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# Venlafaxine alleviates complete Freund's adjuvant-induced arthritis in rats: Modulation of STAT-3/IL-17/RANKL axis

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ABSTRACT

*Aims:* Rheumatoid arthritis is usually accompanied by various comorbidities especially on the psychological side such as depression. This study aimed at revealing the potential curative effects of venlafaxine (VFX), a serotonin/norepinephrine reuptake inhibitor (SNRI), on experimentally-induced arthritis in rats.

*Methods:* Arthritis was induced by injecting complete Freund's adjuvant (CFA, 0.1 ml, *s.c.*). One day thereafter, VFX (50 mg/kg, *p.o.*) was given for 21 days. Methotrexate was used as a standard disease modifying anti-rheumatic drug.

*Key findings:* CFA injection caused prominent arthritis evident by the increase in the hind paw and ankle diameter accompanied by elevating tumor necrosis factor-alpha, interleukin-6, interleukin-17 and matrix metalloproteinase-3 levels, effects that were diminished by VFX. Moreover, VFX down regulated gene expressions of receptor activator of nuclear factor kappa-B (NF-κB) ligand and signal transducer and activator of transcription-3 beside hampering immunohistochemical expression of vascular endothelial growth factor and NF-κB. This SNRI also improved the oxidant status of the hind limb as compared to the arthritic group. Nonetheless, MTX was better in amendment of arthritis authenticated by its effect on some inflammatory and oxidative stress biomarkers.

*Significance:* This study provides a novel therapeutic use of VFX as a considerable anti-arthritic drug and offers an incentive to expand its use in RA.

## 1. Introduction

Although rheumatoid arthritis (RA) mainly affects joints, it is often accompanied by other systemic complications which deteriorate patients' quality of life [1]. Increased incidence of fatigue and depression are among the common comorbidities [2,3].

VFX is a serotonin/norepinephrine reuptake inhibitor (SNRI), frequently used for the treatment of depressive disorders [4]. Vollmar et al. proved that VFX exhibits an anti-inflammatory effect via suppressing several cytokines like interleukin-6 (IL-6) in vitro [4] and unveiled its immunomodulatory activity in vivo via affecting several pro-inflammatory cytokines as tumor necrosis factor-alpha (TNF-α) [5]. Based on these evidences, VFX can be considered as a promising candidate for hindering RA inflammatory cascade and/or the escorted depression.

It is interestingly being reported that inflammation of the synovial membrane and proliferation of the synovial lining is a characteristic of RA [6]. Synovial lining macrophages release the pivotal cytokine IL-6 which plays an initiative role in eliciting the uncontrolled pathological cascade of RA [7]. In this context, IL-6 matures naïve CD4+ T cells into T helper 17 lymphocytes (Th17) [8] by intensifying the expression of signal transducer and activator of transcription-3 (STAT-3) [9]. Consequently, STAT-3 will further activate Th17 cells to release interleukin-17 (IL-17) [9] which is responsible for triggering the nuclear factor (NF-κB) cytokine downstream mediators [10] and stimulation of various immune cells including B cells, macrophages, neutrophils and mast cells to release inflammatory mediators mainly; IL-6 and tumor necrosis factor-alpha (TNF-α) [11]. These cytokines trigger synovial fibroblasts to unleash its destructive enzymes including; matrix metalloproteinases such as matrix metalloproteinase-3 (MMP-3); which are the main cause for cartilage destruction as they can degrade all components of the extracellular matrix [12]. This milieu will stimulate synoviocytes to

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release, receptor activator of nuclear factor kappa-B ligand (RANKL); that proliferates inactive mononucleated osteoclast precursor into active mature polynucleated osteoclast cells [8] and necessarily, causes bone erosion [13].

Noteworthy, IL-17 endorses the enrollment and invasion of myeloid-derived suppressor cells (MDSCs) at the affected joints yielding nitric oxide and reactive oxygen species (ROS) [9]. Moreover, IL-17 can stimulate vascular endothelial cells and hasten the angiogenic advance by expanding the production of vascular endothelial growth factor (VEGF) [9]; this situation leads to pannus formation and sustains the inflammatory cycle. [12–14].

In an attempt to prospect new agents with capacity to ameliorate RA complications, VFX has been investigated as a promising tool as it can target the culprit cytokines responsible for initiating immune reactions and deteriorating inflammation (e.g. IL-6 and TNF-α) as well as relieving depression, an important RA comorbidity. To the best of the authors' knowledge, this is the first report demonstrating the possible mechanistic cassette underlying the ameliorative effect by venlafaxine administration in an arthritic model involving STAT-3/IL-17/RANKL cascade.

## 2. Materials and methods

### 2.1. Animals

Animal house breeding colony of the National Organization for Drug Control and Research (NODCAR, Cairo, Egypt) provided this study with adult male Sprague-Dawley rats (160–180 g). Free access for food and water was allowed. Prior to experiments, rats were exposed to 2 weeks accommodation stage in the animal house. This study followed strictly the ethical guidelines for laboratory animals investigations, approved by the Ethical Committee of Faculty of Pharmacy, Cairo University, Egypt (PT-1781) and in line with the Guide for the Care and Use of Laboratory Animals (ILAR, 1996) [15].

### 2.2. Reagents and chemicals

A parenteral vial of 1 mg/ml complete Freund's adjuvant was provided from DIFCO LABORATORIES (Detroit, Michigan, USA). Furthermore, EIMC United Pharmaceuticals “EUP” (Cairo, Egypt) was the source of 25 mg/ml MTX vial while VFX was kindly gifted from International Drug Agency for Pharmaceutical Industry “IDI Pharmaceutical” (Cairo, Egypt). All other chemicals were of analytical grade or equal quality.

### 2.3. Induction of arthritis

Induction of arthritis was carried out via subcutaneous injection of 100 µl CFA into the sub-plantar surface of the left hind paw. On the same day and one day thereafter, intradermal injection of 100 µl ml CFA was given into the root of the tail as a booster dose. [16].

### 2.4. Experimental design

Animals were divided randomly into five groups (14 rats/each), as follows: the first group; served as normal control and was given saline only. The second group; served as positive control (arthritic rats) and were given CFA (100 µl, s.c.) in a single dose [17]. The third group; rats were injected with a single dose of CFA (100 µl, s.c.), one day thereafter methotrexate (0.75 mg/kg, i.p.) was administered once weekly for three successive weeks [16]. The fourth group; animals were administered CFA (100 µl, s.c.) in a single dose and one day thereafter venlafaxine (50 mg/kg, p.o.) was given by gavage once daily for three successive weeks [18].

### 2.5. Assessment of arthritis

Determining arthritic index as well as paw and ankle diameters by Vernier caliper on specific days in a manner described in previous study [16]. Relative paw weight was estimated as the percent of the left hind limb weight over the total body weight.

The progression of CFA induced arthritis was evaluated on days 0, 1, 3, 6, 8, 10, 13, 15, 17 and 21 after adjuvant injection. Paws were examined and graded for severity of erythema and swelling using a 5-point scale: 0 = no signs of inflammation, 1 = swelling and erythema of the digit, 2 = moderate swelling and erythema, 3 = severe swelling and erythema of the limb and 4 = severe swelling, erythema, gross deformity and disability to use the limb. Ankle diameter was determined by measuring the antero-posterior diameter, using Vernier caliper which is accurate to 0.02 mm [16].

### 2.6. Sample preparation

By the end of the experiment, animals were decapitated under anesthesia to isolate, weigh and examine their left hind limbs. The isolated organs were homogenized for biochemical investigations (IL-6, IL-17, TNF-α, MMP-3, MPO, MDA and GSH) and for RANKL and STAT-3 gene expression. Finally, two randomly chosen hind limbs were preserved in 10% formalin/saline for further histopathological and immunohistochemical investigations.

### 2.7. Biochemical investigations

#### 2.7.1. Homogenate preparation

Left hind paws were rapidly washed with ice-cold saline after isolation. Then homogenized with Glas-Col motor driven homogenizer (USA) in phosphate buffered saline (50 mM, PH 7.4) to obtain 10% (w/v) tissue homogenate, and centrifuged at 1000*g* for 10 min to separate clear supernatants for speculation of biochemical parameters.

#### 2.7.2. Inflammatory biomarkers assessment in paw tissue

The company catalogs for MMP-3, IL-6 and IL-17 ELISA kits (Cusabio®, China) were our guide to assess these biomarkers. Correspondingly, TNF-α was estimated in accordance with that of the manufacturer guide (RayBiotech®, USA).

#### 2.7.3. Estimation of oxidative stress biomarkers in paw homogenate

In accordance with manufacturer (CELL BIOLABS®, USA) protocol, MDA level was evaluated by (OxiSelect™) ELISA Kit. Similarly, the instructions enclosed by the vendor of GSH ELISA Kit (BlueGene Biotech®, China) were followed to estimate rat reduced glutathione homogenate content. Likewise, (Hycult®, USA) MPO rat ELISA kit was used to assess MPO homogenate activity.

#### 2.7.4. Quantitative RT-PCR for RANKL and STAT-3

Total RNA from hind limb tissue was extracted using SV Total RNA Isolation system (Promega, Madison, WI, USA) and the purity of obtained RNA was verified spectrophotometrically at 260 nm. The extracted RNA was reverse transcribed into cDNA using reverse transcriptase polymerase chain reaction (RT-PCR) kit (Stratagene, Santa Clara, CA) according to the manufacturer's instructions. To assess the expression of the target genes, quantitative real-time PCR was performed using SYBR green PCR Master mix (Qiagen, Germany) as described by the manufacturer. Briefly, 25 µL of QuntiFast SYBR Green PCR Master Mix, 22.5 µL dH2O, 2 µL primer pair mix (5 pmol/µL each primer), and 0.5 µL cDNA in a final reaction volume of 50 µL. The sequences of primers of RANKL, STAT-3 and housekeeping gene (GAPDH) are listed in Table 1 [19]. PCR reactions included 10 min at 95 °C for activation of AmpliTaq DNA polymerase, followed by 40 cycles at 95 °C for 15 s (denaturing) and 60 °C for 1 min (annealing/extension). The data were expressed in cycle threshold ($C_t$), where the increased

**Table 1**
Primers used for RT-PCR of studied genes.

| | Primer sequence | Gene bank accession number |
|---|---|---|
| *RANKL* | Forward primer: 5-CACACCTCACCATCAATGCTGC-3 Reverse primer: 5-GAAGGGTTGGACACCTGAATGC-3 | AF_019048.1 |
| *STAT-3* | Forward primer: 5′-GTAGGGGTTCCTCACCCTTC-3<br>Reverse primer: 5′-TGACCTGCCACCTGACAGTA-3 | NM_031784.2 |
| *GAPDH* | Forward primer: 5′-GGTCGGTGTGAACGGATTTGG-3<br>Reverse primer: 5′-ATGTAGGCCATGAGGTCCACC-3 | XM_017593963.1 |

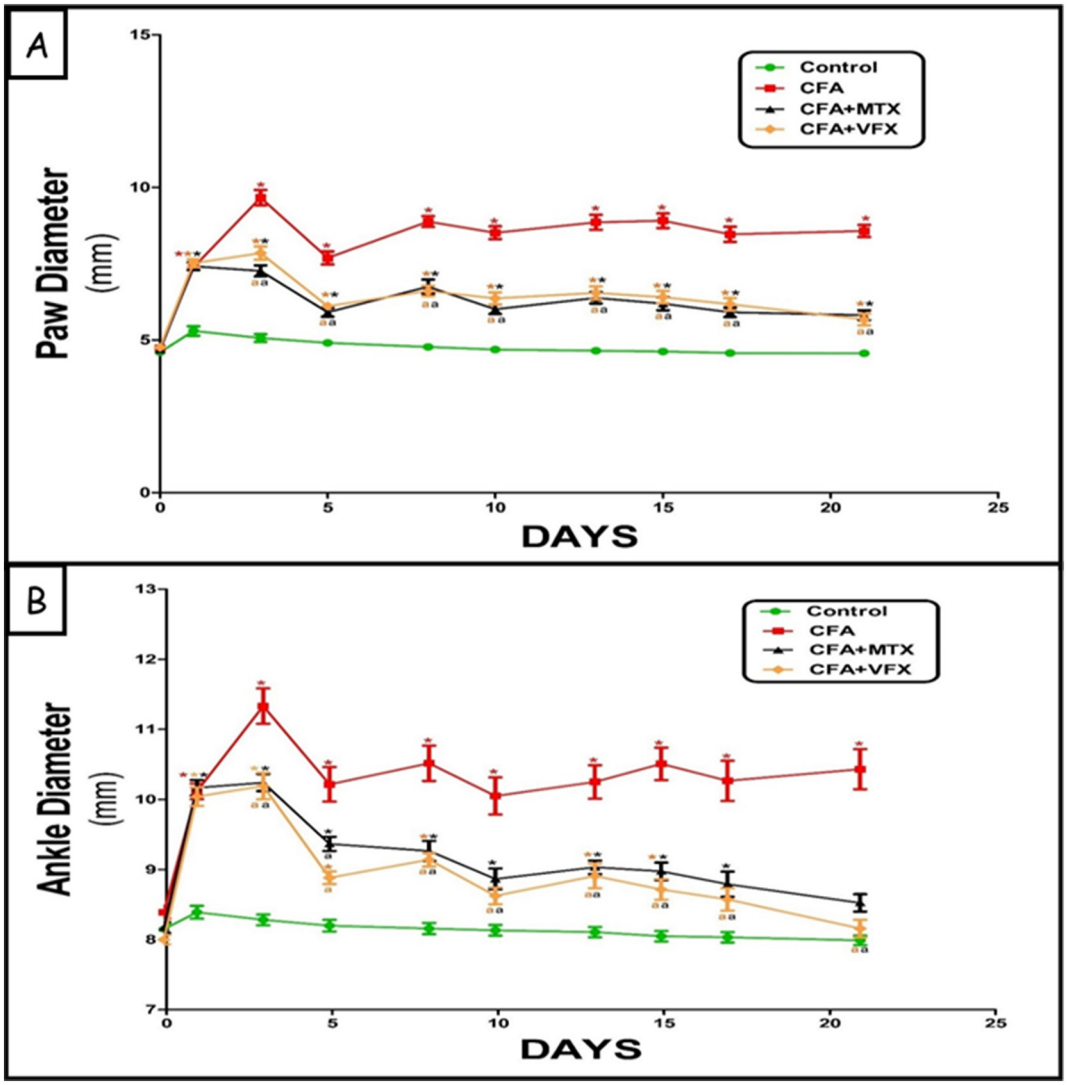



**Fig. 1.** Effect of VFX on paw diameter (A) and ankle diameter (B) of the left hind limb in complete Freund's adjuvant-induced arthritis in rats was evaluated on days 0, 1, 3, 6, 8, 10, 13, 15, 17 and 21. Each point with vertical line represents the mean ± SE (n = 6–8). * or [a] Significantly different from normal control (saline) or CFA control at $p < 0.05$, respectively. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test at $p < 0.05$.

fluorescence curve passes across a threshold value. The relative expression of target gene was obtained using comparative $C_t$ ($\Delta\Delta C_t$) method. The $\Delta C_t$ was calculated by subtracting RANKL or STAT-3 $C_t$ from that of target gene whereas $\Delta\Delta C_t$ was obtained by subtracting the $\Delta C_t$ of reference sample (internal control) from that of test sample. The relative expression ratios were calculated by the $2_t^{-\Delta\Delta C}$ [20].

### 2.8. Histopathological examination

Left hind limbs, including paws and knees, were randomly taken from different groups immersed in 10% neutral formalin to be post-fixed for 48 h. Subsequently, the fixed tissue was moved into decalcifying solution (4 M formic acid) for 35 days to make the bone completely demineralized [21]. During this period, the decalcifying solution was changed once a week then washed by water, dehydrated by alcohol, cleared in xylene and fixed in paraffin for one day using a hot air oven at 56 °C. Specimens were sectioned using a sledge microtome adjusted at a thickness of 4 μm then placed on slides for deparaffinization and staining by hematoxylin & eosin. Finally, the light electric microscope (Olympus CX21, Japan) was used for examination after staining by hematoxylin & eosin (X 40) [22].

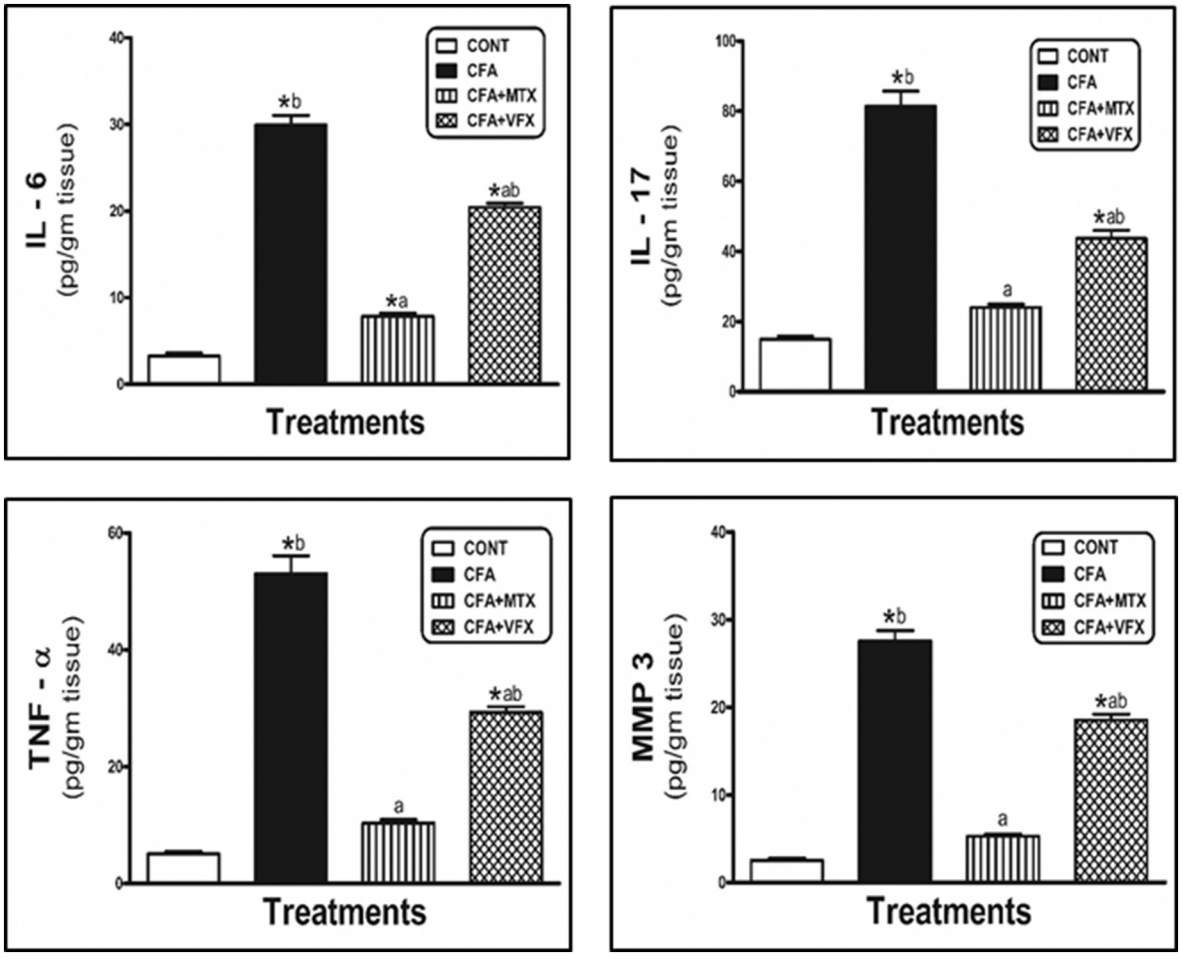



**Fig. 2.** Effects of VFX on inflammatory mediators; IL-6, IL-17, TNF-α and MMP-3 in left hind paw homogenate in complete Freund's adjuvant-induced arthritis in rats by ELISA. Each bar with vertical line represents the mean ± SE (n = 6). *, [a] or [b] Significantly different from normal control (saline), CFA control or CFA + MTX, respectively. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test at $p < 0.05$.

**Table 2**
Effects of VFX on percent change in body weight, relative left hind limb weight and arthritic index in complete Freund's adjuvant-induced arthritis in rats.

| Treatment / Parameter | Normal Control | CFA | | |
|---|---|---|---|---|
| | | Control Arthritic Group | + MTX | + VFX |
| % Change in body weight | 34.38 ± 2.04 | 20.11*[b] ± 2.86 | 35.71[a] ± 2.17 | 33.03[a] ± 1.84 |
| Relative left hind limb weight | 0.59 ± 0.09 | 1.41*[b] ± 0.05 | 1.07*[a] ± 0.03 | 1.06*[a] ± 0.02 |
| Arthritic index | ———— | 10.33[b] ± 0.21 | 3.5[a] ± 0.85 | 3.83[a] ± 0.91 |

### *2.9. Immunohistochemical examination*

In order to assess the immunohistochemical reactivity of nuclear factor kappa-B (NF-κB) and vascular endothelial growth factor (VEGF) the used specimens were prepared by deparaffinization and rehydration using xylene and alcohol. Samples were incubated in citrate buffer (Thermo Fisher Scientific, Fremont, CA; pH 6.0) for 20 min at the boiling point and left to cool down then exposed to overnight re-incubation period with rabbit polyclonal anti-NF-κB (1:200; Abcam®, USA [ab28835]), or anti-VEGF (1:200; Abcam®, USA [ab46154]) primary antibody at 4 °C. Slides were washed with PBS and incubated at 37 °C for 30 min with biotinylated secondary antibody and the Vector Elite ABC kit (Elite reagent avidin DH and biotinylated horseradish peroxidase H reagents; Vector Laboratories Inc., Burlingame, CA). One more wash with PBS was done and diaminobenzidinetetrahydrochloride substrate (DAB Substrate Kit, Vector Laboratories Inc.) was added to develop the antibody–biotin–avidin–peroxidase complex. Hematoxylin counterstaining was performed for all slides then dehydrated and cleared in xylene. A positive immunohistochemical reaction appears as a brown colour in the cytoplasm (X 40) and the quantification of the positive areas were implemented via image analysis software (Image J, 1.50i, NIH, USA).

### *2.10. Statistical analysis*

All values were presented as means ± standard error of the means (SEM). One-way analysis of variance (ANOVA) was the used method to compare between different groups followed by the post-hoc test Tukey-Kramer's. When $P \leq 0.05$, difference was considered significant for all compared groups. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad, San Diego, CA, USA).

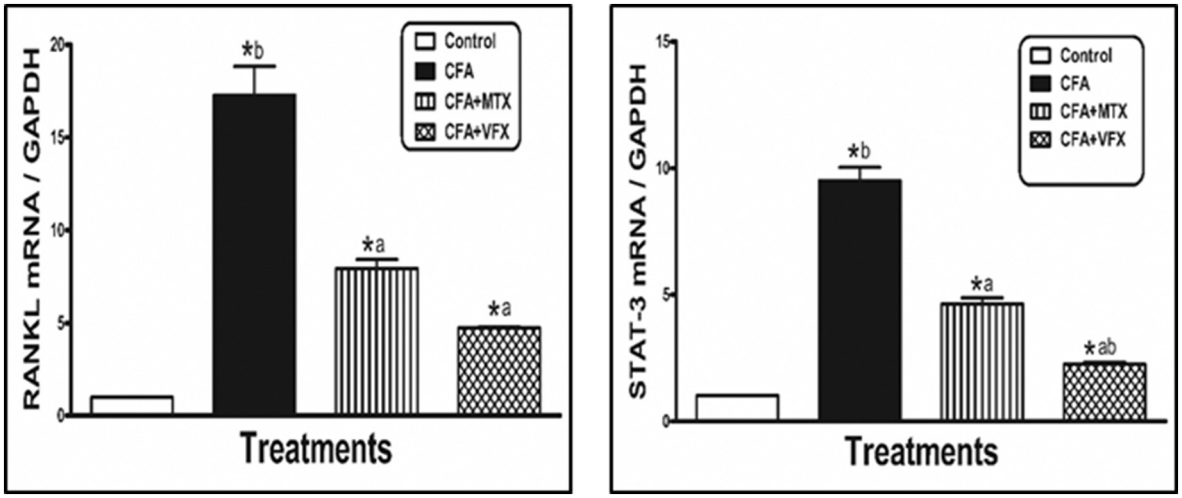



**Fig. 3.** Effects of VFX on gene expression of RANKL and STAT-3 in left hind paw homogenate in complete Freund's adjuvant-induced arthritis in rats. Each bar with vertical line represents the mean ± SE (n = 6). *, [a] or [b] Significantly different from normal control (saline), CFA control or CFA + MTX at $p < 0.05$, respectively. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test at $p < 0.05$.

## 3. Results

### 3.1. Arthritic signs

CFA significantly increased left hind paw diameter (87%) and left hind ankle diameter (31%). Moreover, MTX and VFX significantly decreased left hind paw diameter (32% and 33%, respectively) and left hind ankle diameter (18% and 23%, respectively) as compared to their first day correspondings, respectively (Fig. 1).

On the other hand, MTX and VFX almost normalized body weight (78% and 64%, respectively) but significantly decreased relative left hind limb weight (24% and 25%, respectively) as compared to CFA (arthritic) group. Moreover, MTX and VFX significantly decreased left hind paw diameter (22% and 25%, respectively) and left hind ankle diameter (16% and 20%, respectively) as compared to their first day correspondings, respectively. In addition to arthritic index which was also decreased significantly by MTX (66%) and VFX (63%) as compared to CFA (arthritic) group (Table 4).

### 3.2. Inflammatory biomarkers

Administration of CFA significantly elevated TNF-α (approx. 9 folds), IL-6 (about 8 times), IL-17 (nearly 4 folds) and MMP-3 (around 9 folds) levels in left hind paw homogenate as compared to normal control group. However, MTX and VFX significantly mitigated the arthritic effect of CFA on inflammatory mediators and decreased the elevated TNF-α (80% and 45%, respectively), IL-6 (74% and 32%, respectively), IL-17 (71% and 46%, respectively) and MMP-3 (81% and 33%, respectively) levels in left hind paw homogenate as compared to CFA (arthritic) group (Fig. 2).

### 3.3. Oxidative stress biomarkers

Arthritic rats incurred oxidative stress manifested by a significant surge in left hind limb MPO activity and MDA level (around 9 folds) paralleled by marked depletion of GSH content (92%) as compared to normal control group. On the other hand, MTX and VFX diminished oxidative insult caused by CFA as demonstrated by the marked decrease in MPO activity (76% and 45%, respectively) and MDA level (82% and 47%, respectively) accompanied by restoration of GSH content (approximately 6.5 and 1.5 folds, respectively) as compared to CFA (arthritic) group (Table 2).

### 3.4. Quantitative RT-PCR gene expression

Induction of arthritis caused prominent increase in RANKL (almost 16 times) and STAT-3 (approximately 8 folds) gene expression in the left hind limb as compared to the normal control. In contrast, treatment with MTX and VFX antagonized CFA effect on the aforementioned gene expressions where animals exhibited a significant decrease in RANKL (54% and 73%, respectively) and STAT-3 (51% and 76%, respectively) gene expression as compared to arthritic group (Fig. 3).

### 3.5. Histopathological findings

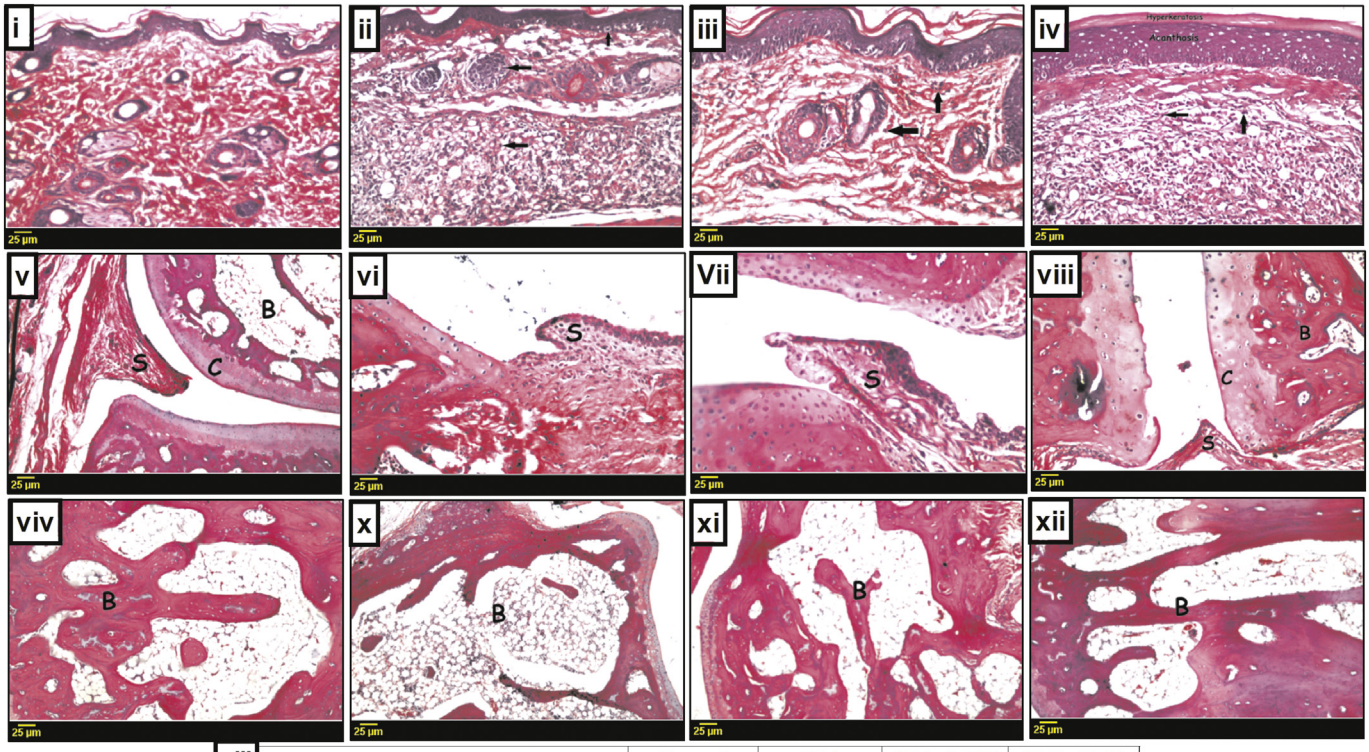
The histopathological findings of the left hind limb demonstrated in Fig. 4 showed that administration of CFA induced various degenerative changes revealed as massive inflammatory cells infiltration all over the dermal layer that reached the subcutaneous tissue. In addition, thickening in periosteum, hyperplasia of the covering cells of synovial membrane (*S*) with inflammatory cells infiltration in underlying tissue, resorption as well as osteoporosis of bone trabeculae (*B*) of knee joint bone were depicted. Moreover, degeneration and resorption of articular cartilaginous surface (*C*) were obviously observed. These histological changes corroborated the biochemical, genetical and immunohistochemical evidences of arthritis. In contrast, treatment with MTX or VFX obviously attenuated the histopathological changes induced by CFA. Noteworthy, VFX group showed hyperkeratosis and acanthosis in epidermis as an adaptation for inflammation and normal intact bone trabeculae (Fig. 4 & Table 3)

### 3.6. Immunohistochemical findings

Immunohistochemical analysis revealed that normal rats showed negative immunostaining for both VEGF and NF-κB (Fig. 5A and E) while arthritic non-treated rats exhibited severe expression in left hind paw tissues (Fig. 5B and F). Treatment of animals with MTX and VFX significantly hampered the arthritic effect of CFA exhibited as decrease in the positively reacted immunostained area percent. MTX decreased area percent for VEGF and NF-κB by 58% and 45%, respectively while VFX decreased the area percent for the same parameters by 43% and 60% (Fig. 5C, D, G and H).

## 4. Discussion

This investigation provides novel insights upon the anti-




xiii

| | Control | CFA | CFA+MTX | CFA+VFX |
|---|---|---|---|---|
| Inflammatory cells infiltration in dermis of the paw | - | ++ | - | - |
| Inflammatory cells infiltration in subcutaneous tissue of the paw | - | +++ | ++ | + |
| Periosteal inflammatory reaction | - | ++ | - | - |
| Hyperplasia of synovial membrane | - | ++ | ++ | - |
| Inflammatory cells infiltration in tissue underlying synovial membrane | - | ++ | - | - |
| Osteoporosis and resorption of bone trabeculae | - | +++ | - | + |

**Fig. 4.** Effect of 3 weeks administration of methotrexate (MTX; 0.75 mg/kg/week, i.p.) and venlafaxine (VFX; 50 mg/kg/day, p.o.) on histopathological changes in complete Freund's adjuvant-Induced arthritis in rats using hematoxylin and eosin (H&E). Photomicrographs of skin section, knee joint and bone of the left hind paw (X 40) showing i) normal histological structure of the epidermis and dermis with sebaceous glands and hair follicles in normal control rats, ii) massive number of inflammatory cells infiltration in diffuse manner all over the dermal layer till the subcutaneous tissue in arthritic rats, iii) intact epidermis and dermis with inflammatory cells infiltration in the subcutaneous tissue in arthritic + MTX rats, iv) mild hyperkeratosis and acanthosis in epidermis with inflammatory cells infiltration the subcutaneous tissue in arthritic + VFX rats, v) normal histological structure of the synovial membrane (*S*), articular cartilaginous surface (*C*) and underlying bone (*B*) in normal control rats, vi) hyperplasia of the covering cells of synovial membrane (*S*) with inflammatory cells infiltration in underlying tissue in arthritic rats, vii) degeneration and resorption of articular cartilaginous surface (*C*) with proliferative hyperplasia in cells covering synovial membrane (*S*) in arthritic + MTX rats, viii) degeneration and resorption of articular cartilaginous surface (*C*) with intact synovial membrane (*S*) in arthritic + VFX rats, viv) normal histological structure of bone trabeculae (*B*) in normal control rats, x) s resorption and osteoporosis of bone trabeculae (*B*) in arthritic rats, xi) intact trabeculae (*B*) in arthritic + MTX rats, xii) intact histological structure of trabeculae (*B*) in arthritic + VFX rats. + + + severe, + + moderate, + mild, − nil.

**Table 3**
Effects of VFX on oxidative stress biomarkers; MPO activity, MDA level and GSH content in left hind paw homogenate in complete Freund's adjuvant-induced arthritis in rats.

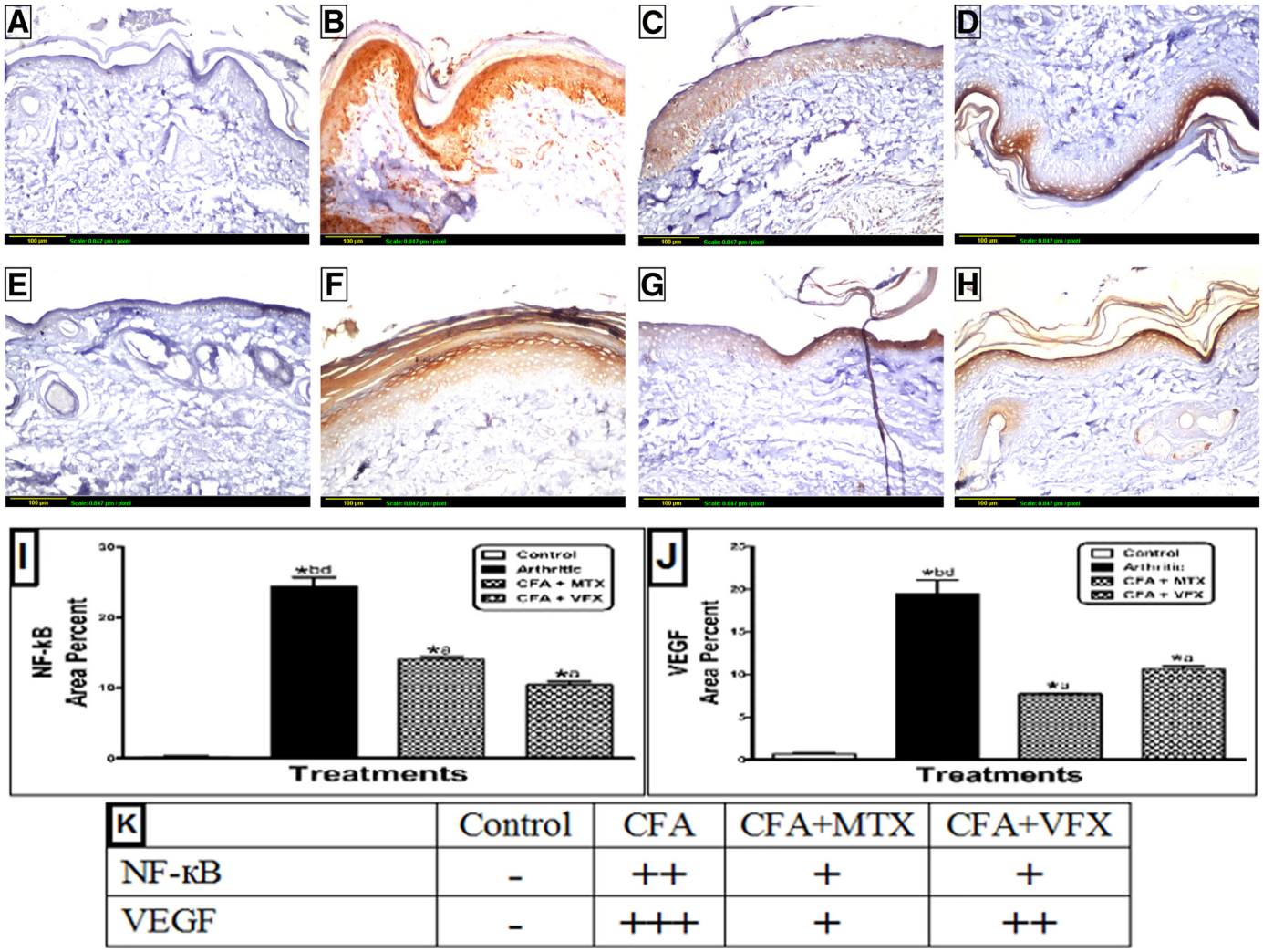
| Parameter | Treatment | | | |
|---|---|---|---|---|
| | Normal control | Control Arthritic group | CFA + MTX | CFA + VFX |
| MPO (U/g tissue) | 1.6 ± 0.07 | $15.76^{*,b}$ ± 0.81 | $3.74^{*,a}$ ± 0.17 | $8.74^{*,a,b}$ ± 0.29 |
| MDA (µmol/g tissue) | 2.99 ± 0.24 | $28.94^{*,b}$ ± 0.87 | $5.32^{*,a}$ ± 0.24 | $15.42^{*,a,b}$ ± 0.65 |
| GSH (pg/g tissue) | 31.52 ± 1.67 | $2.66^{*,b}$ ± 0.2 | $20.08^{*,a}$ ± 0.77 | $6.24^{*,a,b}$ ± 0.23 |

Arthritis was induced by injecting a single dose (0.1 ml, s.c.) of CFA in the left hind paw. Paws were excised after anesthesia. Each value represents the mean ± SE (n = 6). *, $^{a}$, $^{b}$ Significantly different from normal control (saline), CFA (arthritic) control and CFA + MTX, respectively. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test at $p < 0.05$.

**Table 4**
Effect of 3 weeks administration of methotrexate (MTX; 0.75 mg/kg/week, i.p.) and venlafaxine (VFX; 50 mg/kg/day, p.o.) on the severity histopathological alterations in left hind paw sections of complete Freund's adjuvant-induced arthritis in rats.

| | Control | CFA | CFA + MTX | CFA + VFX |
|---|---|---|---|---|
| Inflammatory cells infiltration in dermis of the paw | − | + + | − | − |
| Inflammatory cells infiltration in subcutaneous tissue of the paw | − | + + + | + + | + |
| Periosteal inflammatory reaction | − | + + | − | − |
| Hyperplasia of synovial membrane | − | + + | + + | − |
| Inflammatory cells infiltration in tissue underlying synovial membrane | − | + + | − | − |
| Osteoporosis and resorption of bone trabeculae | − | + + + | − | + |

+ + + severe, + + moderate, + mild, − nil.




| K | Control | CFA | CFA+MTX | CFA+VFX |
|---|---|---|---|---|
| NF-κB | - | ++ | + | + |
| VEGF | - | +++ | + | ++ |

**Fig. 5.** Effect of 3 weeks administration of methotrexate (MTX; 0.75 mg/kg/week, i.p.) and venlafaxine (VFX; 50 mg/kg/day, p.o.) on immunohistochemical expression of NF-κB and VEGF in left hind paw tissues in complete Freund's adjuvant-induced arthritis in rats. Photomicrographs of left hind paw section (X 40) showing A) negative expression of nuclear factor kappa B (NF-κB) in normal control rats, B) severe expression of NF-κB in arthritic rats, C) mild expression of NF-κB in arthritic + MTX rats, D) mild expression of NF-κB in arthritic + VFX rats, E) negative expression of vascular endothelial growth factor (VEGF) in normal control rats, F) severe expression of VEGF in arthritic rats, G) mild expression of VEGF in arthritic + MTX rats, H) moderate expression of VEGF in arthritic + VFX rats, panels (I and J, respectively) represent quantitative image analysis for immunohistochemical staining expressed as area percent across 10 different fields for each rat section. Values are given as mean ± SEM ($n$ = 2 rats, per group). *, [a], [b], [d] Significantly different from normal control (saline), CFA (arthritic) control, CFA + MTX and CFA + VFX, respectively. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test at $p < 0.05$.

inflammatory and the immunomodulatory effects of VFX against CFA-induced arthritis in rats as depicted by reducing paw and ankle diameter, effects that are verified by improvement of hind paw histological features as compared to arthritic group. However previous investigations had proved the anti-inflammatory [4], immunomodulatory [5] and anti-oxidant capacities [23] of VFX, none has shed light on its anti-arthritic effect against CFA-induced arthritis. Nonetheless, the empirical results reported herein should be considered in the light of a specific limitation which is studying STAT-6 conversion into its activate form (p-STAT6) as STAT6 phosphorylation is the phenomenon responsible for the activation of IL-6/STAT3/IL-17 axis.

In this study, VFX reduced IL-6 content in the limb homogenate. Such a finding corroborates the work of Vollmar et al. revealing the decreased level of IL-6 in murine experimental autoimmune

encephalomyelitis following VFX treatment [5]. This could be attributed to the decreased migration of activated lymphocytes into inflamed tissues through the alteration of expression of genes involved in activation and ionic transport of lymphocytes [24]. STAT-3 is also a critical signaling molecule that participates in creating an inflammatory milieu through regulating downstream pro-inflammatory cytokines [9]. VFX obviously suppressed STAT-3 gene expression as compared to arthritic group. IL-6 decline observed herein probably contributed to this effect due to the notion that STAT-3 is activated by IL-6 [25,26]. In context, it has been suggested that interference with STAT-3 signaling could be a good therapeutic strategy to mitigate autoimmune diseases, including RA [26].

The anti-inflammatory effect of VFX was additionally emphasized via the significant hampering of the inflammatory cytokine IL-17. This is ascribed to the downregulation of STAT-3 expression in Th17 lymphocytes [27]. In support, it was previously reported that IL-17 plays a cardinal role in the development of arthritis by activating immune cells such as T cells, B cells, and macrophages to stimulate T cell priming, antibody formation and pro-inflammatory cytokine release, respectively [28,29]. Moreover, this cytokine triggers nonimmune cells to unleash more cytokines and chemokines, including MMPs, VEGF, RANKL and antimicrobial peptides. Altogether, these series of mediators induce immune cells recruitment at inflammatory sites, promote local tissue destruction, induce neovascularization, and enhance osteoclastogenesis, resulting in RA development [10]. Thus, IL-17 is an essential target for arthritis treatment [28,29].

In the arthritic milleu, T helper-17 macrophages release IL-17 which causes nuclear factor (NF-κB) overexpression [10]. Conceivably, NF-κB cascade stimulates various immune cells including B cells, macrophages, neutrophils and mast cells to release many types of inflammatory mediators mainly; IL-6 and TNF-α [9]. These cytokines trigger synovial fibroblasts to unleash its destructive enzymes and cytokines including; matrix metalloproteinases and RANKL responsible for cartilage destruction and bone erosion, respectively [8,12].

In this study, VFX-treated animals exhibited marked decline in tissue NF-κB probably denoting that inhibition of the key pro-inflammatory cytokine IL-17 as well as other inflammatory mediators, such as TNF-a and IL-1b and scavenging reactive oxygen species (ROS) by VFX reported herein are responsible for such effect being required for NF-κB activation [9,30]. As expected, mitigation of NF-κB conceivably resulted in reduction of TNF-α in VFX-treated rats. NF-κB is a specific transcription factor that promotes joint inflammation as well as synovial hyperplasia in arthritis [11]. Noteworthy, activation of NF-κB in the inflamed synovium protected the cells against apoptosis, thereby providing a putative link between inflammation and hyperplasia [31]. Previous studies stated that NF-κB blockade significantly decreased arthritis severity in different animal models [11,31–34]. TNF-α plays a key role in the complex pathology of RA including increased proliferation of human synovial fibroblasts which is the key factor in the invasion of the joint spaces, resulting in destruction of connective tissues and subchondral bone [35].

Disability and joint destruction is the characteristic hallmark of RA due to cartilage destruction and bone erosion [36]. Matrix metalloproteinases are the enzymes that degrade some collagen and all non-collagen matrix components causing cartilage damage while RANKL causes osteoclastogenesis and bone erosion [8,12,35]. High levels of MMP-1 and MMP-3 as well as up regulation of RANKL are predominant in synovial tissues of RA patients [28,35]. In this work, VFX caused a noticeable reduction in MMP-3 and RANKL. Such finding is linked to the fact that VFX diminished several key cytokines namely; IL-6, IL-17 and TNF-α which stimulate synovial fibroblasts to release RANKL and metalloproteinases including MMP-3 [8,12,13,28].

VEGF is an angiogenic growth factor regulating vascular permeability [35]. This angiogenic cytokine have been demonstrated to be highly expressed in inflamed synovium of RA patients [35] contributing to the chronic nature of the disease [37]. Thereby, strategies intervening angiogenesis may exert favorable effects on the progression of RA [35]. In the present investigation, VFX caused substantial decrease in VEGF expression eventually due to hampering IL-6, IL-17 and TNF-α as these cytokines synergistically intensify VEGF expression [9,37].

This study additionally revealed that VFX is endowed with antioxidant properties as shown by replenishing GSH content and decreasing MPO activity associated with substantial fall in MDA levels compared to arthritic group. This could be explained by the fact that IL-17 augments recruitment, infiltration and development of myeloid-derived suppressor cells (MDSCs) which are accused of ROS output [9]. Moreover, VFX inhibition of inflammatory cytokines may be responsible for halting ROS production. Former studies proved that VFX has antioxidant properties exerted by enhancing the antioxidant defenses besides attenuation of oxidative stress and lipid peroxidation as verified by the up-regulation of glutathione peroxidase and GSH as well as vitamins A and C levels [23,38].

## 5. Conclusion

This study authenticates the anti-arthritic potential of VFX in CFA-induced arthritis rats. Possibly, modulation of IL-6/STAT3/IL-17 axis may lend support for the anti-inflammatory and immunomodulatory effects of this serotonin norepinephrine reuptake inhibitor (SNRI). Further investigations should be carried out to approve this mechanism.

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## Competing interests

The authors declare that they have no competing interests.

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