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Trikatu, an herbal compound ameliorates rheumatoid arthritis by the suppression of inflammatory immune responses in rats with adjuvant-induced arthritis and on cultured fibroblast like synoviocytes via the inhibition of the NFκB signaling pathway

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ABSTRACT

The present study was designed to investigate the potential therapeutic effect of trikatu, an herbal compound and its underlying molecular mechanism in rats with adjuvant-induced arthritis (AIA). Our results indicate that trikatu (1000 mg/kg/b.wt. oral) administration suppressed the production of pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein (MCP)-1) and downregulated the mRNA expression levels of inflammatory mediators (TNF-α, IL-1β, IL-6, IL-17, MCP-1, receptor activator of nuclear factor kappa B ligand (RANKL), cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS)) and transcription factors (nuclear factor kappa B 65 (NFκB-p65) and activator protein-1 (AP-1)) in cultured AIA-fibroblast like synoviocytes and synovial tissue of AIA rats. Consistently, the protein expression of NFκB-p65, IL-17, TNF-α, COX-2, and RANKL was also dramatically reduced in cultured AIA-fibroblast like synoviocytes and synovial tissue of AIA rats by trikatu treatment. In addition, trikatu suppressed the expression and phosphorylation of NFκB-p65 similar to the Bay 11–7082 (NFκB inhibitor) in cultured AIA-fibroblast like synoviocytes. Furthermore, trikatu alleviated the histopathology of joint of arthritic rats. Overall, these data highlights that trikatu could be a promising alternative modality for the possible treatment of rheumatoid arthritis and other inflammatory diseases.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease mainly affecting joints, characterized by the migration of inflammatory cells and proliferation of fibroblast-like synoviocytes (FLS) leading to synovial hyperplasia, progressive cartilage and bone destruction and functional deformity [1]. RA-FLS, a major constituent of the synovial hyperplasia are an important class of cells that contributes to the pathogenesis of disease progression by regulating the secretion of inflammatory mediators: tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17), monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS)] [2–4]. The strongest evidences have suggested that intracellular signaling molecules such as nuclear factor κB (NFκB) and activator protein-1 (AP-1) are highly activated in RA-FLS and involved in the pathogenesis of RA [5,6]. NFκB and AP-1 are key transcription factors that control the expression of a plethora of genes involved in fundamental cellular processes, such as cell proliferation, differentiation, immunity and apoptosis. In response to the pro-inflammatory stimuli such as TNF-α and IL-1β, NFκB and AP-1 are potentially activated in RA-FLS and produce proinflammatory cytokines, chemokines, inflammatory enzymes, matrix-metalloproteinases (MMPs), angiogenic factors, and adhesion molecules that contribute to chronic inflammation, neovascularization, and arthritic joint destruction [7]. In addition to their central role in inflammation, several preclinical and human studies have demonstrated that RA-FLS display prominent effect in RA via production of RANKL (receptor activator of nuclear factor-κB ligand) resulting in the excessive stimulation of osteoclast formation and bone erosion [8].

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Current therapeutics mainly include the use of cytokine blockers, non-steroidal anti-inflammatory drugs (NSAIDs) and the disease modifying anti-rheumatic drugs (DMARDs) that have improved the treatment scenario in the management of RA [9,10]. However, clinical trial data show about 30–50% of patients do not achieve clinically meaningful responses to these standard therapies due to their associated adverse effects, including gastrointestinal lesions, cardiovascular complications, and hepatotoxicity and substantially decrease the overall benefits of long-term treatment [11]. Under these circumstances, the use of natural products that are capable of exerting anti-inflammatory effects and reducing the toxicity of pharmacological agents could serve as a potential alternative [12]. Recent studies have reported that the use of herbal and plant based products for the treatment of RA has shown promising results in disease attenuation [13].

Trikatu is a polyherbal compound consisting of three components, namely the dried fruits of black pepper (*Piper nigrum* Linn.), dried fruits of long pepper (*Piper longum* Linn.) and dried rhizomes of ginger (*Zingiber Officinalis* Rosc) in the equal weight ratio of 1:1:1 [14]. Trikatu has been used to treat a wide range of ailments and to increase the bioavailability of other compounds, including herbal, nutrients and pharmaceutical drugs [15]. Recently, trikatu and its major components (piperine, 6-shogaol and 6-gingerol) were found to inhibit the onset and severity of disease through the reduction of paw edema and vital biochemical parameters in both AIA and acute gouty arthritis animal models [15–18]. However, the precise molecular mechanisms of trikatu have not been studied yet. Therefore, the objective of the study was to investigate the anti-arthritic effect of trikatu and its underlying therapeutic mechanisms in cultured FLS cells and in synovial tissues of AIA rats. For comparison purpose, methotrexate (MTX), a common DMARD was used as a reference drug.

2. Materials and methods

2.1. Drug and chemicals

Black pepper (dried fruit of *Piper nigrum* Linn.), long pepper (dried fruit of *Piper longum* Linn.), and ginger (dried rhizome of *Zingiber officinale* Rosc.) were procured from an herbal drug supplier, Chennai, India and authenticated by our chief botanist at VIT University, India. Trikatu was prepared by mixing the three powdered fruits in equal proportions according to the Indian Ayurvedic formula. Methotrexate (MTX), gallic acid, vanillic acid, ferulic acid, *p*-coumaric acid, morin, 6-gingerol, piperine, catechin, and quercetin were obtained from Sigma Aldrich (St. Louis, USA). Antibodies against *p*-NFκB-p65, NFκB-p65 and COX-2 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against IL-17, TNF-α, and RANKL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ELISA kits for TNF-α, IL-1β, IL-6, and MCP-1 were purchased from PeproTech (NJ, USA).

2.2. Preparation of trikatu extract

Trikatu was prepared by mixing the three powdered fruits of black pepper (dried fruit of *Piper nigrum* Linn.), long pepper (dried fruit of *Piper longum* Linn.), and ginger (dried rhizome of *Zingiber officinale* Rosc.) in equal proportions (w/w) according to the Indian Ayurvedic formula. 100 g of trikatu were extracted in sterile milli-Q water using ultra sonication at room temperature for 1 h. Trikatu extract was then dried using a rotary evaporation under reduced pressure at a temperature of 45 °C and lyophilized. The extract final yield after lyophilization was stored at −20 °C until use. The extract was further dissolved in culture medium and filtered through a 0.22 μm filter before treatment to the cells.

2.3. High performance liquid chromatography (HPLC) analysis of trikatu

The qualitative phytochemical screening of trikatu was tested for the presence of polyphenols and flavanoids. The HPLC was performed with the prominence HPLC system (Shimadzu, Tokyo, Japan) consisting of Phenomenox Luna 5-μm C_{18} column (250 × 4.6 mm). A standard for gallic acid, vanillic acid, ferulic acid, *p*-coumaric acid, morin, 6-gingerol, piperine, catechin, and quercetin was prepared using HPLC grade methanol for calibration. The lyophilized trikatu extract was dissolved in HPLC grade methanol and filtered into HPLC vials. The optimized HPLC conditions were: flow-rate, 1 ml/min; injecting volume, 20 μl; run time, 45 min; mobile phase gradient between acetonitrile and 2% acetic acid-water. The chromatograms were recorded at 280 nm and 360 nm for the detection of phenolic acids and flavonoids respectively. Active components were identified by comparing with the retention time (RT) of their respective standards.

2.4. Gas chromatography –Mass spectrometry (GC-MS) analysis of trikatu

GC-MS analysis of trikatu was carried out on a Perkin Elmer, Clarus680 GC equipped and coupled to a Clarus600 mass spectrometer, in the EI mode with electron energy set at 70 eV. The column used for GC-MS analysis was Elite-5MS (30.0 m × 0.25 mm × 25 μm). Helium was used as the carrier gas at a constant flow rate of 1 ml/min. Splitless injection of 2 μl of the sample was employed. Injector temperature was set at 250 °C and detector temperature at 280 °C. The column temperature was set at 60 °C and ramped at 10 °C/min to 300 °C. The component identification was achieved by comparison of the retention time and mass spectra available in the NIST (National Institute Standard and Technology) library. The measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

2.5. Animals

Wistar albino rats of either sex weighing between 150 and 180 g were procured from the animal house at VIT University, India. The animals were acclimatized for a week in a light and temperature - controlled room with a 12- hour light and dark cycle and free access to food and water. The animals were treated and cared for in accordance with the stipulated guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. All experimental procedures were approved by the Institutional Animal Ethical Committee (IAEC), VIT University, India.

2.6. Arthritis induction and drug treatment

Arthritis was induced by a single intra dermal injection of 0.1 ml of Freund's complete adjuvant (CFA) into the foot-pad of the right hind paw [19]. The adjuvant contained heat killed *Mycobacterium tuberculosis* (10 mg) in paraffin oil (1 ml). The rats were randomly divided into four groups (n = 6): normal rats served as control, AIA rats served as arthritic control, AIA rats treated daily with trikatu (1000 mg/kg/b.wt, oral), and AIA rats treated daily with methotrexate (3 mg/kg/b.wt, i.p). Trikatu was prepared as an aqueous suspension in 0.9% saline solution. All rats except control underwent drug treatment for 10 days from day 11–20 post arthritis induction. Control and AIA (arthritic control) rats received vehicle only (0.9% saline) during the 10 days of treatment. On day 21, animals were sacrificed by euthanasia and blood was collected for

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