Chapter 4

In vivo studies

Biochemical and immunological evaluation of the ameliorating effect of triphala in adjuvant induced arthritic rats

4.1 BACKGROUND

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune inflammatory disorder of the joints associated with inflammatory synovitis and cartilage degradation that may lead to severe disability when left untreated (Firestein, 2003). Apart from the inflammatory mediators like reactive oxygen species that participate in the pathogenesis of RA, the cytokines such as TNF-α, IL-1β, IL-6 and IL-17 are considered to play an important role in the development of full-blown arthritis (Dinarello, 2001). The bone-destructive factors other than inflammatory cytokines are found to exist in inflamed joints during the RA. Activated immune cells like Th17 cells secrete IL-17, which stimulates osteoblasts and synoviocytes to produce RANKL, initiating RANKL-associated osteoclastogenesis and bone destruction (Kotake et al., 2001). NO and PGE₂ are involved in the regulation of certain physiological processes which includes fibroblast proliferation, recruitment of chemokines (IL-8 and MCP-1) and activation of transcription factors (NF-κB and AP-1) (Smolen et al., 2012). Since inflammation is the first process that underlies the destructive mechanism in joints, effective blockade of these inflammatory responses could lead to the development of potential anti-inflammatory drug (Lubberts and Van Den Berg, 2003). The pharmacological management of RA principally relies on treatment regimens that include non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatoid drugs (DMARDs). These agents mainly target the symptoms of the diseases rather than the underlying cause. Though these drugs provide the relief of pain and inflammation, their long-term use is overshadowed by their untoward effects such as perforation, cardiovascular complications, renal morbidity and gastrointestinal ulcers. In addition, non-steroidal anti-inflammatory drug (NSAIDs) treatments are shown to perpetuate the bone destruction by inhibiting the synthesis of glycosaminoglycans (Feist and Burmester,
2009). Thus, by considering these side effects, low efficacy, and high costs of drugs, it has become pertinent and indispensable to develop a plant-based herbal therapy to alleviate the symptoms of arthritis.

Triphala has shown wide range of pharmacological properties like anti-inflammatory and immunomodulation. But the underlying mechanism for anti-arthritic effect of triphala with reference to biochemical and molecular events involved in the pathogenesis of RA was not reported till date to the best of our cognition. Therefore, the stupendous medicinal properties of triphala exhorted us to investigate its efficacy against adjuvant-induced arthritis in rats with respect to biochemical and molecular events involved in the inflammatory cascade. Indomethacin, a common NSAID, was used for comparison purposes.

4.2 MATERIALS AND METHODS

4.2.1 STUDY DESIGN AND METHODS

4.2.1.1 ANIMALS

Wistar rats of either sex weighing 125-150 g were used for the study, which was procured from Animal house, VIT-University, Vellore, India. The rats were supplied with commercial balanced diet in regular pellets tap water were provided _ab libitum_ and maintained in polypropylene cages and under standard conditions of 12 h dark/light cycle at 27 ± 1°C. The animals were treated and cared for in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The study was conducted after obtaining approval from Institutional Animal Ethical Committee.

4.2.2 PREPARATION OF AQUEOUS EXTRACT OF TRIPHALA

The commercially available triphala powder (a mixture of dried and powdered fruits of three plants, i.e. _T. chebula, E. officinalis_ and _T. bellerica_ in equalproportions [1:1:1]) was obtained from the Indian Medical Practitioners Cooperative Stores and Society (IMCOPS), Chennai, India. The aqueous extract of triphala was prepared as described earlier in chapter 3, section 3.2.2.
4.2.3 EXPERIMENTAL GROUPS

Rats were divided into four groups, each comprising of six animals

Group I: Control rats administered with saline.

Group II: Arthritis-induced rats.

Group III: Arthritic rats administered with triphala (100 mg/kg body weight [bwt]).

Group IV: Arthritic rats administered with standard drug indomethacin (3 mg/kg bwt),

The dosage of triphala and standard drug indomethacin used in this study was selected based on our previous report and preliminary studies (Kalaiselvan and Rasool, 2015).

4.2.4 INDUCTION OF ARTHRITIS

Arthritis was induced by a single intradermal injection of complete Freund’s adjuvant (0.1 ml) into the footpad of right hind paw. The adjuvant contained heat-killed *Mycobacterium tuberculosis* (10 mg) in paraffin oil (1 ml).

4.2.5 DRUG TREATMENT AND ASSESSMENT

Triphala (100 mg/kg bwt) and indomethacin (3 mg/kg bwt) were administered intraperitoneally once daily for 8 days (from day 11 to 18) after the administration of complete Freund’s adjuvant. On the day before sacrifice, rats were housed in metabolic cages for 24 h urine collection provided with water but without feed. Urine was collected in the bottle, which was maintained at 0°C and urine was free of fecal contamination. The samples were subjected to centrifugation in order to remove sediments. Estimation of hydroxyproline and total glycosaminoglycans was then done using urine samples as alcian blue precipitate polyanions. On day 21, at the end of the experimental period, the animals were killed by euthanasia and the blood was collected without anticoagulant for plasma and serum separation respectively. The paw tissues were immediately dissected out and homogenized in ice-cold 0.01 M, Tris HCl buffer and pH 7.4 to give a 10% homogenate. The hind limbs were dissected out and bone collagen was extracted and hydroxyproline level was determined. Homogenized paw tissues were used for
biochemical estimation of lipid peroxidation, lysosomal enzymes, protein bound carbohydrates, antioxidant status and inflammatory mediators.

4.2.6 ASSESSMENT OF OXIDATIVE STRESS

4.2.6.1 ESTIMATION OF LIPID PEROXIDATION

Lipid peroxidation was measured in terms of thiobarbituric acid (TBA) reaction in paw tissue homogenates. It was estimated by the method of Ledwozyw et al. (1986) and Högberg et al. (1974). To 0.1 ml of distilled water, 0.75 ml of 20% acetic acid, 0.2 ml of sodium dodecyl sulphate, 0.75 ml of 1% thiobarbituric acid and 0.1 ml of enzyme preparation were added. The blank tube contained 0.1 ml distilled water instead of the enzyme preparation. The contents were mixed and heated in a water bath for 60 minutes at 95°C. The tubes were cooled, mixed and centrifuged at 1,000 x g for 15 minutes. The thiobarbituric acid was measured spectrophotometrically at 532 nm based on the purple colour generated by the reaction of thiobarbituric acid (TBA) with malondialdehyde. The values are expressed as nm of MDA released/mg of protein.

4.2.6.2 NITRIC OXIDE ESTIMATION

Nitric oxide (NO) was estimated by the detection of its stable oxidative metabolite nitrite by using Griess method. Equal volume of paw tissue supernatant and Griess reagent (a mixture of 0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid: 1:1) was mixed in 96-well plate. After 20 min of incubation at room temperature in the dark, the absorbance was measured at 540 nm and the nitrite concentration determined based on a standard curve which was constructed using serially diluted sodium nitrite (Grisham et al., 1996).

4.2.7 ASSAY OF ANTIOXIDANTS

4.2.7.1 SUPEROXIDE DISMUTASE

Measurement of enzyme activity was performed by method described by Marklund and Marklund (1974). The ability of super oxide dismutase to inhibit the auto-oxidation of pyrogallol was expressed as enzyme activity. Absolute ethanol and
chloroform were added to the enzyme preparation and mixed by vigorous shaking for 15 minutes. The contents were centrifuged for 10 minutes at 2500 rpm and the supernatant was assayed for SOD activity. Complete auto-oxidation of pyrogallol can be achieved by addition of 2 ml of Tris-HCl and 0.5 ml of pyrogallol and the rate of auto-oxidation were measured at 1 or 3 minutes. The assay was carried out when Tris-HCl buffer and pyrogallol were added with enzyme preparation and the contents were made up to 4 ml using distilled water. The inhibition of auto-oxidation was monitored at 470 nm, in the presence of enzyme and 50% of inhibition of pyrogallol autooxidation was given as 1 unit of SOD activity. The enzyme activity was expressed as units/mg of protein.

4.2.7.2 CATALASE

Catalase activity in the control and experimental rats were measured by the method described by Sinha (1972), where reduction of chromic acetate from chromate by H$_2$O$_2$ was carried out by heating the contents and the amount of chromic acetate formed was estimated by measuring the absorbance at 570 nm. The assay mixture consisting 0.5 ml of H$_2$O$_2$, 1 ml of sodium phosphate buffer, and 0.4 ml of water were added with 0.1 ml of sample to initiate the reaction. 2 ml of dichromate-acetic acid reagent was added to the content after 15, 30, 45 and 60 s to stop the reaction. Control tube was first added with dichromate-acetic acid reagent followed by enzyme preparation. The tubes were then heated for 10 minutes and the colour developed was measured at 570 nm after allowing the contents to cool. The catalase activity was measured as µM of H$_2$O$_2$ consumed/min/mg of protein.

4.2.7.3 GLUTATHIONE PEROXIDASE

The activity of glutathione peroxidase was measured by Rotruk et al. (1973) method. The enzyme preparation was added with 0.5 ml of sodium phosphate buffer and 0.1 ml of sodium azide. The content was thoroughly mixed and was added with 0.2 ml of reduced glutathione and 0.1 ml of H$_2$O$_2$. After incubating the contents for 10 mins at 37°C, 0.5 ml of 10% TCA was added to arrest the reaction and the contents were centrifuged to separate the supernatant. Disodium hydrogen phosphate (4 ml) was added to the supernatant, followed by 1 ml of 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB)
reagent for development of coloured complex which was read at 412 nm. The activity of glutathione peroxidase was expressed as µg of GSH utilized/min/mg of protein.

4.2.7.4 GLUTATHIONE-S-TRANSFERASE

A method developed by Habig et al. (1974), was utilized to measure the level of Glutathione-S-Transferase activity. The enzymatic conjugation of glutathione with 1-chloro-2,4 nitrobenzene (CDNB-aromatic substrate) leads to development of coloured complex, which was read at 340 nm. Briefly, 0.1 ml of reduced glutathione and 0.1 ml of CDNB were added with 1 ml of phosphate buffer. The contents were made up to 2.9 ml using distilled water. After incubating the reaction mixture at 37°C for 5 minutes, 0.1ml of enzyme preparation was added to it to initiate the reaction. The absorbance was measured at 340 nm for every 30 s. Glutathione-S-Transferase activity was expressed as nmol of CDNB-GSH conjugate formed/min/mg of protein.

4.2.7.5 REDUCED GLUTATHIONE

Reduced glutathione was determined by the method developed by Moron et al. (1979). Reaction between GSH and DTNB result in the formation of a yellow coloured complex, which can be measured at 412 nm. 0.2 ml of sample was added with 2 ml of 10% TCA and the contents were centrifuged to obtain 1 ml of supernatant. To this, 0.5 ml of DTNB, 1% sodium citrate and 3 ml of phosphate buffer was added, which led to yellow colouration. The optical density was measured at 412 nm and the reduced glutathione level was nM/mg of protein.

4.2.8 ASSAY OF LYSOSOMAL ENZYMES

4.2.8.1 ACID PHOSPHATASE

Measurement of acid phosphatase activity was performed by the method previously described by king (1965b). Reaction mixture containing enzyme preparation (0.1 ml), citrate buffer (1.5 ml) and distilled water (0.4 ml) was incubated at 37°C for 1 h. After terminating the reaction by addition of folin’s reagents, 15% sodium carbonate (1 ml) was added and the contents were incubated for 10 minutes at 37°C. The colour
developed was measured at 640 nm. The enzyme activity was expressed as μmoles phenol liberated/min/mg protein.

4.2.8.2 β-GALACTOSIDASE

The activity of β-galactosidase was measured by Rosenblit et al. (1974) method. Briefly, 0.2 ml of enzyme preparation was added with 0.5 ml of p-nitrophenyl- β-galactopyranoside (substrate) and 0.3 ml of citrate buffer. The contents were gently shaken and incubated for an hour at 37°C. To this 3 ml of glycine-NaOH buffer was added to arrest the reaction and colour developed was read at 410 nm. The β-galactosidase activity was expressed as μmoles of p-nitrophenol formed/h/mg of protein.

4.2.8.3 N-ACETYL GLUCOSAMINIDASE

A method developed by Maruhn (1976) was used to estimate the enzymatic activity of N-Acetyl glucosaminidase. Sample (0.2 ml) was added with 0.1 ml of buffered substrate (p-nitrophenyl N-acetyl glucosaminide) and the contents were incubated for 30 mins at 37°C. The reaction was ended by adding 2.2 ml of glycine-NaOH buffer. The yellow colour obtained was measured at 405 nm. The enzyme activity was expressed as μmoles of p-nitrophenol formed/h/mg of protein.

4.2.8.4 CATHEPSIN D

The activity of cathepsin D was estimated by Biber et al. (1981) method. Reaction mixture 0.9 ml of buffered substrate and enzyme preparation was added (0.1 ml) and incubated at 37°C for 2 h. 1 ml of 5% TCA was added to terminate the reaction and contents were centrifuged for 10 minutes. Control tubes were added with enzyme after adding TCA. 5% NaOH was added to the supernatant followed by 4.5 ml of alkaline copper reagent. Further, 0.5 ml of Folin’s reagent was added and the absorbance was read at 640 nm. The activity was displayed as μmoles of tyrosine liberated/h/mg of protein.

4.2.9 ESTIMATION OF PROTEIN BOUND CARBOHYDRATES

Defatted tissue was added in to 3 ml of 2M HCl and the contents were boiled at 90°C for 4 h. The contents were let to become cool and then, it was neutralized by
addition of 2 M NaOH. Aliquots were taken from this and used for sialic acid and hexosamine. Methanol (5 ml) was added to 0.1 ml of plasma and the contents were centrifuged for 10 minutes at 3000xg. The pellet was washed with 95% ethanol to obtain glycoproteins. This was used to measure hexosamine, hexose and sialic acid contents.

4.2.9.1 SIALIC ACID

Sialic acid content in plasma and tissue was measured by the method developed by Aminoff (1961) and modified by Niebes (1972). To the plasma or neutralized tissue sample, 0.25 ml of periodate was added and the mixture was incubated at 37°C for 30 minutes. Arsenite was added to stop the reaction and 2 ml of TBA was added after thoroughly shaking the contents. The tubes were heated in a water bath for 6 mins and the pink colour developed was read at 540 nm. Silalic acid content in paw tissues was expressed as mg/g tissue.

4.2.9.2 HEXOSAMINE

Wagner (1979) method was used to estimate the hexosamine content in tissue samples. Neutralized sample (0.5 ml) was made up to 1 ml with distilled water and 0.6 ml of acetyl acetone reagent was added to the tubes and boiled in a water bath for 30 minutes. Ehrlich’s reagent (2 ml) was added to tubes after cooling it and the contents were mixed. The colour was measured at 540 nm. Galactosamine was used a standard. Hexosamine level in paw tissues were expressed as mg/g of tissue.

4.2.9.3 HEXOSE

Hexose content in tissue was estimated by method already described by Niebes (1972). Briefly, 0.5 ml of sample was added with 1 ml of chilled H₂SO₄ and the contents were heated for 3 minutes in boiling water bath and cooled instantaneously. To this 0.2 ml of CPS reagent (1% L-cysteine-HCl and 0.075% of phenol mixture) was added and vortexed. The tubes were left for 1 h in an ice bath and the absorbance was measured at 490 nm. Amount of hexose was expressed as mg/g of tissue.
4.2.10 ASSESSMENT OF BONE COLLAGEN AND URINARY CONSTITUENTS

4.2.10.1 ESTIMATION OF BONE COLLAGEN

Collagen was estimated in terms of hydroxyproline: the procedure of Neuman and Logan (1950) with the modification of Leach (1960) was followed. Collagen was extracted from bone samples in which 50 to 100 mg of bone was autoclaved for 3 hours at 15 pounds pressure in 4 ml of water and centrifuged. The residue was then washed with 4 ml of water, centrifuged and the supernatant was transferred into a clear tube. The residue was autoclaved again with 4 ml of water. The supernatant collected was subjected to drying using boiling water bath. The hydrolysate was prepared by adding 1.0ml of 6 N HCL and autoclaved for 3 hours at 50 pounds pressure and neutralized with NaOH solution. This neutralized solution was used for hydroxyproline estimation.

To estimate the hydroxyproline content, to 1 ml of test solution, 1 ml of 0.05 M CuSO4, 1 ml of 2.5 N NaOH was added and placed in a water bath for 5 minutes at 40°C. 1 ml of 6% H2O2 was added and immediately mixed and left in water bath for 10 minutes. Later to the cool tubes 4 ml of 3 N H2SO4 and 2 ml of 5% p-dimethylaminobenzaldehyde solution was added and kept at 70°C for 16 minutes. The test solution was read at 550 nm along with standard L-hydroxyproline with varying concentrations to determine the hydroxyproline content. The calculated hydroxyproline was multiplied by a constant factor of 7.46 to convert hydroxyproline to collagen. Results were expressed as percentage of collagen mg/g cartilage.

4.2.10.2 ESTIMATION OF URINARY CONSTITUENTS

4.2.10.2.1 HYDROXYPROLINE

The rats were housed in metabolic cages 24 h prior to sacrifice for urine collection. The animals were provided water ad libitum but without any feed. The urine samples were centrifuged to remove sediments followed by estimation of hydroxyproline by the procedure of Neuman and Logan (1950) with the modification of Leach (1960). The hydroxyproline content was expressed as mg/100mg creatinine.
4.2.11 TOTAL GLYkosaminoglycans

Total glycosaminoglycan was estimated by the method of Whiteman (1973). 100 μl of 24 h urine sample was added to 2 ml of alcian blue solution (50 mg of alcian blue in 0.05 mol/L of sodium acetate buffer, containing 0.05 mol of magnesium chloride per liter {pH 5.8}) and incubated at room temperature for 2 h. The mixture was centrifuged at 2000 x g for 10 minutes and the pellet was washed twice with 2 ml of ethanol and resuspended in 2 ml of 50 g/L sodium dodecyl sulfate reagent and measured the absorbance at 620 nm. The absorbance values were compared with the standard curve for chondroitin sulfate (5-100 mg/L) for calibration. The total glycosaminoglycan was expressed as mg of GAGs excreted/24 h.

4.2.12 ASSESSMENT OF ARTICULAR ELASTASE

Activity of elastase (ELA) was evaluated as an index of polymorphonuclear leukocyte accumulation and activation in cartilage tissues (Umar et al., 2012). The analysis was performed using N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide, a highly specific synthetic substrate for neutrophil articular elastase assay. The amount of p-nitroanilide liberated was measured spectrophotometrically at 405 nm and was considered as neutrophil ELA activity. The results were expressed as ng/g protein.

4.2.13 ASSESSMENT OF INFLAMMATORY MEDIATORS

Cytokine levels (TNF-α, IL-1β, VEGF, MCP-1, and PGE₂) in serum and paw tissue homogenates were determined by using commercially available ELISA kits according to the manufacturer’s instructions (Peprotech, Rocky Hill, NJ).

4.2.14 QUANTITATIVE REAL-TIME PCR ANALYSIS

Total RNA was extracted from the paw tissues of control and arthritic rats by using TRIzol reagent (Sigma Aldrich, St. Louis, MO). The 2 μg RNA was reverse transcribed by using the higher capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) and the mRNA expression was amplified by Quantitect SYBR PCR kit (QIAGEN, Valencia, CA). Gene specific primers were designed manually by using NCBI/primer-BLAST tool software and were purchased from Sigma Aldrich.
Quantitative RT-PCR (qRTPCR) was performed to measure TNF-α, IL-6, AP-1, MCP-1, IL-1β, COX-2, iNOS, IL-17, p65 and RANKL, respectively. Transcription levels were assessed utilizing the step one real-time thermal cycler with SYBR Green PCR Master Mix according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA). Amplification was performed in the following cycling conditions: 94°C for 15 s, 60°C annealing for 30 s, and 72°C extension for 30 s. The fold change in gene expression levels of target genes was calculated with normalization to β-actin values using the 2^{-ΔΔCt} comparative cycle threshold method. Each gene analysis was performed in triplicate and the primer sequences of the targeted genes are listed in Table 4.1.

Table 4.1: Primer sequences used for quantitative real-time PCR analysis of RNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>F- 5’GGCATGGATCTCAAGACAAACC3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’ AAATCGGCTGACGGTGG 3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F- 5’ TCTCACAGCAGCATCTCGAC3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’GGTCGTCATCATCCCACAG3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>F- 5’ TACCACATTCAAGGTCGAGG 3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’CAATCAGAATTGGCCATTGCACAAC 3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>F- 5’CACTCACCTGCTGCTACTCAT3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’CTACAGCTTCTTTGAGACACCT3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>F-5’GGCTACCAGATGCCCAGATG3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’CCACTCGTACTTGGGATGCTC3’</td>
</tr>
<tr>
<td>COX-2</td>
<td>F-5’CTCTTCCGAGCTGTGCTGC3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’TGTGTTGTTGTTGCTGCTC3’</td>
</tr>
<tr>
<td>NFκB-p65</td>
<td>F- 5’CTCACCAGGCCTCATCCACAT3’</td>
</tr>
<tr>
<td></td>
<td>R- 5’TGGCTAATGGCTCGCTCCAG3’</td>
</tr>
</tbody>
</table>
AP-1  F- 5’-GACTGCAAAGATGGAAACGACC 3’
      R-5’- AGAAGGTCCGAGTTCTTGGC 3’
RANKL  F- 5’- CCGAGACTACGGCAAGTACC 3’
       R-5’- CTGCCTCGAAAGTACAGGA 3’
IL-17  F- 5’- TCCAGAAGGCCCTCAGACTACC3’
       R- 5’- TCCAGAAGGCCCTCAGACTACC3’
ACTIN  F-5’- ACCACCATGTACCCAGGCATT3’
       R-5’- CCACACAGAGTACTTGCGCTCA3’

4.2.15 WESTERN BLOT ANALYSIS

Paw tissue lysates were prepared in ice-cold RIPA buffer (150 mM NaCl, 1%
IGEPA CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8]) mixed
along with protease inhibitors. Protein content in lysates was measured using a Bradford
protein assay kit (BioRad, Hercules, CA). For the analyses, lysates (30 µg protein/lane)
were electrophoretically resolved over 12% sodium dodecyl sulfate-polyacrylamide gels.
Proteins were then electrotransferred to polyvinylidene fluoride membranes (PVDF); the
blots were then blocked overnight at 4°C in a solution of 5% (w/v) bovine serum albumin
in TBST (Tris-buffered saline containing 0.1% Tween-20). The membranes were then
incubated overnight at 4°C with the desired primary polyclonal antibody individually
against NFκB p65 (1:1000), p-NFκB p65 (1:1000); COX-2 (1:1000); TNF-α (1:1000),
IL-17 (1:1000), iNOS (1:1000), and β-actin (1:2000); all dilutions were in TBST with 1%
BSA. After washing with TBST, membranes were probed for 1 h with appropriate
secondary antibody (HRP-conjugated goat anti-rabbit IgG antibody, 1:10000 in TBST
with 1% BSA). Protein bands were then visualized using an enhanced
chemiluminescence detection system (BioRad). Densities of the bands were measured
using a BioRad Chem DOCTM XRS luminescent image analyzer and accompanying
Imagelab [v.2.0.1] software.
4.2.16 IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis of NFκB p65 and COX-2 was performed on paw tissue sections of each animal from different experimental groups. Tissue sections (5–7 µm) were fixed in 10% formalin, decalcified, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections were quenched for endogenous peroxidase activity with methanol 3% H$_2$O$_2$ in microwave oven using 10 mM citrate buffer at pH 6.0, and were blocked with goat serum for 30 min to minimize the non-specific staining and successively incubated with rabbit anti-COX-2 and rabbit anti-NFκB p65 monoclonal antibody (Cell Signaling Technology, Beverly, MA), respectively, at 4°C overnight. Subsequently, after washing in PBS, the slides were incubated with secondary antibody anti-rabbit IgG coupled with horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX) for 1 h at room temperature. Later on, the resultant chromogenic reactions were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin for the nucleus.

4.2.17 RADIOGRAPHICAL ASSESSMENT

Radiographs of control and experimental rat joints were performed to evaluate the cartilage and bone damage. Prior to sacrifice, the joints of the hind limb were imaged using an X-Ray apparatus (Seimens, Erlangen, Germany) and industrial X-Ray film (Fuji Photo Film, Tokyo, Japan) for assessing the joint damage. The X-ray apparatus was operated at 220V, with a 40V peak, 0.2 s exposure time and a 60-cm tube-to-film for anterior-posterior projection. Images were read independently in a blinded fashion. The degree of joint destruction and bone erosion was scored on a scale of 0-3, where 0 being normal; 1, low destruction; 2, moderate changes; and 3, severe erosion.

4.2.18 HISTOLOGICAL ASSESSMENT

At the end of an experimental period, rats were euthanized and tissue samples of the hind paw were fixed in 10% neutral buffered formalin and decalcified with formic acid (10%) for a minimum of 8 d. After decalcification, the joints were dehydrated using ethanol gradients on paraffin blocks, following sectioning at 5-7 µM. The sections were stained with hematoxylin-eosin and the histological damage was evaluated.
microscopically for the presence of mononuclear cell infiltration, hyperplasia of synovium, pannus formation and bone destruction. Histopathological changes were graded using the observed parameters. Images were read independently in a blinded fashion and histological scores were graded as follows (0-3): 0 = normal, 1 = mild cell proliferation or mild infiltration of mononuclear cells, 2 = modest inflammatory cell infiltration and reasonable cartilage destruction, 3 = massive pannus formation with extensive invasion of inflammatory cells into synovium and joint space.

4.2.19 STATISTICAL ANALYSIS

The results were statistically analyzed using one-way analysis of variance followed by student’s Newman-Keul’s test (Graphpad Software, La Jolla, CA) for individual comparison of groups with control. Results are expressed as mean ± SD. The $p$-value<0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 EFFECT OF TRIPHALA ON OXIDATIVE STRESS

In order to attain a better perspective on the oxidative stress, we investigated whether triphala could modulate the oxidative stress in the paw tissues of arthritic rats. As indicated in the Table 4.2, the lipid peroxidation and NO were significantly increased in the paw tissues of arthritic rats as compared to the control group. Treatment with triphala decreased lipid peroxidation and NO in the paw tissues of arthritic rats similar to indomethacin.

4.3.2 EFFECT OF TRIPHALA ON ANTIOXIDANT STATUS

Table 4.3 represents the effect of triphala on the antioxidant profile (SOD, CAT, GPx, GSH, and GST) in the paw tissues of control and experimental rats. The results obtained in our study showed a substantial decrease in the levels of antioxidants in the paw tissues of arthritis-induced rats compared with the control rats. On the contrary, after triphala treatment, antioxidant status (SOD ~75.6%, CAT ~62.7%, GPx ~55.8%, GST ~82.1%, and GSH ~72.7%) was recouped to near normal levels.
4.3.3 EFFECT OF TRIPHALA ON LYSOSOMAL ENZYMES

The effect of triphala on lysosomal enzymes in paw tissues of control and experimental animals is delineated in Table 4.4. A significant increase in the activities of acid phosphatase, N-acetyl glucosaminidase, β-galactosidase, and cathepsin D was observed in the paw tissues of arthritis-induced rats compared with the control rats. Nevertheless, the administration of triphala to arthritic rats reduced the lysosomal enzyme activities (acid phosphatase ~52.4%, β-galactosidase ~22.9%, N-acetyl β-glucosaminidase ~22.1%, and cathepsin-D ~27.7%) comparable with the indomethacin treatment.

4.3.4 EFFECT OF TRIPHALA ON PROTEIN BOUND CARBOHYDRATES

Table 4.5 portrays the effect of triphala on the protein bound carbohydrates in paw tissues of control and experimental animals. The sugar components of glycoproteins—hexose, sialic acid, and hexosamine were increased in the paw tissues of arthritis-induced rats compared with the control rats. These changes observed in the arthritic rats were recouped back to near normal levels (hexose ~43.3%, hexosamine ~36.5%, and sialic acid ~33.7%) on triphala administration.

4.3.5 EFFECT OF TRIPHALA ON ARTICULAR ELASTASE

The effect of triphala on polymorphonuclear leukocyte accumulation and activation was studied by estimating the activity of ELA in the paw tissues of arthritic rats. As shown in Table 4.6, the ELA activity was increased in arthritic rats as compared to the control group. However, the administration of triphala significantly decreased the ELA activity in arthritic rats similar to indomethacin.

4.3.6 EFFECT OF TRIPHALA ON BONE COLLAGEN AND URINARY CONSTITUENTS

To assess the suppressive effect of triphala on cartilage and bone degradation, the bone collagen and urinary constituents (hydroxyproline and total glycosaminoglycans) were estimated in arthritic rats. As shown in Table 4.7, our results demonstrated significant reduction levels of bone collagen and increased level of urinary constituents.
(hydroxyproline and total glycosaminoglycans) in arthritic rats compared to control group. However, the triphala administration significantly inhibited the cartilage and bone degradation by regulating the bone collagen and urinary constituents to near normal in arthritic rats.

4.3.7 EFFECT OF TRIPHALA ON INFLAMMATORY MEDIATORS

Figures 4.1 and 4.2 show the effect of triphala on inflammatory mediators like TNF-α, IL-1β, VEGF, MCP-1, and PGE₂ in sera and the paw tissue of control and experimental rats. As per the results obtained in our study, there was significant increase in the levels of inflammatory mediators (TNF-α, IL-1β, VEGF, MCP-1, and PGE₂) in serum and paw tissues of arthritis-induced rats compared with the control animals. In contrast, triphala-administrated arthritic rats exhibited significant reduction in the serum (TNF-α ~75.5%, IL-1β ~99%, VEGF ~75.2%, MCP-1 ~76.4%, and PGE₂ ~69.9%) and paw tissue (TNF-α ~71.6%, IL-1β ~75.5%, VEGF ~55.1%, MCP-1 ~69.1%, and PGE₂ ~66.8%) inflammatory mediator levels as compared with the arthritic control.
Table 4.2: Effect of triphala and indomethacin on oxidative parameters in paw tissues of control and experimental rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Arthritis</th>
<th>Arthritis + triphala (100mg/kg b. wt)</th>
<th>Arthritis + indomethacin (3mg/kg b. wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide (µ mol nitrite/mg of wet tissue)</td>
<td>5.23 ± 1.90</td>
<td>9.33 ± 1.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.04 ± 1.70&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.98 ± 2.1&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBARS (n mol of TBARS formed/hr/mg/protein)</td>
<td>6.53 ± 1.67</td>
<td>9.36 ± 0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.89 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.06 ± 0.86&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean± SD of six animals. Comparisons were made as follows: (a) Control versus arthritis, arthritis + triphala, arthritis + indomethacin; (b) Arthritis versus arthritis + triphala, arthritis + indomethacin. The symbols (a and b) represent statistical significance at: p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Table 4.3: Effect of triphala and indomethacin on antioxidant status in paw tissues of control and experimental rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Arthritis</th>
<th>Arthritis+triphala (100mg/kg b. wt.)</th>
<th>Arthritis+indomethacin (3mg/kg b. wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (units/mg protein/min)</td>
<td>16.20 ± 0.92</td>
<td>4.21 ± 2.47(^a)</td>
<td>13.05 ± 1.38(^{ab})</td>
<td>13.8 ± 1.70(^b)</td>
</tr>
<tr>
<td>CAT (μmol of H(_2)O(_2) consumed/min/mg protein)</td>
<td>68.02 ± 11.97</td>
<td>24.88 ± 5.63(^a)</td>
<td>41.81 ± 4.12(^{ab})</td>
<td>49.31 ± 11.50(^{ab})</td>
</tr>
<tr>
<td>GPx (μg of GSH utilized/min/mg protein)</td>
<td>24.54 ± 2.67</td>
<td>10.64 ± 1.92(^a)</td>
<td>20.54 ± 1.40(^b)</td>
<td>19.87 ± 1.86(^{ab})</td>
</tr>
<tr>
<td>GST (nmol of 1-chloro-2,4 dinitrobenzene-GSH conjugate formed/min/mg protein)</td>
<td>3.78 ± 0.61</td>
<td>0.44 ± 0.12(^a)</td>
<td>2.03 ± 0.54(^{ab})</td>
<td>2.35 ± 1.00(^{ab})</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>79.90 ± 15.40</td>
<td>22.40 ± 3.60(^a)</td>
<td>59.75 ± 15.77(^b)</td>
<td>61.33 ± 19.60(^b)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis+triphala, arthritis+indomethacin; (b) arthritis versus arthritis+triphala, arthritis+indomethacin. The symbols (a and b) represent statistical significance at: p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Table 4.4: Effect of triphala and indomethacin on the activities of lysosomal enzymes in paw tissues of control and experimental rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Arthritis</th>
<th>Arthritis+triphala (100mg/kg b. wt)</th>
<th>Arthritis+indomethacin (3mg/kg b. wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase (µmolx10^{-2} of phenol)</td>
<td>0.27 ± 0.05</td>
<td>0.61 ± 0.13(^a)</td>
<td>0.32 ± 0.07(^b)</td>
<td>0.27 ± 0.03(^b)</td>
</tr>
<tr>
<td>β-galactosidase (µmolx10^{-2} of p-nitrophenol liberated/h/mg protein)</td>
<td>1.37 ± 0.18</td>
<td>4.69 ± 1.20(^a)</td>
<td>1.40 ± 0.61(^b)</td>
<td>1.39 ± 0.19(^b)</td>
</tr>
<tr>
<td>N-acetyl β-glucosaminidase (µmolx10^{-2} of p-nitrophenol liberated/h/mg protein)</td>
<td>1.06 ± 0.13</td>
<td>3.98 ± 1.29(^a)</td>
<td>1.13 ± 0.23(^b)</td>
<td>1.15 ± 0.10(^b)</td>
</tr>
<tr>
<td>Cathepsin-D (µmolx10^{-2} of tyrosine liberated/h/mg protein)</td>
<td>0.27 ± 0.10</td>
<td>0.91 ± 0.11(^a)</td>
<td>0.35 ± 0.05(^b)</td>
<td>0.37 ± 0.41(^b)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis+triphala, arthritis+indomethacin; (b) arthritis versus arthritis+triphala, arthritis+indomethacin. The symbols (a and b) represent statistical significance at: p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Table 4.5: Effect of triphala and indomethacin on protein bound carbohydrates in paw tissues of control and experimental rats

<table>
<thead>
<tr>
<th>Parameter (mg/g defatted tissue)</th>
<th>Control</th>
<th>Arthritis</th>
<th>Arthritis+triphala (100mg/kg b. wt)</th>
<th>Arthritis+indomethacin (3mg/kg b. wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose</td>
<td>1.04 ± 0.03</td>
<td>1.58 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.21 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.26 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>1.39 ± 0.62</td>
<td>14.29 ± 1.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.23 ± 0.59&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.97 ± 0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>186.52 ± 1.18</td>
<td>387.92 ± 46.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>197.56 ± 5.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200.36 ± 3.78&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis+triphala, arthritis+indomethacin; (b) arthritis versus arthritis+triphala, arthritis+indomethacin. The symbols (a and b) represent statistical significance at: p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Table 4.6: Effect of triphala and indomethacin on activities of articular elastase in paw tissues of control and experimental rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Arthritis</th>
<th>Arthritis+triphala (100mg/kg b. wt)</th>
<th>Arthritis+indomethacin (3mg/kg b. wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular elastase (ng/g protein)</td>
<td>62.1 ± 0.92</td>
<td>221.1 ± 2.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.1 ± 1.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>85.4 ± 1.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis+triphala, arthritis+indomethacin; (b) arthritis versus arthritis+triphala, arthritis+indomethacin. The symbols (a and b) represent statistical significance at: p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Table 4.7: Effect of triphala and indomethacin on bone collagen and urinary constituents in paw tissues of control and experimental rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Arthritis</th>
<th>Arthritis+triphala (100mg/kg b. wt)</th>
<th>Arthritis+indomethacin (3mg/kg b. wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone collagen (mg/g cartilage)</td>
<td>172.52 ± 11.97</td>
<td>102.37 ± 6.63a</td>
<td>135.32 ± 7.12ab</td>
<td>112.53 ± 11.50ab</td>
</tr>
<tr>
<td>Hydroxyproline (mg/100 mg creatinine)</td>
<td>2.25 ± 0.40</td>
<td>5.25 ± 1.60a</td>
<td>3.5 ± 0.77b</td>
<td>4.0 ± 0.60b</td>
</tr>
<tr>
<td>Total Glycosaminoglycans (mg/24 h)</td>
<td>2.32 ± 0.20</td>
<td>4.68 ± 0.14a</td>
<td>3.55 ± 0.17b</td>
<td>3.42 ± 0.18b</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis+triphala, arthritis+indomethacin; (b) arthritis versus arthritis+triphala, arthritis+indomethacin. The symbols (a and b) represent statistical significance at: p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Figure 4.1: Effect of triphala on cytokine level in serum of control and experimental rats

A

![Graph showing TNF-α level in control and experimental groups.](image)

Control  Adjuvant-induced arthritis  Arthritis+triphala (100 mg/kg bwt)  Arthritis+indomethacin (3 mg/kg bwt)

B

![Graph showing IL-1β level in control and experimental groups.](image)

Control  Adjuvant-induced arthritis  Arthritis+triphala (100 mg/kg bwt)  Arthritis+indomethacin (3 mg/kg bwt)
(A) TNF-α, (B) IL-1β, (C) MCP-1, (D) VEGF and (E) PGE₂. Values are expressed as mean±S.D. for six animals. Comparisons were made as follows: (a) Control versus arthritis, arthritis + triphala, arthritis + indomethacin; (b) Arthritis versus arthritis + triphala, arthritis + indomethacin. The symbols (a and b) represent statistical significance at p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman Keul’s test.
Figure 4.2: Effect of triphala on cytokine level in paw tissues of control and experimental rats
(A) TNF-α, (B) IL-1β, (C) MCP-1, (D) VEGF, and (E) PGE₂. Values are expressed as mean ±S.D. for six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis + triphala, arthritis + indomethacin; (b) arthritis versus arthritis + triphala, arthritis + indomethacin. The symbols (a and b) represent statistical significance at p<0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
4.3.8 GENE EXPRESSION ANALYSIS VIA REAL-TIME PCR

The pathobiology of RA is multifaceted and involves a complex interaction of many pro-inflammatory cytokines, RANKL, inflammatory marker enzymes and transcription factors. As shown in Figure 4.3, the mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17, and MCP-1), inflammatory marker enzymes (iNOS, and COX-2), RANKL, and transcription factors (NFκB p65 and AP-1) were significantly upregulated in the paw tissues of arthritic rats compared to the control group. In contrast, triphala treatment markedly downregulated the expression of these genes in the paw tissues of arthritic rats as similar to indomethacin.

4.3.9 WESTERN BLOT ANALYSIS

To ascertain if many of the anti-inflammatory effects of triphala seen in the in vitro studies might translate to the in vivo scenario, effects of the agent on select proteins in adjuvant-induced arthritic rats were evaluated. Specifically, expression levels of NFκB p65, p-NFκB p65, IL-17, COX-2, and RANKL were evaluated in paw tissues of adjuvant-induced arthritic rats by Western blot analysis. As shown in Figure 4.4, the expression of NFκB p65, p-NFκB p65, IL-17, COX-2, and RANKL proteins were significantly elevated in the paw tissues of the arthritic rats when compared with tissues from control rats. Triphala treatment significantly decreased expression levels of p-NFκB p65, IL-17, COX-2, and RANKL proteins in the arthritic rat tissues; except for with IL-17, effects were often on par with that induced by use of indomethacin.

4.3.10 HISTOLOGICAL EVALUATION

As shown in Figure 4.5, the histological assessment of paw tissue section from the arthritic rats showed an abnormal joint architecture with massive influx of inflammatory cells, prominent synovial proliferation, pannus formation and bone erosion. In contrast, triphala-treated arthritic animals revealed pronounced reduction in inflammation and cartilage degradation with the evidence of less inflammatory cell infiltration and minimal synovial hyperplasia, whereas indomethacin-treated arthritic animals showed moderate synovial hyperplasia and infiltration of inflammatory cells. As shown in Figure 1(E), the histological scores of inflammation, pannus formation, and bone erosion observed in
arthritic rats were significantly diminished in the triphala-treated arthritic rats similar to indomethacin.
Figure 4.3: The mRNA expression of inflammatory mediators by real time-PCR
Relative Quantification

Control
Adjuvant-induced arthritis
Arthritis+triphala (100 mg/kg b wt)
Arthritis+indomethacin (3 mg/kg b wt)

C

D

Relative Quantification

Control
Adjuvant-induced arthritis
Arthritis+triphala (100 mg/kg b wt)
Arthritis+indomethacin (3 mg/kg b wt)
The mRNA expression was analyzed by real time-PCR. (A-J) Displays the effect of triphala on mRNA expression levels of **TNF-α**, **IL-1β**, **IL-6**, **IL-17**, **iNOS**, **COX-2**, **MCP-1**, **RANKL**, **NFκB-p65** and **AP-1** in control and experimental animals. Relative gene expression was normalized to β-actin and compared with control. Data represent the mean±SD of four independent experiments. Symbols (b and c) represent statistical significance at: P < 0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Figure 4.4: Treatment effects on arthritic rat paw levels of select proteins

NFκB-p-p65

NFκB-p65

β-actin

Control  AIA  AIA+Triphala (100mg/kg)  AIA+Indomethacin (3mg/kg)

Relative expression of NFκB-p-p65 protein

*  †  †
(a) **NF-κB p65 and p-NF-κB p65.** Representative Western blot images are shown. Relative expression values shown are means ± SD of data from three experiments. (b) **IL-17, RANKL, and COX-2.** Representative Western blot image. Relative expression values shown are means ± SD of data from three experiments. *Value significantly different (p < 0.05) vs. all other treatments. Among all AIA rats’ samples, values significantly different at p < 0.05: ‡ vs. AIA-only hosts, and # triphala-treated vs. indomethacin-treated hosts.
Figure 4.5: Histopathological evaluation in paw tissue sections of control and experimental animals

Histopathological changes in paw tissue sections of control and experimental animals A) Control animal showing intact articular cartilage with no evidence of inflammation (AC- articular cartilage, JS- joint space, SL- synovium lining); B) Arthritic induced animal showing synovial hyperplasia, pannus formation (single headed arrow) and intense infiltration of inflammatory cells (block arrow); C) Triphala (100 mg/kg) treated animal showing intact articular cartilage and less infiltration of immune cells (double headed arrow); D) Indomethacin (3 mg/kg) treated animal showing moderate evidence inflammation and reduced amount of immune cell in filtration(double headed arrow). E) Plot of the histological scores. Values are expressed as mean ± SD of six animals. Comparisons are made with: (a) control rats versus arthritic rats, triphala-treated arthritic rats and indomethacin-treated arthritic rats; (b) arthritic rats versus triphala-treated arthritic rats and indomethacin-treated arthritic rats; (c) triphala-treated arthritic rats versus indomethacin-treated arthritic rats. Symbols (b and c) represent statistical significance at P < 0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
4.3.11 RADIOGRAPHICAL ASSESSMENT

Radiographic analysis remains one of standard therapeutic monitoring options for investigating the disease progression in RA. As shown in Figure 4.6 adjuvant-induced arthritic rats showed joint edema, diminished joint space, osteophyte formation along with articular deterioration. However, the radiographical indices and scores observed in arthritic rats were considerably diminished on treatment with triphala similar to indomethacin.

4.3.12 IMMUNOHISTOCHEMICAL ANALYSIS

To explore the possible molecular mechanism of the suppressive effects of triphala on arthritic rats, we investigated NF-κB and COX-2 protein expression by immunohistochemical analysis. As shown in Figure 4.8, immunohistochemical analysis revealed massive protein expression of NFκB p65 and COX-2 in the paw tissues of arthritic rats compared to the control group. However, NFκB p65 and COX-2 protein expressions were found to decrease in the arthritic rats treated with triphala similar to the indomethacin.
Radiographical images of the joints of control and experimental animals. a) Control animal showing normal architecture of joints; b) Arthritic induced animal showing soft tissue swelling with diffused joint in phalangeal region and narrowing of joint space; c) Triphala (100 mg/kg) treated animal showing no narrowing of joint space and resembling near normal radiographic pattern of the control animals; d) Indomethacin (3 mg/kg) treated animal showing diminished narrowing of joint space and moderate soft tissue swelling. e) Plot of the radiographic scores. Values are expressed as mean ± SD of six animals. Comparisons are made with: (a) control rats versus arthritic rats, triphala-treated arthritic rats and indomethacin-treated arthritic rats; (b) arthritic rats versus triphala-treated arthritic rats and indomethacin-treated arthritic rats; (c) triphala-treated arthritic rats versus indomethacin-treated arthritic rats. Symbols (b and c) represent statistical significance at P < 0.05. Statistical analysis was calculated by a one-way ANOVA followed by Student’s Newman–Keul’s test.
Figure 4.7: Effects of triphala on the protein expression of NFκB-p65 and COX-2 in paw tissue sections of experimental animals
Effects of triphala on the expression of NFκB-p65 and COX-2 in paw tissue sections of experimental animals. Sections are 6μm thick and photomicrographs are taken at 10x. A, control animal; B, adjuvant-induced animal; C, triphala (100 mg/kg) treated animal; D, indomethacin (3 mg/kg) treated animal; 1, NFκB-p65 expression in the tissue sections; 2, COX-2 expression in the tissue sections; The NFκB-p65 and COX-2 positive cells were stained brown (hematoxylin counterstained) depicting presence of respective inflammatory mediators.
4.4 DISCUSSION

RA is a chronic systemic autoimmune disorder manifested by severe synovial inflammation and infiltration of immune cells, which contributes to cartilage and bone destruction. We previously reported that triphala exhibits anti-inflammatory effect against adjuvant-induced arthritic rats (Kalaiselvan and Rasool, 2015a). However, its underlying mechanism has not been reported so far. Therefore, we decided to investigate the potential anti-arthritic effect of triphala and its possible molecular mechanism in adjuvant-induced arthritic rats. In the present study, our results have demonstrated that triphala has the potential to suppress the various aspects of inflammatory immune responses and molecular events in adjuvant-induced arthritic rats.

Cells in the body generally maintain dynamic equilibrium in quenching of free radicals formed during oxidative stress. However, disproportionate productions of ROS lead to damage of tissue architecture. It is reported that synovial fluid in 90% RA patients found to contain the boundless production of oxygen free radicals (Kurien et al., 2006). Pro-inflammatory cytokines like TNF-α and IL-1β are also entangled in the production of hydroxyl radicals and hydrogen peroxide by stimulating chondrocytes and synoviocytes. Lipid peroxidation is considered as a vital marker in oxidative stress of RA. As observed in the present study, the paw tissues of arthritis-induced rats displayed elevated levels of MDA indicating the enhanced production of free radicals leading to oxidative damage to lipid, DNA and proteins present in the cell. However, triphala treatment significantly prevented the production of free radicals and eventually decreased the levels of lipid peroxidation in arthritic rats. This can be attributed due to the presence of phytochemicals like flavonoids and phenolic content present in the triphala extract which is in compliance with our earlier reports (Sabina and Rasool, 2008).

In order to impede the toxicity produced by free radicals, bone cells maintain a well co-ordinated free radicals cavenging system formed by antioxidants like superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and glutathione. Superoxide dismutase and catalase are the first and second line defense antioxidant system, which interacts with superoxide toxicity and forms hydrogen peroxide. Catalase
reacts with hydrogen peroxide to liberate water and oxygen with no free radical formation. In our study, the declined activity of superoxide dismutase and catalase was observed in the paw tissues of arthritic rats which might be due to the high level of free radical formation during phagocytosis and saturation of antioxidant enzymes (Babior et al., 1973). Glutathione peroxidase scavenges lipid peroxides in the cell membrane and act as a primary defense in mitochondria. The decreased glutathione peroxidase activity observed in arthritic rats might be due to the accumulation of hydrogen peroxide and deficiency of selenium. The glutathione redox cycle is an essential part of the antioxidant system, which comprises enzymatic and non-enzymatic glutathione. Glutathione acts as a substrate for glutathione peroxidase and glutathione-S-transferase during the removal of \( \text{H}_2\text{O}_2 \) and lipid peroxides. Thus, a decrease in the level of glutathione will lead to the reduction in glutathione peroxidase and glutathione-S-transferase activity (Mythilipriya et al., 2007). Glutathione-S-transferase is involved in the detoxification of xenobiotics. This enzyme plays a role in catalyzing the glutathione to electrophilic substrates (Veal et al., 2002). In our present study, owing to the enhanced oxidative stress and reduced ability to resist the attack by radicals in arthritic rats, the glutathione and glutathione-S-transferase levels were significantly decreased compared with the control rats, which are in line with our previous findings (Rasool and Sabina, 2007). However, the administration of triphala to arthritic rats improved the levels of antioxidant status by inhibiting the free radical production, which is apparently revealed in the current investigation as evidenced by decreased lipid peroxidation. This free radical scavenging property of triphala might be speculated due to its constituents such as flavonoids and polyphenolic compounds. These compounds are found to scavenge the singlet oxygen; increase the redox potential, inhibition of neutrophil respiratory burst, and lysosomal enzymes release (Ronzio, 2000).

Any immunogenic or toxic insult to lysosomes results in destabilization and rupture of the lysosomal membrane. Subsequently, there is an extrusion of their contents such as hydrolytic enzymes, glycosaminoglycans, and other proteases leading to degradation of the extracellular matrix and perpetuation of inflammatory condition as observed in arthritis (Vijayalakshmi et al., 1997). Thus, impeding the activity of lysosomal enzymes in arthritic condition would be beneficial. In this study, lysosomal enzyme activities were elevated in paw tissues of arthritic rats. However, triphala
administration considerably reduced the activities of lysosomal enzymes in the arthritis-induced rats, which indicates its anti-inflammatory effect. Glycoproteins are carbohydrate-linked protein macromolecules, which is a principal component of connective tissue that is responsible for the differentiation of cells and maintenance of structural integrity in collagen fibrils. During arthritic condition, synoviocytes activated monocytes, chondrocytes, and infiltrating neutrophils aids in the release of acid hydrolases and altered the metabolism of glycoproteins. Data obtained from this study showed an increase in the levels of hexose, hexosamine, and sialic acid in paw tissues of arthritic rats. The elevated glycoprotein level is attributed due to increased release of lysosomal enzymes during arthritic condition, which are found to amplify the metabolic turnover of structural macromolecules in connective tissue and cartilage proteoglycans. However, after triphala treatment, the glycoprotein levels were recouped to normal levels in the paw tissues of arthritis-induced rats. This membrane stabilizing property of triphala could be due to its antioxidant property of its constituents, which has been already well established (Hari et al., 2004; Lee et al., 2005; Naik et al., 2005; Sivasankar et al., 2015).

In acute inflammation, neutrophils are the cells primarily recruited to the site of injury. Their massive influx into the synovium is the hallmark of RA. During oxidative burst, neutrophils produce reactive oxygen species and proteases, which involve in the degradation of fibronectin, elastic fibers and type II collagen. Thus, in the current study, we estimated the activity of ELA, which is directly relative to the accumulation and activation of polymorphonuclear leukocytes in the inflamed tissue and pathological degradation of cartilage in RA. In our study, articular cartilage degradation resulted in increased ELA activity in arthritic rats when compared to control rats. Administration of triphala brought a substantial reduction in ELA activity, indicating its role in the inhibition of inflammatory cell invasion and neutrophil activation in arthritic rats.

Articular cartilage is a dense connective tissue composed of proteoglycans, collagen, non-collagenous proteins and glycoproteins. Any alteration in the metabolism of connective tissue during inflammatory conditions like RA leads to the excessive urinary excretion of hydroxyproline and glycosaminoglycans and reduction in bone collagen (Babu et al., 2014). In the present study, reduction in bone collagen was
observed in arthritic rats in association with the increased urinary excretion of hydroxyproline and glycosaminoglycans. This result indicates an alteration in the metabolism of collagen and support the fact that increased collagen degradation is associated with the severity of disease. On the other hand, the administration of triphala significantly regulated the collagen metabolism and maintained normal bone collagen levels that indicate prevention of cartilage degradation in arthritic rats as evidenced by the histopathological and radiographical analysis.

NO is an indispensable signaling molecule, excessively produced by inducible nitric oxide synthase (iNOS) (Esplugues, 2002). It is directly or indirectly engaged in damaging the articular constituents and leads to the initiation of pathogenesis in inflammatory arthritis (Bezerra et al., 2004). COX-2 is a rate-limiting enzyme upregulated in the RA synovium and responsible for the production of PGE₂ at the sites of inflammation (Ricciotti and FitzGerald, 2012; Shyni et al., 2015). COX-2 acts as a modulator during systemic inflammation by causing detrimental effects, including vasodilation, fever, pain and swelling. Therefore, restraining the activities of iNOS and COX-2 expression at transcriptional level can serve as a better therapeutic approach for inflammatory diseases. Interestingly, in the present work, we observed that administration of triphala decreased the mRNA expression of iNOS and COX-2 in the paw tissues of adjuvant-induced arthritic rats.

An imperative part of the inflammation is an imbalance and disruption in the homeostasis of pro-inflammatory and anti-inflammatory cytokines. Cytokines perpetuate the disease pathogenesis by the production of various inflammatory mediators like TNF-α, IL-1β, VEGF, MCP-1, and PGE₂. These cytokines facilitate in maintaining the synovial inflammation, activation of synovial macrophages, and neutrophils infiltration that ultimately leads to degradation of the extracellular matrix, complete destruction of cartilage, and bone loss (Zhang et al., 2013). Furthermore, pro-inflammatory cytokines and inflammatory catabolic mediators are activated by different transcription factors like NFκB and AP-1. NFκB is activated by ubiquitination and proteolytic degradation of IκBs and the AP-1 is being activated by the phosphorylation of mitogen-activated protein kinases (MAPKs) (Acquisto et al., 2002).
The pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β produced by the activated immune cells appear to be involved in the pathogenesis of joint inflammation and cartilage degradation during RA (Moelants et al., 2013). The progression of arthritis is further mediated by MCP-1, a chemokine that influence leukocyte trafficking and migration of activated immune cells, such as T and B cells, macrophages and neutrophils to the site of inflammation (Conti et al., 2002). In addition, evidences indicate that IL-17 produced mainly by the Th17 cells in the synovium manifests a crucial role in synovial inflammation and joint destruction in RA patients (Sarkar et al., 2010).

Inflammation and bone resorption are important features involved in the pathogenesis of RA. RANK and RANKL are members of the TNF superfamilies that play a crucial role in inflammation and osteoclast differentiation. RANKL is normally expressed in osteoblasts and activated immune cells, including T cells, B cells and dendritic cells. RANKL mediates osteoclastogenesis by binding to its receptor RANK on osteoclast precursor cells (Souza and Lerner, 2013). Recently, it has been proved that RA patients showed higher levels of RANKL (Xu et al., 2012). In the present study, triphala administration markedly downregulated the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17, and MCP-1), RANKL, and transcription factors (NFκB p65 and AP-1) in the paw tissues of arthritic rats. In addition, immunohistochemical analysis showed decreased protein expression of NFκB and COX-2 in triphala-treated arthritic rats. The transcription of pro-inflammatory cytokines, RANKL and COX-2 has been shown to be dependent on NF-κB activation. In this study, triphala administration significantly decreased NF-κB protein and gene expression in the paw tissues of arthritic rats, suggesting that suppressive effect of triphala on pro-inflammatory cytokines, inflammatory enzymes (iNOS, and COX-2), and RANKL could be due to its inhibitory action on NFκB signaling pathway.
4.5 CONCLUSION

In conclusion, triphala ameliorated bone and cartilage degradation observed in adjuvant-induced arthritic rats by the down-regulation of pro-inflammatory cytokines, inflammatory marker enzymes, RANKL and transcription factor NFκB and AP-1. Taken together, the present results demonstrate that triphala has the potential to be used as an anti-arthritic drug for the treatment of RA.