Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, Early Online: 1–10
 DOI: 10.3109/13880209.2014.910237

informa
healthcare

ORIGINAL ARTICLE

The anti-inflammatory effect of *triphala* in arthritic-induced rats

Sowmiya Kalaiselvan and Mahaboob Khan Rasool

Immunopathology Lab, School of Bio-Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

Abstract

Context: Triphala, an Indian Ayurvedic herbal formulation which contains *Terminalia chebula* Retz. (Combretaceae), *Terminalia bellerica* (Gaertn.) Roxb. (Combretaceae) and *Emblica officinalis* L. (Phyllanthaceae), is used for treating bowel-related complications, inflammatory disorders, and gastritis.

Objective: To determine the anti-arthritic effect of *triphala* in arthritis-induced rats. For comparison purpose, the non-steroidal anti-inflammatory drug indomethacin was used.

Materials and methods: Arthritis was induced in Wistar albino rats by intradermal injection of complete Freund's adjuvant (0.1 ml) into the foot pad of right hind paw. *Triphala* (100 mg/kg b wt, i.p.) was administered from day 11 to 18 after the administration of complete Freund's adjuvant. The activities/levels of lysosomal enzymes, glycoproteins, antioxidant status, and lipid peroxidation were determined in the paw tissues of arthritic rats. In addition, the inflammatory mediators were also measured in both the serum and the paw tissue of arthritic rats.

Results: The levels/activities of lipid peroxidation (∼41.5%), glycoproteins (hexose ∼43.3%, hexosamine ∼36.5%, and sialic acid ∼33.7%), lysosomal enzymes (acid phosphatase ∼52.4%, β-galactosidase ∼22.9%, *N*-acetyl β-glucosaminidase ∼22.1%, and cathepsin-D ∼27.7%) were found to be decreased and the antioxidant status (SOD ∼75.6%, CAT ∼62.7%, GPx ∼55.8%, GST ∼82.1%, and GSH ∼72.7%) was increased in the paw tissues of *triphala*-treated arthritic rats. In addition, the inflammatory mediator levels in serum (TNF-α ∼75.5%, IL-1β ∼99%, VEGF ∼75.2%, MCP-1 ∼76.4%, and PGE_2 ∼69.9%) and in paw tissues (TNF-α ∼71.6%, IL-1β ∼75.5%, VEGF ∼55.1%, MCP-1 ∼69.1%, and PGE_2 ∼66.8%) were found to be suppressed.

Conclusion: Triphala has a promising anti-inflammatory effect in the inflamed paw of arthritis-induced rats.

Keywords

Inflammatory mediators, lysosomal enzymes, rheumatoid arthritis

History

Received 8 January 2014
Revised 3 March 2014
Accepted 26 March 2014
Published online 7 October 2014

Introduction

Inflammation is one of the initial and crucial aspects of defense mechanism against pathogens, damaged cells, and toxic insults to the host. Thereby, it aids in restoring the normal function and structure of tissue. However, its deregulation contributes to the pathogenesis of many inflammatory disorders (Heo et al., 2012). Rheumatoid arthritis (RA) is a chronic, progressive, and systemic, inflammatory autoimmune disease affecting 1% of the world's population widely, whose etiology still remains perplexing. RA progression is portrayed by infiltration of activated immune cells in the synovial membrane and tissue of inflamed joint, notably macrophages and T-cells. In RA, the activated T-cells and macrophage result in discharge of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interferon gamma (IFN-γ) and other inflammatory mediators such as prostaglandin E_2 (PGE_2) which induce the expression of growth factors and chemokines such as vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1). Reactive oxygen species (ROS) like nitric oxide, superoxide anion radical, hydroxyl radical, and lysosomal enzymes make a close interrelationship for the propagation of inflammation and act as biochemical mediators for pathogenesis of RA. An increase in the ROS production serves in the diminution of endogenous antioxidants that eventually leads to cellular disruption of cells in rheumatoid arthritis condition. Also, the alterations in the levels of lysosomal enzymes, glycoproteins, and collagen are apparently observed in the elevated conditions of rheumatoid arthritis. Lysosomal enzymes are involved in discharge of glycohydrolases due to their decrease in stability which leads to degradation of structural macromolecules in connective tissue and proteoglycans in cartilage (Pragasam et al., 2013). Collectively, the release of pro-inflammatory mediators and lysosomal enzymes during arthritic condition plays a vital role in the formation of synovial hyperplasia, pannus, and unremitting inflammation in arthritic joints (Rasool et al., 2007).

Therefore, the agents conducting these inflammatory and biochemical mediators in harmony have potential therapeutic effects. Evolution of clinical practice and treatment stratagems for arthritis has been modified from non-steroidal

Correspondence: Dr. M. Rasool. Ph.D., Immunopathology Lab, School of Bio-Sciences and Technology, VIT University, Vellore 632 014, Tamil Nadu, India. Tel: +91 9629795044. E-mail: mkr474@gmail.com

anti-inflammatory drugs (NSAIDs) to novel biologics such as TNF-α and IL-1β antagonists in past few decades. In spite of these advancements made in treatment of RA, the side effects like gastrointestinal bleeding, renal morbidity, and hormonal disturbances still persists in their long-term usage (Umar et al., 2012). Considering the 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 is an ayurvedic poly herbal formulation consisting of the powdered component of three plant fruits, namely, *Terminalia chebula* Retz. (Combretaceae), *Terminalia bellerica* (Gaertn.) Roxb, and *Emblica officinalis* L. (Phyllanthaceae); in equal proportions (1:1:1) (Rasool et al., 2007). Intensive investigations on this formulation have shown a broad range of biological activities such as antioxidant, antitumor (Kaur et al., 2005), antidiabetic (Sabu et al., 2002), antiproliferative, antimutagenic (Kaur et al., 2002), and radioprotective effects (Jagetia et al., 2002). The individual plant constituents of *triphala* have also been reported to exhibit antiviral (Valsaraj et al., 1997), antibacterial (Ahmad et al., 1998), antifungal, antimalarial (Valsaraj et al., 1997), and antiallergic properties. Furthermore, our preliminary studies with *triphala* have proved its anti-inflammatory, immunomodulatory, and lysosomal membrane stabilizing effects on arthritis-induced animals (Sabina et al., 2009; Rasool et al., 2007).

To extend and correlate these findings, in this study, we have evaluated the anti-inflammatory effect of *triphala* in the inflamed paw of arthritis-induced rats with respect to oxidative damage, lysosomal destabilization, and inflammatory mediators. Indomethacin, a common NSAID, was used for comparison.

Materials and methods

Drug

Commercially available *triphala* powder [mixture of dried and powdered fruits of three plants, *T. chebula, E. officinalis*, and *T. bellerica* in equal proportions (1:1:1)] was obtained from Indian Medical Practitioners Cooperative Stores and Society (IMCOPS), Adyar, Chennai, India. The drug was prepared by removing the seeds from individual fruits and the dried fruit pulp was crushed to powder using a grinder. These powders were mixed in equal proportion (1:1:1) based on the formula of Ayurvedic Formulary of India. From the powder, the aqueous extract was prepared as described previously (Rasool & Sabina, 2007). The *triphala* powder used in this study was found to contain approximately 50% polyphenols as investigated by high-performance thin layer chromatography (HPTLC) densitometer analysis [toluene:ethyl acetate:glacial acetic acid–formic acid (20:45:20:05) solvent system]. Indomethacin was obtained from Tamil Nadu Dadha Pharmaceuticals, Chennai, India. All other reagents were of analytical grade and purchased locally.

GC-MS analysis

GC-MS was performed for the analysis of major compounds present in *triphala* extract. A Perkin Elmer, Clarus680 GC equipped and coupled to a Clarus600 mass spectrometer, was used in the EI mode with electron energy set at 70 eV was used. The column used in GC/MS was Elite-5MS (30.0 m, 0.25 mmID, 250 µmdf). Carrier gas used was helium at a constant flow rate of 1 ml/min. An injection volume of 1 µl was employed (split ratio of 10:1). Injector temperature 250 °C and column initial temperature 60 °C for 2 min, ramp 10 °C/min to 300 °C held for 6 min and the total run time was 32 min. The extract (1 µl) diluted with methanol was injected and the compounds were identified based on the molecular structure, molecular mass, and calculated fragment ratio of resolved spectra with that of mass spectra available from the library. Interpretation was conducted using the database of National Institute Standard and Technology (NIST) having more than 62 000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

Animals

Wistar albino rats of either sex, 125–150 g, were procured from the Animal House, VIT University, Vellore, India. They were acclimatized for a week in a light- and temperature-controlled room with a 12 h dark-light cycle and fed with commercial pellet feed from Hindustan Lever Ltd. (Mumbai, India); water was freely available. 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 experimental protocol was approved by our departmental ethics committee.

Experimental groups

Rats were divided into four groups each comprising six animals.

Group I: control rats treated with saline.

Group II: arthritis-induced rats.

Group III: arthritic rats treated with *triphala* (100 mg/kg b wt, i.p.).

Group IV: arthritic rats treated with standard drug indomethacin (3 mg/kg b wt, i.p.).

The dosage of *triphala* and standard drug indomethacin used in this study was selected based on our previous report and preliminary studies (Sabina et al., 2009).

Induction of arthritis and assessment

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

All treatments were administered intraperitoneally from day 11 to 18 after the administration of complete Freund's adjuvant. On day 19, at the end of the experimental period, the animals were killed by euthanasia and the blood was collected. The paw tissues were immediately dissected out and homogenized in ice-cold 0.01 M Tris HCl buffer, pH 7.4 to give a 10% homogenate. Tissue homogenate of paw tissues

and serum was used for biochemical and inflammatory mediator analysis.

Assessment of lipid peroxidation and antioxidant status

Lipid peroxidation in paw tissues was estimated by the method of Ohkawa et al. (1979). Malondialdehyde (MDA) produced during peroxidation of lipids served as an index of lipid peroxidation. MDA reacts with TBA to generate a color product, which absorbs at 532 nm. Superoxide dismutase (SOD) activity in paw tissues was determined by the method of Marklund and Marklund (1974). The degree of inhibition of the auto-oxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. Catalase and glutathione peroxidase activities in paw tissues were estimated by the method of Sinha (1972) and Rotruk et al. (1973). Glutathione-*S*-transferase (GST) was assayed by the method of Habig et al. (1974). Reduced glutathione was determined by the method of Moron et al. (1979). The protein content was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Assay of lysosomal enzymes

Acid phosphatase was assayed by the method of King (1965) using disodium phenyl phosphate as the substrate. The enzyme activity was expressed as μmoles of phenol liberated/min/mg protein. The activity of β-galactosidase was assessed by the method of Rosenblit et al. (1974) using 4-nitrophenyl-*N*-acetyl galactopyranoside as the substrate and its activity was expressed as μmoles of *p*-nitrophenol liberated/h/mg protein. *N*-Acetyl glucosaminidase activity was assessed by the method of Maruhn (1976) using 4-nitrophenyl-*N*-acetyl glucosaminide as the substrate, and its activity was expressed as μmoles of *p*-nitrophenol formed/h/mg protein. Cathepsin D activity was assayed by the method of Biber et al. (1981) using hemoglobin as the substrate and the activity was expressed as μmoles of tyrosine liberated/h/mg of protein. Protein content was measured by the method of Lowry et al. (1951).

Estimation of protein-bound carbohydrates

The paw tissues samples were defatted before estimation. A weighed amount of defatted tissue was suspended in 3 mL of 2 M HCl and heated at 90 °C for 4 h. The sample was cooled and neutralized with 3 mL of 2 M NaOH. Aliquots from this were used for the estimation of sialic acid, hexose, and hexosamine. To 0.1 mL of tissue homogenate, 5 mL of methanol was added, mixed well and centrifuged for 10 min at 3000 *g*. The supernatant was decanted, and the precipitate was again washed with 5 mL of 95% ethanol, recentrifuged, and the supernatant was decanted to obtain the precipitate of glycoproteins. Hexose was estimated by the method of Niebes (1972). The neutralized sample was mixed with orcinol–H_2SO_4 reagent, heated at 80 °C, cooled and left in the dark for 25 min for color development. The absorbance was read at 540 nm. Hexosamine was estimated by the method of Wagner et al. (1979). The acetylacetone reagent consisting of trisodium phosphate and potassium tetraborate with acetyl acetone was added to the plasma or tissue homogenate and boiled. After cooling, Ehrlich's reagent was added and the pink color developed was measured at 540 nm. Sialic acid was determined by the method of Aminoff (1961) with modifications by Niebes (1972). The neutralized sample was mixed with 0.25 M periodate (in 0.1 N H_2SO_4), and the reaction was inhibited after 30 min by the arsenite solution. Then thiobarbituric acid was added and the contents were heated. The pink color that developed on cooling was measured at 540 nm.

Assessment of inflammatory mediators

Cytokine levels (TNF-α, IL-1β, VEGF, MCP-1, and PGE_2) in serum and paw tissue homogenates were determined by using commercially available ELISA kits according to the manufacturer's instructions (Peprotech, Rocky Hill, NJ).

Results

Identification of bioactive compounds from *triphala* extracts by GC-MC analysis

Identification of bioactive compounds present in the *triphala* aqueous extract has been performed by gas chromatography/mass spectrometry (GC-MS). *Triphala* extract contains many phytoconstituents like flavonoids, tannins, terpenoids, polyphenols, alkaloids, and glycosides. Nevertheless, it is positively rich in tannins and polyphenols and the chromatogram reveals three major peaks corresponding to gallic acid, ellagic acid, and chebulagic acid (Figure 1).

Effect of *triphala* on lipid peroxidation level

Table 1 illustrates the effect of *triphala* on the levels of lipid peroxidation in the paw tissues of control and experimental rats. The levels of lipid peroxides were significantly increased in arthritic rats compared to the control group. In contrast, the administration of *triphala* to arthritis-induced rats reinstated the lipid peroxide level (∼41.5%) to nearly that of normal levels.

Effect of *triphala* on antioxidant status

Table 2 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.

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 3. 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

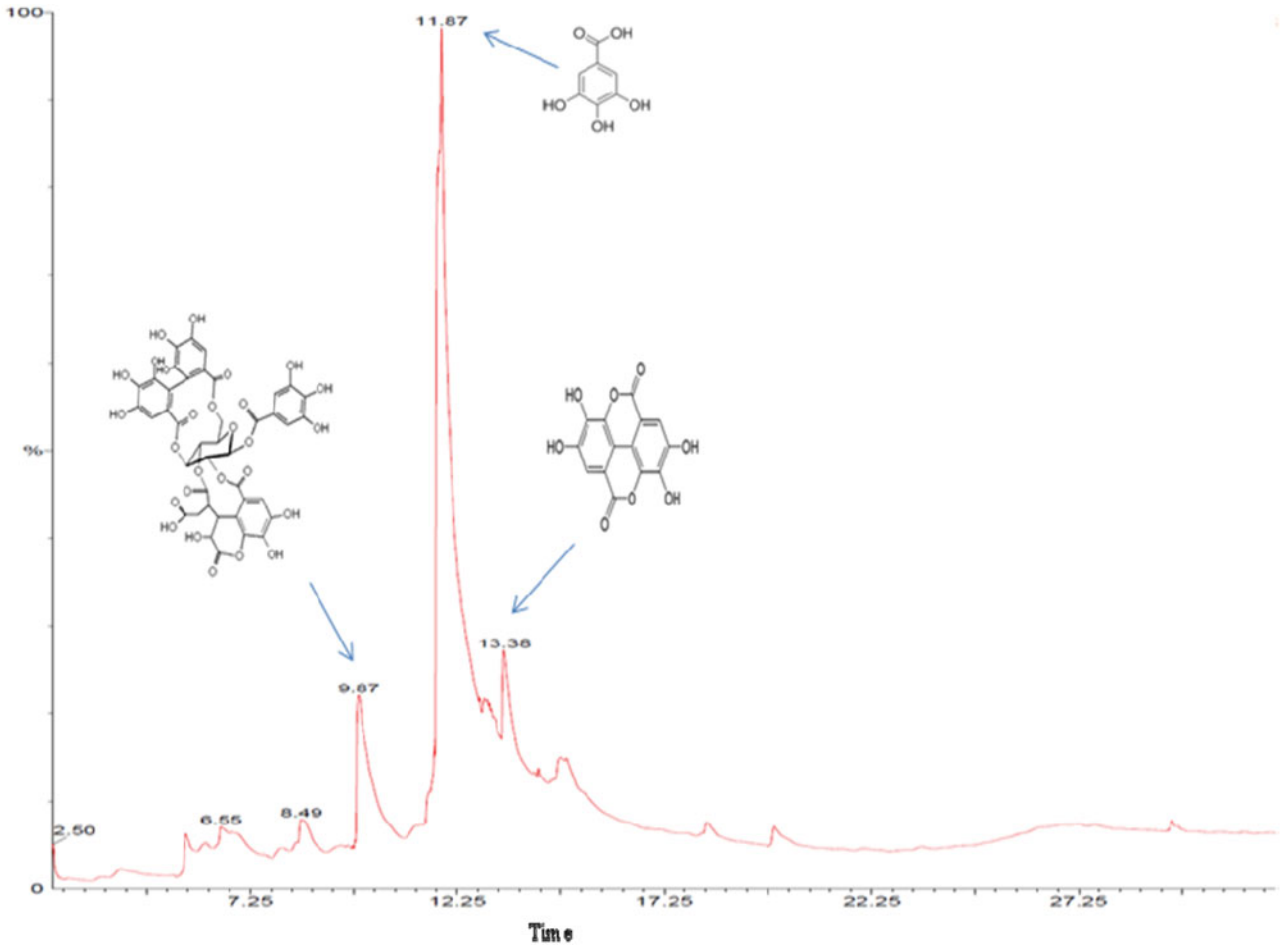

Figure 1. Gas chromatography/mass spectrometry analysis of *triphala* extract results in chromatogram of three major peaks (retention time 11.87), corresponding to gallic acid, (retention time 13.38), corresponding to ellagic acid, and (retention time 9.87) corresponding to chebulagic acid.

Table 1. Effect of *triphala* and indomethacin on lipid peroxidation in paw tissue of control and experimental rats.

Parameter	Control	Arthritis	Arthritis + *triphala* (100 mg/kg b wt)]	Arthritis + indomethacin (3 mg/kg b wt)
Lipid peroxidation (malonaldehyde formed/ mg protein)	40.83 ± 4.90	110 ± 9.5[a]	78.3 ± 17.75[ab]	73.3 ± 2.5[ab]

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 2. Effect of *triphala* and indomethacin on antioxidant status in paw tissues of control and experimental rats.

Parameter	Control	Arthritis	Arthritis + *triphala* (100 mg/kg b wt)	Arthritis + indomethacin (3 mg/kg b wt)
SOD (units/mg protein/min)	16.20 ± 0.92	4.21 ± 2.47[a]	13.05 ± 1.38[ab]	13.8 ± 1.70[b]
CAT (μmol of H_2O_2 consumed/min/mg protein)	68.02 ± 11.97	24.88 ± 5.63[a]	41.81 ± 4.12[ab]	49.31 ± 11.50[ab]
GPx (μg of GSH utilized/min/mg protein)	24.54 ± 2.67	10.64 ± 1.92[a]	20.54 ± 1.40[b]	19.87 ± 1.86[ab]
GST (nmol of 1-chloro-2,4-dinitrobenzene-GSH conjugate formed/min/mg protein)	3.78 ± 0.61	0.44 ± 0.12[a]	2.03 ± 0.54[ab]	2.35 ± 1.00[ab]
GSH (nmol/mg/protein)	79.90 ± 15.40	22.40 ± 3.60[a]	59.75 ± 15.77[b]	61.33 ± 19.60[b]

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 3. Effect of *triphala* and indomethacin on the activities of lysosomal enzymes in paw tissues of control and experimental rats.

Parameters	Control	Arthritis	Arthritis + *triphala* (100 mg/kg b wt)	Arthritis + indomethacin (3 mg/kg b wt)
Acid phosphatase (μmol × 10^{-2} of phenol)	0.27 ± 0.05	0.61 ± 0.13[a]	0.32 ± 0.07[b]	0.27 ± 0.03[b]
β-Galactosidase (μmol × 10^{-2} of *p*-nitrophenol liberated/h/mg protein)	1.37 ± 0.18	4.69 ± 1.20[a]	1.40 ± 0.61[b]	1.39 ± 0.19[b]
N-Acetyl β-glucosaminidase (μmol × 10^{-2} of *p*-nitrophenol liberated/h/mg protein)	1.06 ± 0.13	3.98 ± 1.29[a]	1.13 ± 0.23[b]	1.15 ± 0.10[b]
Cathepsin-D (μmol × 10^{-2} of tyrosine liberated/h/mg protein)	0.27 ± 0.10	0.91 ± 0.11[a]	0.35 ± 0.05[b]	0.37 ± 0.41[b]

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. Effect of *triphala* and indomethacin on protein bound carbohydrate in paw tissues of control and experimental rats.

Parameter (mg/g defatted tissue)	Control	Arthritis	Arthritis + *triphala* (100 mg/kg b wt)]	Arthritis + indomethacin (3 mg/kg b wt)
Hexos	1.04 ± 0.03	1.58 ± 0.17[a]	1.21 ± 0.07[ab]	1.26 ± 0.04[ab]
Hexosamine	1.39 ± 0.62	14.29 ± 1.20[a]	8.23 ± 0.59[ab]	7.97 ± 0.55[ab]
Sialic acid	186.52 ± 1.18	387.92 ± 46.3[a]	197.56 ± 5.31[b]	200.36 ± 3.78[b]

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.

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.

Effect of *triphala* on protein bound carbohydrates

Table 4 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.

Effect of *triphala* on inflammatory mediators

Figures 2 and 3 show the effect of *triphala* on inflammatory mediators like TNF-α, IL-1β, VEGF, MCP-1, and PGE_2 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 PGE_2) 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 PGE_2 ~69.9%) and paw tissue (TNF-α ~71.6%, IL-1β ~75.5%, VEGF ~55.1%, MCP-1 ~69.1%, and PGE_2 ~66.8%) inflammatory mediator levels as compared with the arthritic control.

Discussion

Adjuvant induced arthritis is a severe experimental arthritis model for rats and mice widely used for studying the pathogenesis of human rheumatoid arthritis and for searching new drugs for the treatment of rheumatoid arthritis. This model describes the induction of rheumatoid arthritis by injecting adjuvant (*Mycobacterium butyricum* suspended in mineral oil) in base of the tail or in one of the foot pads in rats which produce an immune reaction that characteristically involves inflammatory destruction of cartilage and bone of the distal joints. The immunological response is due to a cross reaction between a joint molecule and the mycobacterium antigen (Bendele, 2001). In the present study, we evaluated the anti-inflammatory effect of *triphala* in the inflamed paw of arthritis-induced rats with respect to oxidative damage, lysosomal destabilization, and inflammatory mediators.

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 et al., 2008).

In order to impede the toxicity produced by free radicals, bone cells maintain a well-coordinated free radical

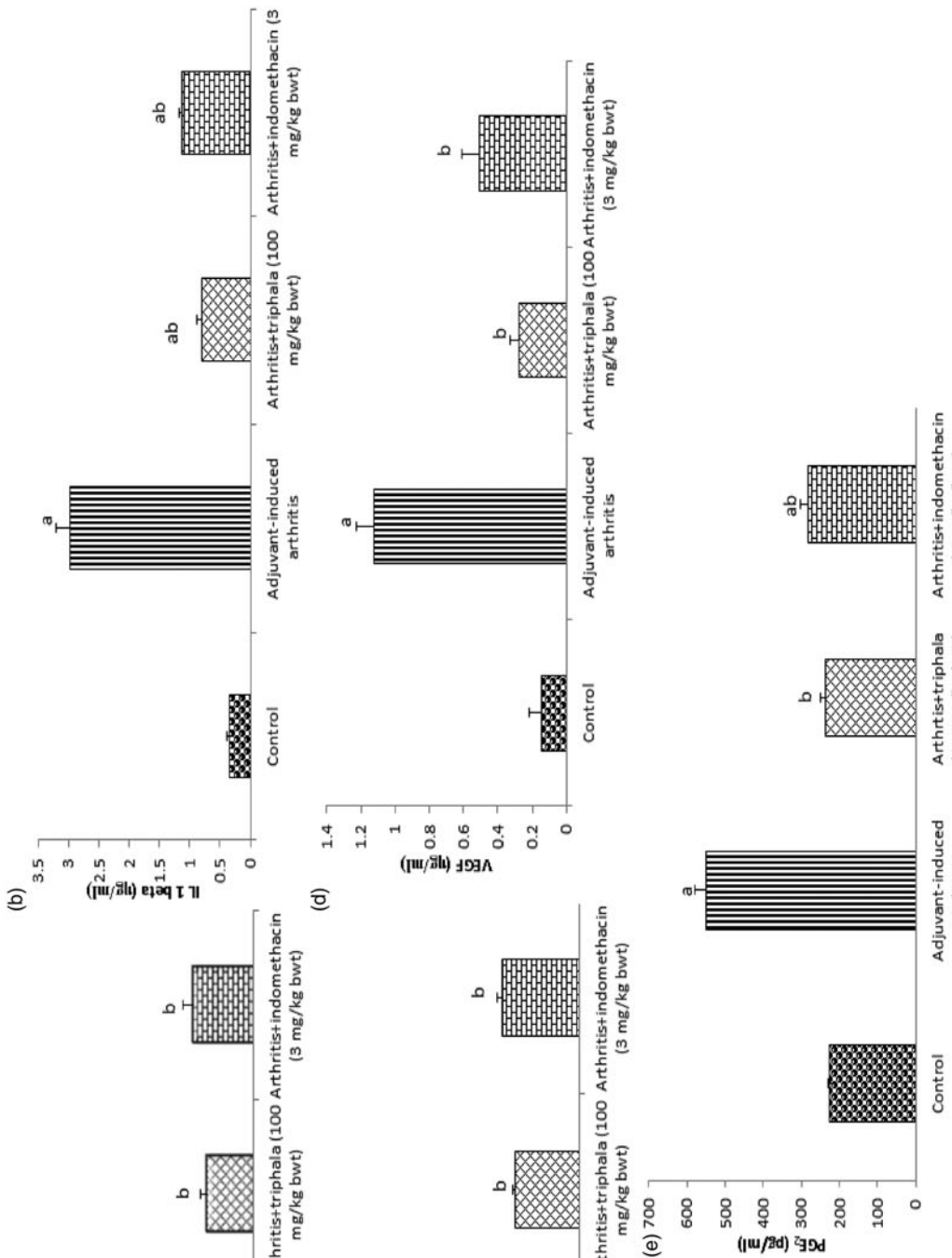

Figure 2 (a–e). Effect of *triphala* on cytokine level in serum of control and experimental rats of (a) TNF-α, (b) IL-1β, (c) MCP-1, (d) VEGF, (e) PGE_2. Values are expressed as mean ± S.D. for six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis + indomethacin; (b) Arthrtis 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.

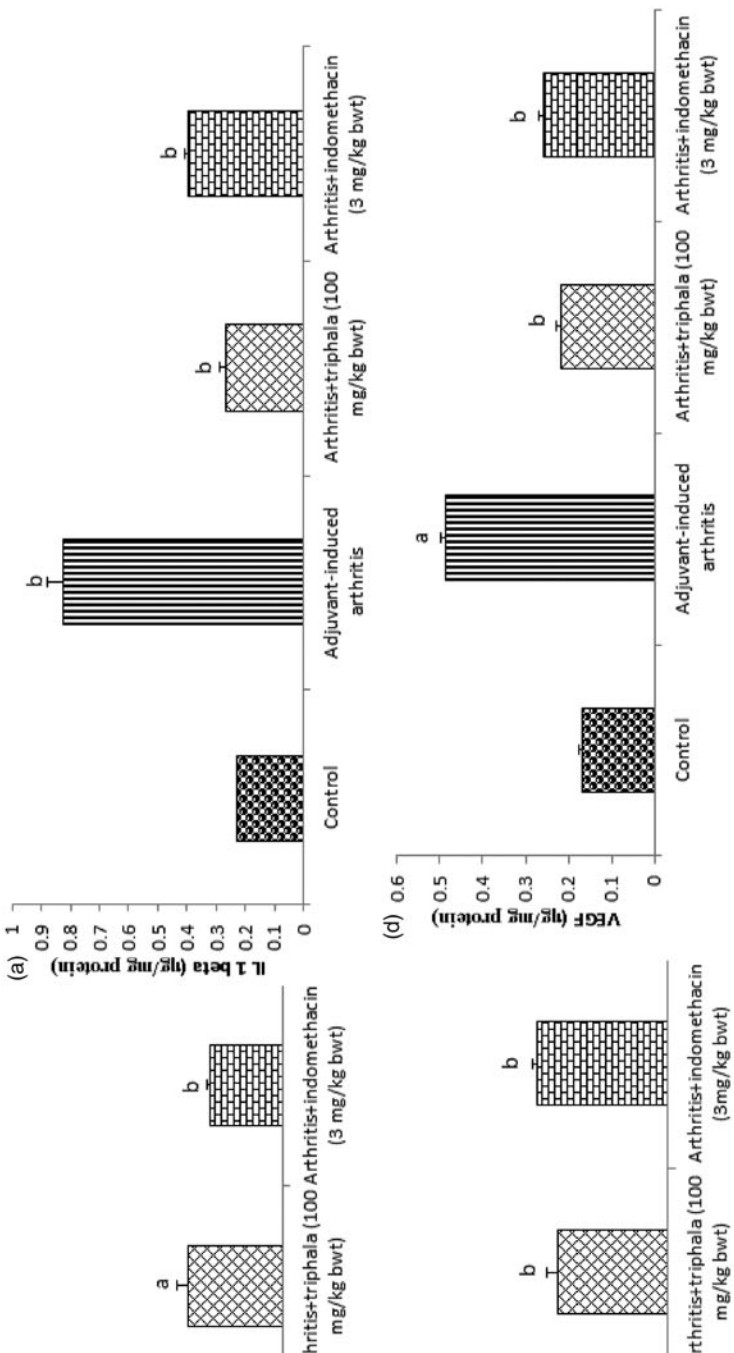

Figure 3 (a–e). Effect of *triphala* on cytokine level in paw tissue of control and experimental rats of (a) TNF-*α*, (b) IL-1β, (c) MCP-1, (d) VEGF, and (e) PGE_2. Values are expressed as mean ± S.D. for six animals. Comparisons were made as follows: (a) control versus arthritis, arthritis + indomethacin; (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.

scavenging 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 H_2O_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*-transeferase levels were significantly decreased compared with the control rats which are in line with our previous findings (Rasool et al., 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 et al., 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, the activities of lysosomal enzymes in paw tissues were elevated in arthritic rats compared with the control 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 (Cheng et al., 2003; Hari Kumar et al., 2004; Lee et al., 2005; Naik et al., 2005).

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 PGE_2. These cytokines facilitate in maintaining the synovial inflammation, activation of synovial macrophages, and neutrophils infiltration which ultimately leads to degradation of the extracellular matrix, complete destruction of cartilage, and bone loss (Zhang et al., 2013).

TNF-α is a major pleiotropic cytokine which plays a prominent role in all inflammatory disorders. The major sources of TNF-α are macrophage like synoviocytes, fibroblast-like synoviocytes, and activated T-cells. These cell types exhibit activated phenotype and produce other pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α itself, thus creating a positive feedback mechanism for the perpetuation of synovial inflammation (Li et al., 2008). IL-1β another major pro-inflammatory cytokine plays a key role in the activation of synovial cells, osteoclasts for the bone resorption, matrix metalloproteases, and proteases for the cartilage erosion in the joints (Iwakura, 2002). These two cytokines act synergistically in attracting and activating the immune cells in the synovium and is also involved in oxidative stress for degradation of articular cartilage and eventually complete bone loss (Chen & Wei, 2003). In this study, it was found that both TNF-α and IL-1β were seen in high levels in both serum and paw tissues of arthritis-induced rats. However, *triphala*-administered arthritic rats exhibited significant reduction in TNF-α and IL-1β levels in serum as well as in paw tissues. This anti-inflammatory property of *triphala* might be due to its active components like ellagic acid, gallic acid, 4-*O*-methylgallic acid, and bellericanin which have shown inhibitory action on TNF-α and IL-1β (Na et al., 2006; Yu et al., 2007; Zhao et al., 2008).

MCP-1 plays a key role in pathogenesis of rheumatoid arthritis by recruiting neutrophils, monocytes, and B-cells to the site of inflammation. Besides, MCP-1 is involved in the migration of activated T-cell and attracts the activated monocytes/macrophages to the site of inflammation for the production of TNF-α and IL-1β (Reale et al., 2001). Based on this report, it is appropriate to study the levels of MCP-1 in inflamed paw tissue and serum of arthritis-induced rats. Data observed in our present study showed increased levels of MCP-1 in arthritis-induced rats compared with the control animals. Angiogenesis plays a key role in normal vascular

development and is a critical factor in cancer, wound healing, and inflammation. Synovial lining angiogenesis is a prerequisite for pannus formation in rheumatoid arthritis. Although several pro-angiogenic factors have been identified, the most potent of these angiogenic factors appears to be vascular endothelial growth factor (VEGF) (Cho et al., 2006). VEGF plays a critical role in the chronic edema and swelling of rheumatoid arthritis, since it increases vascular permeability (Malemud, 2007). In the present study, our results revealed that *triphala*-administrated arthritic rats exhibited reduction in MCP-1 and VEGF levels in serum and paw tissues compared with the arthritic control animals. This anti-inflammatory effect might be due to the inhibition of TNF-α by the phytochemicals present in *triphala* which is in consistent with the previous reports (Lee et al., 2005; Sabina et al., 2008).

Prostaglandin E_2 (PGE_2) is a primary product of arachidonic metabolism and is synthesized via the cyclooxygenase and prostaglandin synthase pathways. Over production of PGE_2 is found to result in pain, fever, swelling, and major structural change in cartilage cells of arthritic rats. In our present study, we found that administration of *triphala* to arthritic rats brought a reduction in the level of PGE_2 in both the serum and paw tissues. This is in concordance with the previous reports that chebulagic acid one of the constituents of *triphala* has shown to inhibit the COX-2 expression by blocking NF-κB mediated inflammatory pathway (Karlsson et al., 2010; Reddy et al., 2009).

In the present study, GC-MS analysis of *triphala* revealed the presence of many bioactive compounds like flavonoids, alkaloids, glycosides, polyphenols, sterols, and terpenoids. The predominant among them are gallic acid, ellagic acid, and chebulagic acid and they are shown to exhibit antioxidant, immunomodulatory, antibacterial, and anticancer properties (Lee et al., 2005). Hence, the anti-inflammatory effect of *triphala* observed in our study might be due to the combined interactions of these phytochemicals. However, further experimentation is required to examine the efficacy of the *triphala* and its active principles in molecular level to prove its mechanism of action whose progress is underway.

Acknowledgements

The authors extend their gratitude to the VIT management for supporting the research work by facilitating the necessary requirements.

Declaration of interest

The authors report no declarations of interest. The authors are grateful for the financial assistance (59/2/2011/BMS/TRM) provided by Indian Council of Medical Research, Government of India, to carry out this research work.

References

Ahmad I, Mehmood Z, Mohammad F. (1998). Screening of some Indian medicinal plants for their antimicrobial properties. *J Ethnopharmacol* 62:183–93.

Aminoff D. (1961). Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem J* 81:384–92.

Babior BM, Kipens RS, Curnutte JT. (1973). The production by leukocytes of superoxide dismutase, a potential bactericidal agent. *J Clin Invest* 52:741–4.

Bendele AM. (2001). Animal models of rheumatoid arthritis. *J Musculoskelet Neuronal Interact* 4:377–85.

Biber J, Stieger B, Haase W, Murer H. (1981). A high yield preparation for rat kidney brush-border membranes, different behaviors of lysosomal markers. *Biochim Biophys Acta* 647:169–76.

Chen Q, Wei W. (2003). Effects and mechanisms of glucosides of *Chaenomeles speciosa* on collagen-induced arthritis in rats. *Int Immunopharmacol* 3:593–608.

Cheng HY, Lin TC, Yu KH, et al. (2003). Antioxidant and free radical scavenging activities of *Terminalia chebula. Biol Pharm Bull* 26: 1331–5.

Cho ML, Jung YO, Moon YM, et al. (2006). Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblasts via AP-1-dependent pathways. *Immunol Lett* 103:159–66.

Habig WH, Pabst MJ, Jakoby WB. (1974). Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249:7130–9.

Hari Kumar KB, Sabu MC, Lima PS, Kuttan R. (2004). Modulation of haematopoetic system and antioxidant enzymes by *Emblica officinalis* Gaertn and its protective role against gamma-radiation induced damages in mice. *J Radiat Res (Tokyo)* 45:549–55.

Heo SJ, Yoon WJ, Kim KN, et al. (2012). Anti-inflammatory effect of fucoxanthin derivatives isolated from *Sargassum siliquastrum* in lipopolysaccharide-stimulated RAW 264.7 macrophage. *Food Chem Toxicol* 50:3336–42.

Iwakura Y. (2002). Roles of IL-1 in the development of rheumatoid arthritis: Consideration from mouse models. *Cytokine Growth Factor Rev* 13:341–55.

Jagetia GC, Baliga MS, Malagi KJ, Kamath SM. (2012). The evaluation of the radioprotective effect of *Triphala* (an ayurvedic rejuvenating drug) in the mice exposed to gamma-radiation. *Phytomedicine* 9: 99–108.

Karlsson S, Nanberg E, Fjaeraa C, Wijkander J. (2010). Ellagic acid inhibits lipopolysaccharide induced expression of enzymes involved in the synthesis of prostaglandin E2 in human monocytes. *Br J Nutr* 103: 1109–19.

Kaur S, Arora S, Kaur K, Kumar S. (2002). The *in vitro* antimutagenic activity of Triphala – an Indian herbal drug. *Food Chem Toxicol* 40: 527–34.

Kaur S, Michael H, Arora S, et al. (2005). The *in vitro* cytotoxic and apoptotic activity of Triphala – an Indian herbal drug. *J Ethnopharmacol* 97:15–20.

King J. (1965). The hydrolases-acid and alkaline phosphatases. In: Van D, ed. *Practical Clinical Enzymology*. London: Nostrand Company Ltd, 191–208.

Kurien BT, Hensley K, Bachmann M, Scofield RH. (2006). Oxidatively modified autoantigens in autoimmune diseases. *Free Radic Biol Med* 41:549–56.

Lee SI, Hyun PM, Kim SH, et al. (2005). Suppression of the onset and progression of collagen induced arthritis by chebulagic acid screened from a natural product library. *Arthritis Rheum* 52:345–53.

Li H, Zhang YY, Tan HW, et al. (2008). Therapeutic effect of tripterine on adjuvant arthritis in rats. *J Ethnopharmacol* 118:479–84.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–75.

Malemud CJ. (2007). Growth hormone, VEGF and FGF: Involvement in rheumatoid arthritis. *Clin Chim Acta* 375:10–19.

Marklund SL, Marklund G. (1974). Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 47:469–74.

Maruhn D. (1976). Rapid colorimetric assay of beta-galactosidase and *N*-acetyl-beta-glucosaminidase in human urine. *Clin Chim Acta* 73: 453–61.

Moron MS, Depierre JW, Mannervik B. (1979). Levels of glutathione, glutathione reductase and glutathione-*S*-transferase activities in rat lung and liver. *Biochem Biophys Acta* 582:67–78.

Mythilipriya R, Shanthi P, Sachdanandam P. (2007). Restorative and synergistic efficacy of Kalpaamruthaa, a modified siddha preparation, on an altered antioxidant status in adjuvant induced arthritic rat model. *Chem Biol Interact* 168:193–202.

Na HJ, Lee G, Oh HY, et al. (2006). 4-*O*-Methylgallic acid suppresses inflammation-associated gene expression by inhibition of redox-based NF-κB activation. *Int Immunopharmacol* 6:1597–608.

Naik GH, Priyadarsini KI, Bhagirathi RG, et al. (2005). *In vitro* antioxidant studies and free radical reactions of Triphala, an Ayurvedic formulation and its constituents. *Phytother Res* 19:582–6.

Niebes P. (1972). Determination of enzymes and degradation products of glycosaminoglycan metabolism in the serum of healthy and varicose subjects. *Clin Chim Acta* 42:399–408.

Ohkawa H, Ohishi N, Yagi K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95:351–8.

Pragasam SJ, Murunikkara V, Sabina EP, Rasool M. (2013). Ameliorative effect of *p*-coumaric acid, a common dietary phenol, on adjuvant-induced arthritis in rats. *Rheumatol Int* 33:325–34.

Rasool M, Sabina EP. (2007). Anti inflammatory effect of the Indian Ayurvedic herbal formulation Triphala on adjuvant-induced arthritis in mice. *Phytother Res* 21:889–94.

Rasool M, Varalakshmi P. (2007). Protective effect of *Withania somnifera* root powder in relation to lipid peroxidation, antioxidant status, glycoproteins and bone collagen on adjuvant-induced arthritis in rats. *Fund Clin Pharmacol* 2:157–64.

Reale M, Barbacane RC, DiGioacchino M, et al. (2001). Differential expression and secretion of RANTES and MCP-1 inactivated peripheral blood mononuclear cell cultures of atopic subjects. *Immunol Lett* 76:7–14.

Reddy DB, Reddanna P. (2009). Chebulagic acid (CA) attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages. *Biochem Biophys Res Commun* 381:112–17.

Ronzio B. (2000). Polyphenols as anti-inflammatory agents. *J Naturopathic Med* 9:44–50.

Rosenblit PD, Metzyer RP, Wick AN. (1974). Effect of streptozotocin diabetes on acid phosphatase and selected glycosidase activities of serum and various rat organs. *Proc Soc Exp Biol Med* 14:244–8.

Rotruk JT, Pope AL, Ganther HE, et al. (1973). Selenium, biochemical role as a component of glutathione peroxidase purification and assay. *Science* 179:588–90.

Sabina EP, Rasool MK, Mathew L. (2009). *In vivo* and *in vitro* immunomodulatory effects of Indian Ayurvedic herbal formulation Triphala on experimental induced inflammation. *Pharmacologyonline* 2:840–9.

Sabina EP, Rasool M. (2008). An *in vivo* and *in vitro* potential of Indian ayurvedic herbal formulation Triphala on experimental gouty arthritis in mice. *Vasc Pharmacol* 48:14–20.

Sabu MC, Kuttan R. (2002). Antidiabetic activity of medicinal plants and its relationship with their antioxidant property. *J Ethnophamacol* 81:155–60.

Sinha AK. (1972). Colorimetric assay of catalase. *Anal Biochem* 47: 389–94.

Umar S, Zargan J, Umar K, et al. (2012). Modulation of the oxidative stress and inflammatory cytokine response by thymoquinone in the collagen induced arthritis in Wistar rats. *Chem Biol Interact* 197: 40–6.

Valsaraj R, Pushpangadan P, Smitt UW, et al. (1997). New anti-HIV-1, antimalarial, and antifungal compounds from *Terminalia bellerica*. *J Nat Prod* 60:739–42.

Veal EA, Toone WM, Jones N, Morgan BA. (2002). Distinct roles for glutathione *S*-transferases in the oxidative stress response in *Schizosaccharomyces pombe*. *J Biol Chem* 277:35523–31.

Vijayalakshmi T, Muthulakshmi V, Sachdanandam P. (1997). Effect of milk extract of *Semecarpus anacardium* nuts on glycohydrolases and lysosomal stability in adjuvant arthritis in rats. *J Ethnopharmacol* 58: 1–8.

Wagner WD. (1979). A more sensitive assay discriminating galactosamine and glucosamine in mixtures. *Anal Biochem* 94:394–6.

Yu YM, Wang ZH, Liu CH, Chen CS. (2007). Ellagic acid inhibits IL-1beta-induced cell adhesion molecule expression in human umbilical vein endothelial cells. *Br J Nutr* 97:692–8.

Zhang Y, Xu W, Li H, et al. (2013). Therapeutic effects of total alkaloids of *Tripterygium wilfordii* Hook f. on collagen-induced arthritis in rats. *J Ethnopharmacol* 145:699–705.

Zhao L, Zhang SL, Tao JY, et al. (2008). Preliminary exploration on anti-inflammatory mechanism of corilagin (β-1-*O*-galloyl-3, 6-(*R*)-hexahydroxydiphenoyl-D-glucose) *in vitro*. *Int Immunopharmacol* 8:1059–64.