Journal of Ethnopharmacology 305 (2023) 116032

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

miRNA-15/IL-10Rα axis promotes Kabasura Kudineer (Indian traditional Siddha formulation) induced immunomodulation by suppressing oxidative stress

Jyotirmaya Behera [a,1], M. Pitchiah Kumar [b,1], A. Ireen Femela [a], Govindan Senguttuvan [c], M.S. Ramasamy [d,*]

[a] Department of Cell and Molecular Biology, Sanjeev Biomedical Research Centre, 1/15, Kumaran Nagar, Keelkattalai, Chennai, 600117, Tamilnadu, India
[b] State Licensing Authority, Directorate of Indian Medicine (Govt. of Tamilnadu), State Licensing Authority (IM), Chennai, 600106, Tamilnadu, India
[c] Department of Physics, University College of Engineering BIT Campus, Anna University, Tiruchirappalli, 620 024, Tamilnadu, India
[d] Head, Research & Development, Sanjeev Biomedical Research Centre, 1/15, Kumaran Nagar, Keelkattalai, Chennai, 600117, Tamilnadu, India

ARTICLE INFO

Handling Editor: Thomas Efferth

Keywords:
miRNA regulation
Oxidative damage
Immunomodulation
IL-10Rα-3′ UTR
Anti-inflammation

ABSTRACT

Ethnopharmacological relevance: Kabasura Kudineer (KK), the traditional Indian medicine of Siddha, effectively manages common respiratory symptoms such as flu, cold, and fever. However, there is no evidence of the immunomodulatory capacity of KK in the cultured Jurkat T-lymphocytes under the LPS insult studied.
Aim of the study: Assess the effect of the traditional Indian medicine of Siddha, Kabasura Kudineer (KK) on immunomodulation by suppressing oxidative damage in cultured Jurkat T cells *in vitro*. The miRNA activity on anti-inflammatory gene receptors and cellular nitric oxide levels also was studied.
Materials and methods: Jurkat T cells were exposed to LPS treatment in the presence or absence of KK. Cell viability and nitric oxide (NO) were measured with MTT and Griess assay. Cellular antioxidant systems (glutathione and SOD) were determined using glutathione and SOD assay. Lipid peroxidation was measured using an MDA assay. MiRNA-15a-5p expression was performed using microRNA qPCR Assays. Both inflammatory and anti-inflammatory genes (IL-6, IL-1, IL-10, IL-13) were performed using a qPCR and ELISA assay.
Results: The data showed that reduced cell proliferation and exaggerated NO production was observed in LPS treated condition compared to the control condition. Further, LPS treatment increased lipid peroxidation and reduced antioxidant enzyme activities (SOD and glutathione) in cultured Jurkat T cells. However, treatment with KK or N-acetyl cysteine (NAC; antioxidant) treatment mitigates the above effect. Mechanistically, LPS-induced oxidative stress upregulated miR- 15-5p expression and suppressed IL-10 Receptor alpha (IL-10Rα) by binding to its 3′-UTR region. The deregulated expression of IL-10Rα expression leads to increased IL-6 and IL-1β expression in LPS-induced Jurkat T cells; however, treatment with KK or NAC reversed the above effects.
Conclusion: Collectively, our study revealed the previously undefined mechanistic role of Kabasura Kudineer (KK) that alleviates the LPS-induced oxidative damage associated with inflammation by inhibiting the miRNA-15-5p/IL-10Rα axis.

1. Introduction

Siddha and Ayurvedic medicine are the Indian traditional medicine that comes under the AYUSH (Ayurveda, yoga and naturopathy, Unani, Siddha, and homeopathy) system of India. Several Siddha formulations from the Siddha system had broader applications due to the various outbreaks of infectious diseases (Jose et al., 2022). Among them, Kabasura Kudineer (KK) is one of the best and most well-known traditional medicine used over centuries and the most prescribed poly-herbal Siddha drug in India for the prevention of disease outbreaks (Kiran et al., 2022; Jain et al., 2020). KK is widely used for Aiya suram (fever) and Aiya noigal (respiratory diseases) in Indian Siddha medicine and possesses antiviral activity (Vanan, 2015). KK is composed of 15 poly-herbal ingredients (Table 1 and all the plant name has been checked with

* Corresponding author. Sanjeev Biomedical Research Centre, Keelkattalai, Chennai, 600117, Tamilnadu, India.
E-mail address: ramasamy.mani@gmail.com (M.S. Ramasamy).
[1] Equal contribution.

https://doi.org/10.1016/j.jep.2022.116032
Received 22 July 2022; Received in revised form 18 November 2022; Accepted 3 December 2022
Available online 29 December 2022

Table 1
A list of the ingredients is present in Kabasura Kudineer (KK).

Sl. No	Kabasura Kudineer ingredients	Tamil Name	Common Name
1	*Zingiber officinale* Roscoe	Chukku	Ginger
2	*Piper longum* L	Thippili	Long pepper
3	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Kirambu	Clove
4	*Tragia involucrata* L	Sirukanchori	Indian stinging nettle, climbing nettle
5	*Anacyclus pyrethrum* (L.) Lag.	Akkirakaram	Pellitory
6	*Andrographis paniculata* (Burm.f.) Nees	Nilavmebu	Andrographis
7	*Terminalia chebula* var. *tomentella* (Kurz) C.B.Clarke	Kadukkaithol	Yellow myroblan
8	*Adhatoda vasica* Nees	Adathodai	Adathodai
9	*Coleus amboinicus* Lour	Karpuravalli	Bishop's weed
10	*Saussurea lappa* (Decne.) Sch. Bip	Kostam	Spiral Ginger
11	*Tinospora cordifolia* (Willd.) Miers	Seendhil	Heart-leaved moonseed
12	*Clerodendrum serratum* (L.) *Moon*	Siruthekku	Beetle killer
13	*Sida acuta Burm.*f	Vattathiruppi	Morning mallow
14	*Cyperus rotundus* L	Korai kizhangu	Cocograss
15	*Hygrophila auriculata* (Schumach.) Heine	Mulliver	Vajradanti

#All the plant name has been checked with the http://www.theplantlist.org database.

http://www.theplantlist.org database) developed by the Siddha Formulary of India (Sathiyarajeswaran et al., 2021). The study has suggested that the ethnopharmacological properties of these ingredients in KK had analgesic, antiviral, anti-fungal, anti-bacterial, anti-asthmatic, hepatoprotective, and anti-diabetic (Thillaivanan et al., 2015). Recent studies have also reported that KK has a strong antiviral effect against SARS- CoV-2 through the In-silico method and thrombolytic activity (Pitchiah et al., 2020, Sathiyarajeswaran et al., 2021; Shree Devi et al., 2021). Others have shown that combinatorial supplementation of KK with Zinc and Vitamin C significantly reduces SARS-COV-2 viral load in affected patients. (Natarajan et al., 2020). Therefore, KK would be a prescribed drug as a good immune booster and against the COVID-19 infection, according to the guideline of the Ministry of AYUSH, the Government of India. Despite much having been studied about the KK against the various outbreak; still, the mechanistic insight of KK-mediated recovery of disease pathology and post-transcriptional gene regulation of target gene expression that modulates the anti-inflammation needs to be further investigated.

MicroRNAs (miRNAs) are endogenous, short (about 18–22 nucleotides), tissue-specific non-coding RNA, which is transcribed but not translated. miRNAs regulate gene expression by restricting target mRNA translation or degradation by binding to its 3′-UTR region (Behera et al. 2021a, Behera et al., 2021b). The study has reported that over 50% of all human protein-coding genes are targeted by miRNAs, found to modulate their regulatory roles in many pathophysiological processes. Approximately more than 1000 miRNAs have been identified and characterized in the human genome (http://www.mirbase.org/) for their regulation of

mRNA/gene degradation and pathogenesis (Zeng, 2006). However, to date, there have been no reports have been published to describe miRNA that can regulate anti-inflammatory gene receptor expression under KK administration.

The study has demonstrated that cytokines and chemokines are indispensable in recruiting immune cells and subsequent regulation of inflammatory responses. T-lymphocytes (T cells) secrets a wide range of inflammatory cytokines such as IL-6, IL-1β, TNF-α, and CXCL8, which are indeed essential for cellular proliferation, differentiation, intercellular communications and mediate inflammatory responses (Gonzalo et al., 1996). on the other hand, exaggerated secretion of these cytokines is attributed to various human diseases, such as cystic fibrosis, pulmonary disease, and the onset of autoimmune disorders (plasmacytosis and hyperplasia) (Carpagnano et al., 2003). Induction of IL-6 has been governed through NF-Kb and STAT3 signaling. Although various signaling has been developed, still, non-coding mediated regulation of inflammatory cytokine response has not yet been studied.

No experimental evidence is available *in vitro* on the effect of Kabasura Kudineer (KK) on immunomodulation. Since T cells regulate immunomodulation, we used Jurkat T cells, a widely accepted experimental model to study immunomodulation (Castiglioni et al., 2017; Amirghofran et al., 2011). Using Jurkat T-cells exposed to LPS and combined with KK, the study demonstrated that LPS significantly increased oxidative stress-dependent miRNA-15a- 5p upregulation. However, inhibition of oxidative stress via KK or NAC suppressed miRNA- 15a-5p and indeed promoted IL-10R-α mRNA expression. Our study provides a piece of novel information that KK treatment-mediated inhibition of oxidative stress and inflammation is an effective strategy for countering inflammation and promotes immunomodulation in Jurkat T cells.

2. Materials and methods

2.1. Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) is obtained from Sigma, (St. Louis, USA). Dulbecco's modified Eagle's medium (DMEM), 100 U/mL penicillin, 100 mg/mL streptomycin, bovine serum albumin (BSA), and fetal bovine serum (FBS) are purchased from Gibco (USA). Trizol reagents are obtained from Sigma (USA). Hi-Quanti One- Step RT-PCR Kit (Real-time PCR Based) is obtained from HiMedia. Liposaccharide (LPS), concanavalin A (Con A), N-acetyl cysteine (NAC), and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) are purchased from Sigma (USA).

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

KK is a polyherbal formulation that was obtained from Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd. (Impcops), Chennai. KK consists of fifteen different phytochemicals or ingredients listed in Table 1. To prepare the extract, 100g of dried powder of KK was weighed and mixed with an aqueous solution (double distilled water) and kept overnight. The next day, the extract was filtered, concentrated, and dried using a vacuum evaporator and stored below 4 °C till further use.

2.3. Cell lines and culture conditions

A human T lymphoblastoid cell line (Jurkat Clone E6-1) was obtained from American Type Culture Collection (ATCC), USA. Jurkat cells were cultured in (RPMI) 1640 Medium (Sigma), supplemented with 10% Foetal bovine serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Sigma, P4333) at 37 °C, with 5% CO2 and 95% relative humidity. The cells were divided into the following groups.

1. Control
2. Lipopolysaccharides (LPS; 75 ng/mL)
3. Kabasura Kudineer (KK; 100 μg/ml)
4. KK treated with LPS-induced cells (LPS + KK (75 ng/ml +100 μg/ml))
5. Concanavalin A treated with cells (Con A; 20 μg/ml)

Briefly, Jurkat T cells were pretreated with KK and then stimulated with LPS or Con A for 24 h at 37 °C. Both cells and conditional medium (CM) were harvested at the end of the experimental procedures and performed various cellular, biochemical and molecular analyses.

2.4. Cell proliferation by MTT assay

Cell viability/proliferation was determined in cultured Jurkat T-cells using the MTT assay as previously described (Behera et al., 2018a,b). Briefly, cultured Jurkat T cells were administrated with various concentrations of KK (0, 50, 100, 150, and 200 μg/mL) with/without LPS (75 ng/mL) or Con A (20 μg/ml) treatment. At the end of the treatment procedures, cells were added with tetrazolium agent (MTT, 1 mg/ml) and incubated for 3 h at 37 °C. Then, the culture medium was then washed away, followed by the addition of 100ul of DMSO to the formazan crystals formed in the well and incubated for 45 min to 2hrs at 37 °C in a 5% CO2 incubator. After the incubation period, supernatants were collected, and the absorbance was measured at OD 570/630 nm using an ELISA reader.

2.5. Nitric oxide production by griess assay

NO metabolite, nitrite in cultured Jurkat T cells was measured by Griess assay as previously described (Behera et al., 2021c). Briefly, cell culture supernatants as CM were collected and performed Griess assay. 100 μl of the cell supernatant was added with 100 μl of Griess reagents (58 mM sulphanilamide in 2.5% phosphoric acid plus 12 mM N-(1-naphthyl) ethylenediamine in 2.5% phosphoric acid) and allowed the samples to incubate for 20 min at the room temperature. The absorbance was taken at 570 nm using an ELISA reader.

The sodium nitrite standards curve (in a range of 1–100 μg) was performed to calculate the unknown nitrite levels in various experimental samples.

2.6. Lipid peroxidation (MDA) assay

A lipid peroxidation (MDA) assay was performed as previously described (Behera et al., 2018d). This assay measures the cellular total lipid peroxide level through the generation of malondialdehyde (MDA) content in the cultured Jurkat T cells under LPS stress using a lipid peroxidation (MDA) assay kit procedure.

2.7. Superoxide dismutase (SOD) assay

SOD activity was measured in cultured Jurkat T cells using a SOD activity assay. Briefly, 300 μl of T-cells-lysates were mixed with 300 μl of chilled ethanol and 170 μl chloroform. The cellular contents were vortexed and centrifuged at 3000 rpm for 7 min. Following centrifugation, the supernatants were collected and mixed with 150 μl Tris-HCl buffer (75 mM, 8.2 pH), 25 μl EDTA (25 mM), and 120 μl pyrogallol (1.5 mM). Further, the cellular contents were transferred into a 96-well plate, and absorbance was taken at 405 nm.

2.8. Glutathione (GSH) assay

Glutathione level was determined in Jurkat T-cells lysate using the glutathione (GSH) assay method. Briefly, 1 ml of 5% TCA solution was added to 250 μl of cell lysates and spun at 3000 rpm for 7 min. The supernatant was collected and mixed with 500 μl of DTNB and 500 μl 1xPBS and allowed to stand for 5 min at room temperature. Later, the samples were further added with 3.5 ml of methanol and incubated for 10 min at room temperature. The experimental sample was turned to yellow colored appearance and transferred to a 96-well plate, and absorbance was taken at 578 nm.

2.9. ELISA analysis of secretary cytokines

The secretary cytokines (IL6, IL-1β, and IL-10) were analyzed in cultured Jurkat T cells supernatants using a commercial Multiplex Human Cytokine ELISA Kit (Pro-Inflammatory Cytokine) (Catalog# EM10001) from Biocompare, as per the manufacturer's instructions.

2.10. Total RNA isolation and gene expression analysis by qPCR assay

Total RNA was extracted using TRIzol™ (Sigma, USA) method according to the manufacturer's specifications. Total RNA (1 μg) was used to synthesize cDNA using Hi-cDNA Synthesis Kit from HiMedia (Cat No: MBT076-100R) (Behera et al., 2019). cDNA was used to amplify the target gene expression using the Hi-Chrom PCR Master Mix in Insta Q96 Plus Real-Time PCR Machine (HiMedia). All the primer sequence of the target gene was obtained. The detailed primer sequences are provided in Table 2. For quantitative measurement and calculation of target mRNA expression, housing keeping gene, GAPDH expression was performed. Likewise. For miRNA preparation, total RNA was isolated using miRNeasy Kit and followed by cDNA synthesis using miScript II RT kit. Using miScript SYBR Green PCR Kit, amplification of target miRNA expression was performed. The detailed primer sequences are provided in Table 2.

2.11. Anti-miRNA inhibitor delivery in vitro

To study the role of miR-15a-5p function in Jurkat T cells function *in vitro*, the miRNA-15 inhibitor and scramble control were purchased (Sigma, USA). Briefly, miRNA-15 inhibitor/anti-miR-15 (50 nM) was transfected to the cultured Jurkat T cells for 48 h under serum-free conditions using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After 48 h of post-transfection, cell lysates were prepared, and performed both gene expression and cytokine profiling under various experimental conditions.

We also tested the efficacy of IL-10Rα in cultured Jurkat T cells, which modulate anti-inflammation. Moreover, an IL-10Rα neutralizing antibody that targets IL-10Rα was applied to the culture condition and examined immunomodulatory activities.

2.12. In silico analysis of miRNA binding site

To predict potential targets of miR-15a-5p at the mouse IL-10Rα mRNA region, we performed *in silico* analysis using Targetscan (http://www.targetscan.org/). After analysis, the miRNA binding seed region was obtained in the 3′-UTR region of the IL-10Rα gene. Using lncLocator Database (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/), we analyzed the sub-cellular localization of miR- 15.

Table 2
Sequences of PCR primers used for real-time quantitative PCR.

Gene	Primer Sequences (5′→3′)
Human IL-1	FP: CACAGACCTTCCAGGAGAATG
	RP: GCAGTTCAGTGATCGTACAGG
Human IL-6	FP: AACAACCTGAACCTTCCAAAGATG
	RP: TCAAACTCCAAAAGACCAGTGATG
Human IL-13	FP: ACGGTCATTGCTCTCACTTGCC
	RP: CTGTCAGGTTGATGCTCCATACC
Human IL-10	FP: TCTCCGAGATGCCTTCAGCAGA
	RP: TCAGACAAGGCTTGGCAACCCA
Human IL-10Rα	FP: ATGCTGCCGTGCCTCGTAGTGC
	RP: ACTCTGGCCCG GTAGCCATTGC
Human Ki67	FP: GAAAGAGTGGCAACCTGCCTTC
	RP: GCACCAAGTTTTACTACATCTGCC
Human CASP3	FP: GGAAGCGAATCAATGGACTCTGG
	RP: GCATCGACATCTGTACCAGACC
Human CASP7	FP: AGTGACAGGTATGGGCGTTC
	RP: CGGCATTTGTATGGTCCTCT
Human TLR4	FP: CCCTGAGGCATTTAGGCAGCTA
	RP: AGGTAGAGAGGTGGCTTAGGCT
Human miR-15-5p	FP: TCAGTTAACCAATAAAAAGGTCAGC
	RP: GCCTGGGTCTCACCATGTAG
Human GAPDH	FP: CTCCCACTCTTCCACCTTCG
	RP: GCCTCTCTTGCTCAGTGTCC

2.13. Statistical analysis

All statistical analyses and graphical presentations were performed with GraphPad Prism software, v.9.2.0. All experimental data are expressed as the mean ± S.E.M. Significant differences between the two groups were determined by a Two-tailed, unpaired Student's t-test. The significant differences in more than two experimental groups were compared by one-way analysis of variance (ANOVA) in combination with Tukey's multiple comparison test. P <

0.05 was considered statistically significant. Experiments were repeated three times independently.

3. Results

3.1. Effect of Kabasura Kudineer (KK) on LPS-induced decrease of cell proliferation in cultured Jurkat T cells

To test the viability/cell proliferation capacity of Kabasura Kudineer (KK), Jurkat T cells were cultured and treated with KK at different concentrations for 24 h as indicated in Fig. 1A. The data demonstrate that cell proliferation remained unaffected at the concentration of 50 μg/mL compared to the untreated control condition; however, a significant effect on cell proliferation was observed when cells were treated with 100 and 150 μg/mL of KK (Fig. 1A). However, the cell proliferation capacity was reduced at the concentration of 200 μg/mL KK. As Jurkat T cells showed maximum proliferation at 150 μg/mL of KK, therefore, the subsequent experiments were set at the same concentration. Next, the effect of KK on LPS and concanavalin A (Con A) was tested on cell proliferative capacity. The data revealed that the decrease in cell proliferation by LPS or Con A was attenuated by the pre-treatment with KK when compared to the LPS or Con A condition (Fig. 1B). Indeed, there was no significant cell proliferation observed among the LPS and Con A conditions.

Fig. 1C depicts the cellular death, which was observed in the cultured Jurkat T cells under treatment of LPS and was found to be improved in KK treatment. In addition, the expression of Ki67 mRNA transcript, a cell proliferation marker, was reduced in the LPS condition. However, treatment of KK improved the Ki67 mRNA transcript expression in the LPS + KK condition compared to the LPS condition (Fig. 1D). On the other hand, the study also confirmed that apoptosis marker genes (CASP3 and CASP7) were found to be reduced in the LPS + KK condition compared to the LPS condition (Fig. 1E). To investigate LPS/TLR4 signaling and LPS-mediated biological effect, we tested TLR4 mRNA transcript expression under various experimental conditions. Interestingly, the study confirmed that TLR4 expression was not changed among experimental groups (Fig. S1B). Therefore, our result shows the TLR4-independent role of LPS in the induction of oxidative damage and inflammation in the cultured Jurkat T cells.

3.2. Kabasura Kudineer (KK) Supplementation inhibits exaggerated nitric oxide (NO) production under LPS insult in cultured T cells

As NO is an important mediator in regulating inflammation (Zamora et al., 2000), however, a link between NO production and LPS insult in Jurkat T cells is still not known. Therefore, it prompted us to study the effects of the LPS on the NO production in the Jurkat T-cells. Using the Griess assay method, the level of nitrite, or NO production, was measured after 24 h of treating Jurkat T cells with both LPS and KK. The data showed that NO production via nitrite was increased under the LPS-induced condition when compared to the control condition. However, the above effect was mitigated under KK treatment in LPS-induced Jurkat T cells culture (Fig. 2A). To further confirm the remarkable role of

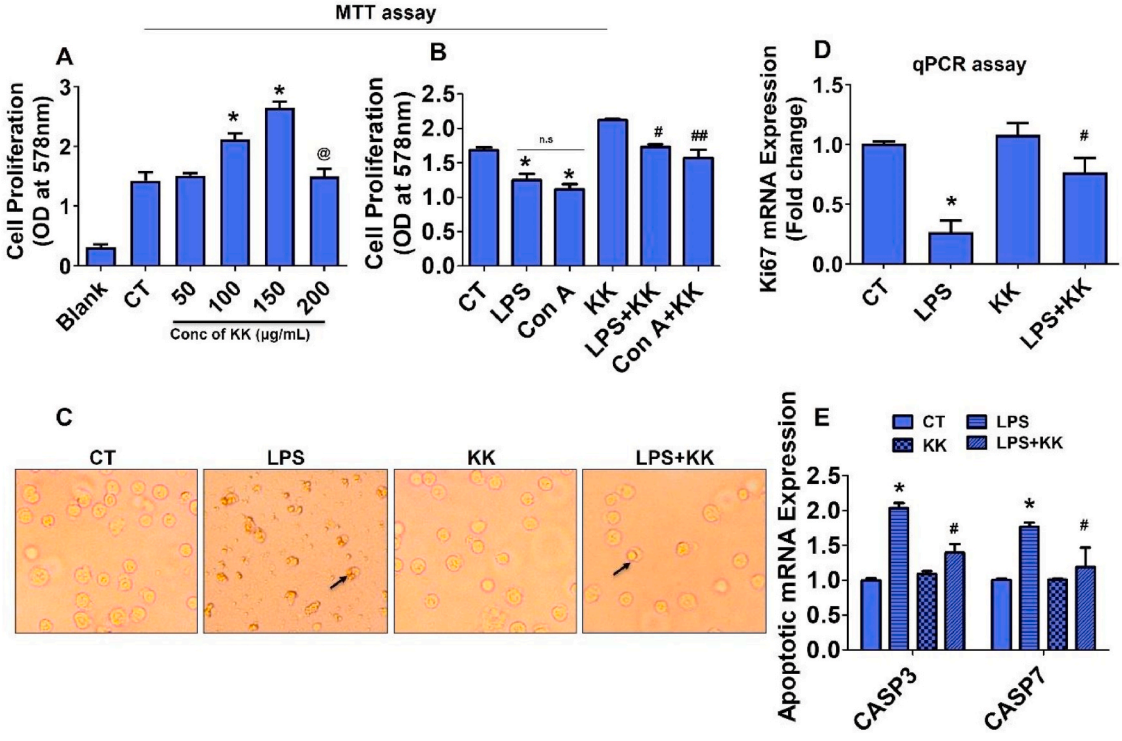

Fig. 1. Effect of the KK extracts on LPS-regulated Jurkat T cells proliferation/viability.
A. Dose-dependent effect of the KK extract on proliferation of the Jurkat T cells. **B.** Effect of the KK extract on LPS-induced diminution of cell proliferation/viability. Concanavalin A used as a positive control to study the cell viability. **C.** Representative phase-contrast microscopy image of cultured Jurkat T cells under various experimental conditions. The arrow (black) represents cell death in various experimental conditions. **D.** mRNA transcript expression of proliferation marker, Ki67 using qPCR. **E.** qPCR analysis of apoptotic marker genes (CASP3 and CASP7). Experiments were repeated at least three times. Data are expressed as mean ± SEM. n = 5 samples per group. *p < 0.05 compared with the control, $^{\#}$p < 0.05 compared with the LPS, $^{@}$p < 0.05 compared with the KK (150 μg/mL), and n.s denotes no statistical significance.

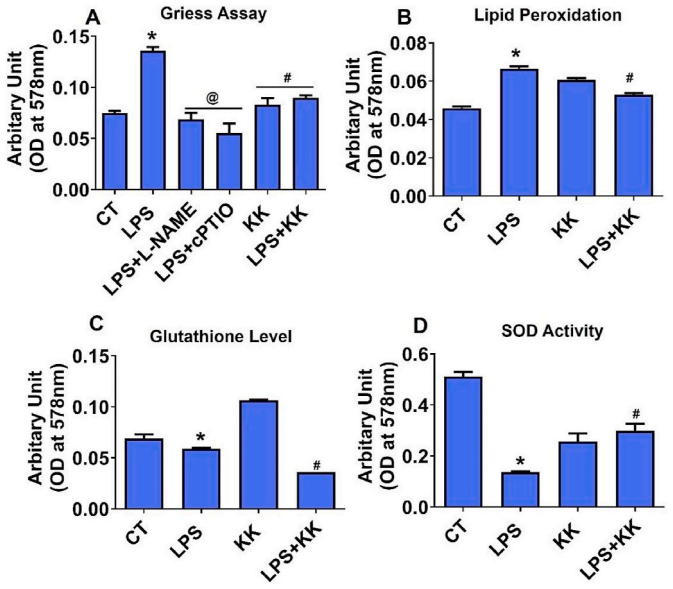

Fig. 2. Effect of the KK on redox homeostasis in LPS induced Jurkat T cells culture.

A. Griess assay was performed to measure the nitrite level from Jurkat T cells culture. **B.** Lipid peroxidation activity of culture Jurkat T cells using Malondialdehyde (MDA) assay. **C.** Glutathione (GSH) level was measured using glutathione assay **D.** Superoxide dismutase (SOD) activity level was measured using SOD assay. Experiments were repeated at least three times. Data are expressed as mean ± SEM. n = 5 samples per group. *p < 0.05 compared with the control, #p < 0.05 compared with the LPS, @p < 0.05 compared with the LPS.

NO in maintaining the Jurkat T cells function, we supplemented both NOS inhibitor (L-NAME) and NO quencher (cPTIO). The data found that inhibition of NOS via L-NAME or quenching of the bioavailability of NO via cPTIO, substantially prevented nitrite production in the LPS + L-NAME or LPS + cPTIO conditions compared to the LPS condition (Fig. 2A). This data suggested that the KK has a strong immunomodulating activity by regulating cellular NO production and preventing exaggerated NO-dependent inflammation.

3.3. Kabasura Kudineer (KK) treatment augments redox homeostasis in cultured T cells

Several parameters were assessed in cultured Jurkat T cells to study the anti-oxidative properties of KK on LPS-induced oxidative damage. Since lipid peroxidation is an essential indicative marker of oxidative damage, we demonstrate that MDA levels are found to be significantly enhanced in LPS-treated Jurkat T cells compared to the control condition. However, the KK administration reverses the above increase in MDA level (Fig. 2B). On the contrary, cellular antioxidant enzyme systems, such as superoxide dismutase (SOD) activity and glutathione level (GSH), were tested. The data showed that KK administration was sufficient to mitigate the LPS-mediated effect in cultured Jurkat T cells (Fig. 2C and D). This finding suggests that KK is an important polyherbal formulation that indeed improves and balances the redox homeostasis in cultured Jurkat T cells.

3.4. Kabasura Kudineer (KK) treatment enhances anti-inflammatory IL-10Rα expression by inhibiting oxidative stress-dependent miR-15a upregulation

Various studies have suggested that IL-10Rα is associated with anti-inflammation by binding to IL-10 cytokine and suppressing pro-inflammatory cytokine induction (Moore et al., 2001, Saraiva and O'Garra, 2010). However, the link between the KK treatment and IL-10/IL-10Rα axis was established so far under LPS insult in cultured Jurkat T cells. On the other hand, the link between miRNAs regulation that targets the IL-10Rα needs to be investigated. Therefore, it prompted us to understand the above mechanism under KK in cultured Jurkat T cells. Using qPCR analysis, we confirmed that miRNA-15a-5p was upregulated ($p < 0.0001$) in LPS-treated Jurkat T cells. However, miRNA-15a expression was normalized ($p < 0.0001$) in the LPS + KK condition (Fig. 3A). To study further the oxidative damage-dependent upregulation of miRNA-15a expression, the study administered antioxidant N-acetyl cysteine (NAC) in LPS- treated Jurkat T cells. Interestingly, the study showed the expression of miR-15a-5p reduced in the LPS + NAC condition (Fig. 3A), indicating that suppression of miR-15a-5p expression is oxidative damage-dependent in LPS-treated Jurkat T cells. Using the lncLocator *insilico* tool database (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/), the study also confirmed that the predicted subcellular location of miR-15a-5p in the cytoplasm (Fig. 3B).

To gain insights into whether miR-15a could modulate IL-10Rα expression in cultured Jurkat T cells, we ran *in silico* TargetScan analysis. The study explored the miR-15a sequence recognized the 933–940 bp of the conserved sequence of the IL-10Rα mRNA (Fig. 3C). Besides, our qPCR data confirmed that the IL-10Rα expression was significantly reduced in the LPS condition compared to the control condition (Fig. 3D). However, administration of KK or NAC improved IL-10Rα expression in the LPS + KK or LPS + NAC condition compared to the LPS condition (Fig. 3D). In addition, transfection of miR-15a inhibitor (anti-miR-15a) in the cultured Jurkat T cells further improved the expression of the IL-10Rα in LPS + anti-miR-15a condition compared to LPS condition (Fig. 3D). The efficacy of the anti-miR-15-based inhibition of miR-15 expression was confirmed qPCR analysis (Fig. S1B). These data suggest that LPS-induced upregulation of miR-15a was associated with complementary binding to the 3′-UTR regions of the IL-10Rα gene and led to suppression of its expression.

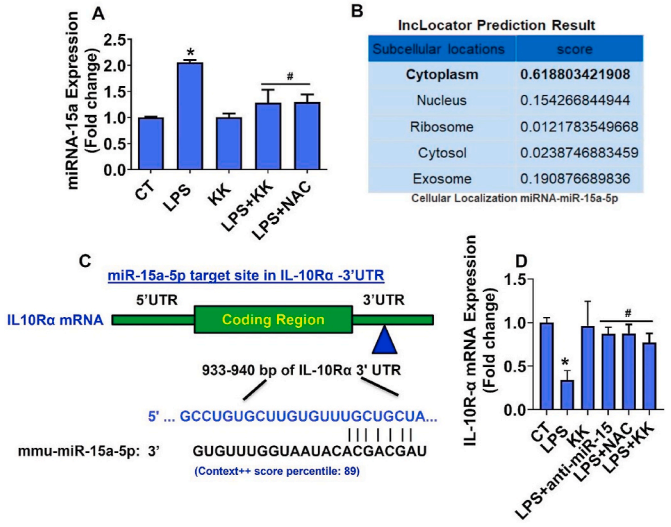

Fig. 3. Effect of the KK on IL-10Rα expression via miR-15a dependent manner in cultured Jurkat T cells. A. qPCR analysis of miR-15a expression in the culture Jurkat T cells. *B. in silico* lncLocator analysis of sub-cellular localization of miR-15. **C.** Targetscan analysis of miR-15a binding site at 3-UTR region of IL-10Rα. **D.** qPCR analysis of IL-10Rα expression in the culture Jurkat T cells. Experiments were repeated at least three times. Data are expressed as mean ± SEM. n = 5 samples per group. *p < 0.05 compared with the control, #p < 0.05 compared with the LPS.

3.5. Kabasura Kudineer (KK) treatment suppresses inflammatory response by upregulating anti-inflammatory gene expression

Using qPCR analysis, inflammatory cytokines such as IL-6, and IL-1β, were found to be upregulated in the LPS-induced Jurkat T cells when compared to the control condition. In contrast, KK or NAC supplementation suppressed the above inflammatory cytokine gene expression in the LPS + KK or LPS + NAC condition compared to the LPS condition (Fig. 4A- B). Furthermore, anti-inflammatory gene (IL-10, IL-13) expression was upregulated in the LPS + KK or LPS + NAC condition compared to the LPS condition (Fig. 4C and D). In addition, inflammatory cytokines (IL-6 and IL-1β) secretion was assessed using ELISA assay, and it found that all these inflammatory cytokines were reduced in the culture supernatant of LPS + KK and LPS + NAC supernatants compared to LPS supernatant (Fig. 4E). To further validate the involvement of IL-10Rα signaling in KK-induced immunomodulation, we treated neutralizing antibodies against IL-10Rα (IL-10Rα neutralization antibody) in cultured Jurkat T cells. The data demonstrated that IL-10Rα neutralization further prevented KK-mediated suppressive inflammatory effect and indeed accentuated inflammatory cytokines (IL-6 and IL- 1β) production. Interestingly, IL-10 treatment significantly reduced inflammatory cytokines production in the LPS + KK + IL-10 condition compared to the LPS + KK condition (Fig. 4E). Furthermore, the study also confirmed that the anti-inflammatory cytokine IL-10 level was found to be increased in LPS + KK and LPS + NAC conditions compared to the LPS condition (Fig. 4F). These results proved that KK administration was associated with improving anti-inflammatory response by suppressing inflammatory cytokines expression in LPS-treated conditions.

4. Discussion

In the present study, we found that treatment of Kabasura Kudineer (KK) improved cell proliferation capacity in LPS-induced Jurkat T cells culture compared to the LPS control condition. The study also detected LPS-induced oxidative damage was associated with exaggerated nitrite production. Importantly, we demonstrate for the first time that miR-15a-5p was upregulated under LPS-induced oxidative stress conditions. However, the administration of KK or antioxidant NAC mitigated the above effect. miR-15a-5p expression was inversely correlated with the anti-inflammation receptor, IL-10Rα expression in LPS-induced Jurkat T cells. Moreover, miR-15-5p directly reduced IL-10R α mRNA levels by binding to its 3′-UTR regions, indicating that aberrant miR-15a-5p expression may be a novel mechanism underlying IL-10Rα down-regulation and induced inflammation under LPS conditions. Additionally, KK and NAC treatment improved IL-10Rα mRNA by reducing the miR-15a-5p expression and promoting an anti-inflammatory response. These findings suggest that KK would be an inflammation reducer and subsequently improve immunomodulatory activity in Jurkat T cells via regulation of oxidative stress-dependent miR-15a-5p/IL-10Rα signaling axis.

Various studies have suggested a role for IL-10 and downstream IL-10R signaling as major regulators of the anti-inflammatory response in mucosal compartments (Shouval et al., 2014; Engelhardt et al., 2013). Loss of function mutations of IL10 or IL10R in rare patients represents inflammatory bowel disease (Engelhardt et al., 2013). However, the role of IL-10R in LPS- induced Jurkat T cells inflammation needs to be investigated. Whether KK plays any role in reversing inflammatory response in LPS-induced conditions. KK treatment might promote anti-inflammation or blocks the oxidative damage mechanism through its antioxidative-immunomodulatory mechanism. Therefore, we have tested the expression of both inflammatory (IL-6, TNF-α) and anti-inflammatory (IL-10, IL-13) genes in the KK-treated LPS cultures. Interestingly, we observed a robust reduction of inflammatory cytokines expression and upregulation of anti-inflammatory cytokines expression. Additionally, the study also found that KK has the capacity to reduce the cytotoxicity potential of Jurkat T cells under LPS insult as determined by the MTT assay.

Previous studies have demonstrated that miR-15a was associated with growth/proliferation inhibition and induction of apoptosis in cultured cancer cells (Luo et al., 2013; Cimmino et al., 2005). However, the function of miR-15a role in immunomodulatory function in Jurkat T cells is not studied yet. Here, we demonstrated the interplay between miRNA-15a function and IL- 10Rα signaling on Jurkat T cells under LPS toxicity. Using *in silico* Targetscan analysis, we confirmed that miR-15a has a binding site on the 3′-UTR regions of IL-10Rα mRNA. Further, our data showed that LPS-induced upregulation of miRNA-15 was negatively correlated with IL-10Rα expression leading to an imbalance in the anti-inflammation process (Fig. 4). This caused marked expression of inflammatory cytokine expression (Figs. 3 and 4). On the other hand, inhibition of oxidative damage by NAC or KK restored the IL-10Rα expression by reducing miR-15a expression.

In summary, KK treatment plays a crucial role in regulating Jurkat T cells' function through cellular proliferation, controlling NO production, and modulating anti-inflammation through orchestrating the miR-15a/

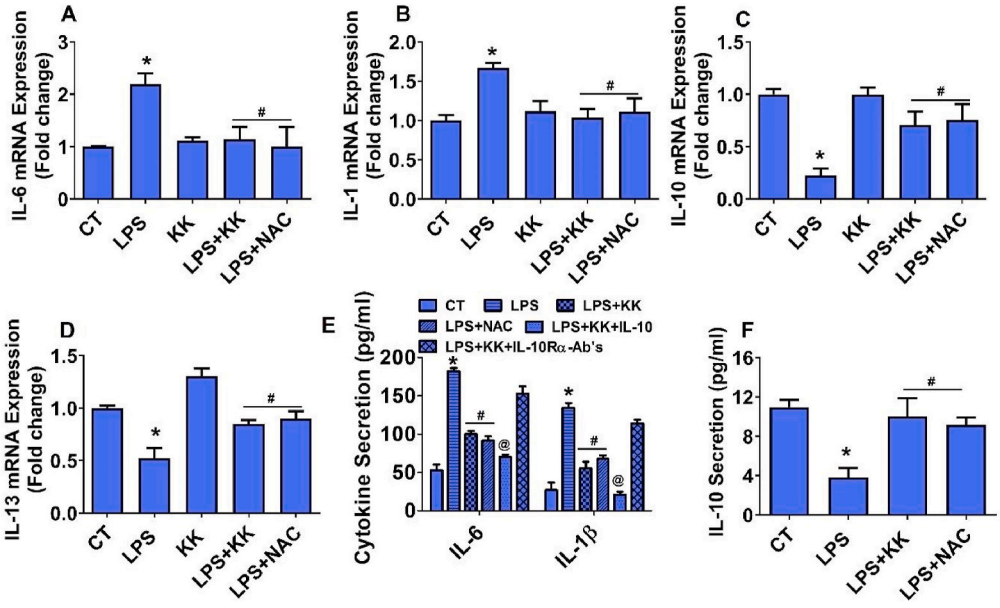

Fig. 4. Effect of the KK on cellular immune modulation and inflammation in cultured Jurkat T cells. A-B. qPCR analysis of the inflammatory genes (IL-6, IL-1β) expression in the experimental conditions. **C-D.** qPCR analysis of anti-inflammatory genes (IL-10, IL-13) expression in the experimental conditions. **E.** ELISA analysis of secretary inflammatory cytokines (IL-6, IL-1β) expression in conditioned medium obtained from cultured Jurkat T cells. **F.** ELISA analysis of secretary anti-inflammatory cytokine, IL-10 expression. Experiments were repeated at least three times. Data are expressed as mean ± SEM. n = 5 samples per group. $^{*}p < 0.05$ compared with the control, $^{\#}p < 0.05$ compared with the LPS, $^{@}p < 0.05$ compared with the LPS + KK.

IL-10Rα axis-dependent immunomodulation. Briefly, the study found that KK mitigates LPS-induced cytotoxicity and inflammatory NO production and also balances anti-inflammatory cytokine production. Mechanistically, LPS-induced oxidative stress promotes upregulation of miR-15a expression and substantially induces Jurkat T cells inflammation via inhibition of IL-10Rα. However, KK treatment prevented the above changes in cultured Jurkat T cells. Therefore, our study strongly suggests that KK could be a potential immunomodulatory therapy against cytokine storm-dependent inflammatory immune deregulation through a distinct miRNA-15α/IL-10Rα regulatory axis in Jurkat T cells. However, much research is warranted to investigate the mechanism of the KK in regulating the immune-modulatory axis in an animal model to counteract the cytokine storm-dependent inflammation.

CRediT authorship contribution statement

Jyotirmaya Behera: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: M. S. Ramasamy reports financial support was provided by Sanjeev Biomedical Research Centre. M. S. Ramasamy reports a relationship with Sanjeev Biomedical Research Centre that includes: employment and funding grants.

Data availability

Data will be made available on request.

Acknowledgment

This research study was supported, in part, by the Sanjeev Biomedical Research Centre (SBRC) foundation grant SBRC-21-0030 to MSR. We also thank Dr. Deepa for approving the intramural fund to conduct the research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.116032.

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