

Evaluation of *in-vitro* Immunomodulatory Activity and Thrombolytic Potential of Kabasura Kudineer (KSK): An Official Siddha Polyherbal Formulation

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ABSTRACT

Aim: Coronavirus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), attacking mainly on the immune system of a body. This has spread mortality and morbidity all over the world. During this dreadful situation there is an urgent need for the development and rapid dissemination of COVID-19 treatment. Siddha's traditional medicine system can be used as preventive care to boost the immune system. **Materials and Methods:** The immense treasure of knowledge found in Siddha medicine can help mortality. Kabasura Kudineer (KSK) is one of the Siddha poly herbal formulation used as an immune-boosting agent against several diseases. **Results:** In the present study the KSK has been investigated for its effects of immunomodulatory and thrombolytic potential. The KSK at the concentrations of 12.5, 25, 50, and 100 $\mu\text{g/ml}$ showed % immune-stimulations of 12.40 %, 20.81, 33.53, and 43.20 and for NBT showed 19.00, 25.50, 64.00, 71.00 % respectively. Moreover, similarly, the thrombolytic activity showed 50, and 100 $\mu\text{g/ml}$ concentration showed 43.83 %, 71.83 % clot lysis, respectively, and the control value for the Streptokinase showed 83.78 %. **Conclusion:** Hence, it can be confirmed that KSK has immunomodulatory and thrombolytic properties *in vitro* models. Immunomodulatory and anti-thrombolytic are the steps to create a stable, safe, and efficient COVID-19 cure.

Key words: Kabasura Kudineer (KSK), Immunomodulatory, Thrombolytic, COVID-19, Siddha formulation.

Submission Date: 02-01-2021;

Revision Date: 03-03-2021;

Accepted Date: 02-06-2021

DOI: 10.5530/ijper.55.3.150

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INTRODUCTION

As a rule, current human healthcare services are significantly tested and challenged by the SARS-CoV-2 with its indisputable complex biochemical architecture. Its present momentum is very much persistent, making a predictable second wave.¹ The human respiratory system is very much vulnerable to different viral infections starting from coronavirus, rhinovirus, human metapneumovirus, and the human immune system is significantly affected during COVID-19 progression in the infected host.² Skowronski,

Astell³ had reported respiratory disease result in a cytokine-chemokine reaction resulting in severe damage to the host.

The role of immunology was the most rapidly developing scientific area and showed an evolving opportunity in the treatment and prevention of disorders, inflammatory reactions of different parts of the human body. Similarly, the infections are considered immunological diseases, whereas the neoplastic and autoimmune diseases occur in immunosuppressed state.⁴ It is reported



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that many of the synthetic, semi-synthetic and natural therapeutic agents have a suppressive and cytotoxic nature which support the immune system.^{1,5-7}

In today's health wellness commerce, the role of immunomodulators is well-established as a critical component. These immunomodulators are grouped into three main classes: immunosuppressants, immunostimulants, and immunoadjuvants, and their applications in medicine and pharma industries for stimulation and suppression of the immune system. Also, these are used as both prodrugs and prophylactic drugs for the healthy populace.^{8,9} Also, the plant kingdom's immunomodulators seem to be a good substitute for the synthetic chemical compounds.¹⁰

The World Health Organization has put a public health emergency by putting COVID-19 as a transnational threat.^{11,12} There is no medicine or prophylactic treatment for this disease and has been constrained towards palliative help to the affected people. Hence, there is a dire need to produce a safe and stable COVID-19 immunisation.

The current trending strategies for the COVID-19 treatment plan have been focused on immunisation against the virus and head-on attack on the virus particle. This makes the host a vital factor in ailment's subtleties. Siddha medicine is always aimed towards a healthy routine rather than just an issue of medicine.

Immunity is termed *Vanmai* in Siddha, and it has a direct association with *Uyir thathukkal* (Vali, Azhal and Aiyam) and Seven *Udal thathukkal* (Body tissues). Natural immunity of the human body by birth is called *Iyarkai Vanmai*, its improvement with the help of intake of balanced food and medicines is called *Seyarkai Vanmai* and *Kala vanmai*, which is further defined as the change of physical state under the effects of seasons and in their affected state there might be possibilities of disease.¹³⁻¹⁵ Human beings are the subtotal of *Uyir thathukkal* and *Udal thathukkal* forming his/her physical solid and mind results in a robust immune system. Individuals with the *Vali* trait have lesser immunity, while persons of *Aiyam* have moderate immunity, and persons are having *Aiyam* have stronger immunity compared to each other. The Siddha medicinal system has thoroughly tested the herbs and the polyherbal formulations via *in vitro* and *in vivo*, including the Urai mathirai, Saya chooranam, Nilavembu kudineer, which are very much beneficial.¹⁶⁻¹⁸ The botanicals used in Kayakalpa are effective in immunomodulation and restoration of immune homeostasis.¹⁹ Most of the Siddha medicines are found to be present in the mixture or consists of more than plant or their extracts. Some of the recent

literatures have stated the importance of synergistic effects of the traditional medicines and plant extracts. Yang, Zhang²⁰ and his co-workers have detailed about the Chinese herbs and their synergistic effects on different biological pathways. Similarly, the potential synergistic effects of plant based biomolecules have been well studied on SARS-CoV-2 by Prasad, Muthamilarasan.²¹ The synergistic combination of molecules interacts with target-disease networks which provide novel, mechanistic insights towards understanding their therapeutic potentials.²² The docking studies carried out by us for better understanding KSK extract revealed a pathway to understanding the Siddha in a scientific manner.²³

The COVID-19 infection cycle has two distinct phases in which the first protective phase of the adaptive immune response in a host might eliminate the virus.²⁴ In the current situation, hydroxychloroquine is considered a candidate for COVID-19 treatment due to its Immunomodulatory and antiviral effects.^{25,26} The COVID-19 leads to blood clots in people with a severe form of the COVID-19 disease. Blood clots cause a severe problem in the blood circulatory system. Blood clots in the form of thrombus hamper blood flow in blood vessels, reducing the oxygen intake to the tissues. The fibrinolytic drug dissolves the clot trapped in coronary vessels, restoring the heart's blood, limiting the necrosis.²⁷ The tissue plasminogen activator, urokinase and Streptokinase are drugs prescribed as thrombolytic agent nowadays by physicians. The Indian population has been prescribed Streptokinase and urokinase due to their low cost,^{25,26} and other drugs with the hyper risk of haemorrhage.^{28,29} The COVID-19 is a trending research topic that is being researched again in every developed country. The research papers are being published in almost every branching scientific field from biotechnology, bioinformatics, physics, chemistry and many others. The traditional medicines are also involved in this research racetrack to curb the pandemic COVID-19. Siddha Medicine is a treasured healing desire that is classically used for treating viral pulmonary infections; this precept of drugs is confirmed to incorporate antiviral compounds. The Siddha medicine is prescribing KSK for the treatment of fever and as prophylactic antiviral agents.³⁰ At present, the Ministry of AYUSH's guidelines, Government of India, KSK, is given for boosting immunity among the ordinary people³⁰ but not limited to prophylaxis and so that we can take to the integrative model of therapeutics. For selecting Siddha Medicine's safety, Efficacy and availability have to be addressed. However, the immunomodulatory activity and thrombolytic activity of Kabasura Kudineer has not been reported or scientifically investigated. Therefore,

the present study focused on investigate the immunomodulatory and thrombolytic potential of KSK.

MATERIALS AND METHODS

Kabasura Kudineer Chooranam is a compound formulation consisting of fifteen ingredients which are given in Table 1. Kabasura Kudineer Chooranam was purchased from Tamil Nadu Medicinal Plant Farms and Herbal Medicine Corporation Limited (TAMPCOL). All the chemicals and solvents are of analytical grade, obtained and used in the same condition. The *Candida albicans* suspension (MTCC-183) was purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

Extraction of the KSK and sample preparation

The dried KSK powder was weighted and was packed in Soxhlet apparatus and refluxed with distilled water. The extracts were pooled, filtered, dried, and stored below 5°C till further use. Doses such as 12.5, 25, 50, and 100 µg/ml were prepared in the isotonic solution for *in vitro* immunomodulatory activity.

In vitro immunomodulatory activity by Phagocytosis of *Candida albicans* assay

Phagocytosis of *Candida albicans* test was carried out according to method.³¹⁻³³ The Sabouraud's dextrose broth was inoculated with *C. albicans* (MTCC-183) and was incubated overnight. The *C. albicans* was then washed with Hank's balanced salt solution and was subjected to centrifugation four times, and the final cell pellet was again mixed sterile Hank's balanced salt solution and

human serum ratio of 4:1. In the present experimentation, the concentration of cells used was 1x10⁸.

Evaluation of Phagocytosis

As per Ponkshe and Indap,³¹ the estimation of the phagocytosis was performed. The finger prick method was employed to assess the phagocytosis by placing a drop of blood sterile glass slide preincubated at 37°C for 25 min. Sterile saline was used to isolate the clot; care was taken not to wash away adhered neutrophils. The KSK extract was tested in 12.5, 25, 50, and 100 µg/ml concentrations and pooled serum was used a standard and were incubated at 37°C for 15 min. This step was followed by predetermined *C. albicans* suspension concentrations and was further incubated at 37°C for 60 min. After this, slides were drained, fixed using methanol and were stained using Giemsa stain. The assessment of the phagocytosed number of *C. albicans* cells by neutrophils was carried out microscopically. The number of *Candida* cells phagocytosed/engulfed by a neutrophil is Phagocytic index (P.I.), and the study was performed in triplicates. Immunostimulation was calculated in percentage using the following equation.

$$\% \text{ of Simulation} = \frac{\text{PI (samples)} - \text{PI (control)}}{\text{PI (control)}} \times 100 \quad (1)$$

Where, the Immunostimulation % = PI (samples) - PI (control) / PI (control) × 100. Were, P.I. of samples: Phagocytic index of the test sample, P.I. of control: Phagocytic index without the test sample (i.e., normally by neutrophils).

Nitroblue Tetrazolium Assay

The test was performed as described as Mali, Hatapakki³⁴ described with minor modification. Leucocyte suspension (5×10⁶/ml) in phosphate buffer saline (PBS) was taken in all Eppendorf tubes as per Dagur and McCoy.³⁵ 100 µl of PBS was added into first Eppendorf tube and was used as control, second Eppendorf tube was added with 100 µl of lipopolysaccharide (10 µg/ml) was used as standard, and the remaining Eppendorf tubes were added with 100 µl of different concentration (12.5, 25, 50, and 100 µg/ml) of the Kabasura Kudineer extract. All these Eppendorf tubes were further added with 200 µl of 0.15% NBT solution and were incubated for 20 min at 37°C. After incubation, the Eppendorf tubes were centrifuged for 3-4 min at 400 g, and the supernatant was discarded. Further, the cells were treated with a small PBS solution, and a thin film was made with the drop on the clean glass slide. The slides were then dried, fixed by heating, and were counterstained with carbol-fuchsin for 15s. The percentage of NBT

Table 1: Kabasura Kudineer ingredients.

S.No.	Ingredients
1	<i>Zingiber officinale</i> Rosc
2	<i>Piper longum</i> L
3	<i>Syzygium aromaticum</i>
4	<i>Tragia involucrata</i> L
5	<i>Anacyclus pyrethrum</i>
6	<i>Andrographis paniculata</i>
7	<i>Hygrophilla auriculata</i> (Schum.)Heine
8	<i>Terminalia chebula</i> Retz.
9	<i>Justicia adhatoda</i> L.
10	<i>Plectranthus amboinicus</i> (Lour) Spreng
11	<i>Costus speciosus</i>
12	<i>Tinospora cordifolia</i> (Willd.) Miers ex Hook.f&Thoms
13	<i>Clerodendrum serratum</i> L.
14	<i>Sida acuta</i> Burm. f.
15	<i>Cyperus rotundus</i> L.

positive cells with blue lumps or granules was determined by observing the stained slides for blue colour cells/lumps/granules under 40 X objective for 200 cells. All the experiments were carried out in triplicates, and the results are expressed as mean $\pm$ S.D.

$$\% \text{ of NBT positive cells} = \frac{\text{observing blue color cells}}{200 \text{ cells}} \times 100 \quad (2)$$

In vitro thrombolytic activity of KSK

Preparation of Streptokinase (S.K.)

The lyophilised S.K. vial of 15,00,000 I.U was correctly mixed with 5 ml phosphate-buffered saline. This suspension was labelled stock from which dilutions were made to thrombolytic activity as per the *in vitro* model developed in our lab.^{36,37}

Determination of thrombolytic activity

Three millilitres of venous blood were distributed in four different Eppendorf tube. The thrombolytic activity was performed by preincubating the Eppendorf tubes at 37°C for 45 mins. Subsequently, the clot formation was followed with the removal of serum without disturbing the clot. The clot weight was determined using the formula; Clot weight = weight of clot filled tube - Weight of empty tube alone. With pre-weighted clot, 100 μ l of KSK extract was added to these tubes, and for the standard, 100 μ l of Streptokinase and negative nonthrombolytic control - 100 μ l of distilled water were separately added to the control Eppendorf tubes. Incubation followed for 90 min at 37°C and was observed for clot lysis. After which, the fluid was removed, and the tubes were weighted to observe a weight difference of.³⁷ The difference obtained in weight taken before and after clot lysis was expressed as the percentage of clot lysis is shown below:

$$\% \text{ of clot lysis} = \left(\frac{\text{Weight of lysis clot}}{\text{Weight of clot before lysis}} \right) \times 100 \quad (3)$$

Statistical analysis

Tests were carried out in triplicate for three separate experiments. Results were expressed as Mean $\pm$ standard deviation. $P < 0.05$ was considered significant and was expressed graphically.

RESULTS AND DISCUSSION

An immunomodulatory agent from the plant or animal kingdom increases the human body's immune system with the activation of non-specific immune responses. Different plants have tested for their immunostimulant

and immunosuppressive properties. In support of this statement, many traditional medicine system concepts of preventive health care and the therapeutic potential have been tested and reviewed in detail.^{32,38} The Ministry of AYUSH has issued guidelines for the Siddha practitioners for COVID-19 for different antiviral and immunity booster formulations, including KSK and Nilavembu Kudineer. We have reported docking studies of bioactive compounds from KSK,²³ which confirmed that this extract has excellent binding efficiency with spike protein of SARS-CoV-2. Further, in this study, we also explored the immunomodulatory and thrombolytic activity of KSK.

The *in vitro* immunomodulatory activity of the KSK extract have illustrated in Figure 1. The percentage of killed *C. albicans* have found to be near to the control sample (serum). This graph substantiates the immunomodulatory property of KSK. Similar results have been observed in *Rhododendron arboreum* leaves,³² *Euphorbia hirta*,³³ similarly, many plant isolated compounds have been reported to immunomodulating nature. The vincristine as an immunosuppressant has been employed for treating thrombotic thrombocytopenic purpura or chronic idiopathic thrombocytopenic purpura.³⁹ This alkaloid compound has also been utilised to treat many more diseases: idiopathic thrombocytopenia purpura, bladder cancer, cervical cancer, non-small-cell lung cancer, autoimmune haemolytic anaemia, neck cancer, and head cancer.^{39,40}

Nitroblue tetrazolium test assesses the test compound's immunomodulatory activity by determining its ability to stimulate the phagocytic activity in leucocytes. Once stimulated, the membrane-permeable, water-soluble, yellow-coloured nitroblue tetrazolium is reduced to blue NBT formazan crystals by the leucocytes. The KSK extract stimulated the leucocytes' phagocytic activity in a concentration-dependent manner, as seen by the

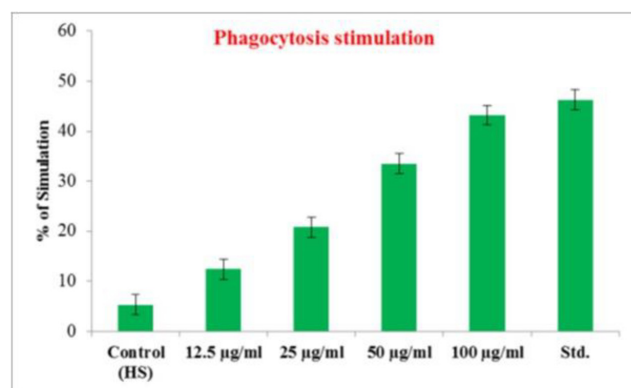


Figure 1: Percentage of killed Candida after treatment with extract by phagocytosis stimulation.

increased percentage of NBT positive cells, shown in Figure 2. The immunomodulatory effect with the aid of nitro blue assay has been observed in *Ficus glomerata* Roxb.⁴¹ *Nelumbo nucifera* Gaertn.⁴² *Pouteria cambodiana*.⁴³ The study's result indicates the functionality of the neutrophils in the process of phagocytosis is high, creating a proactive environment from the infection. The COVID-19 patients have shown thrombosis as one of the symptoms.^{44,45} Also, thrombus formation leads to progressive respiratory failure,⁴⁶ myocardial infarction, systemic arterial embolism in COVID-19 patients.⁴⁷ The effective thrombolytic percentages with different concentration of the KSK extract, control, 50 and 100 µg/ml and standard (S.K.) showed 22.36, 43, 71.83 and 83.75 %, respectively has been illustrated in Figure 3. From Figure 3, it is evident that the percentage of the thrombolytic activity was 71.83 % at 100 µg/ml compared to the 100 µl Streptokinase. From the different samples, the 50µg/ml showed 43% thrombolytic activity, which is higher than the distilled water

(negative control). Kiran²³ have already reported the phytoconstituents of Siddha formulation KSK. These compounds have been detailed of their biological activities; some of them were found to have thrombolytic, immunomodulatory, anti-inflammatory, and fibrinolytic activity, for example, β-bisabolene,⁴⁸⁻⁵¹ piperine,⁵²⁻⁵⁴ Squalene^{55,56} Chebulagic acid,⁵⁷ Carvacrol,^{58,59} Luteolin^{60,61} Magnoflorine.⁶²⁻⁶⁴

As per Siddha, stickiness, mucilaginous, rounded, little hard are listed as characters of *Aiyam*. A thrombus has all the qualities of increased *Aiyam*, most of the thrombolytic drugs are pungent and bitter. Kabasura Kudineer has already been screened for its anti-atherogenic property. The results also suggest the thrombolytic potential of Kabasura Kudineer owing to its fire-based elements in the ingredients. We have reported docking studies of bioactive compounds from Kabasura Kudineer,²³ which confirmed that this extract has excellent binding efficiency with spike protein of SARS-CoV-2.

CONCLUSION

Siddha medicine is one of the best ways to control the COVID-19. The immunomodulatory and anti-thrombolytic are the stepping stones to developing a stable, safe, and working cure for COVID-19. Kabasura kudineer is a polyherbal decoction with fifteen different components, and each of them is in itself a firmly established herbal plant whose synergistic activity might probably improve human immune response and lead the human body to healthiness. The immunomodulatory property and thrombolytic activity of this miracle Siddha medicines have been studied using *in vitro* experiments but still require *in vivo* animal model experiments to understand better. This research paper has indicated and supported the notion of using the KSK extract to improve the immune response in this COVID-19 infected time.

ACKNOWLEDGEMENT

The authors thank all the institutes for their support to carry out this research and thanks to Central Council for Research in Siddha, Ministry of AYUSH, Chennai, Tamilnadu, India and Salem Microbes Private Limited Salem, Tamil Nadu, India.

CONFLICT OF INTEREST

The authors declare no Conflict of interest.

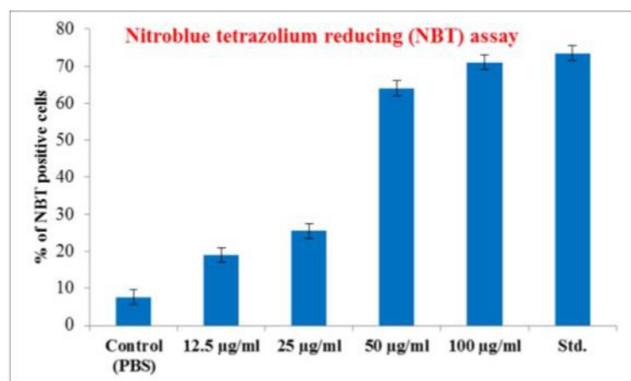


Figure 2: Percentage of NBT positive cells after treatment with extract by Nitro blue Tetrazolium Test (NBT).

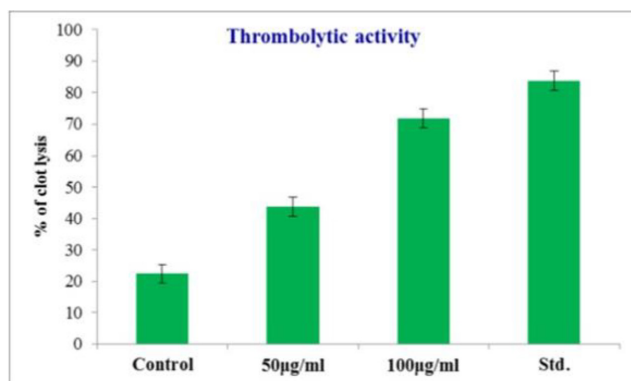


Figure 3: Thrombolytic activity (in terms of % clot lysis) of sample.

ABBREVIATIONS

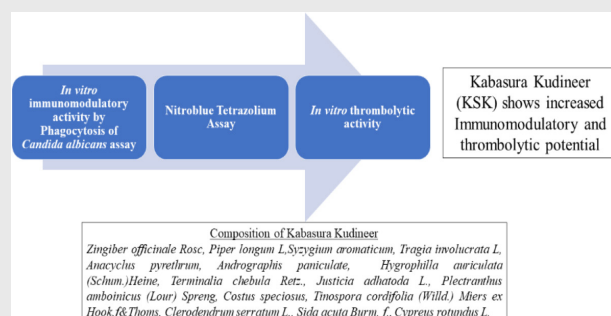
KSK: Kabasura Kudineer; **MTCC:** Microbial Type Culture Collection and Gene Bank; **C. albicans:** *Candida albicans*; **P.I:** Phagocytic index; **PBS:** Phosphate buffer saline; **NBT:** Nitroblue Tetrazolium; **S.K:** Streptokinase; **I.U:** International unit.

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PICTORIAL ABSTRACT



SUMMARY

The present experimental paper details the utilisation and application of the KSK extract as an immunomodulatory substance for the improvement in human immune response

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Cite this article: Parameswaran S, Sampangi Ramulu SDM, Arivarasan VK, Kadarkarai K, Dhanakoti RK, Loganathan K. Evaluation of *in-vitro* Immunomodulatory Activity and Thrombolytic Potential of Kabasura Kudineer (KSK): An Official Siddha Polyherbal Formulation. Indian J of Pharmaceutical Education and Research. 2021;55(3):774-81.