Pharmacology & Pharmacy, 2016, 7, 106-116
Published Online February 2016 in SciRes. http://www.scirp.org/journal/pp
http://dx.doi.org/10.4236/pp.2016.72014

Sphaeranthus indicus: Traditional Wisdom to Modern Medicine—An Orally Active, Potent Cytokine Inhibitor for the Management of Inflammatory Disorders

Jacqueline Trivedi, Firuza Kharas, Sapna Parikh, Roda Dalal, Aurelio Lobo, Lyle Fonseca, Mahesh Jadhav, Shruta Dadarkar, Asha Almeida, Ankita Srivastava, Ashish Suthar*

Department of Herbal Development, Piramal Life Sciences India Limited, Mumbai, India
Email: *ashish.suthar@piramal.com

Received 17 August 2015; accepted 21 February 2016; published 24 February 2016

Copyright © 2016 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).
http://creativecommons.org/licenses/by/4.0/
Open Access

Abstract

Tumor necrosis factor (TNF-α) is a key regulator of the inflammatory and tissue destructive pathways in rheumatoid arthritis (RA). The clinical success of anti-TNF-α and anti-IL-17 biologics has validated the concept that cytokine blockade is beneficial in RA. However, as these drugs are parenterally administered, our efforts are directed at identifying a novel orally active TNF-α inhibitor with a therapeutic profile similar to that of biologics. Since plants are natural immunomodulators, we explored the immunomodulatory potential of *Sphaeranthus indicus* extract. In our studies, the extract dose-dependently inhibited the release of cytokines in stimulated human peripheral blood mononuclear cells (hPBMCs), and their spontaneous release in synovial cells derived from patients suffering from RA. TNF-α and IFN-γ induced release of p40 subunit of IL-12/IL-23, and p19 subunit of IL-23 in differentiated THP-1 cells is potently blocked. The expression of endothelial cell adhesion molecules in TNF-α-stimulated HUVECs was also potently inhibited. The oral treatment significantly and dose-dependently reduced LPS-induced TNF-α and IL-1β production in mice. Disease regression was seen in collagen-induced arthritis in DBA/1J mice, which was validated along with radiological and histopathological evaluation. Therefore, the extract of *Sphaeranthus indicus* could be used in the management of inflammatory conditions.

Keywords

Sphira, Rheumatoid Arthritis, Psoriasis, Cytokine Inhibition

*Corresponding author.

How to cite this paper: Trivedi, J., *et al.* (2016) *Sphaeranthus indicus*: Traditional Wisdom to Modern Medicine—An Orally Active, Potent Cytokine Inhibitor for the Management of Inflammatory Disorders. *Pharmacology & Pharmacy*, **7**, 106-116.
http://dx.doi.org/10.4236/pp.2016.72014

1. Introduction

Immune mediated inflammatory disease is a group of unrelated inflammatory conditions that share common inflammatory pathways. This definition encompasses disorders as diverse as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriatic arthritis (PsA), type 1 diabetes, multiple sclerosis, vasculitis, ankylosing spondylitis (AS), and juvenile chronic arthritis (JCA). Treatment of these conditions incurs substantial costs to patients and society at large. Numerous cytokines have been identified in such diseased tissues across a range of immune mediated inflammatory diseases. Since then, cytokine mediated strategy is one of the most successful remedial efforts that have resulted in regression of IMIDs. One such example is RA.

In the case of RA, laboratory and preclinical animal model studies in the late 1980s and early 1990s identified tumor necrosis factor-α (TNF-α) as a key pathogenic molecule [1]. Data from numerous clinical trials, initially with the anti-TNF agents infliximab and etanercept [2] and later with adalimumab [3], have confirmed the validity of TNF-α as a therapeutic target in RA. The success of clinical trials in RA has prompted investigation of the therapeutic efficacy of anti-TNF-α agents in several other immune-mediated inflammatory diseases in which its pathogenicity has been implicated.

In a psoriatic skin plaque, there is a predominance of interferon-γ, IL-2, IL-12, IL-17 and IL-23 [4]. TNF-α is expressed in the epidermis and dermis and potentially contributes to the accumulation of inflammatory cells in these tissues by inducing expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and keratinocytes [5]. The validity of TNF-α as a therapeutic target in psoriasis has been confirmed in double-blind, placebo-controlled randomized trials using the anti-TNF agents etanercept (25 mg or 50 mg twice weekly) and infliximab (5 mg/kg) [6] [7], where patients have experienced a rapid and high degree of clinical benefit. Recent data on IL-17 and IL-23 have indicated that these cytokines also play a significant role in inducing and maintaining the inflammatory nexus in skin lesions of psoriatic patients. Clinical data emerging with antibodies directed towards each of these cytokines have shown marked disease regression and better efficacy than with anti-TNF-α antibodies [8] [9]. Hence, modern approaches towards therapeutic enhancement of inflammatory diseases include targeting both cytokines, and combinatorial approaches involving TNF-α and IL-17.

Plant sterols and sterolins are natural immunomodulators found in some raw fruits and vegetables and in the alga, spirulina. *S. indicus* is a well-known Indian herb known as Gorakhmundi, Mundi or Munditika, which is also known to have immuno-modulatory as well as anti-inflammatory activities. According to Ayurvedic literature, *S. indicus* is useful in bronchitis, tuberculosis, elephantiasis, anemia, inflammatory conditions of pelvis in women, asthma and glandular swelling in the neck [10] [11]. In our approach to explore the anti-inflammatory potential of this plant, we have initiated studies to evaluate the holistic extracts prepared from fruiting and flowering bodies and their active constituents for anti-TNF activity by using various pharmacological models *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Extraction and Preparation of Sphira (NPS31807)

The extraction and preparation of *S. indicus* was performed as described previously [12]. Various extracts were evaluated for their anti-TNF activity. The methanolic extract was found to be the most potent (data not shown). This extract was further evaluated for its anti-inflammatory potential, and will henceforth be referred to as Sphira (NPS31807).

2.2. *In Vitro* Screening of Sphira for Its Anti-Inflammatory Potential

The anti-inflammatory potential of Sphira was evaluated using various *in vitro* screening models of inflammation. Blood was collected from healthy donors after obtaining Independent Ethics Committee approval and written informed consent. Briefly, peripheral blood was collected in potassium EDTA vacutainer tubes (BD Biosciences). hPBMC were isolated by density gradient centrifugation using Histopaque-1077 solution (Sigma Aldrich; St. Louis, MO). Isolated hPBMC were re-suspended in Rosewell Park Memorial Institute (RPMI) 1640 culture medium (Sigma Aldrich) containing 10% heat inactivated fetal bovine serum (FBS; JRH), 100 U/ml penicillin (Sigma Chemical Co.; St. Louis, MO) and 100 µg/ml streptomycin (Sigma Chemical Co.). The hPBMCs were uniformly plated in 96-well tissue culture plates at a seeding density of 1×10^6 cells/ml. Cells were then treated to different concentrations of Sphira (0.03 - 100 µg/ml) dissolved in DMSO (the final concentration of

DMSO maintained at 0.5%), or 0.5% DMSO (control), and incubated for 30 minutes. Incubation in all the *in vitro* screening assays was in a humidified atmosphere at 37˚C, 5% CO_2.

Cytokine release assay: This assay measures LPS-induced cytokine release from hPBMCs, specifically to evaluate TNF-*α* inhibition. The procedure followed was similar to that described previously [13]. After being exposed to the test compounds, 1 µg/ml LPS (*Escherichia coli* 0127:B8, Sigma Chemical Co., St. Louis, MO) was added per well to stimulate cytokine production. The cells were further incubated for 5 h, following which supernatants were collected, stored at −70˚C and assayed later for TNF-*α*, IL-1*β*, IL-6 and IL-8 by ELISA (OptiEIA ELISA sets, BD Biosciences, Pharmingen). In all experiments, a parallel plate was run to ascertain the toxicity of test extracts. The toxicity was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium) reagent.

IL-17 release assay: After treatment with test compounds, the cells were stimulated with 25 ng/ml of PMA (phorbol 12-myristate 13-acetate; SIGMA) and 1 µM ionomycin. The plates were further incubated for 48 h, after which the supernatant from the cells were collected and ELISA was performed to detect the levels of IL-17.

2.3. Evaluation of Sphira in Synovial Cells from RA Patients

The ability of Sphira to inhibit the spontaneous release of cytokines from freshly isolated human synovial tissue cells was determined in a manner similar to that described by Brennan *et al*. [14]. After informed consent and Independent Ethics Committee approval, synovial tissue was obtained from RA patients undergoing knee replacement surgery. The tissue was minced and digested in RPMI medium containing 100 U/ml penicillin-G, 100 µg/ml streptomycin, 50 ng/ml amphotericin B (GIBCO), 1.33 mg/ml collagenase Type I (Worthington Biochemical Corporation, New Jersey), 0.5 µg/ml DNase Type I (Sigma Aldrich) and 8.33 U/ml heparin (Biological E. Limited, India) for 3 h at 37˚C, 5% CO_2. Isolated cells were uniformly plated in 96-well tissue culture plates at a seeding density of 1×10^6 cells/ml and exposed to various concentrations of Sphira (0.03 - 100 µg/ml) dissolved in DMSO. The plates were incubated for 16 h. Subsequently, the supernatants were harvested and stored at −70˚C. The amounts of TNF-*α*, IL-1*β*, IL-6 and IL-8 in the supernatants were assayed using OptiEIA ELISA sets (BD BioSciences Pharmingen). The protocol followed was as per manufacturer's instructions.

2.4. *In Vitro* Screening of Sphira for Its Anti-Psoriatic Potential

The anti-psoriatic potential of Sphira was evaluated in an *in vitro* screening model targeting p40, a subunit shared by human interleukin-12 and human Interleukin-23 (IL-12/IL-23 p40). Differentiated THP-1 cells were treated with varying concentrations of Sphir aranging from 0.1 to 100 µg/ml, and later on with a combination of TNF-*α* and IFN-*γ*. The levels of IL-12/IL-23p40, IL-23p19 and IL-12p70 were estimated by ELISA [15].

2.5. NF*κ*B Transcription Assay

CEM-*κ*B (a kind gift from Dr. Shigeki Miyamoto, University of Wisconsin, Madison, WI) is a CEM cell line transfected with *κ*B binding element linked to green florescence protein (GFP) promoter. CEM-*κ*B was maintained in RPMI-1640 supplemented with 10% FBS and 0.1% G418 (Sigma Aldrich). The experiment was done as described previously [16]. Briefly, the cells were seeded at a density of 50,000 cells/ml, in a 6-well plate. The cells were treated with 0.1, 0.5, 1, 3, 10 and 30 µg/ml of Sphira dissolved in DMSO, 0.5% DMSO (vehicle control) or BAY 11-7082, an IKK*α* inhibitor (positive control), and incubated for 30 minutes. The cells were then stimulated with 0.5 ng/ml TNF-*α* and incubated for 16 h. After 16 h, the shift in NF*κ*B expression was measured using BD FACS Calibur. The GFP fluorescence measured is directly proportional to NF*κ*B expression.

2.6. Evaluation of Sphira for Its Potential to Affect Cell Adhesion Molecules

The surface expression of endothelial cell adhesion molecules was quantified using cell ELISA, as described previously [17]. Briefly, confluent HUVECs (Cascade biologics) in 96-well fibronectin-coated plates were pretreated with various concentrations of Sphira for 30 minutes, before being stimulated with 1 ng/mL TNF-*α* for the indicated time. The expressions of ICAM-1 and VCAM-1 were evaluated after TNF-*α* stimulation for 4 h, and expression of E-selectin was evaluated after 6 h of stimulation. The cells were fixed with 1% paraformaldehyde and blocked using bovine serum albumin (2% in DPBS). The cells were then washed and incubated with monoclonal mouse anti-human ICAM-1, VCAM-1, E-selectin or the isotype control mouse IgG1 for 2 - 4 h at

4˚C. Subsequently, cells were washed and incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG for 90 minutes. Binding of the secondary antibody was determined by incubating with 3, 3, 5, 5' tetramethylbenzidine (TMB) substrate and then terminating the reaction by 2N sulphuric acid. Surface expression of adhesion molecules was quantified by measuring absorbance at 490 nm in an automated microtitre plate reader.

Monocytic cells and endothelial cell adhesion assay: The human monocytic leukemia cell line, THP-1 was used as a model for monocytic cells. Leukocyte adhesion assays were performed under static conditions as previously described by Kim HK [18], with minor modifications. Briefly, THP-1 cells were labeled with BCECF-AM in serum-free RPMI 1640 media for 30 minutes at RT in the dark, then washed with DPBS to remove surplus dye and suspended in M200 medium. Confluent HUVEC monolayers were pretreated with various concentrations of Sphira for 30 minutes, prior to 4 h exposure to 1 ng/mL TNF-α. Labeled THP-1 (6×10^5 cells/ml) were seeded onto control or treated HUVECs, and incubated for 10 minutes at RT in the dark. The unbound cells were removed by gentle washing with medium and the images were obtained at 485 nm excitation and 538 nm emission using a NIKON Eclipse 80i fluorescence microscope. For adhesion quantification, THP-1 cells bound to HUVECs were lysed with 0.1% Triton-X-100 in 0.1 M Tris buffer, pH 8.8, and fluorescence intensity was measured by a spectrofluorometer with emission at 520 nm and excitation at 485 nm. Adherent cells were quantified using standard curves of BCECF and expressed as % adhesion of added cells. Furthermore, a duplicate set of flasks were kept aside and counterstained with hematoxylin to simultaneously evaluate the morphology of HUVECs and monocytic cells.

2.7. Anti-Inflammatory Effect of the Oral Formulation of Sphira in Balb/c Mice

To evaluate the anti-inflammatory effect of Sphira, the "Inhibition of Lipopolysaccharide (LPS)-induced TNF-α release in BALB/c mice" model was used. All animal experiments were approved by Institutional Animal Ethics Committee of Piramal Life Sciences Limited. The procedure followed is as described in Moreira, AL [19]. Balb/c mice (procured from The Jackson Laboratory, U.S.A.) of either sex weighing between 18 - 22 g (n = 10) were orally administered Sphira at a dose of 30, 100 and 300 mg/kg. All suspensions were freshly prepared in 0.5% CMC. One hour later, LPS (1 and 2 mg/kg, i.p. for TNF-α and IL-1β release, respectively) (*Escherchia coli*, serotype 0127:B8, Sigma Chemical Co., St. Louis, MO) dissolved in sterile pyrogen-free saline was administered intra-peritoneally to the control group, standard treatment group (Rolipram, 30 mg/kg, p.o.) and test groups (Sphira), except the negative control group which received only normal saline. Post LPS-challenge (1.5 h for TNF-α and 4 h for IL-1β), blood samples were collected from anesthetized mice, with heparin as an anti-coagulant (25 IU per sample), centrifuged at 10,000 rpm for 10 min, and the resultant plasma samples were analyzed for levels of cytokines by ELISA, as described by the manufacturer (OptiEIA ELISA sets, BD BioSciences Pharmingen). Percent inhibition of TNF-α and IL-1β release was calculated by comparing the levels of the treatment groups with those of the control group.

2.8. Anti-Arthritic Activity of Sphira in Collagen-Induced Arthritis Model

The anti-arthritic potential of Sphira was evaluated using collagen-induced arthritis mouse model in a manner similar to that described previously [20]. Male DBA/1J mice (procured from The Jackson Laboratory, U.S.A.), aged 8 - 10 weeks were immunized with an emulsion equivalent to 200 µg of type II collagen in Freund's Complete Adjuvant, injected intra-dermally at the base of the tail. A booster shot with the same emulsion was given 21 days later. Sphira was tested in the therapeutic regime, from day 23 following primary immunization. Mice were examined once daily for signs of RA, using the articular index and paw thickness as parameters. Articular index scoring was performed employing the following criteria:

Scoring for forelimbs: Scale 0-3, where, 0: No redness or swelling; 1: Redness, but no swelling; 2: Redness and swelling of the paw; 3: Redness and severe swelling of the paw. *Scoring for hind limbs*: Scale 0-4, where, 0: No redness or swelling; 1: Redness and mild swelling of paw; 2: Redness and moderate swelling of paw and/or swelling of at least one of the digits; 3: Redness and moderate/severe swelling of paw, swelling of ankle joint and/or swelling of one or more digits; 4: Redness and severe swelling of paw, digits and ankle joint, with joint stiffness and altered angle of digits.

Mice with a minimum hind paw score of 2 were inducted into the study. Mice were randomized into the various study groups and orally administered the vehicle (0.5% CMC, 1 ml/kg), test compound (Sphira, 600 mg/kg,

twice daily) or standard compound (Enbrel, 3 mg/kg, s.c., once daily). Each group had a minimum of 8 - 10 mice. The dosing of the compounds was done for 13 days. Parameters such as body weight, articular index, paw thickness (in mm, using a tension-free caliper) and any significant observation regarding the condition of the animal were observed and recorded daily. On the last day, 1 h after the compound treatment, the animals were sacrificed, blood withdrawn, and plasma collected for drug level analysis. Also, the limbs of all the animals were preserved for histopathological evaluations.

Histological analysis: Mice were humanely euthanized and the hind paws were harvested from each animal, fixed in 10% neutral buffered formalin, decalcified in 10% EDTA and embedded in paraffin. Section were stained with either hematoxylin and eosin or safranin O and evaluated microscopically. Histopathological changes were scored as follows: mild (score = 1), moderate (score = 2) or severe (score = 3) for the parameters of cellular infiltration, bone erosions and cartilage damage, graded separately. Cartilage depletion was indicated visually by diminished safranin O staining of proteoglycan matrix. The mean total score was compared to that of vehicle treated group. In case of histological scoring, Kruskal-Wallis analysis was followed by Dunn's multiple comparison tests to evaluate the statistical difference between two groups. Values of $P < 0.05$ were considered significant.

2.9. Statistical Analysis

All statistical analysis were performed by Student's t-test, where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, were considered statistically significant.

3. Results

3.1. Sphira Inhibits the Release of Cytokines in LPS-Induced hPBMC

The anti-inflammatory potential of methanolic extract of *S. indicus* was confirmed *in vitro*. Sphira was studied in 12 donors at eight different concentrations (0.3 - 100 µg/ml) in LPS-induced TNF-α, IL-1β, IL-6 and IL-8 release in hPBMCs. It was observed that it dose-dependently inhibits the release of LPS-induced TNF-α (IC_{50} 4.31 ± 0.78 µg/ml), IL-1β (IC_{50} 1.96 ± 0.56 µg/ml), IL-6 (IC_{50} 10.17 ± 10.47 µg/ml) and IL-8 (IC_{50} 28.33 ± 2.97 µg/ml) in hPBMCs. It was also found to be non-toxic at the doses tested (**Figure 1(A)**). Thus, our data clearly demonstrated that the extract could significantly modulate inflammatory cytokine secretion from monocyte-macrophage lineage in the advent of LPS mediated stimulatory signals.

3.2. Sphira Inhibits IL-17 Production in hPBMCs Stimulated with PMA-Ionomycin

To study the effect of the extract on T-cell mediated cytokines such as IL-17, hPBMC were treated with different doses of Sphira, and the supernatant evaluated for secreted IL-17 levels in presence of PMA-ionmycin stimulus. The effect of the extract on the viability of these cells was also measured simultaneously. It is well demonstrated that Sphira blocked IL-17 (IC_{50} 2.1 ± 0.67 µg/ml) and TNF-α (IC_{50} 1.8 ± 0.9 µg/ml) production by PMA-ionomycin stimulated hPBMCs. The extract did not have any toxic manifestations on normal resting cells, but effectively blocked proliferation of stimulated cells (**Figure 1(C)**).

3.3. Sphira Inhibits the Release of Cytokines in Synovial Cells Isolated from RA Patients

Sphira was evaluated at eight different concentrations in synovial cells obtained from RA patients undergoing knee replacement surgery [14]. This study was performed in 10 donors. The extract inhibited the spontaneous release of TNF-α (IC_{50} 13.10 ± 3.19 µg/ml), IL-1 β (IC_{50} 5.33 ± 1.76 µg/ml), and IL-6 (IC_{50} 16.33 ± 2.91 µg/ml) in a dose-dependent manner, in synovial cells. It was found to be toxic at the highest concentration (100 µg/ml) (**Figure 1(B)**).

3.4. Sphira Inhibits the Release of IL-12/IL-23 p40 and IL-23 p19 from TNF-α and IFN-γ Stimulated THP-1 Cells

Anti-IL-12/23 antibodies have successfully been targeted to the common IL-12p40 subunit; both cytokines are implicated in inflammatory diseases. Ustekinumab and ABT-874, inhibitors of IL-12p40, have shown efficacy in psoriasis [21] [22]. Our studies show that Sphira potently inhibits the release of IL-12/IL-23 p40 (IC_{50} 1.0 ±

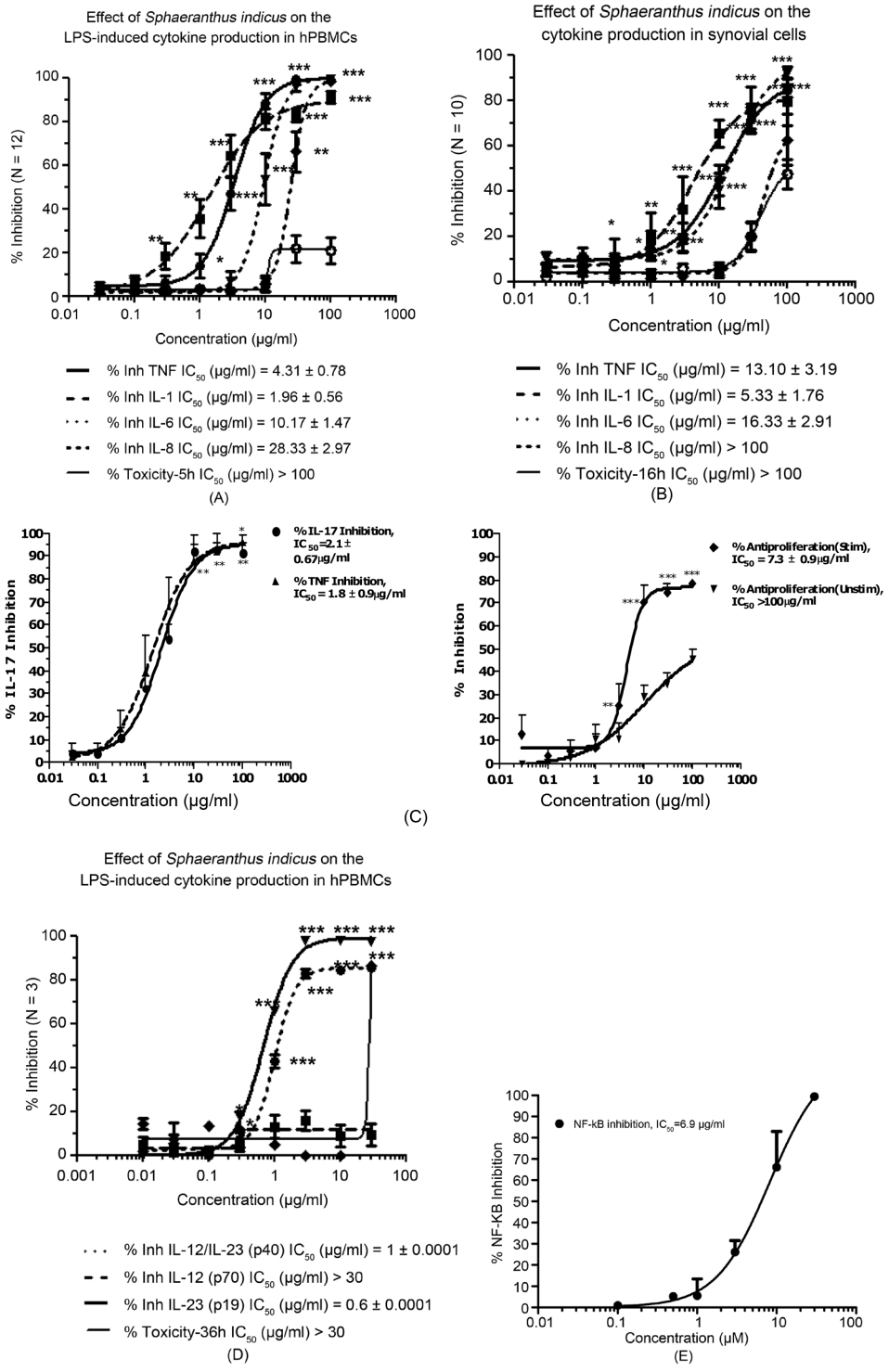


Figure 1. Sphira potently inhibits pro-inflammatory cytokine release. (A) Pretreatment with Sphira induced a concentration dependent reduction in levels of TNF-α, IL-1β, IL-6 and IL-8 in LPS stimulated hPBMCs; (B) Sphira inhibits the spontaneous release of cytokines from synovial cells isolated from RA patients; (C) Pretreatment with Sphira induced a concentration dependent reduction in levels of IL-17 in PMA + ionomycin stimulated hPBMCs; (D) Sphira inhibits the release of IL-12/IL-23 p40 and IL-23 p19 from TNF-α and IFN-γ stimulated THP-1 cells; (E) Sphira inhibits TNF-α induced NFκB activation in CEM cell line transfected NFκB promoter. The graph represents the percent inhibition (mean ± SD). Statistical significance was associated with $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

0.0001 µg/ml) and IL-23 p19 (IC_{50} 0.6 ± 0.0001 µg/ml), with no toxicity up to the highest concentration tested (**Figure 1(D)**).

3.5. Sphira Inhibits TNF-α Induced NFκB Activation in CEM Cell Line Transfected NFκB Promoter

NF-κB is an important transcription factor required for T-cell proliferation and other immunological functions. Sphira dose dependently inhibited NFκB activation (IC_{50} 6.9 µg/ml) in TNF-α stimulated CEM-κB cells (**Figure 1(E)**).

3.6. Sphira Decreases the Cell Surface Expression of ICAM-1, VCAM-1 and E-Selectin in TNF-α-Stimulated HUVECs

As cell adhesion molecules play an important role during inflammation, we analyzed the effect of different concentrations of Sphira on TNF-α-induced cell surface expression of these molecules. In accordance with previous studies, ICAM-1 and E-selectin were expressed at low levels in unstimulated HUVECs, but their expression increased after TNF-α stimulation (data not shown). As shown in **Figure 2(A)**, Sphira, at the highest concentration of 10 µg/mL, significantly inhibited the expression of TNF-α-induced ICAM-1 (61% ± 14%), VCAM-1 (69% ± 8%) and E-selectin (90% ± 7%) respectively. The IC_{50} values of ICAM-1, VCAM-1 and E-selectin expression

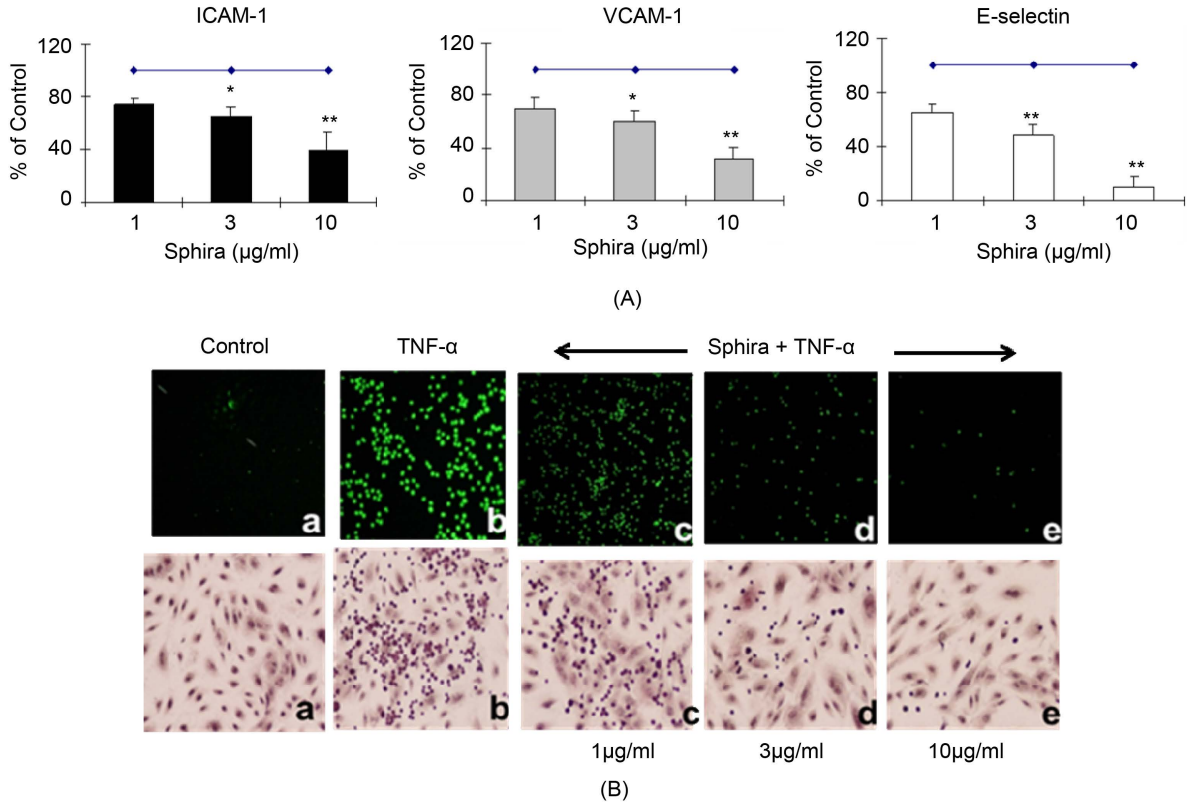


Figure 2. Sphira potently inhibits the expression of cell adhesion molecules. (A) The expression of ICAM-1, VCAM-1 and E-selectin in TNF-α-stimulated HUVECs determined by cell ELISA. HUVECs were pretreated with Sphira (1, 3 and 10 µg/mL) for 30 min, followed by TNF-α stimulation (1 ng/mL) for 4 h (ICAM-1, E-selectin) and 6 h (VCAM-1). Data are expressed as the mean ± SEM of 6 experiments (VCAM-1) and 4 experiments (ICAM-1 and E-selectin) in triplicates. $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared with TNF-α -stimulated HUVECs; (B) Upper panel: Representative fluorescence photomicrographs showing effects of Sphira on TNF-α induced adhesion of BCECF-labeled human monocytic cells to HUVECs (40×). Lower panel: Bright-field images showing the population of intact endothelial cells and bound monocytic cells counterstained with hematoxylin (100×). (a) Untreated control HUVEC; (b) HUVECs stimulated by TNF-α; (c)-(e) HUVECs treated with 1, 3 and 10 µg/ml Sphira, followed by TNF-α stimulation (1 ng/ml).

were 7.6, 3.7 and 2.9 µg/ml, respectively. Taken together, these findings indicate that Sphira specifically inhibits the cytokine-induced expression of ECAMs in a dose-dependent manner.

3.7. Sphira Inhibits the Binding of THP-1 Cells to TNF-*α*-Stimulated HUVECs

The functional significance of inhibition of cell adhesion molecules was evaluated by analyzing the adhesion of monocytic cells to endothelial cells in the presence of Sphira (**Figure 2(B)**). Unstimulated confluent HUVEC monolayers exhibited minimal binding to THP-1 (**Figure 2(B)**, Panel a). However, there was a marked increase in the THP-1 cell adherence to HUVECs treated with TNF-*α* (**Figure 2(B)**, Panel b). Pretreatment of confluent HUVECs with Sphira (1, 3 and 10 µg/mL) drastically inhibited THP-1 adhesion to HUVECs in a dose-dependent manner (**Figure 2(B)**, Panels c-e), with maximum inhibition at 10 µg/mL ($P < 0.01$; $n = 5$). By counterstaining with hematoxylin, the bright-field images (100×) show that the morphology of HUVECs was maintained, and monocytic cells were shown to be intact in each individual condition (**Figure 2(B)**, lower panel). These results demonstrate that Sphira is effective in blocking adhesion of THP-1 to endothelial cells by inhibiting the TNF-*α*-induced expression of ICAM-1, VCAM-1 and E-selectin.

3.8. Sphira Extract Inhibits the Release of Cytokines in the *in Vivo* LPS Model of Inflammation

The anti-inflammatory effect of oral formulations of Sphira was evaluated in the *in vivo* LPS model of inflammation [23]. Percent inhibition of TNF-*α* and IL-1*β* release was calculated by comparing the cytokine levels in the Sphira-treated groups with those of the control group. Dose-dependent inhibition of cytokine release was noted (**Figure 3(A)**).

3.9. Sphira Extract Arrests Collagen-Induced Arthritis When Administered Orally in the Therapeutic Regimen

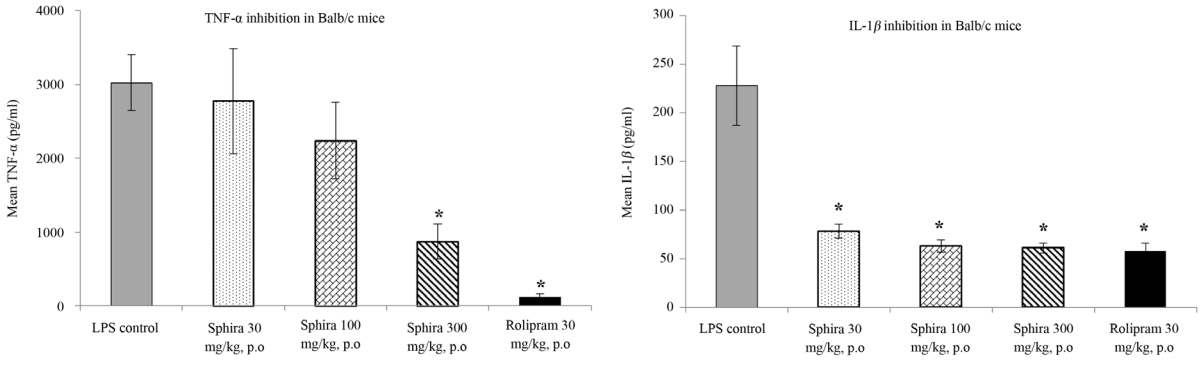
The extract was evaluated in a chronic model of arthritis, *i.e*. the well-established mouse collagen-induced arthritis (CIA) model. As reported earlier [24], the CIA in DBA/1J mice was manifested with significant increases in paw thickness and articular index, and these clinical signs of arthritic disease were significantly reduced in mice receiving Sphira 600 mg/kg, orally, twice daily (**Figure 3(B)**). Histological analyses of paw tissues of diseased mice treated with placebo revealed severe destruction in the joints characterized by synovitis, pannus formation, articular cartilage erosion and pronounced infiltration of inflammatory cells invading bony cortex at multiple foci (**Figure 3(C)**). In contrast, the hind paws of treated mice (either with 600 mg/kg Sphira orally, or Enbrel) showed maintenance of joint architecture with diminished pannus formation and reduced infiltration of inflammatory cells (**Figure 3(C)**). The degree of macroscopic protection provided by Sphira 600 mg/kg, p.o., was statistically similar to the protection demonstrated by Enbrel treated with placebo control (**Figure 3(C)**).

4. Discussion

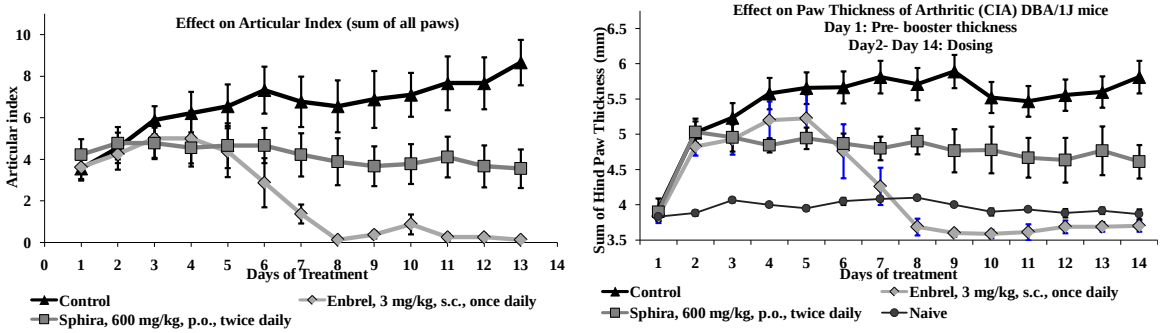
All drugs which modify immune response are generally categorized as immunomodulators. Since plants are known to be immunomodulators, we investigated the methanolic extract of *S. indicus* (Sphira) for its immunomodulatory potential. Our data clearly demonstrates that Sphira robustly treats CIA by selectively inhibiting a spectrum of signal transduction pathways central to the pathogenesis of RA. Sphira abrogates multiple cytokines from human peripheral blood mononuclear cells as well as human RA synovial cells. Abrogation of these cytokines is mediated by mitigating NF-*κ*B activation, which is a transcription factor central to all inflammatory signaling cascades. Sphira potently inhibits diverse cellular responses that play critical roles in driving synovitis, pannus formation, and joint destruction in RA, and hence exerts a multitude of responses which are successful in protecting against inflammation.

In the synovial cells of patients with RA, activation of the NF-*κ*B pathway results in the transactivation of a multitude of responsive genes that contribute to the inflammatory phenotype, including TNF-*α* from macrophages, matrix metalloproteinases from synovial fibroblasts and chemokines that recruit immune cells to the inflamed pannus [25]. Using CEM cell line transfected NF*κ*B promoter, we demonstrated that Sphira inhibits TNF-*α*-induced NF*κ*B activation.

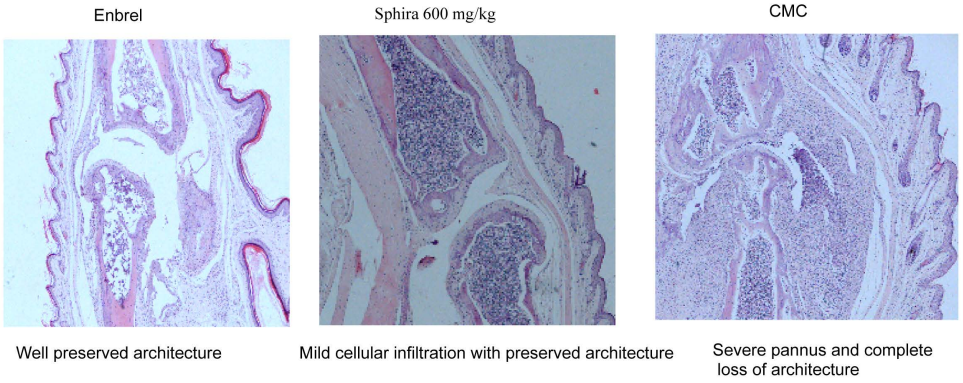
The destruction of synovial tissue in RA is mediated by cytokines and matrix metalloproteinases produced by



(A)



(B)



(C)

Figure 3. Effect of Sphira in *in vivo* models of inflammation. (A) Sphira inhibits the release of cytokines in the *in vivo* LPS model of inflammation: Sphira was orally administered 1 hr before LPS challenge. Plasma levels of TNF-α and IL-1β were evaluated by ELISA. Data represents mean ± SEM (n = 10). Statistical evaluation was performed by Student's t-test. Statistical difference was observed between control and treated mice, where $^{*}P < 0.05$; (B) Sphira treats CIA: Following the development of clinical arthritis (average visual arthritis score of 4), DBA/1J mice with CIA were randomized and treated with placebo (n = 10), 600 mg/kg Sphira (n = 10), or 3 mg/kg enbrel (n = 10). The disease was monitored using a visual arthritis scoring system and paw thickness measurements. Values from the presented results are the mean ± SEM for this representative experiment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with placebo-treated mice; (C) Histological effects of Sphira in CIA: The vehicle control shows extensive pannus formation. Enbrel treated mice show well-preserved joint architecture. Sphira treated mice (600 mg/kg, p.o., twice daily) show mild cellular infiltration and a preserved architecture, comparable to Enbrel.

macrophages and fibroblasts, and seems to be controlled by lymphocytes. Activation, circulation, and migration of mononuclear cells to inflammatory sites are regulated by adhesion molecules such as ICAM-1, VCAM-1, or E-selectin [26]. Therefore, cell adhesion molecules (CAMs) and endothelial growth factors have an important role in the infiltration of rheumatoid synovium with mononuclear cells and seem to play a part in the initiation

and progression of the disease. In our study, we have shown that Sphira inhibited the expression of cell adhesion molecules, thus implying its protective role in RA.

Evidence shows that IL-17 is present at sites of inflammatory arthritis and that, in synergistic interactions, it amplifies the inflammation induced by other cytokines, primarily TNF-α. In several animal models of arthritis, inhibition of IL-17 limits inflammation and joint erosion. Initial observations from phase I trials show that signs and symptoms of RA are significantly suppressed following treatment with anti-IL-17 antibodies, without notable adverse effects [27]. Our studies showed that Sphira inhibits IL-17 production in hPBMCs stimulated with PMA-ionomycin. Sphira also inhibits IL-6, TGF-β and IL-1β induced IL-17 production and accumulation in CD4+ T-cells (data not shown). mRNA expression studies done showed that Sphira inhibits Th17 differentiation markers in normal CD4 cells like IL-17A, IL-21 and IL-22 (data not shown).

In vivo studies in mice using the CIA model showed that treatment with Sphira reduced arthritic clinical score and paw swelling significantly. Histopathologic analysis was performed on hind paws harvested from mice receiving Sphira. The evaluation, by an investigator blinded to treatment group, demonstrated that Sphira resulted in statistically significant reductions in synovitis, pannus, and erosion scores in established CIA treatment.

In addition to potentially providing benefit in RA, it is anticipated that Sphira could also provide efficacy in other autoimmune diseases like psoriasis, as exhibited by our experiments which show a reduction in expression of IL-12/IL-23. Previous studies done at our research center has shown that Sphira inhibits the inflammatory, migratory and proliferative activity in keratinocytes and immune cells [12].

5. Conclusion

In conclusion, we have shown that Sphira potently treats CIA and inhibits multiple signal transduction pathways that drive pathogenic cellular responses in RA. Our results provide further rationale for prospective clinical trials to determine whether Sphira provides efficacy in RA and other autoimmune diseases.

Conflict of Interest

The authors declare no commercial or financial conflict of interest.

References

[1] Feldmann, M. (2008) Many Cytokines Are Very Useful Therapeutic Targets in Disease. *The Journal of Clinical Investigation*, **118**, 3533-3536. http://dx.doi.org/10.1172/JCI37346

[2] Taylor, P.C. (2001) Anti-TNF Therapy for Rheumatoid Arthritis and Other Inflammatory Diseases. *Molecular Biotechnology*, **19**, 153-168. http://dx.doi.org/10.1385/mb:19:2:153

[3] Weinblatt, M.E., Keystone, E.C., Furst, D.E., Moreland, L.W., Weisman, M.H., Birbara, C.A. and Teoh, L.A. (2003) Adalimumab, a Fully Human Anti-Tumor Necrosis Factor α Monoclonal Antibody, for the Treatment of Rheumatoid Arthritis in Patients Taking Concomitant Methotrexate: The ARMADA Trial. *Arthritis & Rheumatism*, **48**, 35-45. http://dx.doi.org/10.1002/art.10697

[4] Uyemura, K., Yamamura, M., Fivenson, D.F., Modlin, R.L. and Nickoloff, B.J. (1993) The Cytokine Network in Lesional and Lesion-Free Psoriatic Skin Is Characterized by a T-Helper Type 1 Cell-Mediated Response. *Journal of Investigative Dermatology*, **101**, 701-705. http://dx.doi.org/10.1111/1523-1747.ep12371679

[5] Ettehadi, P., Greaves, M., Wallach, D., Aderka, D. and Camp, R. (1994) Elevated Tumour Necrosis Factor-Alpha (TNF-α) Biological Activity in Psoriatic Skin Lesions. *Clinical & Experimental Immunology*, **96**, 146-151. http://dx.doi.org/10.1111/j.1365-2249.1994.tb06244.x

[6] Mease, P.J., Goffe, B.S., Metz, J., Vander Stoep, A., Finck, B. and Burge, D.J. (2000) Etanercept in the Treatment of Psoriatic Arthritis and Psoriasis: A Randomised Trial. *The Lancet*, **356**, 385-390. http://dx.doi.org/10.1016/S0140-6736(00)02530-7

[7] Reich, K., Nestle, F.O., Papp, K., Ortonne, J.-P., Evans, R., Guzzo, C., Li, S., Dooley, L.T. and Griffiths, C.E. (2005) Infliximab Induction and Maintenance Therapy for Moderate-to-Severe Psoriasis: A Phase III, Multicentre, Double-Blind Trial. *The Lancet*, **366**, 1367-1374. http://dx.doi.org/10.1016/S0140-6736(05)67566-6

[8] Leonardi, C., Matheson, R., Zachariae, C., Cameron, G., Li, L., Edson-Heredia, E., Braun, D. and Banerjee, S. (2012) Anti-Interleukin-17 Monoclonal Antibody Ixekizumab in Chronic Plaque Psoriasis. *New England Journal of Medicine*, **366**, 1190-1199. http://dx.doi.org/10.1056/NEJMoa1109997

[9] Yeilding, N., Szapary, P., Brodmerkel, C., Benson, J., Plotnick, M., Zhou, H., Goyal, K., *et al*. (2011) Development of the IL-12/23 Antagonist Ustekinumab in Psoriasis: Past, Present, and Future Perspectives. *Annals of the New York*

Academy of Sciences, **1222**, 30-39. http://dx.doi.org/10.1111/j.1749-6632.2011.05963.x

[10] Rohit, R., Mahendra, S., Richa, S., Kavitha, M. and Vikram, S.S. (2014) A Clinical Study on the effect of *Punarnavadi churna*, *Singhanada guggulu* in the management of *Amavata* (Rheumatoid Arthritis). *International Journal of Ayurveda and Pharma Research*, **2**, 46-54.

[11] Ramachandran, S. (2013) Review on *S. indicus* Linn. (Koṭṭaikkarantai). *Pharmacognosy Reviews*, **7**, 157-169. http://dx.doi.org/10.4103/0973-7847.120517

[12] Chakrabarti, D., Suthar, A.S., Jayaraman, G., Muthuvelan, B., Sharma, S. and Padigaru, M. (2012) NPS31807, a Standardized Extract from *Sphaeranthus indicus*, Inhibits Inflammatory, Migratory and Proliferative Activity in Keratinocytes and Immune Cells. *Pharmacology & Pharmacy*, **3**, 178-194. http://dx.doi.org/10.4236/pp.2012.32025

[13] Jansky, L., Reymanova, P. and Kopecky, J. (2003) Dynamics of Cytokine Production in Human Peripheral Blood Mononuclear Cells Stimulated by LPS, or Infected by Borrelia. *Physiological Research*, **52**, 593-598.

[14] Brennan, F.M., Chantry, D., Jackson, A., Maini, R. and Feldmann, M. (1989) Inhibitory Effect of TNF Alpha Antibodies on Synovial Cell Interleukin-1 Production in Rheumatoid Arthritis. *Lancet*, **334**, 244-247. http://dx.doi.org/10.1016/S0140-6736(89)90430-3

[15] Kamata, M., Tada, Y., Tatsuta, A., Kawashima, T., Shibata, S., Mitsui, H., Asano, Y., Sugaya, M., Kadono, T., Kanda, N., Watanabe, S. and Sato, S. (2013) Ciclosporin A Inhibits Production of Interleukin-12/23p40 and Interleukin-23 by the Human Monocyte Cell Line, THP-1. *Clinical and Experimental Dermatology*, **38**, 545-548. http://dx.doi.org/10.1111/ced.12110

[16] Balachandran, S., Gadekar, P.K., Parkale, S., Yadav, V.N., Kamath, D., Ramaswamy, S., Sharma, S., Vishwakarma, R.A. and Dagia, N.M. (2011) Synthesis and Biological Activity of Novel MIF Antagonists. *Bioorganic & Medicinal Chemistry Letters*, **21**, 1508-1511. http://dx.doi.org/10.1016/j.bmcl.2010.12.127

[17] Béchard, D., Scherpereel, A., Hammad, H., Gentina, T., Tsicopoulos, A., Aumercier, M., Pestel, J., *et al*. (2001) Human Endothelial-Cell Specific Molecule-1 Binds Directly to the Integrin CD11a/CD18 (LFA-1) and Blocks Binding to Intercellular Adhesion Molecule-1. *The Journal of Immunology*, **167**, 3099-3106. http://dx.doi.org/10.4049/jimmunol.167.6.3099

[18] Kim, K., Choi, Y., Lee, N., Park, K., Kim, G., Park, J., Kim, B., Lim, Y., *et al*. (2011) 5-Hydroxymethylfurfural from Black Garlic Extract Prevents TNFα-Induced Monocytic Cell Adhesion to HUVECs by Suppression of Vascular Cell Adhesion Molecule-1 Expression, Reactive Oxygen Species Generation and NF-κB Activation. *Phytotherapy Research*, **25**, 965-974. http://dx.doi.org/10.1002/ptr.3351

[19] Moreira, L., Wang, J., Sarno, N. and Kaplan, G. (1997) Thalidomide Protects Mice against LPS-Induced Shock. *Brazilian Journal of Medical and Biological Research*, **10**, 1199-1207. http://dx.doi.org/10.1590/s0100-879x1997001000010

[20] Terato, K., Karen, H., Michael, C., John, S., Alexander, T. and Andrew, K. (1985) Collagen-Induced Arthritis in Mice. Localization of an Arthritogenic Determinant to a Fragment of the Type II Collagen Molecule. *The Journal of Experimental Medicine*, **162**, 637-646. http://dx.doi.org/10.1084/jem.162.2.637

[21] Papp, A., Langley, G., Lebwohl, M., Krueger, G., Szapary, P. and Yeilding, N. (2008) Efficacy and Safety of Ustekinumab, a Human Interleukin-12/23 Monoclonal Antibody, in Patients with Psoriasis: 52-Week Results from a Randomised, Double-Blind, Placebo-Controlled Trial (PHOENIX 2). *Lancet*, **371**, 1675-1684. http://dx.doi.org/10.1016/S0140-6736(08)60726-6

[22] Ding, C., Xu, J. and Li, J. (2008) ABT-874, a Fully Human Monoclonal Anti-IL-12/IL-23 Antibody for the Potential Treatment of Autoimmune Diseases. *Current Opinion in Investigational Drugs*, **9**, 515-522.

[23] Fukuda, T., Sumichika, H., Murata, M., Hanano, T., Adachi, K. and Hisadome, M. (2000) A Novel Dual Regulator of Tumour Necrosis Factor-α and Interleukin-10 Protects Mice from Endotoxin-Induced Shock. *European Journal of Pharmacology*, **391**, 317-320. http://dx.doi.org/10.1016/S0014-2999(00)00096-0

[24] Wooley, H., Luthra, S., Krco, J., Stuart, M. and David, S. (1984) Type II Collagen—Induced Arthritis in Mice. *Arthritis & Rheumatism*, **27**, 1010-1017. http://dx.doi.org/10.1002/art.1780270907

[25] Simmonds, R.E. and Foxwell, B.M. (2008) Signalling, Inflammation and Arthritis: NF-κB and Its Relevance to Arthritis and Inflammation. *Rheumatology*, **5**, 584-590. http://dx.doi.org/10.1093/rheumatology/kem298

[26] Klimiuk, P., Sierakowski, S., Latosiewicz, R., Cylwik, J., Cylwik, B., Skowronski, J. and Chwiecko, J. (2002) Soluble Adhesion Molecules (ICAM-1, VCAM-1, and E-Selectin) and Vascular Endothelial Growth Factor (VEGF) in Patients with Distinct Variants of Rheumatoid Synovitis. *Annals of the Rheumatic Diseases*, **9**, 804-809. http://dx.doi.org/10.1136/ard.61.9.804

[27] Van den Berg, B. and Miossec, P. (2009) IL-17 as a Future Therapeutic Target for Rheumatoid Arthritis. *Nature Reviews Rheumatology*, **10**, 549-553. http://dx.doi.org/10.1038/nrrheum.2009.179