



Anti-inflammatory effect of Kaba Sura Kudineer (AYUSH approved COVID-19 drug)-A Siddha poly-herbal formulation against lipopolysaccharide induced inflammatory response in RAW-264.7 macrophages cells

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ABSTRACT

Ethnopharmacological relevance: Medicinal importance and potential activity of Siddha herbal formulations have proved over several centuries against a wide range of causative agents as Influenza, Dengue, Chikungunya, and Tuberculosis. The traditional medicine system of Siddha is a valuable therapeutic approach for treating viral respiratory infections like Coronavirus disease 2019 (COVID-19) and can be effectively employed to target the host response and preventive care to boost the immune system. Kaba Sura Kudineer (KSK), an official polyherbal formulation has been used in Siddha traditional medicine for centuries. However, the role of KSK in regulating inflammation and the underlying molecular mechanisms has remained elusive.

Aim of the study: The goal of this study was to evaluate the anti-inflammatory effect of KSK using lipopolysaccharide (LPS) stimulated RAW 264.7 murine macrophage cells.

Materials and methods: Raw 264.7 murine macrophage cells were used for this study. The Inflammatory mediators and cytokines were measured by enzyme-linked immunosorbent assay (ELISA). The NF- κ B nuclear translocation and protein expression of iNOS, COX-2 was analyzed with western blot.

Results: KSK supplementation decreased LPS mediated TLR-4 production and secretion of pro-inflammatory mediators and cytokines including IL-6, TNF- α , COX-2 and PGE-2. Moreover, it inhibited the production of nitric oxide (NO) and thereby inhibited the expression of iNOS in the cell. The Western blot analysis further confirmed that KSK strongly prevented the LPS-induced degradation of I κ B which is normally required for the activation of NF- κ B and hereby suppressed nuclear translocation of NF- κ B. The protein expression of iNOS, COX-2 was significantly decreased with the presence of KSK treatment. Results suggested that KSK manipulates its anti-inflammatory effects mainly through blocking the TLR mediated NF- κ B signal transduction pathways.

Conclusions: Together, this study has proven that KSK could be a potential therapeutic drug for alleviating excessive inflammation in many inflammation-associated diseases like COVID-19.

1. Introduction

Siddha medicine is one of the ancient traditional systems of medicines that come under the AYUSH system of India and the efficacy of herbal formulations from the Siddha system is become popular nowadays because of various outbreaks of communicable and infectious diseases. Kaba Sura kudineer (KSK) is one such well-known traditional

medicine described in *Siddha manuscript 22 Citta Vaittiyattirattu (Siddha formulary of India Part II, 2011)*, used in India over centuries and most prescribed official poly-herbal Siddha drug for the management and prevention of various febrile episodes of outbreaks caused by swine flu, dengue virus and chikungunya virus (Kiran et al., 2020; Jain et al., 2018; Jain et al., 2020). This poly-herbal formulation consists of fifteen herbs developed by the Siddha Formulary of India (Sathiyarajeswaran et al.,

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Table 1
Ingredients present in KSK.

Sl. No	Kabasura Kudineer ingredients
1.	Zingiber officinale Rose
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.	Cyperus rotundus L

2020). The ethnopharmacological actions of these fifteen ingredients demonstrated that KSK having anti-inflammatory, antipyretic, analgesic, anti-viral, anti-bacterial, anti-fungal, anti-oxidant, hepatoprotective, anti-diabetic, anti-asthmatic, immunomodulatory, anti-diarrheal and anti-oxidant activities (Thillaivanan et al., 2015). Recent studies on KSK showed its significant antiviral effect as well as its efficacy against SARS-CoV-2 through the In-silico method, Immunomodulatory action, thrombolytic activity, antioxidant and anti-atherogenic activity method (Shree Devi et al., 2021; Vincent et al., 2020; Sathiyarajeswaran et al., 2020; Rajalakshmi et al., 2020). In Silico computational screening, Molecular docking and Clinical studies on the anti-viral effects of phytoconstituents (Table 1) present in KSK has been well established by various studies (Kiran et al., 2020; Vincent et al., 2020; Maideen, 2021). Comparative clinical studies of KSK with Zinc and Vitamin C supplementation on SARS-Cov-2 subjects confirms significant down reduction of SARS-COV-2 viral load in patients. (Natarajan et al., 2020).

According to the guideline of the AYUSH ministry, the Government of India, KSK is prescribing for COVID-19 as well as a good immune booster (AYUSH Ministry of Health Corona Advisory—D.O. No. S. 16030/18/2019—NAM; 06th March 2020).

The recent studies have been emphasizing that above 50% of all the death happening in worldwide is mainly due to chronic inflammatory diseases (GBD Causes of Death Collaborators, 2018). Inflammatory diseases are commonly characterised by exaggerated production of reactive oxygen or nitrogen species (ROS/RNS) which is the source of free radicals. Normally, the overproduction of free radicals is counterbalanced by the antioxidant system but the imbalance between the scavenging and production of free radicals will lead to oxidative stress and this will trigger the activation of several inflammatory signalling cascades. Depending upon the range of variation in the equilibrium, the impact of OS will be either subtle or very severe (Hendrayani et al., 2016; Halliwell and Whiteman, 2004). During a pathogenic inflammatory condition, there will be an overwhelming ROS which inflicts severe oxidative stress and damages the tissue (Fialkow et al., 2007). In such cases, the inflammation begins with innate immune cells possessing pattern recognition receptors (PRRs) such as Toll-like receptor (TLR), NOD-like receptor (NLR) etc. that recognize the pathogen-associated molecular pattern (PAMP) which is a microbial structure (Gudkov and Komarova, 2016) Upon binding, PRR initiates signal transduction pathways and thereby activates the transcription factors, nuclear factor κ B (NF- κ B) and activating protein 1 (AP-1). These factors are responsible for the initiation of inflammatory responses by inducing the gene expression of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) and inducible nitric oxide (iNOS), antimicrobials agents and recruit more immune cells (Stender et al., 2012; Obermeier et al., 1999), along with these certain anti-inflammatory cytokines (IL-10, IFNs, TGF- β) will also produce to regulate the damages. But, certain pathogens will escape

from the immunological responses and developed their strategies for their existence. Such pathogens may keep themselves hidden from the immune system or will overstimulate the immune system which is known as ‘cytokine storm’ that can cause severe infection and damages (Finlay and McFadden, 2006).

Concerns in the treatment of this type of inflammatory condition are that the common steroidal and non-steroidal drugs cannot be used efficiently against it. Hence, there is a need for a therapeutic agent which can efficiently target inflammation, oxidative stress and also involving in immunomodulation. In this regard, traditional medicines are now widely established as an alternative to conventional drugs which is also accepted by the World health organisation (WHO) (Zhang, 1998). In this scenario, Kaba sura Kudineer is one of the best-treasured healing desire that is classically used for treating viral pulmonary infections, because this drug has several antiviral compounds. However, the molecular mechanism underlying the anti-inflammatory action of KSK has not been reported. Therefore, in the present study, we attempted to evaluate the anti-inflammatory effect of KSK using LPS-activated RAW 264.7 cells and the underlying mechanisms associated with inflammation and oxidative stress in RAW 264.7 cells.

2. Materials and methods

2.1. Chemicals

All the chemicals and solvents are of analytical grade, obtained and used in the same condition. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin and bovine serum albumin (BSA) (Gibco BRL, Gaithersburg, MD, USA), Griess reaction kit for Nitric Oxide, ELISA kits for detecting TNF- α and IL-6 (R&D Systems, Inc., USA), PGE₂ ELISA Kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA), Trizol reagent (Invitrogen, Carlsbad, CA, USA), Antibodies specific for COX-2, iNOS, κ B, NF- κ Bp65, and glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

2.2. Extraction and preparation of Kaba Sura Kudineer (KSK)

KSK is a polyherbal formulation consisting of fifteen ingredients listed in Table 1 was obtained from Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd. (Impcops) Thiruvananthapuram. Dried powder of KSK was weighted and filled in the Soxhlet apparatus and then refluxed with double distilled water. The extracts were collected, filtered, dried, and stored below 5 °C till further use.

2.3. Cell culture and viability assay

RAW264.7, a mouse macrophage cell line was obtained from National Centre for Cell Science (NCCS) Pune, India and was cultured in high-glucose DMEM with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in the presence of 5% CO₂ incubator at 37 °C. Before treatment, cells (5×10^4 cells/well) were incubated overnight at 37 °C in a 96-well plate.

After overnight culture, cells were treated with different concentrations (3.125, 6.25, 12.5, 25, 50 and 100 μ g/mL) of KSK in the absence or presence of 1 μ g/mL lipopolysaccharide (LPS) for 24h. For the cell viability analysis, MTT assay was carried out. Thereafter, culture termination, MTT (5 mg/mL) was added to each well, and then incubated for 3 h at 37 °C in a 5% CO₂ incubator. Then 100 μ L/well of solubilized solution (DMSO) was added to each well. Finally, the absorbance of each well was recorded at 570 nm using a microplate reader.

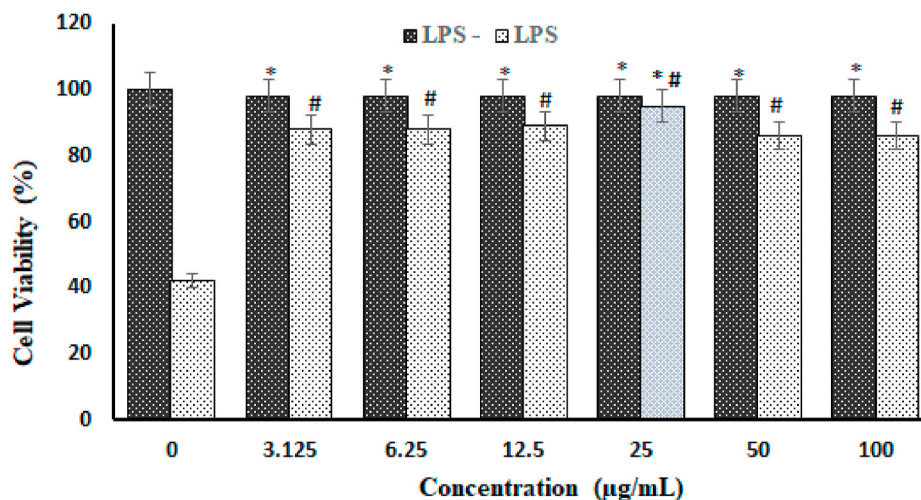


Fig. 1. Cell viability assay was done by MTT assay. RAW 264.7 cells were incubated with different concentrations of KSK (3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL) for 24 h* Compared with LPS treated group, $P \leq 0.05$. # denotes LPS treated group compared with control group, $P \leq 0.05$. Values are expressed as mean \pm SD (n = 3).

2.3.1. Morphological analysis

The observation of morphological changes of RAW 264.7 cells was performed using a phase-contrast inverted microscope. Briefly, cells were incubated for 24 h at various concentrations of KSK in 60 mm diameter tissue culture dishes. The medium was discarded and cells were washed once with PBS. The changes in the morphology of cells were observed to determine the alterations in RAW 264.7 cells and the images of the cells were grabbed at 20x by using the phase-contrast inverted microscope (Labomed, USA).

2.3.2. Measurements of intracellular ROS level

Intracellular ROS levels were measured by the 2', 7'-dichlorofluorescein diacetate (DCF-DA) assay (Leloup et al., 2006). DCFDA can be deacetylated in cells, where it can react quantitatively with intracellular radicals to convert into its fluorescent product, DCF, which is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in oxidative stress. Thereafter, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated with 5 µL DCF-DA for 30 min at room temperature. Fluorescence was measured using a fluorescence plate reader.

2.3.3. Determination of TNF- α and IL-6 levels

The effect of KSK on the production of inflammatory markers like TNF- α and IL-6 were measured by ELISA kits according to the manufacturer's instructions. After overnight culture in a 24-well plate, (1×10^5 cells/well) the cells were pre-treated with KSK at various concentrations for 1 h and lipopolysaccharide for an additional 24 h. Then, the supernatants from each well were collected at the end of scheduled experiments by centrifugation for 10 min at 4 °C and the absorbance was then measured at 520 nm using a microplate reader.

2.3.4. Determination of nitric oxide production

Using the Griess method, the Nitric oxide (NO) production assay was determined and RAW264.7 cells (2.5×10^5 cells/mL) were cultured in 6-well plates with or without LPS pre-treatment for 30 min and then incubated with various concentrations of KSK for another 24 h. 50 µL of supernatants were collected and mixed with equal volumes of Griess reagent were reacted for 10 min at room temperature and the absorbance was then measured at 540 nm using a microplate reader.

2.3.5. Determination of prostaglandin (PGE₂) levels

Cells were cultured in 24-well plates and pre-treated with or without

KSK at various concentrations for 12 h, then incubated with LPS (1 µg/mL) for 20 h. The accumulated PGE₂ in the culture medium was measured using an ELISA kit (Cayman Chemical Company, USA) according to the manufacturer's instructions.

2.3.6. Measurement of antioxidant enzyme activities

Cells (2×10^4 cells/well) in a 96 well plate were pre-incubated with the concentration at 25 µg/mL of KSK for 1 h, then further incubated with LPS for 24 h. The medium was removed and the cells were washed twice with PBS. 1 mL of 50 mmol/L potassium phosphate buffer with 1 mmol/L EDTA (pH 7.0) was added and the cells were scraped. Cell suspensions were sonicated three times for 5 s on the ice each time then centrifuged at $10,000 \times g$ for 20 min at 4 °C. Cell supernatants were used for measuring antioxidant enzyme activities. The protein concentration was measured by using the Lowry method with bovine serum albumin as the standard. Superoxide dismutase (SOD) activity was determined by monitoring the auto-oxidation of pyrogallol. A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol. Catalase activity was measured according to the method of Aebi by following the decreased absorbance of H₂O₂. The decrease of absorbance at 240 nm was measured for 2 min (Aebi, 1984). Glutathione peroxidase (GSH-px) activity was measured by using the method of Lawrence and Burk. One unit of GSH-px was defined as the amount of enzyme that oxidized 1 nmol of NADPH consumed per minute (Lawrence and Burk, 1976).

2.3.7. Western blotting analysis

After treatment with KSK at 25 µg/mL concentration in the presence or absence of 1 µg/mL LPS, cells were analyzed by immunoblotting. The treated cells were washed and scraped into cold phosphate-buffered saline (PBS) and centrifuged at $500 \times g$ at 4 °C. The cell pellets were resuspended in lysis buffer and centrifuged to yield whole-cell lysates. 20 µg protein for each sample was separated by SDS-polyacrylamide gels with electrophoresis and the gel was transferred to the PVDF membrane. The membrane was blocked with 10% skim milk for 1 h and then incubated overnight at 4 °C with 1:2000 dilution of the corresponding primary antibody. After washing, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. The membrane was immersed in the enhanced chemiluminescence solution for 60 s. The gel images were visualized using Chem-Doc (Bio-Rad, CA). The results are representative of three independent experiments.

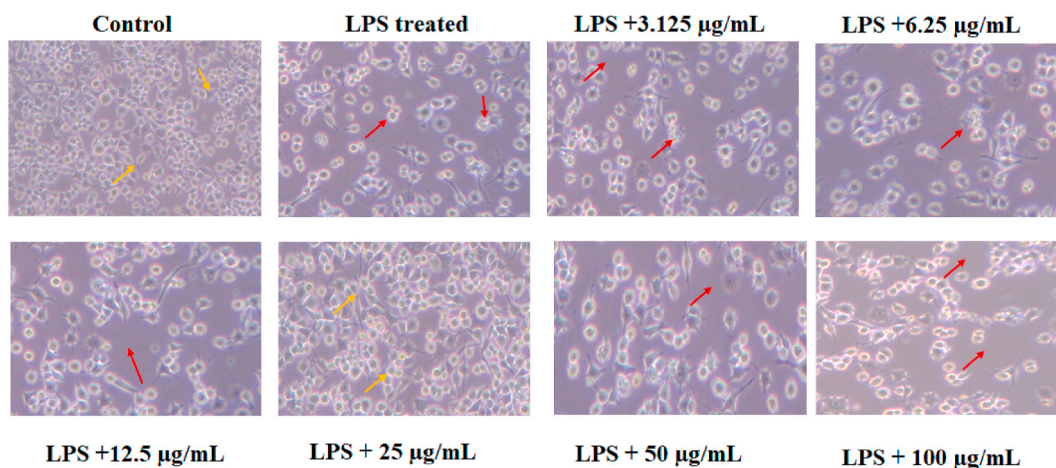


Fig. 2. Morphological analysis of KSK at 20x by using the phase-contrast inverted microscope.

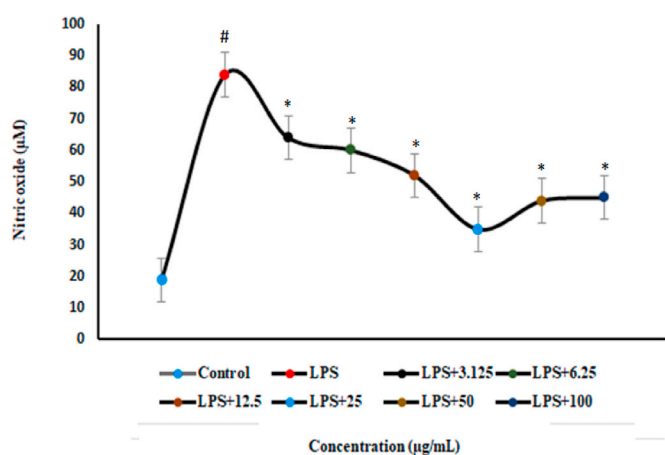


Fig. 3. Effect of KSK on NO production in LPS-induced RAW 264.7 macrophages. # -Statistical difference with the control group at $P \leq 0.05$. * -Statistical difference with LPS at $P \leq 0.05$.

2.4. Statistical analysis

Results are expressed as means and standard deviations of the control and treated cells from triplicate measurements ($n = 3$) of three different experiments. Data were subjected to one-way ANOVA and the significance of differences between means was calculated by Duncan's multiple range test, using SPSS for Windows, standard version 16 (SPSS, Inc.), and significance was accepted at $P \leq 0.05$.

3. Results and discussion

3.1. Cell viability

Determination of the MTT assay evaluates the possible toxicity of KSK extract on Raw 264.7 cells, thereby, it ensures the cells were healthy before performing the molecular assays. The cell viability of Raw 264.7 cells in the presence of KSK extract is shown in Fig. 1. Results indicate that KSK does not show any cytotoxic effect on the viability of Raw 264.7 cells at various concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL. Moreover, the various concentration of the KSK that yielded viability higher than 85% and was found that at 25 µg/mL showed more cell viability higher than 90%.

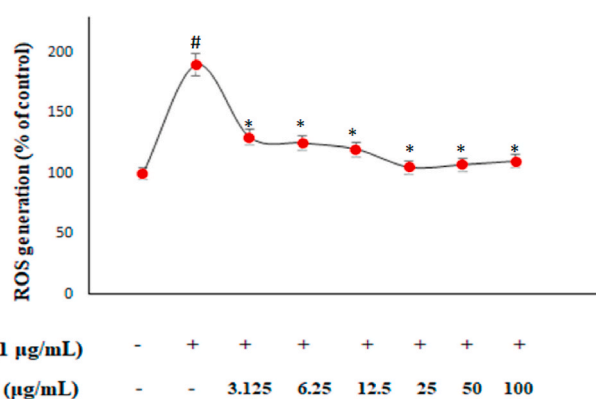


Fig. 4. Effect of KSK on ROS production in LPS-induced RAW 264.7 macrophages. # -Statistical difference with the control group at $P \leq 0.05$. * -Statistical difference with LPS at $P \leq 0.05$.

3.2. Morphological analysis

As shown in Fig. 2 the cell morphology was changed in LPS stimulated RAW264.7 cells as compared with the untreated control group. The cell size was reduced and shrinkage occurs, necrosis and apoptosis are seen. Treatment of KSK revert normal cell morphology at the concentration of 25 µg/mL as compared with the LPS stimulated group. There was no shrinkage, apoptosis, necrosis was observed.

3.3. Effect of KSK on the production of NO

In most tissues, NO acts as an important pluripotent signalling molecule, which plays a valuable role in host defence system and viral replications and are synthesized by nitric oxide synthase isoforms (NOS) (Kin et al., 2011). Notably, it also acts as a mediator and regulator of various inflammatory responses. To investigate the anti-inflammatory effect of KSK, we examined NO production in LPS induced RAW264.7 cells. The levels of NO was determined by Griess reagent assay. As shown in Fig. 3 LPS significantly enhanced NO production when compared with the control group, whereas KSK supplementation markedly inhibited NO production at 25 µg/mL.

3.4. KSK inhibits the LPS induced ROS generation in RAW 264.7 cells

Intracellular ROS generation and oxidative stress are known to be major factors involved in the pathogenesis of inflammation. During an inflammatory response, the overproduction of ROS induces cell damage,

Table 2

Effects of KSK extract on antioxidant enzyme like SOD, CAT and Gpx activities in LPS-stimulated Raw 264.7 cells. a' -Statistical difference with control group at $P \leq 0.05$. 'b' -Statistical difference with LPS at $P \leq 0.05$. SOD- Superoxide dismutase, Gpx- Glutathione peroxidase.

Groups	SOD ($\mu\text{M}/\text{mg}$ protein/min)	Catalase ($\mu\text{M}/\text{mg}$ protein/min)	Gpx (unit/mg protein)
Normal cells	70.37 ± 3.65^a	1.75 ± 0.04^a	4.45 ± 0.01^a
LPS treated cells	25.08 ± 2.45^b	1.22 ± 0.01^b	2.47 ± 0.04^b
LPS + KSK treated	67.74 ± 1.15^a	1.73 ± 0.03^a	5.10 ± 0.02^a

which can lead to an oxidative burst inside the cell which stimulates various inflammatory reactions like DNA damage and mutations (Lonkar and Dedon, 2011). In the present study, the protective action of KSK on intracellular ROS generation in LPS stimulated RAW 264.7 cells was determined using DCFH-DA assay and is shown in Fig. 4. Results indicates that stimulation of LPS significantly exhibited higher ROS generation when compared with the normal cells and thereby decreased the antioxidant level. However, the KSK effectively attenuated the intracellular ROS generation level at 25 $\mu\text{g}/\text{mL}$ concentration. Treatment of LPS stimulated cells with 25 $\mu\text{g}/\text{mL}$ concentration of KSK downregulated ROS level to 112.2% respectively. These results confirmed that KSK might effectively prevent the inflammatory condition by decreasing ROS content and reversed the antioxidant status, which may be associated with its antioxidant activity. Hence, KSK can be used as an anti-inflammatory drug.

3.5. Endogenous antioxidant enzyme activity

During the inflammatory response of several microbial attacks, the generation of ROS leads to cell damage and elevated oxidative stress condition inside the cell, thereby the antioxidant enzymes get depleted.

Due to the depletion of antioxidant enzymes, the antioxidant defence system of living organisms against free radicals was downregulated and hereafter, the suppression of the primary defence of the body against bacterial/viral attacks occur. There are several antioxidant enzymes such as SOD, CAT and Gpx that provide cells with a primary defence mechanism against oxidative stress and ROS production (Vivancos and Moreno, 2005). Upon LPS stimulation the antioxidant enzymes such as SOD, CAT and Gpx level was drastically decreased whereas treatment of KSK significantly increased the antioxidant enzymes, are effectively reduced the oxidative stress and ROS generation. Our result suggested that KSK enhanced antioxidant enzyme activities that may help attenuate inflammation (Table 2).

3.6. KSK suppress LPS induced pro-inflammatory cytokines TNF- α , IL-6 and PGE2 levels

The inhibitory effect of KSK against LPS induced PGE2 level was analyzed using ELISA assay. In Fig. 5 shown that LPS stimulation significantly upregulated the level of PGE-2 in RAW 264.7 cells as compared with the control group whereas treatment of KSK at 25 $\mu\text{g}/\text{mL}$ drastically downregulated the secretion of PGE-2 level as compared with other concentrations of KSK.

3.7. KSK prevented LPS-Induced expression of Cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) in RAW 264.7 cells

Macrophages play a major role in inflammation-related diseases and are associated with the uncontrolled production of inflammatory mediators and cytokines. Upon the activation of NF- κB , the expression of COX-2 and iNOS were upregulated and the level of NO inside the cell is controlled by iNOS and are highly expressed during the inflammatory process thereby overproduction of NO seen inside the body (Beinke and Ley, 2004). The activated inflammatory cells produce NO in high content, which is necessary for the over production of COX-2 enzyme (Bosca

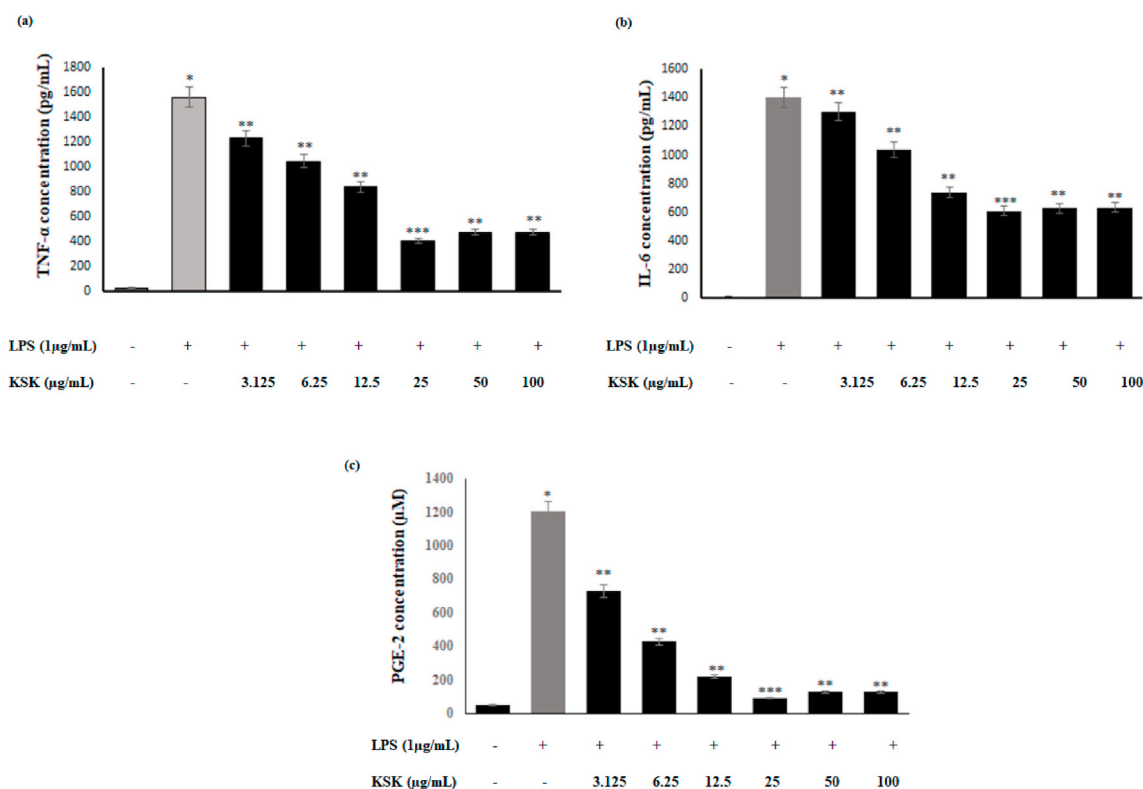


Fig. 5. Effect of KSK on Pro-inflammatory cytokines in LPS-induced RAW 264.7 macrophages. *-Statistical difference with control group, ** -Statistical difference with LPS treated group. *** -Statistical difference with LPS and other KSK doses.

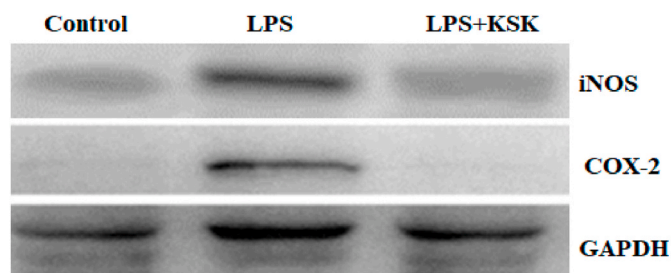


Fig. 6. Western blot analysis for the inhibitory effects of KSK on the protein expression level of iNOS and COX-2 level. GAPDH was used as an internal control.

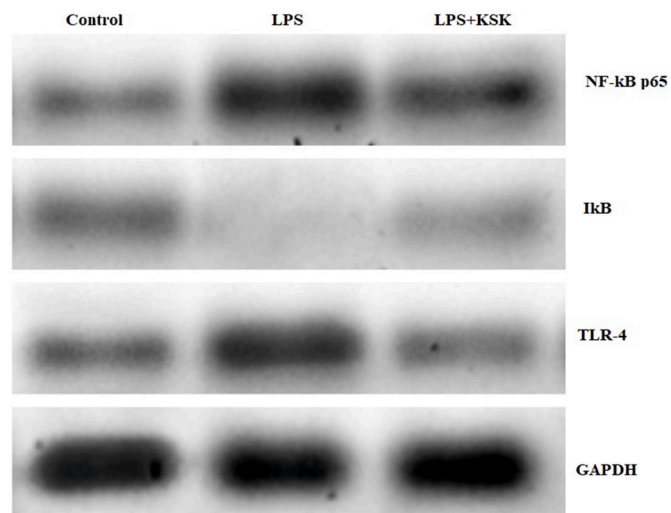


Fig. 7. Western blot analysis for the inhibitory effects of KSK on the degradation of IκB, protein expression of NF- κ B p65 and TLR-4. GAPDH was used as an internal control.

et al., 2005). COX-2 is an inducible form of enzymes seen during inflammation and is responsible for catalysing the conversion of arachidonic acid to prostaglandins. The Western blot analyses explained the expression of iNOS and COX-2 in LPS induced RAW264.7 cells. As shown in Fig. 6 LPS stimulation of the Raw 264.7 cells strongly upregulated the iNOS and COX-2 protein expression levels whereas KSK treated cells, the iNOS and COX-2 activities were significantly suppressed.

3.8. KSK might exert an anti-inflammatory effect by inhibiting the TLR4/NF- κ B signalling pathway

The most important class of pattern recognition receptors are toll-like receptors (TLRs), which are required for the activation of pro-inflammatory cytokines and NF- κ B signalling pathways in response to endogenous molecules (Shang et al., 2018). TLR-4 is one of the crucial signal-transducing receptors found in the cell membrane, which recognizes the endogenous molecules like LPS thereby activating the innate immune system and triggers pro-inflammatory cellular signalling pathways (Shang et al., 2018; Wang et al., 2019). Also, TLR-4 stimulation with LPS induces ROS generation (West et al., 2011). Overproduction of ROS triggers NF- κ B-p65 activation by increasing the phosphorylation of I κ B α and thereby upregulated the expression of pro-inflammatory cytokines (Schmid et al., 2012). In this study, we explored the protein expression of TLR-4 upon treating LPS-induced RAW264.7 was performed by Western blot. Results obtained showed that exposure of KSK at 25 μ g/mL suppressed LPS induced TLR-4 expression and attenuated

the cascade of TLR-mediated inflammatory cellular pathway, which indicates that KSK exerted an anti-inflammatory effect. NF- κ B plays an important role in the regulation of inflammatory mediators (Lawrence et al., 2001), hence it considers as a new target for treating various inflammatory conditions. Upon certain stimuli, such as LPS, NF- κ B was phosphorylated and transported into the nucleus to regulate inflammatory cytokines expression, including iNOS, NO, COX-2, IL-6 and TNF- α and thereby stimulates the NF- κ B signalling pathway (Zhao et al., 2016; Rajakumar et al., 2014). Once, it's get activated then followed by the phosphorylation and degradation of I κ B, which is a crucial step for NF- κ B activation in activated RAW 264.7 cells. The most common NF- κ B dimer is the p65/p50 heterodimer, which is bound to an inhibitor of NF- κ B (I κ B α) and is inactive in the cytoplasm (Lawrence, 2009). Stimulation with LPS phosphorylates p65 and I κ B α , which enhances NF- κ B activation during inflammatory processes (Lawrence, 2009). As shown in Fig. 7, we found that the elevated protein expression levels of NF- κ Bp65 were significantly inhibited by the introduction of KSK at the concentration of 25 μ g/mL. Also, in this study, we investigated whether KSK inhibits the LPS-induced degradation of I κ B in RAW 264.7 macrophages by Western blot analysis. The present study examined whether KSK inhibits the LPS-induced degradation of I κ B or the activation and translocation of NF- κ B p65. These results showed that KSK inhibits significantly the LPS-induced activation and nuclear translocation of NF- κ B p65 in RAW264.7 cells. It was confirmed that the anti-inflammatory effect of KSK through the NF- κ B signalling pathway could be mediated by TLR4. In conclusion, the present study has provided insights into the molecular mechanism of action of KSK as an anti-inflammatory drug and could be an effective therapeutic drug for controlling inflammation-associated diseases like COVID-19.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Svenia P. Jose: Writing – original draft, Writing – review & editing, Data curation. **Ratheesh M:** Conceptualization, Methodology, Writing – original draft, Validation, Formal analysis, Writing – review & editing. **Sheethal S:** Investigation, Resources, Data curation. **Sony Rajan:** Data curation, Resources. **Sangeeth Saji:** Resources, Software. **Vimal Narayanan:** Supervision, Writing – review & editing. **Sandya S:** Supervision, Writing – review & editing.

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