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ANTI-ARTHRITIC EFFECT OF *TRIGONELLA FOENUM GRAECUM* L. LEAVES IN ACUTE AND CHRONIC MODELS OF ARTHRITIS IN ALBINO RATS

V.G. GAJERA*, DR. A. YADAV, DR. C. SHAH, DR. D.P. SHAH

Department of Pharmacy, OPJS University, Churu, Rajasthan- India

"*Address For correspondence"

Vipulkumar G. Gajera ,Assistant Professor ,E-mail: gajera15184@gmail.com

Aim of the study: *Present study investigates the effects of aqueous and chloroform fraction of fenugreek on joint inflammation using different models of acute and chronic inflammation.*

Materials and methods: *Collagen-induced arthritic rat model, a model of chronic joint inflammation, was used to evaluate the anti-arthritic effects of plant extracts. Paw volume, body weight, arthritic index, blood parameter and histopathological evaluation (H & E staining) were performed to measure the severity of arthritis. Acute inflammatory models like, carrageenan induced paw edema models were used to evaluate anti-inflammatory effects of fenugreek.*

Results: *Both fractions significantly inhibited paw edema and decrease RF, CRP, liver parameters, cytokines and increase body weight, A/G ratio. Treatment with fenugreek fractions resulted in almost normalization of altered structure of joint fenugreek significantly attenuated carrageenan-induced paw edema. They also possess anti-oxidant properties.*

Conclusions: *This study showed that plant possessed anti-arthritic and anti-inflammatory properties which might be due to alkaloids and flavonoids.*

Key Words: *Trigonella Foenum Graecum L., Arthritis, Collagen, Carrageenan, Chloroform fraction, Aqueous fraction*

INTRODUCTION: Rheumatoid arthritis (RA) is a common chronic and systemic autoimmune disorder characterized by inflammation of the synovial joints, destruction of cartilage and bone; it involves a complicated pathogenesis, with pathological changes in multiple targets [1, 2]. RA affects about 1% of the world population, in a female/male ratio of 3/1. The disease can occur at any age, its incidence increasing with age [3]. The pro-inflammatory cytokines (e.g., tumor necrosis factor alpha, interleukin-6 and interleukin-1beta) and other mediators (prostaglandins and leukotrienes) play critical roles in the development and perpetuation of tissue inflammation and damage in joint tissue such as articular cartilage and meniscus [4, 5]. The collagen induced arthritis (CIA) model in the rat is in many aspects similar to RA, which is perhaps the most commonly used model for RA today. Intradermal injection in rats with collagen emulsified in IFA leads to a severe, erosive poly-arthritis developing within 2–3 weeks after immunization followed by a subsequent chronic relapsing phase [6]. The primary drugs used in the treatment of RA are nonsteroidal anti-inflammatory (NSAIDS) and disease-modifying antirheumatic drugs (DMARDs). In most cases, these drugs have been proved to be of only limited value. They often suppress the symptoms, but accelerate factors that promote the disease. However, patients frequently become unable to continue long-term treatment with these agents due to toxicity and/or loss of benefit. Anti-TNF, anti-IL-1, and anti-IL-6 therapies have been also reported to be effective in the treatment of RA [7, 8]. Despite increased use of these combination therapies; new treatments for active RA are clearly needed.

Trigonella foenum graecum Linn.commonly known as Fenugreek belongs to the family Fabaceae, which is an annual, herbaceous and aromatic plant. It is one of the oldest medicinal plants, originating in India and Northern Africa. The leaves and seeds, which mature in long pods, are used to prepare extracts or powders for medicinal use. In India, fenugreek seeds are commonly consumed as a condiment. Fresh and dried fenugreek leaves and tender stems are edible which are widely used as a vegetable. Fenugreek is reported to have anti-diabetic, anti-fertility, anticancer, anti-microbial, anti-parasitic, lactation stimulant and hypocholesterolemic effects [9]. Therefore, the present study was designed to explore anti-arthritic effect of fenugreek fractions on carrageenan and collagen induced arthritis.

MATERIALS AND METHODS:

Collection of plant material: The whole plant of *Trigonella foenum graecum* L. (fenugreek) were purchased from local market in Bardoli, (GUJ) and then leaves were carefully separated from whole plant. Authentication of plant was carried out by Dr. B. R. Patel, Associate Professor of Botany, The Patidar Gin Science College, Bardoli, Dist. Surat, Gujarat.

Extraction and fractionation:

Fenugreek dried leaves were coarsely powdered (1500g), extracted with ethanol (95%) after standing for 48h at room temperature, the hydroalcoholic extract was drained off. This process of extraction at ambient temperature was repeated four times. The combined hydroalcoholic extracts were filtered through filter paper and evaporated to dryness under reduced pressure. The extracts were then freeze-dried which were further used for screening purposes.

Fractionation

The crude hydroalcoholic extract (300g) was suspended in distilled water (500ml) and sequentially partitioned with petroleum ether (2 × 500), chloroform (2 × 500), ethyl acetate (2 × 500) and aqueous fraction [10].

Preliminary phytochemical screening: The Preliminary phytochemical screening of the methanolic extract (ME) and fractions of fenugreek were carried out according to previously described method [11].

Animals: Adult healthy Albino rats of Wistar strain of either sex weighing 200–250 g with no prior drug treatment were used in the present study. Animals were maintained at 22±2 ° C with 12 h light and dark cycle. The animals were fed on standard pellet diet and had free access to diet and water throughout the experiment. Animal study was performed in Animal Facility (898/PO/Re/5/05/CPCSEA), Shree N.L. Patel College of pharmacy, Umrakh, Surat, Gujarat with due permission from Institutional Animal Ethics Committee (CPCSEA/SNLPCP/IAEC/17/01/94A).

Acute oral toxicity study: Acute oral toxicity study was performed as per OECD 423 guidelines. Albino mice (n = 6) of either sex selected by random sampling technique were used for acute toxicity study. The animals were kept fasting overnight providing only water, after which the methanolic extract was administered orally at the dose level of 5 mg/kg body weight by gastric intubation and observed for 14 days. If mortality was observed in two out of three

animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose would be repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2000 mg/kg body weight. According to the results of the acute toxicity test, the doses were chosen for experiments [12].

Carrageenan-induced arthritis

Rats were divided in six groups of 6 animals each. Group A: saline control; Group B: 10 mg/kg indomethacin; Group C: chloroform – 100 mg/kg; Group D: chloroform – 200 mg/kg; Group E: Aqueous fraction – 100 mg/kg; Group F: Aqueous fraction – 200 mg/kg. One hour after the oral administration of drugs, acute paw oedema was induced by injecting 0.1 ml of 1% carrageenan in 0.9% saline. Paw volume was measured with the help of plethysmometer from 0 to 3 hours. The percentage inhibition of paw oedema in treated groups was then calculated by using the formula:

$$\text{Percentage inhibition} = (1 - Vt/Vc) \times 100$$

Where Vt = is the oedema volume in the drug treated

Vc = is the oedema volume in the control group

the whole right hind paw and liver tissues were taken at the third hour. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline four times their volume and finally homogenized at 4º C. Then, the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained for the TNF-α, NO and malondialdehyde (MDA) assays.

On the other hand, the whole liver tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline of the same volume and finally homogenized at 4º C. Then, the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained for the anti-oxidant enzyme Like Superoxide dismutase - SOD, Glutathione peroxidase - GPx activity assay [13].

3.1.1.1 Tissue TNF-α by ELISA

Tissue levels of TNF-α were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction. The

measurements were performed according to the manufacturer's protocols. The absorbance at 450 and 540nm was measured on a microplate reader. TNF-α was determined from a standard curve for the combination of these cytokines [14].

3.1.1.2 NO Assay

NO was measured according to the method of Moshage *et al.* (1995). For nitrite determination, NO3− was converted into nitrite after enzymatic conversion by nitrate reductase; NO2− was measured by using the Griess reaction. Values obtained by this procedure represented the sum of nitrite and nitrate [15,16].

3.1.1.3 MDA Assay

MDA was evaluated by the thiobarbituric acid-reacting substance (TRARS) method (Nakhai et. al., 2007) first, the paw tissues were homogenized in buffered saline (1:4); and then, 400 ml of 1, 1, 3, 3-tetraethoxypropan trichloroacetic acid (28% w/v) was added to 200 ml of this mixture and centrifuged in 3000g for 30 min. After that, 300 ml of the supernatant was added to 150 ml of 2-thiobarbituric acid (1% w/v). The mixture was incubated for 45 min in a boiling water bath, and then 450 ml n-butanol was added; the solution was centrifuged and cooled, and absorption of the supernatant was recorded at 532nm. Tetramethoxypropane was used as standard. MDA levels were expressed as nanomoles per milligram of protein. Protein concentration was measured by Lowry method (Lowry et. al., 1951). Bovine serum albumin was used as standard [17,18].

3.1.1.4 Anti-Oxidant Enzymes' Activities

SOD enzyme activity was determined at room temperature. One hundred microliters of tissue extract were added to 880 ml (pH 10.2, 0.1mM EDTA) of carbonate buffer. Twenty microliters of 30mM epinephrine (in 0.05% acetic acid) was added to the mixture at 480nm for 4 min on a Shimadzu model 1800 Spectrophotometer. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.

GPx enzyme activity was determined at 37° C. The reaction mixture composed of 500 ml phosphate buffer, 100 ml 0.01M GSH (reduced form), 100 ml 1.5mM NADPH and 100 ml GRx (0.24 units). One hundred microliters of the tissue extract were added to the reaction mixture and incubated at 37ºC for 10 min. Then, 50 ml of 12mMt-butyl hydroperoxide was added to 450 ml of tissue reaction mixture and measured at 340nm for 180 s. The molar

extinction coefficient of 6.22 X 10^{-3} was used to determine the enzyme activity. One unit of activity is equal to the millimolar of NADPH oxidized per minute per milligram of protein.

Collagen induced arthritis

Rats were divided into seven groups (n = 6). Group A: saline control; Group B: disease control Group C: 10 mg/kg Prednisolone; Group D: chloroform – 100 mg/kg; Group E: chloroform – 200 mg/kg; Group F: Aqueous – 100 mg/kg; Group G: Aqueous – 200 mg/kg.

Arthritis was induced using Chicken Sternal Collagen type–II with Incomplete Freund's Adjuvant. Collagen was dissolved in ice- cold 0.1 M acetic acid at a concentration of 2 mg/mL, kept over-nightly and stored at 4ºC. On day 1, collagen in acetic acid was emulsified with equal volumes of incomplete Freund's adjuvant to produce the inducing agent and stored on ice before use. Rats were immunized intradermally with 0.5 mL of the emulsion (0.1 mL each of the emulsion was injected to five sites; root of tail and regions above each limb). On day 7, after the primary immunization all animals were given booster injection with 0.1 mL of chicken collagen emulsified with Incomplete Freund's adjuvant in the same manner. The vehicles and the drug/extract were administered orally from day 20 to day 40 after the primary immunization with emulsion. The synovial tissues were taken on day 41 from each rat for biochemical examination. Samples of ankle joints were collected on day 41 from the rats after euthanasia by cervical dislocation for histological examination [19].

Body weight: Body weight was recorded on day 0 just before FCA injections and thereafter on day 7, 14, 21 day 28. Percentage body weight change was calculated [20].

Paw volume: The left hind paw volumes of all animals were measured just before CFA injection on day 0 and thereafter at 7,14,21,28 day using a plethysmometer (UGO Basile,). The percentage inhibition of paw oedema in treated groups was then calculated by using the formula [21]:

$$\text{Percentage inhibition} = (1 - Vt/Vc) \times 100$$

Where Vt = is the oedema volume in the drug treated

Vc = is the oedema volume in the control group

Arthritic score: The morphological feature of the arthritis like redness, swelling and erythema was monitored by set visual criteria as follows: normal paw = 0, mild swelling and erythema of digits = 1, swelling and erythema of the digits = 2, severe swelling and erythema = 3, gross deformity and inability to use the limb = 4 on respective days. Thus, the maximum possible score for both hind paws was 8 [21].

Haematological and serum parameters: On day 28, haematological parameters like red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb), and platelets (PLT) were determined by usual standardized laboratory method[11]. Erythrocyte sedimentation rate (ESR) was determined using the Westergren's method. Serum rheumatoid factor (RF) and C-reactive protein was determined by turbidimetric method.

Biochemical parameters: On day 28, blood of the rats was withdrawn by retro-orbital puncture and serum was used for the estimation of serum AST, ALT, ALP and total protein levels [22].

Determination of serum IL-6 and TNF-α levels: Serum was separated from blood samples by centrifugation (3000 rpm for 10 min) and stored at -20ºC. IL-6 and TNF-α were determined by ELISA kits according to the manufacturer's protocol [23].

Anti-oxidant parameters: The levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and lipid peroxidation (LPO) in serum were determined [24].

Measurement of spleen and thymus weight: At day28 after immunization, the animals were sacrificed and the thymus and spleen were removed and weighed [25].

Histopathological study of joints: The animals were sacrificed on day 28 by cervical dislocation. Ankle joints were separated from the hind paw, weighed and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 5µ thickness. The sections were stained with haematoxylin and eosin and evaluated under light microscope for the presence of hyperplasia of synovium, pannus formation and destruction of joint space.

Statistical analysis: All the results were expressed as mean ± S.E.M. Statistical comparisons were made between drug-treated groups and arthritic control groups. The data of disease activity index was statistically analyzed by two-way ANOVA followed by Bonferroni test. The data of biochemical estimation was analyzed by one-way ANOVA followed by Dunnett's multiple range tests using Graph Pad Prism 8.0 software. The values of $P < 0.01$ were considered statistically significant.

RESULTS:

Preliminary phytochemical screening: Result of preliminary phytochemical analysis conducted on methanolic extract and chloroform fraction of *Trigonella foenum graecum* L. showed presence of flavonoids, steroids, alkaloids and triterpens.

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING

Test	Chloroform	Aqueous
Carbohydrade	-	+
Alkaloids	+	
Saponins	+	++
Steroids	+	-
Terpenoids	+	+
Tanins	-	+
Flavonoids	+	+
Protein/ Amino acid	-	+
Glycosides	-	+

Acute toxicity studies showed that the alcoholic extracts did not cause any mortality up to 2000 mg/kg and were considered as safe.

Effect of methanolic extract and various fractions of *trigonella foenum graecum* L. on inhibition of right hind paw volume against carrageenan induced acute inflammation in rats

Treatment	Dose mg/kg	Paw volume (ml)				
		0 hr	1/2 hr	1 hr	2 hr	3 hr
Control	-	0.24 ± 0.02	0.4 ± 0.02	0.53 ± 0.01	0.62 ± 0.01	0.72 ±0.01

Indomethacin	10	0.23 ± 0.01	0.28 ± 0.01	0.31 ± 0.02*	0.24 ± 0.02*	0.17 ± 0.01*
Chloroform fraction	100	0.21 ± 0.03	0.31 ± 0.02	0.32 ± 0.01*	0.27 ± 0.02*	0.23 ± 0.01*
Chloroform fraction	200	0.23 ± 0.01	0.34 ± 0.01	0.36 ± 0.02*	0.26 ± 0.03*	0.21 ± 0.02*
Aqueous fraction	100	0.22 ± 0.02	0.31 ± 0.03	0.34 ± 0.03*	0.25 ± 0.02*	0.22 ± 0.03*
Aqueous fraction	200	0.23 ± 0.02	0.31 ± 0.02	0.33 ± 0.02*	0.26 ± 0.01*	0.20 ± 0.02*

Effect of methanolic extract and various fractions of *trigonella foenum graecum* L. on % inhibition of right hind paw volume against carrageenan induced acute inflammation in rats

Treatment	**Dose mg/kg**	**% Inhibition**			
		1/2 hr	**1 hr**	**2 hr**	**3 hr**
Control	-	0.0	0.0	0.0	0.0
Indomethacin	10	30.00	41.51	61.29	76.39
Chloroform fraction	100	22.50	39.62	56.45	68.06
Chloroform fraction	200	15.00	32.08	58.06	70.83
Aqueous fraction	100	22.50	35.85	59.68	69.44
Aqueous fraction	200	22.50	37.74	58.06	72.22

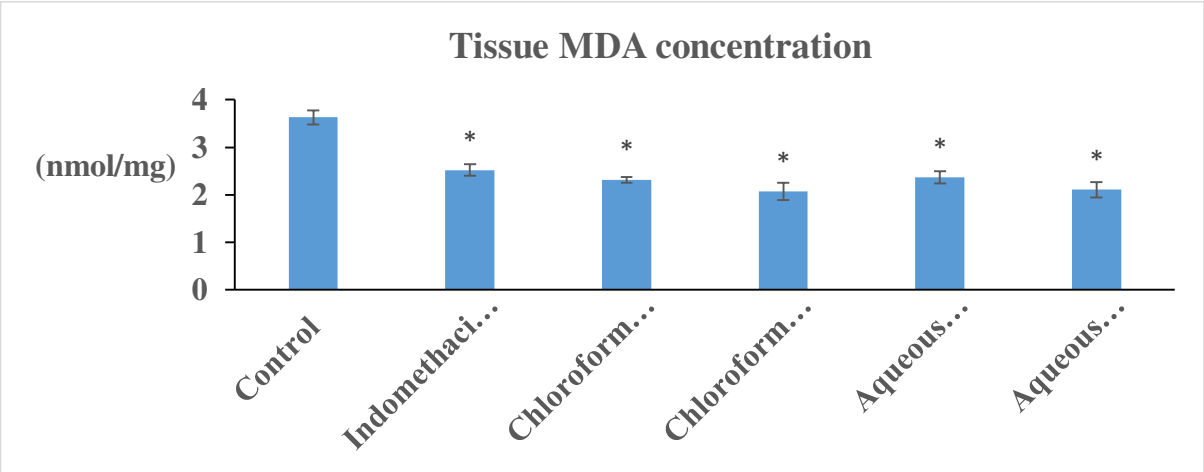
Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on Tissue MDA concentration against carrageenan induced acute inflammation in rats

Treatment	**Dose mg/kg**	**Tissue MDA concentration (nmol/mg) mean ± SEM**
Control	-	3.63 ± 0.15
Indomethacin	10	2.52 ± 0.12*
Chloroform fraction	100	2.32 ± 0.06*
Chloroform fraction	200	2.07 ± 0.18*
Aqueous fraction	100	2.37 ± 0.13*

Aqueous fraction	200	2.11 ± 0.16*

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on Tissue MDA concentration against carrageenan induced acute inflammation in rats



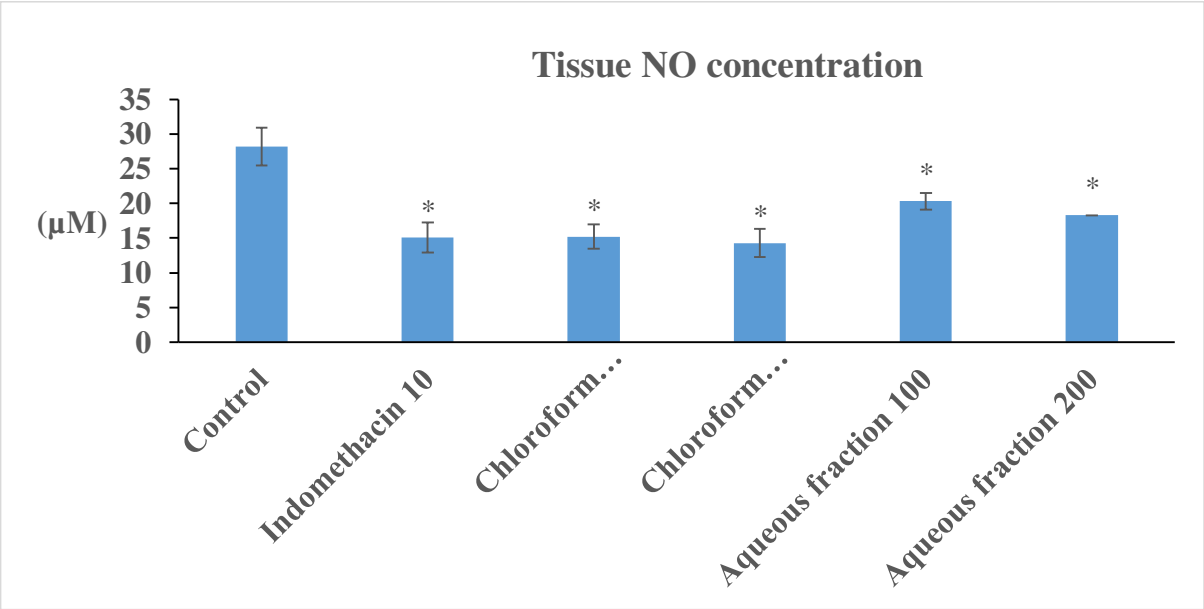
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on Tissue NO concentration against carrageenan induced acute inflammation in rats

Treatment	Dose mg/kg	Tissue NO concentration (µM) mean ± SEM
Control	-	28.16 ± 2.73
Indomethacin	10	15.06 ± 2.17*
Chloroform fraction	100	15.21 ± 1.72*
Chloroform fraction	200	14.29 ± 2.03*
Aqueous fraction	100	20.33 ± 1.20*
Aqueous fraction	200	18.30 ± 2.19*

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on Tissue NO concentration against carrageenan induced acute inflammation in rats



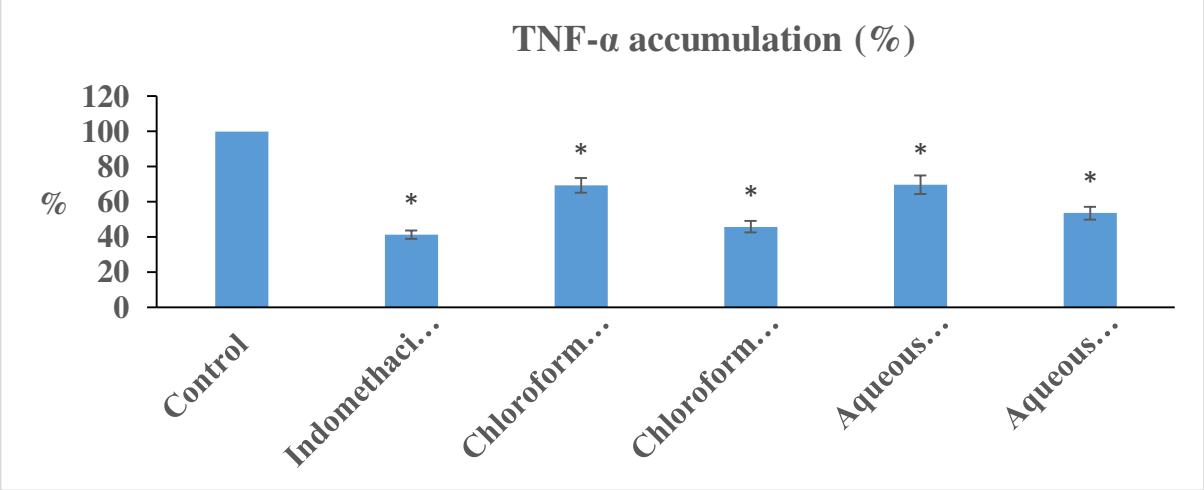
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on % TNF-α against carrageenan induced acute inflammation in rats

Treatment	Dose mg/kg	TNF-α accumulation (%) mean ± SEM
Control	-	100
Indomethacin	10	41.16 ± 2.27**
Chloroform fraction	100	69.24 ± 4.23*
Chloroform fraction	200	45.72 ± 3.36**
Aqueous fraction	100	69.53 ± 5.26**
Aqueous fraction	200	53.43 ± 3.47*

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on % TNF-α against carrageenan induced acute inflammation in rats



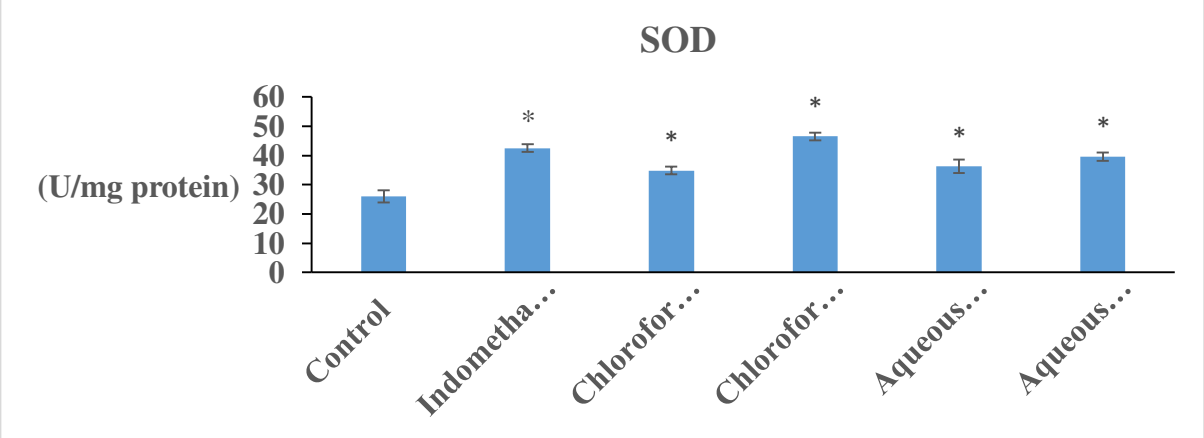
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on liver SOD and GPx activities against carrageenan induced acute inflammation in rats

Treatment	Dose mg/kg	SOD (U/mg protein)	GPx (U/mg protein)
Control	-	26.01 ± 2.14	0.037 ± 0.001
Indomethacin	10	42.51 ± 1.28*	0.065 ± 0.001*
Chloroform fraction	100	34.86± 1.31*	0.057 ± 0.002*
Chloroform fraction	200	46.52± 1.28*	0.063 ± 0.003*
Aqueous fraction	100	36.35 ± 2.30*	0.053 ± 0.001*
Aqueous fraction	200	39.58 ± 1.32*	0.060 ± 0.001*

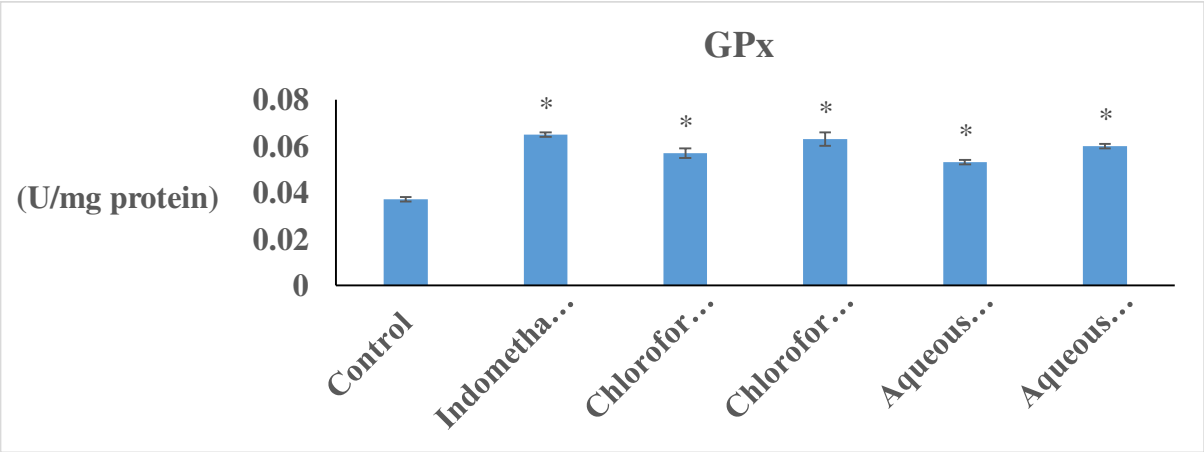
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on liver SOD activities against carrageenan induced acute inflammation in rats



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum graecum* L. on liver GPx activities against carrageenan induced acute inflammation in rats



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

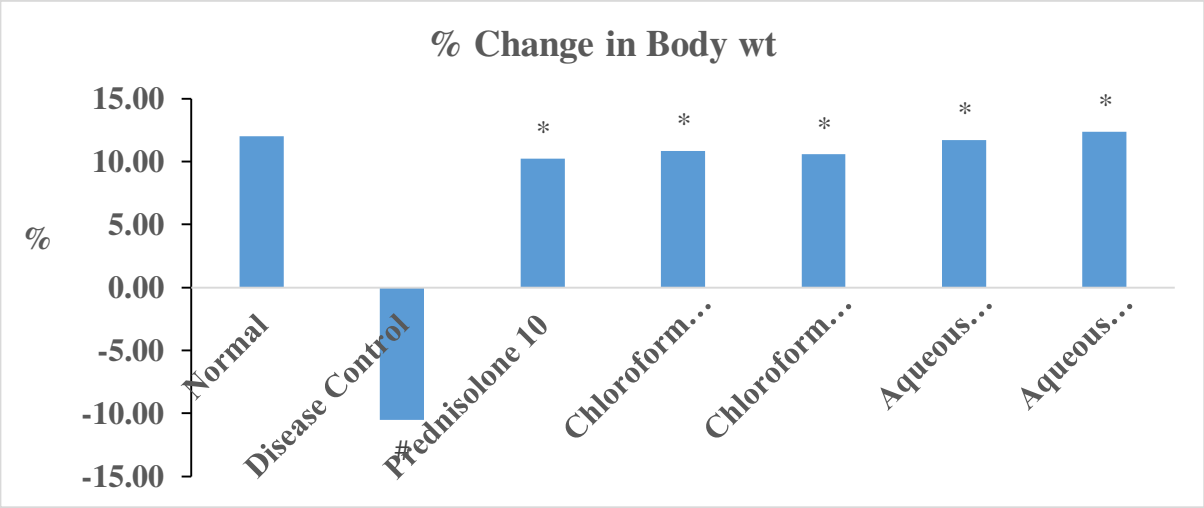
Table 4.26: Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on body weight against Collagen induced arthritis in the rat

Treatment	Dose mg/kg	Body Weight (gm)							% change in body weight
		0 day	7 day	14 day	21 day	28 day	36 day	40 day	
Normal	-	208 ± 2.05	211 ± 3.02	214 ± 1.20	219 ± 2.06	225 ± 1.26	230 ± 2.37	233 ± 1.35	12.02

Control	-	200 ± 1.53	204 ± 2.64	209 ± 2.36	207 ± 1.95	204 ± 2.38	194 ± 3.02	179 ± 1.52	-10.50
Prednisolone	10	215 ± 2.36	218 ± 2.43	222 ± 2.23	226 ± 3.04	229 ± 2.61	233 ± 2.08	237 ± 2.06	10.23
Chloroform fraction	100	212 ± 1.82	216 ± 2.50	219 ± 2.16	223 ± 2.65	227 ± 2.43	231 ± 2.06	235 ± 2.61	10.85
Chloroform fraction	200	208 ± 3.28	213 ± 1.53	216 ± 3.04	220 ± 2.48	224 ± 1.68	227± 2.14	230 ± 1.06	10.58
Aqueous fraction	100	205 ± 2.67	209 ± 1.09	213 ± 2.06	217 ± 3.03	221 ± 1.32	225 ± 2.43	229 ± 1.53	11.71
Aqueous fraction	200	210 ± 1.03	213 ± 1.24	218 ± 2.54	223 ± 3.24	228 ± 1.30	232 ± 2.51	236 ± 2.04	12.38

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on % change in body weight against Collagen induced arthritis in the rat

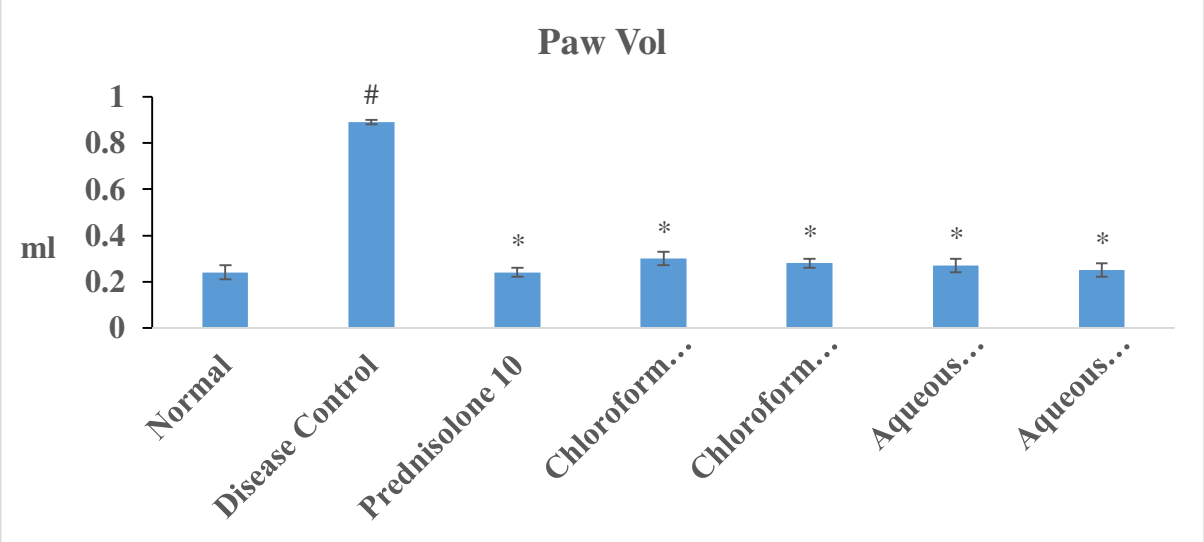


Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on paw volume (ml) against Collagen induced arthritis

Treatment	Dose mg/kg	Paw Volume (ml)						
		0 day	7 day	14 day	21 day	28 day	36 day	40 day
Normal	-	0.22 ± 0.01	0.21 ± 0.02	0.22 ± 0.03	0.21 ± 0.01	0.22 ± 0.02	0.23 ± 0.02	0.24 ± 0.03
Control	-	0.21 ± 0.02	0.57 ± 0.03#	0.64 ± 0.04#	0.71 ± 0.02#	0.80 ± 0.03#	0.86 ± 0.01	0.89 ± 0.01
Prednisolone	10	0.23± 0.01	0.41 ± 0.01*	0.44 ± 0.01*	0.39 ± 0.03*	0.28 ± 0.02*	0.26 ±0.03	0.24 ± 0.02
Chloroform fraction	100	0.22 ± 0.02	0.42 ± 0.01	0.46 ± 0.01	0.42 ± 0.02	0.32 ± 0.01	0.31 ± 0.01	0.3 ± 0.03
Chloroform fraction	200	0.23 ± 0.03	0.39 ± 0.01	0.45 ± 0.02	0.41 ± 0.02	0.33 ± 0.02	0.30 ± 0.02	0.28 ± 0.02
Aqueous fraction	100	0.21 ± 0.12	0.41 ± 0.03	0.48 ± 0.02	0.40 ± 0.01	0.36 ± 0.02	0.32 ± 0.02	0.27 ± 0.03
Aqueous fraction	200	0.22 ± 0.23	0.43 ± 0.02	0.47 ± 0.01	0.41 ± 0.01	0.37 ± 0.02	0.30 ± 0.02	0.25 ± 0.03

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on paw volume (ml) against Collagen induced arthritis



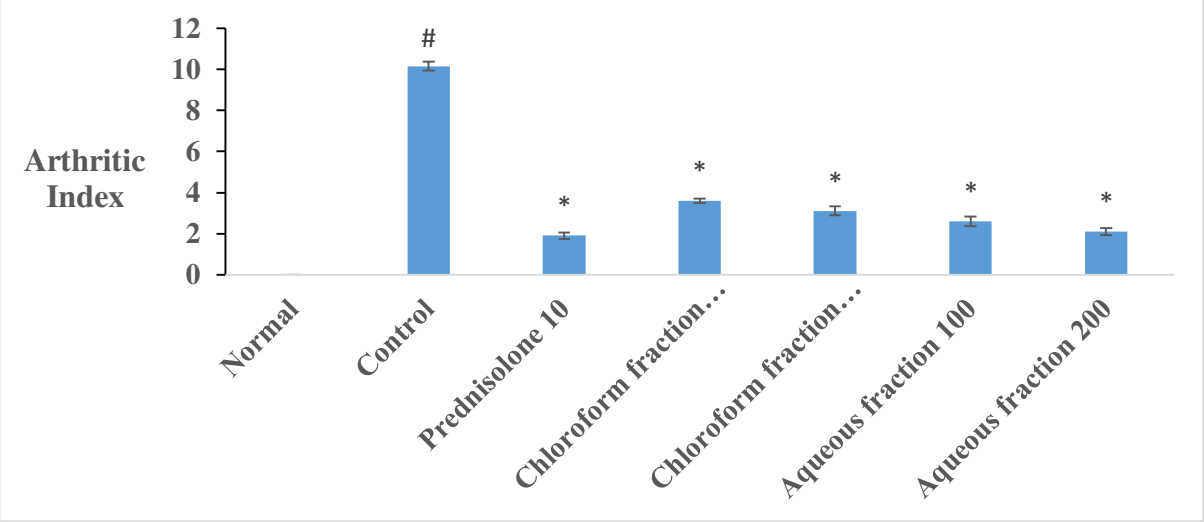
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on arthritic index against Collagen induced arthritis

Arthritic index on Day 28		
Treatment	**Dose (mg/kg)**	**Arthritic index**
Normal	-	0 ± 0
Control	-	10.16 ± 0.22
Prednisolone	10	1.90 ± 0.16
Chloroform fraction	100	3.60 ± 0.11
Chloroform fraction	200	3.10 ± 0.22
Aqueous fraction	100	2.60 ± 0.23
Aqueous fraction	200	2.10 ± 0.17

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on arthritic index against Collagen induced arthritis

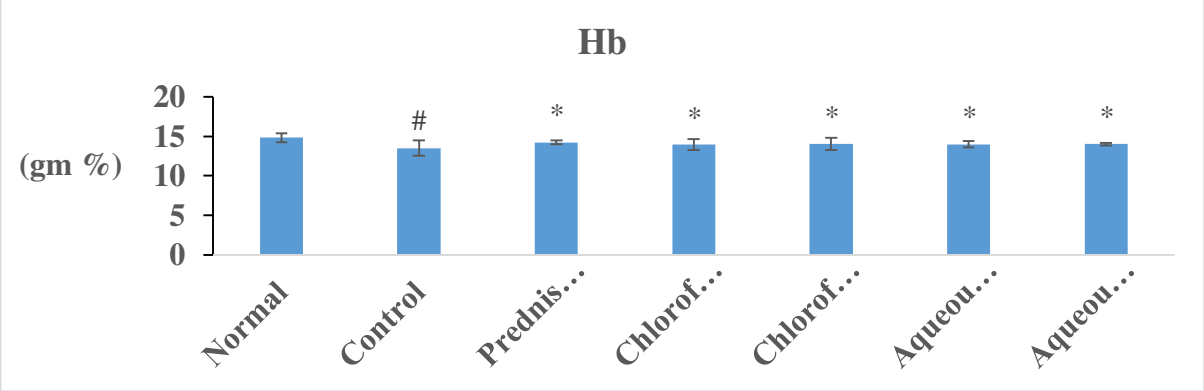


Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on Hb count, total RBC count and Total WBC count against Collagen induced arthritis

Treatment	Dose (mg/kg)	Haemoglobin (Hb) (gm%)	RBC count (million/cmm)	Total WBC count (10^3/µl)
Normal	-	14.85 ± 0.58	6.12 ± 0.30	7.16 ± 0.022
Control	-	13.52 ± 0.96$^{\#}$	5.02 ± 0.14	11.6 ± 0.026$^{\#}$
Prednisolone	10	14.25 ± 0.24*	5.93 ± 0.12	6.05 ± 0.014*
Chloroform fraction	100	13.96 ± 0.72*	5.58 ± 0.21	7.85 ± 0.020*
Chloroform fraction	200	14.05 ± 0.81*	5.71 ± 0.16	7.50 ± 0.020*
Aqueous fraction	100	14.00 ± 0.42*	5.63 ± 0.22	7.15 ± 0.012*
Aqueous fraction	200	14.02 ± 0.17*	5.93 ± 0.21	7.05 ± 0.013*

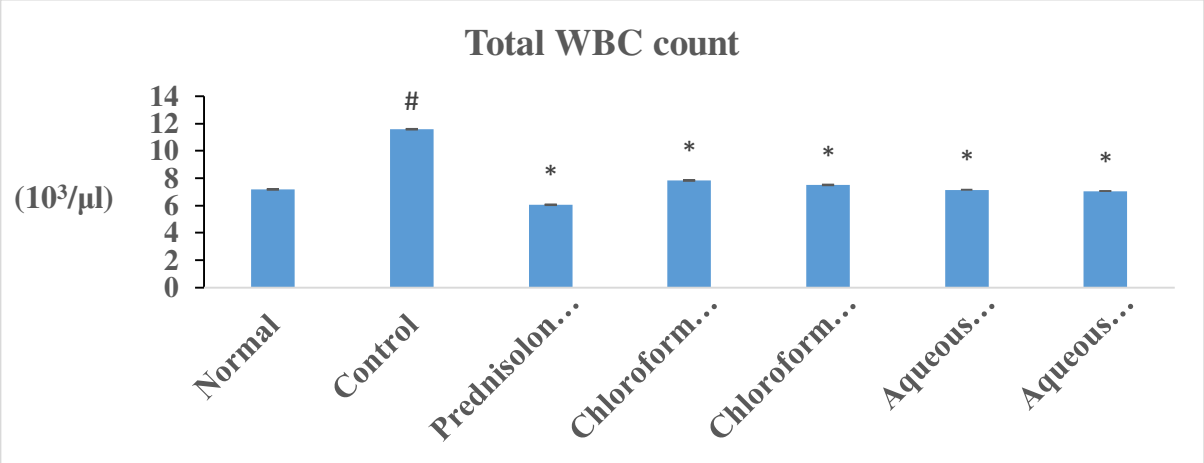
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on Hb count against Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on Total WBC count against Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

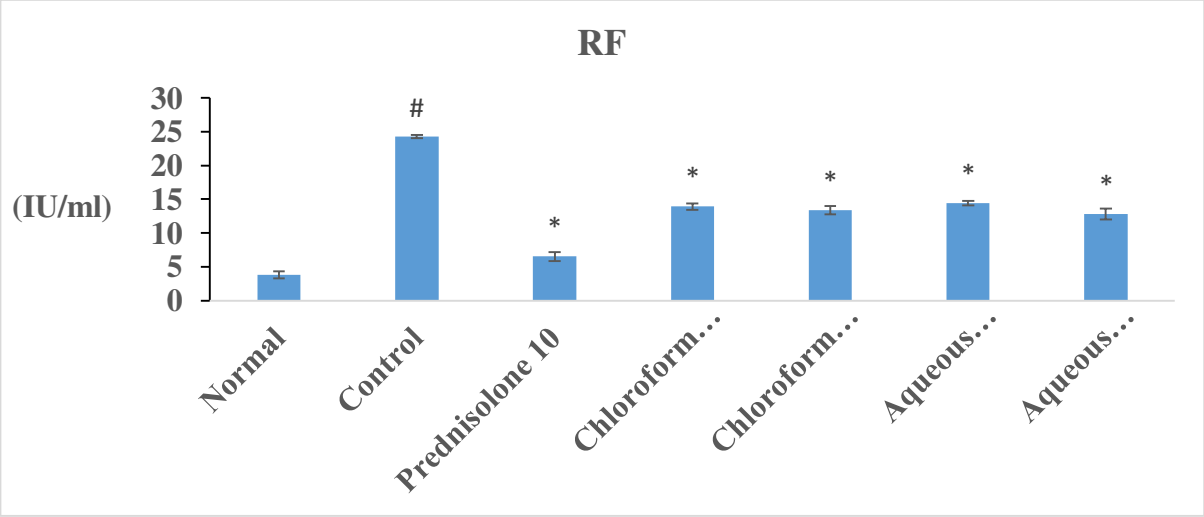
Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on RF, CRP and ESR against Collagen induced arthritis

Treatment	Dose (mg/kg)	RF (IU/ml)	CRP (mg/L)	ESR (mm/hr)
Normal	-	4.80 ± 0.51	4.03±0.10	0.59 ± 0.11

Control	-	24.30 ± 0.24#	14.60±0.53#	4.62 ± 0.22#
Prednisolone	10	6.51 ± 0.64*	5.20±0.21*	1.11 ± 0.03*
Chloroform fraction	100	13.35 ± 0.51*	6.88±0.32*	1.46 ± 0.10*
Chloroform fraction	200	10.20 ± 0.62*	5.76±0.31*	1.36 ± 0.12*
Aqueous fraction	100	14.45 ± 0.34*	6.12±0.32*	1.41 ± 0.03*
Aqueous fraction	200	12.82 ± 0.80*	5.52±0.12*	1.30 ± 0.20*

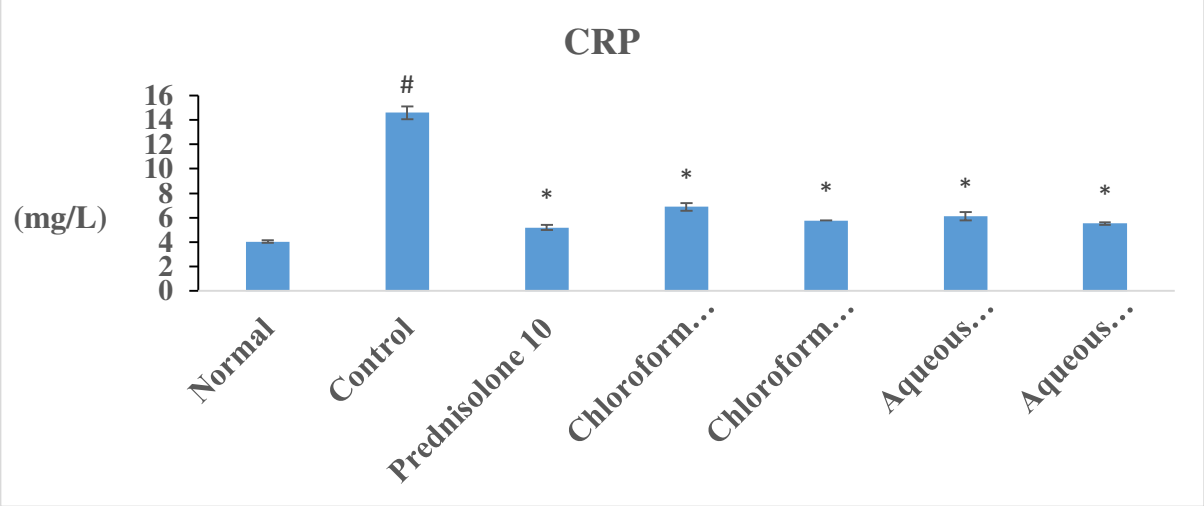
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on RF against Collagen induced arthritis



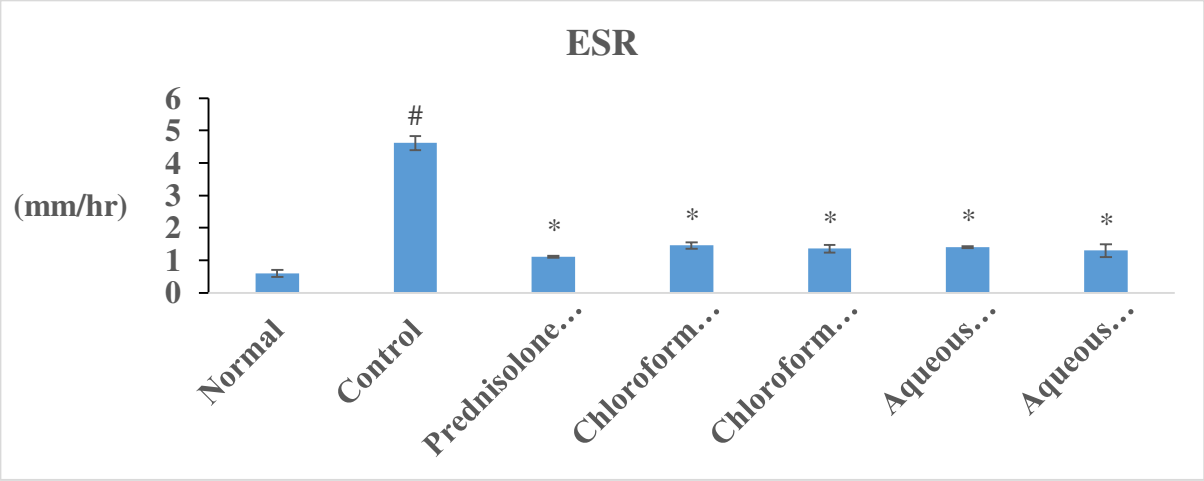
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on CRP against Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on ESR against Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

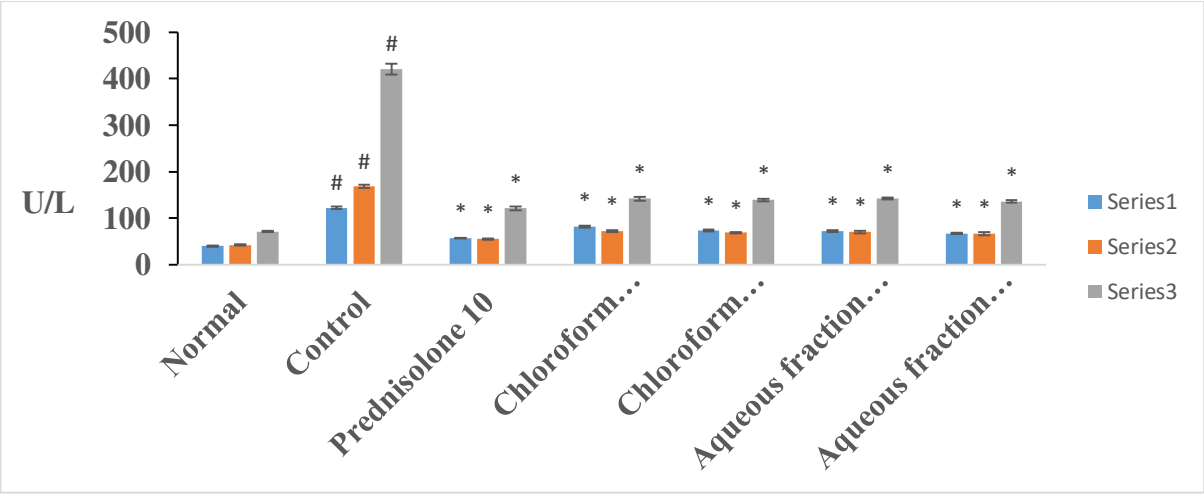
Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on AST, ALT, ALP and A/G ratio in Collagen induced arthritis

Treatment	Dose (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	Total protein (g/dL)	A/G Ratio

Normal		40 ± 1.20	42 ± 1.53	71 ± 1.30	6.4 ±0.05	1.72 ± 0.04
Control		122 ± 3.10#	168 ± 3.20#	420 ± 12.0#	4.5 ± 0.03#	0.72 ± 0.11#
Prednisolone	10	57 ± 1.20*	55 ± 1.00*	121 ± 4.50*	6.2 ± 0.02*	1.51 ± 0.13*
Chloroform fraction	100	82 ± 2.42*	72 ± 2.25*	142 ± 4.23*	6.0 ±0.04*	1.25 ± 0.04*
Chloroform fraction	200	73 ±2.02*	69 ±1.37*	139 ± 3.13*	6.1 ±0.04*	1.30 ± 0.06*
Aqueous fraction	100	72 ±2.21*	70 ± 2.32*	142 ± 2.28*	6.2 ±0.03*	1.32 ± 0.07*
Aqueous fraction	200	67 ±1.02*	66 ± 3.56*	136 ± 3.03*	6.3 ±0.02*	1.34 ± 0.06*

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on AST, ALT and ALP in Collagen induced arthritis



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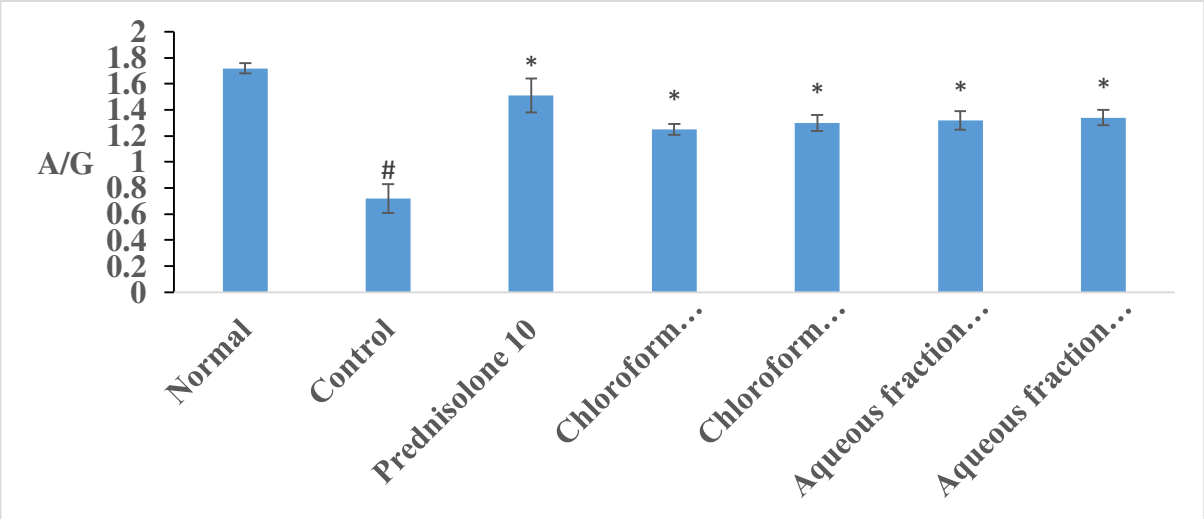
Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on A/G ratio in Collagen induced arthritis



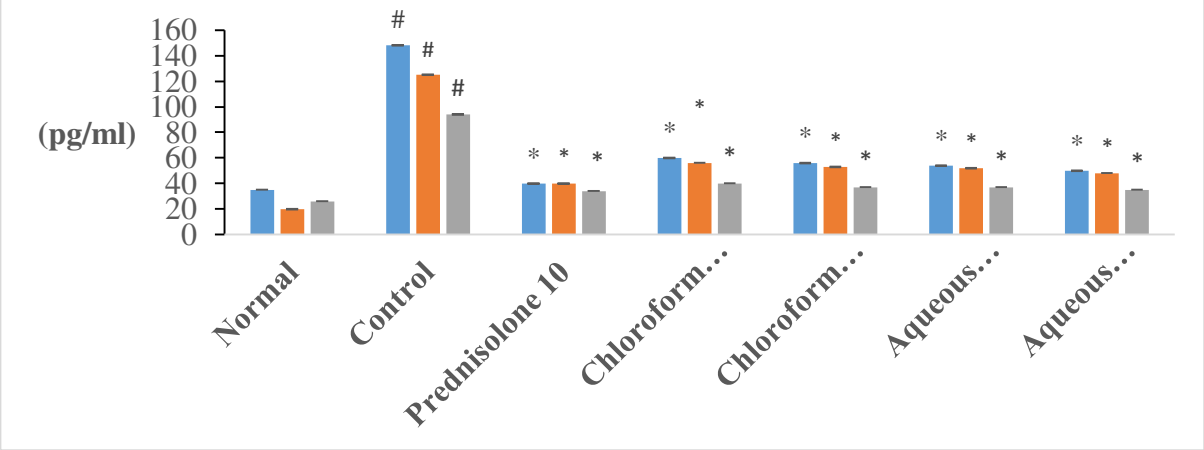
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on TNF-α levels, IL-1β levels and IL-6 levels in Collagen induced arthritis

Treatment	Dose (mg/kg)	TNF-α levels (pg/ml)	IL-1β levels (pg/ml)	IL-6 (pg/ml)
Normal	-	35 ± 0.04	20 ± 0.08	26 ± 0.04
Control	-	148 ± 0.10#	125 ± 0.12#	94 ± 0.13#
Prednisolone	10	40 ± 0.05*	40 ± 0.10*	34 ± 0.03*
Chloroform fraction	100	60 ± 0.12*	56 ± 0.02*	40 ± 0.04*
Chloroform fraction	200	56 ±0.14*	53 ± 0.09*	37 ± 0.03*
Aqueous fraction	100	54 ±0.10*	52 ± 0.11*	37 ± 0.01*
Aqueous fraction	200	50 ±0.04*	48 ± 0.02*	35 ± 0.04*

n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on TNF-α levels, IL-1β levels and IL-6 levels in Collagen induced arthritis



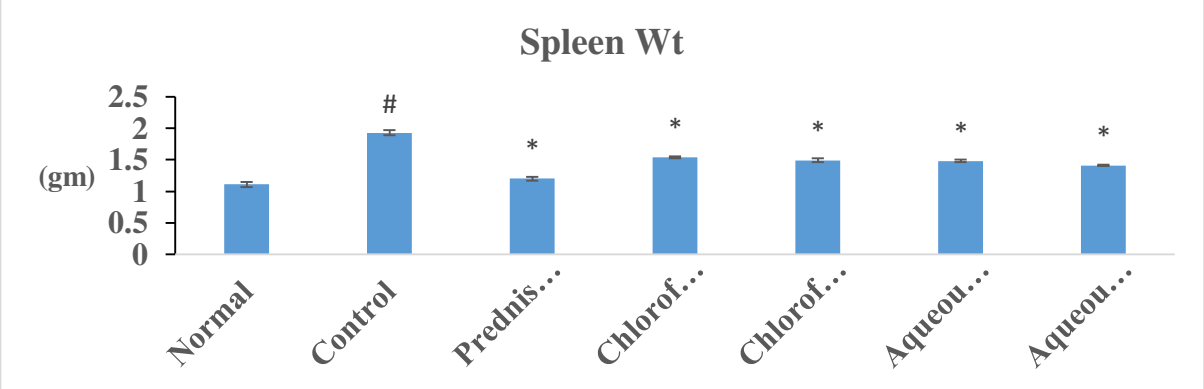
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. On Organ Weight in Collagen induced arthritis

Treatment	Dose (mg/kg)	Organ Weight	
		Spleen weight (g)	Thymus weight (mg)
Normal	-	1.11 ± 0.04	90.43 ± 2.61
Control	-	1.93 ± 0.04	65.32 ± 2.67
Prednisolone	10	1.20 ± 0.03	88.07 ± 1.80
Chloroform fraction	100	1.54 ± 0.02	81.26 ± 1.02
Chloroform fraction	200	1.49 ± 0.03	83.16 ± 2.02
Aqueous fraction	100	1.48 ± 0.02	85.25 ± 1.01
Aqueous fraction	200	1.41 ± 0.01	87.31 ± 2.01

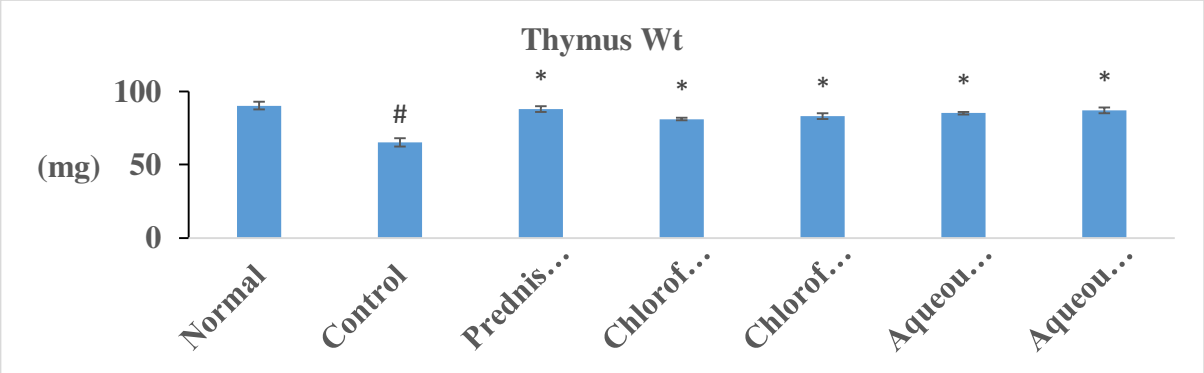
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on spleen Weight in Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on thymus Weight in Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

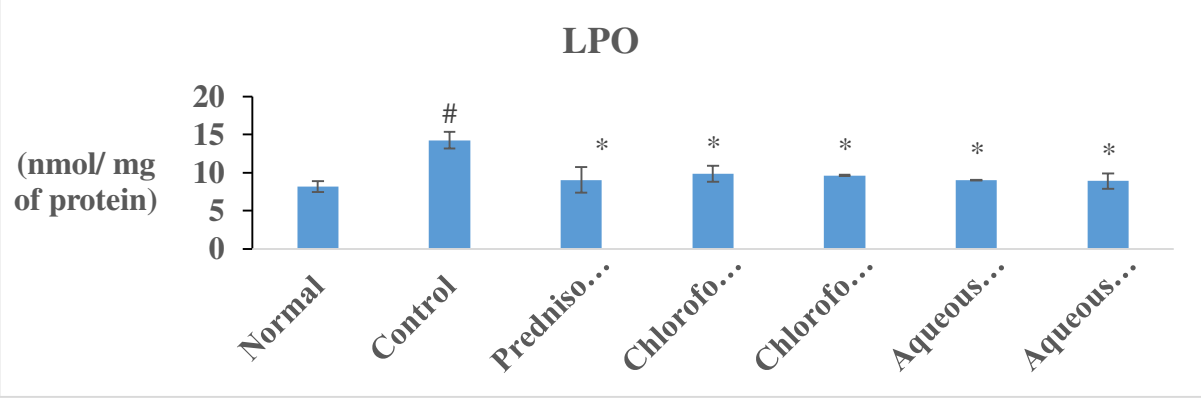
Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on In-vivo antioxidant levels in Collagen induced arthritis

Treatment	Dose (mg/kg)	LPO (nmol/ mg of protein)	SOD (U/mg of protein)	CAT (nmol/ min /mg of protein)	GPx (nmol/ min/mg protein)
Normal	-	8.20 ± 0.71	98 ± 0.23	43 ± 0.27	340 ± 1.05

Control	-	14.24 ± 1.10	84 ±0.41	30 ± 1.40	290 ± 1.06
Prednisolone	10	9.05 ± 1.70*	110 ±1.31	42 ± 0.91	320 ± 2.05
Chloroform fraction	100	9.84 ± 1.03	102 ±0.48	40 ± 1.06	318 ± 0.64
Chloroform fraction	200	9.61 ±0.08	107 ±1.72	42 ± 1.54	320 ± 0.48
Aqueous fraction	100	9.05 ±0.04	105 ± 1.42	42 ± 0.64	330 ± 1.08
Aqueous fraction	200	8.92 ±1.02	108 ±0.56	45 ± 0.43	335 ± 1.05

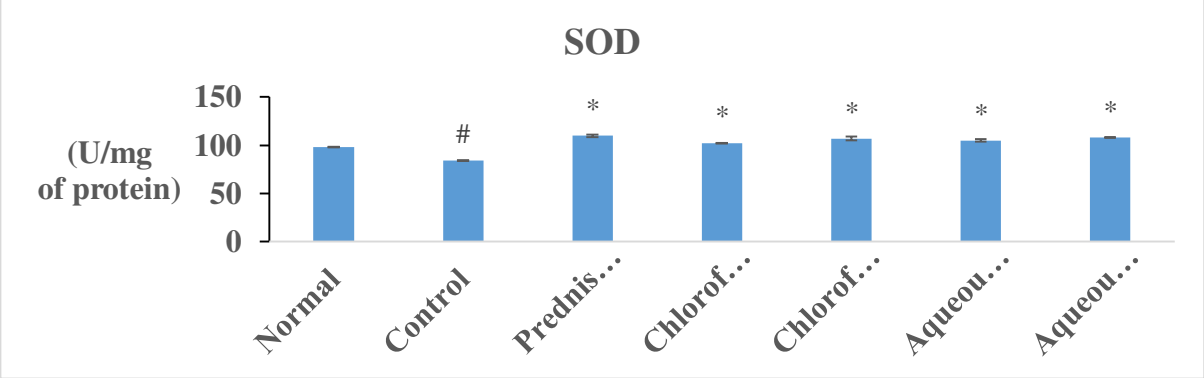
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on LPO levels in Collagen induced arthritis



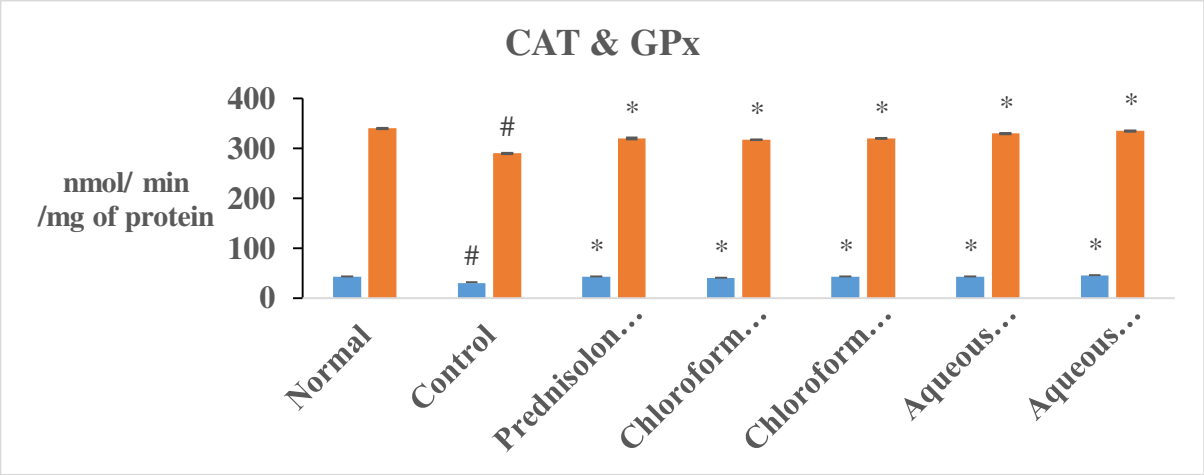
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on SOD levels in Collagen induced arthritis



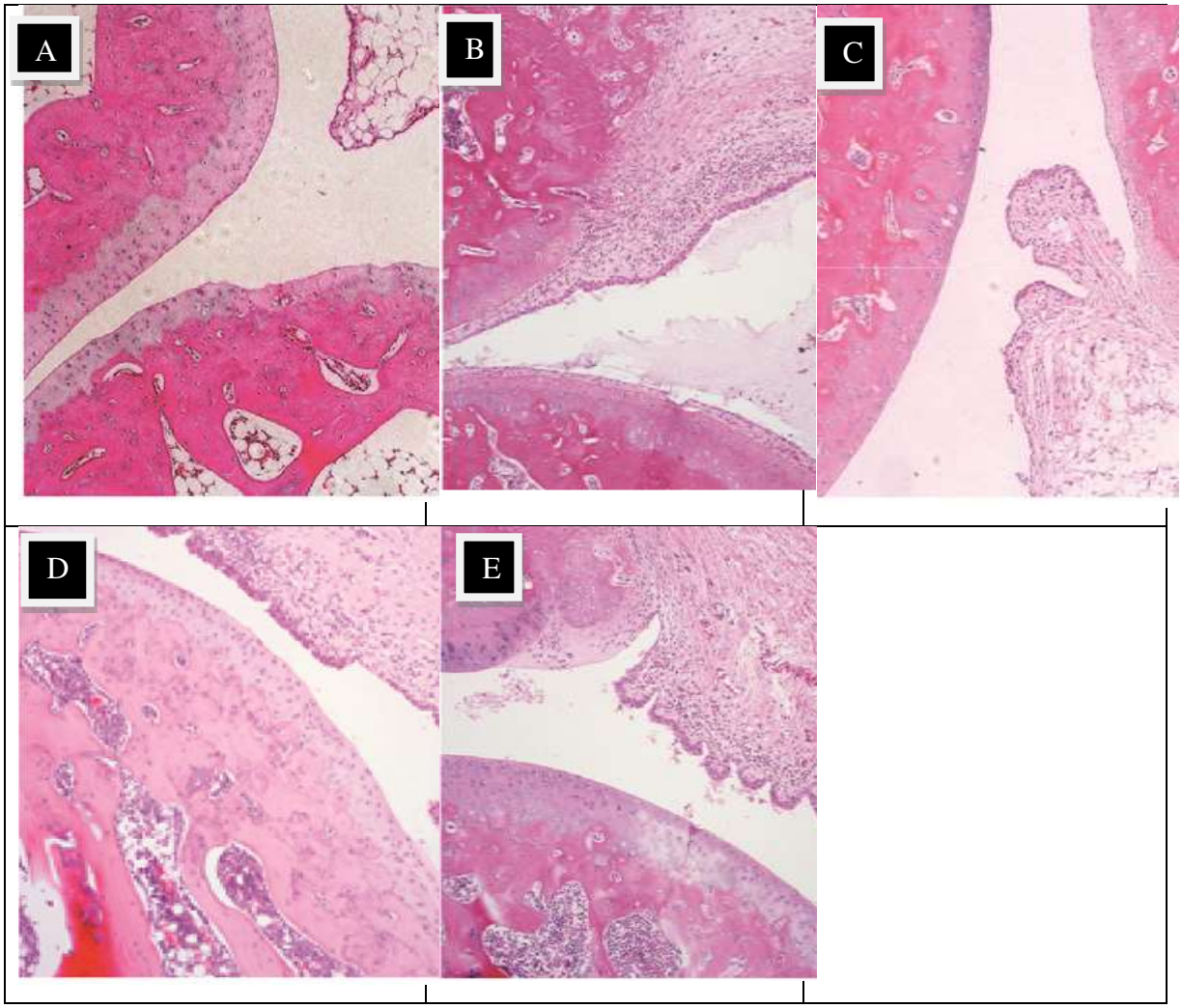
n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on CAT & GPx levels in Collagen induced arthritis



n = 6, each value represents mean ± SEM. *P < 0.01 as compared with the control group (One-way ANOVA followed by Dunnett's test).

Effect of chloroform and aqueous fractions of *trigonella foenum-graecum* L. on histology of synovial membrane of Collagen induced arthritis



A – Normal control; B – Disease control; C – Prednisolone treated; D – CF 200 mg/kg treated; E – AF 200 mg/kg treated

DISCUSSION

RA is a systemic inflammatory disorder that mainly affects the diarthrodial joint. It is the most common form of inflammatory arthropathy worldwide and affects up to 0.75% of the Indian population. The spectrum and disease progression of RA is governed by multiple factors including immune, genetic and environmental factors. Rodent models of RA serve as valuable tools to investigate the underlying mechanisms at early, intermediate and late stages of RA. The goal of generating new and improved treatments for arthritic diseases of the joints has not yet been achieved, although progress is being made. Destruction of articular cartilage is the hallmark of many of these disabling conditions, and an imbalance of pro-inflammatory

cytokines over their anti-inflammatory counterparts promotes the disease process. CIA in rodents, an experimental disease model with a number of pathological, histological, immunological and genetic parameters common with RA was used in the present study [26].

Carrageenan-induced paw edema model is not only a well-established model but also a very commonly used model for screening anti-inflammatory potential of a drug. The triphasic inflammatory changes produced by injecting carrageenan are quite similar to the early exudative stage of inflammation in human. This triphasic inflammatory change is because of the involvement of multiple autacoids. The first phase which lasts for ~1 h following carrageenan administration is characterized by a sudden increase in paw volume induced by the action of histamine, the second phase which lasts for ~2–3 h post carrageenan administration is because of generation of serotonin and kinins, and the third phase which is attributed to the generation of prostaglandins and leukotrienes which lasts from ~4–6 h after post carrageenan administration. This study shows that indomethacin and CFTF and AFTF (100 and 200 mg/kg) treatment produces significant inhibition of inflammation at all observation period suggesting its modulatory activity against several autacoids [27].

In the present study, a significant loss in the body weight was also observed in the arthritic rats when compared with their normal control counterparts. However, fenugreek treated arthritic rats at a dose of 200mg/kg and 100mg/kg showed a significant increase in their body weight in comparison to their arthritic control counterparts.

No obvious redness or joint swelling was observed in normal rats. However, after CII injection, peripheral paw edema was observed in rats within 24 h. Furthermore, after 21 days of treatment by CFTF and AFTF (100 and 200 mg/kg) and Prednisolone (10 mg/kg), paw edema was significantly decreased ($p < 0.01$), compared with rats in the RA group.

Arthritis score is a clinical assessment of joint swelling [28]. In this study, CIA rats showed a significant increase ($p<0.05$) in arthritis scores compared with the control group. The alteration in plasma protein induces the synthesis of proinflammatory cytokines, prostaglandins, leukotrienes and matrix metalloproteinases that caused fluid accumulation in the synovium. This results in an increase in arthritis scores due to damage in joints and bones of the rat's paw [29].

In the present investigation, significant rise in RF, CRP and ESR level and liver parameters was observed higher in disease control animals as compared to normal control, which was counteracted by treatment groups.

In chronic conditions of arthritis, in arthritic condition, there is a mild to moderate rise in WBC count due to inflammatory response by released IL-Iβ. IL-Iβ increases the production of both granulocytes and macrophages colony stimulating factor. The present study shows that CFF and AFF treatments tend to normalize the WBC count and suppresses the migration of leucocytes into the inflamed area. In addition, the decrease in Hb and RBC is indicative of anemia in rats due to endothelial dysfunction. hematological alteration such as the decreased Hb count was also restored by treatment of chloroform and aqueous fraction of fenugreek. We propose that the reduction in the Hb count during arthritis results from reduction in erythropoietin levels, a decreased response of the bone marrow erythropoietin and premature destruction of red blood cells. Thus, increase in the Hb count brought about by test treatment further supports the anti-arthritic effect of chloroform and aqueous fraction of fenugreek [30].

In the present study, CFF and AFF significantly decreased the LPO level in collagen-induced arthritis rats probably indicating the prevention of the cell damage by reducing oxidative stress. In present study CFF and AFF significantly increased the levels of SOD, CAT, and GSH possibly by preventing the inactivation of these enzymes by H_2O_2 or by reducing the oxidative stress. GSH reflect the endogenous defence against damage caused by ROS and organic peroxides as they act as an intracellular reductant in oxidation-reduction processes. The decreased levels of GSH in liver of arthritic rats might be due to the excessive consumption of GSH by the system to defend oxidative damage. The production of oxygen free radicals that occurs with the development of arthritis leads to decreased GSH and SOD levels as a consequence of their consumption during oxidative stress and cellular lysis, which is evident by decreased levels of GSH and SOD in arthritic control group. administration of test drug to the rats significantly re-established the depleted levels of GSH and SOD, probably by competing for scavenging of free radicals[31].

It has been reported that pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 play crucial roles in the development of RA [32,33]. TNF-α has been considered to be on top of a cytokine cascade, which can increase the releases of IL-6 and IL-1β, and stimulate cartilage

matrix degradation. In addition, IL-1β and IL-6 have been reported to contribute to the development of arthritis [34,35]. Interestingly, in our study, both fractions significantly decreased the serum levels of TNF-α, IL-1β, and IL-6. Thus, the potential mechanism of the therapeutic effect of fenugreek on RA may involve in down-regulating of the level of TNF-α, IL-1β, and IL-6.

Spleen is a vital organ involved in immune responses. In adjuvant arthritis, spleen serves as the reservoir for the cells and antibody formation which involved in the immune response. Increased in the weight of spleen is associated with the splenomegaly, generalized lymphadenopathy and altered hepatic function [36]. In Collagen induced arthritis significantly increased weight of spleen and decreased the weight of thymus which is in accordance with previous studies of [37]. Decrease in spleen weight and increase in thymus weight might be due to immune-modulatory effect of fractions of fenugreek.

Results of histological examinations were shown in Fig., which indicated that there were no pathological changes in joint and cartilage of the normal rats. In contrast, CIA animals' slices exhibited visible and massive inflammatory cells infiltration, synovial hyperplasia and bone destruction with fibroblasts proliferation, compared to the normal animals. After administration of CFTF and AFTF (100 and 200 mg/kg), our results showed that the inflammation and destruction of joints as well as synovial hyperplasia was significantly alleviated compared with the control CIA rats.

Conclusion

In conclusion, our present study demonstrated that fractions of fenugreek leaves have promising anti-arthritic effects on type II collagen induced arthritis in rats via suppressing the releases of TNF-α, IL-1β and IL-6 and due to anti-oxidant properties. Consequently, our results suggested that fenugreek have anti-inflammatory activity in carrageenan induced inflammation. It could be regarded as a potential candidate for RA treatment, which merited further studies as regards to fully elucidate the active constituent and its mechanism of action, as well as evaluating its preclinical safety for further anti-RA drug development.

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