ORIGINAL ARTICLE

Membrane stabilizing evaluation of non-polar and polar fractions from *Trigonella foenum-graecum* (Linn.)in arthritic rats

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Abstract

Objective: The present study was aimed to assess the anti-arthritic activity of petroleum ether and n-butanolic fraction of *Trigonella foenum-graecum* (Linn.) seed extract against Freund's complete adjuvant induced arthritis in rats. **Materials and Methods:** Methanolic extract of *T. foenum-graecum* (Linn.) seeds were fractionated in various organic solvents and on the basis of results of *in vitro* protein denaturation and proteinase inhibitory activity (data not shown here), petroleum ether and n-butanolic fractions were selected for anti-arthritic activity. The active petroleum ether and n-butanolic fraction were administered at the dose of 50 and 100 mg/kg body weight. The effects of both fractions on liver alkaline phosphatase, acid phosphatase and lactate dehydrogenase levels and malondialdehyde, glutathione (GSH) and superoxide dismutase (SOD) from articular cartilages in arthritic animals were studied. Prednisolone (10 mg/kg) was used as a standard. **Results:** In arthritic rats, the petroleum ether and n-butanolic fraction showed a highly significant reduction in paw volume (100 mg/kg - 68.37 and 75.21%) (P<0.01, 0.001). Membrane marker enzymes and oxidative free radicals were significantly decreased in the both fraction treated groups and GSH and SOD activities were significantly increased compared with the arthritic control. Among these two active fractions, n-butanolic fraction showed best activity as compared to petroleum ether fraction. **Conclusion:** The n-butanolic fraction of *T. foenum-graecum* (Linn.) seed extract significantly reduced the level of antioxidant enzymes as well as membrane stabilizing markers. The possible mechanism may be due to both stabilizing actions of membrane marker enzymes or inhibition of oxidative free radicals.

Key words: Inflammation, n-butanolic fraction, membrane stabilizing activity, oxidative free radicals, *Trigonella foenum-graecum*

INTRODUCTION

Inflammation is a biological response to noxious stimuli such as pathogens that cause tissue and cell damage.[1] It is considered a protective measure taken by the organism to eliminate harmful stimuli and to commence the healing process. It is classified as either acute or chronic, depending on whether it involves a short response or a prolonged one, respectively.[2] The acute inflammatory response is initiated by plasma and leukocytes penetration to the site of injury or infection.[3] It can be triggered by receptors of the innate immune system, for example, the toll-like receptors.[4] In the first steps of infection, resident macrophages and mast cells release inflammatory mediators, such as cytokines (e.g., interleukin-1β, IL-6, IL-12, and the chemokines IL-8), tumor necrosis factors (e.g., TNF-α and TNF-β), interferon (e.g., IFN-γ), eicosanoids (e.g., prostaglandins and leukotrienes) and vasoactive amines (e.g., histamine). These mediators exert complex regulatory roles in the inflammatory process in order to reinstate tissue homeostasis. Chronic inflammation, on the other hand, is a deregulated response to constant noxious stimuli and seems to be related to tissue breakdown. This prolonged inflammatory condition is linked with a large number of chronic human disorders, including cancer, allergy, arthritis, atherosclerosis and autoimmune diseases.[3]

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Non-steroidal anti-inflammatory drugs (NSAIDs) reduce the pain and inflammation by blocking the metabolism of arachidonic acid by isoforms cyclooxygenase enzyme (COX-1 and/or COX-2), and thereby reduce the production of prostaglandin.[5] Many researchers and practitioners are in quest of alternative approaches to provide an efficient cure in the treatment of ailment and to overcome the serious drawbacks like gastrointestinal bleeding and gastric ulcer. Hence, there is an immense need to find safe compounds for the management of chronic inflammation.[6]

Trigonella foenum-graecum L (Fenugreek; family: Leguminosae) is one such plant, whose seeds and leaves are not only used as food but also as an ingredient in traditional medicine.[7,8] Seeds of fenugreek are used as a condiment with wheat and maize flour for bread making and as a constituent of the daily diet of general population in Indian subcontinent. Seeds of *T. foenum-graecum* Linn. contain tannic acid, fixed and vegetable oils, diosgenin, trigonelline, trigocoumarin, trigomethyl coumarin, steroidal saponin such as gitogenin and traces of trigogenin and vitamin A.[9] A some of the therapeutic uses of *T. foenum-graecum* Linn. include its use as antiulcer, wound healing, central nervous system stimulant, immunomodulatory, antioxidant, antidiabetic, antineoplastic, anti-inflammatory, and antipyretic.[9]

In our previous study, the petroleum ether and butanolic fractions from the methanolic extract of *T. foenum-graecum* seeds has showed potent anti-arthritic activity by protein denaturation methods and proteinase inhibitory activity.[10] However, there is no scientific evidence of these fractions in chronic inflammatory condition, i.e., arthritis. Based on the above perspectives, the present study was designed to explore the most promising fraction responsible for anti-arthritic activity. Our research outcome may also provide scientific data to support the folk medicinal consumption of seeds of *T. foenum-graecum* for the treatment of arthritis.

MATERIALS AND METHODS

Procurement and Authentication of Plant

The seeds of *T. foenum-graecum* Linn. were purchased from the local market of Mandsaur, India. The plant material was taxonomically identified by Dr. Gynendra Tiwari, Scientist, KNK College of Horticulture, Mandsaur, India and the voucher specimen is submitted in Department of Pharmacognosy, B R Nahata College of Pharmacy, Mandsaur for future reference.

Fractionization of Methanolic Extract of *T. foenum-graecum* Linn.

The dried methanolic extract (50 g) was suspended in water and filtered to remove the insoluble material. The water fraction was taken in separating funnel and fractionated by various organic solvents to get petroleum ether (non-polar), chloroform (non-polar), butanol (polar), and water (polar) soluble layer. Each fraction was dried under vacuum to obtain petroleum ether (2 g), chloroform (5.8 g), n-butanolic (6.4 g), and water fractions (2.8 g). Preliminary phytochemical studies of petroleum ether and n-butanolic fractions were performed for the presence of steroids, fatty acids, terpenoids, flavonoids, tannins and glycosides.[11]

Animals

Wistar albino rats (200-250 g) of either sex were used for the study. The animals were maintained under the environmental condition and fed with standard pellet diet and water *ad libitum*. The study protocol was approved by Institutional Animal Ethical Committee (IAEC, 156/PhD/10/IAEC/BRNCP/10-11/Mandsaur). CPCSEA guidelines were adhered to during maintenance and experiment.

Acute Toxicity Studies

Acute toxicity studies were carried out for petroleum ether and butanolic fractions of methanolic extract of *T. foenum-graecum* Linn. according to OECD guidelines 423.[12] The petroleum ether, butanolic fractions were administered orally in a dose of 1000 mg/kg body weight. The animals (n = 3) were observed 24 h for the signs of toxicity. The attention was directed on convulsion, diarrhea, coma, respiratory depression, salivation, and perspiration.

Freund's Complete Adjuvant-induced Arthritis

Arthritis was induced by the injection of 0.1 mL of FCA (Sigma-Aldrich, USA) containing 10 mg of heat killed *Mycobacterium tuberculosis*, in 1 mL of paraffin oil into the right hind paw of the rat intradermally. The animals were divided into seven groups and each group containing six animals. Group I served as normal control; Group II served as arthritic control; Group III was treated with prednisolone (Wyeth Pvt. Ltd., India) 10 mg/kg, standard anti-arthritic drug; Groups IV and V were treated with petroleum ether fraction in dose of 50 and 100 mg/kg; Groups VI and VII were treated with n-butanolic fraction in dose of 50 and 100 mg/kg of methanolic extract of *T. foenum-graecum* Linn. The treatment was given orally daily after 14 days from the day of adjuvant injection for 35 days. The volume of the paw was measured before induction, before treatment and after treatment; the percentage inhibition was determined.[13]

Arthritis Assessment

The severity of arthritis in each paw was quantified daily by a clinical score measurement from 0 to 4 as follows: 0 – No macroscopic signs of arthritis (swelling or erythema),

1 – swelling of one group of joints (namely, wrist or ankle joints), 2 – swelling of two groups of swollen joints, 3 – swelling of three groups of swollen joints, and 4 – welling of the entire paw.[14]

Biochemical Estimation

At the end of the experimental period, rats were fasted overnight, and an anesthetized rats were sacrificed by cervical decapitation. Liver homogenates were centrifuged at 600 g for 10 min. The sediment which containing nuclei, unbroken cells and plasma membranes (nuclear fraction) were separated and the supernatant was subjected to centrifugation at 16,000 g for 30 min. Enzyme activity in the supernatant was determined. The marker enzymes alkaline phosphatase (ALP),[15] acid phosphatase (ACP)[15] lactate dehydrogenase (LDH)[16] were estimated by liver. Malondialdehyde (MDA),[17,18] glutathione (GSH)[16,19] and superoxide dismutase (SOD)[16,19] were estimated by articular cartilages.

Data Analysis

All values are presented as mean±standard error of the mean. Differences between the drug-treated groups and the control group were evaluated by independent unpaired sample *t*-tests using the Prism Software 5.0 version. $P < 0.05$ was considered significant.

RESULTS

Preliminary Phytochemical Screening

The preliminary phytochemical screening of the petroleum ether fraction strongly indicated the presence of steroids, triterpenoids, and n-butanolic fraction showed the presence of flavonoids, polyphenolics and glycosides.

Acute Toxicity Studies

As suggested by OECD guidelines, the tested animals were observed individually for 24 h after single dosing. The animals did not exhibit any symptoms and survived beyond the recommended duration of observation with a dose of 1000 mg/kg of petroleum ether and n-butanolic fractions. Therefore, 50 and 100 mg/kg used for the pharmacological activity.

Freund's Complete Adjuvant-induced Arthritis

In adjuvant induced arthritic animals, a dose-dependent reduction in paw swelling was exhibited in petroleum ether and n-butanolic treated fraction of *T. foenum-graecum*. As shown in Table 1, at the doses of 100 mg/kg of petroleum ether fraction and n-butanolic fraction, arthritic swelling was inhibited by 68.37 and 75.21 % ($P < 0.01$, 0.001), respectively, compared to the adjuvant control on the 35th day. Prednisolone treated group showed an inhibition of 78.63%.

Arthritis Assessment

The treatment of petroleum ether and n-butanolic fraction was initiated at the onset stage of polyarthritic development, i.e., day 14. During the initial phase of treatment, the articular indexes of the treated groups showed moderately significant ($P < 0.01$) difference with those of arthritic control group. However, after this phase, the indexes started to highly significant decrease ($P < 0.001$) in petroleum ether treated rats and moderately significant decrease ($P < 0.01$) in n-butanolic fraction treated rats [Figure 1] .

Table 1: Effect of petroleum ether and n-butanolic fractions of *Trigonella foenum-graecum* on paw volume

Groups and treatments	Paw volume in (ml)					
	0 day	7th day	14th day	21st day	28th day	35th day
Normal Control	0.35±0.04	0.32±0.06	0.31±0.04	0.35±0.05	0.36±0.08	0.34±0.08
Arthritic Control	0.34±0.07	0.80±0.13	1.50±0.12	1.85±0.11***	2.33±0.17***	2.34±0.10***
Prednisolone 10 mg/kg	0.33±0.08	0.76±0.15	1.50±0.22	1.07±0.20** (44.14)	0.68±0.13*** (71.86)	0.51±0.16*** (78.63)
Trigonella foenum-graecum, Pet. ether fraction 50 mg/kg	0.34±0.03	0.77±0.11	1.57±0.14	1.31±0.15* (29.18)	0.90±0.15** (61.37)	0.85±0.16** (63.67)
Trigonella foenum-graecum, Pet. ether fraction 100 mg/kg	0.35±0.04	0.78±0.14	1.56±0.12	1.23±0.15* (33.51)	0.82±0.14** (64.80)	0.74±0.10*** (68.37)
Trigonella foenum-graecum, n-butanolic fraction 50 mg/kg	0.34±0.9	0.76±0.10	1.56±0.11	1.22±0.11* (34.05)	0.73±0.11** (68.66)	0.64±0.11** (72.64)
Trigonella foenum-graecum, n-butanolic fraction 100 mg/kg	0.33±0.11	0.78±0.10	1.55±0.14	1.11±0.15** (40.00)	0.67±0.12*** (71.24)	0.58±0.11*** (75.21)

Values are expressed as mean±SEM, n=6 in each group; *P<0.05, compared to arthritic control. **P<0.01, compared to arthritic control. ***P<0.001, compared to arthritic control. SEM: Standard error of the mean

Oxidative Stress Parameters

As shown in Table 2, MDA levels were observed to increase in Group II when compared with Group I. However, GSH levels and SOD activities were observed to decrease in Group II when compared with Group I. Administration of n-butanolic fraction at dose of 100 mg/kg causes highly significant decrease ($P < 0.001$) in MDA levels and increase in GSH and SOD activities however, petroleum ether fraction showed moderate decrease ($P < 0.01$) in MDA level and increase in GSH and SOD activities [Table 2] .

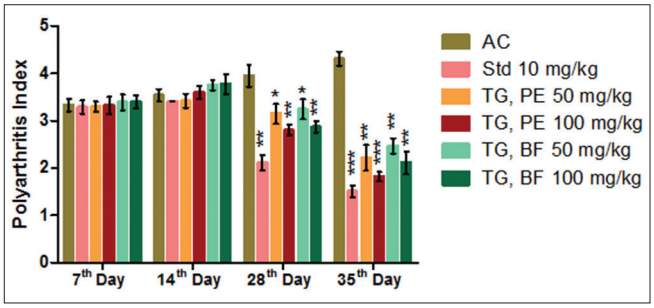

Figure 1: Effect of petroleum ether and n-butanolic fractions of *Trigonella foenum-graecum* on polyarthritic index. Values are expressed as mean ± standard error of the mean, *n*=6 in each group; **P* < 0.05, compared to arthritic control. ***P* < 0.01, compared to arthritic control. ****P* < 0.001, compared to arthritic control

Membrane Marker Enzymes

There was marked increase in the activity of membrane marker enzymes (ALP, LDH, and ACP) in the arthritic rats when compared to control rats. There is a significant increase in membrane marker enzymes of arthritic rats. Treatment with petroleum ether and n-butanolic fraction showed a moderately significant ($P < 0.01$) decrease in the activity of membrane marker enzymes was seen in animals treated at 100 mg/kg [Table 2] .

DISCUSSION

The word medicinal plants include various types of plants used in herbalism and some of these plants have medicinal activities. These medicinal plants consider as rich resources of ingredients which can be used in drug development and synthesis.[20] In arthritic conditions, apart from the crosslink resorption at the site of inflamed joints, there may be increased resorption due to general bone loss associated with disease activity.[19]

In adjuvant model, the affected cartilages are infiltrated by blood-derived cells, mainly neutrophils, macrophages and dendritic cells.[21]

Table 2: Effect of petroleum ether and n-butanolic fractions of *Trigonella foenum-graecum* on oxidative stress and membrane marker enzymes

Groups and treatments	Oxidative stress parameters			Membrane marker enzymes		
	MDA nmol/mg of protein	GSH µmol/g of protein	SOD U/mg of protein	ALP (µmoles of phenol formed/h/mg protein)	LDH (µmoles of pyruvate liberated/min/mg protein)	ACP (×10^{-2} µmoles of phenol formed/min/mg protein)
Normal control	4.51±0.22	6.47±0.20	7.80±0.30	0.40±0.05	7.43±0.19	2.10±0.12
Arthritic control	13.15±0.41***	3.30±0.14***	2.14±0.13***	0.95±0.01***	17.84±0.22***	5.22±0.14**
Prednisolone 10 mg/kg	6.52±0.15***	6.20±0.10***	5.18±0.40**	0.55±0.02***	10.30±0.11***	2.30±0.17**
Trigonella foenum-graecum, Pet. ether fraction 50 mg/kg	9.67±0.21*	5.78±0.39*	4.89±0.56*	0.78±0.06*	12.98±0.30**	3.74±0.23*
Trigonella foenum-graecum, Pet. ether fraction 100 mg/kg	8.48±0.34**	5.90±0.36**	5.12±0.43**	0.66±0.03**	11.13±0.17**	2.37±0.23**
Trigonella foenum-graecum, n-butanolic fraction 50 mg/kg	9.34±0.34**	5.18±0.22**	4.88±0.21*	0.65±0.01**	12.20±0.24**	3.10±0.12*
Trigonella foenum-graecum, n-butanolic fraction 100 mg/kg	8.14±0.21***	6.13±0.40***	5.45±0.27***	0.55±0.01**	10.18±0.14***	2.96±0.14**

Values are expressed as mean±SEM, *n*=6 in each group; **P*<0.05, compared to arthritic control. ***P*<0.01, compared to arthritic control. ****P*<0.001, compared to arthritic control. MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase, ALP: Alkaline Phosphatase, LDH: Lactate Dehydrogenase, ACP: Acid Phosphatase, SEM: Standard error of the mean

Chronic joint inflammation and erratic degrees of bone and cartilage corrosion are the characteristic of RA. Oxygen metabolism has an important role in the pathogenesis of rheumatoid arthritis. Reactive oxygen species are produced in several normal and abnormal processes in humans, counting atheroma, asthma, joint diseases, aging, and cancer. In rheumatoid arthritis, oxidative stress has been described as an important mechanism that underlies vicious proliferative synovitis and tissue damage.[22,23] One of the several approaches for the treat treatment of rheumatoid arthritis is to employ the use of various anti-oxidants. The seed of *T. foenum-graecum* is the well-documented medicinal plant reported to relieve rheumatic pains in the traditional system of medicine.

Preliminary phytochemical analysis of *T. foenum-graecum* showed the presence of steroids and terpenoids in petroleum ether fraction and flavonoids and glycosides in n-butanolic fractions.

It has been reported that adjuvant diseases can be induced by either Freund's complete agents supplemented by mycobacterium or N, N-dioctylddecyl-N', N-bis (2-hydroxy-ethyl) propanediamine. In the present study, chloroform and n-butanolic fractions were accessed in FCA induced arthritis. Butanolic fractions highly significantly reduced paw swelling on the 35th day as compared to petroleum ether fractions. This effect may be due to inhibitory effects on prostaglandin mediated pathways.[24]

The severity of arthritis was expressed as the arthritic score for each individual animal, being the sum of the measures of the four paws subtracted by the measures recorded before the immunization. The arthritic score of n-butanolic fraction was significantly lower as compared to petroleum ether fractions, indicating their anti-arthritic activity.

Lipid peroxidation is a critical mechanism of the injury that occurs during rheumatoid arthritis. The large amount of MDA in arthritic control group is consistent with the occurrence of damage mediated by free radicals. Treatment with petroleum ether and butanolic fractions produced a significant attenuation of MDA and effect is more significant with n-butanolic fraction as compared to petroleum ether fraction. The decrease in neutrophil accumulation by n-butanolic fraction treatment might be due to the inhibition of lipid peroxidation and the consequent decrease in the chemo tactic decrease of peroxide.[25] The production of free radicals that occurs with the progress of arthritis in the articular cartilage leads to decreased GSH and SOD levels as a result of their consumption during oxidative stress and cellular lysis,[26] which is evident by decreased levels of GSH and SOD in arthritic control group. Butanolic fraction causes significantly inhibited the decrease of GSH and SOD, probably by challenging for scavenging of free radicals, which in turn resulted in healing of antioxidant enzyme levels.

The altered enzyme activities in arthritis can be regarded as a guide of membrane marker enzyme commencement occurring in response to the metabolic need of degrading various constituents of cells such as mucopolysaccharides and glycoprotein accumulated in tissues due to arthritis linked with vasculopathies.[27]

Various membrane marker enzymes such as ACP, LDH, and ALP were used to be an important index for the assessment of the integrity of the membrane and are dependable for the tissue damage and necrosis of hepatic tissue.[28,29] Increased activities of liver LDH, ALP and ACP were observed in arthritic rats and may be attributed toward persistent inflammation.

In the present study, the activity of membrane marker enzymes was markedly increased in the arthritic rats and significantly reduced after treatment. Butanolic fraction had significantly lowered the enzymatic activity as compared to petroleum ether fraction and it may be due to inhibition of rupture of membrane and release of membrane marker enzymes.

Butanolic fraction of *T. foenum-graecum* shown more effective anti-arthritic activity as compared to petroleum ether fraction. As in phytochemical analysis, n-butanolic fraction showed the presence of flavonoids and glycoside compounds. It is previously reported that various phytoconstituents like alkaloids, terpenoids, steroids and flavonoids have potent anti-inflammatory and anti-arthritic activity. Hence, the anti-arthritic potential of n-butanolic fraction may be due to the presence of flavonoids and glycoside compounds.

CONCLUSION

The severity of arthritic disease was correlated with the weakness of the cellular membranes, subsequent expulsion of the enzymes and generation of reactive free radical species.

Our results suggest that treatment of n-butanolic fraction has prominent anti-arthritic effect on adjuvant induced arthritis in rats. The mechanism of the effect might be due to either by modifying the cellular membrane in such a way that it is capable of preventing the release of membrane enzymes, and could retard spread of the inflammatory mediators or inhibition of production of free radicals. Hence from the basis of above studies, we concluded that n-butanolic fraction promote the stabilization of lysosomal membrane and modify the progression of disease.

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