

## Synergistic Ameliorative Effects of Resveratrol with Leflunomide on Serum Levels of Inflammatory Biomarkers and Joint Damage in Rats with Adjuvant Arthritis

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### Abstract

**Background:** Rheumatoid arthritis (RA) is chronic irreversible inflammatory disorder and characterized by the thickening of synovial tissue with pannus formation and the destruction of joint structure; due to persistent overproduction of proinflammatory cytokines (TNF- $\alpha$  and IL-6), proinflammatory enzymes such as cyclooxygenase II (COX-2) and matrix metalloproteinases. Leflunomide, potent pyrimidine biosynthesis inhibitor, exhibiting anti-inflammatory and immunosuppressive actions and resveratrol is a potent antioxidant and anti-inflammatory agent; exerting potential antiarthritic effects and also can protect diverse tissue types as heart and colon in different experimental animal models.

**Objective:** Evaluating outcomes of combinational use of both resveratrol and leflunomide on serum levels of proinflammatory biomarkers contributing to progression and severity of joint damage of adjuvant- induced arthritis in rats.

**Materials and Methods:** 50 male Wistar rats divided into 5 equal groups, rheumatoid arthritis was induced by Complete Freund's adjuvant. Arthritic rats were subdivided into 4 equal groups and taken the tested drugs daily for two weeks. Blood samples were taken for assay of serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6, TNF- $\alpha$  and inflamed arthritic rat joints of hind paw from different rat groups were used for histological evaluation.

**Results:** Arthritis rat group treated by concomitant resveratrol and leflunomide showed significant reduction in their serum levels C-reactive protein, MDA, MMP-3, PGE2, IL-6, TNF- $\alpha$ , with histological improvement in comparison to others rat groups.

**Conclusion:** Both drugs resveratrol and leflunomide exerted potent anti-inflammatory effects on serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6, TNF- $\alpha$  and their concomitant use showed more significant ameliorative effects against joint damage reflected from histopathological analysis, with more additive inhibitory effects on serum levels of these cytokines and biomediators, that are strongly implicated in the pathogenesis and progression of RA and their combinational use is recommended for better management of RA in patients.

**Keywords:** Rheumatoid arthritis; Leflunomide; Resveratrol; Complete Freund's adjuvant; Inflammatory cytokines; TNF- $\alpha$

### Introduction

Rheumatoid arthritis (RA) is a chronic degenerative, systemic inflammatory disorder, most probable of autoimmune origin, characterized by chronic inflammation of multiple joints, with disruption of joint cartilage and structure due to destructive effects of a variety of pro-inflammatory cytokines and chemokines. The role of inflammatory chemokines and cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukins (IL-1  $\beta$ , IL-6); proinflammatory enzymes such as cyclooxygenase (COX-1 and COX-2), 5-lipoxygenase (5-LOX) and matrix metalloproteinase (MMP-9) and adhesion molecules in the pathogenesis of arthritis is well documented [1-4].

The subsequent release of prostaglandins promotes, sustains and enhances additional cytokine production and inflammation, leading to the destruction and degeneration of the cartilage. Most of these inflammatory mediators regulated by the transcription factor Nuclear factor- $\kappa$ B (NF- $\kappa$ B), that also can initiate the destruction and degeneration of the cartilage by promoting the overproduction of these inflammatory mediators [5,6]. Thus, agents that suppress the expression of tumour necrosis factor- $\alpha$ , interleukin-1 $\beta$ , cyclooxygenase-2, lipoxygenase, matrix metalloproteinases and adhesion molecules, or suppress the activation of NF- $\kappa$ B, all have potential for the treatment of arthritis.

Leflunomide exhibits anti-inflammatory, anti-proliferative, and immunosuppressive effects through different cellular mechanisms that are not fully understood but most probably via suppressing lymphocyte proliferation and reducing inflammatory TNF gene expression with a potential anti-rheumatic activity [7-12]. Some recent studies suggest TNF may be the biomediator of pathogenesis of RA [13], and agents that can down-regulate TNF-mediated cellular inflammatory responses or antibodies against TNF, have been approved for treatment of RA [14,15]. Previous studies shown that leflunomide blocks TNF-mediated NF- $\kappa$ B activation by suppressing I- $\kappa$ Ba degradation and suppressing TNF gene expression [12]. Hence, leflunomide has been approved for treatment of rheumatoid arthritis [16].

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**Received** February 05, 2015; **Accepted** February 23, 2015; **Published** March 02, 2015

**Citation:** Ahmed A, Bader A (2015) Synergistic Ameliorative Effects of Resveratrol with Leflunomide on Serum Levels of Inflammatory Biomarkers and Joint Damage in Rats with Adjuvant Arthritis. Clin Exp Pharmacol 5: 170. doi:10.4172/2161-1459.1000170

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Numerous agents derived from plants can suppress TNF-mediated cellular inflammatory responses, including resveratrol (or trans-3,5,40-trihydroxystibene) which is a natural phytoalexin (plant polyphenol) found in red grapes, red wine, cranberries and peanuts grapes [17]. Resveratrol possesses potent antioxidant, anti-inflammatory, anti-proliferative and immunomodulatory properties and has the potential for treatment of many of inflammatory disorders such RA and inflammatory bowel diseases [18-20] and so, resveratrol can exert anti-inflammatory properties that had been shown to protect diverse tissue types as joints [21] and more also brain, heart, kidney tissue in experimental models [22].

### Aim of the study

The present study was designed to investigate the outcomes of combined concomitant use of resveratrol and leflunomide on serum levels of proinflammatory biomediators (C-reactive protein, MDA, MMP-3, PGE2, IL-6, TNF- $\alpha$ ) on progression and severity of joint damage of adjuvant-induced arthritis in rats.

### Statistical analysis

The results are expressed as the means  $\pm$  standard deviation. Statistical analyses of significance of differences between different rat groups were performed by Student's *t*-test and one-way analysis of variance. Multiple comparisons of means were done by Bonferroni multiple comparison test as a post ANOVA test. The *P* value of less than 0.05 was considered statistically significant, the *p*<0.01 was very significant and the *p*<0.001 was extremely significant.

## Materials and Methods of the Study

### Materials and reagents

Complete Freund's adjuvant was obtained from Sigma Chemical Co., USA, Leflunomide (Arava) 10 mg tablet, Aventis Chemical Co., USA. Resveratrol 250 mg soft gel capsule Sigma Chemical Co., USA. Thiobarbituric acid and Dodesil sodium sulfate from Loba Chemie Ltd., Mumbai, India. The sera levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF- $\alpha$ ; were assayed using a rat ELISA kit, from Sigma Chemical Co., USA. Reagents and chemicals were used as formaldehyde, 10% Ethylene diamine tetra acetic acid, paraffin, hematoxylin and eosin dye, Formalin 10%; for histological assessment of inflamed arthritic joints of different rat groups.

### Experimental design

This study was conducted on Fifty Male Wistar rats with average body weight of 200-250 grams. The rats had free access to standard pellet diet and tap water ad libitum. Prior ethical approval was obtained from the University ethics committee of experimental animal care and uses in experimental researches, for the study. Where 10 rats kept as non-arthritic saline treated group, and remaining 40 rats were subjected to the induction of adjuvant arthritis. Adjuvant arthritis was induced according [23,24] by 0.1 ml of 10 mg/ml *Mycobacterium butyricum* in Freund's complete adjuvant at the base of the right hind paw and the tail of the rats, where systemic polyarthritis, and an increase in the paw thickness; developed after two weeks of adjuvant injection [23,25]. After that, the arthritic rats were subdivided into 4 equal sub-groups:

**Group 1:** Non-arthritic saline treated group of 10 rats; receiving saline by oral feeding gavage for 4 weeks.

**Group 2:** Untreated arthritic group of 10 rats; kept free to food for 4 weeks.

**Group 3:** Arthritic-Resveratrol and Leflunomide treated group of 10 rats; treated at start of 3<sup>rd</sup> week with resveratrol (10 mg/kg/day) and leflunomide (10 mg/kg/day) by oral feeding gavage for 2 weeks.

**Group 4:** Arthritic-Resveratrol treated group of 10 rats; treated at start of 3<sup>rd</sup> week with resveratrol (10 mg/kg/day) by oral feeding gavage for 2 weeks.

**Group 5:** Arthritic-Leflunomide treated group of 10 rats; treated at start of 3<sup>rd</sup> week with leflunomide (10 mg/kg/day) by oral feeding gavage for 2 weeks.

At the end of 4<sup>th</sup> week of study, rats were subjected to the following procedures:

**A) Blood samples:** Blood samples were collected from different rat groups after fasting for 12 hours; from retro-orbital venous plexus of the rats then they had been sacrificed with ether anesthesia. After centrifugation for 20 min at a speed of 3000 rounds per minute, sera were obtained for measurement of the following parameters (by using corresponding parameter rat ELISA kit according to the manufacturer's instructions):

- Serum C-reactive protein (CRP), as marker of acute inflammatory phase proteins, expressed as mg/ml and assayed according to method of [26].
- Serum MDA as marker of oxidative stress and tissues damage, expressed as nmol/ml and assayed according to the method described by Ohkawa et al. [27]. The principle of this method is spectrophotometric measurement of red color, which exists after after binding of MDA from proteins with dodesil sodium sulfate and thiobarbituric acid in the environment where pH is 3.5.
- Serum matrix metallo-proteinase-3(MMP-3), as marker of osteo cartilaginous joint destruction and also, the predictors of progression of joint damage, as expressed as pg/ml and assayed according to method [28].
  - Serum levels of markers of severity of arthritis and also predictors of prognosis of arthritis to therapeutic agents as serum levels of PGE2, expressed as pg/ml and was determined using solid-phase extraction followed by an enzyme immunoassay determination, according to the instructions provided by the manufacturer and according to method of [29], serum IL-6 expressed as pg/ml and was measured as according to [30] and serum TNF- $\alpha$  expressed as pg/ml and was determined using an enzyme-linked immunosorbent assay according to the principle of Takahashi et al. [31].

**B) Histological examination:** of arthritic joints from different rat groups of the study. Rats were sacrificed with ether anesthesia, and the arthritic hind paws from different rat groups were collected for histopathological analysis. Formaldehyde-fixed hind paws were decalcified with a solution containing 10% ethylenediaminetetraacetic acid, and ankle tissues were then sectioned, embedded in paraffin, and sliced for hematoxylin and eosin (H&E) staining by a pathologist who was blinded to different rat groups of the study (Figures 1-3).

## Results

### The laboratory results

- Adjuvant arthritis was producing a significant increase in serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and in the arthritic non-treated rat group as compared to normal saline treated rat group. These results shown that these inflammatory cytokines

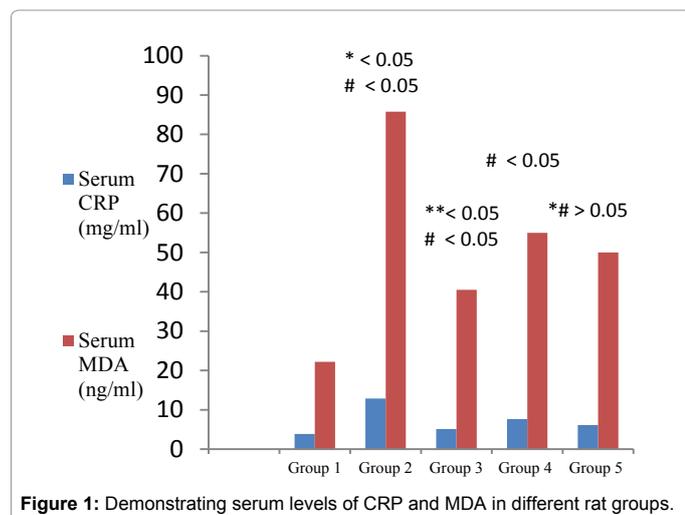


Figure 1: Demonstrating serum levels of CRP and MDA in different rat groups.

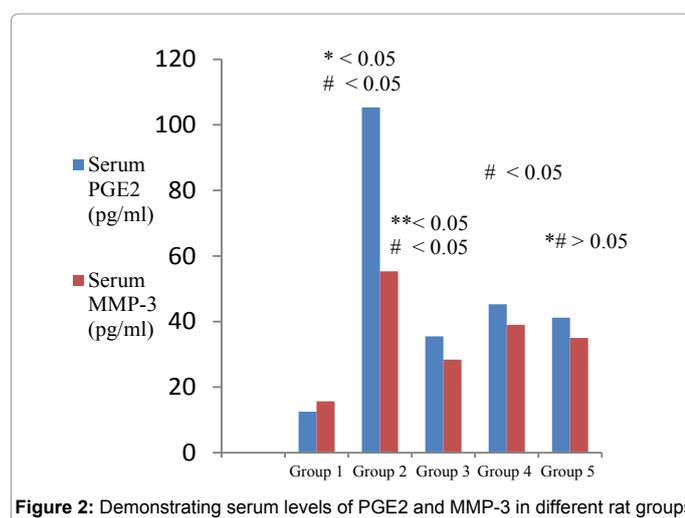


Figure 2: Demonstrating serum levels of PGE2 and MMP-3 in different rat groups.

and oxidative biomarkers of inflammatory response as serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF-alpha are crucial in association with the joint destruction of RA. The significant increase in serum levels of MMP-1 and PGE2 are closely related to the increase in serum level of the inflammatory cytokine IL-6 and TNF-alpha, where these biomarkers could be directly related to damage of collagen and proteoglycan in cartilage and bone matrix of arthritic joints (Table 1).

- Concomitant administration of resveratrol and leflunomide on arthritic rats for two weeks daily; was producing a significant decrease on serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF-alpha in arthritic-resveratrol-leflunomide treated rat group as compared to arthritic non-treated rat group and other rat groups treated with either of resveratrol or leflunomide alone (Table 1).
- Administration of resveratrol alone on arthritic rats for two weeks daily; was producing a significant decrease on serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF-alpha in arthritic-resveratrol treated rat group as compared to arthritic non-treated rat group but not superior to results of arthritic-resveratrol-leflunomide treated rat group (Table 1).
- Administration leflunomide alone on arthritic rats for two weeks

daily; was producing a significant decrease on serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF-alpha in arthritic-leflunomide treated rat group as compared to arthritic non-treated rat group but not superior to results of arthritic-resveratrol-leflunomide treated rat group (Table 1).

### Results of histological assessment of arthritic joints of different rat groups

**Histopathological changes and assessment:** were detected using H&E staining and were evaluated and compared to each other according to the degree of the infiltration of inflammatory cells, synovial hyperplasia, pannus formation, cartilage and bone erosion (Figures 4A-4E).

### Discussion

The aim of the present study was designed to investigate the synergistic ameliorative effects of combined use of resveratrol and leflunomide on inflammatory cytokines and oxidative biomarkers of inflammatory response as serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF-alpha and degree of joint damage in rats with experimental adjuvant arthritis. The results of the study especially of arthritic non-treated rat group confirmed that many of inflammatory cytokines and oxidative biomarkers of chronic inflammatory response such as serum IL-6 and TNF-alpha are crucial in association with the joint destruction of RA as reflected also, from the significant increase in serum levels of predictor biomarkers of erosive bony damage and oxidative tissue damage in RA; such serum MMP-3, PGE2, C-reactive protein, and MDA as evident from some results of previous studies reported that serum levels of the PGE2 and matrix metalloproteinases (as MMP-3, -8, -9) are associated with disease activity and are also, predictors of joint damage progression [32- 34].

The results of present study were in agreement with the outcomes of the previous studies that reported that the proinflammatory cytokines such as TNF-alpha and IL-1beta have been shown to mediate cartilage degradation and apoptosis in chondrocytes in degenerative joint diseases such as RA and Osteoarthritis (OA) in humans as well as in animals with experimental rheumatoid arthritis. Indeed, cytokine-mediated apoptosis of chondrocytes is believed to play a key role in the pathogenesis of such degenerative joint diseases [35-38]. These proinflammatory cytokines are produced by activated synoviocytes, macrophages and chondrocytes [39]. They are well known to activate the ubiquitous transcription factor NF-kB, which leads to further production, expression and upregulation of proinflammatory cytokines and enzymes, which are very closely implicated in the erosive bony and cartilage damage of joints in RA such as Cox-2 and MMPs, which in turn produce prostaglandins especially PGE2, which degrades ECM macromolecules leading to cartilage degradation and further joint inflammation [40].

It is well established that TNF-alpha, plays an important role in the pathology of RA, as it can induce collagenase production that may contribute directly to cartilage destruction and bone resorption that found in RA [41,42] and so the potential therapeutic agents that can suppress the expression of tumour necrosis factor-alpha, interleukin-1b, cyclooxygenase-2, lipooxygenase, matrix metalloproteinases or adhesion molecules, or suppress the activation of NF-kB, having a potential anti rheumatic action for the treatment of arthritis [43].

Leflunomide is the first isoxazole-containing, which is one of disease-modifying anti rheumatic drugs (DMARDs) approved for RA treatment, by its suppressive action of expression of many of

Rat Groups Parameters	Group-1 (Normal saline treated rats)	Group-2 (Arthritic non-treated rats)	Group-3 (Arthritic Resveratrol-leflunomide treated rats)	Group-4 (Arthritic Resveratrol treated rats)	Group-5 (Arthritic leflunomide treated rats)
Serum CRP (mg/ml)	3.86 ± 0.13	12.85 ± 1.14 * < 0.05	5.12 ± 0.43 # < 0.05 ** > 0.05	7.65 ± 0.85 # < 0.05	6.15 ± 0.85 # < 0.05 *# > 0.05
Serum MDA (ng/ml)	22.2 ± 3.00	85.78 ± 5.20 * < 0.01	40.5 ± 3.38 # < 0.05 ** < 0.05	55.0 ± 2.82 # < 0.05	50.0 ± 3.82 # < 0.05 *# > 0.05
Serum MMP-3 (pg/ml)	15.7 ± 3.00	55.30 ± 1.20 * < 0.01	28.4 ± 3.90 # < 0.05 ** < 0.05	39.0 ± 2.50 # < 0.05	35.0 ± 3.70 # < 0.05 *# > 0.05
Serum PGE2 (pg/ml)	12.5 ± 2.00	105.3 ± 4.20 * < 0.01	35.5 ± 3.60 # < 0.01 ** < 0.05	45.3 ± 3.30 # < 0.01	41.2 ± 4.90 # < 0.01 *# > 0.05
Serum TNF-α (pg/ml)	0.010 ± 0.30	90.2 ± 4.80 * < 0.001	40.15 ± 3.40 # < 0.01 ** < 0.05	52.11 ± 4.17 # < 0.05	45.33 ± 5.22 # < 0.05 *# < 0.05
Serum IL-6 (pg/ml)	27.5 ± 3.20	225.2 ± 2.20 * < 0.001	95.2 ± 3.20 # < 0.01 ** < 0.05	120.0 ± 4.10 # < 0.01	110.0 ± 2.70 # < 0.01 *# < 0.05

p-value using one-way ANOVA followed by Bonferroni multiple comparison test as a post ANOVA test.

\*comparing results of group 2 with that of group 1.

#comparing results of group 3, group 4 and group 5 with that of group 2.

\*\*comparing results of group 3 with those of group 4 and group 5.

\*#comparing results of group 5 with that of group 4.

Table 1: Laboratory analysis of serum levels of CRP, MDA, MMP-3, PGE2, TNF-α and IL-6 in different rat groups of the study (Values expressed in mean ± SD).

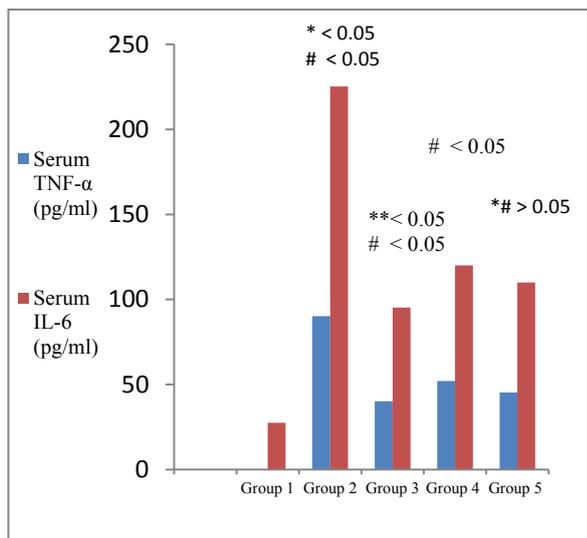


Figure 3: Demonstrating serum levels of TNF-α and IL-6 in different rat groups.

### Different Rat Groups

**Group 1:** Non-arthritic saline treated group

**Group 2:** Untreated arthritic group

**Group 3:** Arthritic-resveratrol and leflunomide treated group

**Group 4:** Arthritic-resveratrol treated group

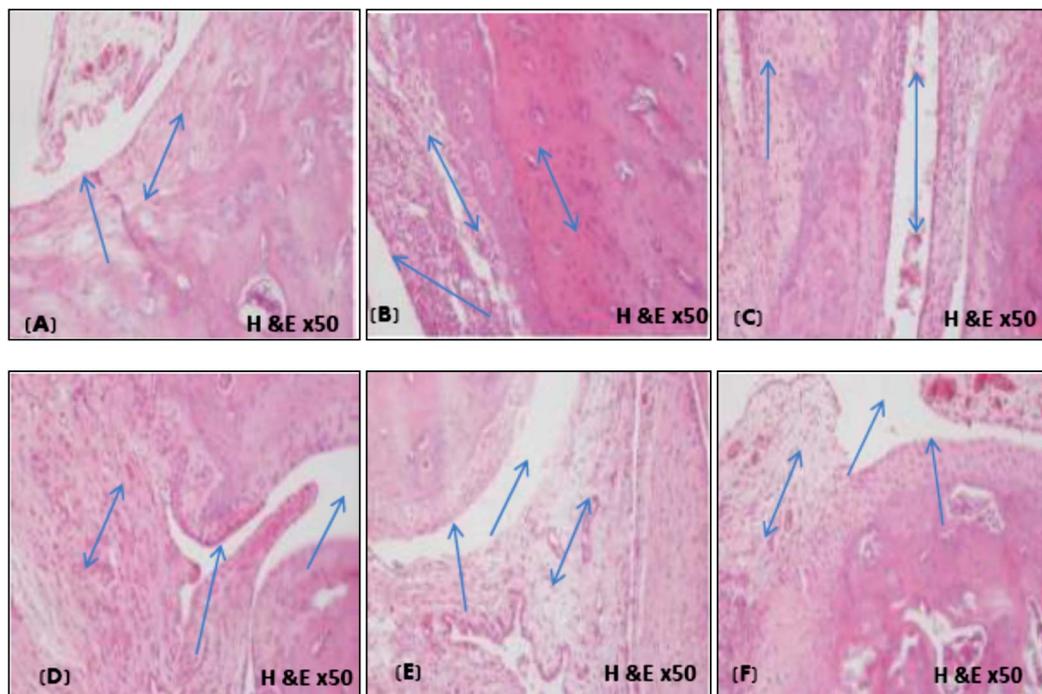
**Group 5:** Arthritic-leflunomide treated group

\*comparing results of group 2 with that of group 1.

#comparing results of group 3, group 4 and group 5 to group 2.

\*\*comparing results of group 3 with those of group 4 and group 5.

\*#comparing results of group 5 with that of group 4.



**Figure 4A:** Normal saline treated rat group showing normal histological picture without any signs of inflammation or bone damage. 4B and 4C: Non-treated arthritic rat group showing severe infiltration of inflammatory cells and cartilage and bone erosion ( middle and right arrows), synovial hyperplasia, pannus formation (right arrow of Figure 4C). 4D: Arthritic-resveratrol-leflunomide treated rat group showing significant less infiltration of inflammatory cells ( left arrow) and absence of synovial hyperplasia and pannus formation ( right arrow) and normal picture cartilage and bone (middle arrow). 4E: Arthritic-resveratrol treated rat group showing very mild infiltration of inflammatory cells (right arrow) and absence of synovial hyperplasia and pannus formation (middle arrow) and very mild damage to cartilage and bone of joints (left arrow). 4F: Arthritic-leflunomide treated rat group showing also a very mild degree infiltration of inflammatory cells (right arrow) and absence of synovial hyperplasia and pannus formation (middle arrow) and a very mild degree of damage of joint's cartilage and bone (left arrow).

proinflammatory biomarkers as TNF- $\alpha$ . Although it is effective in reducing joint inflammation and destruction in clinical trials, leflunomide has numerous dose dependent undesirable side effects preventing it from becoming a widely prescribed treatment of RA and this necessitate reduction of its doses in patients with RA [44-47].

Resveratrol is potent anti-inflammatory dietary phytochemicals that have previously been shown to antagonize some catabolic effects of TNF $\alpha$  and IL-1 $\beta$  via inhibition of NF- $\kappa$ B in different cell types [48-51]. Furthermore, resveratrol is a potent and specific inhibitor of NF- $\kappa$ B activation induced by TNF- $\alpha$  or IL-1, and therefore, resveratrol might be a potential therapy for rheumatoid arthritis [49,52-54].

The present study leads to the following findings, that the concomitant administration of resveratrol and leflunomide on adjuvant arthritic rats for two weeks daily; was producing a significant decrease on serum levels of oxidative stress biomarker MDA, a significant decrease on serum levels of predictor biomarkers of joint destruction as MMP-3, PGE2 and also, a significant decrease in serum levels of the proinflammatory cytokines biomarkers as IL-6 and TNF- $\alpha$ , in addition to this, a significant reduction in acute phase proteins characteristic to inflammatory response of RA as serum C-reactive protein in arthritic-resveratrol-leflunomide treated rat group in comparison to arthritic non-treated rat group and other rat groups of the study. Moreover, arthritic rat group treated by concomitant oral administration of resveratrol and leflunomide showed a significant histopathological improvement in their arthritic joints with a very significant less infiltration of inflammatory cells and absence of synovial hyperplasia and pannus formation, and with a nearly normal

picture of cartilage and bone of their joints in comparison to arthritic non-treated rat group and other rat groups of the study.

Furthermore, the results of the present study are consistent with findings of a previous study reported that the anti-inflammatory and anti-apoptotic effects of resveratrol and/or curcumin are mediated through the inhibition of the IKK induced and proteasome-induced NF- $\kappa$ B pathway, which are activated by a wide variety of proinflammatory agents, and concluded that treatment with curcumin and resveratrol suppressed NF- $\kappa$ B-regulated gene products involved in inflammation such as (cyclooxygenase-2, matrix metalloproteinase (MMP)-3, MMP-9, vascular endothelial growth factor), inhibited apoptosis and prevented activation of caspase-3 and hence, resveratrol and curcumin can block NF- $\kappa$ B and NF- $\kappa$ B-regulated gene expression in arthritis [55].

Leflunomide is a pyrimidine biosynthesis inhibitor, approved for treatment of rheumatoid arthritis since more than 15 years. Manna et al. [56] reported that therapeutic efficacy of leflunomide against RA was related to its suppressive action on generation of TNF- $\alpha$ , with also, prevention of reactive oxygen metabolites generation and lipid peroxidation produced by TNF- $\alpha$  and the suppressive effects of leflunomide on TNF signaling were completely reversible by uridine, indicating a critical role for pyrimidine biosynthesis in TNF-mediated cellular responses, and concluded that the suppression of TNF signaling is one of the possible mechanisms for inhibitory activity of leflunomide against rheumatoid arthritis. Moreover, a similar previous experimental study we showed that UTL-5b, a novel leflunomide analogue, was exerted an effective anti-inflammatory and anti-arthritic

actions on rats with adjuvant arthritis and these actions of UTL-5b were mediated through the reduction of TNF- $\alpha$  in vivo resulting in amelioration of arthritis in animal model [57].

All of these findings of previous studies are in accordance with results of present study regarding effects of leflunomide on laboratory and histological parameters of different rat groups treated with this drug. Furthermore, the present study findings of leflunomide are in agreement with the histological and laboratory finding of previous experimental reported that leflunomide treated arthritic rat group showed the best improvement of synovial pathology and significant reduction in serum level of oxidative MDA marker when compared to other rat groups [58]. In addition, a previous study showed that some of the beneficial effect of leflunomide in RA patients may be due to the inhibition of PGE2, IL-6 and MMP-1 production in synoviocytes, which can produce a significant reduction in the rate of disease progression in RA patients when with leflunomide [59]. These findings are consistent with laboratory findings of present study on serum levels of C-reactive protein, MDA, MMP-3, PGE2, IL-6 and TNF-alpha of arthritic rat groups treated with leflunomide.

Furthermore, the results of study are in accordance with the findings of a previous study reported that resveratrol was capable of to prevent hyperplasia of synovial cells in human rheumatoid arthritis by inducing apoptosis in human rheumatoid arthritis synovial cells, and suggested that resveratrol could be a promising drug for treatment of rheumatoid arthritis [60]. In addition, some previous studies reported that resveratrol can suppress the activation of NF-kB and downregulate inflammatory gene products such as COX-2, 5-LOX, IL-1b, and IL-6 released from synoviocyte, where all of which play a crucial role in arthritis [61,62] and all of these finding are similar to the finding of present study. Also a previous study showed that pre-ischemic infusion of resveratrol protected the spinal cord from IR injury in rabbits, and this protection was attributed to antioxidant and anti-inflammatory effects of resveratrol that lead to a significant decrease in oxidative stress in the ischemic spinal cord tissue and a decrease in neutrophil infiltration [63].

Thus, the results of resveratrol on different serum parameters of the study could be explained by its potential antiarthritic action on RA that, most probably attributed to its ability to inhibit protein degradation by inhibiting the activation and translocation of NF-kB to the nucleus [64]. Moreover, Tang et al. [65] showed that resveratrol can suppress the proliferation and can induce caspase-3-mediated apoptosis of synoviocytes in vitro. Elmali and colleagues determined the in vivo effects of intra-articular injections of resveratrol on cartilage and synovium in an experimental osteoarthritis (OA) model in rabbits, where they found that resveratrol significantly reduced cartilage tissue destruction, and hence concluded that resveratrol could protect cartilage against the development of experimentally induced OA and a potential novel nutraceutical agent for treatment of arthritis [49,66-68].

All of these previous results are consistent with present study laboratory and histological results, and recommending the resveratrol for the prevention and treatment of arthritis. Also, they are in accordance with the recent studies that reported resveratrol showed significant cerebroprotective action against global cerebral ischemic/reperfusion injury mediated by an antioxidant and anti-inflammatory mechanisms, and manifested as a significant reduction in oxidative stress markers as MDA, and inflammatory biomarkers as TNF- $\alpha$ , IL-6 and 10 and MPO enzymes [69,70]. Moreover, the laboratory and histological results of present study, are in agreement with other recent experimental study reported that the articular cartilage degeneration

with synovial hyperplasia and inflammatory cell infiltration was significantly suppressed with also, a significant reduction in serum levels of COX-2 and PGE2 in adjuvant arthritic rats treated with resveratrol and suggested that resveratrol has a significant anti-inflammatory effects; mediated by its ability to reduce the production of inflammatory biomediators COX-2 and PGE2 [71].

The combinational use of both resveratrol and leflunomide not evaluated before and the results of their supplemental use concomitantly in the present study showed a synergism between them on inhibition of increase in serum levels of many of proinflammatory cytokines and enzymes that underlying the pathogenesis of RA, and so the nutraceutical agent, resveratrol has a several advantages like effectiveness as anti-arthritic agent, cost effective and lack of side effect. Thus, resveratrol together with leflunomide are the gold mine for treatment of arthritis [72].

It can be concluded from the present study that resveratrol and leflunomide are potent inhibitors of many potential proinflammatory cytokines especially TNF- $\alpha$  and IL-6, proinflammatory biomediator enzymes such as COX-2, matrix metalloproteinases and their concomitant use showed an additive inhibitory effects on serum levels of MMP-3, PGE2, IL-6, TNF- $\alpha$ , that are strongly implicated in the pathogenesis an progression of RA, and with a significant ameliorative and protective effects against joint damage reflected from histopathological assessment. Thus, the concomitant use of resveratrol with leflunomide is recommended for better management of many of degenerative joint diseases especially rheumatoid arthritis in human.

#### Acknowledgements

The author would like to acknowledge Dr. Tark Salam (Central laboratory of Pharmacy College) for his technical laboratory assistance and Dr. Gamal Nassar (Pathology laboratory of College of Medicine) for his histological processing and histopathological assessment of the arthritic rat joints.

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