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

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Anti-arthritic activity of *N*′-[(2,4-dihydroxyphenyl)methylidene]-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-c][1,2]benzothiazin-1(4*H*)-yl)acetohydrazide

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ABSTRACT

Benzothiazine and pyrazole derivatives possess anti-inflammatory properties. Previously, synergism of both heterocyclic moieties into a single nucleus has shown to produce biologically active *N*′-arylmethylidene-2-(3,4-dimethyl-5,5-dioxidopyrazolo(4,3-c)(1,2)benzothiazin-2(4*H*)yl)acetohydrazides) compound. Present study investigates the anti-arthritic effect and possible mechanism of 2,4-dihydroxyphenyl derivative (DHP) in Freund's complete adjuvant-induced arthritic rat model. Ankle joint histopathology was performed with Hematoxylin & Eosin staining, while serum C-reactive protein (CRP) levels were measured by agglutination method. mRNA expression levels and protein levels of proinflammatory markers were measured by real time reverse transcription polymerase chain reaction and Enzyme linked immunosorbent assay (ELISA), respectively. *in vitro* Concanavalin A (ConA)-stimulated splenocyte proliferation was also measured by ELISA reader. DHP treatment reduced the macroscopic arthritic score, CRP levels, synovial inflammation, bone erosion and pannus formation. Levels of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), Prostaglandin-E2 (PGE2), and 5-lipoxygenase (5-LOX), were significantly attenuated by DHP. It also significantly decreased the levels of toll-like receptor 2, nuclear factor-kappaB (NF-κB), and tissue necrosis factor-α (TNF-α) and non-significantly elevated interleukin-4 (IL-4) levels. Piroxicam, used as reference drug, significantly reduced the levels of COX-1, COX-2, PGE2, NF-κB, and TNF-α, but did not show reduction in 5-LOX and toll-like receptor 2 levels. However piroxicam significantly enhanced the levels of IL-4. Both DHP and piroxicam suppressed ConA-stimulated splenocyte proliferation. DHP normalized all altered hematological markers and did not show any sign of hepatotoxicity or nephrotoxicity as determined by alanine transaminase, aspartate aminotransferase, urea, and creatinine levels. Results showed that DHP possesses significant anti-arthritic activity which may be attributed to its immunomodulatory and anti-inflammatory effects.

1. Introduction

Rheumatoid arthritis (RA) is categorized as an autoimmune disorder which results in synovial inflammation, hyperplasia, deformity of cartilage and bone, and systemic illnesses (Mclnnes and Schett, 2011). According to the report by World Health Organization, RA accounts for 0.8% of total global YLD (Years lived with disability), and is the 31st leading reason of YLDs globally. The incidence and prevalence are greater in women than men and increase with age (Symmons et al., 2012).

Disease-modifying anti-rheumatic drugs (DMARD), tumor necrosis factor (TNF) inhibitors, monoclonal antibodies directed against B cells, and nonsteroidal anti-inflammatory drugs (NSAID) are most commonly used therapies of RA (Scott et al., 2010; Sundy, 2001). NSAIDs are the mainstream therapy of RA, and considered as the "first line" therapy. They effectively ameliorate the symptoms of RA, although they are not capable of changing the course of the disorder (Grover et al., 2011). They are effective in relieving the stiffness, swelling, and pain of joints, and exert their anti-inflammatory and analgesic action by inhibiting PGE2 via inhibition of cyclooxygenase (COX) enzyme (Crofford, 2013). Different factors, such as, improving the adverse effects and increasing the selectivity have kept pharmaceutical chemists interested in the continuous development of new compounds.

Benzothiazine derivatives are known for their role in the treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis (Gennari et al., 1994). Piroxicam, meloxicam, and ampiroxicam are 1,2-benzothiazine-3-carboxamide-1,1 dioxide derivatives of

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benzothiazines, possess anti-inflammatory and analgesic properties, and have been used globally as NSAIDs (Zia-ur-Rehman et al., 2009). Different other derivatives have also demonstrated antioxidant, calpain 1 inhibitory (Bihovsky et al., 2004; Zia-ur-Rehman et al., 2009), and significant anti-bacterial activities against *Bacillus subtilis* (Zia-ur-Rehman et al., 2006). Pyrazole and its derivatives is another vital class of heterocyclic compounds. Different pyrazole derivatives are known to possess antiviral (Baraldi et al., 1998), anti-inflammatory (Smith et al., 2001a), hypoglycemic (Cottineau et al., 2002), and local anesthetic and antiarrhythmic (Iovu et al., 2003) properties. Considering the potential pharmacological properties of both heterocyclic moieties, it was perceived that synergism of 1,2-benzothiazine-1,1-dioxide and pyrazole moieties into a single nucleus might result in the synthesis of valuable compounds from pharmacological point of view (Ahmad et al., 2010).

Previously, the synthesis of novel *N′*-arylmethylidene-2-(3,4-dimethyl-5,5-dioxidopyrazolo(4,3-c)(1,2)benzothiazin-2(4*H*)yl) acetohydrazides) compounds were reported and preliminary antioxidant and antibacterial activities were found. 2,4-dihydroxyphenyl derivative (DHP) has demonstrated highest free radical scavenging activity and antibacterial activity against *B. subtilis* and *Escherichia coli*, followed by 3-ethoxy-4-hydroxyphenyl derivative (Ahmad et al., 2010).

In present study, we evaluated the anti-arthritic activity of DHP in a rat model of FCA-induced arthritis by measuring various immunomodulatory and anti-inflammatory parameters.

2. Materials and methods

2.1. Animals

Sprague Dawley rats of 6–8 weeks age and either sex, weighing 150–250 g, were kept at animal house, University of Health Sciences, Lahore. Rats were provided water and standard pellet diet *ad libitum*. Animals were kept at 12 h dark/light cycles and standard conditions of humidity (60–70%) and temperature (28 °C ± 2 °C). All animals were acclimatized to the environment for a period of one week before the beginning of experiment. All the experiments were approved by Ethical Review Committee, University of Health Sciences, Lahore.

2.2. Experimental design and induction of arthritis

The rats were randomly divided by balloting method into four groups, each containing ten rats.

Group-I: Vehicle control (0.1 N NaOH 10 mg/kg i.p.).
Group-II: Arthritic control (0.1 N NaOH 10 mg/kg i.p.).
Group-III: Arthritic DHP treated (DHP 10 mg/kg i.p. dissolved in 0.1 N NaOH).
Group-IV: Arthritic piroxicam treated (Piroxicam 10 mg/kg i.p. dissolved in 0.1 N NaOH).

Arthritis was induced by injecting 0.2 ml FCA (*Mycobacterium butyricum* suspended in mineral oil) in sub plantar region of the left hind rat paw on day 0 in all groups except vehicle control group. Treatment was initiated at the 8th day of arthritis induction and all animals were killed at day 28. All the doses were selected by a pilot study (Jawed et al., 2010).

2.3. Assessment of the arthritis development

Arthritic scoring method was used for the determination of incidence and severity of arthritis. Rats were examined for the appearance of visible signs which were characterized by erythema and edema in the hind rat paws. Arthritic score was measured at days 0, 4, 8, 12, 16, 20, 24, and 28 by macroscopic criteria (Jawed et al., 2010), 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.

2.4. Histopathological examination of ankle joints

The ankle joints of rat were removed, fixed in 10% formalin, and subsequently decalcified using hydrochloric acid, ethylenediaminetetra acetic acid, sodium tartrate, and potassium sodium tartrate containing decalcifying solution. Tissues were embedded in paraffin, and stained with hematoxylin and eosin (H&E) after slicing into 5 μm thick sections. Bone erosion, pannus formation, and infiltration of inflammatory cells were examined by blinded histopathologist. Results were semi-quantified by giving 0, 1, 2, 3, and 4 numbers to normal, minimal, mild, moderate, and severe changes, respectively (Cai et al., 2007).

2.5. Determination of C-reactive protein levels

CRP levels were measured based on the agglutination method by using commercial kit and was conducted according to the manufacturer's protocol (Antec diagnostic products-UK). Test relies on an immunological reaction between CRP in the serum and CRP antisera bound to biologically inert latex particles. Visible agglutination was observed when serum sample contained high levels of CRP reacted with antisera. CRP levels were semi-quantified according to the protocol.

2.6. Biochemical parameters

Various biochemical parameters, such as, AST, ALT, urea, and creatinine levels were determined in blood by using autoanalyser (Humalyzer 3500). Commercially available kits were used for the detection according to manufacturer's protocol. Various hematological parameters like, WBC, RBC, and Platelet counts, along with Hb content were also determined by using automated hemocytometer (Sysmex XT-1800i).

2.7. Enzyme-linked immunosorbent assays for PGE2 and 5-LOX

PGE2 and 5-LOX levels were measured by EISA according to kit manufacturer's protocol (Glory Science Co., Ltd.). Briefly, samples were added in pre-coated wells of a 96 well microtiter plate. Then PGE2/5-LOX antibody was added, followed by streptavidin–horseradish peroxidase. Plate was gently shook, sealed, and incubated at 37 °C for 1 h. Plate was washed for 3 times with washing solution and chromogen solution A and B were added subsequently. An incubation period of 10 min was allowed at 37 °C away from light. After this, stop solution was added and optical density was measured at 450 nm wavelength.

2.8. Determination of mRNA expression levels of toll-like receptor 2, NF-κB, TNF-α, IL-4, COX-1, and COX-2

Total RNA from blood was extracted by TRIzol method, while, purity and yield was quantified through nanodrop. cDNA was synthesized by reverse transcription according to kit manufacturer's protocol (Thermo Scientific, America). Briefly, 500 ng total RNA was used per reaction and mixed with 0.5 μg of 100 μM oligo dt_{18} and nuclease free water. Mixture was heated at 65 °C for 5 min and subsequently chilled down by placing on ice. Then 5 × reaction buffer (20 mM $MgCl_2$, 250 mM KCl, 250 mM Tris–HCl (pH = 8.3), and 50 mM DTT), 10 mM dNTP mix, 20 units of RiboLock RNase inhibitor, and 200 units of M-MuLV reverse

transcriptase enzyme were added and mixture was placed at 42 °C for one hr. Reaction was terminated by heating at 70 °C for 5 min.

Real time polymerase chain reaction (qPCR) was used to amplify and quantify the product using Bio-Rad system. Briefly, cDNA template was mixed with gene specific primer, SYBR Green (2 ×) PCR master mixed with ROX (internal dye; Thermo Scientific, America), and nuclease free water and placed in a thermal cycler with 45 cycles of denaturation (95 °C), annealing (60 °C), and termination (72 °C). Genes of the various markers were picked from Ensembl Genome Browser and gene specific primers were designed manually and by using Primer3 Input (v. 0.4.0) software. Contamination from genomic DNA was avoided by choosing only intron-overlapping primers, which are presented in Table 1.

2.9. Concanavalin A-induced splenocyte proliferation

Spleen cells were isolated according to the "protocol for the isolation of splenocytes from murine spleens, ProImmune limited UK". Whole spleens of normal rats were removed and washed with antibiotic containing (penicillin–streptomycin) phosphate buffer saline (PBS). Spleens were placed in 70 μ cell strainer and meshed with black rubber of syringe plunger to release the splenocytes into the petri dish. Resulting cell suspension was centrifuged at 400*g* for 10 min at room temperature and ammonium chloride lysing reagent (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA) was added. Suspension was left for 3 min in the dark at room temperature followed by centrifugation at 400*g* for 5 min. Pellet was again suspended and washed with PBS. Suspension was again centrifuged at 400*g* for 5 min, and pellet was re-suspended in cell culture medium (RPMI-1640, L-Glutamate, 10% fetal bovine saline, penicillin–streptomycin).

A total of 2×10^4 cells per well were seeded with 100 μl cell culture medium in 96-well cell culture flat bottom plate and incubated at 37 °C for 24 h. Then, the cells were treated with 10 μg of ConA (a proliferation stimulant), BrdU (BromodeoxyUridine) dissolved in cell culture media (label), and 1 mg of experimental and reference drugs. The plate was incubated for 24 h at 37 °C.

BrdU-DNA assay was conducted according to kit manufacturer's protocol (Calbiochem, Merck Millipore). After incubation, contents of the 96 well plate were removed by inverting over sink and gentle tapping over tissue paper. 200 μl of fixative/denaturing solution was added to each well and incubated at 37 °C for 30 min. Contents of plate were again removed and anti-BrdU antibody was added to each well. Plate was incubated for 1 h at 37 °C. After incubation each well was washed 3 times by wash buffer (1 ×) and peroxidase goat anti-mouse IgG HRP conjugate was added to each well. Plate was again incubated for 30 min at 37 °C followed by 3 times washing with wash buffer (1 ×) to remove unbound antibody. Whole plate was flooded with dH_2O and contents were removed by inverting over sink. Substrate solution was then added and incubated in the dark for 15 min. Stop solution was added in each well in the same order as substrate solution was added. Absorbance was measured at 450 nm wave length within 15 min of adding stop solution by using ELISA reader (BioTek, ELx-800).

Table 1
Primer sequences and amplified band (base pair).

Marker	Forward/ reverse	Sequence	Amplified band (base pair)
Toll-like receptor 2	Forward	5′-CCAGATGGCCAGAGGACTCA-3′	212
	Reverse	5′-TGTGAGTCCCGAGGGAATAGA-3′	
NF-κB	Forward	5′-CAAGGAAGAGGATGTGGGGTT-3′	207
	Reverse	5′-AGCTGAGCATGAAGGTGGATG-3′	
TNF-α	Forward	5′-CCTCTTCTCATTCCTGCTCGT-3′	266
	Reverse	5′-TGAGATCCATGCCATTGGCC-3′	
IL-4	Forward	5′-GGATGTAACGACAGCCCTCT-3′	219
	Reverse	5′-ACCGAGAACCCCAGACTTGT-3′	
COX-1	Forward	5′-CTGCCCTCTGTACCCAAAGA-3′	200
	Reverse	5′-GGACCCATCTTTCCAGAGGT-3′	
COX-2	Forward	5′-CCAGATGGCCAGAGGACTCA-3′	237
	Reverse	5′-TGTGAGTCCCGAGGGAATAGA-3′	
GAPDH	Forward	5′-TCTCTGCTCCTCCCTGTTCT-3′	229
	Reverse	5′-CTTGCCGTGGGTAGAGTCAT-3′	

2.10. Statistical analysis

Data was presented as Mean ± S.E.M and analyzed using GraphPad Prism (v. 5) software. Quantitative variables were compared and differences among groups were analyzed by using one way ANOVA followed by Tukey's test or student's *t*-test where applicable. *P* value ≤ 0.05 was considered as statistically significant.

3. Results

3.1. DHP suppressed arthritic score

Sub plantar injection of FCA produced arthritic signs and lesions on both injected and non-injected paws which are indicative of primary and secondary arthritic lesions, respectively. Unilateral inflammatory edema was observed around days 1–9 while arthritic signs in non-injected contra lateral paw were observed around days 10–27. No signs of paw swelling were observed in vehicle control group. DHP treatment was started at the 8th day of arthritis induction and significantly ($P < 0.001$) reduced arthritic score (2.400 ± 0.1633) at 12th day as compared with arthritic control group (3.200 ± 0.1333). Piroxicam also showed significant ($P < 0.05$) suppression (2.800 ± 0.1333) on 12th day. Arthritic scores determined at different days are presented in Fig. 1A.

3.2. DHP reduced histopathological score

Ankle joints of ipsilateral rat paws were subjected to histopathological evaluation and we found significant ($P < 0.001$) bone erosion (1.778 ± 0.1470), inflammation (2.556 ± 0.2940), and pannus formation (3.111 ± 0.2606) as compared with vehicle control group. Treatment with DHP significantly attenuated bone erosion (0.8889 ± 0.1111; *P* value < 0.001), pannus formation (1.556 ± 0.1757; $P < 0.001$) and inflammation (1.444 ± 0.1757; $P < 0.01$). Piroxicam also demonstrated significant reduction in inflammation (1.571 ± 0.2020; $P < 0.01$), pannus formation (1.429 ± 0.2020; $P < 0.001$), and bone erosion (1.143 ± 0.1429; $P < 0.01$) (Fig. 1C–E).

3.3. Treatment with DHP alleviated CRP levels

Increased levels of CRP were found in arthritic control group (38.40 ± 3.919) 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 DHP (22.80 ± 3.323; $P < 0.01$) and piroxicam (24.00 ± 3.098; $P < 0.05$) showed significant suppression of CRP levels (Fig. 1B).

3.4. DHP suppressed levels of PGE2 and 5-LOX

We found increased ($P < 0.05$) serum levels of PGE2 (1.019 ± 0.03781) in arthritic control group as compared to vehicle control group (0.8029 ± 0.05727). Treatment with both DHP (0.8052 ± 0.04184) and piroxicam (0.9182 ± 0.02298) significantly ($P < 0.05$) suppressed PGE2 levels as compared with arthritic control group.

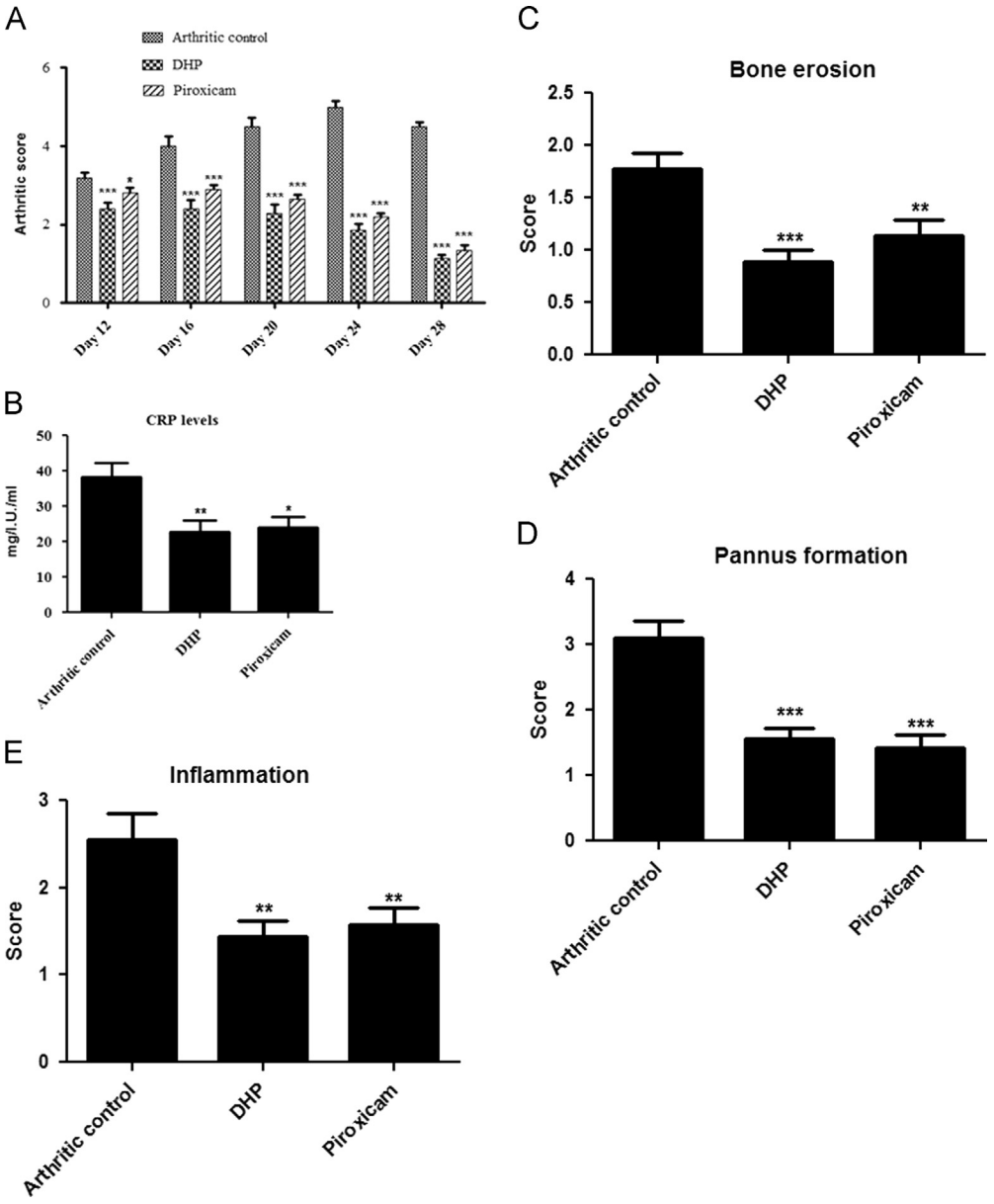

Fig. 1. DHP and piroxicam significantly suppressed arthritic score at days 12, 16, 20, 24, and 28 (A), CRP levels in serum samples (B), bone erosion (C), pannus formation (D), and inflammation (E). Results are presented as Mean ± S.E.M for 7–10 rats in each group. $P < 0.05$, $P < 0.01$, and $P < 0.001$ are presented by *, **, and ***, respectively, which denote comparison of experimental drugs groups with arthritic control group.

Similarly, elevated ($P < 0.05$) levels of 5-LOX were also observed in arthritic control group (0.8966 ± 0.03418) as compared to vehicle control group (0.7845 ± 0.03967). We observed significant reduction ($P < 0.05$) in 5-LOX levels after treatment with DHP (0.7096 ± 0.04337), whereas, non-significant suppression was found in by piroxicam (0.8422 ± 0.03233) (Fig. 2C and D).

3.5. DHP suppressed mRNA expression levels of COX-1, COX-2, toll-like receptor 2, NF-κB, and TNF-α

Significantly elevated ($P < 0.05$) mRNA expression levels of COX-1 and COX-2 were found in arthritic control group (1.635 ± 0.2213 and 1.382 ± 0.1615 respectively) as compared with vehicle control group (1.000 ± 0.1464 and 1.000 ± 0.05166, respectively). Both DHP (0.7471 ± 0.2214 and 0.5714 ± 0.0790) and piroxicam (0.6311 ± 0.2360 and 0.8100 ± 0.1497) significantly attenuated ($P < 0.05$) the mRNA expression levels of COX-1 and COX-2, respectively (Fig. 2A and B).

Data showed a significant ($P < 0.05$) increase in toll-like receptor 2 mRNA expression levels in arthritic control groups (2.147 ± 0.4315) as compared with vehicle control group (1.000 ± 0.2799). Treatment with DHP (0.9126 ± 0.1896) exhibited significant reduction ($P < 0.05$), whereas, we did not find suppression of toll-like receptor 2 mRNA expression levels in piroxicam treated group (2.184 ± 0.3917) when compared with arthritic control group. Similarly, we also observed significant ($P < 0.05$) elevation in the mRNA expression levels of NF-κB in arthritic control group (2.351 ± 0.4469) as compared with vehicle control group (1.000 ± 0.1469). A significant ($P < 0.05$) attenuation was found after treatment with both DHP (1.231 ± 0.2274) and piroxicam (0.9656 ± 0.3241) (Fig. 3A and B).

Increased TNF-α expression levels ($P < 0.05$) were also detected in arthritic control group (2.263 ± 0.4144) as compared with vehicle control group (1.000 ± 0.2090). Treatment with DHP (1.256 ± 0.1475) and piroxicam (0.9434 ± 0.2657) produced significant suppression ($P < 0.05$) when compared with arthritic control group (Fig. 3C).

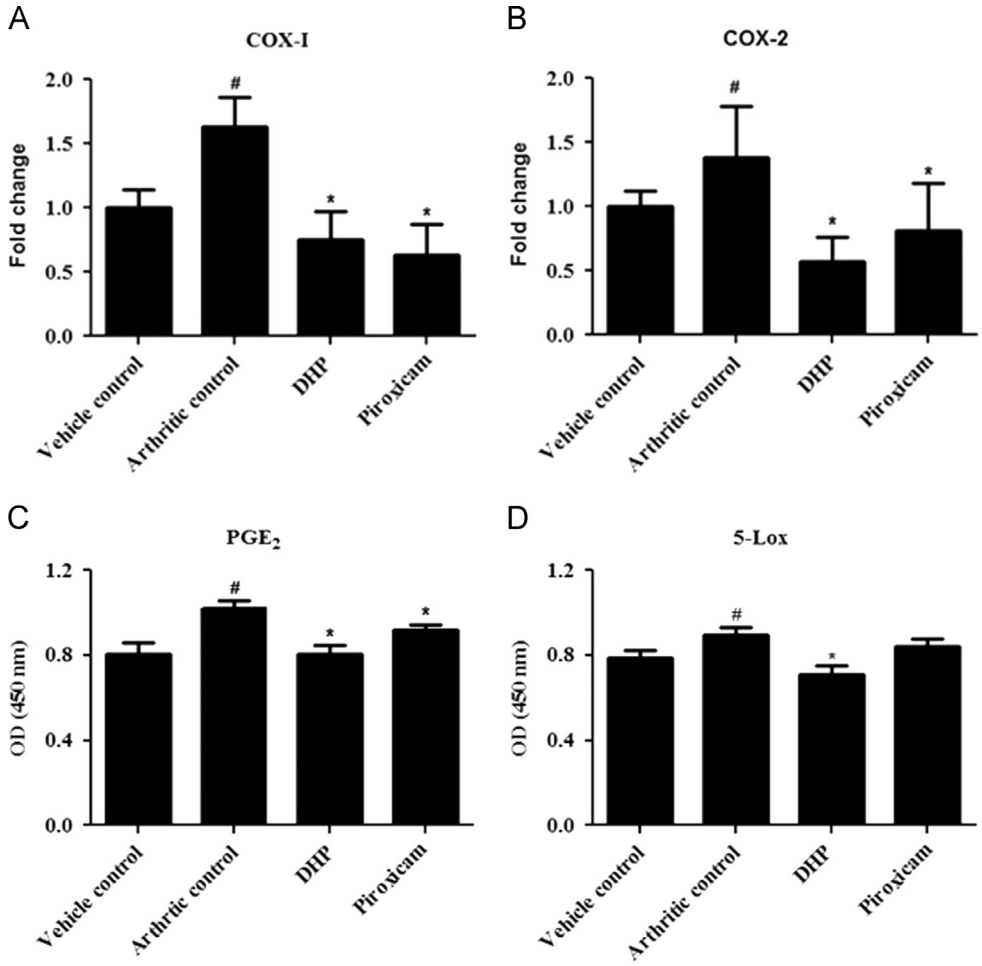

Fig. 2. DHP and piroxicam significantly reduced expression levels of COX-1 (A) and COX-2 (B), and protein levels of PGE2 (C) and 5-LOX (D). Results are presented as Mean ± S.E.M for 06 rats in each group. *P* values < 0.05 are presented by * and #, which denote comparison of experimental drugs groups with arthritic control group and comparison of vehicle control group with arthritic control group, respectively.

3.6. IL-4 mRNA expression levels were non-significantly elevated by DHP

We observed significant ($P < 0.05$) alleviation in the expression levels of IL-4 in arthritic control group (0.4837 ± 0.1204) as compared with vehicle control group (1.000 ± 0.1715). Treatment with piroxicam (2.283 ± 0.7511) resulted into significant ($P < 0.05$) increase in IL-4 levels, whereas, DHP showed non-significant elevation (1.468 ± 0.4979) (Fig. 3D).

3.7. DHP inhibited ConA stimulated splenocyte proliferation

ConA significantly ($P < 0.05$) stimulated splenocyte proliferation (0.3377 ± 0.01073) as compared to negative control (0.2903 ± 0.01268). Both DHP (0.1463 ± 0.009701) and piroxicam (0.1430 ± 0.02254) showed marked ($P < 0.001$) inhibition of proliferation (Fig. 3E).

3.8. DHP normalized hematological parameters

Data exhibited significant elevation of WBC ($P < 0.01$) and Platelet ($P < 0.05$) counts in arthritic control group (17.39 ± 1.081 and 1877 ± 90.18 respectively) as compared with vehicle control group (12.67 ± 0.3567 and 1534 ± 117.8, respectively). Treatment with DHP significantly ($P < 0.05$) normalized WBC and platelet counts (13.55 ± 1.263 and 1523 ± 98.69, respectively). Similarly, piroxicam also decreased the WBC and platelet counts to normal (13.33 ± 0.1620 and 1457 ± 64.42, respectively) (Fig. 4A and B).

Significant ($P < 0.05$) reduction in RBC count and Hb content were observed in arthritic control group (7.206 ± 0.2937 and 13.06 ± 0.4313 respectively) as compared with vehicle control group (8.420 ± 0.3694 and 15.48 ± 0.8291, respectively). Both markers were significantly ($P < 0.05$) normalized by treatment with DHP (8.082 ± 0.2441 and 14.78 ± 0.5354 respectively) and piroxicam (8.335 ± 0.3202 and 14.61 ± 0.3458, respectively) (Fig. 4C and D).

3.9. DHP did not show hepatotoxicity and nephrotoxicity

We measured alanine transaminase (ALT) and aspartate aminotransferase (AST) levels, and data showed normal values in DHP treated group when compared with vehicle control group. Similarly urea and creatinine levels were also measured and no difference of all groups was found as compared with vehicle control group (Fig. 5A–D).

4. Discussion

Pharmacologically, benzothiazine and pyrazole are important drug classes which are known for their anti-inflammatory and anti-arthritic effects (Gennari et al., 1994; Smith et al., 2001a, 2001b) Synergism of both heterocyclic moieties into a single nucleus produced new pharmacologically active compounds. Among those, 2,4-dihydroxyphenyl derivative was found to

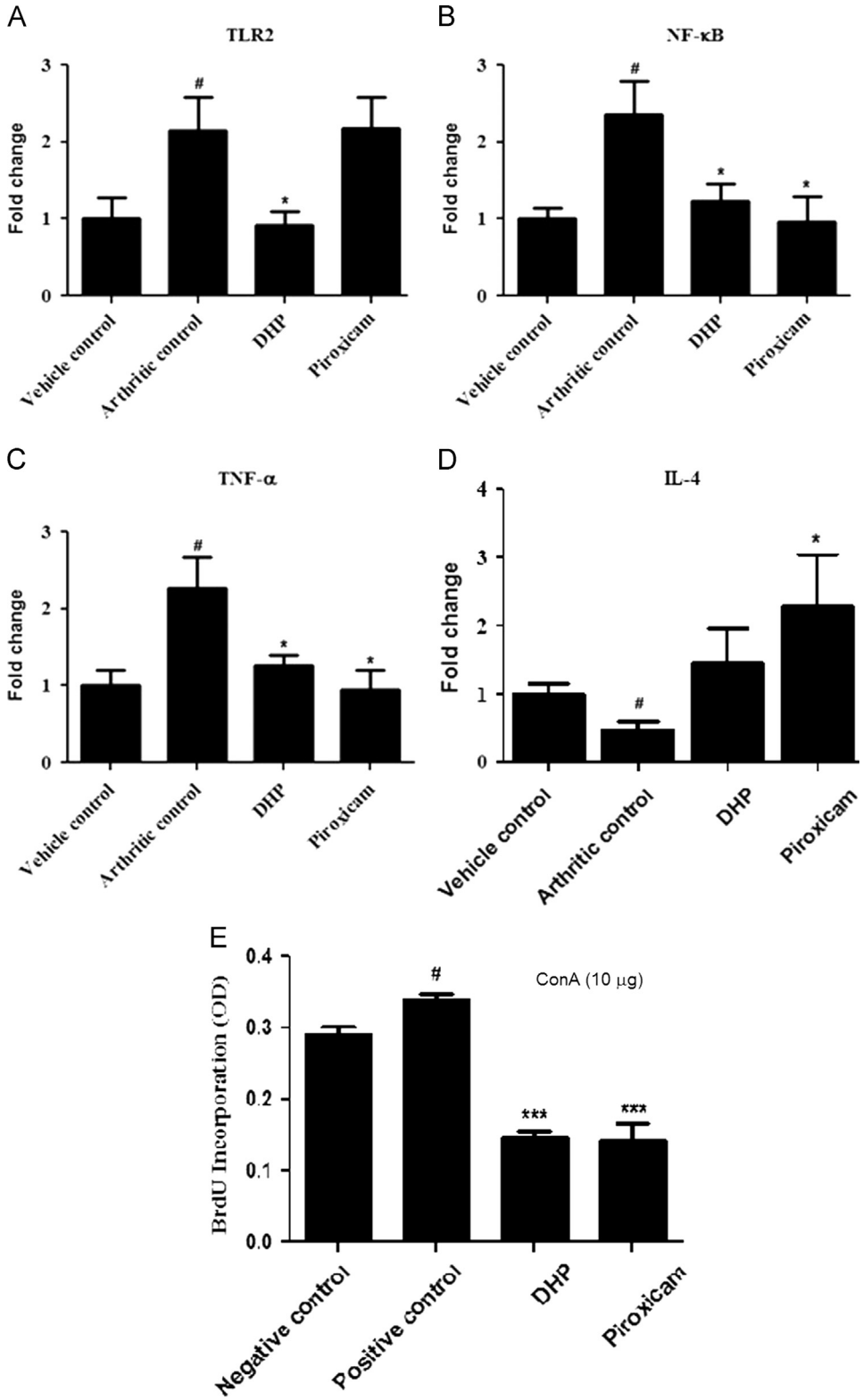

Fig. 3. DHP significantly attenuated the expression levels of toll-like receptor 2 (A), NF-κB (B), and TNF-α (C), while non-significantly elevated the levels of IIL-4 (D). ConA-stimulated splenocyte proliferation is also significantly inhibited by DHP (E). Results are presented as Mean ± S.E.M for 06 rats in each group. *P* values < 0.05 are presented by * and #, which denote comparison of experimental drugs groups with arthritic control group and comparison of vehicle control group with arthritic control group, respectively.

possess highest anti-oxidant and anti-bacterial activity (Ahmad et al., 2010).

We used FCA-induced arthritis rat model, and piroxicam, a commonly used drug in the treatment of rheumatoid arthritis, was used as a reference drug. Mycobacterial constituents of FCA have been recognized to involve toll like receptors and stimulate the production of TNF-α and IL-12, later is known to deviate the differentiation towards Th1 response (Billiau and Matthys, 2001).

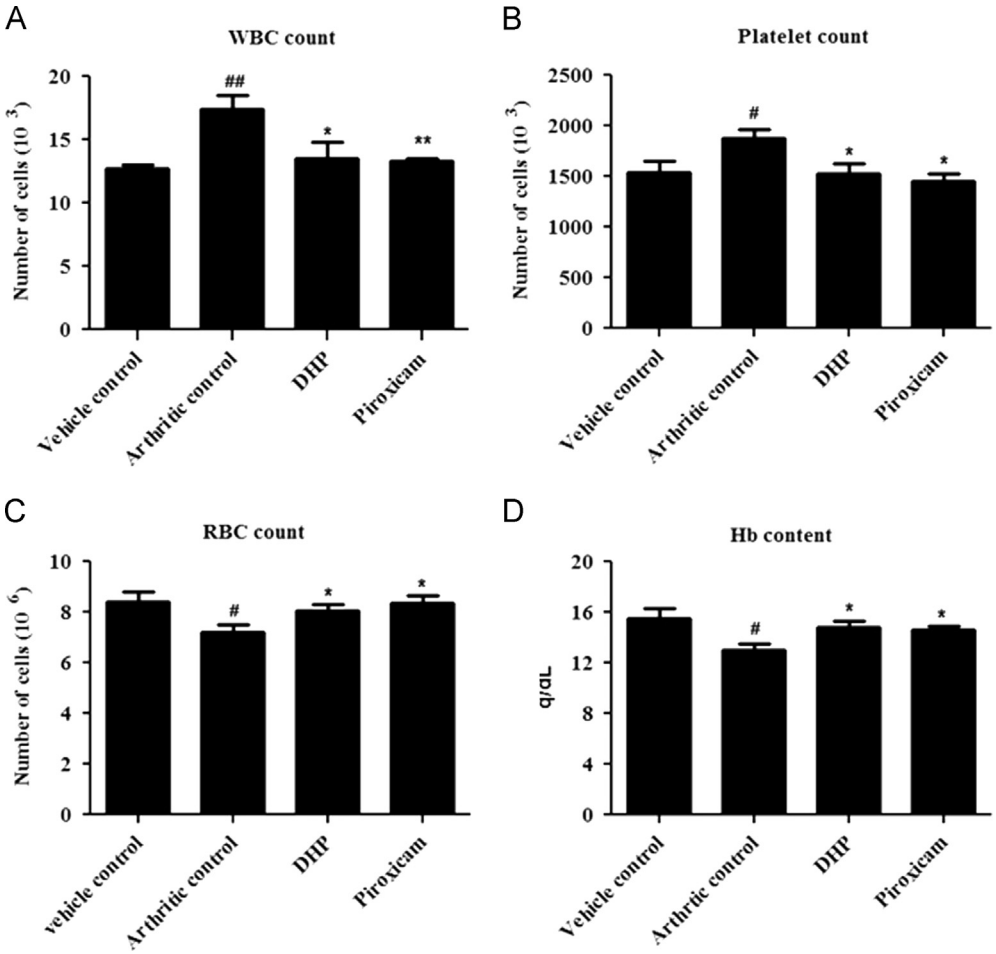

Fig. 4. DHP and piroxicam significantly reduced WBC (A) and platelet counts (B), and significantly elevated RBC counts (C) and Hb levels (D). Results are presented as Mean ± S.E.M for 10 rats in each group. *P* values < 0.05 are presented by * and #, which denote comparison of experimental drugs groups with arthritic control group and comparison of vehicle control group with arthritic control group, respectively.

Our results showed that treatment with DHP significantly reduced macroscopic arthritic score and histopathological score.

Cyclooxygenase (COX) is an enzyme responsible for the production of prostaglandins from arachidonic acid, and NSAIDs exhibit anti-inflammatory property through inhibition of COX activity (Dugowson and Gnanashanmugam, 2006). Our data showed that DHP significantly suppressed expression levels of both COX-I and COX-2 enzymes, similar to the effect of piroxicam. COX-1 and COX-2 are two leading isoforms in mammals. COX-1 is generally recognized as a "housekeeping" enzyme and is involved in physiological functioning of prostaglandins (Fattahi and Mirshafiey, 2012). COX-2 is mostly known to be upregulated in RA, which leads to angiogenesis and persistent vascularization in synovium (Woods et al., 2003). However, different evidences suggest that both isoforms of cyclooxygenase are inflammatory modulators as well as are involved in homeostasis (Martel-Pelletier et al., 2003). Different studies have also shown normal response towards inflammatory stimuli in COX-2 knockout mice and nonexistence of gastrointestinal problems in COX-1 knockout mice. Considering these results and many others, finding a balance between COX-2 and COX-1 suppression was emphasized (Lee et al., 2000).

Lipoxygenases are essential enzymes in the production of leukotrienes that are important in the pathophysiology of various inflammatory disorders (Gheorghe et al., 2009). 5-lipoxygenase is responsible for the generation of LTB_4, LTC_4, LTD_4, and LTE_4, which are potent mediators of inflammation. COX suppression may cause shifting of arachidonic acid metabolism towards lipoxygenase pathway. Drugs which inhibit only COX pathway can lead to gastrointestinal problems due to over production of leukotrienes. Therefore, it can be anticipated that inhibition of both pathways may yield a protective effect on gastrointestinal mucosa (Hudson et al., 1993; Laufer, 2001; Martel-Pelletier et al., 2003; Rainsford, 1993, 1987; Tries et al., 2002). Bronchoconstriction is another severe adverse effect which is attributed to the shift in metabolic pathway towards 5-lipoxygenase pathway (Dannhardt and Kiefer, 2001). Present study demonstrated that DHP attenuated 5-lipoxygenase levels, whereas, treatment with piroxicam did not exhibit reduction in the levels of 5-lipoxygenase. This indicates that DHP may possess less adverse effects and high safety profile than piroxicam. Previous studies suggest that piroxicam is a non-selective COX inhibitor and does not reduce lipoxygenase activity or leukotriene levels (Carty et al., 1980; French et al., 1987; Rao and Knaus, 2008). However, different derivatives of pyrazole containing compounds have been shown to possess 5-lipoxygenase inhibitory property (Ergün et al., 2010). Suppressive effect of DHP on 5-LOX activity could possibly be the result of synergism of pyrazole and oxicam moieties in a single nucleus; however, further studies are required to confirm the structure activity relationship.

Increased levels of prostaglandins can mediate vasodilation, extravasation of fluid, and pain. Especially PGE2 is involved in the

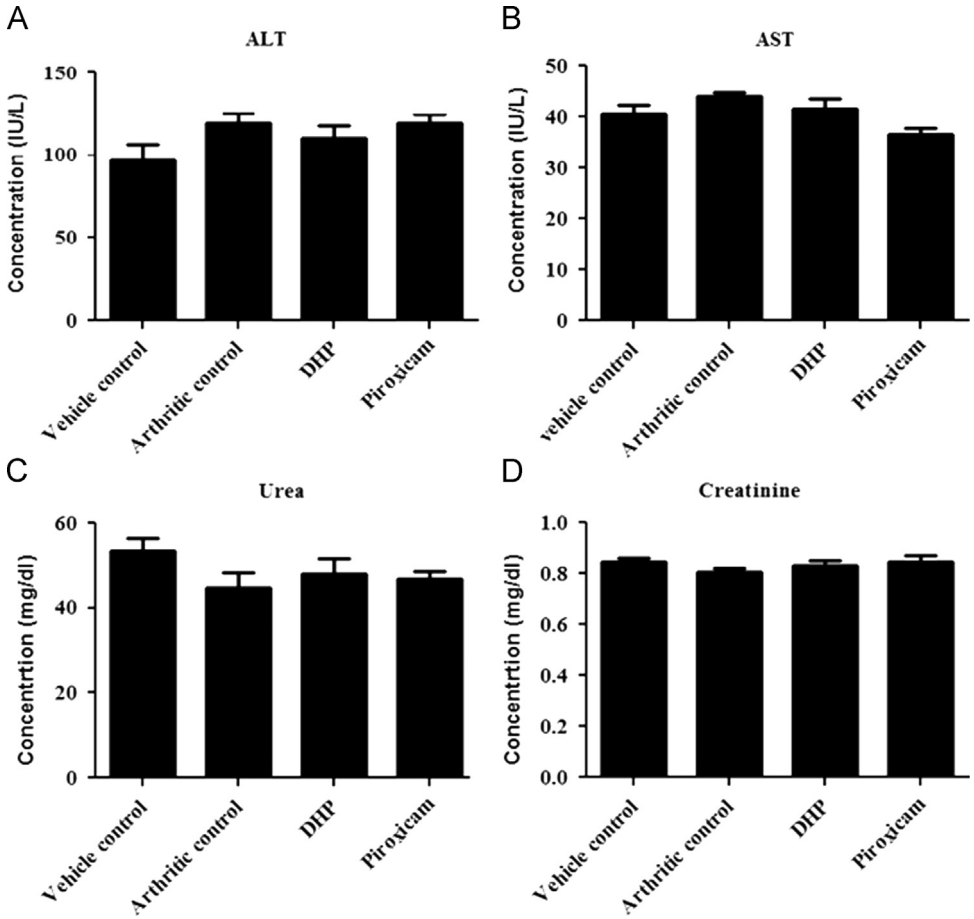

Fig. 5. Non-significant difference was observed in all rat groups when compared with vehicle control group for the determination of ALT (A), AST (B), urea (C), and creatinine (D) levels.

erosions of bone and articular cartilage (Crofford et al., 1994; Fattahi and Mirshafiey, 2012). Our results showed that DHP significantly suppressed activity of PGE2. This decreased activity of PGE2 can possibly be attributed to the inhibitory effect of DHP on the expression of cyclooxygenase enzyme.

Toll like receptors recognize pattern recognition receptors which are expressed by cells that undergo stress (Huang and Pope, 2009). Various studies have shown the increased expression of toll-like receptor 2 in RA synovial fibroblasts, RA synovial fluid macrophages, rheumatoid joint, and also in the pannus (Huang et al., 2007; Kim et al., 2007; Seibl et al., 2003). Macrophages may be activated by endogenous toll-like receptor ligands and result in increased expression of pro-inflammatory cytokines, which lead to the destruction of cartilage and bone. Attenuation of toll-like receptor signaling is considered as novel therapeutic approach in patients suffering from RA (Huang and Pope, 2009). In present study, we found increased toll-like receptor 2 expression levels in the blood of FCA-induced arthritic rats and treatment with DHP significantly decreased the expression, however, piroxicam did not reduce toll-like receptor 2 expression. Toll-like receptor 2 utilizes MyD88 pathway and causes the activation of nuclear factor-kappaB (NF-κB) through interaction of various proteins (Abdollahi-Roodsaz et al., 2012; Takeda and Akira, 2004).

NF-κB is a well-known inflammatory regulator and is involved in various features of RA pathology, such as, differentiation and activation of osteoclasts which cause bone resorbtion, abnormal apoptosis, development of Th1 response, and proliferation of RA synovial cells. Inhibitors of NF-κB are considered to have high therapeutic efficacy and are feasible as RA therapy for human disease (Makrov, 2001). Our study showed that DHP, as well as, piroxicam significantly reduced NF-κB expression levels. NF-κB resides in cytoplasm and moves to nucleus when activated. Activation of NF-κB is the result of enzymatic degradation of IκB, which is responsible for NF-κB retention in cytoplasm (Gilmore, 2006). Role of NF-κB is important in maximal expression of many pro-inflammatory cytokines including TNF-α (Blackwell and Christman, 1997). It is also known that activation of toll-like receptor 2 results in spontaneous discharge of pro-inflammatory cytokines, including TNF-α by synovium (Abdollahi-Roodsaz et al., 2012). Present study showed that DHP significantly inhibited TNF-α expression which might be the result of decrease in expression levels of toll-like receptor 2 and NF-κB. It has been demonstrated that inhibition of toll-like receptor 2 attenuates spontaneous release of TNF-α in synovial explant cultures (Ultaigh et al., 2011). Our results also revealed that DHP clearly abrogated the splenocyte proliferation. Previous experiments have shown that inhibition of splenocyte proliferation is also associated with inhibition of TNF-α secretion from the splenocytes activated by ConA (Ma et al., 2013).

Presence of TNF-α in large concentration in the synovial fluid of RA patients results in joint damage and inflammation which are considered as hallmark of RA (Vasanthi et al., 2007). Our results also displayed increased infiltration of inflammatory cells in synovium, bone erosion, and pannus formation which were reduced by treatment with DHP. TNF-α also activates NF-κB (Aghai et al., 2007; Taylor et al., 2009), therefore, inhibition of TNF-α causes global effect by decreasing the levels of pro-inflammatory cytokines, which require NF-κB for their expression. Various studies have shown that suppression of TNF-α leads to the reduction of other pro-inflammatory cytokines, such as, IL-8,

IL-6, IL-1 and GM-CSF (Vasanthi et al., 2007). It is also known that TNF-α causes damage to connective tissue by inducing the synthesis of PGE2 and collagenase (Hauptmann et al., 1991).

RA is considered as Th1 mediated autoimmune disorder (Irmler and Bräuer, 2007). Substantial evidences show that Th1 and Th2 pathways are antagonistic to each other and tend to repress each other response. IL-4 is one of the primary cytokines which mediate Th2 type response (Rosloniec et al., 2002). It promotes the production of Th2 cells while inhibiting Th1 generation. It is considered as one of the most successful tools for the attenuation of Th1 mediated autoimmune disorders and has been used as a part of therapy for many *in vivo* autoimmune experimental disorders in animals (Schulze-Koops and Kalden, 2001). Results showed significant decrease in IL-4 level in animals of arthritic control group and its levels were significantly enhanced in piroxicam treated group, whereas, a non-significant elevation was also observed in DHP treated group.

Our results displayed reduction in Hb content and RBC count in arthritic rats which are indicative of anemic condition. Failure of bone marrow to produce adequate cells in anemia and anomalous iron storage in synovial tissue and reticuloendothelial system are the important reasons. Increase in WBC and platelet counts is also observed in this study and may be attributed to the induction of immune response against assaulting pathogen (Ekambaram et al., 2010; Mowat, 1971). To establish the safety of DHP, we measured serum levels of ALT, AST, creatinine and urea in all groups. Results showed no difference among all groups suggesting that DHP does not possess hepatotoxic and nephrotoxic effects.

5. Conclusion

The study suggests that DHP possesses significant anti-arthritic property in rats with FCA-induced arthritis and is attributed to its immunomodulatory and anti-inflammatory activities. To what extent, DHP clinically decreases the levels of inflammatory markers of immune response is unclear and requires further clinical studies.

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References

Ahmad, M., Siddiqui, H.L., Zia-ur-Rehman, M., Parvez, M., 2010. Anti-oxidant and anti-bacterial activities of novel *N*′-arylmethylidene-2-(3,4-dimethyl-5,5-dioxidopyrazolo(4,3-c)(1,2)benzothiazin-2(4*H*)-yl)acetohydrazides. Eur. J. Med. Chem. 45, 698–704.

Abdollahi-Roodsaz, S., van de Loo, F.A.J., Koenders, M.I., et al., 2012. Destructive role of myeloid differentiation factor 88 and protective role of TRIF in interleukin-17-dependent arthritis in mice. Arthritis Rheum. 64 (6), 1838–1847.

Aghai, Z.H., Kode, A., Saslow, J.G., Nakhla, T., Farhath, S., Stahl, G.E., Eydelman, R., Strande, L., Leone, P., Rahman, I., 2007. Azithromycin suppresses activation of nuclear factor-kappa B and synthesis of pro-inflammatory cytokines in tracheal aspirate cells from premature infants. Pediatr. Res. 62 (4), 483–488.

Baraldi, P.G., Manfredini, S., Romagnoli, R., Stevanato, L., Zaid, A.N., Manservigi, R., 1998. Synthesis and anti-HSV-1 activity of 6 substituted pyrazolo(3,4-d) pyridazin-7-one. Nucleosides Nucleotides Nucleic Acids 17, 2165–2171.

Bihovsky, R., Tao, M., Mallamo, J.P., Wells, G.J., 2004. 1,2-Benzothiazine 1,1-dioxide α-ketoamide analogues as potent calpain I inhibitors. Bioorg. Med. Chem. Lett. 14, 1035–1038.

Billiau, A., Matthys, P., 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. J. Leukocyte Biol. 70, 849–860.

Blackwell, T.S., Christman, J.W., 1997. The role of nuclear factor-kB in cytokine gene regulation. Am. J. Respir. Cell Mol. Biol. 17, 3–9.

Cai, X., Zhoua, H., Wong, Y.F., Xie, Y., Liu, Z.Q., Jiang, Z.H., Bian, Z.X., Xub, H.X., Liu, L., 2007. 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, 39–48.

Carty, T.J., Stevens, J.S., Lombardino, J.G., Parry, M.J., Randall, M.J., 1980. Piroxicam, a structurally novel anti-inflammatory compound. Mode of prostaglandin synthesis inhibition. Prostaglandins 19 (5), 671–682.

Cottineau, B., Toto, P., Marot, C., Pipaud, A., Chenault, J., 2002. Synthesis and hypoglycemic evaluation of substituted pyrazole-4-carboxylic acids. Bioorg. Med. Chem. Lett. 12, 2105–2108.

Crofford, L.J., 2013. Use of NSAIDs in treating patients with arthritis. Arthritis Res. Ther. 15 (Suppl. 3), S2.

Crofford, L.J., Wilder, R.L., Ristimaki, A.P., et al., 1994. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1β, phorbol ester, and corticosteroids. J. Clin. Invest. 93 (3), 1095–1101.

Dannhardt, G., Kiefer, W., 2001. Cyclooxygenase inhibitors—current status and future prospects. Eur. J. Med. Chem. 36, 109–126.

Dugowson, C.E., Gnanashanmugam, P., 2006. Nonsteroidal anti-inflammatory drugs. Phys. Med. Rehabil. Clin. N. Am 17, 347–354.

Ekambaram, S., Perumal, S.S., Subramanian, V., 2010. Evaluation of antiarthritic activity of *Strychnos potatorum* Linn seeds in Freund's adjuvant induced arthritic rat model. BMC Complement. Altern. Med 10, 56.

Ergün, B.C., Nuñez, M.T., Labeaga, L., Ledo, F., Darlington, J., Bain, G., Cakir, B., Banoglu, E., 2010. Synthesis of 1,5-diarylpyrazol-3-propanoic acids towards inhibition of cyclooxygenase-1/2 activity and 5-lipoxygenase-mediated LTB4 formation. Arzneimittelforschung 60 (8), 497–505, http://dx.doi.org/10.1055/s-0031-1296318.

Fattahi, M.J., Mirshafiey, A., 2012. Prostaglandins and rheumatoid arthritis. Arthritis, http://dx.doi.org/10.1155/2012/239310 (Article ID 239310).

French, J.K., Hurst, N.P., McColl, S.R., Cleland, L., 1987. Effects of piroxicam on superoxide generation, phospholipid methylation and leukotriene production by human blood mononuclear cells. J. Rheumatol. 14 (5), 1018–1021.

Gennari, C., Salom, B., Potenza, D., Williams, A., 1994. Synthesis of sulfonamido-pseudopeptides: new chiral unnatural oligomers. Angew. Chem. Int. Ed. Engl. 33, 2067–2069.

Gheorghe, K.R., Korotkova, M., Catrina, A.I., Backman, L., Klint, E., Claesson, H.-E., Rådmark, O., Jakobsson, P.-J., 2009. Expression of 5-lipoxygenase and 15-lipoxygenase in rheumatoid arthritis synovium and effects of intraarticular glucocorticoids. Arthritis Res. Ther. 11, R83, http://dx.doi.org/10.1186/ar2717.

Gilmore, T.D., 2006. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 25, 6680–6684.

Grover, H.S., Gaba, N., Gupta, A., Marya, C.M., 2011. Rheumatoid arthritis: a review and dental care considerations. Nepal Med. Coll. J 13 (2), 74–76.

Hauptmann, B., Van Damme, J., Dayer, J.M., 1991. Modulation of IL-1 inflammatory and immunomodulatory properties by IL-6. Eur. Cytokine Netw. 2 (1), 39–46.

Huang, Q.Q., Pope, R.M., 2009. Role of toll like receptors in rheumatoid arthritis. Curr. Rheumatol. Rep 11 (5), 357–364.

Huang, Q., Ma, Y., Adebayo, A., Pope, R.M., 2007. Increased macrophage activation mediated through toll like receptors in rheumatoid arthritis. Arthritis Rheum. 56 (7), 2192–2201.

Hudson, N., Balsitis, M., Everitt, S., Hawkey, C.J., 1993. Enhanced gastric mucosal leukotriene B4 synthesis in patients taking non-steroidal anti-inflammatory drugs. Gut 34, 742–747.

Iovu, M., Zalaru, C., Dumitrascu, F., Draghici, C., Moraru, M., Criste, E., 2003. New substituted 2-(pyrazol-1-yl)-dialkylacetanilides with potential local anesthetic and antiarrhythmic action. Part II. Farmaco 58, 301–307.

Irmler, I., Bräuer, R., 2007. Paradoxical role of interferon-gamma in arthritis. Z. Rheumatol. 66, 591–594.

Jawed, H., Shah, S.U.A., Jamall, S., Simjee, S.U., 2010. *N*-(2-hydroxy phenyl) acetamide inhibits inflammation-related cytokines and ROS in adjuvant-induced arthritic (AIA) rats. Int. Immunopharmacol. 10, 900–905.

Kim, K.W., Cho, M.L., Lee, S.H., Oh, H.J., Kang, C.M., Ju, J.H., et al., 2007. Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-activation. Immunol. Lett. 110 (1), 54–64 (PubMed: 17467812).

Laufer, S., 2001. Discovery and development of ML 3000. Inflammopharmacol 9, 101–112.

Lee, Y.H., Choi, S.J., Kim, A., Kim, C.H., Ji, J.D., Song, G.G., 2000. Expression of cyclooxygenase-1 and -2 in rheumatoid arthritis synovium. J. Korean Med. Sci. 15, 88–92.

Ma, J., Song, B., Ruan, H., Zhou, P., Cui, Y., 2013. Effect of berberine hydrochloride on the proliferation, apoptosis and cytokine secretion of splenocytes in mice. Xi. Bao. Yu. Fen. Zi. Mian. Yi. Xue. Za. Zhi 29 (8), 802–804.

Makrov, S.S., 2001. NF-kB in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. Arthritis Res. 3, 200–206.

Martel-Pelletier, J., Lajeunesse, D., Reboul, P., Pelletier, J.-P., 2003. Therapeutic role of dual inhibitors of 5-Lox and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Ann. Rheum. Dis. 62, 501–509.

Mclnnes, I.B., Schett, G., 2011. The pathogenesis of rheumatoid arthritis. N. Engl. J. Med. 365, 2205–2219.

Mowat, A.G., 1971. Hematologic abnormalities in Rheumatoid arthritis. Sem. Arthritis Rheum 1 (3), 195–219.

Rainsford, K.D., 1993. Leukotrienes in the pathogenesis of NSAID-induced gastric and intestinal mucosal damage. Agents Actions 39, C24–C26.

Rainsford, K.D., 1987. The effects of 5-lipoxygenase inhibitors and leukotriene antagonists on the development of gastric lesions induced by nonsteroidal antiinflammatory drugs in mice. Agents Actions 21, 316–319.
Rao, P.N.P., Knaus, E.E., 2008. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. J. Pharm. Pharm. Sci. 11 (2), 81s–110s.
Rosloniec, E.F., Latham, K., Guedez, Y.B., 2002. Paradoxical roles of IFN-γ in models of Th1-mediated autoimmunity. Arthritis Res. 4, 333–336.
Schulze-Koops, H., Kalden, J.R., 2001. The balance of Th1/Th2 cytokines in rheumatoid arthritis. Best Pract. Res. Clin. Rheumatol. 15 (5), 677–691.
Scott, D.L., Wolf, F., Huizinga, T.W.J., 2010. Rheumatoid arthritis. Lancet 376 (9746), 1094–1108.
Seibl, R., Birchler, T., Loeliger, S., Hossle, J.P., Gay, R.E., Saurenmann, T., et al., 2003. Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium. Am. J. Pathol. 162 (4), 1221–1227 (PubMed: 12651614).
Smith, S.R., Denhardt, G., Terminelli, C., 2001a. The anti-inflammatory activities of cannabinoid receptor ligands in mouse peritonitis models. Eur. J. Pharmacol. 432 (1), 107–119.
Smith, S.R., Terminelli, C., Denhardt, G., 2001b. Modulation of cytokine responses in *Corynebacterium* parvum-primed endotoxemic mice by centrally administered cannabinoid ligands. Eur. J. Pharmacol. 425, 73–83.
Sundy, J.S., 2001. COX-2 inhibitors in rheumatoid arthritis. Curr. Rheumatol. Rep 3, 86–91.
Symmons, D., Mathers, C., Pfleger, B., 2012. World Health Organisation. The Global Burden of Rheumatoid Arthritis in the Year 2000. (Online) Available at: ⟨http://www.who.int/healthinfo/statistics/bod_rheumatoidarthritis.pdf⟩ (accessed 07 March 2012).
Takeda, K., Akira, S., 2004. TLR signaling pathways. Sem. Immunol. 16, 3–9.
Taylor, S.L., Krempel, R.L., Schmaljohn, C.S., 2009. Inhibition of TNF-alpha-induced activation of NF-kappaB by hantavirus nucleocapsid proteins. Ann. N.Y. Acad. Sci 1171 (Suppl 1), E86–E93, http://dx.doi.org/10.1111/j.1749-6632.2009.05049.
Tries, S., Neupert, W., Laufer, S., 2002. The mechanism of action of the new antiinflammatory compound ML3000: inhibition of 5-Lox and COX-1/2. Inflamm. Res. 51, 135–143.
Ultaigh, S.N.A., Saber, T.P., McCormick, J., Connolly, M., Dellacasagrande, J., Keogh, B., McCormack, W., Reilly, M., O'Neill, L.A., McGuirk, P., Fearon, U., Veale, D.J., 2011. Blockade of Toll-like receptor 2 prevents spontaneous cytokine release from rheumatoid arthritis ex vivo synovial explant cultures. Arthritis Res. Ther. 13, R33, http://dx.doi.org/10.1186/ar3261.
Vasanthi, P., Nalini, G., Rajashekhar, G., 2007. Role of tumor necrosis factor-alpha in rheumatoid arthritis: a review. APLAR J. Rheumatol 10, 270–274.
Woods, J.M., Mogollon, A., Amin, M.A., Martinez, R.J., Kocha, A.E., 2003. The role of COX-2 in angiogenesis and rheumatoid arthritis. Exp. Mol. Pathol. 74, 282–290.
Zia-ur-Rehman, M., Choudary, J.A., Elsegood, M.R.J., Siddiqui, H.L., Khan, K.M., 2009. A facile synthesis of novel biologically active 4-hydroxy-N0-(benzylidene)-2*H*-benzo(e)(1,2)thiazine-3-carbohydrazide 1,1-dioxides. Eur. J. Med. Chem. 44, 1311–1316.
Zia-ur-Rehman, M., Choudary, J.A., Ahmad, S., Siddiqui, H.L., 2006. Synthesis of potential biologically active 1,2-benzothiazin-3-yl-quinazolin-4(3*H*)-ones. Chem. Pharm. Bull. 54, 1175–1178.