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Scientific validation of anti-arthritic effect of *Kashayams* – A polyherbal formulation in collagen induced arthritic rats

Aswathy I. S., Santhi Krishnan, Jasmine Peter, Vidya Sabu, A. Helen*

Department of Biochemistry, University of Kerala, Kariavattom, Thiruvananthapuram, 695581, Kerala, India

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ABSTRACT

Background: Toll-like receptor-4 (TLR-4) mediates activation of nuclear factor *kappa*-light-chain-enhancer of activated B cells (NF-κB) resulting in induction of proinflammatory genes such as that encoding tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) which played a significant role in cartilage destruction of rheumatoid arthritis (RA). Low risk and better efficacy made herbal drugs more reliable than nonsteroid anti-inflammatory drugs (NSAIDS) in RA treatment. *Gugguluthiktam Kashayam* (GuK), *Punarnavadi Kashayam* (PuK) and *Balaguluchiadi Kashayam* (BgK) are ayurvedic polyherbal formulations prescribed in classical ayurvedic texts *Sahasrayogam* and *Ashtangahridayam* as medicines for the treatment of RA.

Objective: The objective of the present study was to elucidate the molecular mechanism of anti-arthritic effect of these *Kashayams* on TLR-4 signal transduction pathway in collagen induced arthritic rats.

Material and methods: The wistar rats grouped into group I - Normal, group II- Collagen induced arthritis (CIA), group III- CIA + BgK, group IV– CIA + PuK, group V- CIA + GuK, group VI - CIA + Indomethacin (3 mg/kg b.wt.). Treatment with *Kashayam* (2 ml/kg b.wt) started after 14 days of primary immunization with type II collagen and continued for a period of 45 days.

Results: Arthritis index, C-reactive protein (CRP), rheumatoid factor (RF) and myeloperoxidase (MPO) in serum and protein level of TLR-4, myeloid differentiation factor 88 (MYD88), NF-κB, TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 COX-2) and prostaglandin E-2 (PGE-2) in cartilage were significantly elevated in CIA rats. Further, treatment with *Kashayams* downregulated all these inflammatory mediators hitherto TLR-4-NF-kB signal transduction pathway except IL-10, an anti-inflammatory cytokine which showed a reverse effect.

Conclusion: This molecular mechanism of the investigation confirmed the clinical efficacy of *Kashayams* in preventing the progression of RA and gave an intuition of the scientific validation of *Kashayams*, an Ayurvedic classical medicine.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation and progressive destruction of joint cartilage and bone tissues [1]. If left untreated, it may affect the cardiovascular system, haematological system, liver, respiratory system, eyes, muscles, kidneys and the neurological system [2] consequently one's life quality. Currently, 1% of the world population is affected by rheumatoid arthritis and the incidence of this degenerative disease keeps increasing [3,4]. Chronic inflammation is the essential pathological change of RA [5] and it occurs in the synovial region lining the joints. Immune cells infiltration into synovial fluid occurs; synovium expands and invades the cartilage results in cartilage destruction and pannus formation.

The involvement of toll-like receptor 4 (TLR4) in the pathogenesis of RA has been well established [6,7]. TLR4 is a member of the toll-like receptor (TLR) family of transmembrane proteins that recognize conserved pathogen-associated molecular patterns like lipopolysaccharide (LPS), viral double-stranded RNA, bacterial flagella, viral and bacterial CpG DNA and cause chronic

* Corresponding author.
E-mail: helenbios@yahoo.com
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inflammation by activating a cascade of proinflammatory events [8]. When a ligand binds to TLR4 various adaptor molecules are recruited to the TIR domain of TLR4 and enables downstream signaling. Multiple sequence of events gets orchestrated beginning from the binding of myeloid differentiation factor-88 (MYD88) protein to the TIR domain of this receptor. MYD88 is an essential molecule that binds in pair having a binding domain for another MYD88 molecule. Enlargement of recruitment platform through MYD88 dependent pathway promotes the activation of downstream molecules like IRAK1 and IRAK4 [9]. This mediates activation of nuclear factor *kappa*-light-chain-enhancer of activated B cells (NF-κB) thereby promoting transcription of proinflammatory genes such as those encoding tumor necrosis factor (TNF-α), interleukin-1β which played a significant role in the induction of cartilage destruction [10] associated with RA. It is then translocated from the cytoplasm into the nucleus to mediate transcription of proinflammatory cytokines [11] including inducible enzymes cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS). Prostaglandin E-2 (PGE-2) is the most important prostanoids released during inflammatory conditions by the action of COX-2 [12]. This inducible COX-2 gene expression can be persuaded by multiple cytokines and growth factors, which in turn lead to PGE-2 production [13].

Although nonsteroid anti-inflammatory drugs (NSAIDS) and disease modifying drugs (DMARDS) could manage RA symptoms the reported side effects from its longterm use are quite detrimental. In Indian traditional system of medicine, Polyherbalism has been given noteworthy importance because of their therapeutic efficiency and in '*Sarangdhar Samhita*' an ayurvedic literature efficiency of polyherbal formulations was described as when combining the multiple herbs in a particular ratio, it will give a better therapeutic effect and reduce the toxicity [14]. The use of polyherbal formulations as antiarthritic drugs [15] was also reported. *Kashayams* otherwise called herbal decoctions are polyherbal formulations with water soluble principles used in Indian traditional system of medicine and for the current study *Balaguluchiadi Kashayam* (BgK), *Punarnavadi Kashayam* (PuK) and *Gugguluthiktam Kashayam* (GuK) were taken. Accumulating evidence have shown anti-inflammatory, antioxidant, immunomodulatory properties of *Tinospora cordifolia* [16,17], an essential herb of three *kashayams* and also *Indian bdellium* [18,19], *Azadirachta indica* [20], *Justicia adhathoda* [21] which forms the components of GuK. *Balaguluchiadi Kashayam* consists of 10 herbs including *Sida cordifolia* [22], *Adenanthera pavonina* [23], *Cedrus deodara* [24] whose anti-inflammatory properties have been evidenced while *Punarnavadi Kashayam* was made from 6 plants like *Boerhavia diffusa* [25], *A. indica* [26], *Adhathoda vasicca* [27]. Hence, the objective of the study was to elucidate the molecular mechanism of anti-arthritic effect of these *Kashayams* on TLR-4 signal transduction pathway in collagen induced arthritic rats for scientific rationalization.

2. Materials and methods

2.1. Preparation of Kashayams

Raw materials collection and *Kashayams* preparation following classical guidelines [28] was done under the supervision of Dr. Sukumara Varier, Senior Ayurvedic Physician, Kottakkal Arya Vaidyasala. Carefully collected plant parts were shade dried, coarsely powdered and stored. From this single batch, *Kashayams* were prepared for the whole study and steps followed were one part of this powder was soaked in 16 parts of water, boiled till its volume reduced to 1/4th which was filtered and used.

2.2. Experimental animals

Adult female Wistar rats of weight 100–200 g, which were bred and reared in the department animal house, was used for the work. They were provided with laboratory chow (Hindustan Lever Lab diet) and water ad libitum throughout the experimental period. The rats were housed in polypropylene cages in a room with the temperature maintained at 26 ± 1 °C and a 12-hour light dark cycle. All experiments were conducted as per the guidelines of the Animal Ethics Committee CPCSEA (Registration No. 218/CPCSEA) [IAEC-KU-16/2014-'15-BC-AH (28)] according to Government of India accepted principles for laboratory animal use. Animals were grouped into Group I- Normal, Group II- Collagen induced arthritis (CIA), Group III- CIA + BgK, Group IV–CIA + PuK, Group V-CIA + GuK, Group VI- CIA + Indomethacin (IND) as positive control each having six rats.

2.3. Immunization and treatment protocol

Rheumatoid arthritis was induced in rats with type II collagen (Sigma) as described by Brand et al. [29]. Type II collagen obtained from bovine nasal septum was dissolved in glacial acetic acid at a concentration of 4 mg/ml and emulsified with an equal volume of incomplete Freund's adjuvant for induction. All rats except the normal were immunized with 200 μg of collagen intradermally at the base of the tail and received the same dose as booster injection on 7*th* day. After 7d of secondary immunization treatment started with BgK, PuK and Guk at a daily dose of 2 ml/kg b.wt. and with IND at 3 mg/kg b.wt. *intra-gastrically* to respective groups and continued up to 45 days. Dose of *Kashayams* was calculated from human dose based on body surface area according to Paget and Barne's dose conversion table [30]. After 45 days of treatment rats in six groups were euthanized, blood, cartilage and paw were collected for various analysis.

2.4. Evaluation of paw volume and arthritis scores

Paw volume of rats belonging to all groups was measured starting from the first day of the experiment and at regular intervals until the end on the 60th day of the experiment using paw edema meter. The severity of arthritis was assessed by arthritis score, joint destruction score and inflammation score. Both paw volume and arthritis score were noted. Rats were also monitored for the signs of arthritis from which severity symptoms were assigned different values from 0 to 4 and expressed as arthritis scores with 0 - no signs of arthritis, 1 inflammation in only one paw, 2 –inflammation/redness in more than one paw, 3 -swelling/redness in joint, 4- severe inflammation of joints, entire paw with deformity and ankylosis. The macroscopic arthritic score of each rat was presented as the sum of each score of the four limbs, with the maximum score being 16 for all four limbs.

2.5. Assay of rheumatoid factor, C-reactive protein (CRP) and myeloperoxidase in blood serum

The blood was collected in a centrifuge tube and kept for 30 min undisturbed at room temperature. Then centrifuged at 1000–2000 g for 10 min to remove the clot and serum was collected. The assay of RF in serum was done by quantitative turbidimetric test using RF-Turbilatex assay kit (Euro diagnostic systems) according to manufacturer's instructions. Latex particles got agglutinated on treating with sample and change in absorbance was taken spectrophotometrically at 630 nm. CRP in blood serum was measured using CRP -turbi-kit (Spinreact PVT Ltd, India) by

employing immuno turbidimetric method. Myeloperoxidase activity in serum was determined by Bradley et al. method [31].

2.6. Isolation of peripheral blood mononuclear cells

Isolation of peripheral blood mononuclear cells was carried out as described by Radhika et al [32]. 3 mL Histopaque 1083 solution was placed in a 15 mL tube and 3 mL blood was layered on top of this density gradient. On centrifugation (400 g for 30 min at room temperature) the blood cells were separated into an upper white layer comprising plasma, mononuclear cells plus the majority of platelets, form a buffy coat at the interface region, and a lower layer containing erythrocytes and granulocytes. From the buffy coat, the mononuclear cells were carefully taken off by aspiration and washed with phosphate buffered saline. This was repeated twice. After that, the pellet was resuspended in phosphate buffered saline with tween and subjected to freeze-thaw cycle three times. The resulting lysate was used as the enzyme source for 5-LOX assay.

2.7. Assay of 5-lipoxygenase (LOX) activity

Assay of 5-LOX activity was done by the method of Axelrod et al. [33]. 5- LOX activity was determined in a reaction mixture prepared from phosphate buffer (pH 6.1), enzyme source and sodium linoleate and the increase in OD was measured at 280 nm.

2.8. Nuclear and cytosolic fractionation

Cartilage tissues were trimmed minced and washed in ice-cold PBS buffer and gently spun to pellet the tissue. After pelleting, the tissue was rinsed and homogenized in STM buffer. The homogenate was centrifuged at 800 g for 15 min in ice-cold centrifuge. The supernatant was collected and used as cytosolic fraction while pellet dissolved in nuclear extraction buffer served as nuclear fraction [34].

2.9. Estimation of TLR-4, MYD88, NF-κB, TNF-α, IL-1β, iNOS, and IL-10 by ELISA

ELISA was performed to quantitate the amount of TLR-4, MYD88, NF-κB, TNF-α, IL-1β, iNOS, IL-10 in cartilage and COX-2, PGE-2 in paw tissues [35]. Tissues were grind in lysis buffer and different concentration of lysate precoated onto ELISA plates served as the antigen. After incubating for 3 h at room temperature, the wells were washed with PBS. Free binding sites were blocked with blocking buffer having 0.2% gelatin in PBS and 0.05% tween. After extensive washing the wells were incubated with primary antibody (Sigma) of 1:500 dilution followed by the addition of secondary antibody (Sigma) with 1: 1000. O-dianisidine with citrate phosphate buffer and hydrogen peroxide was the substrate and the coloured horse radish peroxidase (HRP) product was measured spectrophotometrically by an automated microplate reader (Thermo Multiskan System) at 405 nm.

2.10. mRNA expressions by RT- PCR

The total RNA was extracted from cartilage and paw tissue (50–100 mg) using Tri-reagent (Sigma). RNA quantification and purity assessed by UV-Vis spectroscopy at 260–280 nm. Then carried out cDNA synthesis and amplification in eppendorf thermal cycler according to manufacturer's protocol. The RT-PCR products were electrophoresed through a 1.5% agarose gel and band were visualized and quantified using gel doc analyzer (Major Science). The signals were expressed relative to the intensity of GAPDH in each sample. The primer pairs (Sigma) for TLR-4, TNF-α, COX- 2, iNOS and GAPDH were as follow (Table 1).

2.11. Quantitative real time PCR

Quantitative real-time PCR of proinflammatory cytokine IL-1β and anti-inflammatory cytokine IL-10 was performed in an eppendorf thermal cycler. RNA from cartilage was isolated, quantified and used for cDNA synthesis. After that qRT-PCR analysis was carried out, with real time PCR smart mix kit having Eva Green dye according to the manufacturer's protocol. The forward primer was kept at 95 °C for 15 min and reverse primers at 95 °C for 20 sec respectively and their sequences (Sigma) are in Table 1.

2.12. Determination of protein concentration

Protein concentration was determined by Lowry et al. method [36].

2.13. Statistical analysis

The results were analyzed using SPSS/PC+, version 16.0 (SPSS Inc., Chicago, USA). One-way ANOVA was employed for comparison among groups. Pair-fed comparisons between the groups were made by Duncan's multiple range tests and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of BgK, PuK and GuK on paw volume and arthritis index

In collagen induced arthritic rats the symptoms of arthritis appeared on the 14th day of primary immunization. Paw edema was measured by taking paw volume at each time interval from the 0th day onwards. Paw thickness had increased significantly ($p < 0.05$) in CIA rats after 14 days and elevation pattern significantly followed till the end of the experiment (60th day) as compared to normal (Fig. 1a).

The rats in the CIA group had significantly higher arthritis index (on day 21, 45, 60) (Fig. 1b) than the control rats ($p < 0.05$), demonstrate that CII immunization successfully induced arthritis in the animals. Upon *Kashayams* administration from the 14th day for a

Table 1
Forward and reverse primers of inflammatory genes.

Gene	Forward primer	Reverse primer
COX-2	5′CCAAACCAGCAGGCTCATACT3′	5′AGCGGATGCCAGTGATAGAGT3′
TLR4	5′AGTGTATCGGTGGTCAGTGTGCTT3′	5′ATGAAGATGATGCCAGAGCGGCTAG3′
TNFα	5′TGCTCAGAAACACACGAGACGC3′	5′TTCAGCAGCCTTGTGAGCCAGA3′
iNOS	5′CAGCACAGAGGGCTCAAAGG 3′	5′TCGTCGGCCAGCTCTTTCT 3′
IL-1β	5′AACCTGCTGGTGTGTGACGTTC3′	5′CAGCACGAGGCTTTTTTGTTGT3′
IL-10	5′AGAGCCCCAGATCCGATTTT3′	5′CATCAAGGCGCATGTGAACT3′
GAPDH	5′TCAAGAAGGTGGTGAAGCAG3′	5′AGGTGGAAGAATGGGAGTTG3′

treatment period of 45 days rats in the treated groups showed a maximum reduction in paw thickness and arthritis index (on day 25, 45, 60) as compared to the CIA rats ($p < 0.05$). Since *Kashayam* treatment showed retarded paw edema and joint inflammation, arthritis index subsides as shown in Fig. 1a and b. Thus, 45 days treatment with *Kashayams* significantly attenuate CII induced arthritis symptoms in rats belonging to BgK, PuK, GuK treated groups and this reduction was comparable to that shown by indomethacin treated positive control group.

3.2. Effect of Kashayams on serum arthritic biomarkers and myeloperoxidase activity

Arthritic biomarkers like C-reactive protein (CRP), rheumatoid factor (RF), and myeloperoxidase activity were clinically evaluated in rat serum. It was observed that CRP and RF values were increased in RA condition of CIA rats and this change was significant ($p < 0.05$) when compared to normal. At the same time in groups II, IV and V *kashayams* supplementation from 14 th day of collagen primary dose induction for 45 days had showed highly significant decline in the level of these parameters as compared to untreated CIA rats (II group) (Table 2). The insignificant change ($p > 0.05$) were also noticed in three *kashayam* treated groups when related to normal rats and positive control indomethacin treated ones.

As previous data had suggested during a chronic inflammatory condition along with other lymphocytes, neutrophils also moves into the site of attack as first line of defence. Myeloperoxidase enzyme activity was assessed in serum to measure infiltration rate of neutrophils. In CIA rats enhanced MPO activity was observed as indicated in Table 2 and however upon *kashayam* administration strongly inhibited enzyme activity in a significant manner ($p < 0.05$).

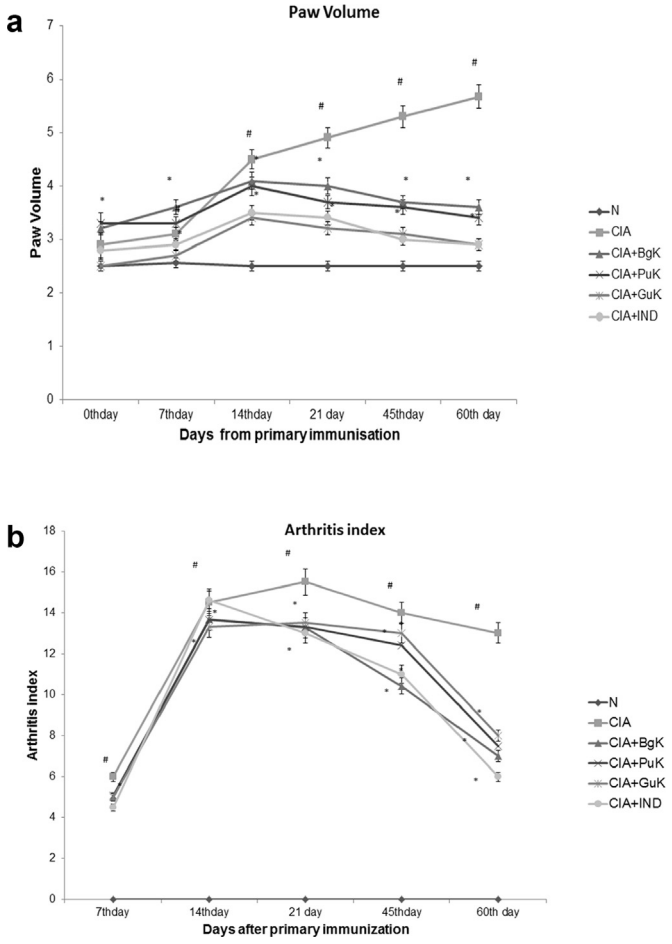

Fig. 1. Changes in Arthritis index and paw volume. The effect of *Kashayams* BgK, PuK, and GuK studied on collagen induced arthritic rats for a period of 45 days and from arthritic scores determined arthritic index. a) Paw volume varies during the treatment period. b) 14 days after primary immunization in all groups except the normal both paw volume and arthritis index rises. Treatment with BgK, PuK, GuK and positive control IND started from 14*th* day and continued for 45 days. # represents statistical significance at $p < 0.05$ when compared to normal (N) and * represents significance at $p < 0.05$ when compared to collagen induced arthritic (CIA) groups. Data were expressed as the mean ± SD from six separate experiments.

3.3. Effect of Kashayams on TLR-4, MYD88 and NFκB

To investigate the anti-inflammatory effects of BgK, PuK and GuK on rheumatoid arthritis model TLR4 -MYD88 dependent signal transduction pathway being focussed to determine the amount of TLR-4 receptor and MYD88. In CIA group the level of TLR-4 and MYD88 significantly ($p < 0.05$) elevated as compared to the normal rats model which revealed significant expression of TLR-4 receptor (Fig. 2a and b). *Kashayams* were administered for the experimental period of 45 days rats showed significant ($p < 0.05$) subsidence in the level of TLR-4 and MYD88. This is confirmed by the relative mRNA expression of TLR-4 that is significantly elevated in CIA rats when compared to normal (Fig. 4c). Thus the *Kashayams* exhibited potent inhibitory role in TLR-4 signaling pathway that was moderated through MYD88.

Transcription factor NF-κB is a key signaling molecule that exists in an inactive state in the cytosol of normal rats, so its p65 subunit amount is estimated in both cytosol and nucleus. As compared to normal rats in arthritic rats by collagen induction cytosolic p65 decreased significantly ($p < 0.05$) Fig. 2b, whereas in BgK, PuK and GuK administered rats p65 quantity significantly elevated in cytosol as compared to diseased rats. On the contrary, nuclear p65 quantification in joint cartilage by ELISA gave opposite results where CIA rats NF-κB p65 amount is significantly higher ($p < 0.05$) in nucleus on comparison with normal rats and treated rats had decreased its translocation significantly. This was represented as significantly lower levels ($p < 0.05$) of subunit concentration in the nucleus.

3.4. Effect of Kashayams on proinflammatory and anti-inflammatory cytokines

Pro and anti-inflammatory cytokines released by the activation of NF-κB was quantified in the cartilage of joint of both diseased and normal rats. The concentration of proinflammatory cytokines IL-1β and TNF-α was significantly elevated ($p < 0.05$) in CIA rats, whereas *Kashayam* treated one showed significant retardation ($p < 0.05$). This is similar to the effect produced by treatment with 3 mg/kg b.wt. indomethacin (Fig. 3a). An anti-inflammatory cytokine IL-10 was also estimated and its quantity was decreased in CIA rats when compared to normal (Fig. 3b). On the other hand, the

Table 2
Arthritic biomarkers CRP, RF and myeloperoxidase activity measured in serum.

Groups	CRP (IU/ml)	RF (IU/ml)	MPO(μmoles/min/mgpotein)
N	15.37 ± 1.4	5.81 ± 0.69	256.25 ± 23.38
CIA	41.92 ± 4.02#	26.73 ± 2.67#	543.25 ± 49.51#
CIA + BgK	15.95 ± 1.73*	8.20 ± 0.83*	299.12 ± 28.03*
CIA + PuK	15.87 ± 1.6*	8.11 ± 0.80*	301 ± 29.1*
CIA + GuK	15.47 ± 1.47*	7.20 ± 0.72*	313 ± 30.75*
CIA + IND	17 ± 1.66*	7.02 ± 0.67*	273.62 ± 25.05*

Results were indicated as mean ± SD and n = 6. #- is the significance compared to normal group at $p < 0.05$ and * represents significance as compared to CIA groups at $p < 0.05$.

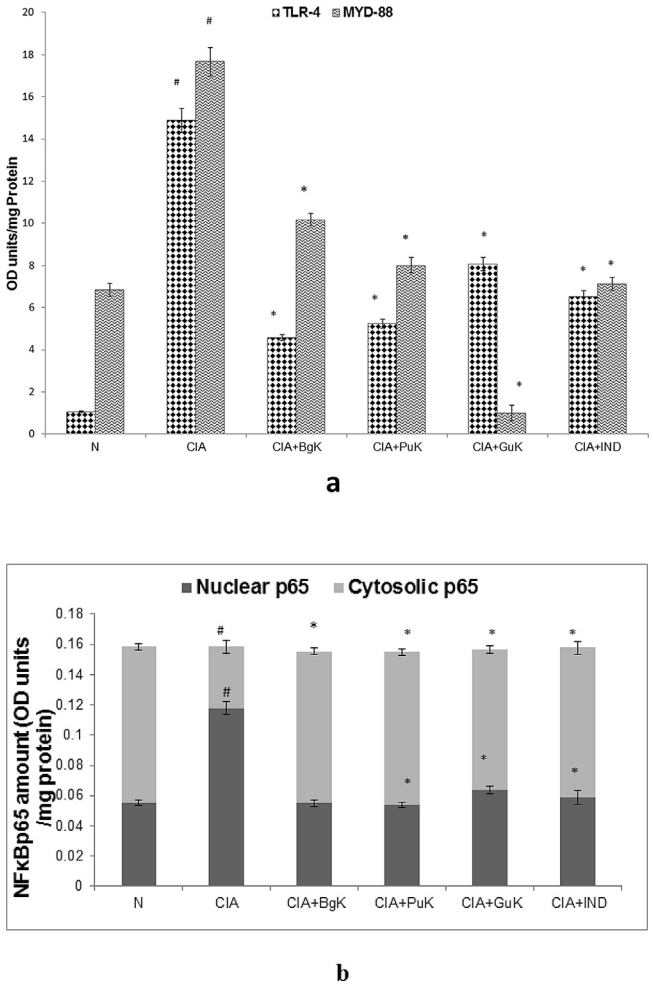

Fig. 2. Effect of BgK, PuK, GuK on TLR4 receptor activation, MyD 88 recruitment and NFκB translocation. a) The quantity of TLR-4 and MyD-88 in the cartilage of joints were determined by ELISA. b) The quantity of cytosolic and nuclear NF-κB p65 transcription factor in cartilage determined by ELISA. The six groups were N-normal groups, CIA-collagen induced arthritic groups, Balaguluchiadi, Punarnavadi, and Gugguluthiktam *Kashayam* treated groups and indomethacin treated positive control groups. # represents statistical significance at $p < 0.05$ when compared to normal (N) and * represents significance at $p < 0.05$ when compared to collagen induced arthritic (CIA) groups. Data were expressed as the mean ± SD from six separate experiments.

significant elevation identified in CIA rats was established by mRNA (qPCR) expression of TNF-α and correlated to a gene fold change of IL-1β and IL-10 (qRT- PCR). Fig. 3c and 3d clearly depict upregulation and downregulation of IL-1β and IL-10 respectively in CIA group when compared to normal and reversed in the case of *Kashayam* given groups. All these experiments suggested that activated TLR4 has been blocked by BgK, PuK and GuK that inhibits further activation and release of TNFα and IL-1β by NF-κB upon which IL-10 have an immune regulatory function.

3.5. Effect on COX- 2, 5-LOX, iNOS and PGE-2 synthesis

Here CIA rats showed a significant increase in cyclooxygenase -2, LOX, inducible nitric oxide synthase and prostaglandins-E2 amount as compared to the normal groups (Fig. 4a and b) While in BgK, PuK, GuK treated groups these inducible enzymes and the product quantity suppressed and exerted highly significant effect to CIA groups.

This obtained effect was again confirmed by its mRNA expression whose relative density is significantly reduced in treatment groups like IND groups (Fig. 4c and d). These data confirmed that *Kashayams* have potent anti-inflammatory nature that inhibits COX 2, 5- LOX and iNOS activities and regulates the release of associated inflammatory mediators and prostaglandins.

4. Discussion

Balaguluchiadi, *Punarnavadi* and *Gugguluthiktam Kashayams* are recommended Ayurvedic classical medicine used in the treatment of rheumatoid arthritis but scientific data regarding its molecular mechanism of action is not yet available. In order to determine the efficacy of *Kashayam* as an antiarthritic medicine in a clinical arthritic rat model, antiarthritic parameters and TLR-4 pathway followed by various cytokines were given particular emphasis in the present study. Even though RA has an unrevealed etiology recent researches have been focussed on to prevent the progression of disease and Ayurveda rigorously aims a holistic approach. CIA model chosen for this work is the widely accepted model since the pathological features attained by joints and synovial fluid is similar to RA [37,38]. The Severity of arthritis is assessed by measuring the paw thickness and arthritis score and on inducing type II collagen from bovine nasal septum different stages of RA were accomplished as represented by clinical scores. Meanwhile, *Kashayam* treatment for a period of 45 days retarded the worsening of disease symptoms like bone degradation and joint deformities. Biochemical markers like C reactive protein (CRP) and rheumatoid factor (RF) were measured to correlate with the rapid and severe progression of joint damage. Persistently high CRP levels are associated with substantial progression in chronic inflammation and joint damage in CIA groups. The pathogenic role played by RF as an autoantibody in rheumatoid arthritis cannot be ruled out, as our study showed a remarkable rise in its level. Contradictory results produced by *Kashayam* administration shall be attributed to its therapeutic potential by slow retrieval from its pathological state.

Infiltration of various cells like neutrophils, macrophages, B cells, T cells into the affected joints is the characteristic feature of RA. This will result in pannus formation and accumulation of cells release cytokines and chemokines which are proinflammatory in nature. Published data had brought about the influence of these cytokines in bone resorption and cartilage devastation [39–42]. For the release of these cytokines TLR-4 an integral membrane protein present on the cells is activated by specific ligand in collagen induced arthritic rats. Proper orientation and conformation are essential for this ligand-receptor interaction [43] and TLR-4 have intracellular TIR domain that dimerizes and helps in MYD88 dependent recruitment of other mediators. From the study we speculated that MYD88 is also an indispensable protein which is downregulated by *Kashayams* and might have affected series of downstream dependent molecules that determine cytokines release.

Since NF-κB is a key regulatory molecule involved in cytokine stimulation its activity inhibition may directly influence the release of TNF-α and IL-1β [44] in signalling pathways of inflammation. Numerous studies highlighted the direct role of NF-κB in bone destruction and osteoclasts activation in rheumatoid arthritic condition. In CIA model, in the present study the translocation of NF-κB molecule were observed and found the significant presence of p65 subunit in the nucleus. This enhanced the transactivation of proinflammatory genes by the binding of p65 fraction on target region of DNA. Thus proinflammatory cytokines TNFα and IL-1β secretion upregulated which is confirmed by its mRNA and fold change in gene. Whereas *kashayams* administration attenuates the perpetuation of signals thereby p65 fractionation and translocation which is affected by I-kb unbinding from NF-κB. Earlier studies [45,46] had shown involvement of these cytokines in bone

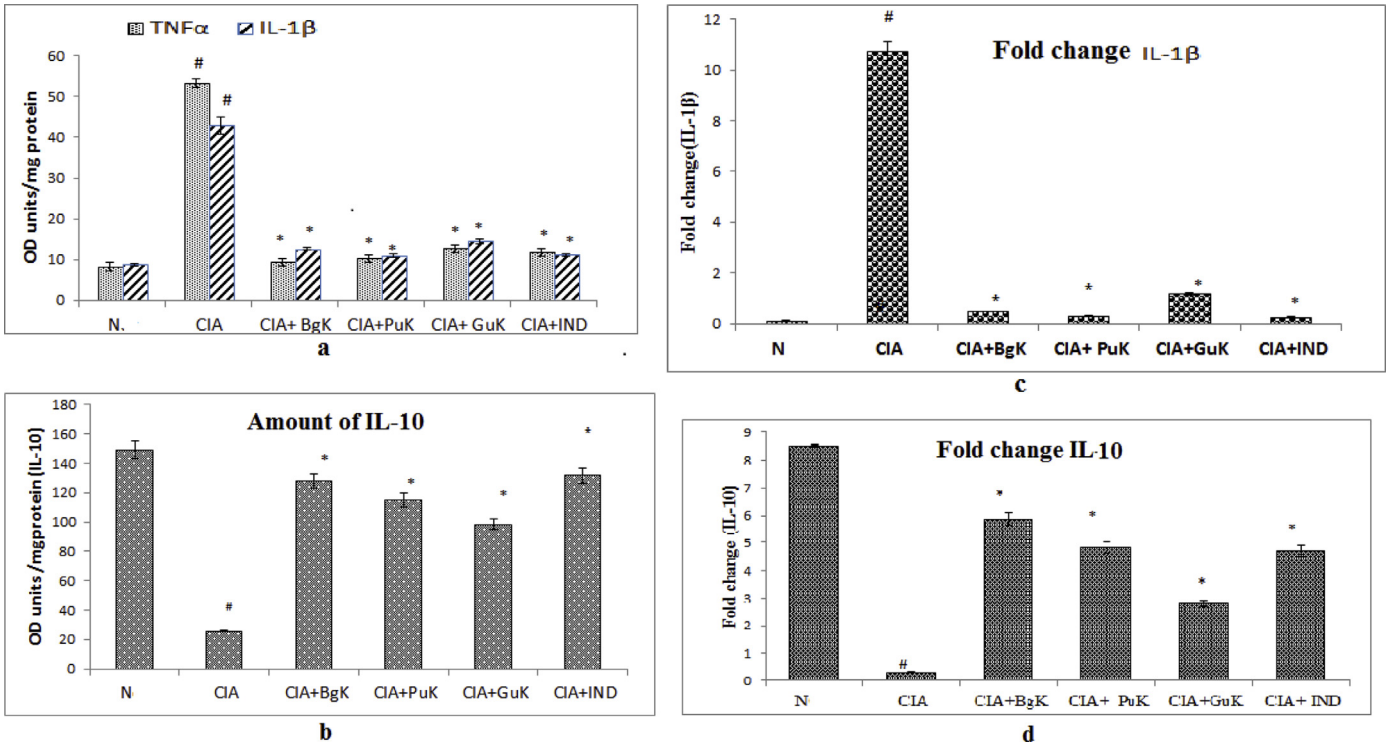

Fig. 3. Effect of *kashayams* on proinflammatory and anti-inflammatory cytokines. a) The quantity of proinflammatory cytokines TNFα and IL-1β in the cartilage of joints were determined by ELISA. b) The quantity of anti-inflammatory cytokine IL-10 in the cartilage of joints determined by ELISA. c) and d) correspondingly represents fold change in the case of IL-1β gene and Il-10 gene respectively as determined by qRT-PCR method. The six groups were N-normal groups, CIA-collagen induced arthritic groups, Balaguluchiadi, Punarnavadi, and Gugguluthiktam *Kashayam* treated groups and indomethacin treated positive control groups. # represents statistical significance at $p < 0.05$ when compared to normal (N) and * represents significance at $p < 0.05$ when compared to collagen induced arthritic (CIA) groups. Data were expressed as the mean ± SD from six separate experiments.

degradation in RA and so its decline contributes to retarded arthritic symptoms. To maintain a healthy immune status anti-inflammatory cytokines induces a regulatory function and so in our study IL-10 release at joint cartilage was noted. In arthritic joints downregulation of IL-10 gene promote TNF, IL-1β and the interplay of these cytokines induces the production of enzyme iNOS [47] which catalyses the formation of nitric oxide (NO). All these experiments suggested that activated TLR4 has been blocked by BgK, PuK and GuK that inhibits further activation of NF-κB thus decelerates transcription and hastens IL-10 gene activation which imparts an immunoregulatory function on synergistic action of cytokines. Thus with *Kashayam* treatment collagen induced arthritis was able to maintain a balance between proinflammatory and anti-inflammatory cytokines via TLR 4-MYD 88 dependent NF-κB signaling pathways.

Transactivation of inflammatory genes NFκB also activates COX-2 gene an important chemical mediator, induced by immune cells in response to inflammatory stimuli [48]. It acts on arachidonic acid of the membrane and synthesize prostaglandins a key inflammatory molecule. So in this present study, we have investigated action of Bgk, PuK and GuK on COX-2 induction and PGE2 production since both are coupled. In converse to CIA rats, COX-2 get inhibited by *Kashayams* which in turn downregulated PGE-2 synthesis and pacified pathogenic symptoms of rheumatoid arthritis. Thus COX-2 was expressed during tissue damage or inflammation in response to proinflammatory cytokines such as IL-1β, interferon gamma and TNF-α as reported earlier. 5-LOX enzymes are another set of enzymes whose target of action were membrane phospholipids and synthesize leukotrienes which are potent proinflammatory mediators [49]. 5-lipoxygenase is reported to be present in RA synovium and mostly expressed in neutrophils and mast cells in the sublining layer [50] and in rat mononuclear cells during chronic inflammatory conditions [51]. The retarded activity of 5-LOX by *Kashayam* ameliorates release of chemical mediators like LTB4 that controlled the overexpression of TNF-α and other proinflammatory cytokines otherwise leading to a chronic inflammatory environment. Inducible nitric oxide synthase which catalyzed the production of nitric oxide was strongly interpreted in cartilage tissues on disease induction with type-II collagen in the current work as implicated in several literature surveys [52,53]. Arthritic rats on treatment with *Kashayam* were able to create a balance between pro and anti-inflammatory cytokines that renders lessening of iNOS thereby causing a healthy restoration.

The presence of polyherbs in *balaguluchiadi*, *punarnavadi* and *gugguluthiktam kashayams* gave these classical medicines its unique therapeutic potential. Rather individual herbs in these polyherbal formulations have reported tocontain active principles flavonoids, alkaloids, terpenoids etc which exert different mechanism of actions. The synergistic effect produced by the combination of these principles may have imparted potent anti-inflammatory and anti-arthritic potential to these classical *Kashayams*.

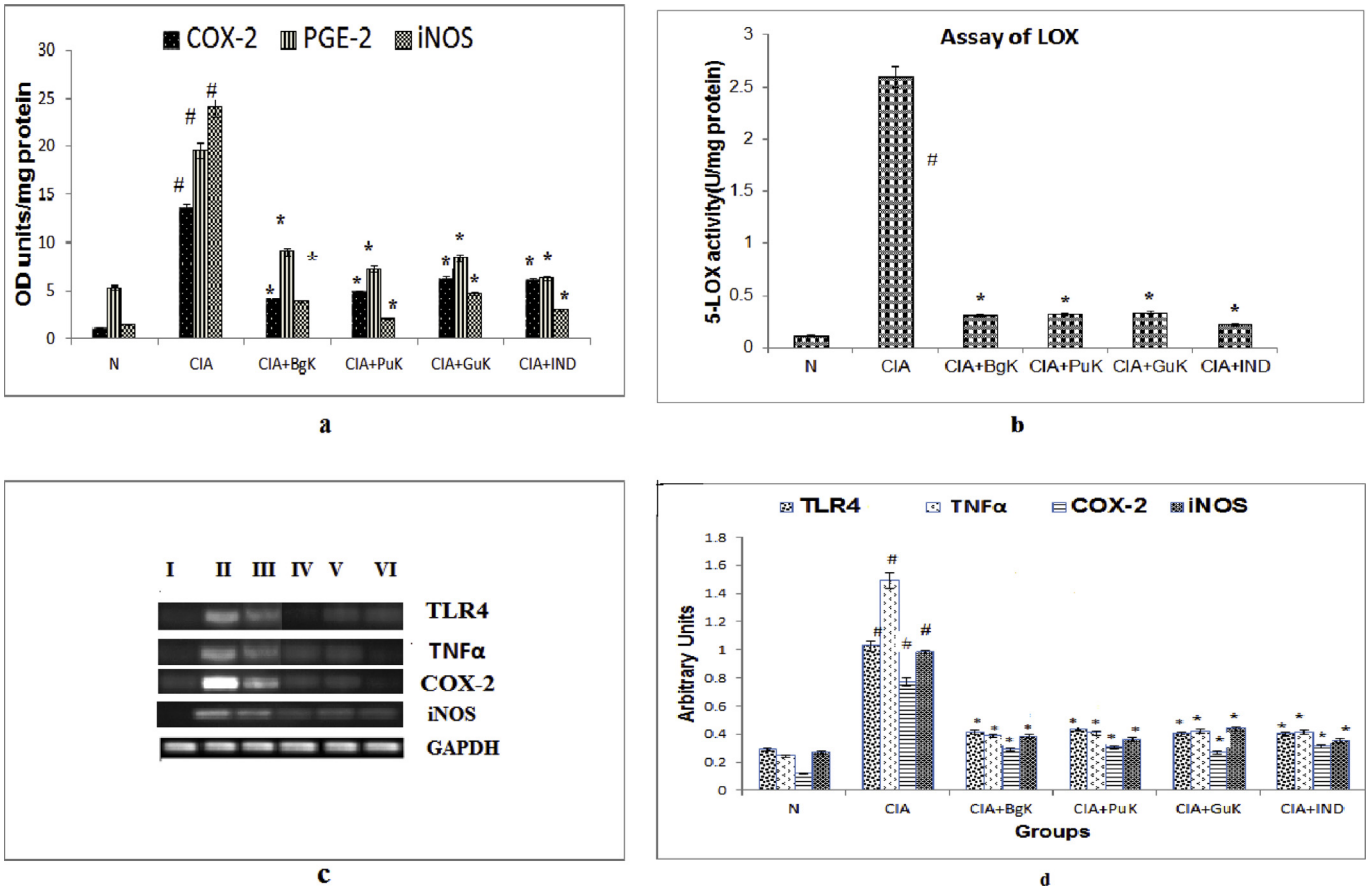

Fig. 4. Effect on COX- 2, 5-LOX, iNOS and PGE-2 synthesis. a) The quantity of COX-2, PGE-2 and iNOS the cartilage of joints were determined by ELISA. b) The activity of anti-inflammatory enzyme 5-LOX in monocytes measured in U/mg protein. c) mRNA expression of TLR-4, TNFα, COX-2, iNOS and their intensity as compared to GAPDH. The six groups were I- normal groups, II- (CIA) collagen induced arthritic groups, III- Balaguluchiadi, IV- Punarnavadi, and V- Gugguluthiktam *Kashayam* treated groups and VI- indomethacin treated positive control groups. #- statistical significance at $p < 0.05$ when compared to normal (N) and *- significance at $p < 0.05$ when compared to collagen induced arthritic (CIA) groups. Data were expressed as the mean ± SD from six separate experiments.

5. Conclusion

In conclusion, the tested *Kashayams* BgK, PuK and GuK acts as a potent anti-inflammatory drug by inhibiting TLR-4 MYD88 dependent signal transduction pathway. The balance between proinflammatory and anti-inflammatory cytokines, its synthesis and secretions were maintained thereby regulating chronic inflammation. This study was the first of its kind that explained the antiarthritic effect of *Kashayams* through the molecular level of organization in biological systems which provides an insight into scientific validation for Indian traditional medicine.

Ethical approval

All applicable institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution in which this study was conducted (Animal Ethics Committee CPCSEA (Registration No. 218/CPCSEA) [IAEC-KU-16/2014-'15-BC-AH (28)]

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Conflict of interest

None.

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