In-vitro Immunomodulatory activity and Thrombolytic potential of Kabasura Kudineer (KSK), an official Siddha polyherbal formulation

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Abstract:

Coronavirus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a pandemic, which has led to the spread of mortality and morbidity all over the globe. In this dire situation, there is an urgent requirement for the development and immediate dissemination of treatment against COVID-19. The traditional medicine system of Siddha can be utilized as preventive care to boost the immune system. The vast treasure of knowledge found in Siddha medicine can help in the betterment of humankind. Kabasura Kudineer (KSK) is from the ancient times present as an immune-boosting agent against several diseases. The present experimental setup to investigate the immunomodulatory and thrombolytic potential of KSK. The *in vitro* immunomodulatory models of phagocytosis of *Candida albicans* assay and nitro blue tetrazolium have demonstrated that KSK is giving better results compared with the controls (pooled serum, lipopolysaccharide, and streptokinase). The KSK at the concentrations of 12.5, 25, 50, and 100 µg/ml showed % immune-stimulations of 12.40 %, 20.81 %, 33.53 %, 43.20 % and for NBT showed 19.00 %, 25.50 %, 64.00 %, 71.00 % respectively. And similarly, the thrombolytic activity showed 50 and 100 µg/ml concentration showed 43.83 %, 71.83 % clot lysis respectively; and the control value for the streptokinase showed 83.78 %. Hence, it can be confirmed that KSK has immunomodulatory and thrombolytic properties in *in vitro* models, although the in vivo and the identification of KSK are to be discovered.

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

Introduction

Current human healthcare services are as a rule 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 (Cyranoski 2020). The human respiratory system is very much vulnerable to different viral infections starting from coronavirus, rhinovirus, human metapneumovirus etc. and the human immune system is very affected during COVID-19 progression in the infected host (Schmidt and Varga 2018). Skowronski et al. (2005) had reported respiratory disease result in cytokine-chemokine reaction resulting in serious 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 are occur in immunosuppressed state (Ziauddin et al. 1996). It is reported that many of the synthetic, semi-synthetic and natural therapeutic agents have the suppressive and cytotoxic nature which support the immune system (Cyranoski 2020; Manderville 2001; Ren and Kinghorn 2019; Shavit et al. 1984).

In todays, health wellness commerce, the role of immunomodulators is well-established as a key component. These immunomodulators are grouped into three main classes: immunosuppressants, immunostimulants, and immunoadjuvants, and their applications in medicine and pharma industries as for stimulation and suppression of the immune system. And used as both prodrugs and prophylactic drugs for the healthy populace (El Enshasy and Hatti-

Kaul 2013; Shukla et al. 2014). In addition, the immunomodulators from the plant kingdom seem to be a good substitute for the synthetic chemical compounds (Patwardhan et al. 1990).

The World Health Organization has put a public health emergency by putting COVID-19 as an transnational threat (WHO 2020; Wu et al. 2020). Until, today there is no medicine or prophylactic treatment for this disease and has been constrained towards the palliative help to the affected people. Hence, there is dire need to produce a safe and stable COVID-19 immunization.

The recent trending strategies for the COVID-19 treatment plan have been focused in the immunization against the virus and head on attack on the virus particle. This makes host as a vital factor in ailment's subtleties. The Siddha medicine is always aimed towards healthy routine rather than just 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 (Govindammal 2016; Rajeshwari 2017; Srinivasan 2007). The human beings are the subtotal of *Uyir thathukkal* and *Udalthathukkal* forming his/her strong physical and mind results in a strong immune system. Individuals with *Vazhi* trait have lesser immunity, while persons of *Azhal* have moderate immunity, and persons having *Aiyam* are having stronger immunity. The Siddha medicinal system has tested thoroughly the herbs and the polyherbal formulations via *in vitro* and *in vivo* which include the Urai mathirai, Saya chooranam, and Nilavembu kudineer, etc., which are very much beneficial (Dayanand et al. 2019; Kavinilavan et al. 2017; Santhammal et al. 2011). The botanicals used in Kayakalpa are

effective in immunomodulation and restoration of immune homeostasis (Vaidya 2010). The docking studies carried out by us for better understanding KSK extract revealed a pathway to understand the Siddha in scientific manner (Kiran et al. 2020).

The COVID-19 infection cycle has two distinct phases in which the first protective phase of the adaptive immune response in host which might eliminate the virus (Shi et al. 2020). In the current situation, hydroxychloroquine is considered as a candidate for COVID-19 treatment due to its Immunomodulatory and antiviral effects (Chen et al. 2020; Liu et al. 2020). The COVID-19 leads to blood clots in people with severe form of the COVID-19 disease. Blood clots causes a severe problem in blood circulatory system. Blood clots in the form of thrombus hampers the flow of blood in blood vessels, reducing the oxygen intake to the tissues. The fibrinolytic drug dissolves clot trapped in coronary vessels, restoring the blood of heart, limiting the necrosis (Mucklow 1995). The tissue plasminogen activator, urokinase and streptokinase are drugs prescribed as thrombolytic agent nowadays by physicians. The Indian population have been prescribed streptokinase and urokinase due to its low cost, (Collen 1990; Martin et al. 1996) in comparison to other drugs which have hyper risk of hemorrhage (Liu et al. 2018; Rouf et al. 1996).

The COVID-19 is the trending research topic which is being researched again in every developed country. The research papers are being published in almost every branching scientific fields from biotechnology, bioinformatics, physics, chemistry and many other. The traditional medicines are also involved in this research race track 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

(AYUSH 2020). At present, the guidelines issued by the Ministry of AYUSH, Government of India, KSK is given for boosting immunity among the common people (AYUSH 2020) but not limited to prophylaxis too. 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, 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 (Ponkshe and Indap 2002; Ramesh and Padmavathi 2010; Rawat et al. 2018). 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 for 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 $1x10^8$.

Evaluation of Phagocytosis

As per Ponkshe and Indap (2002), 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, which was preincubated at 37° C for 25 min. Sterile saline was used to isolate 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 phagocytosed number of *C. albicans* cells by neutrophils was carried out microscopically. The number of *Candida* cells phagocytosed/engulfed by a neutrophil are Phagocytic index (PI) and the study was performed in triplicates. Immunostimulation was calculated in percentage using the following equation.

% of Simulation = PI (samples) - PI (control) / PI (control) x 100 ----------(1)

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

Nitroblue Tetrazolium Assay

The test was performed as described as Mali et al. (2008) described with minor modification. Leucocyte suspension ($5x10^6$/ml) in phosphate buffer saline (PBS) was taken in all

Eppendorf tubes as per Dagur and McCoy (2015). 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 small volume of 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 countered stained with carbol-fuchsin for 15s. The percentage of NBT 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 ± SD.

% of NBT positive cells = observing blue color cells / 200 cells ×100 ------- (2)

***In vitro* thrombolytic activity of KSK**

Preparation of streptokinase (SK)

The lyophilized SK vial of 15,00,000 I.U was mixed properly 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 (Khan et al. 2011; Prasad et al. 2006).

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

minutes. Subsequently, the clot formation was followed with removal of serum without disturbing clot. The clot weight was determined using the formula, Clot weight = Weight of clot filled tube - Weight of empty tube alone. To these tubes, with pre-weighted clot, 100 µl of KSK extract was added and for the standard, 100 µl of streptokinase and negative nonthrombolytic control - 100µl of distilled water were separately added to the control Eppendorf tubes. Incubation followed for 90 minutes at 37 °C and were observed for clot lysis. After which, the fluid was removed and the tubes were weighted to observe weight difference (Prasad et al. 2006). The difference obtained in weight taken before and after clot lysis was expressed as the percentage of clot lysis is shown below:

$$\% \text{ of clot lysis} = (\text{Weight of lysis clot/weight of clot before lysis}) \times 100$$

Statistical analysis

Tests were carried out in triplicate for three separate experiments. Results were expressed as graphically with mean± standard deviation values.

Results and Discussion

An immunomodulatory agent from the plant or animal kingdom increases the responsiveness of immune system of human body with activation of non-specific immune responses. Different plants have tested for their immunostimulant and immunosuppressive properties. In the support of this statement; many of the traditional medicine system concepts of preventive health care and the therapeutic potential have been tested and reviewed in detail (Rawat et al. 2018; Upadhyay 1997). The Ministry of AYUSH has issued guidelines for the Siddha practitioners for COVID-19 for different antiviral and immunity booster formulations which includes KSK, and NilaVembu Kudineer (AYUSH 2020). We have reported docking studies of bioactive compounds from KSK (Kiran et al. 2020), which confirmed that this extract

has a good binding efficiency with spike protein of SARS-CoV-2. Further, in this study we also explored the immunomodulatory and thromolytic 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 (Rawat et al. 2018), *Euphobia hirta* (Ramesh and Padmavathi 2010), Similarly, many plant isolated compounds have been reported to immunomodulating nature. The vincristine as immunosuppressant have been employed for treating thrombotic thrombocytopenic purpura or chronic idiopathic thrombocytopenic purpura (Qweider et al. 2007). Also, this alkaloid compound has utilized for the treatment of many more diseases idiopathic thrombocytopenia purpura, bladder cancer, cervical cancer, non-small-cell lung cancer, autoimmune hemolytic anemia, neck cancer, and head cancer (Dhayalan et al. 2015; Qweider et al. 2007).

Nitroblue tetrazolium test is to assess the immunomodulatory activity of the test compound by determining its ability to stimulate the phagocytic activity in leucocytes. Once stimulated, the membrane permeable, water soluble, yellow-colored, nitroblue tetrazolium is reduced to blue NBT formazan crystals by the leucocytes. The KSK extract stimulated phagocytic activity of the leucocytes in a concentration dependent manner as seen by the increased percentage of NBT positive cells, results shown in **Figure 2**. The immunomodulatory effect with the aid of nitroblue assay have been observed in *Ficus glomerata* Roxb.(Heroor et al. 2013), *Nelumbo nucifera* Gaertn.(Mukherjee et al. 2010), *Pouteria cambodiana* (Manosroi et al. 2006). The result of the study 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 (Connors and Levy 2020; Panigada et al. 2020). And the formation of thrombus leads to progressive respiratory failure (Ackermann et al. 2020), and myocardial infarction, systemic arterial embolism in COVID-19 patients (Klok et al. 2020). The effective thrombolytic percentages with different concentration of the KSK extract, Control, 50 and 100 µg/ml and standard (SK) showed 22.36, 43, 71.83 and 83.75 %, respectively has been illustrated in **Figure 3**. From the **Figure 3**, it is evident that the percentage of the thrombolytic activity was 71.83 % at 100 µg/ml when 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). The phytoconstituents of Siddha formulation KSK have been already reported by Kiran et al. (2020). These compounds have been detailed of their biological activities, some them were found to have thrombolytic, immunomodulatory, ant inflammatory, and fibrinolytic activity for example β-bisabolene (Akbar 2020; Aruna et al. 2014; Li et al. 2019; Sharma 2018), piperine (Meghwal and Goswami 2013; Rather and Bhagat 2018; Zheng et al. 2016), Squalene (Fox et al. 2011; Fox et al. 2012), Chebulagic acid (Shanmuganathan and Angayarkanni 2018), Carvacrol (Ezz-Eldin et al. 2020; Kianmehr et al. 2016), Luteolin (Maatouk et al. 2017; Wangchuk et al. 2018), Magnoflorine (Bala et al. 2015; Sharma et al. 2012; Xu et al. 2020).

*A*s per Siddha stickiness, mucilaginous, rounded, little hard, are listed as characters of *kabam*. A thrombus has all the qualities of increased *kabam*, majority of the drugs which are thrombolytic are pungent and bitter in taste. Kabasura Kudineer has already been screened for its anti-atherogenic property. The data from the results also suggests 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 (Kiran et al. 2020), which confirmed that this extract has a good binding efficiency with spike protein of SARS-CoV-2.

CONCLUSION

Siddha medicine is one of best way to control the COVID-19. The immunomodulatory and anti-thrombolytic are the stepping stones towards the development of a stable, safe and working cure for COVID-19. Kabasura kudineer is a polyherbal decoction with fifteen different components, and each of them are in themselves strongly established herbal plants, 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 has been studied using *in vitro* experiments but still requires *in vivo* animal model experiments for better understanding. This research paper has clearly indicated and has supported the notion of using the KSK extract for improving the immune response in this COVID-19 infected time.

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Conflicts of Interest

None

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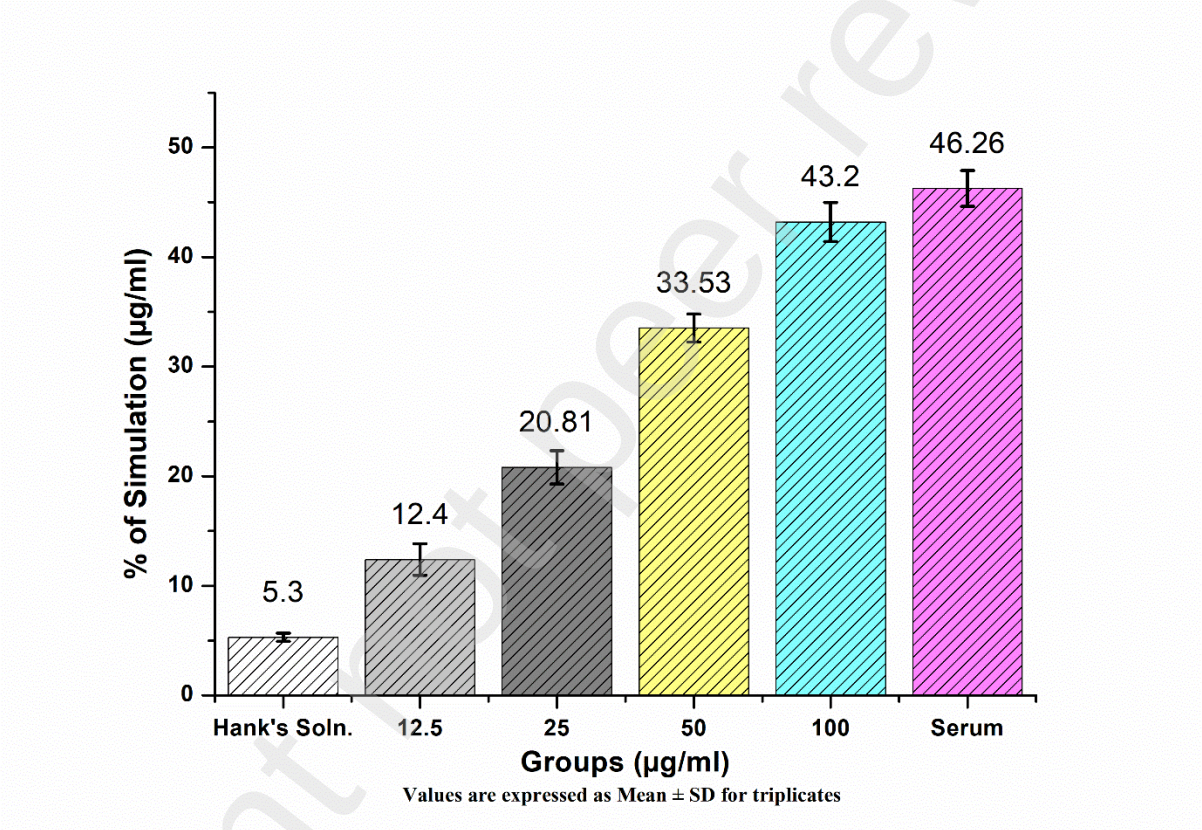

Figure 1. Percentage of killed *Candida albicans* after treatment with KSK extract by Phagocytosis stimulation.

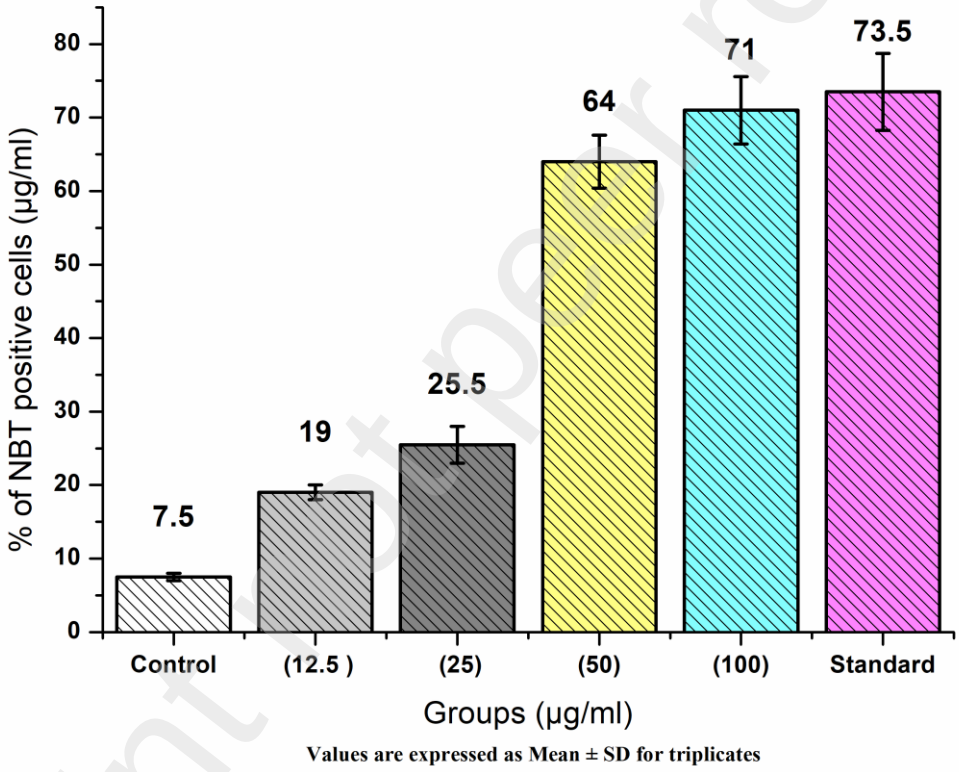

Figure 2. Percentage of NBT positive cells after treatment with KSK extract by Nitroblue Tetrazolium Test (NBT)

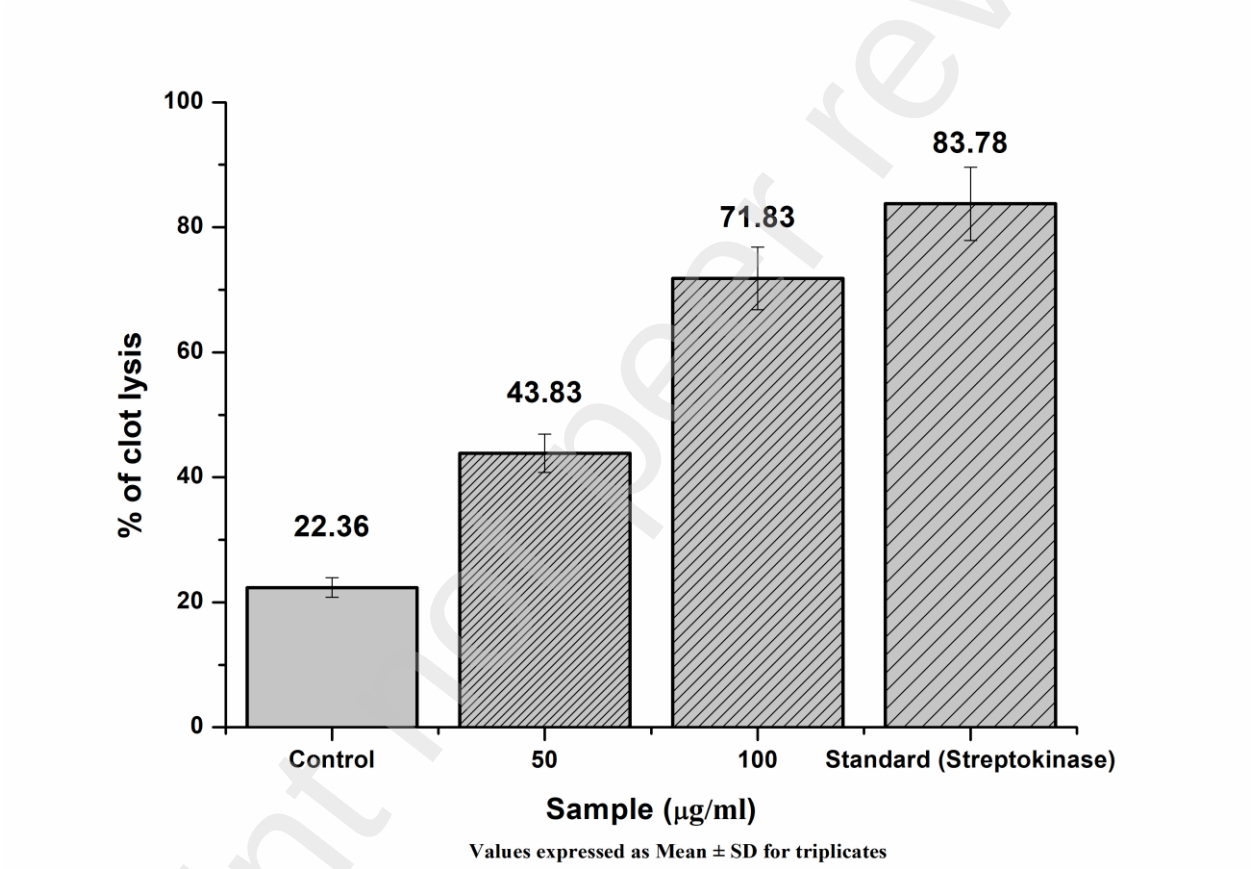

Figure 3. Thrombolytic activity (in terms of % clot lysis) of KSK extract

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

Table.1: Kabasura Kudineer ingredients