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Research Article

Trigonella foenum graecum mitigates proteases and restores antioxidant balance in collagen induced arthritis in rats

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ABSTRACT
Autoimmunity driven immune cell infiltration and associated proteolytic enzyme stimulation with concomitant oxidative stress is responsible for the tissue degradation in human rheumatoid arthritis. In the present study, we investigated the anti-inflammatory and antioxidant potential of hydro alcoholic extract of *Trigonella foenumgraecum* in collagen induced arthritis in Wistar rats. Rats were treated with *Trigonella foenumgraecum* at a dose of 120 mg kg^{-1} b.wt orally for 21 days and effects were assessed by biochemical (elastase, LPO, GSH, SOD, catalase and NO) and histological evaluation in joints.The disease was evident after 13 ± 1 days post induction with increase in paw volume, digital inflammation which progressed to swelling of multiple joints. A single injection of CIA led significant elevation of neutrophil elastase ($p<0.01$). Peroxidation of lipids increased with parallel decrease in reduced glutathione content ($p<0.001$). Enzymatic antioxidants viz. superoxide dismutase and catalase showed a similar decrease in the CIA immunized rats. Moreover, nitrite content was seen to be significantly increased ($p<0.001$) in the arthritic rats. *Trigonella foenumgraecum* extract was able to decrease neutrophil elastase ($p<0.001$) and decrease free radical overload both of oxygen and nitrogen free radicals to a significant extent. All these biochemical changes were reflected in the histology which revealed the decrease in infiltration of inflammatory cells after treatment with *Trigonella foenumgraecum* against collagen induced arthritis. Our investigation supports the antioxidant and anti-inflammatory properties of *Trigonella foenumgraecum* in collagen induced arthritis model and at the same time highlight neutrophil elastase and oxidative stress to be the targets of therapeutic intervention.

Keywords: *Trigonella Foenumgraecum*, articular elastase, nitric oxide, inflammation, Oxidative stress.

INTRODUCTION
Rheumatoid arthritis (RA) is an autoimmune disease in which major cause of morbidity is destruction of bone in the joints [1]. The disease is characterized by synovial hyperplasia, with excessive inflammatory cell infiltration in the joints, leading to erosion of the articular cartilage and bone, and subsequent joint destruction [2]. The autoimmunity generated against the collagen type II attracts several pro-inflammatory immune cells that secrete several reactive intermediates which lead to the degeneration of the joints with a limited or no capacity of self repair [3]. These immune cells especially macrophages have potential of secreting several reactive oxygen species that can lead to the degeneration of the cellular structures and shifting of the redox balance towards the more oxidised state [4, 5].Reactive oxygen species (ROS) and other reactive nitrogen species (RNS) are highly reactive transient chemical species with the potential to initiate cellular damage in cartilage directly and damage components of the extracellular matrix either directly or indirectly by up regulating mediators of matrix degradation [6]. These reactive molecules are formed during normal aerobic metabolism in cells. During infection or inflammation phagocytes activate a cascade of the uncontrolled production of free radicals which damage biomolecules that leads to altered function and disease [7]. It has been reported that ROS destroy antioxidant systems and that RA patients are thus exposed to oxidative stress and lipid peroxidation because of the reduced endogenous antioxidant defence system [8] and may lead to the clinical manifestations.Apart from the oxidative environment generated, the polymorphonuclear cells secrete several proteases that degrade the native structure of collagen leading to the joint degradation [2].Therefore this bimodal role played by the infiltrating immune cells is one of putative mechanisms of the human RA. Many attempts have been made in the past to check this disease using several molecules which include cyclooxygenase inhibitors, anti-inflammatory drugs, corticosteroids and still other prostaglandin synthesis inhibitors. While many of them are still in the market and have been found to be useful for RA patients but at the cost of number of side-effects like gastrointestinal disorders, bleeding and hypertension and weakened immune system [9]. Patients with rheumatoid arthritis rely on other option like use of complementary and alternative medicine (CAM) and according to reports CAM therapy is on rise as 60-90% dissatisfied patients are likely to seek option of CAM therapy.*Trigonella foenum-graecum* L. Leguminosae (Fenugreek) is one of the oldest medicinal plants, originating in India and Northern Africa. Fenugreek have been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antioxidant activity [10,11]. The seeds are reported to have restorative and nutritive properties and to stimulate digestive processes [12]. Seeds are used as a traditional remedy for the treatment of diabetes [13, 14]. Supplementation of fenugreek seed powder in the diet leads to a reduction in biomarkers of oxidative damage in alloxan-diabetic rats [15]. Further fenugreek seed polyphenols prevented hemolysis and lipid peroxidation induced by H_2O_2 *in vitro* in human erythrocytes [16].Therefore because of its wide

pharmacological properties, it was thought worthwhile to evaluate its antioxidant and antiarthritic activity in collagen induced arthritis in Wistar rats.

Materials and methods

Reagents

Freund's adjuvant complete (CFA), *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide and Griess Reagent system were purchased from Sigma Chemical Co. (St Louis, MO, USA). Collagen type II from bovine nasal septum was purchased from Elastin Products Co, INC, Owensville, Missouri, USA. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5-5-dithio-bis-2-nitrobenzoicacid (DTNB), nitrobluetetrazolium (NBT), ethylene diamine tetra-acetic acid (EDTA), xanthine, xanthine oxidase, tris hydrochloride were purchased from SD Fine chemicals India. All other routine chemicals used were of research grade.

Animals

Male Wistar rats (150-170 g) were used and experimental procedures done were as per the animal ethical committee recommendations of Hamdard University. They were kept in the Central Animal House in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity 45-55 % with 12 h light / dark cycles after initial acclimatization for about 1 week. Animals had free access to standard rodent pellet diet and water ad libitum. The experimental study was conducted in accordance with the Institutional Animal Ethics Committee of the University.

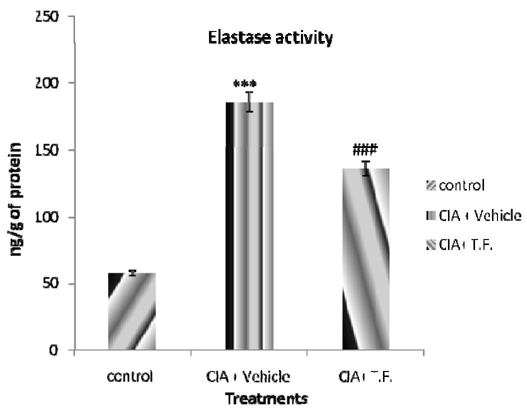


Fig.1 Articular elastase activity in joints of rats immunized with collagen type II after treatment with T.F. (120mg/kg b.wt.). Data are expressed as Mean ± SEM of 6 rats.
*** ($p<0.001$) vs. Control, ### ($p<0.001$) vs. CIA+ Vehicle

Induction of collagen-induced arthritis (CIA) and experimental protocol

Arthritis was induced in rats as described previously [17]. Collagen Type II from bovine nasal septum was dissolved in 0.05 M acetic acid at a concentration of 2 mg/ml emulsified with an equal volume of Freund's complete adjuvant (CFA) containing 1 mg/ml *Mycobacterium tuberculosis* H37 RA and stored on ice before use. Rats were immunized intradermally at about 1.5 cm distal from the base of the tail. The first group served as control, the second was collagen induced arthritis (CIA), the third was administered T. foenumgraecum extract (120 mg kg^{-1} body weight) daily, for 21 days following immunization. The dose of T. foenumgraecum extract was selected after calculation based on prescribed doses of the drug in Indian system of medicine and literature.

Preparation of aqueous methanol extract of T. foenumgraecum

Seeds were procured from local market and authenticated by Dr. H.B Singh, Scientist 'F', Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi with voucher specimen number (ref-NISCAIR/RHMD/Consult/2008-2009/1098/129).The dried seeds were extracted with H_2O: MeOH (20:80) at room temperature. The extract was dried under reduced pressure to a residue.

Measurement of clinical severity of arthritis

Evaluation of joint inflammation was performed by a blinded independent observer with no knowledge of the treatment protocol. The severity of the arthritis was quantified daily by a clinical score measurement [18] from 0 to 4.

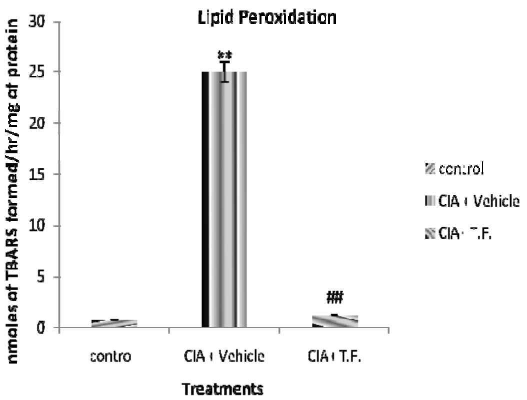


Fig.2 Lipid peroxidation in joints of rats immunized with collagen type II after treatment with T.F. (120mg/kg b.wt.). Data are expressed as Mean ± SEM of 6 rats.
** ($p<0.01$) vs. Control, ## ($p<0.01$) vs.CIA+ Vehicle

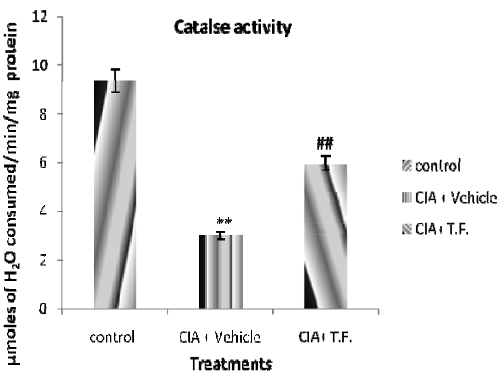


Fig. 3 Catalase activity in joints of rats immunized with collagen type II after treatment with T.F. (120mg/kg b.wt.). Data are expressed as Mean ± SEM of 6 rats.
** ($p<0.01$) vs. Control, ## ($p<0.01$) vs. CIA+ Vehicle

Articular Elastase (ELA)

Elastase levels in the articular joints were evaluated as an index of polymorphonuclear leukocyte (PMNs) accumulation and activation in the inflamed tissue as described earlier [19]. Briefly, tissue samples were homogenized in a solution containing 20 mM potassium phosphate buffer pH 7.0 in a ratio of 1:10 (w/v) and centrifuged for 20 min at 10,000 x g at 4° C. An aliquot of each sample was incubated for 24 h at 37° C with 0.1M Tris – Hcl buffer, (pH 8.0), containing 0.5M NaCl and 1mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, a high specific synthetic substrate for neutrophil elastase (ELA). The amount of *p*-nitroanilide liberated was measured spectrophotometrically at 405 nm and was considered as neutrophil ELA activity. The ELA activity was converted and expressed as ng/g protein using molar extinction coefficient (9500) of substrate as per manufacturer recommendation.

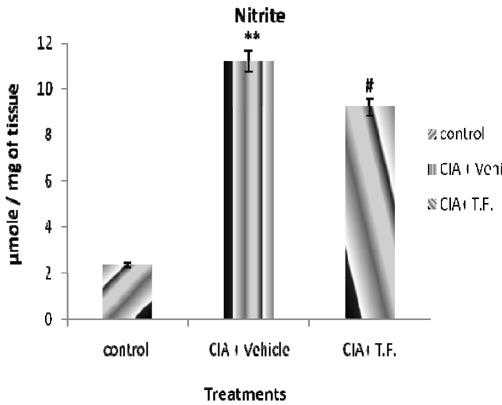
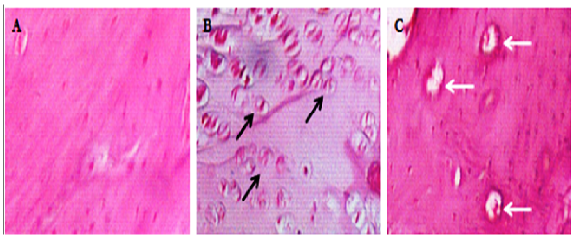


Fig. 4 Effect of T.F. (120mg/kg b.wt.) Treatment on articular nitrite content in joints of rats immunized with collagen type II after treatment with. Data are expressed as Mean ± SEM of 6 rats.
** ($p<0.01$) vs. Control, # ($p<0.05$) vs.CIA+ Vehicle



Histopathological features	Control	CIA + Vehicle	CIA + T.F.
Inflammatory cells	-	+++	+
Necrosis	-	++++	++

(-) Indicates normal, (+) indicates mild, (++) indicates moderate, (+++) indicates severe, (++++) indicates extremely sever

Fig. 5 Histological findings. Massive and diffuse polymorphonuclear cellular flux (black arrows) in the cartilage of collagen induced arthritic rats (B) in comparison to the normal rats (A). Reduction of cellular flux and cartilage erosion in the rats fed with daily aqueous methanol extract of *T. Foenumgraecum* (C). Original magnification 20X.

Preparation of cell - free extract of the knee joints

At the end of experiment animal were sacrificed by cervical dislocation. Arthritic and nonarthritic joints were removed and cut into small pieces and homogenized in 5 vol of 50 mM Tris HCl buffer, pH 7.4 containing 0.1 M NaCl and 0.1% Triton X-100 and 1 vol. of fine glass powder by using a mortar and pestle. The crude extract then was sonicated for 20 sec. The homogenate was centrifuged at 3,000 × *g* for 5 min, and the resulting supernatant was stored at - 20°C until further analysis.

Table 1 Effect of T.F on incidence and severity of arthritis: Rats were immunized intradermally in the tail with CII emulsified with CFA. Arthritis index was calculated by adding the total clinical severity score of each joint in each group of rat and dividing by the total number of arthritic rat in that group.

Group	No. Immunized/ no arthritic	Arthritic index	Arthritic paw in each group	Mean day of onset (range)
CIA+ Vehicle	6/5 (83.3%)	2.42 ± 0.20	7	13 (12-14)
CIA+ T.F.	6/4 (66.6%)	1.85 ± 0.26	5	13 (12-14)

Estimation of thiobarbituric acid reactive substances (TBARS)

The assay of TBARS was done according to the method mentioned earlier [20], adapted to microtiter plates by bringing the final volume to 150 µL. In brief, tissue homogenate was prepared in 0.15 M KCl (5%w/v homogenate) and aliquots of 30 µL were incubated for 0^{o}C and 37^{o}C for 1h. Subsequently, 60 µL of 28% w/v TCA was added and the volume was made up to 150 µL by adding 60 µL of distilled water followed by centrifugation at 3000 x g for 10 min. The supernatant (125 µL) was taken and colour was developed by addition of 25 µL of 1% w/v TBA dissolved in 0.05 N NaOH and kept in boiling water bath for 15 min. The absorbance was read at 532 nm in a plate reader (Bio-Rad, U.S.A). The result was expressed in µmoles TBARS formed /h /g tissue using a molar extinction coefficient of 1.56×10^{5} M^{-1} cm^{-1}.

Reduced glutathione (GSH)

GSH was measured in the groups following the method described earlier [21]. Homogenized joint tissue (10% w/v in phosphate buffer pH 7.4) was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4^{o}C for 2 hrs. The contents were centrifuged at 2000xg for 15 min.50 µL supernatant was added to 200 µL of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA (pH 8.9) followed by the addition 20 µL of 0.01M DTNB. The absorbance was read in a microplate reader at 412 nm and results were expressed as µg GSH/g tissue using a molar extinction coefficient of 13.6×10^{3} M^{-1} cm^{-1}.

Total superoxide dismutases (SOD) activity

Total SOD were measured in joints as described earlier [22] adapted to microtiter plates by bringing the final volume to 100 µL. Reaction mixture consisted of 0.05M phosphate buffer (pH 7.4), 1mM xanthine and 57µM NBT. After incubation at room temperature for 15 min, reaction was initiated by addition of 50 mU xanthine oxidase. The

SOD activity is expressed in Units /mg protein using a molar extinction coefficient of 4.02×10^3 M^{-1} cm^{-1}.

Catalase activity

Catalase activity in the joint tissues was assayed according to method described earlier [23] using H_2O_2 as substrate. The reaction mixture was adjusted to multiwell flat bottom plates by reducing the final volume to 200 µL. Reaction mixture consisted of phosphate buffer (0.01M, pH 7.0), distilled water and 10% homogenate (prepared in 0.1M phosphate buffer). Reaction was started by adding H_2O_2 (0.2M), incubated at 37^oC for 1 min. and stopped by addition of dichromate: acetic acid reagent (1:3). The tubes were kept in a boiling water bath for 15min. and centrifuged for 10min at 1500 x g. The color developed was read at 570 nm in a microplate reader. The enzyme activity was expressed as µmol H_2O_2 consumed/min/mg protein using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Measurement of Nitric oxide (NO): Griess Reaction

After the experiment, animals were sacrificed and the joint tissues were washed with PBS (pH 7.4) and placed on ice as the method described earlier [24]. Briefly a 50µL sample was added with 100µL of Griess reagent and reaction mixture was incubate for about 5-10 minutes at room temperature and protected from light. The optical density was measured at 540 nm in microplate reader according to the manufacturer's protocol. Calculations were done after generating a standard curve from sodium nitrite in the same buffer as used for preparation of homogenate.

Histological examinations

Rats were sacrificed on the day 21 by cervical dislocation. Knee joints were removed and fixed for 4 days in 4% formaldehyde. After decalcification in 5% formic acid, the samples were processed for paraffin embedding [25]. Tissue sections (5 µm thick) were stained with haematoxylin–eosin for light microscope examination.

Protein content

Protein was determined by Bradford method [26] using bovine serum albumin (BSA) as a standard.

Statistical Analysis

Results are expressed as mean ±SEM. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's test for all parameters. Any variation with $P< 0.05$ was considered statistically significant.

Results and Discussion

Rheumatoid arthritis has been demonstrated to harbour many mediators of inflammation viz. macrophages, neutrophils and dendritic cells to the joints apart from different proinflammatory cytokines [27]. The attraction of these cells leads to the generation of reactive oxygen species (ROS) within the joint. The role of reactive oxygen species in rheumatoid arthritis has been highlighted earlier in humans [28].These reactive species not only deplete the antioxidant defense but are also responsible for the degradation of the vital biomolecules [7]. In case of nitrite, which is a reactive oxide of nitrogen, peroxynitrite is formed in presence of superoxides [29] which also degrade protein. The harbored cells not only release ROS but many other proteases like elastase which has been implicated in degrading the native structure of collagen [30]. This inflammation mediated break in the antioxidant defense and associated collagen damage is one of the pathways of the joint destruction in the RA. Our study showed that clinical assessment of animals had marked difference in incidence and severity in the pattern of disease. In two independent experiments, both groups of animals were immunized with collagen type II emulsified with CFA for the induction of arthritis and observed for two weeks for the development of clinical arthritis. As summarized in Table 1, incidence of arthritis was 66.6 % with severity on arthritic index being 1.85 ± 0.26 in drug treated group as compared to 83.3% incidence and arthritic index of 2.42 ± 0.20 observed in CIA treated group.

To further validate the antiarthritic activity of *T. Foenumgraecum* extract, we evaluated neutrophils elastase activity which accounts for ability of synovial tissues to degrade cartilage matrix. It is directly proportional to the activation of polymorphonuclear leukocytes (PMNs) in the inflamed tissue and is an important tissue damage marker [8]. Very low ELA concentrations were measured in the joints of control rats (53.49 ± 1.34 ng/g protein) on the day 21st in the studied groups. However, elevated levels of this enzyme were seen in CIA + vehicle group (169.87 ± 0.44 ng/g protein). In contrast the administration of *T. Foenumgraecum* showed a significant reduction ($p<0.01$) in neutrophil activation and infiltration (Fig.1). The inflammation so caused by the infiltrating cells leads to the release of reactive oxygen and nitrogen species [31, 32]. Lipid peroxidation is considered a critical mechanism of the injury that occurs during RA. Low levels of TBARS (0.67 ± 0.08 µmoles TBARS formed /h /g tissue) were seen in the control group at the end of the experiment and these values were considered normal. However, a significant increases (24.97 ± 0.20 µmoles TBARS formed /h /g tissue) in TBARS production was found in the joints of CIA + vehicle group. Treatment with *T. Foenumgraecum* decreased TBARS concentrations by inhibiting lipid peroxidation in the cartilage tissue in a significant ($p < 0.01$) manner (Fig. 2). We suggest that the decrease in elastase activity observed in our study might be due to the inhibition of lipid peroxidation levels and the consequent decrease in the reduction of chemotactic peroxide [33]. Lipid peroxidation increased significantly with decrease in the non enzymatic antioxidant defense viz. reduced glutathione [34, 35]. Table 2 shows the changes in GSH content evaluated in the joints of the experimental groups. A marked decrease (0.625 ± 0.002 µg GSH /g tissue) in GSH concentrations was found in the joint of CIA + vehicle group. Treatment with *T. Foenumgraecum* significantly ($p < 0.001$) inhibited the decrement in (0.729± 0.005 µg GSH /g tissue) GSH levels. *T. Foenumgraecum* extract reduced the lipid peroxidation significantly and replenished the reduced glutathione. The reduction in lipid peroxidation may be due to free radical scavenging property of saponins and flavonoids present in *T. Foenumgraecum* extract [36]. The break in the non enzymatic defense was also reflected in the enzymatic defense where catalase activity decreased with parallel decrease in superoxide dismutase activity (defense against superoxide ions). Table 2 summarizes the articular content of SOD in the experimental groups. In control animals, SOD activity was 17.58 ± 0.059 Units / mg of protein. On the other hand, a significant decrease in this antioxidant was seen in CIA group given vehicle alone (4.44 ± 0.075). Administration of the *T. Foenumgraecum* significantly ($p < 0.001$) replenished SOD level. Fig.3 shows cartilage catalase activity evaluated at the end of experiment. In the control group catalase activity was 9.35 ± 0.013μmol H_2O_2 consumed/min/mg protein. However, a substantial reduction in this enzyme level was observed in the cartilage of CIA group (3.01 ± 0.007 μmol H_2O_2 consumed / min / mg protein). Treatment with *T. Foenumgraecum* was significantly ($p < 0.01$) effective as compared to CIA group. This break in the antioxidant defense was replenished by the treatment with *T. Foenumgraecum* extract where SOD and catalase activity increased indicating the ability of drug to ameliorate these degenerative changes. Nitric oxide is an important signaling molecule. It can react with molecular oxygen and superoxide anion to produce reactive species that can modify various cellular functions [37, 38]. These indirect effects of NO have significant role in inflammation [39]. Since peroxide level increases in the inflamed tissues along with nitrite content, protein degradation in the joint tissue is unavoidable. Result of nitrite estimation is summarized in Fig. 4. In the control group, the nitrite concentration was 3.4 ± 0.014 μmol/mg wet tissues whereas the CIA group showed high nitrite content (11.20 ± 0.028 μmol/mg wet tissue). Treatment with *T. Foenumgraecum* lowered nitrite level significantly ($p < 0.05$) as compared to the CIA group. In our study, nitrite increased in the vehicle treated group, but *T. Foenumgraecum* extract treatment decreased the concentration of nitrite in articular tissue. The biochemical alterations found in our study were further supported by histopathological observations of the joint tissues. The CIA + vehicle treated group showed higher number of infiltrating cells, extensive bone degradation, and synovial hyperplasia which are hallmarks of RA (Fig. 5 and Table 3). Bone degradation was characterised by absence of the trabecular structure in the bone whereas synovial hyperplasia was noticed as the proliferation of synoviocytes to the cartilages and bone. Treatment with *T. foenumgraecum* was able to reverse the histological changes to almost normal condition.

Conclusion

Present study highlights the role of inflammatory cells in conjunction with reactive oxygen and nitrogen species in the development of the pathology of RA. Moreover this study also provides biological basis for the use of *T. Foenumgraecum* as an alternative but safer therapy for inflammatory disorders like rheumatoid arthritis.

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