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Original Article

Evaluation of the anti-arthritic activity of *Cinnamomum cassia* bark extract in experimental models

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ABSTRACT

Background: *Cinnamomum cassia* iswidely used as a traditional medicinal plant for the treatment of rheumatoid arthritis.
Objective: The present study aimed to assess the anti-arthritic activity of *C. cassia* bark hydroalcoholic extract (CCHE) in different arthritic animal models.
Methods: In formaldehyde model, sub-plantar administration of 0.1 ml of formaldehyde (2% v/v) into the right hind paws of Wistar albino rats on days 0 and 3. The rats were divided into six groups as follows: normal control, disease control, indomethacin group (3 mg/kg, p.o.) and three groups, treated with 50, 100 and 200 mg/kg CCHE (p.o.). Joint diameter was measured, and ankle joints were collected for MDA and GSH measurements. In complete Freund's adjuvant (CFA)-induced arthritis model, CFA was injected into the sub-plantar surface of the right hind paw in rats. Joint diameter was measured, and serum TNF-α and IL-1β were measured. Histopathological and immunohistochemical analyses were also performed.
Results: CCHE treatment significantly ($p < 0.01$) reduced MDA levels and joint swelling in a concentration-dependent manner in rats with formaldehyde-induced arthritis, in which GSH levels were elevated ($p < 0.01$). In rats with CFA-induced arthritis, CCHE treatment significantly reduced joint swelling as well as IL-1β and TNF-α levels ($p < 0.01$). TNF-α receptor expression was decreased in rats treated with indomethacin or CCHE.
Conclusion: Based on these findings, it can be concluded that *C. cassia* possesses anti-arthritic properties.

1. Introduction

Rheumatoid arthritis (RA) is one of the most prevalent chronic autoimmune diseases. Its early stages involve local swelling and stiffness in synovial joints before advancing to a chronic multisystem disease. RA causes symmetrical polyarthritis of large and small joints, typically presenting between 30 and 50 years of age.[1] Symptoms are severe in the morning because of inactivity. Increases in both the cellularity of synovial tissue and joint damage due to inflammatory reactions are the

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pathological features of RA.[2] The key inflammatory cascades involved in RA include the increased production and expression of tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). The primary cause of augmented TNF-α and IL-1β production is the interaction among T and B lymphocytes, synovial-like fibroblasts and macrophages. This development leads to further increases in the formation of other cytokines such as IL-1β, IL-6 and IL-18, leading to continuous inflammation and joint damage.[3]

Although currently available treatments have improved efficiency, the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin; disease-modifying anti-rheumatoid drugs (DMARDs), such as methotrexate, sulfasalazine, leflunomide and hydroxychloroquine and corticosteroids, such as prednisolone and methylprednisolone is associated with several adverse reactions. Hence, patients with musculoskeletal disorders have sought alternative methods for symptomatic relief. The published literature reveals that patients prefer complementary and alternative medicine.[4]

Cinnamomum cassia, also named Chinese cassia or Chinese cinnamon, is an evergreen plant. The genus *Cinnamomum* is a member of Lauraceae family, and it contains approximately 250 species that are distributed in China, India, Indonesia, South America, Hawaii and Australia.[5] *C. cassia* is an herbaceous tree, having leathery leaves with an opposing arrangement, and it is up to 10 cm in length with a long acuminate tip. It possesses active constituents such as cinnamic aldehyde, cinnamic acid and coumarin, which are responsible for most of its protective activities. It is commonly used to treat conditions, such as dyspepsia, gastritis, blood circulation disturbances and inflammatory diseases, in traditional Chinese medicine. The bark extract of *C. cassia* has potent anti-inflammatory activity,[6] and it also exerts anti-bacterial effects against clinically antibiotic-resistant isolates of *Bacillus megaterium* and *Enterococcus faecalis*.[7] Other parts of *C. Cassia*, such as its leaves, buds and bark, have displayed prominent antioxidant activities.[8] In one study, *C. cassia* extract exhibited anti-proliferative activity in Hep G2 cells.[9] SiHa cells treated with *Cinnamomum* water extract displayed reduced colony formation compared to the control cells.[10] The hexane extract of *C. cassia* exhibited high anti-proliferative activity against the human breast cancer cell lines MCF-7 and MDA-MB-231.[11] In addition to these activities, *C. cassia* additionally possesses anxiolytic effects.[12] The aqueous extract of the bark of *C. cassia* Blume displayed protective effects against neuronal cell death.[13] Some studies also reported that the essential oil of *C. cassia* potentiated the anti-fungal effect of amphotericin B.[14] The methanolic extract of *C. cassia* concentration-dependently decreased melanin content in B16 cells.[15] Cinnamaldehyde has been found to be responsible for most of the activities of *C. cassia* including its anti-tyrosinase,[16] anti-oxidant[17] and anti-cancer activities.[18] *C. cassia* administration was illustrated to decrease triglyceride and low-density lipoprotein levels in hypercholesterolaemic rats.[19] In TNF-α-activated endothelial cells, *C. cassia* treatment decreased the expression of vascular cell adhesion molecule-1.[20]

The use of natural medicines and other complementary therapies in the management of chronic conditions is well researched. However, despite their increased use, data supporting the efficiency and safety of many complementary therapies are lacking.[21] Thus, our study evaluated the potential effects of a standardized hydroalcoholic extract of *C. cassia* bark (CCHE) in experimental models of arthritis.

2. Methods

2.1. Plant material

CCHE was procured from Natural Remedies Pvt. Ltd. (Bangalore, India; Batch No. PC/BCCEX/2013LOT005). The coarsely powdered bark of *C. cassia* (10 kg) extract with deionised water (50 l) by reflux at 85-90° for 1 h. Filter and repeated the process by adding deionised water (40 l). The combined filtrate was distillate at 75 °C under vacuum to acquire whole solids of 25–30% and spray the solution at inlet temperature of 180 °C and out let temperature of 100 °C and obtained 1 kg brown powder was *C. cassia* extract.

High Performance Liquid Chromatographic (HPLC) System Shimadzu LC 2010 A with UV and PDA detector in combination with LC solution was software used for the analysis. Phenomenex-luna/hibar column was used; 5 u C-18; Size: 250 × 4.60 mm having column oven temperature at 25 ± 1 °C. The mobile phase comprised methanol–acetonitrile–water (35:20:45). The solution was filtered through 0.45 μm membrane filter and degassed with the help of sonicator for 3 min and 100% acetonitrile used as an eluent B. The detecting wavelength was 254 nm and flow rate remained 1.0 mL/min. HPLC chromatogram of CCHE used in this experiment is available as supplementary figure.

2.2. Chemicals, drugs and instruments

Indomethacin was purchased from Sigma Chemical Company (St. Louis, MO, USA). CFA was procured from Difco (USA). Serum TNF-α ELISA kit was obtained from Diaclone SAS (France; Cat. No. 865.000.096). Serum IL-1β ELISA kit was acquired from Ray Biotech (USA; Cat. No. ELR-IL1b).

2.3. Animals

This study was conducted after obtaining approval from the Institutional Animal Ethics Committee of AIIMS, New Delhi (Approval No. 980/IAEC/16). Adult female Wistar albino rats (age 6–8 weeks), weighing 150–200 g, were obtained from the Central Animal Facility of AIIMS and housed in polypropylene cages with *ad libitum* access to food and water under standard laboratory conditions, including normal 12-h/12-h light-dark cycles, temperature of 25 ± 2 °C and 55–65% relative humidity.

2.4. Formaldehyde-induced arthritis

Wistar rats with formaldehyde-induced arthritis were divided into six groups ($n = 6$) as follows: group I, normal control (saline treatment without formaldehyde exposure); group II, disease control (formaldehyde exposure alone); group III, indomethacin treatment (3 mg/kg, p.o.) and three groups,

treated with 50, 100 and 200 mg/kg CCHE (p.o.), respectively. Each treatment was administered via oral gavage for 10 days. Arthritis was induced via the sub-plantar administration of 0.1 ml of formaldehyde (2%) into the right hind paws of animals in each group (except normal control) on days 0 and 3, 30 min after the administration of vehicle/drugs.[22] Joint diameter was measured at baseline and on days 8–10 using a micrometer screw gauge. A 10% tissue homogenate was prepared in ice-chilled phosphate buffer (0.1 M, pH 7.4) to measure MDA[23] and GSH levels.[24]

2.5. *Complete Freund's adjuvant (CFA)-induced arthritis*

Wistar rats ($n = 6$) were divided into six groups. Micrometer screw gauge was used for baseline recording of the joint diameter. Normal saline was administered to group I and served as the normal control. Group II served as a disease control. Group III received the standard drug indomethacin (3 mg/kg) and three groups, treated with 50, 100 and 200 mg/kg CCHE (p.o.), in geometrically progressive doses. Arthritis was induced via the sub-plantar administration of 0.1 ml of CFA (0.05% w/v *Mycobacterium butyricum* in mineral oil) into the right hind paw of animals in all groups (except normal control) 30 min after the administration of the vehicle/drug. This was designated as day 0. After immunization with CFA, all animals were maintained on vehicle/drug treatment for 21 days. The ankle joints of CFA-injected rats were collected and stored in 10% formaldehyde for histopathological and immunohistochemical evaluation.[25]

The extent of degeneration was determined via histopathological and immunohistochemical analyses.

2.5.1. *Serum TNF-α and IL-1β levels estimation*

Serum TNF-α and IL-1β levels were measured using commercially available ELISA kits in CFA-induced arthritis model. Monoclonal antibody has been coated on microwell plate that was provided with the commercial kit. 100 μl of each samples and standard were added to respective wells. After washing with wash buffer, diluted biotinylated anti rat TNF-α or IL-1β were added to all wells and plate was incubated. Then wash with washing buffer and streptavidin-HRP solution added and incubated, after that, washing repeated. TMB substrate added to all wells and incubated. In the final step, stop reagent was added to each well, and absorbance value of every well was taken at 450 nm. The level of TNF-α or IL-1β is directly proportional to the intensity of colour.[26]

2.5.2. *Histopathological analysis of paw tissue*

The entire paw tissue sections were fixed by immersion at room temperature in 10% neutral buffered formalin solution. The limbs were decalcified in EDTA for 30 days, and then processed for paraffin embedding, sectioned, and stained with hematoxylin–eosin. Scores were given as 0 (not present), 1 (mild), 2 (moderate) and 3 (severe) for bone resorption and inflammation.[27]

2.5.3. *Immunohistochemical analysis of paw tissue*

Immunohistochemical analysis was done by using Vector ABC kit, USA. Decalcification of ankle joints was completed with 10% EDTA (pH 7.4) and frozen sections were prepared.

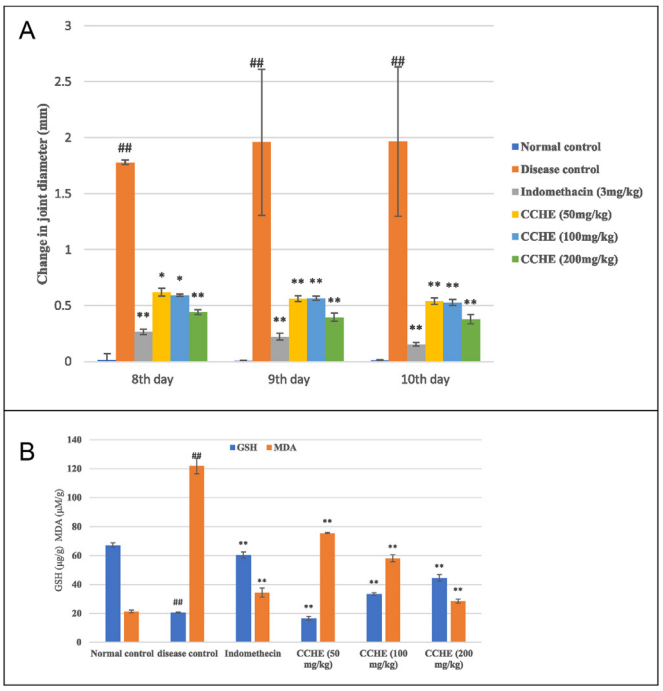

Fig. 1 – (A) Anti-arthritic activity of CCHE in formaldehyde induced arthritis; (B) Effect of CCHE on MDA and GSH levels in formaldehyde induced arthritis. All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons. ##$p < 0.01$, vs. normal control, *$p < 0.05$ **$p < 0.01$ vs. disease control. CCHE, *Cinnamomum cassia* hydroalcoholic bark extract; GSH, glutathione; MDA, malondialdehyde.

After finishing acetone fixation, slides were washed with phosphate-buffered saline (PBS) and blocked for 45 min with blocking kit. After that, slides with primary antibody (TNF-R1; 1:100 dilution) were incubated overnight at 4 °C. Washing was performed again and incubated in 4% H_2O_2 for 20 min. Washing was repeated and incubated with avidin-biotin based detection kit. Washing was repeated and visualization of cytokine receptor expression was done by colour development using diaminobenzidine (DAB) counter staining with haematoxylin and envisaged under microscope.[28] TNF-R1 expression scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining).[29]

2.6. *Statistical analysis*

Data were shown as mean ± standard error of the mean and evaluated by one-way analysis of variance followed by Tukey's post hoc test (SPSS; Version 23.0). $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Formaldehyde-induced arthritis*

Anti-arthritic activity was evaluated by measuring joint swelling. CCHE administration resulted in significant

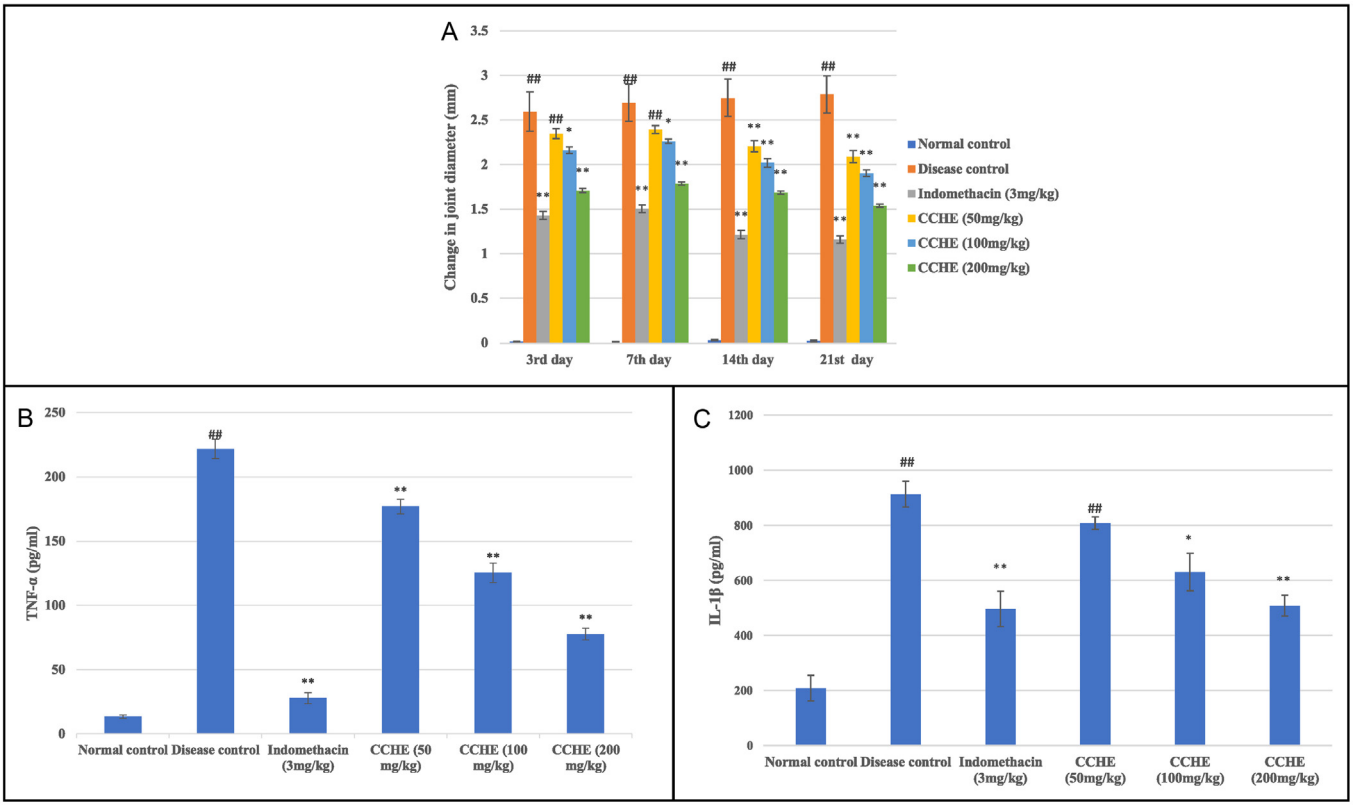

Fig. 2 – (A) Anti-arthritic activity of CCHE in complete Freund's adjuvant induced arthritis; (B) Effect of CCHE on serum TNF-**α** in complete Freund's adjuvant induced arthritis; (C) Effect of CCHE on serum IL-1**β** in complete Freund's adjuvant induced arthritis. All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons. ##$p < 0.01$, vs. normal control, *$p < 0.05$ **$p < 0.01$ vs. disease control. CCHE, *Cinnamomum cassia* hydroalcoholic bark extract; TNF-**α**, tumour necrosis factor alpha; IL-1**β**, interleukin-1 beta.

concentration-dependent reductions in joint swelling compared with the findings in disease control group. Similarly, indomethacin significantly reduced joint swelling on all observation days. Significant anti-arthritic ($p < 0.01$) activity comparable to that in indomethacin group was observed in CCHE 200 mg/kg group. However, indomethacin group displayed approximately 2-fold inhibition of paw oedema relative to the findings in CCHE 200 mg/kg group (Fig. 1A).

MDA and GSH levels were estimated to further evaluate the anti-oxidative activity of CCHE in rats with formaldehyde-induced arthritis. MDA levels were significantly increased in disease control group. All CCHE-treated groups exhibited significant declines in MDA levels ($p < 0.01$). However, MDA levels were lower in normal and indomethacin groups than in all CCHE-treated groups. GSH levels were significantly ($p < 0.01$) elevated in all CCHE-treated groups compared with those in disease group, with the greatest effect observed in CCHE 200 mg/kg group (Fig. 1B).

3.2. Complete Freund's adjuvant (CFA)-induced arthritis

Immunization with CFA-induced arthritis and a significant increase in the ankle joint diameter, as observed in disease control group. Maximum joint swelling was observed in all six groups on day 3, after which gradual decreases were observed through day 21. The exception to this trend was observed in disease control group, in which joint swelling was slightly elevated after day 14 due to intensified cell-mediated immune response. CCHE exerted concentration-dependent inhibitory effects on joint swelling at all concentrations versus the findings in disease control group throughout the observation period ($p < 0.01$, Fig. 2A). Two lower doses (50 and 100 mg/kg) of CCHE showed nearly similar inhibition of joint diameter on days 7, 14, and 21. Meanwhile, the greatest inhibitory effects on joint swelling were observed in indomethacin group on days 3, 7, 14, and 21. CCHE 50, 100, and 200 mg/kg showed 2.09, 1.90, and 1.54 mm of increase in joint swelling, respectively on day 21.

3.3. Serum TNF-α and IL-1β level measurements

CFA administration resulted in increased serum TNF-α and IL-1β levels, whereas these effects were reversed by CCHE treatment in a concentration-dependent manner when compared with disease control group ($p < 0.01$, Fig. 2B,C). However, serum TNF-α and IL-1β levels in disease control group were increased almost twofold when compared with indomethacin and CCHE (200 mg/kg) groups. CCHE (50 mg/kg) group showed less decrease in TNF-α and IL-1β levels as compared with disease control group. TNF-α level was 221.74 pg/ml in disease

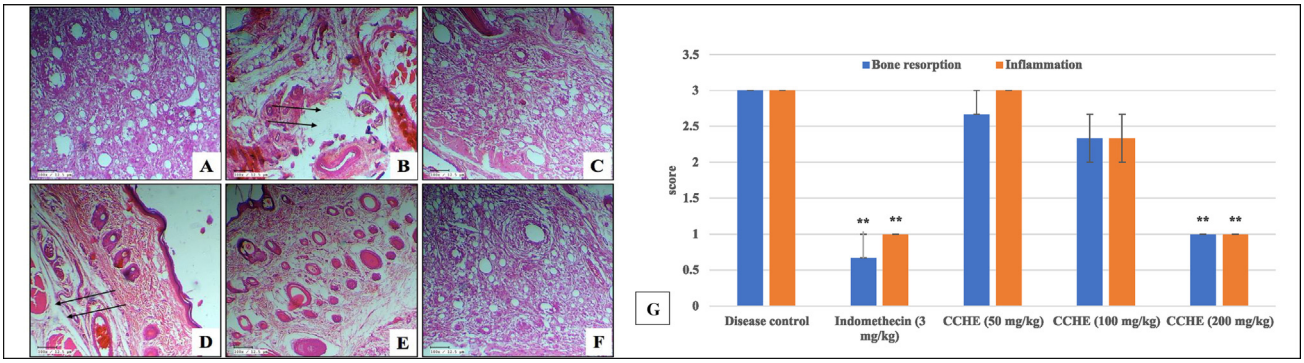

Fig. 3 – Histopathological analysis of ankle joints in complete Freund's adjuvant induced arthritis: (A) normal control; (B) disease control; (C) indomethacin 3 mg/kg; (D) CCHE 50 mg/kg; (E) CCHE 100 mg/kg; (F) CCHE 200 mg/kg. Black arrow shows architectural loss. Total magnification 100×; (G) Histopathological scoring of ankle joints in complete Freund's adjuvant induced arthritis (all values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons. $^{*}p<0.05$ $^{**}p<0.01$ vs. disease control). CCHE, *Cinnamomum cassia* hydroalcoholic bark extract.

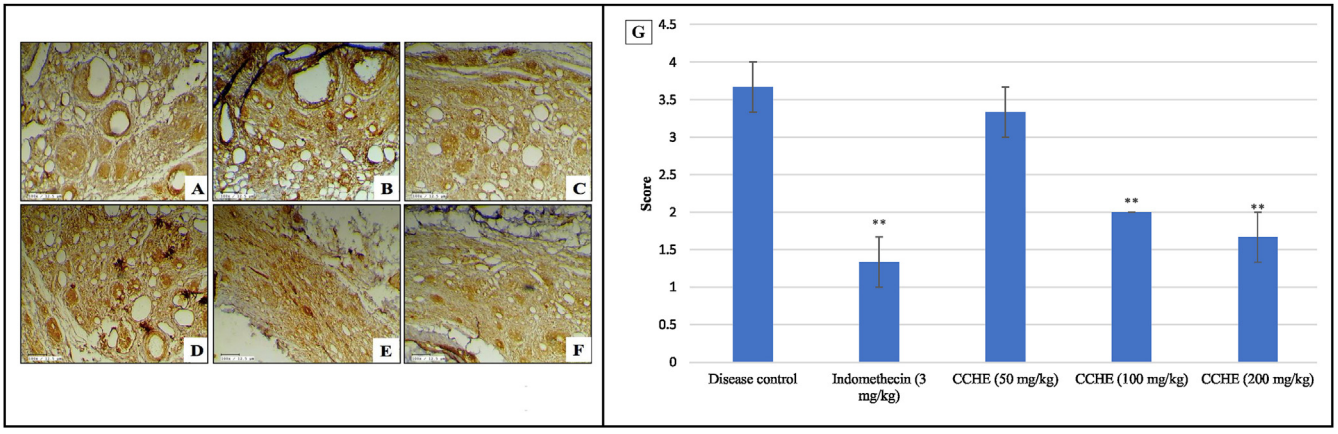

Fig. 4 – Immunohistochemical analysis of ankle joints for expression of TNF-R1. (A) Normal control; (B) disease control; (C) indomethacin 3 mg/kg; (D) CCHE 50 mg/kg; (E) CCHE 100 mg/kg; (F) CCHE 200 mg/kg. Total magnification 100×. CCHE denotes *Cinnamomum cassia* hydroalcoholic bark extract; TNF-R1 denotes tumour necrosis factor receptor 1; (G) Immunohistochemical scoring of ankle joints for expression of TNF-R1 (all values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons. $^{*}p<0.05$ $^{**}p<0.01$ vs. disease control). CCHE, *Cinnamomum cassia* hydroalcoholic bark extract; TNF-R1, tumour necrosis factor receptor 1.

control group and reduced significantly in CCHE 200 mg/kg group, where it was to be found 77.61 pg/ml. Serum level of IL-1β was 507.77 pg/ml in high dose group of CCHE, which was approximately similar to indomethacin group, where in disease control group it was found to be significantly higher at 913.27 pg/ml.

3.4. *Ankle joint histopathology*

In histopathological analysis, disease control group revealed damage articular cartilage with synovial hyperplasia, lymphocytes and macrophages cell infiltration. Architectural damage was also present as compared with the findings in normal control group. Indomethacin-treated group showed less cell influx and synovium has gained the normal architecture. In CCHE 50 mg/kg group, proliferating cells were seen but less concentrated than disease control group. Synovial architectural loss was still detected in CCHE 100 mg/kg group; however, less bone damage was observed. CCHE 200 mg/kg group displayed significant reduction of arthritic severity and inflammatory cell infiltration in comparison to the findings in disease control group, synovial lining was intact with few infiltrating cells. Decline in the thickness of outer membrane with less proliferating blood vessels. In CCHE 200 mg/kg group, Cartilage damage was less intense with respect to other CCHE treated group, similar to the observations seen in indomethacin group (Fig. 3).

3.5. *Immunohistochemical analysis*

The effects of CCHE on TNF-R1 expression were assessed in CFA-injected ankle joints. Normal control group exhibited non-appearance of TNF-R1 expression in synovial tissue. In disease control group, significant expression of TNF-R1 in the

synovial and adjacent tissue is noted. The results illustrated that TNF-R1 expression was decreased in both indomethacin- and CCHE (200 mg/kg) treated rats compared with the findings in disease control group (Fig. 4). Moreover, appearance of TNF-R1 was present in CCHE 50, 100 mg/kg groups but less concentrated than the disease control group.

4. Discussion

In the present study, anti-arthritic activity of *C. cassia* bark extract was evaluated by using formaldehyde and CFA-induced arthritis models and treatment with CCHE resulted decline in the production of inflammatory mediators and oxidative stress marker. The formaldehyde-induced model of arthritis mimics human arthritis, thus permitting assessment of the potential anti-arthritic and anti-inflammatory properties of test compounds.[30] The injection of formaldehyde into the rat paw produces localized inflammation and pain, which are biphasic in nature, i.e., an early neurogenic component followed by a later tissue-mediated response. The finding that *C. cassia* treatment significantly decreased joint diameter in rats suggests that components of the medicinal plant target these biphasic processes.

Oxidative stress is augmented in several pathological conditions such as RA, diabetes mellitus, Alzheimer's disease, endothelial dysfunction and malignancy.[31] Free radicals have crucial roles as secondary messengers in inflammation, and they can exacerbate joint damage.[32] Specifically, free radicals amplify the inflammatory response by inducing protein, collagen and DNA degradation and lipid peroxidation. The oxidative stress level depends on both MDA and GSH. MDA mainly formed due to lipid peroxidation and increases ROS generation. GSH play a crucial role as a scavenger of ROS and hinders oxidative stress.[33] Our findings supported an anti-inflammatory role of CCHE as indicated by the decrease in MDA levels and increase in GSH levels in rats with formaldehyde-induced arthritis, in line with a previous study by Liao et al.[6] Some studies have reported that ROS plays a role of sending a stimulatory signal for NF-κB activation that further induced the expression of pro-inflammatory cytokine.[34] Furthermore, Kwon et al. reported anti-NF-κB activity of *C. cassia*, that may establish plant have dual activity.[35]

To further establish the anti-arthritic activity of CCHE, a CFA-induced arthritis model was also used. This model has many clinical similarities with RA in human patients, including pathological and immunological features.[36] CFA injection caused activation of immune system, resulting in abnormal leukocyte proliferation and differentiation. Dendritic cells react with adjuvants components, it enhanced phagocytosis, proliferation of CD4+ lymphocytes, and secretion of cytokines (TNF-α and IL-1β).[37] Several studies have reported that TNF-α and IL-1β play crucial roles in inflammation and synovial tissue damage, and their levels were increased in patients with RA.[38] Thus, TNF-α and IL-1β represent crucial targets for treating RA. It is well established that TNF-α binds to two cytokines receptors, namely TNFR-1 and TNFR-2. However, major inflammatory reactions are facilitated by TNFR-1.[39] It is possible that CCHE prevented joint destruction by suppressing pro-inflammatory cytokine/cytokine receptor levels. In this study, CCHE inhibited both TNF-α and IL-1β, it has been reported that these two cytokines caused activation of Signal transducer and activator of transcription 3 (STAT3). Induction of STAT3 further increases the expression of RANKL,[40] which promotes osteoclastogenesis and joint destruction. Hence sustained inflammation and joint destruction may have suppressed by CCHE. Moreover, a study by Liao et al. concluded that *C. cassia* exerted anti-inflammatory effects by blocking the protein expression of inducible nitric oxide synthase, cyclooxygenase-2, and NF-κB.[6,35] In patients with RA, joint damage, tissue hyperplasia and pannus formation are caused by excessive vascular endothelial growth factor (VEGF) levels.[41] Kim et al. reported that *C. cassia* water extract had anti-inflammatory potential based on its inhibitory effects on VEGF. This suggests that the suppression of VEGF could also explain the inhibitory effects of *C. cassia* on pro-inflammatory cytokines and joint destruction.[18]

Most conventional treatments for RA provide only symptomatic relief. Whereas NSAIDs do not cure the disease, DMARDs can change its course by acting on different inflammatory mediators.[42] NSAIDs, DMARDs and biologics are also associated with a number of side effects such as ulcers, cardiovascular complications, reproductive toxicity, gastrointestinal abnormalities, emergence of opportunistic infections and immunosuppression, thereby increasing the morbidity and mortality of RA.[43,44] Many studies have found significant protective effects of *C. cassia* against peptic ulcer,[45] cardiovascular complications[46] and hepatic injury,[5] indicating that the plant can improve the efficacy and decrease the side effects of conventional treatments for RA.

In conclusion, CCHE exhibited anti-arthritic activity in formaldehyde- and CFA-induced models of arthritis by reducing pro-inflammatory levels similarly as indomethacin.

Conflict of interest

The authors declare no conflict of interest.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.imr.2018.08.002.

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