

# Protective effects of vitamin D and losartan in complete Freund's adjuvant-induced arthritis in rats

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**Abstract:** The current study was designed to explore the protecting effects of vitamin D and losartan in treatment of rheumatoid arthritis (RA). Animals were allotted to five groups: Group I received vehicles only (vehicle control). Group II was administered complete Freund's adjuvant (CFA) and did not receive any medication. The remaining three groups (III, IV, V) were given CFA followed by treatment with leflunomide, vitamin D or losartan, respectively for two weeks. Compelling increment in tumor necrosis factor (TNF- $\alpha$ ), interleukin 6 (IL-6), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), malondialdehyde (MDA) level, white blood cells (WBCs), total cholesterol (TC) and triglycerides (TGs) was revealed in arthritic rats. This was associated with marked decline in glutathione (GSH) level, red blood cells (RBCs), hemoglobin (Hb), Platelets (Plts), hematocrit (Hct) and high density lipoprotein cholesterol (HDL). vitamin D or losartan significantly decreased TNF  $\alpha$ , IL-6, RF, ESR, MDA, TC, TGs, WBCs and significantly increased RBCs, Hb, Hct, Plts and HDL. It could be concluded that vitamin D and losartan are able to repress the alterations associated with adjuvant-induced arthritis (AIA). This preserving effect might be partially attributed to antiarthritic, hypolipidemic and antianemic properties.

**Keywords:** Complete Freund's adjuvant, leflunomide, losartan, rats, rheumatoid arthritis, rheumatoid factor, vitamin D.

## INTRODUCTION

Rheumatoid arthritis (RA) is a persistent auto immune disease of unknown etiology outlined by swelling and tenderness of the joints. In addition to, loss of synovial joints resulting in serious impairment and death (Aletaha *et al.*, 2010). Elevated levels of circulating inflammatory cytokines, including multiple interleukins and tumor necrosis factor- $\alpha$  have been reported (Barrera *et al.*, 1995). There is no cure for RA (Shaw *et al.*, 2011). Non-steroidal anti-inflammatory drugs relieve symptoms of arthritis, still they are unsatisfactory to correct major causes leading the chronic inflammation (Barsante *et al.*, 2005). Disease-modifying anti-rheumatic drugs (DMARDs) for example methotrexate and leflunomide retard joint destruction by means of immunomodulation (Shaw *et al.*, 2011). However, they have serious adverse effects like hepatotoxicity and leukopenia (Dessein *et al.*, 2005).

Vitamin D active form, 1,25-dihydroxyvitamin D<sub>3</sub>, plays a major part regarding cell proliferation and differentiation as well as in immune regulation. This is further supported by the existence of receptors vitamin D (VDRs) on peripheral blood monocytes (Mehta *et al.*, 2010). Andjelkovic *et al.*, (1999) found that, the active form of vitamin D acts as an important paracrine factor in the immune system because it could form by mononuclear cells and exerts powerful effects on the entire immune system.

Angiotensin II up-regulates cytokines and has a crucial role in the development of inflammation. Angiotensin II levels and angiotensin II type1 receptor (AT1R) are increased in synovium (Price *et al.*, 2007) implying that AT1R plays an important role in RA progression. Furthermore, it has been demonstrated that losartan reduced knee joint inflammation and swelling in rats with arthritis (Mackenzie *et al.*, 2013). The main goal of the current investigation was to gain additional insight regarding beneficial effects of vitamin D and losartan against rheumatoid arthritis induced in rats.

## MATERIALS AND METHODS

### *Experimental animals*

Female Wistar albino rats (180-200g) obtained from the Modern Veterinary Office for Laboratory Animals, Cairo, Egypt were used in this study. Animals were allowed to accommodate for one week to all the laboratory conditions prior to experimentation. Rats were maintained on twelve hours dark/ light cycle at 22 $\pm$ 3°C and have free access to food and water ad libitum. The experimental protocol and methods were accepted and supervised by Ethics Committee of Faculty of Pharmacy, Beni-Suef University (Ethical approval no. 14-125).

### *Drugs and chemicals*

Complete Freund's adjuvant (CFA) was obtained from Sigma-Aldrich (MO, USA). Leflunomide, vitamin D and losartan potassium were obtained from Sigma Pharmaceutical Industries, Cairo, Egypt. All drugs were

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dissolved in sterile saline solution and were freshly prepared.

#### **Adjuvant arthritis induction**

Adjuvant induced arthritis was experimentally introduced in rats by subcutaneous injection of 0.25ml of CFA in the palmar surface of the left hind paw (Piliero *et al.*, 1966). CFA was kept at 4°C and was agitated well prior to administration.

#### **Experimental design**

Rats were randomly assigned into four groups (n=8). Group I (vehicle control) received subcutaneous injection of 0.25ml of paraffin oil into the palmar surface of the left hind paw. In the remaining three groups, adjuvant arthritis was induced. Group II was assigned as adjuvant-induced arthritis (AIA) group. Groups III and IV received vitamin D (0.05µg/kg/day, p.o.) (Larsson *et al.*, 1998) and losartan (20mg/kg/day, p.o.) (Refaat *et al.*, 2013) respectively. Four days after induction of arthritis all drug treatment started and continued up to two weeks.

#### **Blood collection and serum preparation**

By the end experimental period, animals were lightly anesthetize, blood samples were obtained from the medial epicanthus of the animal's eyes by non-heparinized capillary tube and subdivided into two portions. The first portion was used for CBC analysis and the second portion was centrifuged at 3000rpm for 30 minutes. Serum samples were collected and kept at -80°C until further use.

#### **Assessment of TNF- $\alpha$ , IL-6, RF and ESR serum levels**

TNF- $\alpha$  was determined utilizing an Enzyme Linked Immunosorbent Assay (ELISA) kit (ID labs, Canada) according to the principle of Takahashi *et al.*, (1996). IL-6 was measured according to the method of Sanchez-Fidalgo *et al.*, (2010) using ELISA kit (Glory Science Co., Ltd, Del Rio, TX, USA). RF expressed as IU/ml was assayed according to method described previously (Waalder, 2007) using rheumatoid factor Elisa kit (MyBioSource, San Diego, California, USA). ESR expressed as mm/hr, was determined according to the method described previously (Kanfer and Nicol, 1997).

#### **Assessment of serum thiobarbituric acid reacting substances (TBARS) and blood glutathione (GSH) levels**

Serum MDA as well as blood GSH were determined using spectrophotometric procedures explained previously by Esterbauer *et al.* (1991) and Agrawal *et al.* (1991) respectively.

#### **Assessment of lipid biomarkers**

Serum TG, cholesterol and HDL were evaluated using reagent kits (Spinreact, Spain).

#### **Assessment of hematological parameters**

Briefly, blood smears were rapidly prepared just after collection of blood on a glass slide, rapidly dried, and

stained with Giemsa and May-Grunwald stain for the differential blood count.

#### **Hind paw processing and histopathological examination**

Soon after blood collection, animals were killed by decapitation under thiopental sodium (50mg/kg) anesthesia and the left hind paw of each rat was collected into 10% neutral buffered formalin and then processed for complete decalcification using a solution containing 10% ethylene di-amine tetra-acetic acid, and ankle joint tissues were then sectioned, fixed in paraffin. Sections were sliced at 5-µm thickness and stained using hematoxylin and eosin (H&E).

### **STATISTICAL ANALYSIS**

All data were expressed as mean  $\pm$  standard error (S.E.) of 8 rats per experimental group. One-way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparisons test was used for statistical analysis.  $P < 0.05$  was considered to indicate statistical significance between groups.

### **RESULTS**

Results of the present study revealed a higher serum level of TNF- $\alpha$ , IL-6, RF and ESR in AIA control group compared to vehicle control group. Arthritic rats treated with leflunomide showed decreased levels of TNF- $\alpha$ , IL-6, RF and ESR. Vitamin D and losartan succeeded to reduce all these biomarkers as compared to arthritic control group without showing any significant difference compared to leflunomide group (table 1).

Estimation of serum MDA level showed an elevation in AIA group as compared to vehicle control group. Furthermore, all the administered drugs diminished serum TBARS level as compared to AIA rats. AIA group exhibited reduction in serum GSH in comparison to normal control group. Leflunomide, vitamin D and losartan significantly elevated serum GSH level compared to arthritic control group ( $P < 0.05$ , table 2).

Adjuvant arthritis significantly increased serum levels of cholesterol, TG and significantly decreased serum level of HDL as compared to normal control group. Administration of leflunomide to arthritic rats markedly decreased serum cholesterol and TG levels and significantly raised serum levels of HDL. However, vitamin D and losartan restored these biomarkers as compared to AIA control group (table 2).

As shown in table 3, adjuvant arthritis developed a notable increment in serum level of, WBCs and a marked reduction in serum levels of RBCs, Hb, platelets and Hct compared to vehicle control. Leflunomide markedly decreased serum level of WBCs and significantly raised serum levels of RBCs, Hb, platelets and Hct as compared to AIA group. However, these biomarkers were

**Table 1:** Effect of vitamin D and losartan on serum level of TNF-  $\alpha$ , IL-6, RF and ESR

	Normal Control (n=8)	Adjuvant Induced Arthritis (n=8)			
		Control	Leflunomide (10mg/kg)	Vitamin D (0.05 $\mu$ g/kg)	Losartan (20mg/kg)
TNF- $\alpha$ (pg/ml)	32.78 $\pm$ 1.25	117.55 $\pm$ 1.75*	52.26 $\pm$ 3.24* <sup>@</sup>	51.95 $\pm$ 3.47* <sup>@</sup>	59.81 $\pm$ 2.56* <sup>@</sup>
IL-6 (pg/ml)	34.76 $\pm$ 1.22	130.95 $\pm$ 4.84*	59.64 $\pm$ 2.15* <sup>@</sup>	69.96 $\pm$ 6.76* <sup>@</sup>	62.93 $\pm$ 1.92* <sup>@</sup>
RF(IU/ml)	11.66 $\pm$ 0.31	50.78 $\pm$ 1.05*	17.78 $\pm$ 0.89* <sup>@</sup>	15.43 $\pm$ 0.96* <sup>@</sup>	18.37 $\pm$ 0.79* <sup>@</sup>
ESR (mm/hr)	6.75 $\pm$ 0.32	44.21 $\pm$ 1.11*	13.61 $\pm$ 1.19* <sup>@</sup>	14.23 $\pm$ 0.95* <sup>@</sup>	14.23 $\pm$ 0.61* <sup>@</sup>

**Table 2:** Effect of vitamin D and losartan on serum level of MDA, GSH, cholesterol, TG and HDL

	Normal Control (n=8)	Adjuvant Induced Arthritis (n=8)			
		Control	Leflunomide (10mg/kg)	Vitamin D (0.05 $\mu$ g/kg)	Losartan (20mg/kg)
MDA(nmol/ml)	1.11 $\pm$ 0.07	12.24 $\pm$ 0.48*	2.78 $\pm$ 0.20* <sup>@</sup>	3.38 $\pm$ 0.33* <sup>@</sup>	2.83 $\pm$ 0.19* <sup>@</sup>
GSH(Mmol/L)	55.38 $\pm$ 0.92	17.05 $\pm$ 0.57*	50.18 $\pm$ 2.78 <sup>@</sup>	42.81 $\pm$ 3.47* <sup>@</sup>	42.99 $\pm$ 2.16* <sup>@</sup>
Cholesterol(mg/dl)	128.71 $\pm$ 1.43	172.59 $\pm$ 8.46*	134.75 $\pm$ 2.03 <sup>@</sup>	135.04 $\pm$ 2.75 <sup>@</sup>	133.36 $\pm$ 2.28 <sup>@</sup>
TG(mg/dl)	65.60 $\pm$ 3.00	108.56 $\pm$ 3.11*	73.84 $\pm$ 2.49 <sup>@</sup>	73.65 $\pm$ 0.84 <sup>@</sup>	77.98 $\pm$ 1.32* <sup>@</sup>
HDL(mg/dl)	53.56 $\pm$ 1.11	29.76 $\pm$ 1.44*	48.04 $\pm$ 1.71 <sup>@</sup>	49.04 $\pm$ 1.26 <sup>@</sup>	47.30 $\pm$ 1.28 <sup>@</sup>

**Table 3:** Effect of vitamin D and losartan on serum level of WBCs, RBCs, Hb, platelets and Hct

	Normal Control (n=8)	Adjuvant Induced Arthritis (n=8)			
		Control	Leflunomide (10mg/kg)	Vitamin D (0.05 $\mu$ g/kg)	Losartan (20mg/kg)
WBCs(10 <sup>3</sup> /Ml)	8.55 $\pm$ 0.61	21.74 $\pm$ 1.14*	10.33 $\pm$ 0.77 <sup>@</sup>	12.01 $\pm$ 1.01* <sup>@</sup>	13.16 $\pm$ 0.50* <sup>@</sup>
RBCs(10 <sup>6</sup> /mcl)	3.18 $\pm$ 0.18	1.63 $\pm$ 0.14*	3.01 $\pm$ 0.14 <sup>@</sup>	3.07 $\pm$ 0.08 <sup>@</sup>	3.24 $\pm$ 0.15 <sup>@</sup>
Hb(g/dl)	13.92 $\pm$ 0.16	8.21 $\pm$ 0.28*	10.09 $\pm$ 0.56* <sup>@</sup>	12.31 $\pm$ 0.54* <sup>@#</sup>	11.65 $\pm$ 0.46* <sup>@#</sup>
Platelets(10 <sup>6</sup> /L)	351.25 $\pm$ 9.95	271.50 $\pm$ .67*	304.38 $\pm$ 11.73* <sup>@</sup>	310.95 $\pm$ 6.27* <sup>@</sup>	332.75 $\pm$ 6.17 <sup>@</sup>
Hct (%)	53.28 $\pm$ 1.22	38.90 $\pm$ 2.23*	49.75 $\pm$ 1.85 <sup>@</sup>	47.38 $\pm$ 2.34 <sup>@</sup>	50.75 $\pm$ 0.75 <sup>@</sup>

Data was expressed as mean  $\pm$  s.e.m.

Statistical analysis was carried out using one way analysis of variance (Anova) followed by Tukey-Kramer multiple comparisons test.

\*Significantly different from normal control group value at  $p < 0.05$

@Significantly different from AIA control group at  $P < 0.05$

attenuated following administration of vitamin D and losartan compared to AIA control group. Vitamin D and losartan showed significant variation in comparison with leflunomide regarding serum level of Hb.

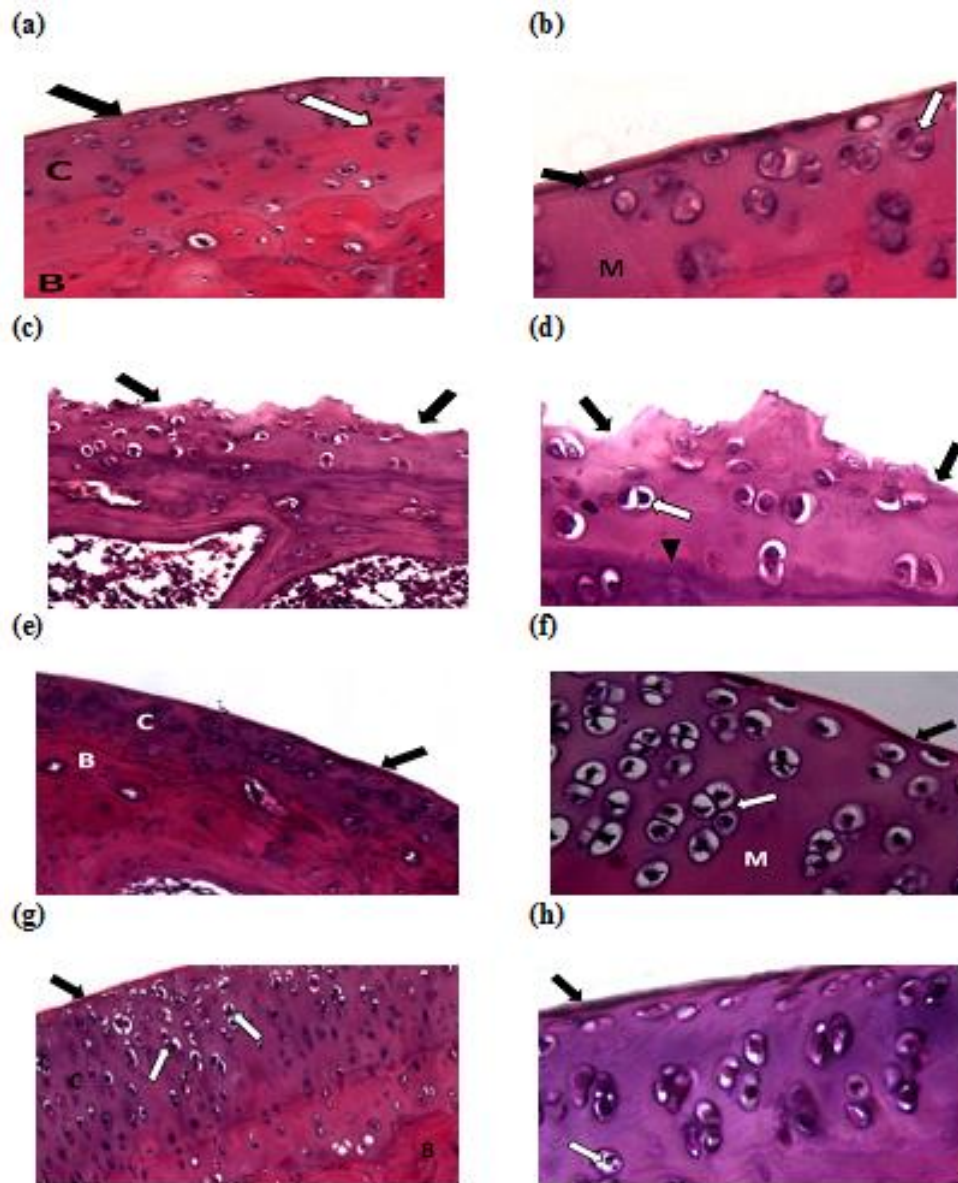
### Histopathological examination

Histopathological examination for H&E stained sections displayed that joint obtained from vehicle control rats had normal structure, smooth articular surface (black arrow) and regular tide mark (white arrow) separating between the articular cartilage (C) and the underlying sub-chondral bone (B) and no inflammatory cells were noticed. All chondrocytes were embedded in a homogenous matrix and regular tide mark can be noticed (fig.1a,b). Joints from AIA showed articular surface of an osteoarthritic joint with a disrupted articular surface (black arrow). Higher magnification showed erosion of the articular cartilage with doubled tide mark and degeneration of chondrocytes with pyknotic nuclei was observed (fig.1c,d). Joints from leflunomide treated rats showed

smooth articular surface (black arrow), thickened articular cartilage(C) and sub-chondral bone (B) can be observed. All chondrocytes were embedded in a homogenous matrix, showing hyper cellularity and cloning (fig 1e,f). Joints from vitamin D-treated rats showed increase in articular cartilage layers, normal chondrocytes were embedded in a homogenous matrix hyper cellularity and aggregation (fig g, h). Losartan treated rats showed Smooth articular surface (black arrow). Thickened articular cartilage (double head arrow) and subchondral bone (B) can be observed. The chondrocytes (white arrow) showing hypercellularity and aggregation (fig. 1 i,j)

### DISCUSSION

Complete Freund's adjuvant arthritic model represents one of the best RA model because it exhibits clinical and pathological characteristics corresponding to human rheumatoid arthritis (Refaat *et al.*, 2013).



(a) Normal architecture of the joint showing smooth articular surface (black arrow) and a regular tide mark (white arrow) separating the articular cartilage (C) from the underlying subchondral bone (B). (b) A higher magnification of the above section showing oval chondrocytes (black arrow) in the superficial layer and round ones (white arrow) in the deep layer. All chondrocytes are embedded in a homogenous matrix (M). A regular tide mark (arrow head) can be noticed. (c) A section in the AIA group showing articular surface of an osteoarthritic joint showing a disrupted articular surface (black arrow) (d) A higher magnification showing erosion of the articular cartilage (black arrow) with doubled tide mark (arrow heads). Degeneration of chondrocytes with pyknotic nuclei (white arrow) is observed. (e) A smooth articular surface (black arrow). Thickened articular cartilage (C) and subchondral bone (B) can be observed in the leflunomide treated group. (f) A higher magnification of the above section showing smooth articular surface (black arrow) with an increase in articular cartilage layers. The chondrocytes (white arrow) showing hypercellularity and cloning. All chondrocytes are embedded in a homogenous matrix (M). (g) A section in the vitamin D group showing smooth articular surface (black arrow). Thickened articular cartilage (C) and subchondral bone (B) can be observed. The chondrocytes (white arrow) showing hypercellularity and aggregation. (h) A higher magnification showing smooth articular surface (black arrow) with an increase in articular cartilage layers. All chondrocytes are embedded in a homogenous matrix (M). Degeneration of chondrocytes with pyknotic nuclei (white arrow) is observed. (i) Losartan treated rats showed smooth articular surface (black arrow). Thickened articular cartilage (double head arrow) and subchondral bone (B) can be observed. The chondrocytes (white arrow) showing hypercellularity and aggregation. (j) Higher magnification showed smooth articular surface (black arrow) with an increase in articular cartilage layers. The chondrocytes (white arrow) showing hypercellularity and aggregation. All chondrocytes are embedded in a homogenous matrix (M). (Photomicrographs (a,c,e,g and i) at x 200; (b,d,f,h, and j) at x 400 magnification.

**Fig. 1:** Photomicrographs for sections stained with hematoxylin & eosin

In the current investigation, subcutaneous injection of CFA in rats caused severe polyarthritis evidenced by significant increase in serum levels of many inflammatory and oxidative stress biomarkers such as TNF- $\alpha$ , IL-6, ESR, MDA, RF, WBCs, cholesterol and TG and markedly reduced serum levels of GSH, RBCs, Hb, platelets, Hct and HDL as compared to normal control group. Furthermore, histopathological examination also supported these findings.

Reduced RBCs count, Hb level and increased ESR reveal anemia which is a characteristic and important factor in the diagnosis of RA. ESR is strongly linked to the size and the number of the RBCs and to the corresponding plasma proteins level, specially  $\beta$  globulins and fibrinogen. ESR increment reveal active but obscure RA progression (Petchi *et al.*, 2015). It is also well known that oxidative damage caused by reactive oxygen species release is a crucial mechanism that determine the propagation of destructive synovitis and joint degradation (Leonaviciene *et al.*, 2012). A noticeable rise in ROS in arthritic rats is observed (Walker *et al.*, 2002). High lipid per oxidation levels were an indicator of reduced antioxidant capacity and increased oxidative stress in RA (Suresh *et al.*, 2012)

TNF- $\alpha$  and interleukins have a major role in the pathology of rheumatoid arthritis. They induce collagenase production that may contribute directly to cartilage destruction and bone resorption observed in RA. Moreover, oxidizing species and the decrease in RBCs value were expected to have a vital part in the pathogenesis of the disease. It has been reported that, circulating RBCs can scavenge ROS released extracellularly by activated neutrophils (Georgiadis *et al.*). This explains the important role of RBC in monitoring oxidative reactions in the surrounding medium and blocking free radical-mediated cytotoxicity.

In the current research, unfavorable lipid pattern was observed in the untreated RA animals as evidenced by a notable elevation in serum cholesterol and TG levels, in addition to marked reduction in serum HDL level. These results are in harmony with that of Georgiadis *et al.*, (2006) who reported that the primary cause for this poor lipid profile is low concentrations of HDL, which results in an unfavorable ratio of total cholesterol (TC) to HDL. Furthermore, circulating inflammatory cytokines, such as IL-6 and TNF- $\alpha$ , promote free fatty acid release and triglyceride synthesis in the liver (Feingold *et al.*, 1989; Khovidhunkit *et al.*, 2000).

The present results revealed that, leflunomide exerted a protective effect in treatment of RA induced by CFA. It significantly decreased TNF- $\alpha$ , IL-6, ESR, MDA, RF, WBCs, cholesterol and TG. Moreover, it significantly increased GSH, RBCs, Hb, platelets, Hct and HDL. These

findings are in accordance with that of (Yao *et al.*, 2003) demonstrating that leflunomide has protective immunological effects. The anti-inflammatory and immunomodulatory effects leflunomide were explained by its ability to restrain chemotaxis of inflammatory cells, expression of inflammatory cytokine (IL-1, IL-6 and TNF- $\alpha$ ) and lymphocyte proliferation and activation (Breedveld and Dayer, 2000).

In the present study, vitamin D exerted a protective effect in treatment of RA induced by CFA where vitamin D significantly decreased TNF- $\alpha$ , IL-6, ESR, MDA, RF, WBCs, cholesterol and TG. These results are in harmony with previous results (Major *et al.*, 2007; Erbas *et al.*, 2014). Moreover, vitamin D significantly increased GSH, RBCs, Hb, platelets, Hct and HDL and these results are in compliance with the results of Garcion *et al.*, (1999) and Shoji *et al.*, (2004). The observed increase in RBCs count, Hb, Hct and platelets explains the role of vitamin D in treatment of anemia induced by CFA, and the observed decrease in number of WBCs compared to RA group indicates that vitamin D has anti-inflammatory and immunomodulatory effects.

In addition, vitamin D-treated group was associated with a favorable serum lipid profile as compared to RA group evidenced by marked elevation in HDL concentration and significant reduction in cholesterol and TG concentrations. This improvement in lipid profile may be due to inhibition of IL-6 and TNF- $\alpha$ . These cytokines promote free fatty acid release and triglyceride synthesis in the liver (Feingold *et al.*, 1989; Khovidhunkit *et al.*, 2000) and reduce the action of lipoprotein lipase in endothelial cells, an enzyme responsible for triglyceride-rich lipid catabolism (Redgrave *et al.*, 1992; Khovidhunkit *et al.*, 2000).

According to the present study, losartan showed a protective effect in treatment of RA where it significantly reduced TNF- $\alpha$ , IL-6, ESR, MDA, RF, WBCs, cholesterol and TG. These findings confirm the work of Kamper *et al.*, (2010) who showed that losartan treatment improved oxidative and nitrosative stress as well as inflammation. In addition, losartan significantly increased GSH, RBCs, Hb, platelets, Hct and HDL and these result are in harmony with that of Bayorh *et al.*, (2003) showing that losartan significantly decreased oxidative stress in rats. Antiarthritic effect exhibited by losartan may be explained by its ability to decrease TNF- $\alpha$  and IL-6 by blocking AT1 receptor (Andrzejczak *et al.*, 2007). It is well documented that angiotensin II can trigger the release of TNF- $\alpha$ , IL-6, monocyte chemo attractant protein-1 in macrophages which possess a crucial role in the inflammation (Nobuhiko *et al.*, 2004; Yang *et al.*, 2010). Both TNF- $\alpha$  and IL-1b can up regulate angiotensin II AT1 receptors (Cowling *et al.*, 2002). Accordingly, targeting angiotensin II AT1 receptors using losartan can

efficiently suppress elevated serum TNF- $\alpha$  and IL-1 $\beta$  levels.

Oxidative stress plays a major role in the pathogenesis of RA and that AT1 receptor stimulation can provoke the production of ROS in vascular cells (Nickenig and Harrison, 2002; Wassmann *et al.*, 2004). Reduction of oxidative stress by antioxidants was shown to be associated with an improvement of inflammation and joint function (Nickenig and Harrison, 2002; Vecchione and Brandes, 2002)

Losartan, markedly declined WBCs count and significantly raised RBCs count, Hb, platelets count and Hct value as compared to RA rats. Blocking AT1 receptor decreases the destruction of RBCs by ROS and thus leads to improvement in anemic condition (Vlahakos *et al.*, 2010).

## CONCLUSION

To conclude, vitamin D and losartan are capable to quench the alterations associated with adjuvant-induced arthritis. This might be attributed partially to their anti-inflammatory, antioxidant and immune-modulatory effect.

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