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Indian Journal of Physiology and Pharmacology

Original Article

Protective effects of the ethanolic fenugreek seeds extract and its potentiation with nitric oxide modulators in adjuvant induced changes in arthritic index, pro-inflammatory/anti-inflammatory cytokines imbalance and oxidative stress markers in rats

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Received : 10 January 2022
Accepted : 30 March 2022
Published : 31 May 2022

DOI
10.25259/IJPP_11_2022

Quick Response Code:

ABSTRACT

Objectives: The current study was designed to evaluate protective role of the ethanolic fenugreek seed extract (FSE) and potentiating its effects with nitric oxide (NO) modulators in experimental arthritis and its comparison with the standard drug methotrexate.

Materials and Methods: The FSE was prepared using standard procedures. Fifty-four male Wistar rats were equally distributed into nine groups of six animals in each group. Rheumatoid arthritis was induced by administration of complete Freund's adjuvant (CFA) in sub-plantar region of rt. hind paw. FSE alone and with L-arginine or N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were administered on day 10 of CFA inoculation, i.p. Animals were evaluated for arthritic parameters, cytokines and oxidative stress markers estimation. Statistics: The data were analysed by two-way ANOVA followed by Newman Keul's *post hoc* test for inter group analysis by GraphPad Prism 6.0 and $P < 0.05$ was taken as significant.

Results: Adjuvant inoculated rat shows significant increase in arthritic and inflammatory parameters as well as oxidative stress biomarkers in serum, paw homogenates and joint synovial fluid. CFA inoculation significantly decreased anti-inflammatory cytokine-10 and SOD activity. These adjuvant-induced arthritic changes were significantly attenuated by ethanolic FSE administration from 10 to 28 days. These results are comparable to standard drug methotrexate. NO modulators further potentiated protective effects of FSE when given in combination. These results were more prominent when ethanolic seed extract was given with iNOS inhibitor, L-NAME.

Conclusion: These findings suggest that FSE shows protective effects in CFA induced arthritic changes that may be mediated through pro-inflammatory/anti-inflammatory cytokines imbalance and it is associated with modulation of oxidative stress and NO-signalling.

Keywords: Cytokines, Fenugreek, Inflammation, Nitric oxide, Oxidative stress, Rheumatoid arthritis

INTRODUCTION

Inflammatory disorder that brings about joint destruction and functional disability is rheumatoid arthritis (RA) and it is characterised by synovial membrane inflammation and erosion of bones

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and cartilages that result into progressive destruction of joint and further, it may involve extra-articular tissue.[1]

The pro-inflammatory and anti-inflammatory cytokines imbalance determines severity of cartilage damage. Tumour necrosis factor alpha (TNF-α) and Interleukin-1 beta (IL-1β) shows significant involvement in the pathogenesis of RA.[2,3] Our previous studies suggest that phosphodiesterase inhibitors such as theophylline,[4] pentoxiphylline,[5] and plant product, mangiferin attenuated adjuvant induced changes in arthritis parameters and protect pro-inflammatory/anti-inflammatory cytokine imbalance in rats.[6]

Recently, reactive nitrogen species (RNS) free radicals in biological system had received much attention in the pathogenesis of various diseases.[7,8] Researchers have now established that the free radicals, involving RNS, play significant role in inflammation and pathogenesis of RA.[9]

Drugs are available to treat symptoms and modify the progression of pathogenesis of RA. NSAIDs are available to treat pain in RA which can cause gastrointestinal bleeding or perforation and disease modifying drugs may cause severe anaemia and even death. Disease-modifying anti-rheumatic drugs, like methotrexate can lead to liver cirrhosis, sulfasalazine can cause thrombocytopenia and chloroquine can lead to maculopathy while leflunomide and etanercept can lead to chest infections in long term use. Due to their adverse effects their use becomes limited. Apart from the allopathic drugs, traditional and complementary and alternative medicines are in use from the ancient time for the management of RA.[10]

In many countries, herbal medicines are gaining popularity as alternative and complimentary therapies. Fenugreek (*Trigonella foenum-graecum* L.) is a leguminous plant its medicinal properties have been found in the extracts of fenugreek seeds and leaves.[11] The secondary metabolites of plants provides humans with numerous biological active products which has been used extensively. Fenugreek seeds contain about 0.1–0.9% of diosgenin which is a phytosteroid sapogenin of fenugreek, Saponin (fenugrin B) and many types of coumarin compound along with alkaloids (e.g., trigonelline, gentianine and carpaine) are also present in fenugreek seeds.[12]

Fenugreek have effective protective role in various diseases.[13] Its seeds are used as a lactation stimulant in India from an ancient time[14] and antidiabetic properties were also found in seeds and leaves of this plant.[15,16] Hypocholesterolaemic effects of fenugreek are also.[17] Its anti-cancer properties were also studied.[18] In a study its protective role in thyroxine-induced hyperglycaemia recently studied.[19] Its also shows protective effect on ethanol induced liver toxicity.[20] Uses of the fenugreek in the traditional Indian and Chinese medicine is well documented. An attempt has been made to combine traditional medicine with modern drugs for better therapeutics for untreatable rheumatoid arthritis in animal model.

Very few studies have been carried out in past to evaluate its role in treatment of RA, but none of them have shown its potential plausible mechanism of RA protection.

Taken together, the current study is designed to evaluate protective role of ethanolic fenugreek seed extract (FSE) and its potentiation by NO modulators is mediated through pro-inflammatory/anti-inflammatory cytokine imbalance and oxidative markers.

MATERIALS AND METHODS

Experimental animals

Fifty-four male Wistar rats (180–225 g, $n = 6$ per group) were utilised for this study. Rats were purchased from certified breeding centre of CSIR-Indian Institute of Toxicological Research, Lucknow. The care of animals was done as per CPCSEA guidelines. Study protocol was approved by Institutional Animal Ethics Committee (IAEC) of KGMU, Lucknow (No. 91/IAEC/2018).

Experimental design

The experiments were conducted for 28 days. All animals were acclimatised for 1 week after purchase from CPCSEA certified animal house. On day "0" all animals were injected 0.2 ml of the complete Freund's adjuvant (CFA) containing mycobacterium tuberculosis bacterial cell wall in mineral oil in sub-planter region of the right paw. The right and non-injected left paw were assessed daily for the development of the RA symptoms which mimic human RA. From day 10^{th} to 28^{th} day, daily once ethanolic FSE which was prepared in the laboratory was administered in a dose of 200 mg/kg and 400 mg/kg, p.o alone and in combination with NO donor, L-argnine (100 mg/kg, i.p.) and inducible nitric oxide synthase (iNOS) inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (10 mg/kg, i.p.).

Wister rats ($n = 6$) are divided into nine groups are as follows:
Group 1: Control (Normal saline)
Group 2: Disease model CFA (0.2 ml, subcutaneous, rt. paw)
Group 3: Adjuvant + Methotrexate (5 mg/kg, i.p.)
Group 4: Adjuvant + FSE (200mg/kg, p.o.)
Group 5: Adjuvant + FSE (400 mg/kg, p.o.)
Group 6: Adjuvant + L-Arginine (100 mg/kg, i.p.)
Group 7: Adjuvant + L-NAME (10 mg/kg, i.p.)
Group 8: Adjuvant + FSE (400mg/kg, p.o.) + L-Arginine (100 mg/kg, i.p.)
Group 9: Adjuvant + FSE (400 mg/kg, p.o.) + L- NAME (10 mg/kg, i.p.).

On day 28th after the daily dose, all animals were assessed for the RA index and paw volume changes. Then all animals were sacrificed by using high dose of anaesthesia, pentobarbitone (100 mg/kg, i.p.). Their blood, paws and synovial fluid were collected for cytokine and oxidative stress markers estimation.

Ethanolic FSE preparation

From the local market, 2.5 kg of fenugreek seeds were purchased and were authenticated by the botanist of CSIR-National Botanical Research Institute Lucknow. Ethanolic FSE was prepared as per the standard protocol. Briefly, 2.5 kg of the powered fenugreek seed material was soaked in 80% alcohol for overnight and then filtered through Whatman filter paper No.41 along with sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulphate was wetted with absolute alcohol. The filtrate was then concentrated using rotatory evaporator.[21] The FSE obtained was used for the experiments.

Drugs and chemicals

Chemicals

CFA was obtained from Sigma Aldrich Co. USA. Hexane, Ethanol, Trichloroacetic acid, Griess's reagent, Tris-HCl buffer, Triton-X, Thiobarbituric acid, Hydrogen peroxide, Pyrogallol, Nitrate reductase and other routinely used chemicals were obtained from TCI chemical Co. Japan.

Drugs

Standard drug (Methotrexate), nitric oxide (NO) donor (L-arginine) and iNOS inhibitor (L-NAME) were obtained from Sigma Chemical Co. USA. Ethanolic FSE was prepared in the departmental chemical laboratory.

ELISA kits

Pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory IL-10, cytokines estimation kits were obtained from Diaclone, France, for their estimations in serum, paw homogenate and joint synovial fluid.

Arthritis

Induction of arthritis in rats

Experimental arthritis was induced in rats by inoculated with 0.2 ml of CFA in sub-plantar region on day "0" as described by Lee and Weinblatt, 2001[22] and Pal *et al*., 2016.[5] Sensitivity of CFA was modified by intradermal injection of 0.1 ml Squalene before CFA inoculation into a different site in the sub-planter surface of the right hind paw on day "0". Development of inflammation and arthritic changes were observed daily. The drug treatments with FSE alone and with NO modulators were given from day 10th to 28 day daily. At the end of the study on 28th day, all animals were sacrificed under high dose of anaesthesia, pentobarbitone 100 mg/kg, i.p. Blood was collected by cardiac puncture and centrifuged at 3000 rpm for 5 min, serum was separated and used for cytokine an oxidative stress biomarkers estimation. Synovial fluids was collected from knee joint and used for TNF-α estimation. Paws were excised and homogenate was prepared for biochemical and cytokine estimation.

Evaluation of arthritis index

Daily evaluation of rats was done for arthritis development. Grading system was used to determine physical symptoms of arthritis and progress of RA development. Rats were scored from 0 to 16 on the basis symptoms of arthritis were developed as normal paw (0); erythema of toe (1); erythema and oedema of paw (2); oedema of ankle (3); complete oedema of whole leg (4); and incapability to bend (6).[22,23] To get arthritis index of rats, score of four individual paws was added as per the procedure described by Wooley 1981.[24] A sensitised animal was considered to have arthritis when at least one of the non-injected paws was inflamed (Philippe *et al*., 1977.[23] The effect of test, drug fenugeek seed extract (FSE) was evaluated and compared with standard drug Methotrexate. The dose of methotrexate was selected based on the previous studies.[25,26] The mean arthritic index score was calculated and presented as line graph.

Paw volume and ankle diameter changes

Paw volume and ankle diameter changes were assessed daily after different drug treatment. Both ipsilateral (injected) and contralateral (non-injected) paws in experimentally induced arthritis rats as per the procedure described by Lee and Weinblatt, 2001[24] measured by using Mitutoyo dial calliper (Model No 505-730, Mitutoyo Corporation, Japan) with graduation 0.02 mm, 2 mm/rev, accuracy: ±0.03 mm. Briefly, the height, length and width of the paw was measured in millimetre. One complete round of the dial measure 1 mm and noted for both paws and paw volume (Length × Height × Width) was calculated.[24] The paw volume changes was calculated using following formula:

$$\%\,Change = \frac{Right\ paw\ volume\left(injected\right) - Left\ paw\ volume\left(non\text{-}injected\right)}{Right\ paw\ volume\left(injected\right)} \times 100$$

and was presented as % change in paw volume

TNF-α, IL-1β and IL-6 and IL-10 cytokines estimation

TNF-α, IL-1β, IL-6 and IL-10 levels were determined in serum following instructions provided in ELISA kits (Diaclone, France).

TNF-α in ankle joint synovial fluid estimation

Joint synovial fluid was collected after each experiment as per the previously described method. Briefly, after treatment on day 28, animals were anesthetised using pentobarbitone sodium 30 mg/kg, i.p. after anaesthesia, their paw was bent over the glass vial in such a way that patella was upward to insert the a perfusion needle. After connecting 23 gauge needles to a roller pump through a rubber perfusion tubing, normal saline was infused at a rate of 100 µl/min after 1 min. of infusion of the normal saline, outflow tube was connected to a 25-gauge needle to reduce the pressure that has built up in the joint space (Liu *et al.*, 2005).[27] Fluid thus drawn was collected in a 2 ml Eppendorf and stored at −20°C. TNF-α level was estimated in the fluid thus collected as per the procedure provided in the ELISA kit. (Diaclone, France).

Oxidative stress markers

Lipid peroxidation

After the experiments, rats were sacrificed using anaesthesia and paws homogenate was prepared as previously described.[5] The extent of lipid peroxidation (Malonylaldehyde [MDA]) was estimated by following method of Ohkawa *et al.*[28] Protein concentration in paw homogenate by determined using Lowry's method.[29]

Superoxide dismutase (SOD) activity

SOD activity was estimated in rats paw homogenates using the standard method.[30] Protein content was estimated using Lowry's method.[29]

Nitrite/nitrate (NOx) estimation

The ratio of NOx was estimated using the Griess's reagent following the protocol described by Green *et al.*[31] The absorbance at 542 nm was taken using the micro-scan spectrophotometer (Microscan-5405A, ECIL). Protein content in paw homogenate was estimated using Folin-phenol reagent as described in Lower's method.[30]

Changes in the body weight measurement

The body weight of each rat was recorded before and daily after CFA inoculation and different drug treatment to assess food intake and changes in their weight throughout the study. Change in the body weight in arthritic and different drug treated rats was calculated to determine effect of CFA inoculation and drug treatment on body weight and food and water intake.

Statistical analysis

The data were analysed by two-way ANOVA followed by Newman Keul's *post hoc* test for in group analysis by GraphPad Prism 6.0 and $P < 0.05$ was taken as significant.

RESULTS

Effects of FSE and NO modulators on adjuvant induced changes in arthritic index

Significant increase in arthritis index after CFA inoculated rats was observed. Arthritis index of adjuvant only group increase from 0 at the start of the study to maximum level of 15.6 ± 1.1 on day 28 of the study as compared to control group ($P < 0.05$). Methotrexate (5 mg/kg) significantly decreased adjuvant induced arthritis index in comparison with adjuvant group ($P < 0.05$) and these results were found comparable with the animal treated with FSE 200–400 mg/kg ($P < 0.05$). L-arginine (100 mg/kg) alone and in combination with FSE 400 mg/kg treated rats shown significant enhancement in arthritic index as compared to FSE (200 mg/kg) treated group ($P < 0.05$). However, maximum protection in arthritis index was shown with FSE (400 mg/kg) plus L-NAME (10 mg/kg) when compared with standard drug methotrexate ($P < 0.05$). Results are summarised in [Figure 1].

Effects of FSE NO modulators on adjuvant induced changes in ankle diameter

CFA inoculated rats shown significant increase in ankle diameter as comparison to saline treated controls ($P < 0.05$). Methotrexate (5 mg/kg) shows protection in ankle diameter and was found comparable with FSE (200 mg/kg) treatment ($P < 0.05$). FSE (200–400 mg/kg) alone significantly attenuated CFA induced increase in ankle diameter comparable with methotrexate group ($P < 0.001$). FSE (200 mg/kg) + L-NAME (10 mg/kg) treated group has shown significant protection and comparable with methotrexate treated group ($P < 0.01$). These results are summarised in [Figure 2].

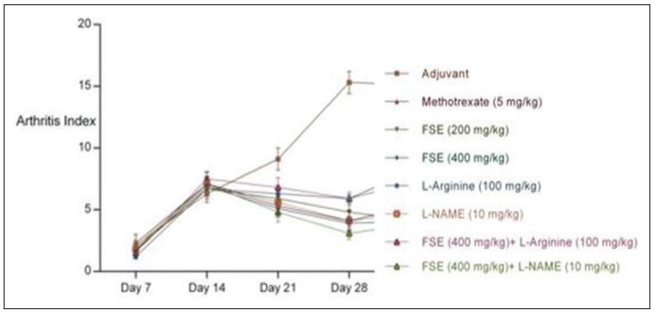

Figure 1: Effect of fenugreek seed extract and NO modulators on arthritic index. NO: Nitric oxide.

Effects of FSE NO modulators on adjuvant induced changes in paw volume

Significant increase in paw oedema of adjuvant inoculated rats was seen in comparison with normal saline treated control group. Significant suppression in % change in paw volume was observed after FSE (200 mg/kg) administration as compared to adjuvant and methotrexate (5 mg/kg) treated group ($P < 0.05$). FSE (200 mg/kg) with L-arginine (100 mg/kg) significantly attenuated these adjuvant induced alteration in % change in paw volume when compared to L-arginine (100 mg/kg) treated rats. Treatments with FSE (400 mg/kg) and or with L-NAME (10 mg/kg), shown maximum protection in paw volume changes in comparison to standard drug methotrexate ($P < 0.01$). These results are summarised in [Figure 3].

Effects of FSE and NO modulators on adjuvant induced changes in pro-inflammatory and anti-inflammatory cytokines imbalance in rat paw homogenate

Pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) levels were significantly increased in paw homogenate and also in serum after adjuvant inoculation in paws as compared to normal saline treated control group ($P < 0.05$). Level of these cytokines was restored toward normalcy after FSE (200–400 mg/kg) treatment, in a dose-dependent manner ($P < 0.01$) in comparison with L-Arginine (100 mg/kg) alone and also with methotrexate treated group ($P < 0.05$). IL-10 level in serum and paw homogenate was found significantly suppressed in adjuvant group as compared to control group. Treatment with FSE (200 mg/kg) in combination with L-NAME (10 mg/kg) significantly reversed pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory cytokine (IL-10) levels in both serum and paw homogenate ($P < 0.05$). The results are comparable with methotrexate (5 mg/kg). These results are summarised in [Table 1].

Effects of FSE and NO modulators on adjuvant induced changes in TNF-α level in ankle joint synovial fluid

The TNF-α levels in joint synovial fluid were measured to assess the impact of the different drug treatment and which was found significantly increased in adjuvant inoculated group as compared to non-inoculated normal saline treated control group. This increase in TNF-α levels was reduced after treatment FSE (200–400 mg/kg) treatment in dose-dependent manner ($P < 0.001$). FSE (200 mg/kg) with L-arginine (100 mg/kg) significantly reduces TNF-α level in synovial fluid. These results were significant and found comparable with standard drug methotrexate treated group [Figure 4]. These protective effects of FSE (200 mg/kg) were found potentiated when treated with L-NAME (10 mg/kg) ($P < 0.01$) [Figure 4 and Table 2].

Effects of FSE and NO modulators on oxidative stress markers

MDA is the marker for lipid peroxidation. MDA levels in paw homogenate were significantly increased after CFA inoculated rat paw as compared to controls (non-inoculated paws)

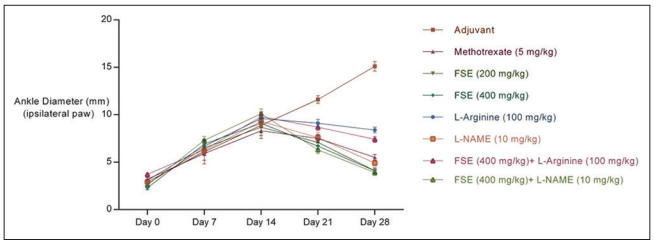

Figure 2: Effect of fenugreek seed extract and NO modulators on ankle diameter. NO: Nitric oxide.

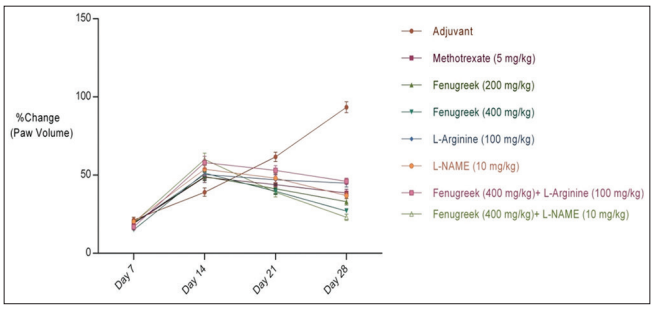

Figure 3: Effect of fenugreek seed extract and NO modulators on % change in paw volume. NO: Nitric oxide.

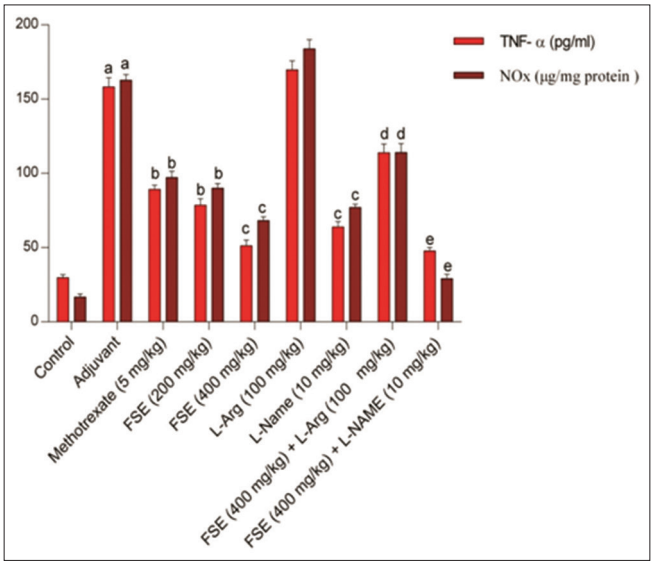

Figure 4: Effect of fenugreek seed extract and NO modulators on TNF-α and NOx levels. [a]$P < 0.05$, compared to control, [b]$P < 0.05$, compared to Adjuvant, [c]$P < 0.02$, compared to Methotrexate, [d]$P < 0.01$, compared to FSE (200 mg/kg), [e]$P < 0.03$, compared to (FSE 400 mg/kg) + L-Arg (100 mg/kg). NO: Nitric oxide, TNF-α: Tumour necrosis factor alpha, FSE: Fenugreek seed extract.

($P < 0.05$). FSE (200–400 mg/kg) significantly attenuated adjuvant induced raised MDA levels in a dose-dependent manner as compared to adjuvant and methotrexate treated group. Combination of FSE (200 mg/kg) with NO precursor, L-Arginine (100 mg/kg) and/or inhibitor, L-NAME (10 mg/kg) significantly further attenuated MDA levels ($P < 0.001$) [Table 3].

SOD activity was observed on the lower side in adjuvant inoculated paw homogenates which is found comparable with controls ($P < 0.05$). FSE (200 mg/kg) with L-Arginine (100 mg/kg) or L-NAME (10 mg/kg) significantly attenuated CFA induced elevation in MDA levels and SOD activity. The increased SOD activity was less marked in rats treated with combination of FSE (200 mg/kg) with L-Arginine (100 mg/kg) as compared to FSE (200 mg/kg) + L-NAME (10 mg/kg) ($P < 0.05$) [Table 3].

In adjuvant inoculated group, significantly enhance NOx levels were found in comparison with control group ($P < 0.05$). Treatment with FSE (200–400 mg/kg) along L-Arginine (100 mg/kg) or L-NAME (10 mg/kg) significantly decreases NO_X levels toward normalcy ($P < 0.001$) [Figure 4 and Table 1].

Effects of FSE and NO modulators on body weight changes

During the course of study, body weight of control group was increased significantly ($P < 0.05$). Body weight of control peaked to a maximum of from 202.0 ± 7.4 g on day "0" of study to 242.0 ± 11 on day 21. Body weight of adjuvant group decreases from 210.9 ± 14.1 g on day 0 of the study to 128.1 ± 7.3g day 28 of the study.

Maximum increase in body weight was observed in the animals which were treated with FSE 400 mg/kg alone. Body weight of these animals increased from 197.6 ± 8.5 g on day 14 to a maximum of 221.7 ± 8.3 ($P < 0.001$) as compared to adjuvant group. Body weight was increased substantially in other treatment groups also, after a brief fall in their body weight; however, the increase in body weight was less prolific than increase in the weight of animals treated with FSE 200 mg/kg.

Body weight of animals receiving methotrexate 5 mg/kg increases from 208.6 ± 7.0 g on day "0" to 211.7 ± 5.9 g on day 28. Minimum increase in body weight was observed in animal group receiving FSE 200 mg/kg alone and combination of FSE 400 mg/kg and L-arginine 100 mg/kg. Body weight of rats treated with FSE 200 mg/kg alone was increased from 220.1 ± 15.1 g to 223.1 ± 15.1 g on day 28. Body weight of rats treated with a combination of FSE 400 mg/kg plus L-arginine 100 mg/kg, increases from 215.1 ± 4.9 g at the start of study to 217.7± 6.3 g on day 28. Bodyweight of group receiving combination of FSE 400 mg/kg and L-NAME 10 mg/kg was increased from 211 ± 4.1 g at the start of study to 215.7± 8.1 g on day 28. These results are summarised in [Table 4].

DISCUSSION

Current investigation was conducted to explore protective roles of ethanolic seed extract of fenugreek and its interaction with NO modulators in CFA-induced animal model of arthritis.[32] Results of present study, clearly suggests that fenugreek has cytokine and oxidative stress modulating as well as NO signalling attenuating properties. Marked reversal of adjuvant-induced changes in paw oedema, arthritis index, ankle diameter and body weight was observed after ethanolic FSE treatment alone and in combination with NO modulators during study.

Arthritis index was significantly increased in adjuvant inoculated rats ($P < 0.05$). This can be explained by the fact that Mycobacterial components present in the CFA led to continued induction of cytokines, which led to reliable onset and progression of bone resorption and proliferation of periosteal bone, synovial proliferation and cartilage destruction resulting in worsening of the arthritic

Table 1: NOx, IL-6, IL-1β and IL-10 level in paw homogenate in adjuvant-induced arthritic rats on day 28 (data are in Mean±SEM) (n=6/group; all treatments administered i.p).

Treatment group	IL6 (pg/ml)	IL-1β (pg/ml)	IL-10 (pg/ml)	NOx (µg/mg protein)
Control	14.8±2.1	17.2±1.2	511.6±13.7	25.8±1.9
Adjuvant	40.2±1.8	65.8±2.8	198.7±8.3*	162.8±3.7*
Adjuvant+Methotrexate (5 mg/kg)	25.2±1.9	50.2±2.9	309.3±23.1	97.3±4.1#
Adjuvant+FSE (200 mg/kg)	34.6±1.9	49.8±2.2[a]	325.4±14.9[a]	90.2±3.3[b]
Adjuvant+FSE (400 mg/kg)	29.7±2.4	39.7±2.9[a]	383.7±21.8[a]	68.3±2.5[a]
Adjuvant+L-Arginine (100 mg/kg)	21.9±2.5	28.7±1.3[a]	435.8±28.3[a]	183.9±6.1#
Adjuvant+L-NAME (10 mg/kg)	39.2±2.7	59.3±2.8	468.7±11.2[a]	77.6±2.3[d]
Adjuvant+FSE (400 mg/kg) + L-Arginine (100 mg/kg)	35.9±2.3[c]	57.4±2.3[c]	338.4±10.3[c]	114.1±5.9*,#
Adjuvant+FSE (400 mg/kg) + L-NAME (10 mg/kg)	15.2±2.1[a,b]	20.3±1.4[a,b]	510.3±108[a,b]	29.1±2.9*,#,d

*P<0.05 compared to control group, #P<0.05 compared to adjuvant group, [a]P<0.02 compared to adjuvant group, [b]P<0.05 compared to FSE (400 mg/kg), [c]P<0.02 compared to L-arginine (100 mg/kg), [d]P<0.001 compared to FSE (400 mg/kg) + L- arginine (100 mg/kg). FSE: Fenugreek seed extract, L-NAME: N^{ω}-Nitro-L-arginine methyl ester hydrochloride, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, IL-10: Interleukin-10

Table 2: TNF-α levels in knee joint synovial fluid and paw homogenate of adjuvant-induced arthritic rats on day 28 (data are in Mean±SEM) (*n*=6/group).

Group (Treatment)	Synovial fluid TNF-α (pg/ml)	TNF-α (pg/kg) in paw homogenate
Control	0.0±0.0	15.2±1.2
Adjuvant	258.4±11.3*	48.7±2.4*
Methotrexate (5 mg/kg)	210.4±3.2#	24.8±2.1#
Adjuvant+FSE (200 mg/kg)	145.2±3.2	23.6±4.2
Adjuvant+FSE (400 mg/kg)	84.2±4.2[a]	21.2±2.1[a]
Adjuvant+L-Arginine (100 mg/kg)	145.2±5.2[b]	52.6±6.4[b]
Adjuvant+L-NAME (10 mg/kg)	45.3±2.6[c]	21.5±4.2[c]
Adjuvant+FSE (400 mg/kg)+L-Arginine (100 mg/kg)	84.3±3.2	50.8±6.1
Adjuvant+FSE (400 mg/kg)+ L-NAME (10 mg/kg)	30.2±1.9[b,c,d]	20.2±1.4[b,c,d]

*P<0.05 compared to control group, #P<0.05 compared to adjuvant group, [a]P<0.02 compared to adjuvant group, [b]P<0.05 compared to FSE (400 mg/kg), [c]P<0.02 compared to L- arginine (100 mg/kg), [d]P<0.001 compared to FSE (400 mg/kg) + L- arginine (100 mg/kg). FSE: Fenugreek seed extract, L-NAME: N^{ω}-Nitro-L-arginine methyl ester hydrochloride, TNF-α: Tumour necrosis factor alpha

Table 3: Effect of Fenugreek on CFA induced changes in oxidative stress markers in paw homogenate (data are in Mean ± SEM) (n = 6/group).

Treatment	SOD (U/gm Hb)	MDA (nM/L)
Vehicle	961.7 ± 17.8	9.9 ± 0.1
Adjuvant	293.1 ± 7.8*	37.1 ± 0.7*
Adjuvant + Methotrexate (5 mg/kg)	511.5 ± 9.2[a]	25.4 ± 0.3[a]
Adjuvant + FSE (200 mg/kg)	538.6 ± 7.9[a]	22.3 ± 0.4[a]
Adjuvant + FSE (400 mg/kg)	715.3 ± 11.8[a]	15.2 ± 0.5[a]
Adjuvant + L-Arginine (100 mg/kg)	339.1 ± 4.9[b]	30.1 ± 0.6[b]
Adjuvant + L-NAME (10 mg/kg)	632.9 ± 17.1[b]	20.9 ± 0.2[b]
Adjuvant + FSE (400 mg/kg)+L-Arginine (100 mg/kg)	437.8 ± 8.1[b]	27.3 ± 1.2[b]
Adjuvant + FSE (400 mg/kg)+L-NAME (10 mg/kg)	836.5 ± 11.1[b,c]	10.3 ± 1.9[b,c]

*P < 0.001 compared to control, [a]P < 0.05 compared to adjuvant; [b]P < 0.05 compared to Methotrexate (5 mg/kg), [c]P < 0.05 compared to Fenugreek Seed Extract (FSE) (400 mg/kg). SOD: Superoxide dismutase, MDA: Malonylaldehyde, FSE: Fenugreek seed extract, CFA: Complete Freund's adjuvant, L-NAME: N^{ω}-Nitro-L-arginine methyl ester hydrochloride

state. Administration of standard drug, methotrexate (5 mg/kg) significantly attenuated arthritis index when compared to only adjuvant treated group (P < 0.05). Significant attenuation in arthritis index was observed when FSE (400 mg/kg) administrated alone or with L-NAME (10 mg/kg) (P < 0.05) which may be due to enhanced production of NO by adjuvant in the paw and which is significantly attenuated by FSE treatment. Inflammatory effect of NO as evidenced by the treatment with L-arginine (100 mg/kg) and anti-inflammatory effects is shown by treatment by fenugreek. L-NAME, a non-specific iNOS inhibitor shown RA protective effects by suppressing NOS activity, as a result NO production is decreased which suppresses ROS/RNS signalling pathways. Various inflammatory mediators such as cytokines, prostaglandins and cyclooxygenases enzymes expression are attenuated by fenugreek treatment.[33]

Significant increase in ankle diameter after CFA inoculation was observed (P < 0.05), this effect may be due to infiltration of various inflammatory cells and secretion of inflammatory cytokines mediates joint destruction at the sites of ankle joints. Treatment with methotrexate (5 mg/kg) shown attenuating effects in ankle diameter, compared to adjuvant and normal saline treated control group (P < 0.05). Ethanolic fenugreek seeds extract (200–400 mg/kg) dose dependently, attenuated ankle joint destruction. Fenugreek with L-NAME (10 mg/kg) significantly attenuated adjuvant induced increased ankle diameter (P < 0.001).

Paw volume of adjuvant inoculated rats was significantly increased. % Changes in paw oedema of rt. paw was found more as in comparison to non-inoculated paws. Methotrexate (5 mg/kg) significantly decreases paw volume (P < 0.05). FSE (200–400 mg/kg) alone and/or with NO modulators significantly reversed these adjuvant-induced % change in paw volume (P < 0.001). Administration of FSE (400 mg/kg) with L-NAME show strong attenuation in % change in paw volume (P < 0.01), which suggests that NO play a significant role in arthritis pathogenesis and when inhibited shows protective effect in RA. Various inflammatory cells infiltrated to the site of adjuvant induced inflammation, which secretes various inflammatory mediators such as NO, ROS/RNS, cytokines and prostaglandins, which were found attenuated by FSE (200–400 mg/kg) treatment and when iNOS was inhibited by L-NAME (10 mg/kg) it shows synergistic effects in RA protection.

Anti-inflammatory cytokine, IL-10 level was significantly decreased in adjuvant-induced rats in comparison with controls (P < 0.05). FSE significantly reversed adjuvant induced decreased in serum IL-10 levels. Which suggests that protective effects of FSE were mediated through suppression of the anti-inflammatory cytokines secretion.

Table 4: Effect of Fenugreek on CFA induced changes in body weight (in gram) (data are in Mean±SEM) (n=6/group).

Day	Control	Adjuvant	Adjuvant+ Methotrexate	Adjuvant+FSE (200 mg/kg, p.o.)	Adjuvant+FSE (400 mg/kg, p.o.)	Adjuvant+L-arginine (100 mg/kg, i.p.)	Adjuvant+L-NAME (10 mg/kg, i.p.)	FSE (400 mg/kg, p.o.)+L-arginine (100 mg/kg)	FSE (400 mg/kg, p.o.)+L-NAME (10 mg/kg, i.p.)
0	202.0±7.4	205.9±14.1	208.6±7.0	220.1±15.1	227.9±5.6	213.1±11.2	225.8±5.3	215.1±4.9	211±4.1
7	213.4±6.1	189.3±11.7	195.3±6.9	216.7±11.8	206.1±5.8	215.8±7.3	217.1±6.3	220.5±6.3	214.3±6.1
14	224.1±8.3	173.1±7.3*,a	187.1±5.9	205.9±7.9	197.6±8.5[a]	200.6±9.3	205.8±9.5	210.3±8.1	200.4±8.1[a,b,d]
21	236.2±5.3	143.9±11.9*,a	194.1±8.0	214.8±9.6	206.9±11[a]	194.8±6.3	209.3±4.9	223.1±5.6[c]	209.3±5.1*,a,b,d
28	242.0±11.3	128.1±7.3*,a	211.7±5.9	223.8±12[d]	221.7±8.3[d]	186.1±8.7[b,c]	213.5±7.3	217.7±6.3[c]	215.7±8.1*,a,b,d

*P<0.05 Compared adjuvant and control day (0): [a]P<0.01 Compared to adjuvant day (0), [b]P<0.05 Compared to methotrexate (10 mg/kg) day (0), [c]P<0.05 Compared to FSE (400 mg/kg+L-NAME 10 mg/kg) day (0), [d]P<0.02 Compared to Adjuvant on day 21 and 28; [e]P<0.05 Compared to Adjuvant+L-arginine 100 mg/kg. FSE: Fenugreek seed extract, CFA: Complete Freund's adjuvant, L-NAME: N^{ω}-Nitro-L-arginine methyl ester hydrochloride

CFA inoculated rat shows significant increased levels of TNF-α in knee joint synovial fluid of rats. The increased level of TNF-α in the group receiving L-arginine was found as compared to only FSE treated group. It may be because, L-arginine further increases the NO synthase activity and also NO metabolites (NOx) as well as increased ROS production.[33] Significant rise in pro-inflammatory cytokines was observed in serum and synovial fluid of animals treated with L-arginine. Although when methotrexate (5 mg/kg) was given to arthritic rats, it decreases levels of TNF-α as methotrexate stimulates adenosine release from cells that produces anti-inflammatory effects. Treatment with the ethanolic FSE (200–400 mg/kg) with iNOS inhibitor, L-NAME, shown both in NO modulators shown inhibitory effects on TNF-α level both in serum and synovial fluid. Methotrexate also significantly decreases serum TNF-α level due to its immunosuppressive action and mild anti-inflammatory activities. These results of methotrexate were found comparable with only FSE treated animals. However, this methotrexate induced suppression in TNF-α level, less marked in comparison to FSE (400 mg/kg) with L-NAME (10 mg/kg) ($P < 0.001$).

The present study demonstrated that the ethanolic seed extract of fenugreek significantly attenuated development and progression of RA pathogenesis. This is evident by the results of this study. Fenugreek treatment is shown significant protective effects on paw oedema infiltration of inflammatory cell, pro-inflammatory, anti-inflammatory cytokines and oxidative stress imbalance in arthritic rats.[34] The present investigation suggests that fenugreek ethanolic seed extract at the dose of (400 mg/kg), significantly attenuated adjuvant induced inflammatory/anti-inflammatory/oxidants imbalance and also results are reflected on arthritic parameters. Fenugreek shows anti-arthritic, anti-inflammatory activities which may be due to inflammatory/anti-inflammatory cytokines and oxidative stress modulation by its antioxidant and immunomodulatory properties. Current findings suggest that due to cytokine modulating and antioxidant activities of fenugreek may be the possible reason behind the observed anti-arthritic and immunomodulatory protective effects. Fenugreek in combination with NO modulators specifically, iNOS inhibitor shows betters anti-arthritic effects. In future, it may be developed as therapeutic natural drug for the treatment of RA. The results of the present study may be utilised to develop a new drug based on the natural source and in combination with the modern drugs for the better and targeted drug therapy for non-curable RA, which may be through anti-oxidant-immunomodulation mediated through NO-signalling pathways. Further, studies are needed to understand molecular signalling pathways involved and also for newer therapeutic drug target for RA.

CONCLUSION

The protective mechanism of ethanolic extract of Fenugreek seeds in CFA induced changes are mediated through modulation of cytokines and NO signalling pathways. In future, FSE may be used to target NO-signalling pathway involved in RA pathogenesis. Treatment of FSE with NO inhibitors may be developed into a new drug and may have better therapeutics to treat RA. Results of the present study suggests that protective role of FSE is mediated through pro-inflammatory/anti-inflammatory cytokine and antioxidant/antioxidant imbalance. Since, NO is free radical molecule and was found suppressed by FSE treatment as evidenced by the result of the present study by using NO-donor and iNOS inhibitor. Thus, the result of the study suggests possible involvement of NO-signalling pathways in modulation of pathogenesis of RA by pro-inflammatory/anti-inflammatory cytokine and antioxidant/oxidant imbalance which is prevented by fenugreek treatment with iNOS inhibitor.

Acknowledgment

The authors thank Mr. Prafulla Chandra Tiwari (SRF-ICMR) and Ms. ShipraKartik (SRF-ICMR) for their technical assistance.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Kamal P, Pal R, Nath R, Sachan AK. Protective effects of the ethanolic fenugreek seeds extract and its potentiation with nitric oxide modulators in adjuvant induced changes in arthritic index, pro-inflammatory/anti-inflammatory cytokines imbalance and oxidative stress markers in rats. Indian J Physiol Pharmacol 2022;66:45-54.