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Anti-inflammatory and antioxidant activity of *Trachyspermum ammi* seeds in collagen induced arthritis in rats.

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Abstract

The generation of reactive oxygen species (ROS) and other reactive nitrogen species (RNS) might be implicated to have a role in the pathogenesis of rheumatoid arthritis. Modulatory agents derived from plants that have properties like scavenging of free radicals clearly have therapeutic potential against these diseases. The present study was aimed to investigate the possible antioxidant potential of *Trachyspermum ammi* on collagen induced arthritis (CIA) in Wistar rat. *Trachyspermum ammi* extract (TAE) in a dose of 100 mg kg^{-1} was orally administered to rat once daily for 21 days after immunization. The estimation of levels of oxidant products and the activities of antioxidant enzymes were carried out in the joints. The induction of arthritis significantly increased the levels of oxidative stress markers like thiobarbituric acid reactive substances and inflammation markers like elastase. The level of non-enzymatic antioxidant, reduced glutathione (GSH) and the activities of enzymatic antioxidants like superoxide dismutase and catalase decreased. The study revealed that the treatment with TAE was effective in bringing significant changes on all the parameters studied as compared with CIA rat. Supplementation with *T.ammi* reversed the oxidative changes in all the parameters suggesting either termination of cellular infiltration or limiting the generation of oxidants following CIA in rats and might have potential value in the treatment of inflammatory disease.

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Introduction

Rheumatoid arthritis (RA) is a well known human autoimmune disease characterised by chronic inflammation of the synovial joints and subsequent progressive, erosive destruction of articular tissue [1-3]. RA affects about 1% of the human population globally [4]. There has been progress in defining aetiology and pathogenesis of this disease but exact mechanism still remains obscure. Recently, some studies have reported the effects of the administration of synthetic and naturally occurring compounds on the progression of collagen induced arthritis in experimental animals [5].

There has been strong growing evidence that the generation of reactive oxygen species (ROS) and other reactive nitrogen species (RNS) might have a role in the pathogenesis of rheumatoid arthritis [6-9]. ROS and 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 [10]. 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 [11]. 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 [12] and may lead to the clinical manifestations.

Current treatment modalities for RA either produce symptomatic relief (NSAIDs) or modify the disease process (DMARDs). Though effective, their use is also limited by their side effects including gastrointestinal ulcers and perforation, cardiovascular complications and emergence of opportunistic infections due to immunosuppressant [13]. Owing to the chronic nature of disease and side effects associated with long-term use of these agents, 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 [14].

Trachyspermum ammi (L.) Sprague ex Turril (Umbelliferae) has been used for centuries as a therapeutic agent for the treatment of inflammatory diseases and disorders of the digestive tract by the practitioners of the Ayurvedic and Unani systems of medicine [15, 16]. The seeds are widely used in India and eastern Asia, both in diet and in traditional system of medicine for its diuretic, anti-emetic, analgesic, antiasthmatic, antidyspnea, and carminative properties [17, 18]. It's antibacterial [19], antihelminthic , antifungal [20], antihypertensive, antispasmodic, bronchodilator, and hepatoprotective activities have been described [21].

Despite its widespread use in traditional medicine for the treatment of pain and inflammatory disorder, there is a dearth of scientific evidence regarding its antioxidant and anti-inflammatory activity. Only a few studies have reported the anti-inflammatory activity of this plant in experimental models [22]. To the best of our knowledge, there is no report available on the antiarthritic and antioxidant activity of *T. ammi* in collagen induced arthritis model. Therefore, we have selected this plant and tried to elucidate the antioxidant and antiarthritic activity of its seed extract.

Materials and methods

Chemicals

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 in this investigation were of research grade.

Test drug and its identification

Seeds were procured from local market, New Delhi and authenticated by Dr. H.B Singh, Scientist 'F', Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi. The drug sample was deposited with voucher specimen number (NISCAIR / RHMD / Consult / 2008-2009 / 1098 / 129). The dried seeds were extracted with H_2O: MeOH (20:80). The extract was dried under reduced pressure to a residue (189 g). Total phenols measured in terms of gallic

acid equivalent were 76.45±6.37 µg/ml and flavonoids in terms of quercetin equivalent were 32.77±2.51 µg/ml. Quantitative estimation of thymol was done by HPTLC densitometry using a precoated silica gel 60 F_{254} TLC plate (Merck) of 0.2 mm thickness. The solvent system was Toluene: ethyl acetate (93:7). The total thymol content in TAE was found Fig. 1 to be 3.17 % (w/w).

Animals

Male Wistar rats (150-170g) were used. 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.

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

Arthritis was induced in rats as described previously [23]. Collagen Type II from bovine nasal septum was dissolved in 0.05 M acetic acid at a concentration of 2 mg/ml was emulsified with an equal volume of Freund's adjuvant complete (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. All rats were randomly assigned to three groups of six animals each. The first group served as control and saline was given orally, the second was collagen induced arthritis (CIA), the third was administered TAE (100 mg kg^{-1} body weight) daily, starting from day 0 following immunization. The dose of TAE was selected from literature and in vivo studies demonstrating the anti-inflammatory efficacy without any resultant toxicity as previously done in carrageenin model [22].

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 [24] from 0 to 4.

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.

Biochemical analyses

Biochemical parameters were carried out in articular joints. The assay of TBARS was done according to Utley et al., 1967 [25], GSH was measured in the groups following the method Sedlak and Lindsay,1968[26]. The supernatant was centrifuged at 12,000 × *g* for 5 min, and resultant PMS is used to carry out elastase, CAT, GSH, SOD and NO. ELA levels in the articular joints were evaluated as an index of polymorphonuclear leukocyte (PMNs) accumulation and activation in the inflamed tissue as described earlier [27].CAT activities were determined by the method of Sinha, 1972[28]. SOD activity was measured according to the method described by Beauchamp and Fridovich, 1971[29]. NO levels were determined with Griess method[30] .The protein concentration of the tissue was measured by the method of Bradford, 1976[31].

Histological examinations

Rats were sacrificed at 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 [32]. Tissue sections (5 µm thick) were stained with haematoxylin–eosin for light microscope examination.

Protein content

Protein was determined by Bradford method [31] 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

Effect of TAE treatment on severity of arthritis.

After immunisation, animals began to show evidence of clinical inflammation at day 9 in one or more hind paws. The first manifestation of disease was erythema of one or more ankle joints, followed by involvement of the metatarsal and interphalangeal joints. In Table 1. We showed the incidence of arthritis with and without

treatment by TAE throughout the study period. The symptoms of arthritis in all groups were evident on 13 ±1 days.

Effect of TAE on articular elastase activity (ELA)

Elastase activity was assayed on the day 21st after completion of experiment in the studied groups. Very low ELA concentrations were measured in the joints of control rats (57.07±0.402 ng/g protein). However, elevated activity of this enzyme was seen in CIA + vehicle group (185.65±0.45 ng/g protein). Administration of the TAE showed a significant decrease ($p<0.001$) in ELA activity resulting reduction in neutrophils activation and infiltration (Fig.2).

TAE treatment decreased TBARS level

The effect of TAE on TBARS level was measured to demonstrate the oxidative damage on lipid (Fig. 3). A significant increase ($p<0.05$) in TBARS level was observed in CIA + vehicle group as compared to the control group. Treatment with TAE decreased TBARS level significantly by inhibiting lipid peroxidation in the cartilage tissue.

TAE restored the GSH level

The concentration of GSH was evaluated to estimate endogenous defences against hydrogen peroxide formation. Table 2. Shows the changes in GSH level evaluated in the joints (day 21) in the experimental groups. A marked decrease ($p<0.01$) in GSH concentrations was found in the joints of CIA + vehicle rats. Treatment with TAE significantly restored the reduction in GSH level ($p<0.01$).

Effect of TAE on SOD activity

SOD activity was evaluated to estimate endogenous defences against superoxide anions. Table 2 summarises the level of SOD. In control animals, normal SOD activity was 17.51 ± 0.059 Unit /mg of protein. In contrast, a significant decrease in this antioxidant level was seen in CIA+ vehicle rats (4.62 ± 0.075). Administration of TAE significantly ($p<0.01$) slowed the decline in SOD activity.

Effect of TAE on Catalase activity

Fig.4 shows Catalase activity evaluated at the day 21st in the joints. In the control group catalase activity was 9.35±0.013 µmol H_2O_2 consumed/min/mg protein. On the contrary, a substantial reduction in this enzyme was observed in the CIA+ vehicle rats (3.03±0.007). Also in this case the treatment with TAE was significantly ($p<0.01$) effective as compared to CIA+ Vehicle group.

Nitric oxide after TAE treatment

Estimation of nitrite is summarised in Fig.5. In the control group, the nitrite concentration was 3.45±0.019 µmol/mg wet tissue while CIA group showed a high nitrite level (12.55±0.035). Treatment with TAE reduced nitrite level significantly ($p<0.001$) as compared to the CIA group.

Histology

Histological studies carried out in rats following treatment with T. ammi extract revealed down regulation of the inflammatory infiltration with associated tissue damages (fig.6). Vehicle injected CIA rats demonstrated severe loss of the normal joint structure which was evident from the disrupted trabeculae within bone and massive cellular build up. These changes were associated with nodular necrosis within the cartilage and may be a driving force behind physical deficit appearing in the CIA rats. Oral supplementation with aqueous methanolic extract of T. ammi within the first acute episode resulted in reversal of the latter discussed changes. The articular tissue showed restoration of the normal structure of the bone and cartilage which could be due to the blockade of the autoimmunity triggered cellular penetration within the joints and is consistent with the restoration of the antioxidant status reflected in the biochemical parameters.

Discussion

Collagen induced arthritis (CIA) is well established animal model widely used for pharmacological evaluation of anti-arthritic agents, as it possesses many of the cellular and humoral immune events associated with human rheumatoid arthritis and therefore has a relatively high degree of validity [33].We investigated the anti-inflammatory and antioxidant potential of *T.ammi* seed extract in this model. Quantitative estimation of thymol in TAE was found to be 3.17 % (w/w).

TAE demonstrated significant antioxidant potential in collagen induced arthritis. Its anti-arthritic efficacy was also evident from the reduction in joint swelling throughout the observation period. To further validate the antiarthritic activity of the TAE, we evaluated elastase activity which is a marker for collagen degradation. Its activity is directly proportional to the accumulation and activation of polymorphonuclear leukocytes in the inflamed tissue [12] as it is released from stimulated granulocytes at the site of injury. We observed the significant decrease in its activity after TAE supplementation suggesting its role in stoppage of the inflammatory cell invasion which may

responsible for the stimulation of several other cell types like macrophages. Our result is in agreement with other studies shows that thymol inhibited elastase activity probably by inactivating calcium channels machinery[34].

The inflammation so caused by the infiltrating cells leads to the release of ROS and RNS [35-37]. Lipid peroxidation is considered a critical mechanism of the injury that occurs during RA. The large amount of TBARS found is consistent with the occurrence of damage mediated by free radicals. 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 [38].

Free radicals production that occurs during development of arthritis in the articular cartilage leads to decreased GSH level and antioxidant activity as a result of their consumption during oxidative stress and cellular lysis [39, 40]. GSH is the main non-enzymatic antioxidant in defending against oxygen free radicals. A reduction in the level of GSH may impair H_2O_2 clearance and promote formation of hydroxyl radical ($^{\bullet}OH$), the most toxic molecule of the cell, leading to oxidative insult [41].This decrease contributes to increased cellular damage by favouring attack by free radicals. Our data has shown that treatment with TAE significantly reversed the depleted level of GSH and SOD, probably by competing with scavenging of free radicals and as a result helped to maintain the integrity of cellular membranes in the injured cartilage. Together with our data and similar evidence in RA model further support the antioxidant property of TAE [22, 42].

Nitric oxide (NO) is an important signalling molecule, produced as part of the inflammatory response from activated cells and macrophages [43]. NO has several biochemical activities including mediating vasodilatation, directly scavenging superoxide, attenuating leukocyte adhesion, and activation and maintaining endothelial integrity [44]. Therefore, compounds that inhibit excessive NO production may have beneficial therapeutic effects in arthritis by blocking cartilage degradation [46]. In the present study, increased NO level has been detected in arthritic group similar to those previously reported in synovial fluids of patients with rheumatoid arthritis [35]. Treatment with TAE produced a significant decrease in nitric oxide level.

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. 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 TAE was able to reverse the histological changes to almost normal condition.

Conclusion:

Our study highlights the role of inflammatory cells regulated free radical production in the development of the pathology of RA and its treatment with TAE. Anti-oxidant and anti-inflammatory activity of the TAE extract may be attributed to the presence of flavonoids and phenols. Therefore, in light of the above facts and with previous studies it can be established that TAE mediates protection in CIA model via its potent antiarthritic and antioxidant effects. Finally we believe that TAE can be used as a favourable remedy for treatment of rheumatoid arthritis, pending further studies to elucidate exact mechanisms.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflict of interest to disclose.

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Effect of TAE on incidence and severity of arthritis in Wistar rats

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+ TAE	6/4 (66.6%)	1.62 ± 0.45	5	13 (12-14)

Table 1. 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.

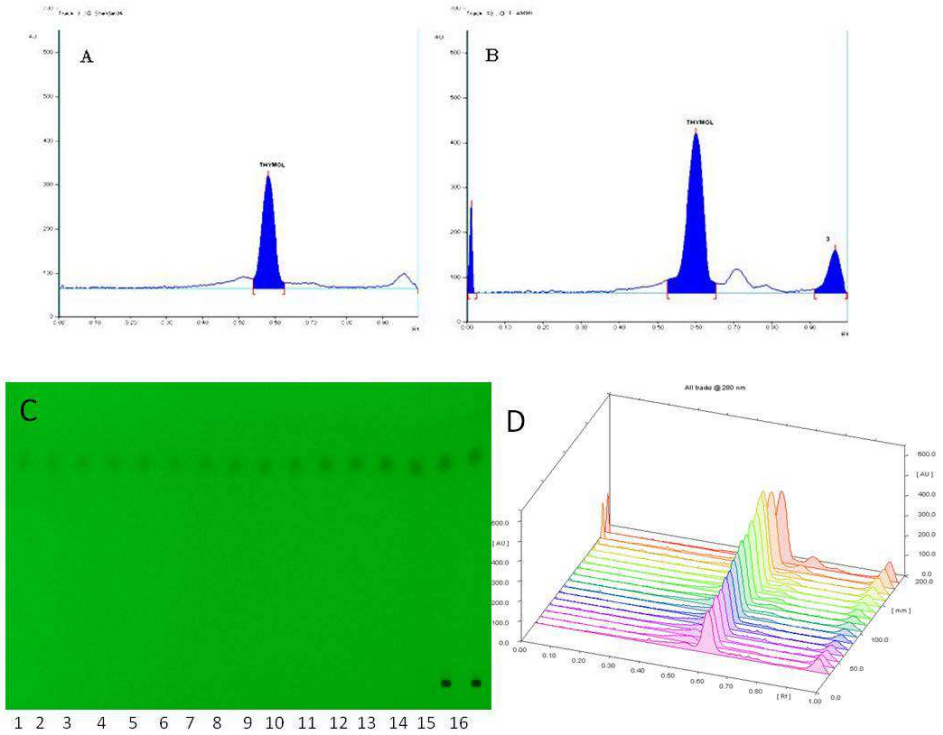

Fig.1 HPTLC chromatogram of Trachyspermum ammi extract (A) Standard Thymol (B) Trachyspermum ammi extract (C) Thymol standard sample from 1-14, T. ammi extract sample 15-16 (D) 3D graph at 280 nm.

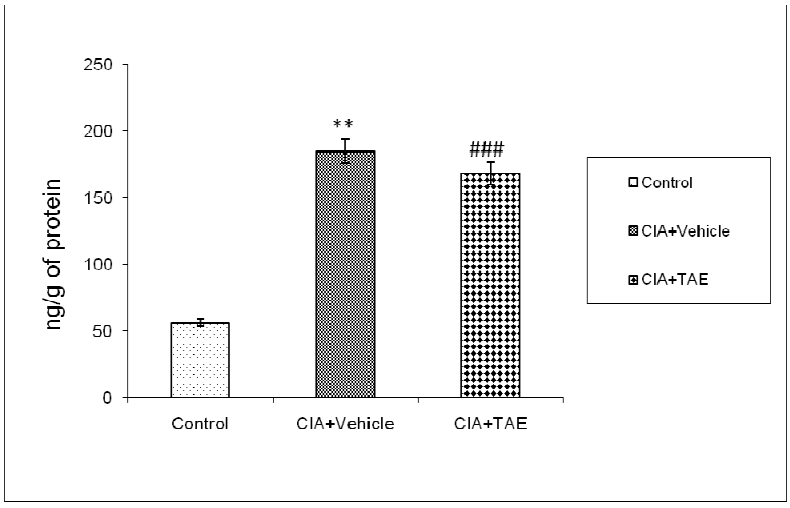

Fig.2 Articular elastase activity in joints of rats immunized with collagen type II after treatment with TAE (100mg/kg b.wt.). Data are expressed as Mean ± SEM of 6 rats.
** ($p<0.01$) Control vs. CIA + Vehicle group
($p<0.001$) CIA+ Vehicle vs. TAE.

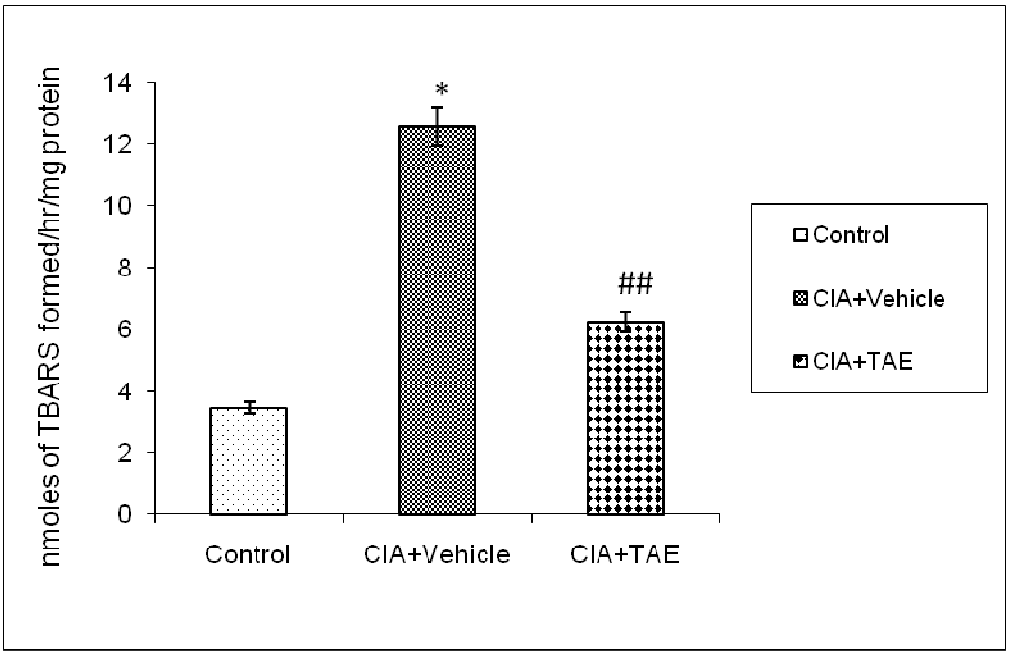

Fig.3 Lipid peroxidation in joints of rats immunized with collagen type II after treatment with TAE (100mg/kg b.wt.). Data are expressed as Mean ± SEM of 6 rats.
* ($p<0.05$) Control vs. CIA + Vehicle group
($p<0.01$) CIA+ Vehicle vs. TAE.

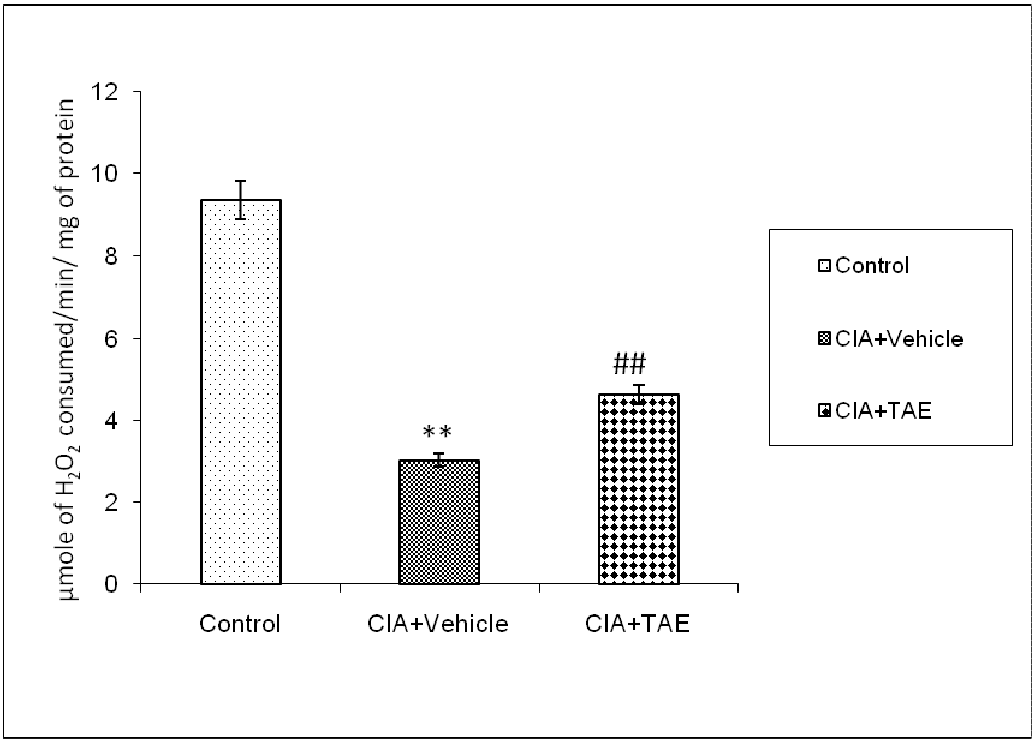

Fig. 4 Catalase activity in joints of rats immunized with collagen type II after treatment with TAE (100mg/kg b.wt.). Data are expressed as Mean ± SEM of 6 rats.
** ($p<0.01$) Control vs. CIA + Vehicle group
($p<0.01$) CIA+ Vehicle vs. TAE.

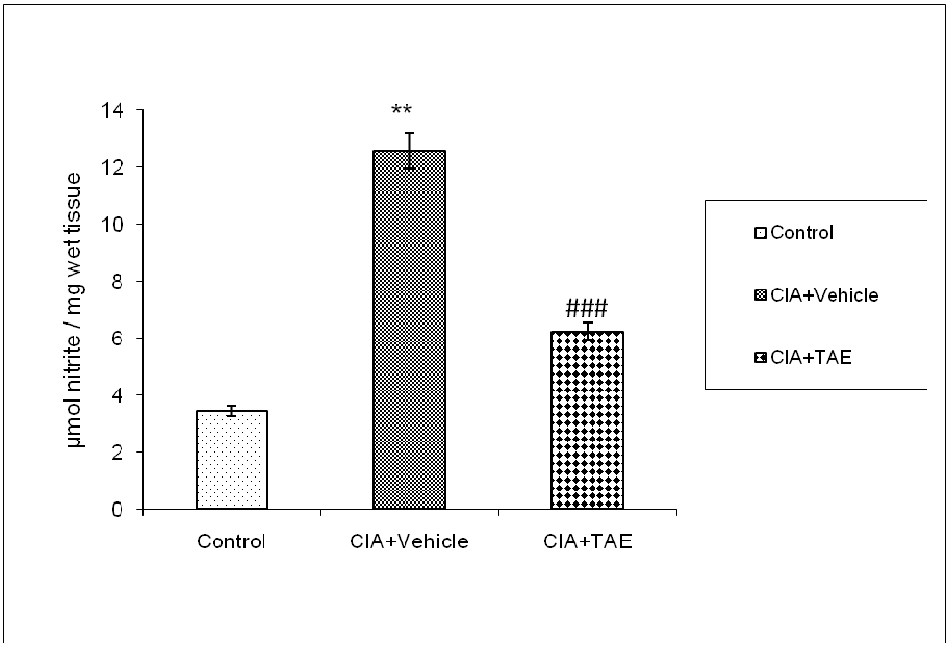

Fig. 5 Effect of TAE (100mg/kg b.wt.) treatment on articular nitrite content in joints of rats immunized with collagen type II after treatment with. Data are expressed as Mean ± SEM of 6 rats.
** ($p<0.01$) Control vs. CIA + Vehicle group
($p<0.001$) CIA+ Vehicle vs. TAE.

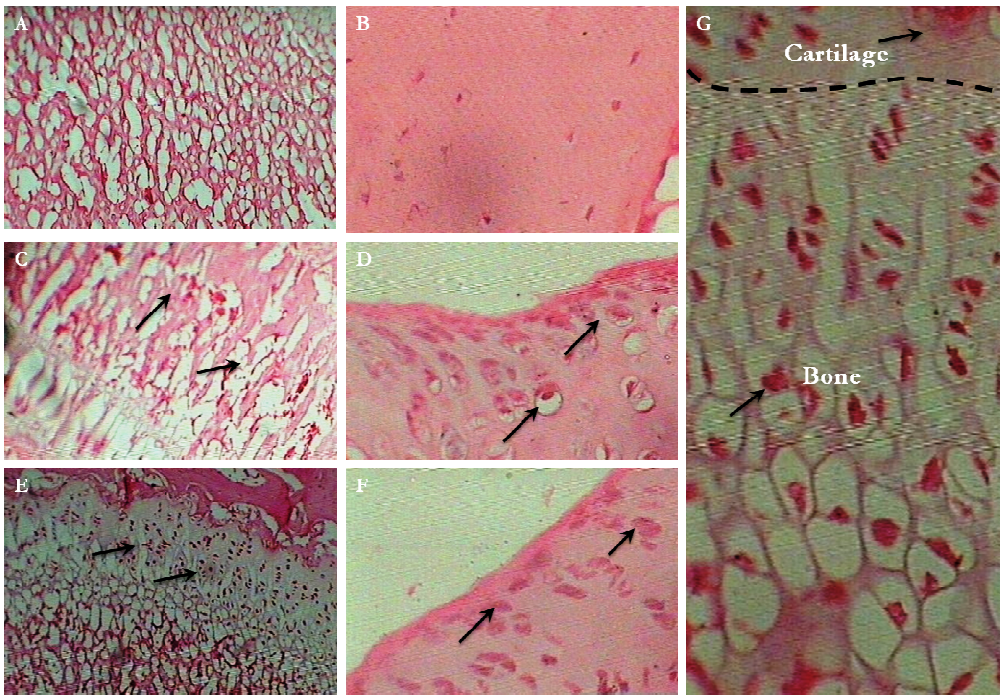

Fig. 6 Histological changes in joints of rats. Normal trabecular structure of the articulated femur (A) and cartilage in the knee sections from normal rats in comparison to the vehicle injected collagen induced arthritic rats demonstrating disruption of the bone articulature along with cellular infiltration in the synoviocyte layer (C) causing disatttchment of the bone from the cartilage. Vehicle injected CIA rat cartilage (D) displaying inflammatory cellular buildup and differentiated osteoclasts and nodular necrosis with pannus reaction. T. ammi (aqueous methanolic extract) reversed the tissue damage evident from the restoration of the trabecular structure with lesser inflammation (E) and articulated cartilage and bone. T. ammi ameliorated the necrosis and limited the cellular infiltration (F). (G) CIA rats demonstrating the inflammatory cellular infiltration within the span of the joint tissue from the cartilage to the sub chondral bone.

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