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Potential anti-inflammatory effect of lemon and hot pepper extracts on adjuvant-induced arthritis in mice

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KEYWORDS

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Abstract Arthritis and related disorders, including rheumatoid arthritis (RA), are common diseases affecting millions of people. The present study aimed to investigate the therapeutic potential of lemon and hot pepper extracts on adjuvant induced arthritis (AIA) in mice. Arthritis was induced by injection of complete Freund adjuvant (CFA) subcutaneously at the planter surface of hind paw, the lemon and hot pepper extracts were administered subcutaneously at the same site twice weekly (100 mg/kg), for 2 weeks starting 2 days after CFA injection. Arthritic scores, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), anti-nuclear antibody (ANA), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and paw histopathology were assessed at the end of the experiment. The extract treatments reduced the severity of arthritic scores in the following order: lemon fruit peel (LFP) > lemon leaf (LL) > hot pepper leaf (HL) during the experimental period as compared with positive control (RA). LFP, LL and HL extracts significantly suppressed ESR, ANA, CRP and TNF-α as compared with RA group. HL, LFP and LL reduced the IL-1β by 63.02%, 47.22%, 44.92%, while IL-6 cytokine production significantly decreased by 29.74%, 28.96%, and 23.93% for IL 6 as compared with RA. Hot pepper fruit (HF) extract treated-group showed a significant decrease for ESR on the other hand there was non-significant difference for TNF-α, IL-6, IL1β, CRP and ANA as compared with RA. Histopathological examination indicated that LFP, LL and HL extracts alleviated infiltration of inflammatory cells and synovial hyperplasia as well as protected joint destruction. The data showed that all extracts except HF have significant anti-arthritic and anti-inflammation effects and suggest that these effects may be mediated via the suppression of pro-inflammatory cytokines.

Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease (Smolen and Steiner, 2003). Several

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prevalence and incidence studies of RA have been reported during the last decades, suggesting a considerable variation of the disease occurrence among different populations (Drosos et al., 1997; Aho et al., 1998; Cimmino et al., 1998; Riise et al., 2001; Andrianakos et al., 2003). A study carried out in Middle East estimates a prevalence of affects about 1% of the general population; RA prevalence in Egypt represents about 0.2% (Alamanosa and Drosos, 2005). The disease is characterized by aggressive synovial hyperplasia (pannus formation) and inflammation (synovitis) (Fournier, 2005). Animal disease models that reproduce the pathology of human RA are of great interest as vehicles for the testing of potential therapeutics designed for treatment. These models are required to evaluate the safety, effectiveness, and toxicity of many new potential RA treatments. Rodent populations with RA-like disease are created by collagen-induced arthritis (CIA) and complete Freund's adjuvant (CFA) which behaves similar to rheumatoid Arthritis within the cells, creating synovitis and erosions (Hegen et al., 2008). Lorenzo et al. (2008) reported that rodent adjuvant arthritis, as an experimental model, resembles RA in histological pathology, pannus formation and a number of angiogenic mediators, including cytokines and growth factors. The similarities in joint pathology between AA and RA could be exerted for screening of new drugs for treatment of RA disease.

Conventional medicine includes treatment with steroids, nonsteroidal anti-inflammatory drugs (NSAIDs) as well as biological agents as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) antagonists (Fleischmann et al., 2004), and disease-modifying anti-rheumatic drugs (DMARDs) (Choy et al., 1998). These treatment agents are associated with unpleasant side effects such as gastrointestinal disturbances (Scheiman, 2001) and have shown only limited success against all forms of arthritis (Chandrashekara et al., 2002). As Alternative therapies are popular among people with rheumatoid arthritis, herbal products are receiving increasing public interest. However, alternative medicine should complement, not replace conventional care (Soeken et al., 2003).

Recently, fruits and vegetables have been recognized as natural sources of various bioactive compounds (Pennington and Fisher, 2010; Dembitsky et al., 2011). The main phytochemical compounds present in fruits and vegetables are flavonoids, anthocyanins, vitamins C and E, phenolic compounds, dietary fiber, and carotenoids (González-Aguilar et al., 2008).

Citrus limon commonly known as lemon belongs to the family Rutaceae (Ghazanfar, 1989). Some species of Citrus have a broad spectrum of biological activities, including antibacterial, antiviral, antioxidant, antifungal, analgesic, and anti-inflammatory (Ezzat, 2000; Luzia and Jorge, 2009). Peppers are consumed worldwide and their importance has increased gradually to place them among the most consumed spice crops and used as additives in the food industry where a variety of antioxidants can be found (Bown, 2001). They also have a significant role in traditional medicine. In Indian, Native American, and Chinese traditional medicine, Capsicum species have been used for the treatment of arthritis, rheumatism, stomach aches, skin rashes, dog/snake bites, and flesh wounds (Meghvansit et al., 2010). These therapeutic applications are related to the capsaicinoid, phenolic compound, and carotenoid content of peppers (Park et al., 2010).

The present study was designed to investigate the potential therapeutic effect of lemon fruit peel (LFP), lemon leaf (LL) hot pepper fruit (HF) and hot pepper leaf (HL) extracts using the mouse of adjuvant induced joint arthritis, as an animal model of autoimmunity diseases with great similarities to human rheumatoid arthritis.

Materials and methods

Male mice weighing 30–35 g were purchased from experimental animal center of Helwan University and allowed to acclimate in the laboratory for 7 days. They were housed in polystyrene cages in which the floor was covered with sawdust to minimize the possibility of painful contact with a hard surface. Mice were kept in a 12 h light–dark cycle with full access to food and water (*ad libitim*).

Preparation of mice AIA model

Adjuvant arthritis was induced on day 0 of the experiment by a single subcutaneous injection of 200 μL of CFA (complete Freund adjuvant) into the plantar surface of right hind paw of the mice with a 25-gauge hypodermic needle (Lao et al., 2001). The inflammation, manifesting as redness, edema, and hyper-responsiveness to noxious stimuli, was limited to the injected paw. Inflammation appeared shortly after the injection, peaked between 12 and 24 h. CFA-injected mice showed routine grooming behavior and levels of activity, and the effect of hyperalgesia on their normal behavior seemed minimal.

Plant extract

Unprocessed lemon fruit peel, hot pepper (*Capsicum annuum* L.) fruit and leaves were collected from Zagazig government (Egypt), allowed to air dry, ground into powder and processed at room temperature with absolute ethyl alcohol for complete extraction (Atta and Alkofahi, 1998). Evaporation temperatures were kept below 50 °C to minimize the possible breakdown of thermolabile compounds that may present in the extracts. This initial crude extract was concentrated under reduced pressure, and the dried residue was coded and weighed for the study. After removing the solvent the percentage of ethanol extract yields were 5.5% for lemon fruit peel, 4.5% for lemon leaf, 20.8% for hot pepper fruit and 9.5% hot pepper leaf.

Toxicity/adverse effects assessment

To evaluate the acute toxicity of the 4 extracts after a single intraperitoneal (IP) dose, 30 mice were treated with 1.0, 2.0, 5.0, 7.5 and 10.0 g/kg (6 mice in each group). Mortality and clinical signs were monitored for 7 days (Shin et al., 2002). Animals were closely monitored for unusual behavioral changes and for symptoms such as loss of appetite, diarrhea, weight loss, fur discoloration, sedation, irritation, and convulsion during the 7 days of the single-dose of all extracts Gad, (2002).

Experimental Protocol

Sixty mice (30 ± 5 g) were divided into 10 groups as follows: group 1: normal control; group 2 (RA): positive control RA

(single dose of complete Freund adjuvant (CFA) 20 µl for each mice subcutaneous at the planter surface of right hind paw); group 3: lemon fruit peel extract (LFP);group 4: lemon leaf extract(LL); group 5: hot pepper fruit extract (HF); group 6:hot pepper leaf extract (HL); group 7: LFP + RA; group 8: LL + RA; group 9: HF + RA; group 10: HL + RA. Lemon, hot pepper extract or vehicle was administered twice weekly (2nd and 5th day of the week) for 2 weeks. Mice of all experimental groups were sacrificed 24 h after the last injection, and blood and tissues were collected. A stock solution of plant extract was prepared by dissolving 500 mg of extract in 5 ml of saline and administered subcutaneously into the plantar surface of hind paw of the mice (30–50 µl, 100 mg/kg). Animals in the vehicle control group received biweekly the same injected amount of saline. At the end of treatment course (2 weeks) animals were sacrificed. Blood samples were collected by cardiac puncture.

Clinical evaluation of arthritis

Mice were examined every 3 days for clinical parameters. Incidence of arthritis was judged macroscopically. Each joint was examined for swelling and redness. The severity of arthritis was graded on a scale of 0–3 for each paw for degree of redness and swelling. Grade 0 indicated normal, grade 1 was given to light swelling of the joint and/or redness of the footpad, grade 2 for obvious swelling of the joint, and grade 3 indicated severe swelling and fixation of the joint. A severity score was calculated for the four limbs (maximum 12 points for individual mice).

Determination of erythrocyte sedimentation rate (ESR)

Modified Westergren method was used for the measurement of erythrocyte sedimentation rate. Exactly 4 volume of blood (anticoagulated blood with EDTA) and 1 volume of normal saline were mixed (Westergren, 1957). Well mixed diluted blood was placed in Westergren pipette and allowed to stand for 1 h in a vertical position. The number of millimeter the red blood cells fall during this time representing ESR.

Determination of serological parameters

Serum interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) were measured by ELISA using solid phase sandwich ELISA test kit obtained from immune-biological laboratories Co. Ltd., Japan (Catalog No. 27193and 27194,respectively). Serum interleukin-6 (IL-6) and C-Reactive protein (CRP) were determined using enzyme linked immunosorbent assay ELISA test kit obtained from IBL-America, Inc., USA (Catalog No. IB49706 and IB66103, respectively). Serum anti-nuclear antibodies' (ANA) level was determined by using enzyme immunoassay (EIA) test kit obtained from DRG international Inc., USA (Catalog No. EIA-3562).

Histological examination

At the end of the experiment hind paws were removed above the knee joint and were fixed in 10% formalin saline solution. The paws were then decalcified in 10% EDTA for 14 days at 4 °C, embedded in paraffin, and sectioned in a mid-sagittal plane. The sections of articulation of the tarsal joints were stained with hematoxylin and eosin. The histological damage evaluated microscopically was defined according to system evaluated cartilage and bone destruction by pannus formation, mononuclear cell infiltration, and vascularity in synovial tissues. The histological damage evaluated microscopically was defined according to Omoto et al. (2005).

Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance (ANOVA) and then differences among means were analyzed using Duncan test. Differences were considered significant at $P \leqslant 0.05$.

Results

Toxicity and adverse effects of lemon and hot pepper extracts

There was no mortality in mice treated with all tested extracts. The behavior and body weight did not change in vehicle control or all treated groups.

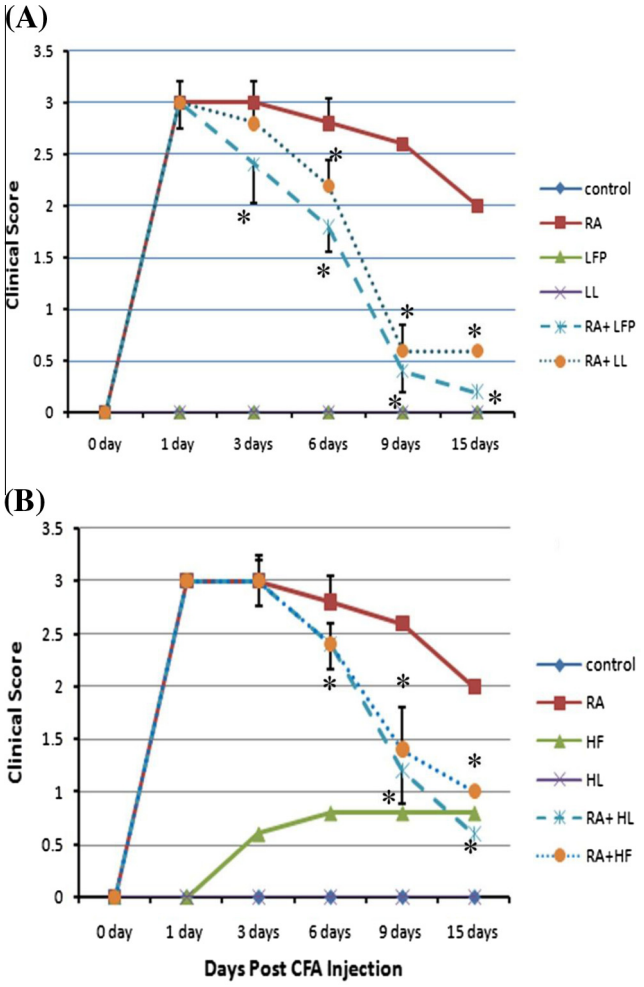

Figure 1 (A) Effect of lemon fruit peel and lemon leaf extracts, (B) hot pepper fruit and leaf extracts on arthritis as assessed with arthritic scores (mean ± S.E., $n = 6$/group) in mice. $^{*}P \leqslant 0.05$ vs. RA.

Clinical evaluation of arthritis

As shown in Fig. 1A and B, lemon fruit treatment remarkably reduced the severity of joint swelling and erythema during the experiment period as compared with RA followed by lemon leaf, hot pepper fruit and hot pepper leaf.

ESR and serological parameters

ESR increased by CFA administration (Fig. 2) in arthritis mice as compared with normal control values. ESR levels decreased significantly in arthritis mice after treatment with lemon fruit peel, lemon leaf, hot pepper leaf, and hot pepper fruit by −79.09%, −77.00%, −77.53% and −77.53% respectively ($P \leqslant 0.05$).

Serum IL-1β level of the positive control (RA) arthritic mice showed a 2.2-fold increase compared to the non-arthritis vehicle group. Hot pepper leaf, lemon fruit peel and lemon leaf extracts significantly suppressed IL-1β of arthritic mice by 63.02%, 47.22% and 44.92%, respectively compared to RA (Fig. 3).

Serum TNF-α level of RA was about two times more than that of the normal control group. There was a significant decrease ($P \leqslant 0.05$) in serum TNF-α level of AIA mice treated with lemon fruit peel, hot pepper leaf and lemon leaf extracts with 42.04%, 27.80% and 26.51% percent of change respectively. However, hot pepper fruit treated-group showed non-significant effect on TNF-α levels as compared with the RA group (Fig. 4).

IL-6 level showed a significant decrease by 29.74% for leaf of hot pepper extract, 28.96% for lemon fruit extract, 23.93%

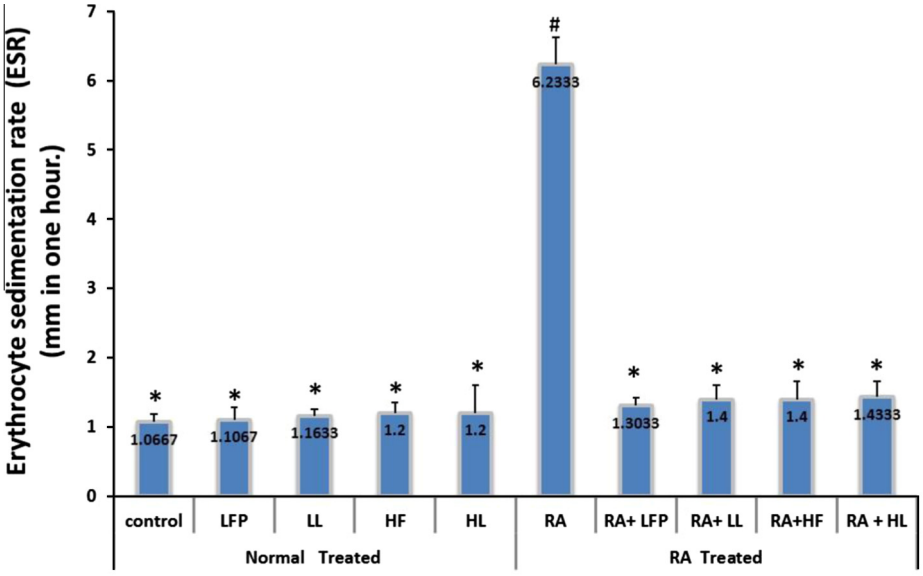

Figure 2 Effects of lemon and hot pepper extracts on the ESR of AIA mice. The data are expressed as mean ± S.E. $^{*}P \leqslant 0.05$ vs. RA $^{\#}P \leqslant 0.05$ vs. normal control.

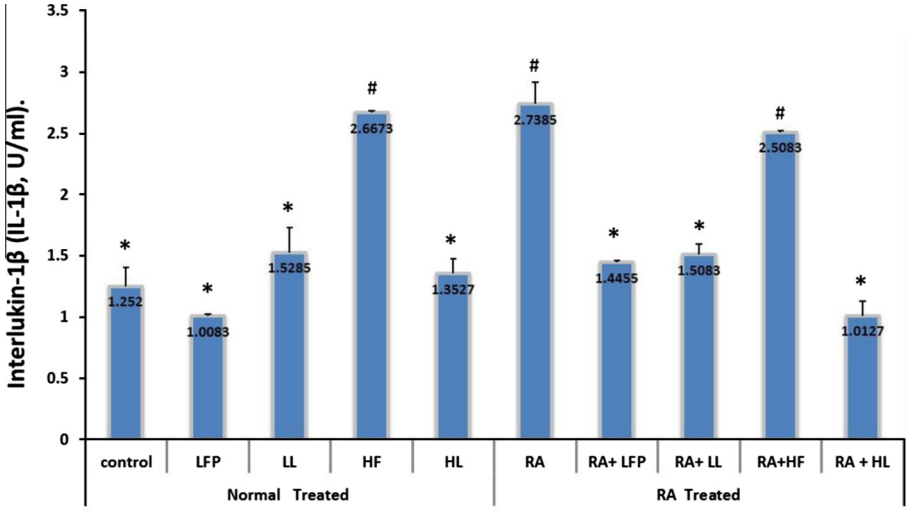

Figure 3 Effects of lemon and hot pepper extracts on serum IL-1β of AIA mice. The data are expressed as mean ± S.E. $^{*}P \leqslant 0.05$ vs. RA $^{\#}P \leqslant 0.05$ vs. normal control.

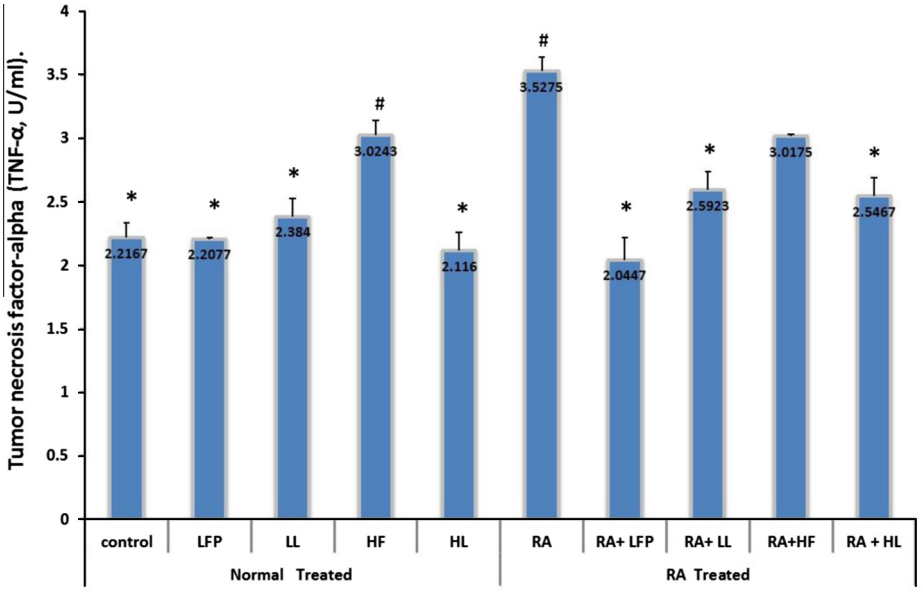

Figure 4 Effects of lemon and hot pepper extracts on serum TNF-α level of AIA mice. The data are expressed as mean ± S.E. $^{*}P < 0.05$ vs. RA $^{\#}P \leqslant 0.05$ vs. normal control.

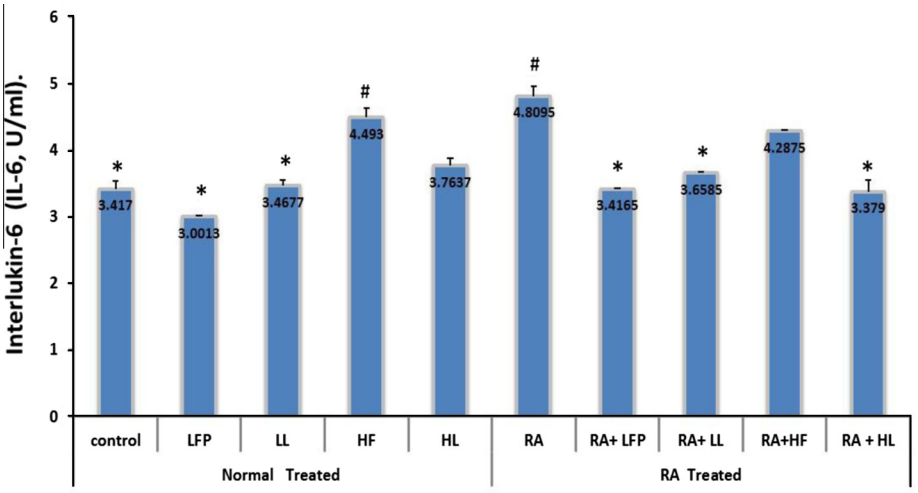

Figure 5 Effects of lemon and hot pepper extracts on serum IL-6 level of AIA mice. The data are expressed as mean ± S.E. $^{*}P < 0.05$ vs. RA $^{\#}P \leqslant 0.05$ vs. normal control.

for lemon leaf extract and hot pepper fruit extract showed non-significant effect as compared with the RA group (Fig. 5).

Lemon fruit, lemon leaf and hot pepper leaf extracts significantly decreased the level of CRP at the end of the experimental period by 32.55%, 44.18% and 40.09% respectively compared to the positive control (RA) (Fig. 6).

Positive control mice showed a higher level of ANA as compared with normal control extracts of lemon fruit, lemon leaf and hot pepper leaf remarkably reduced ANA with inhibitory percentages of 59.64%, 47.83%, 15.66% and 46.63%, respectively RA (Fig. 7). There was no significant change in ANA when the mice were treated with hot pepper fruit extract.

Histological examination

Histological photography examination of ankle joints of normal mice from different plant extract treated-groups showed ankle joints with intact bone, articular cartilages and normal joint space (Fig. 8A, C, E, G, I). The joints of AIA mice presented prominent infiltrating granulocytes and mononuclear cells, synovial hyperplasia and pannus formation, and erosion of bone and cartilage in comparison to the normal group (Fig. 8A). HF-treated group showed mild recovery and reduction in the number of cartilage cells and joint space (Fig 8D), while HL, LFP, LL treated-groups (Fig. 8F, H, J respectively) showed beneficial effects on these pathological manifestations. It showed nearly normal histological features with almost intact bone and cartilage, in addition to normal joint space.

Discussion

The use of NSAIDs cannot block the development and progress of rheumatoid arthritis (Silverstein et al., 2000), and DMARDs have been impeded by their potential of long-term

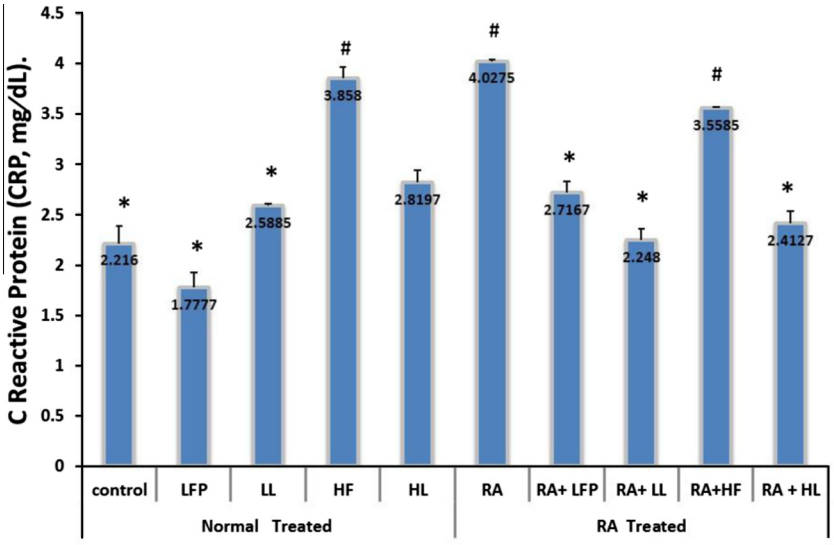

Figure 6 Effects of lemon and hot pepper extracts on serum CRP level of AIA mice. The data are expressed as mean ± S.E.$^{*}P < 0.05$ vs. RA $^{\#}P \leqslant 0.05$ vs. normal control.

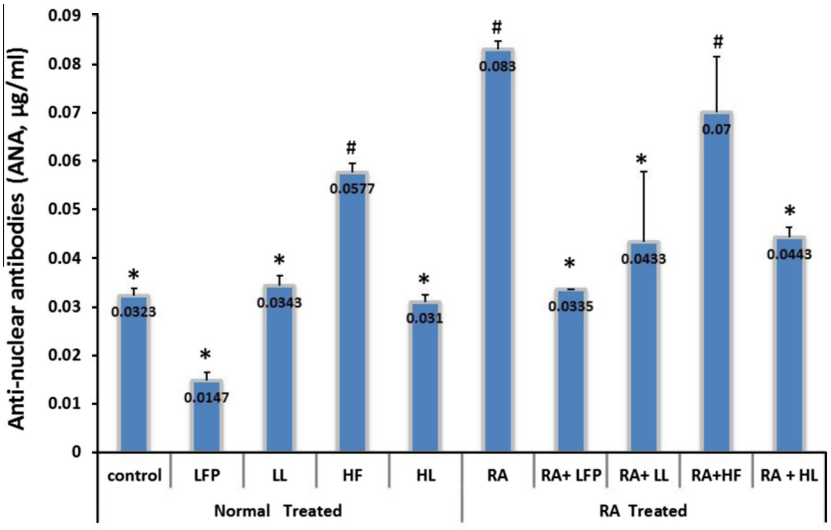

Figure 7 Effects of lemon and hot pepper extracts on serum ANA level of AIA mice. The data are expressed as mean ± S.E.$^{*}P < 0.05$ vs. RA $^{\#}P \leqslant 0.05$ vs. normal control.

side effects, toxicity and immunosuppression (Scanzello et al., 2006). Yao et al. (2011) reported that it is important to search for new therapeutic drugs with greater efficiency and lower toxicity from a natural source. Therefore the present study is concerned with the evaluation of the efficacy of lemon fruit peel, lemon leaf, hot pepper fruit and hot pepper leaf extracts as anti-arthritic factors using AIA model.

The present study demonstrated that a single injection of FCA at the plantar surface of mice developed pronounced arthritis in the paws, showing 100% incidence. In this AIA model, ESR and CRP levels were significantly elevated at the end of the experiment as compared to normal control mice. High values of serum CRP can be used as a useful biomarker for evaluating the active inflammation in arthritic patients (Milovanoic et al., 2004; Yildirim et al., 2004). Moreover, Wolfe (1997) reported that ESR measured clinically to assist in establishing the presence of RA. The present work indicated a significant elevation in antinuclear antibody (ANA) of AIA mice. ANA is considered as a possible diagnostic marker for RA (Verma et al., 2012). The increment in ESR and CRP levels observed in the arthritic animals were found to be significantly reduced in lemon fruit peel, lemon leaf and hot pepper leaf treated mice. There is no data concerning the effect of any parts of lemon and hot pepper extracts on ESR and CRP in the AIA model for this reason it is very difficult to compare our results with those reported by other authors.

In our study, markedly increased levels of sera IL-β1, IL-6, and TNFα were found in AIA mice. The present results agree with the observation of Gonzalez-Gay et al. (2005) who stated that RA is caused by number of pro-inflammatory molecules released by macrophages. These include reactive oxygen species and eicosanoids such as prostaglandins, leukotrienes and

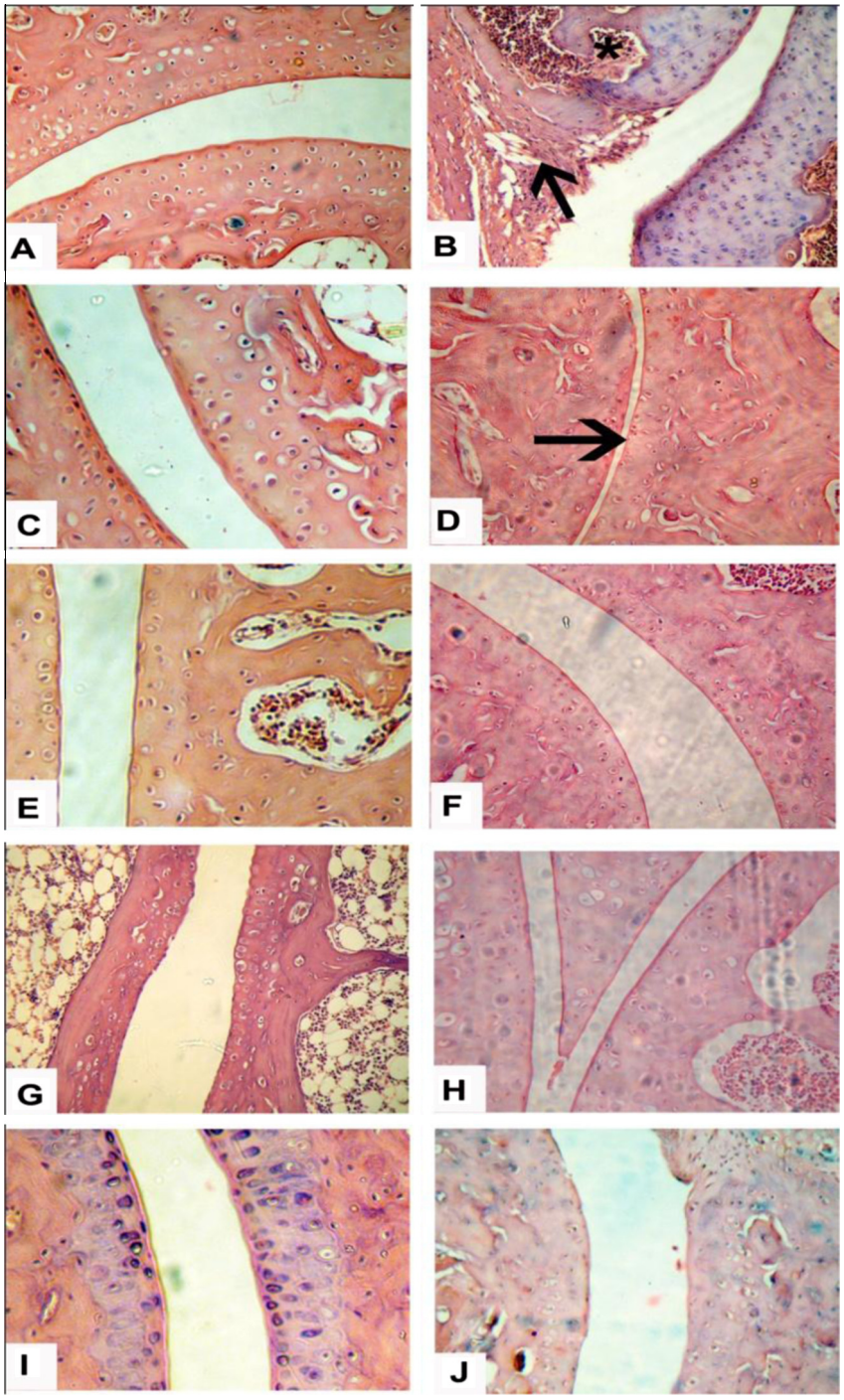

Figure 8 Representative histological changes in hematoxylin and eosin (H&E) stained tarsal joint sections in AIA mice. (A) normal mice, (B) AIA mice showed pannus formation; lymphatic infiltration (arrow), and cartilage erosion (asterisk) (C) hot pepper fruit extract treated mice; (D) AIA mice treated with hot pepper fruit extract showed mild recovery and reduction in the number of cartilage cells and joint space (arrow) (E) hot pepper leaf extract treated mice; (F) AIA mice treated with hot pepper leaf extract showed Arthritis-preventing effect (G) lemon fruit peel extract treated mice, showed maximum recovery (I) lemon leaf extract-treated mice (J) AIA mice treated with lemon leaf showed mild recovery (200×).

cytokines (IL-β1, IL-6, and TNFα) (Bharadwaj et al., 1999). Goldring and Gravallese (2000) also reported that characteristic feature of arthritic joints is the persistence of pro-inflammatory cytokines such as TNF-α and 1L-1 produced by the inflamed synovium as well as by chondrocytes in the affected joints.

The present results of cytokines can be confirmed by Suffredini et al. (1999) who reported that the pro-inflammatory cytokines especially IL-1, TNFα, and IL-6, increase the production of several plasma proteins such as CRP. Lemon fruit peel, lemon leaf and hot pepper leaf treatments significantly inhibited IL-1, IL-6 and TNF-α produced by

synoviocytes which derived from arthritic tissue in AIA mice decreasing the levels of serum CRP levels as compared with the RA group.

Dai et al. (2003) suggested that TNF-α considered as an important factor in promoting mechanisms leading to inflammation, whereas IL-1 led to cartilage and bone destruction. Inhibition of IL-1 reduced the extent of inflammation and bone destruction in adjuvant induced arthritis (Feige et al., 2000).

Further, we found that the hot pepper fruit extract significantly decreased the ESR. However, concentration of CRP and the pro-inflammatory cytokines showed non-significant difference as compared with AIA.

Three types of flavonoids occur in Citrus species are flavanones, flavones and flavonols. Several mechanisms explaining the anti-inflammatory activity of flavonoids have been described, including (a) antioxidative and radical scavenging activities, (b) regulation of cellular activities of inflammation-related cells, (c) modulation of the activities of arachidonic acid metabolism enzymes (phospholipase A2, cyclooxygenase lipoxygenase) and nitric oxide synthase, (d) modulation of the production of other proinflammatory molecules, (e) modulation of proinflammatory gene expression (García-Lafuente et al., 2009), thus may cause decrease in ESR and serum CRP levels.

Galati et al. (2005) stated that lemon mucilage significantly inhibited carrageenan-induced edema in rat paw from 59% to 73.5%. Moreover, in the traditional Chinese medicine the dried mature fruit peels of *Citrus reticulata* and their varieties have been widely used for centuries as remedies to treat indigestion and to improve inflammatory syndromes of the respiratory tract (Huang and Ho, 2010). This result is coincidence with the present study with extract of different parts of lemon.

The results of the present study also indicated that the hot pepper leaf exhibits anti-inflammatory properties in mice with AIA. Several previous studies have reported similar observations (Wu and Liu, 2011; Hernández-Ortega et al. 2012; Zimmer et al., 2012). Hernández-Ortega et al. (2012) evaluated antioxidant, analgesic, and anti-inflammatory activities of carotenoids extracted from dried peppers. They concluded that pepper carotenoid extracts exhibited good antioxidant activity and had the best scavenging capacity for the DPPH + cation (24.2%) and indicated that carotenoids have potential therapeutic significance in pain and inflammation management (Wu and Liu, 2011). Moreover, Zimmer et al. (2012) suggested that the anti-inflammatory activity of *Capsicum baccatum may* be induced by capsaicin. Considering the strong analgesic effect of the capsaicin injection in this pain model, the capsaicin treatment leads to functional/morphological elimination of the whole nerve ending. Intra-articular injection of capsaicin has been shown to result in a partially selective loss of substance P and calcitonin gene-related peptide, two important mediators of neuroinflammation, and immunopositive nerve fibers lining the synovium (Barrot, 2012).

In the present work, the main pathological changes of AIA include synovitis associated with hypertrophy of the synovial lining and increased cellularity and vascularity of the synovial connective tissue. An erosive pannus comprised of fibrovascular connective tissue and mononuclear and polymorphonuclear inflammatory cells infiltrates the articular cartilage and subchondral bone beginning at the joint margins. The therapeutic administration of lemon fruit peel, lemon leaf and hot pepper leaf extracts significantly inhibited synovium hyperplasia which contributed to inhibition of the secondary paw swelling in AIA rats. Yue et al. (2004) suggested that synoviocytes were ultimate effectual cells with pathologic change during this process. Moreover, O'Reilly et al. (1997) stated that activated synoviocytes proliferate and overexpress cytokines such as IL-1 and TNF-α, which are important in the pathogenesis of RA. The present result tends to suggest that the inhibitory effect of the extracts on edema formation is probably due to the inhibition of the synthesis and/or release of the inflammatory mediators, especially cytokinase.

In conclusion, the present study displayed that ethanolic extracts of lemon fruit peel, lemon leaf and hot pepper leaf effectively controlled arthritis development. The arthritis scores of AIA were significantly decreased in lemon fruit peel, lemon leaf and hot pepper leaf- treated mice, as indicated by reduction in ESR, CRP and cytokines' levels. The pathological examination demonstrated that lemon fruit extract showed the maximal prevention of articular cartilage degeneration, inflammatory cells' infiltration in joint cavity, synovial hyperplasia and pannus formation in arthritis mice.

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