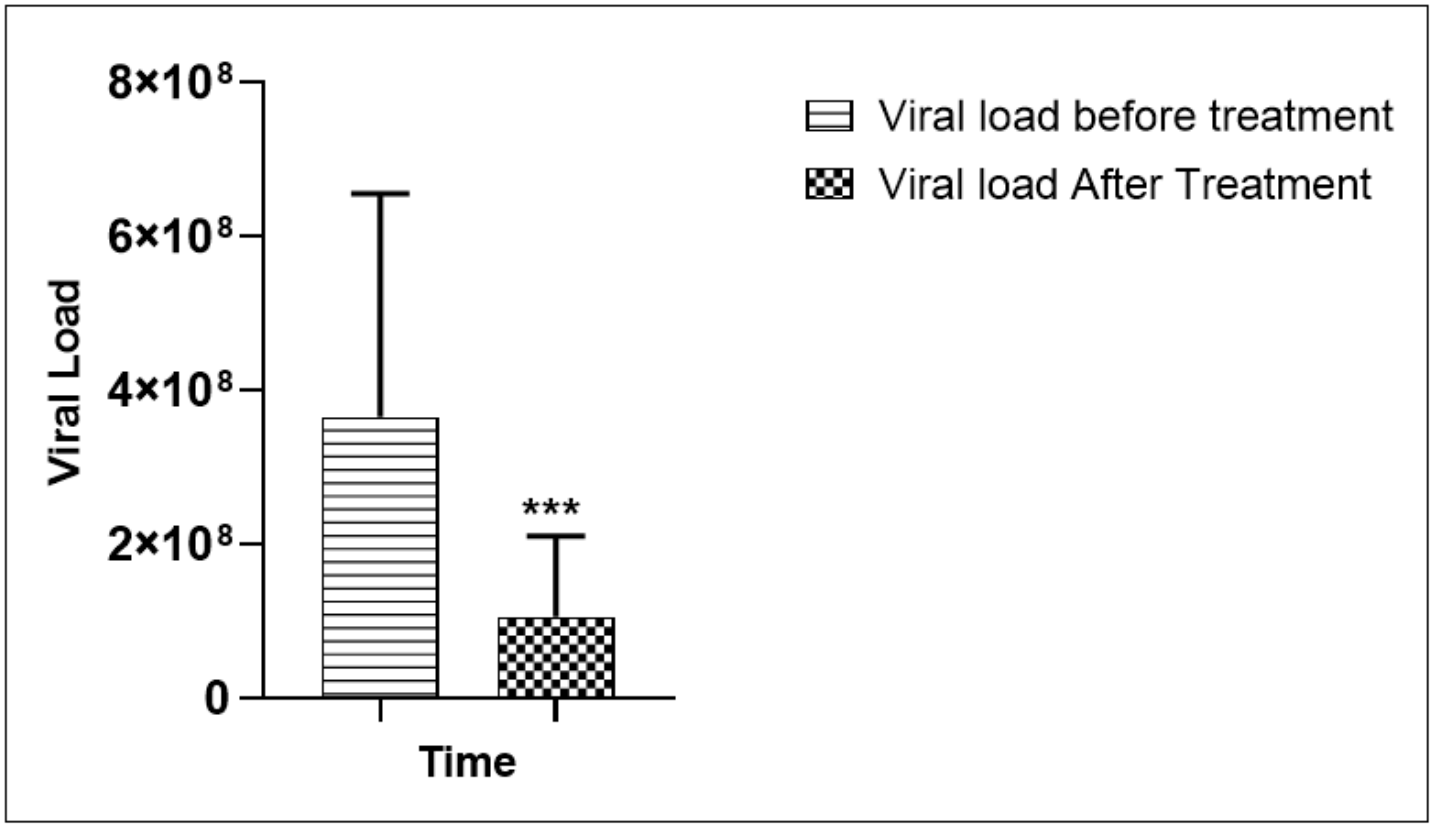

Figure 3

Effect of Thinqure 20 treatment on viral load in COVID 19 patients

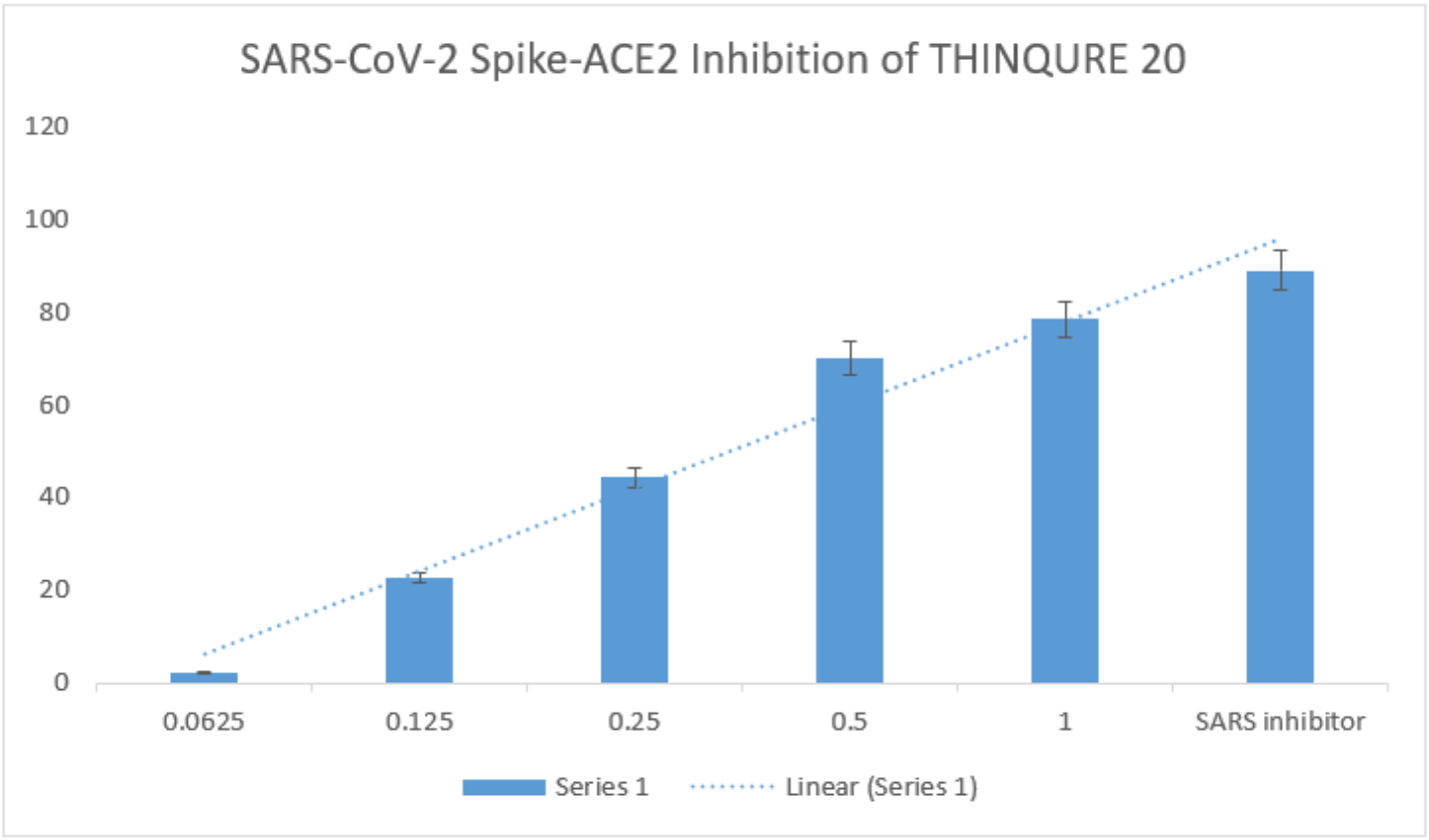

Figure 4

Legend not provided with this version.