Nerolidol: a potential approach in rheumatoid arthritis through reduction of TNF-α, IL-1β, IL-6, NF-kB, COX-2 and antioxidant effect in CFA-induced arthritic model

Shanila Akhter1 · Hafiz Muhammad Irfan1 · Alamgeer2 · Shah Jahan3 · Muhammad Shahzad4 · Muhammad Bilal Latif5

Received: 13 April 2021 / Accepted: 1 February 2022 / Published online: 25 February 2022
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2022

Abstract
Rheumatoid arthritis is primarily associated with inflammation and increased level of proinflammatory cytokines which are released by immune cells, macrophages or activation of arachidonic acid metabolism. The expression of these cytokines, oxidative free radicals and the activation of COX-2 enzymes are crucial targets for chronic inflammation. On the basis of established anti-inflammatory efficacy of nerolidol, the primary study was further appraised to determine its approach against Freund’s complete adjuvant (CFA) rheumatoid model. Arthritis was induced by inoculation of 0.1 mL CFA injection into the left hind footpad of rats. Anti-arthritic potential of nerolidol (at 200, 400 and 800 mg/kg doses) was assessed by measuring the paw volume, body weight, serum analysis, histopathological and radiographs of ankle joints. Expressions of cytokine’s panels such as IL-10, IL-4, COX-2, NF-kB, TNF-α, IL-6, PGE-2 and IL-1β were determined by real-time qPCR. Antioxidant enzyme analyses were conducted by measuring the SOD, POD and catalase activity from serum and equated with arthritic control group. Nerolidol prevented body weight loss, stabilized biochemical and haematological homeostasis and significantly reduced the paw volume. Furthermore, X-ray and histopathological assessment of ankle joints showed an improvement in the joint structure of rats treated with nerolidol. Besides that, overexpression of gene pointers like TNF-α, IL-1β, IL-6, NF-kB, PGE-2 and COX-2 in CFA-treated control rats were also reversed with nerolidol. This anti-arthritic mechanism was further supported by the increased level of IL-10, IL-4 and serum antioxidant activity. The present findings demonstrate that nerolidol reduced adjuvant arthritis by downregulating the proinflammatory cytokines and upregulating the aforementioned anti-inflammatory cytokines and may be used as a therapeutic substance for the management of human rheumatoid arthritis.

Keywords Chronic inflammation · Cytokines · Interleukins · Rheumatoid arthritis · RT-qPCR

Introduction
Cytokines are involved in many biological processes including activation, differentiation and growth of cells, as well as inflammation (Feldmann et al. 1996). Chronic inflammation also allies with dysfunctional effects of adoptive and innate immune responses that lead to further induction of cytokines (Agonia et al. 2020). Arthritis is an autoimmune inflammatory syndrome that disturbs 1% of the whole population and it is two to three times more abundant in women than men (Hegen et al. 2008). The augmented level of proinflammatory cytokines such as IL-1β, TNF-α, NF-kB and interleukins-6 in the acute phase ultimately leads towards bone deformity (Arya et al. 2011). Apart from extended level of proinflammatory cytokines, increased level of oxidative strain is
also an important factor that damages the joints during the pathogenesis of RA. These factors increase the production of inflammatory cells, especially neutrophils and macrophages, accelerate the release of reactive oxygen species in synovial fluid, and cause further tissue destruction (Ulta et al. 2018). Phagocytic macrophages and granulocytes produce hydrogen peroxide and superoxide in excessive amounts, which degrade lipids by a process known as lipid peroxidation (Biemond et al. 1984). Antioxidants defend the cells against oxidative stress through the inhibition of reactive oxygen species formation in targets such as deoxyribonucleic acid, lipids and protein that are linked with diseases including RA, diabetes, cancer and Alzheimer (De Carvalho et al. 2018). Glutathione peroxidase, catalase and superoxide dismutase are the enzymes that catalyse hydrogen peroxides and hydroperoxides into harmless molecules including metal ion binding proteins such as caeruloplasmin and transferrin (that chelates, sesquters iron) and prevent the production of free radicals (Ighodaro and Akinloye 2018).

Conventional treatment of rheumatoid arthritis includes NSAIDs, corticosteroids, and DMARDs that are aimed to decrease joint inflammation and pain, but are not purely anti-arthritic because they do not reduce the B cells and T cells-mediated responses (Agarwal 2010). So there is a need to introduce novel herbal compounds which are most effective and economical against such responses. Phytoconstituents derived from plants that inhibit the expression of cytokines have prospective use against arthritis. Henceforth, efforts should be organized to seek out novel beneficial and efficient phytoconstituents which can be used for long-standing treatment of RA (Arya et al. 2011).

Nerolidol is derived from Peperomia serpens (Da Silva et al. 2006). Despite that, P. serpens was used to treat pain and inflammation and reduce the expression of IL-1β and TNF-α in mouse model for pain (Fonsêca et al. 2016). Nano-encapsulated nerolidol has also been evaluated for its anti-inflammatory role in zymogen-induced arthritis in mice and they have also shown the gastro-protective effect of nerolidol against ulcer (Trindade et al. 2020). The antioxidant effect of nerolidol on the hippocampus of mice shows that nerolidol is beneficial against oxidative stress (Neto et al. 2013). Therefore, the aim of the current investigation was to appraise the effect of nerolidol in a standard model of arthritis (chronic immunological Freund’s complete adjuvant rheumatoid arthritis). The study was also intended to support the possible mechanism of action of nerolidol at molecular levels and to discover its efficacy against joint infection.

## Materials and methods

### Drugs and chemicals

The drugs and chemicals included nerolidol (Sigma-Aldrich, USA), CFA (Sigma-Aldrich, USA), TRIzol solution, cDNA synthesis kit (Gene Direx, USA), forward/reverse primers (Gene Direx, USA), Cybergreen, deionized water, ethanol, and chloroform.

### Animals

Sprague–Dawley rats (150–250 g) of either sex were used for the investigational procedure. Animals were housed in the animal house at the College of Pharmacy, University of Sargodha with recommended housing conditions. All animals were fed a standard diet and water and were controlled by following the guidance in accordance with the National Research Council. All tests were approved by the animal ethics and review committee at the University of Sargodha (Approval NO. SU/Pharm/Animal Ethics Approval/2019/215).

### Complete Freund’s adjuvant-induced rheumatoid arthritis model

Animals were separated randomly into six different groups (n = 5). The first group (normal control group) and the second group (arthritic control group) received 2% Tween 80 (3 mL/kg). The third group (standard group) was given naproxen 20 mg/kg, while fourth, fifth and sixth group served as treatment groups that received 200, 400 and 800 mg/kg of nerolidol, respectively, for 28 days. Arthritis was induced by inoculation of 0.1 mL of CFA injection (containing 1 mg/mL of heat-killed M. tuberculosis in 0.15 mL mono-oleate and 0.85 mL paraffin oil) into the left hind footpad of each rat except normal control group. The day of the CFA shot served as day 0. Arthritis in all groups was evaluated by recording various factors (Mahdi et al. 2018).

### Evaluations of arthritis from body weights and paw volume of rats

During the period of treatments, the body weight of each rat was observed every 7th day. Paw size/oedema was measured using digital plethysmometer. Percent inhibition in paw oedema/size was calculated by this equation:

\[
\text{Percent inhibition} = \frac{\text{paw volume of arthritic control} - \text{paw volume of treatment}}{\text{paw volume of control}} \times 100.
\]
Assessment of arthritis from serum and blood

On day 28, all rats were killed and through cardiac puncture blood was collected for the assessment of biochemical and haematological markers such as WBCs, RBCs, HB, C-reactive proteins, RF, ESR, platelets, ALP, SGOT, SGPT, creatinine and urea. These tests were performed at the diagnostic center, University of Sargodha (Hassan et al., 2019).

Estimation of mRNA expression levels of TNF-α, IL-1β, IL-6, COX-2, IL-4, NF-KB and IL-10

The collected blood samples were used for consideration of mRNA factors such as TNF-α, IL-1β, IL-6, COX-2, NF-kβ, IL-4, and IL-10. TRIzol method was used for the extraction of total RNA from blood. According to this method, into 500 µL of blood, 700 µL of TRIzol solution was added. It was mixed gently and incubated it for 10 min. Then 200 µL of chloroform was added into it, shaken thoroughly and placed in the centrifuge machine for 15 min at 12,000 rpm and 4 °C. The aqueous layer was taken and then 500 µL of isopropanol added and again mixed vigorously. Samples were incubated for 10 min and retained in the centrifuge machine at 12,000 rpm and 4 °C for 15 min. The supernatant solution was discarded and the RNA pellet was washed with absolute ethanol. The RNA pellet was air dried and 30 µL of purified water added and quantified with a Nanodrop reader. After this, cDNA was synthesized by following the kit manufacturer’s procedure (Gene-Direx). In brief, 1 µL of RNA solution, 1 µL of oligo (dT) 20, 1 µL of dNTP mix and then RNA-free water were added into it. The reaction mixture was heated at 65 °C for 3–5 min, spun and promptly in a freezer. Then 4 µL of first strand buffer, 1 µL DTT, 1 µL of Script RTase and were added and finally made up to a volume of 20 µL. It was incubated for 30–60 min at 50 °C and the enzyme was inactivated at 70 °C for 15 min.

Real-time quantitative PCR was used to intensify and quantify the reaction by using the Bio-Rad scheme in the Pharmacology department, University of Health Sciences, Lahore. Afterwards, templates of cDNA were mixed with qPCR master mix and the specific primers of genes and nuclease-free water were added, and then placed in a thermal cycler for 45 cycles with denaturation temperature at 95 °C, annealing at 56 °C, extension at 72 °C and termination of the reaction at 72 °C. Various markers of genes were selected from Ensemble Genome Browser for determination of specific gene primers physically by using Input primer 3 (v. 0.4.0.) which is an available online software. The sequences of primers are provided in Table 1 (Shabbir et al., 2016; Lim et al., 2017).

ELISA (enzyme-linked immunosorbent assay) for prostaglandin E2

ELISA test was performed for quantitative identification of rat prostaglandin E2 in serum samples according to the kit manufacturer’s procedures (Rat Prostaglandin E2 ELISA kit, Bio-assay Technology laboratory having Cat No. E0504Ra, standard curve range = 0.05–15 ng/mL, size = 96 wells, sensitivity = 0.026 ng/mL). By adding an acidic solution, the reaction was terminated and absorbance was measured at 450 nm (microplate reader with 450 ± 10 nm).

Estimation of peroxidase antioxidant enzyme activity

Peroxidase activity was measured by determining its capability to decrease hydrogen peroxide at wavelength 470 nm (Zia et al., 2011). 0.06 mL of enzyme extract was added in 3 mL of buffer substrate solution that comprised 47 mL of phosphate buffer (0.2 M), 0.7 mL of guaiacol and 0.32 mL of H2O2. After 3 min of enzyme reaction, the optical density was measured at 470 nm in a spectrophotometer against blank (phosphate buffer guaiacol). Peroxidase activity was measured by using the undermentioned formula:

\[
\text{Peroxidase activity (U/mL) = } \frac{\Delta A}{26.6 \times 0.06 \times 3.0}.
\]

Table 1 Forward–reverse primers sequences with respect to gene markers used in real-time PCR

| Gene markers | Forward reverse | sequences | Base pair (amplified band) |
|--------------|-----------------|-----------|--------------------------|
| IL-1β        | Forward reverse | 5’-GCTGTCCAGATGAGAGCATC-3’ 5’-GTCAGACAGCAGGACATT-3’ | 293 |
| IL-6         | Forward reverse | 5’-AGACCTCGACAGCAGTGGT-3’ 5’-CTGACAGTGCATCTGCCTG-3’ | 233 |
| COX-2        | Forward reverse | 5’-GCTTCCCTTGCCAGACCTT-3’ 5’-GCTTTGACTGTTGGAGGAT-3’ | 210 |
| TNF-α        | Forward reverse | 5’-AGGACACTCATGACAGCGGAAA-3’ 5’-GGGCCATGGAACCTGTAGGA-3’ | 234 |
| NF-Kβ        | Forward reverse | 5’-GCAACTTCCTTGTCGACCTA-3’ 5’-CTGCTCTGAGCGTTGACTT-3’ | 203 |
| IL-4         | Forward reverse | 5’-CACCATTGCTGCTCAGCTGTTT-3’ 5’-CCTGCAATGACGCTCTTGT-3’ | 195 |
| IL-10        | Forward reverse | 5’-GCCAGAACATCAAGGAGCAT-3’ 5’-CGTAGGCCTCTATGCCGTT-3’ | 210 |
A = absorbance at 470 nm, 26.6 = extinction coefficient of guaiacol (Mm⁻¹ cm⁻¹), 0.06 = volume of enzyme extract (mL), and 3.0 = volume of phosphate buffer (mL).

**Analysis of catalase antioxidant enzyme activity**

Assay was performed for determining the catalase activity of antioxidant enzymes and the ability to reduce H₂O₂ at 240 nm was checked. The reaction mixture comprised 3.0 mL of K₂PO₄ buffer (50 mM, pH 7), 0.1 mL of hydrogen peroxide (30 mM) and 0.1 mL of enzyme extract. Absorbance was observed after 3 min of reaction time at 240 nm (Chance and Maehly, 1955). Catalase activity was determined by the formula:

\[
\text{Catalase activity} = \frac{\Delta A_3}{0.04 \times 0.01} \times 3.
\]

\[A_3 = \text{absorbance at 240 nm, and 0.04 = extinction coefficient for } \text{H}_2\text{O}_2 \text{ (M}^{-1}\text{CM}^{-1})\].

**Evaluation of superoxide dismutase**

The SOD bustle was performed to check its ability to inhibit the photoreduction of nitro-blue tetrazolium. This test was performed by adopting the protocol with slight modification as discussed by Worthington 1988. The assay mixture contained 1 mL of 0.0067 M potassium phosphate buffer (7.8pH), 0.05 mL extract of enzyme and 0.016 mL of 0.012 mM solution of riboflavin. The reaction mixture was incubated in a light box for 12 min. After that, 0.067 mL of EDTA/NaCN solution and 0.033 mL of nitro-blue tetrazolium solution was added into the reaction mix. After 30 s of reaction time, the absorbance was observed against blank through a spectrophotometer at a wavelength of 560 nm. The activity of SOD was calculated by the formula:

\[
\text{Percent inhibition} = \frac{A_\text{(Blank)} - A_\text{(Sample)}}{A_\text{(Blank)}} \times 100.
\]

**Histopathological assessment of ankle joints**

At the end of the treatment, ankle joints of the arthritic control and treated rat paws were collected and immersed in 10% solution of formalin for the assessment of histopathology of the joints (Shabbir et al., 2014).

**Radiographic assessment of joints**

The legs were removed at the knee joints and subjected to radiographic assessment with a computerized radiographic system (Toshiba 630 M) (Uttra and Hasan, 2017).

---

**Results**

**Effect of nerolidol on morphological deviations and paw volume**

The results specified in Fig. 1 describe that the treated groups revealed a significant increase \( p < 0.01 \) in paw oedema on day 1 but paw volume decreased significantly \( p < 0.001 \) throughout the period. A significant reduction (75.99, 79.97, and 81.54%) in paw volume was detected at 28 days of treatment for the dose 200, 400 and 800 mg/kg respectively. Moreover, primary marks of chronic inflammation such as arthralgia, redness, immobility and swelling of joints were substantially low in rats treated with nerolidol and naproxen sodium, respectively, compared to the arthritic control. There was a noteworthy reduction in body weight from 207.00 ± 13.20 to 152.60 ± 15.29 g that was observed in arthritic rats during the treatment period. However, significant upsurge in weight was observed in rats treated with nerolidol at 200, 400 and 800 mg/kg on the 28th day of treatment. Major difference was found in weight gain when the dose was doubled from 200 mg/kg as described in Fig. 2 on day 7.

**Biochemical and haematological parameters**

Haematologic alteration was observed in injected CFA arthritic control rats: among them, significant decline in haemoglobin and RBCs count, increase in value of platelets, WBCs and ESR, ALP, SGPT, SGOT, creatinine, urea and C-reactive proteins were documented. Oral administration of nerolidol significantly increased the RBCs and haemoglobin, but noticeable reduction in the liver enzymes, WBCs, C-reactive proteins, ESR, platelets, creatinine and urea was observed equated to the arthritic control rats as revealed
Nerolidol inhibits the mRNA expression level of TNF-α, IL-6, IL-1β, COX-2, and NF-kB, and induces the expression of IL-10 and IL-4

Nerolidol is a potential approach in rheumatoid arthritis through reduction of TNF-α, IL-1β, COX-2, and NF-kB and induction of IL-10 and IL-4. These results showed protective effects against the signs of nephrotoxicity and hepatotoxicity at doses of 200, 400 and 800 mg/kg. Moreover, nerolidol significantly decreased the RF values in CFA-injected rats.

Effect of nerolidol on prostaglandin E₂

The animals treated with nerolidol at three doses (p < 0.001) revealed a significant decrease in the level of PGE₂: 0.77 ± 0.04, 0.57 ± 0.02 and 0.74 ± 0.03, respectively, as compared to diseased animals (1.18 ± 0.03). Maximum effect was achieved at 400 mg/kg (51.6%) of nerolidol in contrast to other doses (Fig. 4) and at medial dose the effect was 14.6% more than the standard.

Table 2: Effect of oral administration of nerolidol on biochemical and haematological parameters in CFA-induced model

| Biochemical and haematological parameters | Arthritic control | Normal control (20 mg/kg) | Naproxen (200 mg/kg) | Nerolidol (200 mg/kg) | Nerolidol (400 mg/kg) | Nerolidol (800 mg/kg) |
|------------------------------------------|-------------------|---------------------------|----------------------|-----------------------|-----------------------|-----------------------|
| SGOT (U/L)                               | 149.61 ± 0.26     | 110.83 ± 0.09***          | 125.24 ± 0.36***     | 134.62 ± 0.32***     | 127.49 ± 0.26***     | 120.84 ± 0.10***     |
| SGPT (U/L)                               | 49.95 ± 0.04      | 21.05 ± 0.02***           | 35.03 ± 0.02***      | 37.33 ± 0.33***      | 33.72 ± 0.31***      | 32.29 ± 0.28***      |
| Urea (mg/dL)                             | 37.66 ± 0.32      | 18.58 ± 0.26***           | 27.89 ± 0.04***      | 28.36 ± 0.31***      | 26.42 ± 0.21***      | 22.95 ± 0.42***      |
| ALP (U/L)                                | 312.32 ± 0.27     | 158.58 ± 0.25***          | 203.00 ± 1.00***     | 201.65 ± 0.88***     | 195.44 ± 2.35***     | 192.07 ± 0.70***     |
| CREAT (mg/dL)                            | 0.99 ± 0.00       | 0.46 ± 0.00***            | 0.58 ± 0.00***       | 0.63 ± 0.02***       | 0.57 ± 0.00***       | 0.51 ± 0.00***       |
| C-RP (mg/dL)                             | 39.69 ± 0.12      | 3.91 ± 0.07***            | 12.48 ± 0.49***      | 13.94 ± 0.03***      | 10.33 ± 0.32***      | 8.98 ± 0.54***       |
| RF (IU/UL)                               | 35.75 ± 1.2       | 6.38 ± 0.41***            | 9.78 ± 0.11***       | 11.32 ± 0.65***      | 9.62 ± 0.03***       | 8.61 ± 0.31***       |
| ESR (mm/h)                               | 9.58 ± 0.29       | 3.55 ± 0.04***            | 4.88 ± 0.00***       | 4.98 ± 0.00***       | 4.82 ± 0.01***       | 3.96 ± 0.03***       |
| PLAT (10^⁹/UL)                           | 1321.08 ± 0.30    | 941.21 ± 0.33***          | 963.22 ± 3.18***     | 983.66 ± 3.18***     | 963.67 ± 1.20***     | 955.36 ± 0.31***     |
| WBCs (10^³/UL)                           | 14.03 ± 0.22      | 8.08 ± 0.48***            | 12.27 ± 0.64***      | 10.97 ± 0.45***      | 8.33 ± 0.21***       | 6.96 ± 0.29***       |
| RBCs (10^⁶/UL)                           | 5.25 ± 0.25       | 9.30 ± 0.23***            | 7.10 ± 0.06***       | 7.58 ± 0.22***       | 8.10 ± 0.07***       | 8.48 ± 0.33***       |
| Hb (g/dL)                                | 9.13 ± 0.09       | 14.76 ± 0.06***           | 11.69 ± 0.30***      | 11.05 ± 0.03***      | 12.90 ± 0.03***      | 14.26 ± 0.25***      |

Values are expressed as mean ± SEM (n=5) followed by one-way ANOVA with Dunnett’s posttest

***p < 0.001, the values were significant as compared to the arthritic control
Effect of nerolidol on antioxidant (SOD, POD and CAT) activities

Oral administration of nerolidol at the doses of 200, 400 and 800 mg/kg increased the antioxidant enzyme activity of SOD by 28.76, 33.66 and 44.76%, respectively, and induced the protective defence mechanism inside body. Catalase is also an alternative enzyme that works together with SOD and POD through the antioxidant defence system of enzymes that break down the H$_2$O$_2$ into O$_2$ and water that protects the cell from O$_2$ toxicity and lipid peroxidation. Nerolidol at 800 mg/kg dose showed significant increase ($p < 0.001$) in the enzyme activity of catalase (47.41%). Similarly, peroxidase activity was 67.17% at 200 mg dose of nerolidol for 28 days as compared to the arthritic control (Table 3).

Fig. 3 Expression of cytokines assessed by RT-qPCR treated with nerolidol for 28 days in CFA-induced model. Analysed by one-way ANOVA with Dunnett’s posttest. ***$p<0.001$, **$p<0.01$ and ns is non-significant. Key: A (TNF-α); B (IL-6); C (IL-1β); D (COX-2); E (IL-4); F (IL-10); G (NF-Kβ)
Effect of nerolidol on histopathological assessment of rat’s ankle joints

Evaluation of the histopathology of the ankle joint was done on day 28 of treatment. Histopathology of normal control rats showed that they exhibited normal joint space with intact articular cartilage and synovial tissues having no signs of inflammation with compact arrangements of cells. Ankle joint of arthritic control rats showed noticeable synovial lining, distinct propagation of synoviocytes and incursion of inflammatory cells with granulomatous and pannus development. Similarly, the erosive changes in bone and cartilage as well as deposition of collagen fibers were also observed in arthritic control groups (Fig. 5). However, naproxen (20 mg/kg) showed mild incursion of inflammatory cells, cartilage destruction, and thickening of synovial intergalactic space and decreased pannus development. Also, oral administration of nerolidol at 800 mg/kg showed considerable

**Table 3** Effect of nerolidol and other treatments given for 28 days on antioxidant enzyme activities

| Treatment groups   | POD (U/mg protein) | SOD (U/mg protein) | CAT (U/mg protein) |
|--------------------|---------------------|--------------------|--------------------|
| Arthritic rats     | 3.45 ± 0.14         | 9.91 ± 0.21        | 42.18 ± 1.68       |
| Normal rats        | 15.26 ± 0.20 ***    | 20.00 ± 0.00 ***   | 95.33 ± 1.76 ***   |
| Naproxen (20 mg/kg)| 13.83 ± 0.02 ***    | 14.76 ± 0.10 ***   | 67.07 ± 0.41 ***   |
| Nerolidol (200 mg/kg) | 10.51 ± 0.07 ***  | 13.91 ± 0.06 ***   | 63.73 ± 0.96 ***   |
| Nerolidol (400 mg/kg) | 12.95 ± 0.03 *** | 14.94 ± 0.03 ***   | 73.91 ± 1.03 ***   |
| Nerolidol (800 mg/kg) | 14.05 ± 0.03 *** | 17.94 ± 0.03 ***   | 80.21 ± 0.28 ***   |

Values are articulated as mean ± SEM followed by one-way ANOVA with Dunnett’s posttest (**p < 0.001)

**Fig. 4** Effect of daily oral administration of nerolidol (200, 400 and 800 mg/kg) and naproxen for 28 days on prostaglandin E2. The results were analysed by using mean ± SEM, followed by one-way ANOVA (n = 5), where ***p < 0.001

**Fig. 5** Effect of oral administration of nerolidol for 28 days on histological variations in Freund’s induced arthritis. Whereas A represents slide of normal rat possessing no inflammatory cells, B represents histology of arthritic control rat showing vessel invasions and fibrous deposition, while C, D, E and F represent slides of rats treated with naproxen and different doses of nerolidol (200, 400 and 800 mg/kg) showing reduction in inflammatory cells
fortification against proliferation of vascular lesions, limited space between joints, cartilage destruction and small number of inflammatory cells with no pannus development that resembled very much the normal architecture of joints. However, 400 mg/kg dose of nerolidol showed moderate cartilage destruction with little cellular invasion and pannus formation. Alternatively, 200 mg/kg nerolidol-treated rats exhibited little reduction in cartilage with minor invasion of inflammatory cells and no formation of pannus.

Radiographic examination

The radiographic examination is useful for the analysis of rheumatoid arthritis for estimation of severity and progression of disease. It has been found that reduction in joint space results in loss of cartilage which initiates a variety of abnormal mechanisms. X-rays of normal rats showed normal morphology and architecture of cartilage with no swelling around the joints, while X-ray analyses of arthritic control rats showed that they possessed narrowing of joint space, severe swelling of tissues and bone erosion. In contrast, rats treated with nerolidol 800 mg/kg exhibited good inhibition of tissue inflammation, bone injury and narrowing of joint space and joint distortion. Likewise, rats treated with nerolidol 400 mg/kg showed moderate protection against swelling, bone erosion and joint deformity. Similarly, moderate changes occurred in soft tissues of joints with distinct reduction in joint space also observed in naproxen-treated rats as illustrated in Fig. 6.

Discussion

In the present study, we have shown for the first time that nerolidol alleviated CFA arthritis in rat’s model of arthritis with pathological structures similar to those of human rheumatism (Lin et al. 2013). Rheumatism is a chronic demagogic autoimmune disorder that targets the cartilage, bones and synovial membrane (McInnes and Schett 2007). However, the exact aetiopathogenesis is not yet known, but significant validations have confirmed that liberal demolition of bone and cartilage in rheumatism results from synovial neovascularization, increased manifestation of proinflammatory cytokines, osteoclast interceded bone desorption and suspension of cartilage articular matrix mediated by proteinase (Liu et al. 2013). After CFA injection inoculation,

![Fig. 6 Pictorial representation of X-ray examination of treated rats versus arthritic control rat. Whereas A represents ankle joint of normal rat with normal morphology and architecture of cartilage, B represents ankle joint of arthritic control rat showing bone erosion and narrowing of joint space, C represents ankle joint of rat treated with naproxen, while D, E and F represent ankle joints of rats treated with different doses of nerolidol (200, 400 and 800 mg/kg) exhibiting inhibition of space narrowing and tissue swellings](image)
inflammatory reactions begin within a few days, as second-
yary lesions come after primary lesions within 2 weeks (Alamgeer et al. 2017). CFA comprised heat-killed M. tuberculosis dissolved in liquefied paraffin that activates the cell arbitrated immunity, thus accelerating the creation of anti-
bodies (Kim et al. 2016). Mycobacterium is associated in
the formation of oedema that may be due to the invasion of
extracellular fluid and protein debris at the site of inflam-
mation. It comprises three phases (induction phase, early
synovitis and late synovitis) (Bose et al. 2014; Woode et al.
2009). So, nerolidol significantly reduced the paw volume
as compared to arthritic paw volume of rats. It is possible
that the drug prevented the invasion of fluid into the joints
or may be due to its anti-inflammatory effect together with
immunomodulatory property by activating the MAPKs path-
way (Valdivieso-Ugarte et al. 2019; De Càssia Da Silveira
et al. 2015).

Rheumatoid cachexia was observed in rheumatism char-
acterized by appetite and weight loss that is due to increased
production of cytokines, which accelerates the proteolysis
and resting metabolism (Adeneye et al. 2014). Proinflam-
atory cytokines TNF-α, IL-6 and IL-1β are key elements
of synovitis and also stimulate the NF-kB and cause the
proteolysis of muscles by activating the proteasome path-
ways. Overexpression of these proinflammatory cytokines
induces anorexia and ultimately muscle loss. Besides that,
consumption of glucocorticoids as pharmacological symp-
tomatic treatment of RA might aggravate the rheumatoid
cachexia (Masuko 2014). The observed cachexia may also
be due to muscle proteolysis induced by lysosomal protease
that is mediated by decreased absorption of 14C-glucose and
C-leucine as well as prostaglandin-E2 in the intestines
of rats. Anti-inflammatory medications have the capacity
to recover the damage caused by the disease (Alamgeer et al.
2017). Similarly, they also decrease the expression of
proinflammatory cytokines and also restore the intesti-
nal absorption of rats (Ahsan et al. 2021). Additionally, in
CFA-induced arthritis, we also evaluated the biochemical
and haematological tests. Anaemia is the most common hae-
matological abnormality in rheumatoid arthritis. From the
findings of this study, it was apparent that decrease in hae-
moglobin level and RBCs count in arthritic rats signify that
anaemia may be due to the destruction of premature RBCs,
decreased level of erythropoietin and decreased iron loading
in the synovial tissue of the reticuloendothelial system and
synovial tissues (Alamgeer et al. 2017). Nerolidol caused a
notable increase in haemoglobin and RBCs, possibly due
to the decreased manifestation of NF-kB. So it normalized
the invasion of inflammatory cells in the synovial fluid and
ultimately inhibited the cascade of inflammation (Iqubal
et al. 2019). Besides, the level of platelets and WBCs were
augmented in arthritic rats due to the excretion of TNF-α and
IL-6 (Fig. 5A, 5B), as these cytokines initiate acute phase
reaction in rheumatoid arthritis. Though, nerolidol signifi-
cantly decreased the level of platelets and WBCs in rats by
preventing the manifestation of IL-6 and TNF-α, it might be
due to its immunomodulatory effect.

The overexpression of the proinflammatory cytokine
causes the accumulation and release of ROS. When the pro-
duction of reactive species is higher than that of the natural
antioxidant defence system, oxidative stress destroys nor-
mal physiological function of the DNA, proteins and lipids
(Valdivieso-Ugarte et al. 2019). ESR and C-reactive protein
are the primary markers for the early analysis of RA. The
augmentation of C-RP and ESR in the arthritic rats showed
the presence of infective proteins in the blood, while these
levels were considerably reduced (p < 0.001) in rats treated
with naproxen and nerolidol, since its values relate with the
radiological progression of disease (Babu et al., 2014). Treat-
ment with nerolidol prevented the elevated reactions of
the acute phase, when compared with arthritic control rats.
A striking decrease in RF values in the treatment groups
ensured the protective effect of nerolidol against arthritis.
The factor RF is formed against the Fc portion of the autoan-
tibody and initiates the immune cascade towards the pro-
gression of RA. Moreover, excessive concentration of serum
ALP in diseased control group may be due to increased bone
erosion and integrity of lysosomal enzymes (Chakraborty
et al., 2010). However, administration of different doses of
nerolidol to respective groups significantly reduced the ALP,
inhibited the atypical rise in SGPT and SGOT that might be
due to reduction in bone loss and improved the steadiness
of lysosomes.

Proliferating synovial cells, T lymphocytes and mac-
rophages play a crucial role in the progression of arthritis
(Goronzy and Weyand 2001). Proinflammatory cytokines
are produced by macrophages and stimulated T cells that
are involved in the initiation and progression of rheuma-
toid arthritis (Vandooren et al. 2009). T cell-mediated
immune reaction activates the discharge of proinflammatory
cytokines and accelerates the formation of antibodies, which
lead to joint destruction. Overexpression of these cytokines
causes the irreparable proliferation of tissues, tissue eradica-
tion, bone loss and automatic cell death (Zou et al. 2013).
Hence, potential antidotes against these cytokines have the
ability to protect the cells from inflammation (Shin et al.
2016). Therefore, in the current investigation, the effects of
nerolidol on the expression level of proinflammatory (TNF-
α, IL-6, IL-1β), inflammatory cytokines (NF-kB, COX-2)
and anti-inflammatory cytokines (IL-4, IL-10) were studied
by using RT-qPCR. It has been shown that IL-1β induces
the formation of (MMPs) matrix metalloproteinase and
osteoclast and ultimately bone erosions (Barksby et al.
2007). In the manifestation of soluble IL-6R, IL-6 induces
the formation of TIMPs in synovial fibroblast and cultured
chondrocytes. The protective catabolic response produced in
tissues is important for extracellular matrix turnover (Silacci et al., 1998). From the present findings, it was suggested that the anticipatory effect of nerolidol on joint demoli-
tion was arbitrated by the prevention of induction of IL-6
induced RANKL and formation of MMPs via suppression of
IL-6, followed by osteoclastogenesis. Therefore, therapeutic
agents that reduced IL-6, IL-1β and TNF-α categorized a
major intervention in the treatment of RA. Augmented level
of these proinflammatory cytokines was observed in arthritic
control rats; however, significant (p < 0.001) decrease in the
expression of these cytokines was detected in rats treated
with nerolidol at all doses as shown in Fig. (3B, 3C), which
suggested its anti-arthritic and anti-inflammatory role.
NF-kβ is involved in the activation and differentiation of
osteoclasts, which causes the development of Th1 response
and bone resorption. It has been already been shown that
NF-kβ controls the manifestations of TNF-α; later, it works
as a potent inducer for the stimulation of NF-kβ (Shabbir
et al., 2016). The existing study elucidates that increased
level of NF-kβ in diseased rats was significantly inhibited
by nerolidol on the last day of treatment.

The level of COX-2 is increased at the site of injection
and upregulation of the formation of prostaglandin-E2 medi-
ates angiogenesis, vasodilation, extravasation of fluid and
vascularization in the synovial membrane (Shabbir et al.,
2016). Overproduction of inflammatory cytokines causes
the increased expression of PGE2. This augmented level of
PGE2 accelerates the differentiations of osteoclast and
generates the degrading enzymes, and hence stimulates the
bone erosion, vasodilation and migration of leukocytes at
the site of inflammation. So, any substance that prevents
the generation of the aforementioned mediators carries great
importance in the management of rheumatism (Manan et al.,
2020). In the current study, increased expression of COX-2
and PGE-2 were noted in diseased controlled rats; however,
significant decrease in COX-2 and prostaglandin-E2 were
observed in rats treated with nerolidol. Hence, we suggest
that nerolidol protected the animals from joint inflamma-
tion, may be due to decreased production of prostaglandins
and significant inhibition of COX-2 manifestations. Conse-
quently, prevention of arachidonic acid metabolism might be
another mechanism of action of nerolidol possessing anti-
arthritic activity.

Moreover, IL-4 is an anti-inflammatory cytokine that
maintains the production of Th2 cells and inhibits the auto-
mune reaction mediated by Th1 cells (Schulze-Koops and
Kalden 2001). IL-10 also has capability to inhibit the cel-
lar defence mechanism by inactivating the macrophages
in response and modify the synovitis of rheumatoid arthritis
(Bozkurt et al. 2006). In the progression of RA, IL-10 not
only inhibits the Th1-mediated immune response, but also
downregulates the function of antigen presenting cells and
protects the reliability of joint (Uttra et al. 2018) similarly
to that observed with the tested drug as shown in Fig. 3D.

In histopathological assessment, inflamed joints exhib-
ited continuous migration of polymorphonuclear leuko-
cytes, macrophages and lymphocytes into joints and syn-
ovium. They all produce inflammatory cytokines in the
synovial fluid and joints. Thus, inhibition of migration of
macrophages and leukocytes decreased the accumulation
of inflamed debris in joints and may have beneficial effect
for the protection of joints (Gao and Issekutz 1994, Wilder
et al., 1989). Our histopathological examination suggested
that the nerolidol-treated groups inhibited the migration
of these leukocytes and protected the joint from hyperpla-
sia, joint deformity, pannus formation and destruction of
cartilage as shown in Fig. 5. Moreover, X-ray examination
revealed that nerolidol showed significant protection
against the expansion of subordinate lesions, development
of bone desolation and destruction of cartilage in contrast
to arthritic control rats as shown in Fig. 6.

Free radicals and ROS have been stated as mediators
in the pathogenesis of RA and cause tissue destruction.
In rheumatism, the affected parts are penetrated by dif-
ferent cells such as dendritic cells, macrophages and neu-
trophils. These cells generate reactive oxygen species in
greater amount at the site of inflammation and overcome
the antioxidant enzyme defence system. So, they induce
the annihilation of the affected joints because during
phagocytosis superoxide anions are produced that activate
the NF-kβ-dependent manifestation of proinflammatory
cytokines through the second messenger system (Babu
et al., 2014). These reactive species cause the peroxidation
of lipids, enzyme inactivation, oxidation of proteins, DNA
damage and other changes in the cell organelles (Sghaier
et al. 2011); (Valdivieso-Ugarte et al. 2019). Endogenous
enzymes of antioxidant such as catalase protect them
from tissue damage caused by the reactive species. Cata-
lase is involved in lessening the level of hydrogen perox-
ide in synovial tissues. SOD is the antioxidant enzyme
that catalyses the superoxide into hydrogen peroxide and
oxygen free radical which is further catalysed by catalase
enzymes into water and oxygen molecules. In the current
study, nerolidol was able to upregulate the antioxidant
enzyme activity of POD, SOD and CAT and considerably
(p < 0.001) decreased lipid peroxidation in the synovial
fluid induced by the release of ROS. This increase in the
concentration of antioxidant enzymes in the synovial fluid
diminished the destruction of cartilage and tissue deform-
ity in the ankle joint of treated rats. Lipid peroxidation is
the process in which polyunsaturated fatty acids undergo
the oxidation process and cause functional abnormality in
the cell membrane. However, nerolidol decreased the lipid
peroxidation level acted as an in vivo antioxidant agent
and reduced oxidative stress.
Conclusion

In a nutshell, oral administration of nerolidol in arthritic rats significantly reduced the paw volume, regained body weight, normalized the altered biochemical and haematological parameters and also inhibited mRNA expression level of the above-mentioned cytokines. So the anti-arthritic effect of nerolidol might be due to its capability to decrease the level of NF-kB, COX-2, PGE2, TNF-α, IL-1β, and IL-6 and increase the level of IL-4, IL-10 and antioxidant enzyme activity. Hence, nerolidol may be recommended as a therapeutic substance for the treatment and management of severity of rheumatism for clinical trials.

Author contributions The study conceptualization was designed by HMI and A: methodology and research work was developed by Miss SA: manuscript draft was prepared by MBL, and PCR: analysis was performed by SJ and MS.

Funding This study was performed with financial support from Higher Education Commission, Govt. of Pakistan, PIN NO. 518-2MD5-113.

Availability of data and material All the data of this study is transparent.

Code availability Not applicable.

Declarations

Conflict of interest All authors declare that they do not have any known competing interest.

Ethical approval The experiments conducted on animals were performed according to animal ethics guidelines with approval NO. SU/ Pharm/Animal Ethics Approval/2019/215.

Consent to participate We, the authors, give consent as participants of this study.

Consent for publication All the contributing authors provide consent for the publication of this study in your journal.

References

Adeneye AA, Oregba AI, Ishola IO, Kalejaiye HA (2014) Evaluation of the anti-arthritic activity of the hydroethanolic leaf extract of Alchornea cordifolia in rats. Afri J Trade Compl Alter Med 11:402–410

Agarwal S (2010) Newer nutritional basis in the management of rheumatoid arthritis. Elect Phys 2:33–38

Agonia I, Couras J, Cunha A, Andrade AJ, Macedo J, Sousa-Pinto B (2020) IL-17, IL-21 and IL-22 polymorphisms in rheumatoid arthritis: a systematic review and meta-analysis. Cyt 125:813–825

Ahsan H, Irfan HM, Shahzad M, Asim MH, Akram M, Zafar MS (2021) Anti-rheumatic activity of pseudoephedrine (a substituted phenethylamine) in complete Freund’s adjuvant-induced arthritis rats by down regulating IL-1β, IL-6 and TNF-α as well as upregulating IL-4 and IL-10. Inflammopharmacol 29:1–10

Alameer N, S.G., Ultra, A.M., Qaiser, M.N. and Ahsan, H, (2017) Appraisal of anti-arthritic and nephroprotective potential of Curcuma reflexa. Pharm Bio 55(1):792–798

Arya V, Gupta VK, Kaur R (2011) A review on plants having anti-arthritic potential. Int J Pharm Sci Res 7(2):131–136

Babu NP, Saravanan S, Pandikumar P, Krishna KB, Raj MK, Ignacimuthu S (2014) Anti-inflammatory and anti-arthritic effects of 3-hydroxy-2-methoxy sodium butanoate from the leaves of Clerodendrum phlomidis Lf. Inflamm Res 63(2):127–138

Barksby HE, Lea SR, Preshaw PM, Taylor JJ (2007) The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 149(2):217–225

Biemond P, Swaak AJ, Koster JF (1984) Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid. Arthritis Rheum: J Am Col Rheumatol 27(7):760–765

Bose M, Chakraborty M, Bhattacharyya S, Bhattacharjee P, Mandal S, Kar M, Mishra R (2014) Suppression of NF-κ B p65 nuclear translocation and tumor necrosis factor-α by Pongamia pinnata seed extract in adjuvant-induced arthritis. J Immunotoxicol 11(3):222–230

Bozkurt FY, Ay ZY, Berker E, Tepe E, Akkus S (2006) Anti-inflammatory cytokines in gingival crevicular fluid in patients with periodontitis and rheumatoid arthritis: a preliminary report. Cytokine 35(3–4):180–185

Chakraborty M, Bhattacharyya S, Bhattacharjee P, Das R, Mishra R (2010) Prevention of the progression of adjuvant induced arthritis by oral supplementation of Indian fresh water mussel (Lamellidens marginalis) aqueous extract in experimental rats. J Ethnopharmacol 132(1):316–320

Chance B, Maehly AC (1955) [136] Assay of catalases and peroxidases. Elsv 2:764–775

De Silva ACM, Andrade EHA, Carreira LMM, Guimarães EF, Maia JGS (2006) Essential oil composition of Peperomia serpens (Sw) loud. J Essen Oil Res 18(3):269–271

De Carvalho RB, De Almeida AAC, Campelo NB, Lellis DROD, Nunes LCC (2018) Nerolidol and its pharmacological application in treating neurodegenerative diseases: a review. Recent Pat Biotech 12(3):158–168

De Cásia Da Silveira e Sá R, Andrade LN, De Sousa DP (2015) Sesquiterpenes from essential oils and anti-inflammatory activity. Natural Prod Commun 10(10):1767–1774

De Carvalho ACM, Andrade EHA, Carreira LMM, Guimarães EF, Maia JGS (2006) Essential oil composition of Peperomia serpens (Sw) loud. J Essen Oil Res 18(3):269–271

Dellmann M, Brennan FM, Maini RN (1996) Role of cytokines in rheumatoid arthritis. Annual Rev Immunol 14(1):397–440

Enigdemon P, Saha AI, Puri SK (2011) Apoptotic effect of curcumin in an animal model of rheumatoid arthritis. J Ethnopharmacol 134:310–314

Fonsêca DV, Salgado PR, de Carvalho FL, Salvadori MGS, Penha ARS, Leite FC, Borges CJS, Piuvezam MR, Pordeus LCDM, Sousa DP, Almeida RN (2016) Nerolidol exhibits antinociceptive and anti-inflammatory activity: involvement of the GABAergic system and proinflammatory cytokines. Fund Clin Pharma col 30(1):14–22

Gao JX, Issekutz AC (1994) The effect of ebselen on T-lymphocyte activation and leukocyte migration to arthritic joints and dermal inflammatory reactions in rats by down regulating IL-1β, IL-6 and TNF-α as well as upregulating IL-4 and IL-10. Inflammopharmacol 29:1–10

Gorontzy JJ, Weyand CM (2001) Thymic function and peripheral T-cell homeostasis in rheumatoid arthritis. Trends Immunol 22(5):251–255

Hassan UH, Shahzad M, Shabbir A, Jahan S, Saleem M, Bukhari IA, Assiri AM (2019) Amelioration of adjuvant induced arthritis in sprague dawley rats through modulation of inflammatory mediators by Ribes alpestre Decne. J Ethnopharmacol 235:460–471

Hegen M, Keith JC, Collins M, Nickerson-Nutter CL (2008) Utility of animal models for identification of potential therapeutics for rheumatoid arthritis. Annal Rheum Dis 67(11):505–515

Husen ZK, Ahmed M (2014) Anti-inflammatory activity of heartwood extract of Ziziphus nummularia in rat adjuvant arthritis model. Int J Drug Dev Res 6(2):519–528

Karki P, Paudel P, Thapa P (2014) Anti-inflammatory activity of plant extracts in experimental animal models. Asia Pac J Trop Biomed 4(10):785–791

Karthikeyan MG, Gourishankar S, Thyagarajan KS, Anamalai S, Vasantachandra K (2021) Anti-inflammatory and antioxidant activities of lipophilic extract of Ribes alpestre Decne in carrageenan induced paw edema in rats. J Basic Clin Appl Res 9:183–189

Landaia L, Hernández E, Cruz I, García S, Fernández-Fernández E, Rodríguez-Pérez LM, Carballedo J (2019) Anti-inflammatory activity of aqueous extracts of Coriandrum sativum L. and Cuminum cyminum L. as an effective therapeutic strategy for chronic inflammatory diseases. J Ethnopharmacol 227:387–394

Léonard N, Couturier D, Schmitz A, Martin M, Peeters M, Fedorak RN, Lefebvre V (2016) Peptide-based therapy for inflammatory bowel disease: a role for NF-κB inhibitors? Curr Opin Investig Drugs 17(10):603–613

Li Y, Guo H, Fan Q, Guo Y, Wang L, Zhao X, Wang J, Meng X (2018) The anti-inflammatory activity of Euphorbia pulcherrima. J Ethnopharmacol 227:237–243

Lokhande S, Patil SV (2018) Anti-inflammatory activity of the aqueous leaf extract of Alchornea cordifolia. J Ethnopharmacol 215:139–145

Narang Y, Bedi SP (2015) Anti-inflammatory activity of methanolic extract of Ziziphus nummularia in adjuvant arthritis. J Adv Pharm Technol Res 5(3):128–133

Niazi NA, Ehsan M, Shuaib M, Shahzad M, Ahsan H (2017) Anti-arthritic potential of Curcuma reflexa. Int J Pharm Sci Rev Res 7(2):131–136

Nia K, Mirzaei MA, Nezhad M, Gholami A (2016) Protective effects of nerolidol against alloxan-induced diabetes in rats. Biomed Pharmacother 85:80–85

Oliveira AM, Silva JM, Silva SA, De Oliveira AL, Marques SI, Aveiro JG, Ferrão LC, Lopes WC (2017) Antioxidant and anti-inflammatory activities of nigella sativa seed extract in adjuvant-induced arthritis. J Immunotoxicol 14(1):91–96

Passos C, de Faria RB, de Almeida VA, de Moraes CE, Teixeira Júnior J, de Souza DM, Carneiro GD (2017) Pharmacological evaluation of anti-inflammatory and anti-proliferative activity of the anti-inflammatory drug zileuton. J Ethnopharmacol 207:217–225

Phuc DV, Ha LH, Tri DM, Nguyen NH, Nguyen HT, Tran TH (2019) Anti-inflammatory and antipyretic activity of methanolic extract of Pluchea indica. J Adv Pharm Technol Res 8(3):223–227
McInnes IB, Schett G (2007) Cytokines in the pathogenesis of rheumatoid arthritis. Best Pract Res Clin Rheumatol 38(9):1861–1870

Shabbir A, Shahzad M, Ali A, Zia-ur-Rehman M (2016) Discovery of new benzothiazine derivative as modulator of pro-and anti-inflammatory cytokines in rheumatoid arthritis. Inflamm Res 39(6):1918–1929

Shin TH, Kim HS, Kang TW, Lee BC, Lee HY, Kim YJ, Shin JH, Seo Y, Choi SW, Lee S, Shin K (2016) Human umbilical cord blood-stem cells direct macrophage polarization and block inflammatory response to alleviate rheumatoid arthritis. Cell Death Dis 7(12):442–453

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.