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Thymoquinone attenuates rheumatoid arthritis by downregulating TLR2, TLR4, TNF-α, IL-1, and NFκB expression levels

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ABSTRACT

Background: Thymoquinone (TQ), the most important active principle of *Nigella sativa* is known to have anti-inflammatory, analgesic, antimicrobial and antioxidant properties.
Aim: The present study was designed to see the anti-arthritic effect of TQ in rat model of arthritis.
Methods: In the current research, anti-arthritic effect of TQ was determined in Freund's Complete Adjuvant (FCA)-induced arthritic rats by measuring TLRs expression levels. The mRNA expression levels of toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), interleukin-1 (IL-1), nuclear factor-kappa B (NFκB) and tissue necrosis factor-α (TNF-α) were measured by reverse-transcription polymerase chain reaction. Arthritic signs were observed by macroscopic criteria. Hematoxylin and Eosin staining was used to perform ankle joint histopathology and agglutination method was used for measuring C-reactive protein (CRP) levels. Rheumatoid factor, alanine transaminase, aspartate aminotransferase, urea and creatinine were also determined in serum.
Results: TQ treatment reduced the macroscopic arthritic score, levels of CRP, synovial inflammation, pannus formation and bone erosion. It also reduced the mRNA levels of TLR2, TLR4, IL-1, NFκB and TNF-α. Methotrexate, used as a reference drug also significantly decreased their expression levels. TQ also normalized the hematological markers and did not depict any signs of hepatotoxicity and nephrotoxicity as determined by serum levels of alanine transaminase, aspartate aminotransferase, urea and creatinine. Our results showed that TQ possesses significant anti-arthritic activity which can be due to its anti-inflammatory and immunomodulatory effects.
Conclusion: Results indicate that TQ has got the potential to ameliorate rheumatoid arthritis by downregulating TLR2, TLR4, **TNF-α, IL-1, and NFκB expression levels**.

1. Introduction

Rheumatoid arthritis (RA) is a chronic debilitating autoimmune disorder affecting the entire system occurring predominantly in females rather than males and increasing with age [1]. It is the most prevailing form of inflammatory arthritis and globally affects up to 1% of the population in industrialized world, with survival rates comparable to coronary artery disease and cancer [2–4].

In the last few years, many researchers have conjoined in an attempt to figure out the part played by TLRs in the manifestation of rheumatoid arthritis having a pivotal part in the initiation of inflammatory reaction to microbial antigens [5]. These receptors are single, non-catalytic, stretching over the cell membrane whose high expression levels are seen in many cell types of the immune system including macrophages, monocytes, dendritic cells (DCs), neutrophils, B cells, T cells, NK cells and also been detected in synovial cells, fibroblasts, osteoblasts, osteoclasts, chondrocytes and endothelial cells [6]. TLRs recognition of the microbial antigens results in primary immune response, which could end up in chronic inflammation, and may also progress to disease [7]. The inflammation occurs mainly from the stimulation of macrophages and neutrophils that leads to the formation of cytokines like NFκB, TNF-α and IL-1 that have an essential part in the innate immune response [8]. TLRs role in RA pathogenesis is confirmed from the findings that inducible heat shock protein 70 (Hsp70), commonly agreed upon as a TLR4 ligand, is elevated in RA synovium and on dendritic cells segregated from RA synovial fluid along with increased expressions of TLR2, TLR3, TLR4, and TLR7 [9].

Targeting TLRs particularly can be beneficial therapeutically as its

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role in conditions like sepsis syndrome, systemic lupus erythematosus (SLE), asthma and rheumatoid arthritis is arising [10]. Nowadays many steroidal, non-steroidal, disease modifying anti-rheumatic drugs (DMARDs) and immunosuppressive agents are used to control rheumatoid arthritis but they have restricted effectiveness and are potentially harmful [11].

Among many others, *Nigella sativa* is a promising medicinal plant related to family *Ranunculaceae*. People have benefited from the seeds of *N. sativa* for centuries for treating different ailments. *N. sativa* is being widely investigated for its pharmacological properties and potential medicinal value. It's gastroprotective, hepatoprotective, renal protective, immunomodulatory, anti-inflammatory and antioxidant effects are well established [12]. In several researches based on inflammatory models like experimental encephalomyelitis, colitis, peritonitis, edema, and arthritis TQ which the fundamental constituent of the black seed oil has shown strong anti-inflammatory effects by suppressing prostaglandins and leukotrienes [13].

In the present study we evaluated the anti-arthritic effect of TQ in FCA-induced arthritic rats by measuring Toll-like receptors expression levels and other immuno-modulatory parameters.

2. Materials and methods

2.1. Animals

40 male Sprague-Dawley rats, 6–8 weeks old were weighed and placed into four groups I, II, III and IV with 10 rats in each group. They were maintained at animal house of University of Health Sciences, Lahore under standard environmental conditions of 22–24 °C. Animals had free approach to water and standard pellet diet *ad libitum*. Ethical Review Committee, University of Health Sciences, Lahore had permitted all the experiments (UHS/ERC/17-42).

2.2. Experimental design

It was an experimental study and simple random sampling was done by balloting method. Rats were numbered 1–40 and randomly assigned to four groups I, II, III, IV. All the experiments were performed twice. Commercially purchased Thymoquinone (Sigma-Aldrich) was used for the study.

2.2.1. Group I (Vehicle Control)

0.5 ml of distilled water was given intraperitoneally.

2.2.2. Group II (Arthritic control)

0.5 ml of distilled water and 0.1% DMSO was given intraperitoneally.

2.2.3. Group III (TQ treated)

TQ, 10 mg/kg of body weight reconstituted in 0.1% DMSO and dissolved in distill water was given intraperitoneally [14].

2.2.4. Group IV (MTX treated)

Methotrexate, 0.5 mg/kg of body weight dissolved in distilled water was given intraperitoneally [15].

2.2.5. Induction of arthritis

Arthritis was induced by injecting 0.2 ml FCA in sub plantar region of the left hind rat paw on day 0 in all groups except vehicle control group. Treatment with TQ and methotrexate was initiated at the 8th day of arthritis induction as a single dose per day for 20 consecutive days. All animals were sacrificed at day 28 [16].

2.3. Assessment of arthritis development

It was determined by using the modified methods of [17] and [18]. Morphological features of the arthritis such as redness, swelling and erythema were examined. Occurrence and severity of arthritis was assessed through arthritic scoring method. Macroscopic criteria were adopted for grading the arthritic score at 0, 4, 8, 12, 16, 20, 24, and 28 days where 0 was given to normal paw, whereas, score 1 to 4 was given starting from the redness and swelling of one digit or paw to the involvement of all digits and entire paw [4].

2.4. Hematology and biochemical markers

At day 28th, the blood samples were collected by cardiac puncture and hemoglobin levels and inflammatory cells *i.e.* total leucocyte count, neutrophils, lymphocytes and monocytes were checked by automated hematology analyzer (XT-1800i Sysmex Japan). ALT, AST, urea, and creatinine levels (Randox kit) were also analyzed by using chemistry analyzer (Humalyzer 3500).

2.5. Determination of CRP levels and rheumatoid factor

At day 28, CRP levels in blood were determined by agglutination method according to the manufacturer's code (Antech Diagnostic Products-UK). Semi-quantification of CRP results was done as per manufacturer's instructions. RF in blood was also detected by agglutination method using reagent kit (Human Diagnostics).

2.6. Ankle joint histopathology

After sacrificing the rats, ankle joints were removed and placed in 10% neutral buffered formalin for fixation. They were decalcified using hydrochloric acid, ethylenediamine tetra acetic acid, sodium tartrate and potassium sodium tartrate containing decalcifying solution. Analysis of inflammatory cells was done after staining the paraffin sections of 5-um thickness with hematoxylin and eosin (H&E) [17]. Slides were examined by the Pathologist in a blinded fashion. Ankle joints were analyzed for the presence of bone erosion, pannus formation, and infiltration of inflammatory cells. 0, 1, 2, 3, and 4 numbers were given to normal, minimal, mild, moderate, and severe changes respectively and the results were semi-quantified [19].

2.7. Evaluation of mRNA expression levels of TLR2, TLR4, NFκB TNF-α and IL-1

Blood was collected on day 28th of the study and RNA extraction was done by TRIzol method by acquiring standard procedure according to the manufacturer's instructions (Thermo Fisher Scientific, America). Gel electrophoresis confirmed the purity of RNA and quantification of RNA concentration was determined through optical density. Reverse transcription polymerase chain reaction was done to produce cDNA from mRNA. Appropriate primers were made and used for the pro-inflammatory mediators for the production of copies by RT-PCR [16]. GAPDH gene was used as a housekeeping gene.

3. Statistical Analysis

Graph-Pad prism (v. 5) software was used for the data analysis. One-way ANOVA was applied and Post hoc Tukey test was applied to detect which group means is different from other. Mean ± SD was done for quantitative variables. Percentages and frequencies were given for qualitative variables. Statistically p-value of ≤ 0.05 was considered significant.

4. Results

4.1. TQ suppressed arthritic score

Arthritic signs were observed after sub plantar injection of FCA in

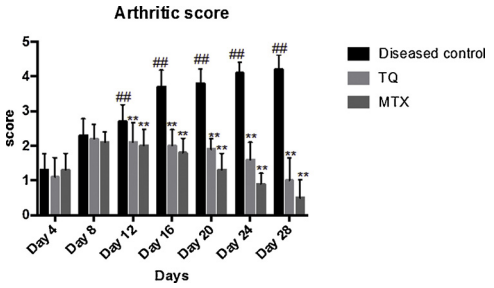

Fig. 1. TQ and MTX significantly suppressed arthritic score at days 12,16, 20, 24, and 28. Results are presented as mean ± SD for 10 rats in each group. ** shows $p < 0.01$ and indicates significant difference compared to arthritic control group. ## shows $p < 0.01$ and indicates significant difference compared to vehicle control group.

injected and non-injected paws indicating primary and secondary arthritic lesions, respectively. Signs of inflammation were observed around days 1–9. Vehicle control group did not show any signs of paw swelling. Treatment with TQ was commenced at the 8th day of induction and notably decreased ($P < 0.01$) the arthritic score (2.1 ± 0.56) at 12th day in comparison to the arthritic control group (2.7 ± 0.48). MTX also showed significant ($P < 0.01$) decrease (2.0 ± 0.47) on 12th day. TQ also significantly ($P < 0.01$) suppressed the arthritic score compared to the arthritic control group on day 16 (2.0 ± 0.47 vs 3.7 ± 0.48), day 20 (1.9 ± 0.31 vs 3.8 ± 0.42), day 24 (1.6 ± 0.51 vs 4.1 ± 0.31) and day 28 (1.0 ± 0.66 vs 4.2 ± 0.42) as well. There wasn't any significant difference in reducing arthritic score between TQ and MTX treated groups (Fig. 1).

4.2. TQ normalized the hematological parameters

The study exhibited significant elevation of total leucocyte count (7.95 ± 1.42; $P < 0.001$), lymphocytes (85 ± 3.22; $P < 0.001$), neutrophils (44.9 ± 5.17; $P < 0.001$) and monocytes (2.5 ± 0.54; $P < 0.01$) of arthritic control group in comparison to vehicle control. Treatment with TQ significantly normalized TLC (6.28 ± 0.42; $P < 0.001$), lymphocytes (78.17 ± 2.63; $P < 0.001$), neutrophils (23.2 ± 4.58; $P < 0.001$) and monocytes (1.5 ± 0.54; $P < 0.01$). Similarly, MTX also significantly reduced TLC (3.41 ± 0.50; $P < 0.001$), lymphocytes (74.83 ± 3.43; $P < 0.001$), neutrophils (21.5 ± 2.46; $P < 0.001$) and monocytes (1.16 ± 0.40; $P < 0.01$) as compared to arthritic control group (Fig. 2).

4.3. Treatment with TQ alleviated CRP levels

Increased levels of CRP were found in arthritic control group (32.2 ± 2.09) as compared with vehicle control group. CRP levels were less than the minimum detectable range (12 mg/I.U./ml) of available CRP kit in vehicle control group. Treatment with TQ (22.1 ± 1.79; $p < 0.001$) and MTX (20.0 ± 2.16; $p < 0.001$) showed significant decrease of CRP levels (Fig. 3).

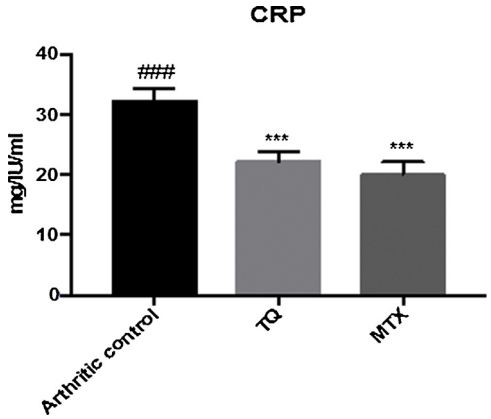

Fig. 3. Effect of TQ and MTX on CRP in serum samples. Results are presented as mean ± SD for 10 rats in each group. *** shows $p < 0.001$ indicating significant difference compared to arthritic control group. ### shows $p < 0.001$ indicating significant difference compared to vehicle control group.

4.4. TQ reduced histopathological score

Our results showed a significant increase in severity of inflammation (2.16 ± 0.40; $P < 0.001$), pannus formation (2.0 ± 0.94; $P < 0.01$) and bone erosion (1.4 ± 0.51; $P < 0.01$) in arthritic control group as compared to vehicle control group. Treatment with TQ significantly attenuated inflammation (1.5 ± 0.54; $P < 0.01$), pannus formation (1.1 ± 0.73; $P < 0.001$) and bone erosion (0.8 ± 0.42; $P < 0.001$). Methotrexate also demonstrated significant decrease in inflammation (1.16 ± 0.40; $P < 0.01$), pannus formation (0.9 ± 0.31; $P < 0.001$) and bone erosion (0.7 ± 0.48; $P < 0.001$) (Table 1 and Fig. 4).

4.5. TQ suppressed mRNA expression levels of TLR2, TLR4, IL-1, NFκB and TNF-α

The results showed that mRNA expression levels of TLR2, TLR4, IL-1, NFκB and TNF-α were significantly ($P < 0.001$) raised in arthritic control group when compared to vehicle control group. Treatment with TQ and MTX significantly ($P < 0.001$) decreased mRNA expression level of all the above-mentioned parameters. There was no significant difference found in the tendency of MTX and TQ in decreasing the mRNA expression levels when compared with one another (Fig. 5).

4.6. Rheumatoid factor

Visible agglutination was seen in blood sera of arthritic control, TQ and Methotrexate treated groups. Treatment with TQ and Methotrexate didn't affect the levels of Rheumatoid factor (Data not shown).

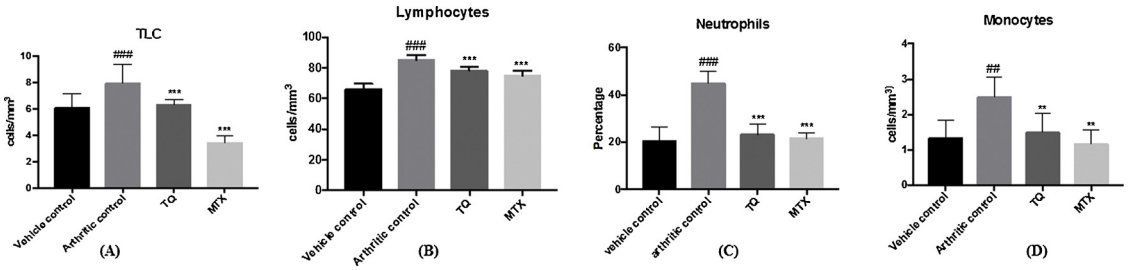

Fig. 2. TQ and MTX significantly reduced TLC (A), Lymphocytes (B), Neutrophils (C), and Monocytes (D). Results are presented as mean ± SD for 10 rats in each group. ** and *** shows $p < 0.01$ and $p < 0.001$ indicating significant difference compared to arthritic control group. ## and ### shows $p < 0.01$ and $p < 0.001$ indicating significant difference compared to vehicle control group.

Table 1
Mean ± SD of Histopathological score in all groups (n = 10).

Parameters	Group I (Vehicle control)	Group II (Diseased)	Group III (TQ)	Group IV (MTX)
Severity of inflammation	0	2.16 ± 0.40[a]	1.5 ± 0.54[b]	1.16 ± 0.40[b]
Pannus formation	0	2.0 ± 0.94[a]	1.1 ± 0.73[b]	0.9 ± 0.31[b]
Bone erosion	0	1.4 ± 0.51[a]	0.8 ± 0.42[b]	0.7 ± 0.48[b]

[a] shows a significant difference with group I.
[b] shows a significant difference with group II.

4.7. TQ didn't show toxic effects

Alanine transaminase (ALT), aspartate amino- transferase (AST), serum urea, creatinine and Hb levels were measured and results demonstrated normal values in all groups except Hb levels which were decreased in the arthritic group and normalized by TQ (Fig. 6).

5. Discussion

In the present study, experimental arthritis was developed by injecting 0.2 ml of FCA in the sub-plantar region of rats resulting in inflammatory response due to immune reaction against the intruding pathogens causing severe pain and swelling of joints [20]. Maximum paw edema was developed within 3–5 days reported similarly by [21].

Treatment with TQ 10 mg/kg/day in FCA-induced arthritic rats profoundly reduced the macroscopic arthritic score by decreasing the paw swelling. TQ is the main bioactive constituent of essential oil of an extensively used medicinal plant *Nigella sativa*. Methotrexate a commonly used DMARD for treating rheumatoid arthritis was a reference drug and we also found that TQ almost unanimously corresponded to the MTX group suggesting that it also exhibit immunomodulatory and anti-inflammatory properties.

The increase in inflammatory cells have a significant pathogenic role in the aggravation of arthritis by discharging pro-inflammatory compounds which results in the pathology of joint tissue. The study revealed that TQ inhibited the proliferation of all inflammatory cells i.e TLC, neutrophils, lymphocytes and monocytes in blood of arthritic rats and also restored the Hb levels. By decreasing inflammation TQ also resulted in reducing the CRP levels in the treated group.

Histopathological studies also justified the possible anti-arthritic effect of TQ. Severe bone erosion, pannus formation and cellular infiltration was seen in the ankle joints of arthritic rats. TQ treatment revealed marked improvement in overall inflammatory signs which is comparable to the reference standard drug MTX. [22].

In the present research, we also observed that TQ treatment significantly decreased the expressions of TLR2 and TLR4 in FCA-induced arthritic rats. Toll-like receptors are germline-encrypted pattern recognition receptors with vital role in identification of host cells and reactions to antigens associated with microbes [23]. Various studies have revealed the raised levels of TLR2 and TLR4 in fibroblasts of RA synovium, synovial fluid, macrophages, arthritic joints and pannus [24]. Both of these receptors are present extracellularly and identify lipids from gram + ve and gram–ve microorganisms [25]. Endogenous TLR ligands stimulate the macrophages causing an increased level of inflammatory cytokines such as TNF-α, IL-1 and IL-6, finally resulting in the destruction of cartilage and bone. Debilitation of TLR signaling is thought to be an innovative treatment strategy in patients affected by arthritis [26]. With the exception of TLR3, all TLRs use MyD88-dependent pathway leading to the stimulation of transcription factors among which NFκB, AP-1 and interferon regulatory factor (IRF) family members are being currently explored regarding RA [25]. The cytokines including TNFα, (pro)IL-1β and IL-6, type-1 IFNs, and chemokines [25] are excited by these transcription factors which in turn produce complex immune response.

Animal studies of inflammatory arthritis *in vivo* also reinforce the view that NFκB exhibit a very major part in the development and growth of arthritis [27]. NFκB transcription factor is mostly found in the cytoplasm as a dormant complex adhered to the IκB (inhibitors of κB) proteins. NFκB moves to the nucleus when activated after the enzymatic degeneration of IκB, which is liable to keep NFκB in the cytoplasm [28]. NFκB signaling pathways resolve many important events in the inflammatory reaction by chondrocytes, resulting in continual destruction of extracellular matrix and cartilage [29]. NFκB inhibitors are known to possess high therapeutic effectiveness and are suitable for treating RA in humans [30]. Our research demonstrated that TQ significantly reduced NFκB expression levels.

Many pro-inflammatory cytokines including TNF-α are maximally expressed in the presence of NFκB which shows its leading role. The stimulation of TLR2 results in abrupt discharge of TNF-α and other pro-inflammatory cytokines from synovium [31]. If TNF-α is present in abundance in the synovial fluid of arthritic patients, destruction of joints and inflammation are surely to occur which are thought to be the trademark of RA [32]. Moreover TNF-α also promotes the synthesis of PGE2 and collagenase resulting in injury to connective tissue. Our research revealed that there was appreciable rise in expression level of TNF-α in arthritic control group as compared to vehicle control. In the current study TQ also markedly reduced the expression levels of TNF-α along with another pro-inflammatory cytokine, IL-1. The results were almost similar to the standard reference drug MTX.

Although a variety of drugs has been developed for the treatment of arthritis; these include NSAIDs, DMARDs, steroids *etc.* but drugs with low cost and minimal side effects are still lacking. Many herbs are also commonly used to treat arthritis but the therapeutic efficacies and mode of action of such medicines are currently unclear. Therefore, large scope of new drug development exists in the treatment of rheumatoid arthritis. This study has provided insight into possible anti-arthritic effect of TQ.

Moreover, TQ does not show any hepatotoxic or nephrotoxic effects according to the levels of ALT, AST, creatinine and urea in the serum.

6. Conclusion

The current study highlights that anti-inflammatory and

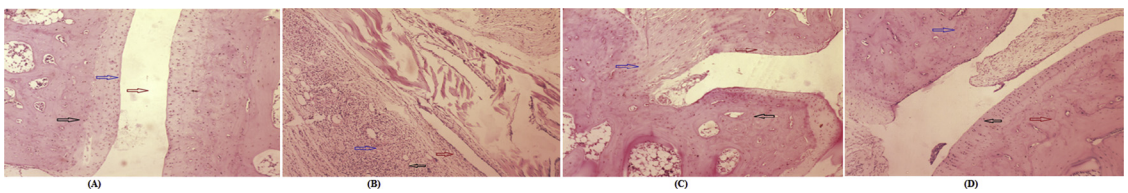

Fig. 4. (A) H & E staining, showing normal ankle joint tissue, normal synovium (red arrow), cartilage (blue arrow), bone (black arrow) and no inflammation (Control). (B) H & E staining, showing severe inflammation (blue arrow), pannus (red arrow), bone erosion (black arrow) (Arthritic). (C) H & E staining showing resolution of inflammation (blue arrow), pannus formation (red arrow) and bony erosion (black arrow) (TQ). (D) H & E staining showing resolution of inflammation (blue arrow), pannus formation (black arrow) and bony erosion (red arrow) (MTX) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

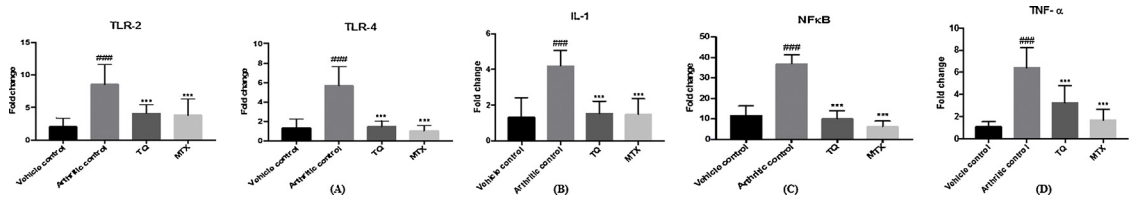

Fig. 5. Graphical representation of mean ± SD relative mRNA expression levels of TLR2, TLR4, NFκB, IL-1 and TNF-α in all groups (n = 10). *** shows $p < 0.001$ and indicates significant difference compared to arthritic control group while ### shows $p < 0.001$ and indicates significant difference compared to vehicle control group.

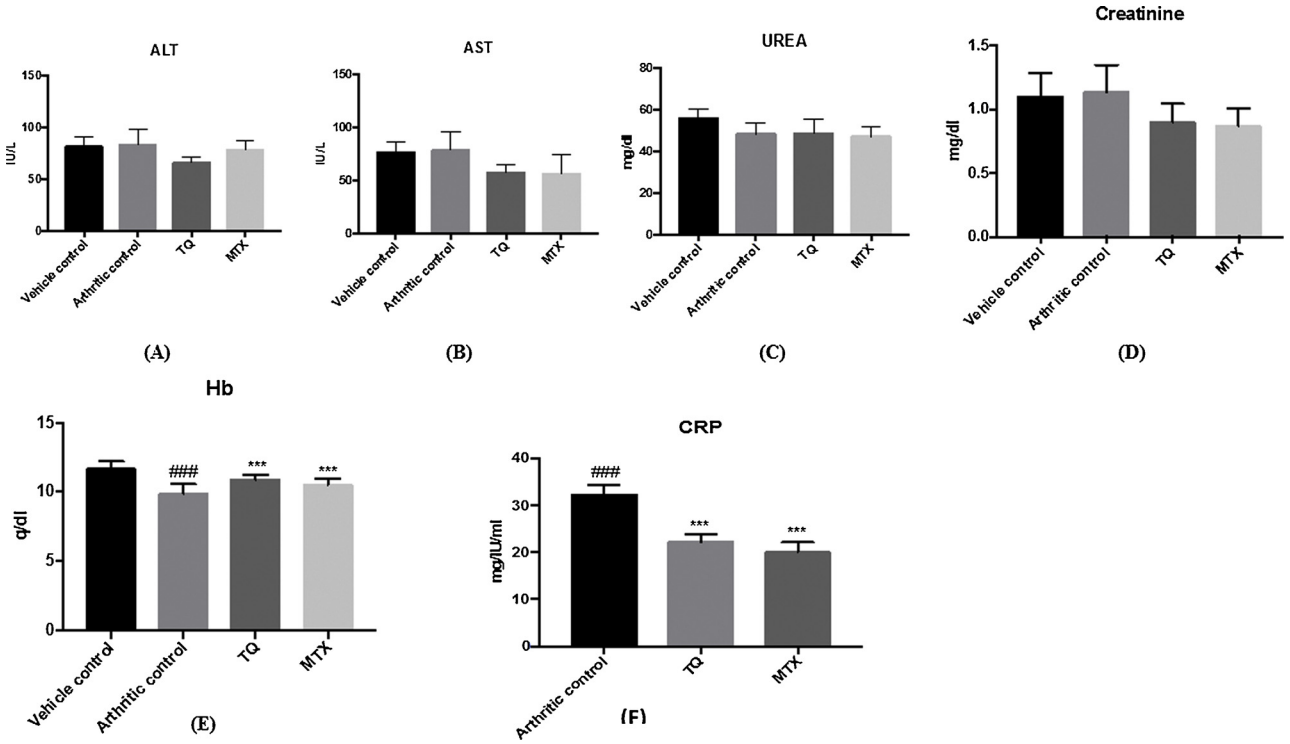

Fig. 6. Graphical representation of mean ± SD of serum ALT, AST, urea, creatinine and Hb levels in all groups (n = 10). *** shows $p < 0.001$ and indicates significant difference compared to arthritic control group while ### shows $p < 0.001$ and indicates significant difference compared to vehicle control group.

immunomodulatory effects of TQ has almost similar therapeutic effects to methotrexate, suggesting that TQ may be an alternative DMARD for treating RA. It significantly decreased arthritic score, the total count of leukocytes in the blood, improved the histopathological changes and reduced the expressions of TLR1, TLR2, IL-1, NFκB and TNF-α. To establish the safety of TQ, liver function tests and renal function tests were also performed and was found to be completely safe. Cell type-specific assessment assay is required in future studies to investigate the cell type modulated by TQ.

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Conflict of interest

Authors declare that they have no conflict of interest.

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References

[1] D. Symmons, C. Mathers, B. Pfleger, The Global Burden of Rheumatoid Arthritis [online], Available at: (2006) (Accessed 15 August 2006), http://www.who.int/healthinfo/statistics/bod_rheumatoidarthritis.
[2] J.A. Markenson, Worldwide trends in the socioeconomic impact and long-term prognosis of rheumatoid arthritis, Semin. Arthritis Rheum. 21 (2 Suppl. 1) (1991) 412.
[3] F.M. Brennan, I.B. McInes, Evidence that cytokines play a role in rheumatoid arthritis, J. Clin. Invest. 118 (2008) 3537–3545.
[4] A. Shabbir, M. Shahzad, A. Ali, M. Zia-ur-Rehman, Discovery of new benzothiazine derivative as modulator of pro- and anti-inflammatory cytokines in rheumatoid arthritis, Inflammation 39 (6) (2016) 1918–1929.
[5] Y. Yang, H. Sun, X. Mo, Y. Liu, H. Jia, X. Li, G. Zhang, Q. Li, Prediction of novel genes associated with negative regulators of toll-like receptors-induced inflammation based on endotoxin tolerance, Inflammation 35 (6) (2012) 1889–1899.
[6] J. Michiaki Takagi, Toll-like receptor—A potent driving force behind rheumatoid arthritis, J. Clin. Exp. Hematop. 51 (2) (2011) 77–92.
[7] K. Lundberg, N. Wegner, T. Yucel-Lindberg, P.J. Venables, Periodontitis in RA—the citrullinated enolase connection, Nat. Rev. Rheumatol. 6 (2010) (2010) 727–730.
[8] A.L. Blasius, B. Beutler, Intracellular toll-like receptors, Immunity 32 (3) (2010) 305–315.
[9] C.A. Martin, S.E. Carsons, R. Kowalewski, D. Bernstein, M. Valentino, F. Santiago-Schwarz, Aberrant extracellular and dendritic cell (DC) surface expression of heat shock protein (hsp)70 in the rheumatoid joint: possible mechanisms of hsp/DC-mediated cross-priming, J. Immunol. 171 (11) (2003) 5736–5742.

[10] M. Gambuzza, N. Licata, E. Palella, D. Celi, V. Foti Cuzzola, D. Italiano, S. Marino, P. Bramanti, Targeting toll-like receptors: emerging therapeutics for multiple sclerosis management, J. Neuroimmunol. 239 (1-2) (2011) 1–12.
[11] K. Soeken, S.A. Miller, E. Ernst, Herbal medicines for the treatment of rheumatoid arthritis: a systematic review, Rheumatology 42 (5) (2003) 652–659.
[12] N.A. Al Rowais, Herbal medicine in the treatment of diabetes mellitus, Saudi Med. J. (11) (2002) 1327–1331.
[13] M.L. Salem, Immunomodulatory and therapeutic properties of the Nigella Sativa L. seed, Int. Immunopharmacol. 5 (13-14) (2005) 1749–1770.
[14] L. Ustyol, K. Demiroren, I. Kandemir, R. Erten, K. Bulan, S. Kaba, N. Demir, M.T. Basunlu, Comparative nephroprotective effects of silymarin, N-Acetylcysteine, and thymoquinone against carbon tetrachloride-induced nephrotoxicity in rats, Iran. Red Crescent Med. J. 9 (Jan. (1)) (2017) e37746.
[15] R. Faisal, U. Imran, Comparative evaluation of thymoquinone and methotrexate in lung inflammation in murine model of rheumatoid arthritis, J. Postgrad. Med. Inst. 29 (2) (2015) 88–92.
[16] A. Shabbir, M. Shahzad, A. Ali, M. Zia-ur-Rehman, Anti-arthritic activity of N-[(2,4-dihydroxyphenyl)methylidene]-2-(3, 4-dimethyl-5,5-dioxidopyrazolo[4,3-c] [1,2] benzothiazin-1 (4H)-yl) acetohydrazide, Eur. J. Pharmacol. 738 (2014) (2014) 263–272.
[17] M.S. Mali, A. Sinnathambi, C.U. Kapase, S.L. Bodhankar, K.R. Mahadik, Anti-arthritic activity of standardized extract of Phyllanthus amarus in Freund's complete adjuvant induced arthritis, Biomed. Aging Pathol. 1 (2011) (2011) 185–190.
[18] W. Zhu, L. Meng, C. Jiang, X. He, W. Hou, P. Xu, H. Du, R. Holmdahl, S. Lu, Arthritis is associated with T-cell-induced up-regulation of Toll-like receptor 3 on synovial fibroblasts, Arthritis Res. Ther. 13 (3) (2011) 103.
[19] X. Cai, H. Zhoua, Y.F. Wong, Y. Xie, Z.Q. Liu, Z.H. Jiang, Z.X. Bian, H.X. Xub, L. Liu, Suppression of the onset and progression of collagen-induced arthritis in rats by QFGJS, a preparation from an anti-arthritic Chinese herbal formula, J. Ethnopharmacol. 110 (2007) 39–48.
[20] Z.Q. Dong, J. Zhu, D.Z. Lu, Q. Chen, Y.L. Xu, Effect of electroacupuncture in "Zusanli" and "Kunlun" acupoints on TLR4 signaling pathway of adjuvant arthritis rats, Am. J. Ther. (2016), https://doi.org/10.1097/MJT.0000000000000477.
[21] M. Summanth, Anusha, S. Swetha, Elucidation of mechanism of antiarthritic action of Arthrosansar, IJKT 11 (4) (2012) 704–713.
[22] S. Umar, J. Zargan, K. Umar, S. Ahmad, C.K. Katiyar, H.A. Khan, Modulation of the oxidative stress and inflammatory cytokine response by thymoquinone in the collagen induced arthritis in Wistar rats, Chem. Biol. Interact. 197 (1) (2012) 40–46.
[23] T. Kawai, S. Akira, Toll-like receptors and their crosstalk with other innate receptors in infection and immunity, J. Immun. 34 (5) (2011) 637–650.
[24] K.W. Kim, M.L. Cho, S.H. Lee, H.J. Oh, C.M. Kang, J.H. Ju, et al., Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-activation, Immunol. Lett. 110 (1) (2007) 54–64.
[25] K.C.M. Santegoets, L. Van Bon, W.B. Van den Berg, M.H. Wenink, T.R.D.J. Radstake, Toll-like receptors in rheumatic diseases: are we paying a high price for our defense against bugs? FEBS Lett. 585 (2011) (2011) 3660–3666.
[26] Q.Q. Huang, R.M. Pope, Role of toll like receptors in rheumatoid arthritis, Curr. Rheumatol. Rep. 11 (5) (2009) 357–364.
[27] A. Mor, S.B. Abramson, M.H. Pillinger, The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction, Clin. Immunol. 115 (2005) (2005) 118–128.
[28] R. Kumar, Q.C. Yong, C.M. Thomas, Do multiple nuclear factor kappa B activation mechanisms explain its varied effects in the heart? Ochsner J. Spring. 13 (1) (2013) 157–165.
[29] J.A. Roman-Blas, S.A. Jimenez, NF-κB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis, Osteoarthr. Cartil. 14 (9) (2006) 839–848.
[30] S.S. Makrov, NF-kB in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction, Arthritis Res. 3 (2001) 200–206.
[31] S. Abdollahi-Roodsaz, M.F. Roelofs, L.A. Joosten, W.B. van den Berg, T. Radstake, The orchestra of Toll-like receptors (TLRs) and their potential role in frequently occurring rheumatic conditions, Arthritis Rheum. 2008 (2008) 338–348.
[32] P. Vasanthi, G. Nalini, G. Rajashekhar, Role of tumor necrosis factor-alpha in rheumatoid arthritis: a review, APLAR J. Rheumatol. 10 (2007) 270–274.