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Research Paper

Anti-arthritic and disease modifying activity of *Terminalia chebula* Retz. in experimental models

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Abstract

Objective This study evaluates the anti-arthritic effect of *Terminalia chebula* hydroalcoholic extract (TCHE) in experimental models and attempts to correlate the effect of treatment on macrophage-derived pro-inflammatory cytokine expression and extent of disease activity.

Methods Arthritis was induced in rats by subplantar administration of either formaldehyde or complete Freund's adjuvant (CFA). Joint size was measured at regular intervals by using a micrometer screw gauge. Serum and ankle joints of rats immunized with CFA were collected and subjected to ELISA for estimation of TNF-α level and immuno-histochemistry for detection of IL-1β, IL-6 and TNF-R1, respectively. An acute and 28-day oral toxicity study was carried out to evaluate the safety of the test drug.

Key findings TCHE produced a significant inhibition of joint swelling as compared with control in both formaldehyde-induced and CFA-induced arthritis. TCHE treatment also reduced serum TNF-α level and synovial expression of TNF-R1, IL-6 and IL-1β. Results of acute toxicity study showed that the oral LD50 of TCHE was >2000 mg/kg. Chronic administration also did not produce any significant physiological changes as compared with normal rats.

Conclusion Results indicate that the anti-arthritic activity of TCHE was at least in part due to its modulatory effect on pro-inflammatory cytokine expression in the synovium. We believe that TCHE has the potential to be used as a disease-modifying agent in treatment of rheumatoid arthritis.

Keywords adjuvant arthritis; formaldehyde arthritis; pro-inflammatory cytokines; *Terminalia chebula*

Introduction

Rheumatoid arthritis is a debilitating autoimmune disorder that has gained scientific interest owing to its progressive nature. It initially emerges as a local swelling and stiffness in the synovial joints and then progresses into a chronic multisystem disease. It is believed to be the result of a systemic reaction of the body against self epitopes in the synovium, leading to a heightened inflammatory response in the body. The exact aetiology of rheumatoid arthritis is not known and it is thought to be triggered by a variety of external and genetic factors.[1] The inflammatory environment in the synovium is primarily conditioned by macrophage cytokines such as TNF-α, IL-6, IL-1, GM-CSF, etc., and manifests as hyperplasia of the synovial membrane.[2] This, and subsequent infiltration of activated fibroblasts, T lymphocytes and plasma cells into the synovial membrane, contributes towards progressive destruction of the articular cartilage and maintenance of the chronic state of the disease.[3,4] Among these cytokines, TNF-α appears to be an important factor as its circulating levels have been shown to correlate with disease activity in patients with rheumatoid arthritis.[5]

Conventional drugs that are used in the treatment of rheumatoid arthritis either produce symptomatic relief (nonsteroidal anti-inflammatory drugs; NSAIDs) or modify the disease process (glucocorticoids, interleukin (IL)-1 receptor antagonist, and anti-tumour necrosis factor-alpha (TNF)-α drugs). Though effective, their use is also associated with a plethora of side effects including gastrointestinal ulcers, cardiovascular complications and emergence of opportunistic infections due to immunosuppression.[1] Owing to the chronic nature of disease and side effects associated with long-term use of these agents, patients with rheumatoid arthritis seek alternative methods of relief and are among the highest users of complementary and alternative medicine.[6,7]

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Terminalia chebula Retz. (Family: *Combretaceae*) is widely distributed in the tropics and is known as 'chebulic/black myrobala' in English and 'haritaki' in Sanskrit. In traditional systems of medicine the unripe fruit of *Terminalia chebula* (TC) is used in the treatment of gastrointestinal diseases, hepatomegaly, splenomegaly, chronic fever, anaemia, polyuria, metabolic disturbances, joint pain and rheumatoid arthritis.[8,9] The principal chemical constituents present in unripe TC fruit are D-galloyl glucose and tannins *viz.* chebulagic acid, chebulinic acid, ellagic acid, syringic acid, gallic acid and chebulic acid.[10]

Despite its widespread use in traditional medicine for the treatment of joint pain and arthritis, there is a dearth of scientific evidence regarding its anti-inflammatory and anti-arthritic activity. Only a few studies have reported the anti-inflammatory activity of this plant in experimental models. TC has been shown to inhibit carrageenan-induced paw oedema and compound 40/80-induced paw oedema in rats,[11] which can be attributed to its dual inhibitory action on COX–5LOX.[12] Additionally, TC has been shown to have a modulatory effect on the activity of regulatory T-cells in collagen-induced arthritis.[13]

Although these studies report the anti-inflammatory and anti-arthritic activity of TC, there is still no confirmatory evidence regarding the mechanism behind its efficacy in rheumatoid arthritis. Therefore, this study was carried out to scientifically evaluate the anti-arthritic and disease-modifying activity of TC with an aim to elucidate its probable mechanism of action.

Materials and Methods

Animals

Adult male Wistar albino rats (150–180 g) from our institutional breeding stock were used in the study. Rats were housed at 25 ± 2°C in clean polypropylene cages in groups of three with free access to food and water throughout the duration of the study. The experimental protocol was approved by the Institutional Animal Ethics Committee, All India Institute of Medical Sciences, New Delhi and all experiments were carried out in accordance with 'Guidelines for care and use of animals in scientific research (Indian National Science Academy 1998, Revised 2000)'.

Test drug

Dried immature fruits of TC were obtained and authenticated by a botanist at Jamia Hamdard University, New Delhi, India and a voucher specimen (PRL/JH/08/01) was submitted at the herbarium for future reference. They were coarsely ground and one kilogram of the powder was extracted by cold maceration with 50% methanol for 72 h. After filtration through cotton wool the solvent was evaporated under reduced pressure till a semisolid residue was obtained. The extract was brownish-black in colour and the total quantitative yield was 32.00% w/w. *Terminalia chebula* hydroalcoholic extract (TCHE) was further subjected to pharmacognostical standardization for detection of secondary plant metabolites[14] and was found to contain tannins and sugars. Quantitative estimation of total tannins was done colorimetrically by using Folin–Denis reagent. The absorbance corresponding to tannins and other oxidizable substances was read at 760 nm and the absorbance corresponding to non-tannins was read after precipitation of the tannins with gelatin. The difference was calculated and the total tannin content in TC was found to be 30.32%. Identification of chebulagic acid was carried out by comparing the retention time of sample with that of the standard. An RP-HPLC system (AGILENT 1200) equipped with C_{18} 4.6×150 mm column was used for separation. HPLC-grade acetonitrile–pH 2.7 phosphoric acid solution (80 : 20) was used as the mobile phase with a flow rate of 1 ml/min. The absorption peaks was detected at 270 nm and identified with authentic standard. The total chebulagic acid content in TC was found to be 10.2% w/w.

Formaldehyde-induced arthritis

Rats were divided into five groups ($n = 6$) and baseline ankle joint diameter was measured by using a micrometer screw gauge. Dose selection for indometacin and test drug was made on the basis of a pilot study carried out in our laboratory. All drug suspensions were freshly prepared in 1% gum acacia (vehicle) at the time of administration. Group I received the vehicle (2 ml/kg body weight) and served as the control, group II received 3 mg/kg indometacin[15] and groups III, IV and V received TCHE in doses of 20 mg, 40 mg and 80 mg/kg, respectively, for a duration of 10 days. Arthritis was induced by subplantar administration of 0.1 ml formaldehyde (2%v/v) into the left hind paw of all the rats on days 1 and 3.[16,17] Increase in joint diameter was measured on days 8, 9 and 10.

Complete Freund's adjuvant-induced arthritis

Grouping of rats, measurement of baseline ankle diameters and drug treatment was carried out as described under 'Formaldehyde-induced arthritis'. Arthritis was induced by the subplantar administration of 0.1 ml of complete Freund's adjuvant (CFA) (0.05% w/v *Mycobacterium butyricum* in mineral oil; Difco Laboratories, Detroit, MI, USA) into the left hind paw of the rat. This was designated as day 0. All groups received respective drug/vehicle treatment for a duration of 21 days starting from the day of immunization. Joint size measurements were carried out on days 3, 7, 14 and 21.[18] On Day 21, the rats were sacrificed and terminal blood collection was carried out. Serum was separated and TNF-α level was estimated by commercial ELISA kit (U-CyTech Biosciences, the Netherlands). The ankle joints of the injected paw were collected and stored at −80°C for immuno-histochemistry studies.

Immuno-histochemistry

The ankle joints were decalcified with 10% EDTA (pH 7.4) and frozen sections (6 μm) were made. After acetone fixation the sections were processed for immuno-histochemical analysis of TNF-R1, IL-1β and IL-6 expression by using anti-rat antibodies (Santa Cruz Biotech Inc, Santa Cruz, CA, USA) and an avidin–biotin based detection kit (Vector Laboratories, Burlingame, CA, USA). Expression of the cytokines/cytokine receptor was visualized by colour development using diaminobenzidine (DAB) substrate kit (Vector Laboratories, USA) and counterstaining with haematoxylin. The specimens were then air dried, cleared in xylene and mounted in DPX mountant.

Toxicity studies

Evaluation of acute oral toxicity of TCHE was carried out according to the OECD guidelines for testing of chemicals – 425.[19] A limit test (2000 mg/kg body weight) was performed using five male Wistar rats (150–180 g) from our breeding stock. All the rats were observed for behavioral changes and mortality for 14 days after administration of the dose.

Evaluation of 28-day oral toxicity of TCHE was carried out according to the OECD guidelines for testing of chemicals – 407.[20] Sixteen male Wistar rats (150–180 g) from our breeding stock were divided into two groups ($n = 8$). Group I received the vehicle (2 ml/kg body weight, 1% gum acacia) and served as normal control and group II received TCHE in a dose of 400 mg/kg body weight (five times the maximum dose tested in anti-arthritic studies). Drug/vehicle was administered daily for a duration of 28 days.

Statistical analysis

Difference between the groups was analysed by one-way analysis of variance followed by Dunnett's Multiple Comparison (GraphPad InStat; Version 3.05). $P < 0.05$ was considered significant.

Results

Effect of TCHE on joint swelling in formaldehyde-induced arthritis

Subplantar administration of formaldehyde produced an increase in ankle joint diameter of all the rats (Figure 1). Although all drug-treated groups showed a decrease in joint swelling when compared with control, the difference was significant only in group V (TCHE 80 mg/kg) on all the observational days. Groups II (indometacin) and IV (TCHE 40 mg/kg) showed significant decrease in joint swelling on days 9 and 10. Maximum inhibition of joint swelling was produced by TCHE (80 mg/kg) throughout the study.

Effect of TCHE on joint swelling in complete Freund's adjuvant-induced arthritis

Immunization with CFA produced an increase in the ankle joint diameter of all the rats. Maximum joint swelling in all the groups was observed on day 3, following which there was a gradual decrease towards day 21. The exception to this trend was group I (control), in which there was a slight increase in joint swelling after day 14 (Figure 2).

Drug treatment produced a significant reduction in joint swelling as compared with control on all observational days. Inhibition of joint swelling by TCHE at the two lower doses (20 and 40 mg/kg) was similar to each other. At the highest dose tested, TCHE (80 mg/kg) was comparable to indometacin in reducing CFA-induced joint swelling.

Effect of *Terminalia chebula* hydroalcoholic extract on serum tumour necrosis factor-α in complete Freund's adjuvant-induced arthritis

Serum TNF-α level in normal rats was not in the detectable range for the ELISA kit that was used in the study (U-CyTech Biosciences, Utrecht, the Netherlands). CFA administration produced an increase in the serum TNF-α levels in both vehicle-treated and drug-treated groups (Figure 3). Although TCHE produced a dose-dependent decrease in the serum TNF-α level as compared with control, the decrease was

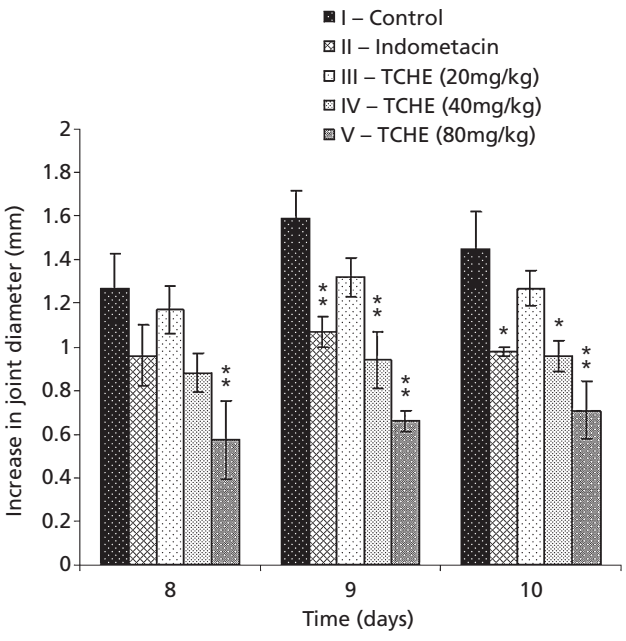

Figure 1 Effect of *Terminalia chebula* extract treatment on joint swelling in formaldehyde-induced arthritis in rats. TCHE = *Terminalia chebula* hydro-alcoholic extract. All values are mean ± SE. Statistical analysis by one-way analysis of variance followed by Dunnett's Multiple Comparison. *$P < 0.05$, **$P < 0.01$ as compared with control.

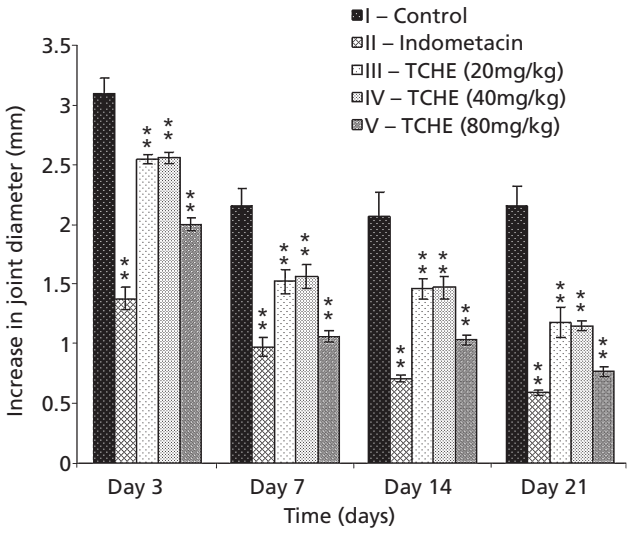

Figure 2 Effect of *Terminalia chebula* extract treatment on joint swelling in complete Freund's adjuvant-induced arthritis in rats. TCHE = *Terminalia chebula* hydro-alcoholic extract. All values are mean ± SE. Statistical analysis by one-way analysis of variance followed by Dunnett's Multiple Comparison. **$P < 0.01$ as compared with control.

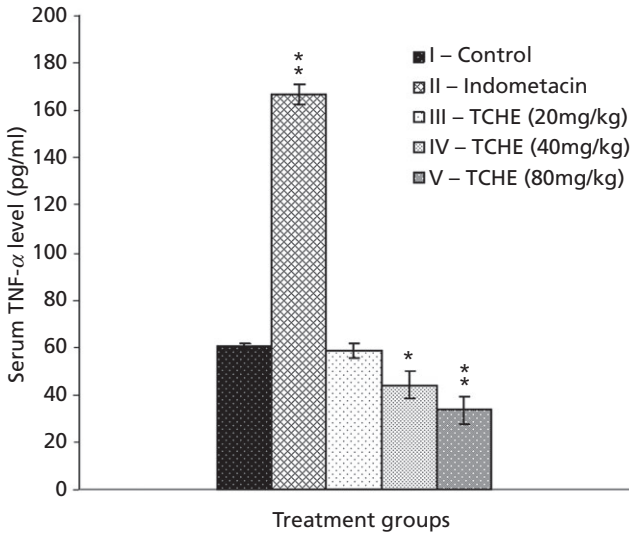

Figure 3 Effect of *Terminalia chebula* extract treatment on serum tumour necrosis factor-α level in complete Freund's adjuvant-induced arthritis in rats. TCHE = *Terminalia chebula* hydro-alcoholic extract. All values are mean ± SE. Statistical analysis by one-way analysis of variance followed by Dunnett's Multiple Comparison. *$P < 0.05$, **$P < 0.01$ as compared with control.

significant only in the groups treated with a dose of 40 mg/kg and 80 mg/kg. However, indometacin treatment of rats produced a significant and >2.5-fold increase in the TNF-α levels as compared with control.

Immuno-histochemistry of the synovial joint

Photomicrographs of synovial joint sections taken after incubation with anti-IL-1β, anti-IL-6, anti-TNF-R1 antibodies and an avidin-biotin detection kit, followed by colour development using DAB substrate, demonstrated the localization of large amounts of corresponding cytokines/cytokine receptor in the synovium of control rats (Figure 4). Drug treatment produced a reduction in the expression of these cytokines/cytokine receptor, with maximum reduction being produced in the group treated with TCHE 80 mg/kg.

Toxicity profile of the test plant

Administration of TCHE at a dose of 2000 mg/kg did not produce any behavioral abnormalities in the rats. No mortality was observed for 14 days after drug administration. As all the tested rats survived, the oral LD50 of TCHE in rats was found to be >2000 mg/kg body weight.

Chronic administration of TCHE at a dose of 400 mg/kg body weight for 28 days also did not produce any significant physiological changes as compared with normal control (data not shown). Although there was a marginal increase in red blood cell count and SGOT (serum glutamate oxaloacetate transaminase) level and a marginal decrease in body weight, white blood cell count, % haemoglobin, SGPT (serum glutamate pyruvate transaminase) level and percentage organ weight of liver, these changes were not statistically significant. However, chronic administration of TCHE (400 mg/kg) produced a statistically significant decrease in SGPT level and an increase in bleeding time as compared with normal control, but these changes were also within physiological limits. All other parameters remained unaltered.

Discussion

This study was carried out to evaluate the anti-arthritic activity of TCHE in two experimental models *viz.* formaldehyde-induced arthritis and CFA-induced arthritis. Additionally, we also evaluated the disease-modifying activity of TCHE by immuno-histochemical analysis of inflammatory cytokine/receptor expression in the synovium of arthritis rats. Formaldehyde-induced inflammatory arthritis is believed to be due to protein denaturation at the site of administration[21] and it is primarily mediated by arachidonic acid-derived autacoids. In our study TCHE demonstrated significant anti-arthritic activity and was found to be superior to indometacin throughout the observation period. This anti-arthritic activity could be attributed to the COX-5LOX dual inhibitory activity of chebulagic acid, which would have resulted in the decreased generation of inflammatory mediators.[12] This same mechanism may also have contributed to the increase in bleeding time seen during the chronic toxicity study.

The CFA-induced arthritis model has been widely used to evaluate drugs with potential anti-arthritic activity. This model has been shown to share a number of clinical and immunological features with human arthritis and is therefore used with a relatively high degree of validity.[18] In this model along with the measurement of joint diameter, we also evaluated three key cytokines that are primarily secreted by macrophages, *viz.* TNF-α, IL-6 and IL-1β. Additionally, we also checked the expression of TNF-R1 in the synovium as most of the pathological effects of TNF-α are known to be mediated through this receptor subtype.[5,22]

Our study showed a consistent decrease in joint swelling with all TCHE doses. None of the treated groups showed an increase in joint diameter from days 14 to 21, as seen in the case of control rats. The increase in joint swelling after day 14 is believed to be the result of a heightened cell-mediated immune response, characteristic of the late phase in this model. The serum TNF-α level was also found to be reduced in all TCHE-treated groups as compared with control. However, indometacin treatment produced an increase in serum TNF-α level, which could have been the result of gastrointestinal damage caused by its long-term use.[23–26]

Immuno-histochemistry studies also correlated with the aforementioned finding, as expression of TNF-R1, IL-1β and IL-6 was found to be reduced in the synovium of rats treated with TCHE (80 mg/kg) and indometacin. Even though indometacin was more effective in reducing joint swelling as compared with TCHE (80 mg/kg), the expression of cytokines/cytokine receptor in the synovium of indometacin-treated rats was intermediate to that of control and TCHE-treated groups. This superior effect on the cytokine profile despite a lower inhibition of joint swelling as compared with indometacin indicates that TCHE may also act through a mechanism other than COX– LOX inhibition.

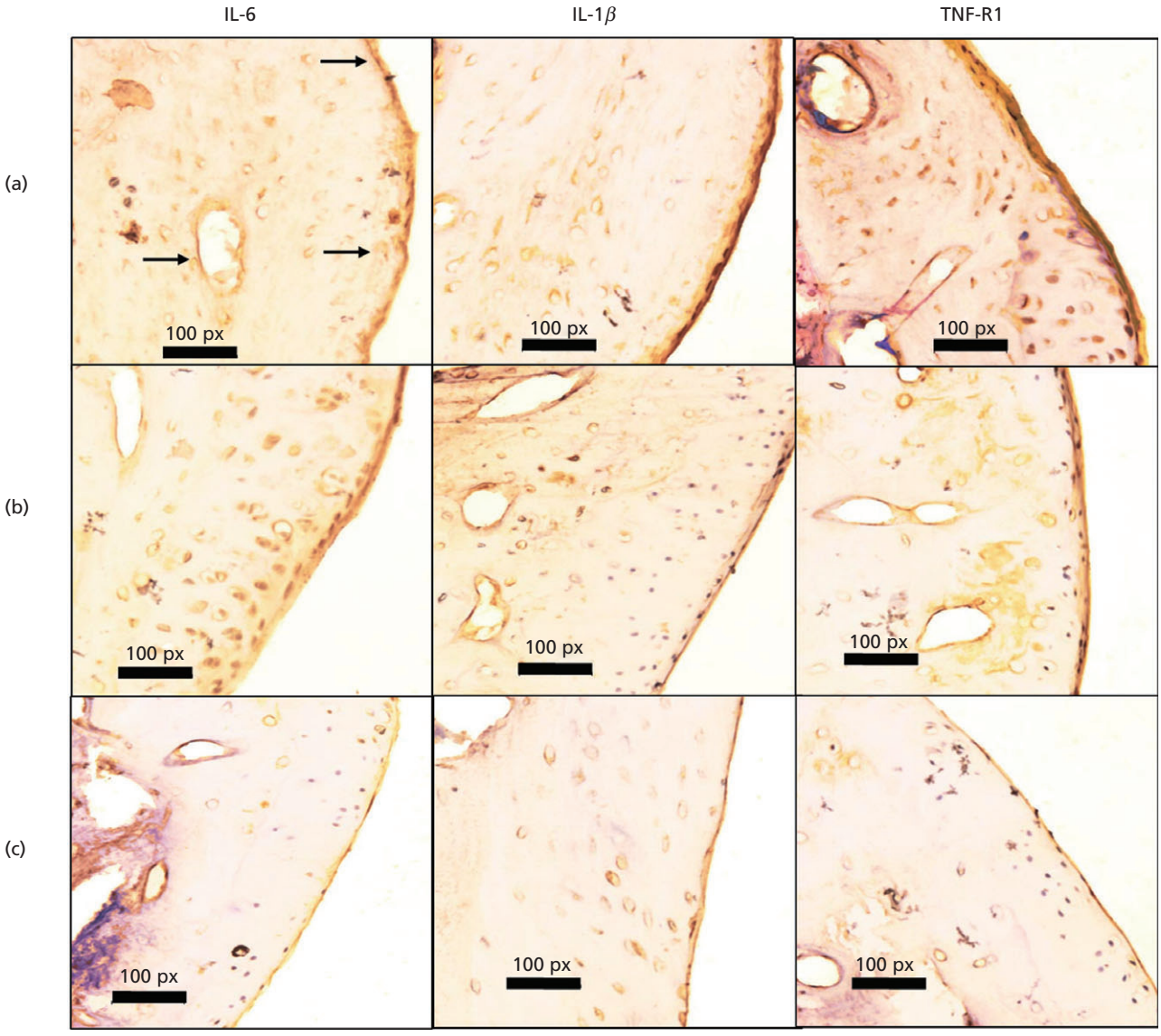

Figure 4 Effect of *Terminalia chebula* extract treatment on cytokine/cytokine receptor expression in rat synovial joint. Sections are 6 μm thick and photomicrographs are taken at 40×. TCHE = *Terminalia chebula* hydro-alcoholic extract. (a) control rat; (b) indometacin-treated rat; (c) TCHE (80 mg/kg)-treated rat. Bold arrows = DAB staining (yellow-brown) of the synovial membrane depicting presence of respective cytokines.

Conclusion

Results of this study indicate that the anti-arthritic activity of TCHE was at least in part due to its modulatory effect on the cytokine profile, along with a decrease in the production of inflammatory arachidonic acid derivatives. Although the exact mechanism underlying decrease in pro-inflammatory cytokine expression by TCHE is elusive at the moment, further studies using in-vitro expression systems may be helpful in identifying the same. In our study TCHE was found to have an oral LD50 value above 2000 mg/kg body weight. Chronic administration also did not produce any pathological changes in the tested rats, thus demonstrating its safety on long-term use. Based on the outcome of this study, we believe that TCHE has the potential to be used as a disease-modifying agent in the treatment of rheumatoid arthritis.

Declarations

Conflict of interest

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

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