

Comparison Of Oxidative Stress Markers Due To Inoculation Of Ethanolic Fenugreek Seed Extract (Fse) And Nitric Oxide (No) Modulators On Adjuvant Induced Rheumatoid Arthritis In Rats

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1. INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease and is posing a great problem to the health of many people all over the world. It is an inflammatory disease that brings about joint destruction and functional disability. RA patients have an increased risk of disability leading to various degenerative disorders and finally death [1].

RA occurs two to four times more frequently in women than in men [2] and occurs in all races and ethnic groups. Although the disease often begins in middle age and occurs with increased frequency in older people, children and young adults also develop it [3]. When it occurs in children it is called JRA [4]. RA is the most common inflammatory joint disease, with prevalence between 0.5% and 1% worldwide [5].

The etiology of RA is not fully understood [6]. It is clear that both genetic and environmental factors play important roles [7]. Now, it has been established that the free radicals, involving reactive oxygen species (ROS) and reactive nitrogen species (RNS), play an important role in RA inflammations [8]. Recently the formation, behaviour and scavenging properties of oxygen and nitrogen free radicals in the biological system had received much attention [9]. Fortunately, the ROS production in healthy individuals is low and lipid peroxidation is inhibited by the combined activities of various antioxidants present in the plasma.

Numerous agent derived from plants have the potential for the treatment of rheumatoid arthritis like *Boswellia Serrata*, *Curcuma longa*, *Zingiber Officinale*, *Withania Somnifera* and *Trigonella foenum graecum*. *Trigonella foenum graecum* also known as "methi" in Hindi or fenugreek in English belong to family *Fabaceae* is an annual plant, with leaves consisting of three small obovate to oblong leaflets. It is cultivated worldwide as a semiarid crop. Its seeds and its leaves are common ingredients in dishes from South Asia.

Various activities are found in seed extract, seed oils, leaves and shows hypoglycaemic [10,11], Antioxidant [12-14], Lactation aid [15-16], and immunomodulatory effect [17]. This pharmacological activity of fenugreek shows potential for the cure of much disease but mechanism of action is not well elucidated. Therefore there is need to understand the molecular mechanism of action of fenugreek. An major function in inflammatory illnesses is played by the free radical molecule nitric oxide (NO). NO is a common signalling molecule that can freely flow across cell membranes and, unlike other classical neurotransmitters, cannot be controlled by the processes of storage, release, and reuptake [18]. NO has many physiological roles; in maintenance of blood pressure in the cardiovascular system [19-21], immune system [19-21], Platelet aggregation [19-21], gastro protection [22], inflammation [23] stimulating host defences in the immune system [24], regulating neural transmission in the brain [25] and male sexual dysfunction [26,27].

Few studies have previously been conducted to assess the effectiveness of *Trigonella foenum graecum* and mangiferin in the management of rheumatoid arthritis, but none have shown its probable mechanism of RA protection [28-30]. NO is a free radical and inflammatory mediators play significant role in various disease but their role in RA is not well understood. The current article compares oxidative stress markers caused by inoculation of ethanolic Fenugreek Seed Extract (FSE) and Nitric Oxide (NO) modulators on adjuvant induced Rheumatoid Arthritis in rats which is a part of previous research that assessed the protective role of ethanolic Fenugreek Seed Extract and its potentiation by NO modulators is mediated through pro-inflammatory/anti-inflammatory cytokine imbalance and oxidative markers [31].

1.1 Materials and Methods:

A. Experimental Animals

For this study, 54 male Wistar rats (weighing between 180 and 225 g; six per group) were utilized. Rats were procured from the Lucknow-based, CSIR-certified breeding facility of the Indian Institute of Toxicological Research (IITR). According to Committee for the purpose of control and supervision of experiments on animals [CPCSEA] regulations,

the animals were taken care of. Institutional Animal Ethics Committee (IAEC) of the KGMU gave its blessing to the study protocol with the approval number 91/IAEC/2018.

B. Methodology/Experimental Design

Prior to the experiment, the animals were acclimatized to their surroundings for 12 days, during which they were fed a normal pellet diet and given unlimited water.

The rats were grouped into nine groups of 6 rats each as follows

Group I	Control (normal saline)
Group II	Arthritis Group (CFA)
Group III	Arthritis Group + Methotrexate (5mg/kg)
Group IV	Arthritis Group + Fenugreek Seed Extract (200mg/kg).
Group V	Arthritis Group + Fenugreek Seed Extract (400mg/kg).
Group VI	Arthritis Group + L- arginine (100mg/kg).
Group VII	Arthritis Group + N^{ω} -Nitro-L-arginine methyl ester hydrochloride (L-NAME)(10mg/kg).
Group VIII	Arthritis Group + Fenugreek Seed Extract (best dose) + L- arginine (100mg/kg).
Group IX	Arthritis Group + Fenugreek Seed Extract (best dose) + L- NAME (10mg/kg).

CFA= Complete Freund's adjuvant

C. Induction of Arthritis and Administration of Drugs

According to Li et al., 2010 and Pal et al., 2016, rats were used to conduct an experimental arthritis study. Rats were inoculated with 0.2 ml of Complete Freund's adjuvant (CFA) FA in the sub-plantar region on day "0." (32,33). Before inoculating CFA into a new spot in the sub-planter surface of the right hind paw on day "0," 0.1 ml of squalene was intradermally injected to change the sensitivity of CFA .

Arthritis was induced in the rats of group II to IX by injecting 0.1 ml of Complete Freund's Adjuvant (CFA) intradermally in the footpad of their left hind paw.

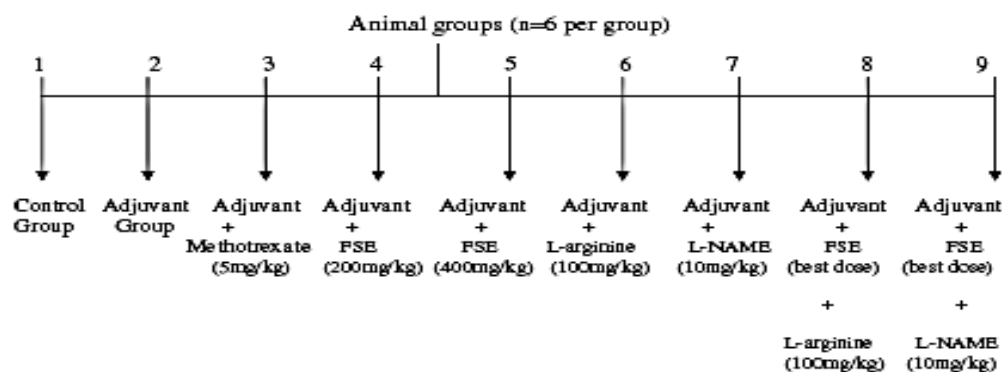
DAY 0-On day zero control group was given normal saline while rest group (group I to group IX) was injected with Complete Freund's Adjuvant 0.1 ml on the left hind paw.

DAY 1-14-As it takes 7-14 days for Complete Freund's Adjuvant to induce arthritis in rats, days 1-14 were spent in observation for the appearance of swelling in the limbs as a marker of induction of arthritis.

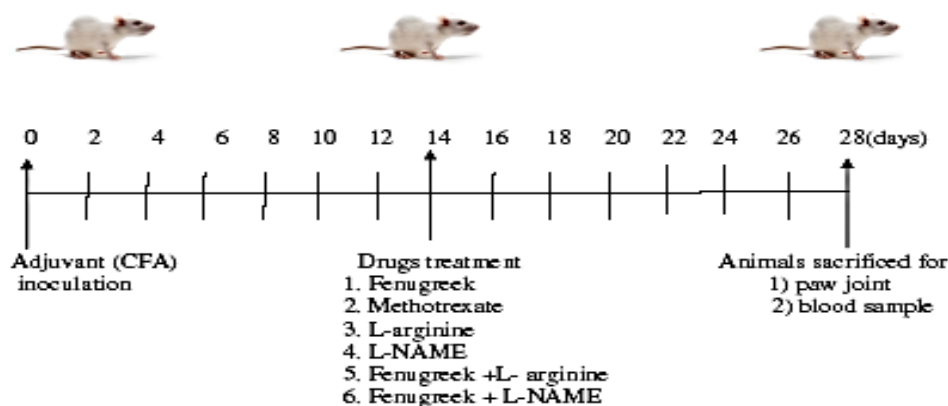
By day 14, almost all the rats developed arthritis which was suggested by the appearance in their limbs. Grading of arthritis was done in all the rats according to the arthritic index. After this, the pharmacological parameters i.e. paw thickness, ankle diameter, paw volume and body weight were again measured.

DAY 14-28-Drug administration was carried out from days 14-28 in groups III-IX. All parameters (arthritic index, body weight, paw volume, ankle diameter) were measured on alternate days of the study from day "0" to day "28". Then, 100 mg/kg of pentobarbitone was administered intravenously to anaesthetize every animal before being slaughtered. In order to estimate the levels of cytokines and oxidative stress indicators, their blood, paws, and synovial fluid were collected.

Flow chart of research methodology



Dose Schedule and Treatment



D. Ethanolic Fenugreek Seed Extract (FSE) Preparation

From the neighborhood market a botanist from the **CSIR-National Botanical Research Institute (NBRI) Lucknow** authenticated 2.5 kilograms of fenugreek seeds. The standard protocol for preparing ethanolic fenugreek seed extract (FSE) was followed. To sum up, 2.5 kg of powered fenugreek seed material was steeped in 80% alcohol for an entire night before being filtered using sodium sulphate-coated Whatman filter paper No. 41 to get rid of any sediments and traces of water in the filtrate. Prior to filtering, pure alcohol was used to moisten the filter paper and sodium sulphate. Following that, a rotatory evaporator was used to concentrate the filtrate [34]. In the experiments, fenugreek seed extract that had been obtained was used.

1.2 Drugs and Chemicals

A. Chemicals

Sigma Aldrich Co., USA provided the Complete Freund's adjuvant (CFA). TCI Chemical Co. Japan provided the hexane, ethanol, trichloroacetic acid (TCA), Griess' reagent, Tris-HCl buffer, Triton-X, Thiobarbituric acid, hydrogen peroxide, pyrogallol, nitrate reductase, and other widely used compounds.

B. Drugs

Sigma Chemical Co., USA provided the standard drug (methotrexate), the Nitric oxide donor (L-arginine), and the iNOS inhibitor (N-Nitro-L-arginine monomethyl lester hydrochloride, L-NAME). In the departmental chemical laboratory, ethanolic fenugreek seed extract (FSE) was created.

1.3 Oxidative Stress Indicators

A. Malondialdehyde (MDA) Level in Paw Homogenate

Rat paw is cut and minced to small pieces and then homogenised in a cold room by using 0.01 M Tris-HCl buffer, pH 7.4. High speed Teflon homogeniser was used to obtain 10 % homogenate. This tissue homogenates of rat paw samples will be analyzed for the lipid peroxidation by the method of Ohkawa [35], and expressed as nmol/mg protein. Chromogen optical density was measured at 532 nm using double beam spectrophotometer. Protein estimation was done by using Folin-phenol reagent [36].

B. Superoxide Dismutase (SOD) Activity in Paw Homogenate

SOD activity was measured in U/gm of protein in rat paw tissue homogenates using the Marklund and Marklund method. Superoxide dismutase inhibits pyrogallol autoxidation in this method. It will be studied in the presence of

EDTA at pH 7.9- 10.6. A spectrophotometer was used to measure the activity at 420 nm. Lowry's method will be used to estimate protein concentrations [36, 37].

C. Catalase Activity in Paw Homogenate

Catalase activity is assayed adapted to micro plate reader with slight modification [38]. The reaction mixture consisted of 195 ml joint buffer (0.1 M, pH 7.4), 100 ml hydrogen peroxide (0.019 M), and 5 ml 10% PMS in a final volume of 300 ml. Changes in absorbance were recorded kinetically by micro plate reader (Bio-Rad, U.S.A.) at 240 nm followed by 30 s shaking. Catalase activity was calculated as micromoles H₂O₂ consumed per minute per milligram of protein by using a molar extinction coefficient of 39.6 M⁻¹cm⁻¹.

D. NOX (NO⁻ /NO³) Level

The sacrificed animals' joint tissue is collected and washed with PBS (pH 7.4). Tissue homogenate (10% w/v) prepared in 0.1 M Joint buffer, pH 7.4 with 50 mM Tris HCl buffer, pH 7.4 with 0.1 M NaCl and 0.1% Triton X-100 [39]. In brief, a 50 ml homogenate sample was mixed with 100 ml of Griess reagent, and the reaction mixture was incubated at room temperature for 5-10 minutes while being kept away from light. The optical density was determined at 540 nm using a micro plate reader (Bio-Rad, USA) and the manufacturer's protocol. After generating a standard curve from sodium nitrite in the same buffer used for homogenate preparation, calculations were performed.

E. Statistical Analysis

The two-way analysis of variance (ANOVA) was used to assess the data, which were presented as Mean SEM. Newman Keul's posthoc test for multiple groups was then performed. In all parameters, p 0.05 was regarded as statistically significant.

2. RESULTS

A. Effect of Fenugreek Seed Extract and NO Modulators on Lipid Peroxidation in Paw Homogenates.

Rat paw tissue homogenates were tested for malonyldehyde (MDA) levels. Due to severe paw inflammation, there was a considerable rise in MDA in the paw tissue homogenate after CFA inoculation (37.1 ± 0.7) as compared to the adjuvant group (p 0.001). Methotrexate (5 mg/kg) considerably lowers the MDA levels in paw tissue homogenate (p 0.05) [28-29].

Treatment with fenugreek seed extract (200, 400 mg/kg) alone or in conjunction with the NO modulators L-arginine (100 mg/kg) and L-NAME (10 mg/kg) (p 0.05, p 0.001) dramatically lowers MDA levels. Table 5 offers an overview of these outcomes [28, 29, 31].

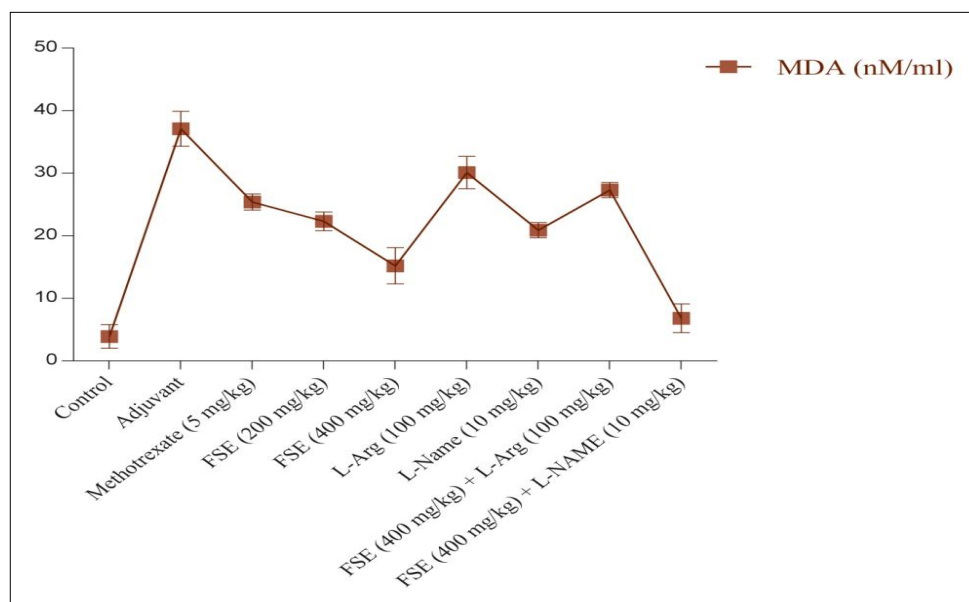


Figure: Effect of fenugreek seed extract on lipid peroxidation level

B. Effect of Fenugreek Seed Extract (FSE) and Nitric Oxide (NO) Modulators on Superoxide Dismutase (SOD) Estimation in Tissue Homogenates of Rat Paws Samples

SOD activity was measured in rat paw tissue homogenates as well. SOD activity in paw tissue homogenates was slightly lower in CFA-inoculated rats (293.1 ± 7.8 U/gmHb) than in control rats (p 0.001). Methotrexate (5 mg/kg) treatment also protects against CFA-induced oxidative stress (511 ± 9.2 U/gmHb) due to an increase in SOD activity (p 0.05) [28,29, 30]. Treatment with fenugreek seed extract alone 200mg/kg has increased SOD activity 538.6 ± 7.9 U/gmHb and FSE 400mg/kg has reduced SOD activity to 715.3 ± 11.8 U/gmHb as compared to adjuvant group (p< 0.05). While the group receiving L-arginine showed the SOD activity of 339.1 ± 4.9 U/gmHb and group receiving L-NAME has shown the

activity 632.9 ± 17.1 U/gmHb as compared to methotrexate group ($p < 0.05$). Group receiving the combination of FSE 400 mg/kg with L-arginine (100 mg/kg) has shown SOD activity to 437 ± 8.1 U/gmHb as compared to methotrexate group ($p < 0.05$). Increase in SOD was less marked in rats treated with combination of fenugreek seed extract alone (400 mg/kg) and L-arginine (100 mg/kg) as compared to other groups ($p < 0.05$). These results are summarized in table 5 [28,29,31].

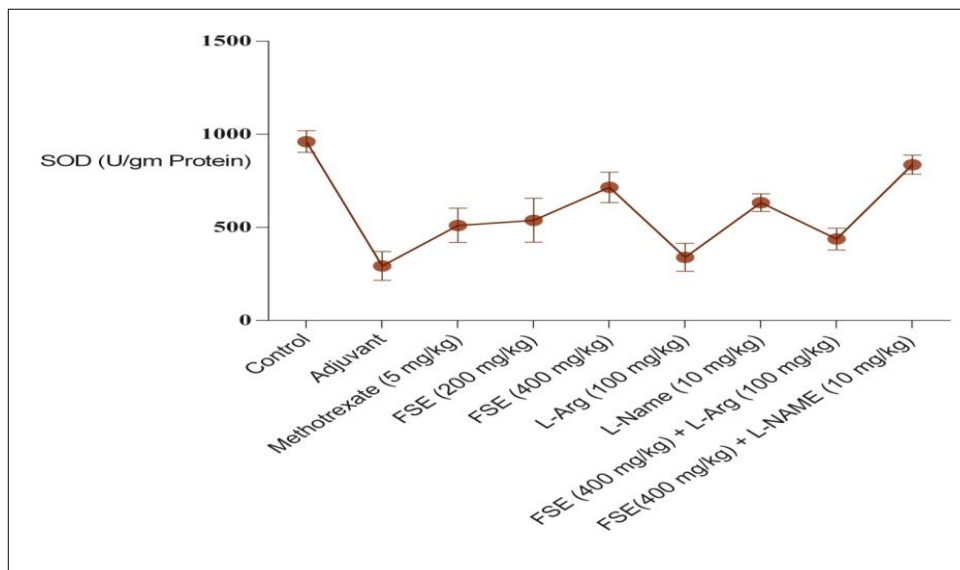


Figure: Effect of Fenugreek Seed Extract (FSE) on superoxide dismutase (SOD)

Table: 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)	CAT (U/gm Hb)	MDA (nM/L)
Vehicle	961.7 ± 17.8	37.9 ± 2.5	9.9 ± 0.1
Adjuvant	293.1 ± 7.8*	7.8 ± 1.9*	37.1 ± 0.7*
Adjuvant + Methotrexate (5 mg/kg)	511 ± 9.2 ^a	13.2 ± 0.9 ^a	25.4 ± 0.3 ^a
Adjuvant + Fenugreek (200 mg/kg)	538.6 ± 7.9 ^a	16.8 ± 1.3 ^a	22.3 ± 0.4 ^a
Adjuvant + Fenugreek (400 mg/kg)	715.3 ± 11.8 ^a	29.1 ± 2.7 ^a	15.2 ± 0.5 ^a
Adjuvant + L-Arginine (100 mg/kg)	339.1 ± 4.9 ^b	10.3 ± 0.13 ^b	30.1 ± 0.6 ^b
Adjuvant + L-NAME (10 mg/kg)	632.9 ± 17.1 ^b	24.5 ± 156 ^b	20.9 ± 0.2 ^b
Adjuvant + Fenugreek (400 mg/kg) + L-Arginine (100 mg/kg)	437 ± 8.1 ^b	17.3 ± 1.6 ^b	27.3 ± 1.2 ^b
Adjuvant + Fenugreek (400 mg/kg) + L- NAME (10 mg/kg)	836.5 ± 11.1 ^{b,c}	34.9 ± 2.1 ^{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)

C. Effect of fenugreek seed extract and Nitric Oxide (NO) modulators on Catalase (CAT) activity

The expression of catalase (CAT) was as follows: RA control group 37.9 ± 2.5 U/gmHb. In adjuvant induced group catalase activity was reduced to 7.8 ± 1.9 U/gmHb ($p < 0.001$) as compared to control group. Treatment with methotrexate activity of catalase was 13.2 ± 0.9 U/gmHb. While treatment with fenugreek 200mg/kg it was increased to 16.8 ± 1.3 U/gmHb and with fenugreek 400mg/kg alone it was increased to 29.1 ± 2.7 U/gmHb ($p < 0.05$) as compared to adjuvant group. Catalase activity was reduced with adjuvant combine to L- arginine to 10.3 ± 0.13 U/gmHb ($p < 0.05$) as compared to group receiving standard drug methotrexate. Maximum increase in catalase activity was seen in fenugreek 400mg/kg combined with L-NAME to 34.9 ± 2.10 U/gmHb ($p < 0.05$) as compared to group receiving methotrexate and group receiving. These results are summarized in table 5 [28,29,31].

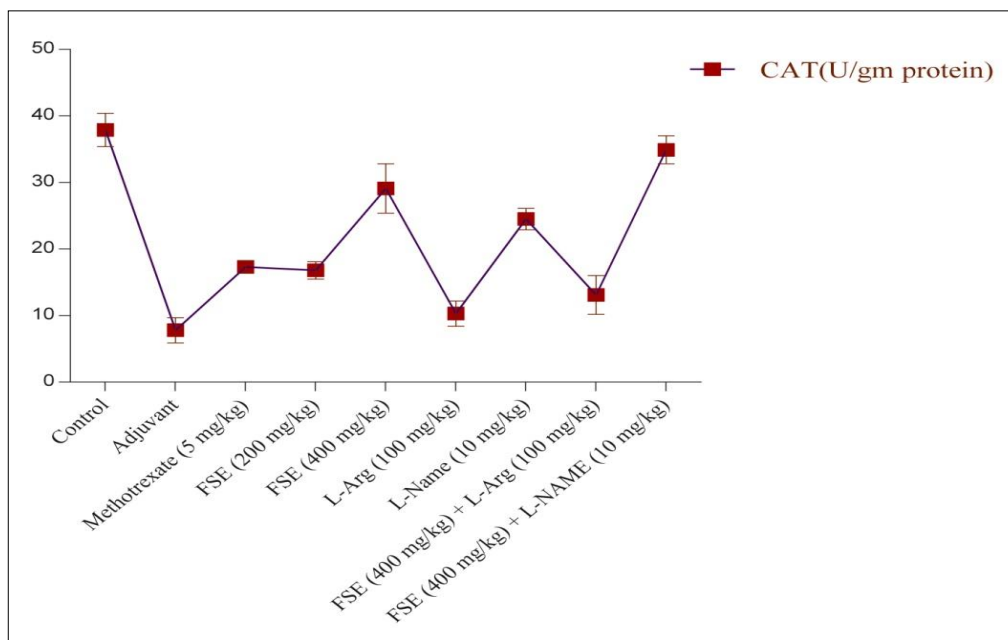


Figure: Effect of fenugreek seed extract (FSE) on catalase activity

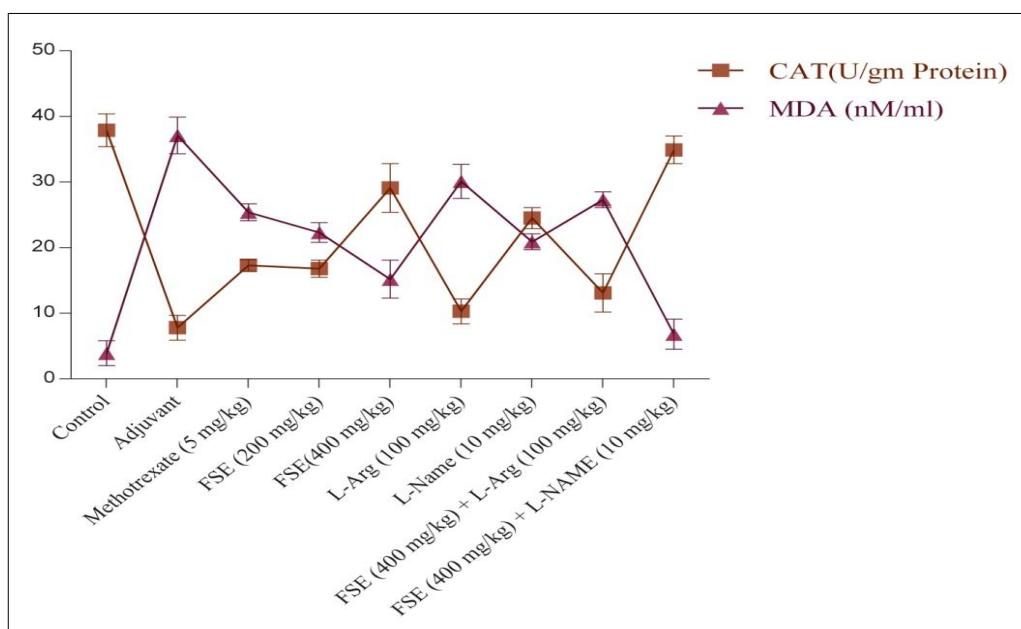


Figure: Comparison of effect of Fenugreek Seed Extract (FSE) on catalase and lipid peroxidation

D. Effect of Fenugreek Seed Extract (FSE) and Nitric Oxide (NO) Modulators on Oxidative Stress Markers NO_x

CFA inoculation significantly enhances nitrite concentration $25.8 \pm 1.9 \mu\text{g}/\text{mg}$ protein as compared to control only group ($p < 0.001$). Methotrexate (5 mg/kg) restores NO₂⁻ levels towards normalcy $97.3 \pm 4.1 \mu\text{g}/\text{mg}$ protein ($p < 0.05$). Treatment with fenugreek seed extract (FSE) 200 mg/kg alone has NO₂⁻ level of $90 \pm 3.3 \mu\text{g}/\text{mg}$ protein, while the FSE 400mg/kg group has NO₂⁻ level of $68.3 \pm 2.5 \mu\text{g}/\text{mg}$ protein ($p < 0.05$) as compared to adjuvant group. The group receiving the L-arginine has significantly increased NO₂⁻ level to $183.9 \pm 6.1 \mu\text{g}/\text{mg}$ protein as compared to methotrexate group ($p < 0.001$), while group receiving L-NAME has reduced NO₂⁻ level to $77 \pm 2.3 \mu\text{g}/\text{mg}$ protein ($p < 0.05$) as compared to methotrexate group. Combination of FSE 400 mg/kg with NO modulators L-arginine (100 mg/kg) group has NO₂⁻ level of $114.1 \pm 5.9 \mu\text{g}/\text{mg}$ protein and the combination of FSE 400mg/kg with L-NAME (10 mg/kg) group has NO₂⁻ level of $29.1 \pm 2.9 \mu\text{g}/\text{mg}$ protein significantly decreases NO₂⁻ concentration ($p < 0.001$). These results are summarized in table 6 [28,29,31].

Table: NOx level in paw homogenate in adjuvant-induced arthritic rats on day 28 (data are in Mean \pm SEM) (n = 6/group; all treatments administered i.p)

Group	NOx ($\mu\text{g}/\text{mg}$ protein)
Control	25.8 \pm 1.9
Adjuvant	162.8 \pm 3.7
Methotrexate (5 mg/kg)	97.3 \pm 4.1
FSE (200 mg/kg)	90 \pm 3.3
FSE (400mg/kg)	68.3 \pm 2.5
L-Arginine (100 mg/kg)	183.9 \pm 6.1
L-Name (10 mg/kg)	77 \pm 2.3
FSE (400 mg/kg) + L-Arg (100 mg/kg)	114.1 \pm 5.9
FSE (400 mg/kg) + L-NAME (10 mg/kg)	29.1 \pm 2.9

3. DISCUSSION

Present article compares malonydehde ,superoxide dismutase and catalases after inoculation oft of ethanolic extract of *Trigoenella foenum graecum* (fenugreek) seed and its interaction with NO modulators, which was compared with the standard drug methotrexate in experimental arthritis in rats .The focus was to see the antioxidant effects of the inoculate measuring the NOx level in paw homogenates. Adjuvant-induced arthritis is a well-established model in rats, has been extensively used in the study of inflammatory process [40] and validated as a model to investigate the pathogenesis of RA and autoimmune diseases and to identify potential therapeutic targets [41]. Complete Freund's adjuvant (CFA) is a reagent frequently used to induce RA in animal models [42].

In terms of clinical and serological alterations, as well as the contribution of inflammatory mediators to the arthritic aetiology, adjuvant-induced arthritis is strikingly similar to human RA [43]. Recently developed anti-arthritic medications have been examined and evaluated using this paradigm. The synthesis of bacterial peptidoglycan and muramyl dipeptide in this condition caused persistent edoema [44]. An indicator of several medications' anti-arthritic activity is the measurement of paw edoema. The release of several mediators, including the cytokines GM-CSF, interferons, and PGDF, is what causes this chronic inflammation [45].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in tissue destruction and inflammation [46]. Experimental and clinical data show that rheumatoid inflammation is associated with increased production of oxidative free radicals and NOx[47].A measure of lipid peroxidation during RA is tissue malonaldehyde analysis [48]. In RA, free radicals cause articular cartilage damage, resulting in malonaldehyde accumulation and decreased activity of catalase (CAT) and superoxide dismutase (SOD). In the current study, the arthritic control had higher LPO (higher TBARS) and lower SOD and CAT levels.Treatment with both doses of ethanolic extract of *Trigoenella foenum graecum* seed (200 and 400 mg/kg) resulted in significant reductions in LPO levels and increases in CAT and SOD levels, indicating that the active components in the plant extract likely act by competing for the scavenging of free radicals, resulting in antioxidant enzyme levels being recovered.

According to studies on how it impacts antioxidant parameters in arthritis-prone rats, *Trigoenella foenum graecum*'s ethanol extract can significantly reduce the development and progression of CFA-induced arthritis. The outcomes demonstrate that 400 mg/kg body weight of *T. foenum graecum* has significant antioxidant activity. Flavonoids, alkaloids, and triterpenoids, which are potential antioxidant moieties, may be responsible for this activity. These findings imply that the anti-arthritic characteristics of *trigoenella foenum graecum* may be the cause of the impact that has been seen.

Through NO signalling pathways, *trigoenella foenum graecum* extract's protective effect in CFA-induced alterations to oxidative stress markers was mediated. In the future, FSE might be clinically applied to block the RA NO signalling pathway. An inflammatory condition like RA may respond more favourably to treatment of FSE with NO inhibitors.

4. CONCLUSION

Complete Freund's Adjuvant (CFA) was used to induce arthritis in rats and changes in inflammatory marker, arthritic index and oxidative stress markers induced arthritis in the rats as compared to control group.Higher doses of fenugreek seed extract (400 mg/kg) was more effective than its lower dose against changes in controlling oxidative stress marker (SOD, CAT, MDA and NOx).Combination of complete freund's adjuvant (CFA) with L-arginine had shown more decrease in SOD, CAT activity and more increase in the lipid peroxidation and NOx as compared to adjuvant only group. In the light of above evidences, the combination of higher dose of ethanolic extract of *Trigoenella foenum graecum* seed and L-NAME might have a potential usefulness as an adjunct to conventional therapy in further management of rheumatoid arthritis. However details of complete mechanism have not been explored. Therefore, further experiments are required to elucidate the exact mechanism of action. Also more specific and longer duration animal and human studies are required to further substantiate the findings of the present study.

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