Allylpyrocatechol attenuates collagen-induced arthritis via attenuation of oxidative stress secondary to modulation of the MAPK, JAK/STAT, and Nrf2/HO-1 pathways

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Abbreviations: ANOVA, Analysis of variance; APC, Allylpyrocatechol; BSA, Bovine serum albumin; CFA, Complete Freund's Adjuvant; CIA, LPS accelerated collagen induced arthritis; CII, Bovine collagen type II; CMC, Carboxy methyl cellulose; DMSO, Dimethyl sulfoxide; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); ERK1/2, Extracellular-signal-regulated kinase; FBS, Fetal bovine serum; GSH, Glutathione; H&E-Haematoxylin and Eosin; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; H2O2, Hydrogen peroxide; HO′, hydroxyl radical; HO-1, Heme oxygenase-1; i.d., Intradermal; i.p., Intraperitoneal; LAL, Limulus Amoebocyte Lysate; LPO, Lipid peroxidation; LPS, Lipopolysaccharide; MDA, Malondialdehyde; MPO, Myeloperoxidase; MTX, Methotrexate; NADPH, β-Nicotinamide dinucleotide phosphate; NEDD, N-1 Naphthyl ethylene diamine dihydrochloride; NfκB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NO, Nitric oxide; Nrf2, Nuclear factor erythroid 2-related factor 2; O2−, Superoxide radical; ONOO−, Peroxynitrite; p.o., per os; IL, Interleukin; PBS, Phosphate buffered saline; RA, Rheumatoid arthritis; RFU, Relative fluorescence units; ROS, Reactive oxygen species; RPMI 1640, Roswell Park Memorial Institute medium; b.w., Body weight; SAPK/JNK, Stress-activated protein kinase/c-Jun N-terminal protein kinase; STAT, Signal transducers and activators of transcription; TBA, Thioarbituric acid; TCA, Trichloroacetic acid; TLR- Toll-Like Receptor; TNF, Tumour necrosis factor.

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Abstract

Rheumatoid arthritis (RA), an inflammatory autoimmune disorder is characterized by synovial hyperplasia and bony destruction. The pathogenesis of RA includes redox dysregulation, concomitant with increased levels of pro-inflammatory mediators. As the ability of Allylpyrocatechol (APC), a phytoconstituent of *Piper betle* leaves to alleviate oxidative stress has been demonstrated in patients with RA, its anti-arthritic activity was evaluated in an animal model of arthritis, along with establishment of the underlying mechanism(s) of action. The animal model was established by immunizing rats with bovine collagen type II (CII) followed by lipopolysaccharide, along with a booster dose of CII on day 15. Rats were treated with APC or Methotrexate (MTX) from days 11 to 27, wherein paw edema, radiography, histopathology and markers of inflammation were evaluated. The pro/anti-inflammatory signaling pathways were studied in a RAW264.7 macrophage cell line. APC prevented the progression of arthritis as evident from the reduction in paw edema, attenuation of damage to bones and cartilage as evident by radiography as also histopathology. Additionally, there was reduction in the levels of pro-inflammatory cytokines (TNF-α and IL-6) and restoration of the redox balance. Importantly, MTX ameliorated the features of arthritis, but not the associated oxidative stress. In RAW264.7, APC inhibited generation of nitric oxide and pro-inflammatory cytokines (TNF-α, IL-6 and IL-12p40), modulated the phosphorylation of pro-inflammatory (ERK1/2, SAPK/JNK, and JAK/STAT) and cytoprotective (Nrf2, HO-1) signaling pathways. Taken together, APC controlled the development of arthritis possibly via modulation of signaling pathways and deserves further consideration in the therapy for RA.
Introduction

Rheumatoid arthritis (RA) is a systemic, destructive autoimmune disease characterized by systemic inflammation that primarily affects the diarthodial joints, synovial membranes, articular cartilages and bones (Scott et al., 2010). RA affects 0.5-1.0% of the adult population worldwide and is associated with physical disability, substantial economic loss, higher mortality and reduced life expectancy (Myasoedova, 2010; Scott et al., 2010; Woolf and Pfleger, 2003; Handa et al., 2016). The generation of pannus/hypertrophied synovium involves infiltration of macrophages and their interaction with fibroblasts translates into an enhanced presence of pro-inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α), interleukin-12p40 (IL-12p40), interleukin-6 (IL-6), and interleukin-1β (IL-1β, McInnes et al., 2016). Accordingly, the therapeutic strategies include approaches that disrupt recruitment of inflammatory cells and limit the pro-inflammatory, tissue destroying milieu. As TNF-α and IL-6 play a dominant role, therapies targeting IL-6 and TNF-α have demonstrated inhibition of disease progression (McInnes et al., 2016).

The pathogenesis of RA is strongly associated with oxidative stress, as the overproduction of reactive oxygen species (ROS) from activated neutrophils and macrophages can mediate tissue injury (Kundu et al., 2012; Datta et al., 2014). In patients with RA, the raised levels of superoxide radical (O$_2^-$), hydroxyl radical (HO’), hydrogen peroxide (H$_2$O$_2$), and peroxynitrite (ONOO’), are strongly associated with oxidative damage (Datta et al., 2014). Furthermore, these elevated levels of ROS via modulation of signaling pathways promote the generation of a pro-inflammatory milieu and sustain disease progression (Filippin et al., 2008; Hitchon and El-Gabalawy, 2004; Shah et al., 2011). Accordingly, pharmacological attenuation of oxidative stress appears a promising approach.

The present therapy of RA is associated with several adverse effects emphasizing the need for safer drugs (Myasoedova, 2010). With the concerted efforts of the World Health Organization to incorporate herbal medicines into standard care, the development of herbal medicines and formulations has gained considerable awareness (World Health Organization, 2013). Allylpyrocatechol (APC), a major phytoconstituent of an ethanolic extract of leaves of *Piper betle* is a perennial climber, whose ethnomedicinal properties can be traced to early Sanskrit literature (FRLHT’s ENVIS Centre on...
Medicinal Plants, Bangalore. http://envis.frlht.org/plant_details.php?disp_id=1666&parname=0). Its anti-inflammatory, anti-oxidant and anti-ulcer activity have been validated (Sarkar et al., 2008; Yadav et al., 2013; Sarkar et al., 2013). Additionally, APC mitigated ex-vivo the oxidative stress generated in the synovial infiltrate of patients with RA (Kundu et al., 2011). The present study demonstrates that administration of APC can significantly decrease the severity of arthritis as also inhibit cartilage and bone destruction in a lipopolysaccharide (LPS)-accelerated collagen-induced model of arthritis (CIA). Additionally, as monocytes/macrophages are major contributors toward disease progression, the impact of APC on inflammatory (MAPK and JAK/STAT) and cytoprotective (Nrf2/HO-1) signaling cascades were investigated in a macrophage cell line (RAW264.7), wherein the results endorse the effectiveness of APC in the treatment of RA.

**Materials and methods**

**Materials**

Normal chow diet was procured from Provimi Animal Nutrition India Pvt. Ltd. (Bangalore, Karnataka, India). All chemicals if not otherwise stated were obtained from Sigma-Aldrich (St. Louis, MO, USA) except Limulus Amoebocyte Lysate (LAL) assay kit, MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Basel, Switzerland), bovine collagen type II solution (CII, Chondrex, Inc. Redmond, WA, USA), heat killed *Mycobacterium tuberculosis H37Ra* powder from DIFCO (Detroit, MI, USA). Thiobarbituric acid (TBA), trichloroacetic acid (TCA), β-nicotinamide dinucleotide phosphate (NADPH), 5,5′-dithio-bis (2-nitro-benzoic acid, DTNB), sulphanalimide, carboxy methyl cellulose (CMC) from Sisco Research Laboratories (Mumbai, Maharashtra, India), N-1 naphthyl ethylene diamine dihydrochloride (NEDD, Loba Chemie Pvt. Ltd., Mumbai, Maharashtra, India), protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin (BSA) from Himedia Laboratories (Mumbai, Maharashtra, India), antibodies for extracellular-signal-regulated kinase (ERK1/2), stress-activated protein kinase/c-Jun N-terminal protein kinase (SAPK/JNK), p38, signal transducers and activators of transcription 1 and 3 (STAT1 and STAT3) and their phosphorylated counterpart along with nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and α-Tubulin from Cell Signalling Technology Inc. (Danvers, MA, USA), and ELISA kits for murine tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-
12p40 (IL-12p40) from eBioscience, Inc. (San Diego, CA, USA) and anti-rat TNF-α and IL-6 from PeproTech (Rocky Hill, NJ, USA).

**Preparation of Allylpyrocatechol (APC)**

The leaves of *Piper betle* were collected from the local market, and identity was authenticated by the Botanical survey of India (Ganguly et al., 2007). An ethanolic extract was prepared, and APC was isolated as previously described (Rathee et al., 2006). HPLC analyses of the compound and peak detection at 254 nm confirmed its purity (Sarkar et al., 2008). The absence of endotoxin was confirmed by the LAL assay.

**Animals**

Adult Sprague-Dawley rats (male or female, aged 8-10 weeks, weight 150-175 g) were obtained from National Institute of Nutrition, Hyderabad, India and housed in groups of 3 in the animal facility of Department of Pharmacology, Institute of Postgraduate Medical Education and Research, Kolkata, India under controlled temperature (22 ± 2°C), humidity (60 ± 5%) and a 12 h light/dark cycle. The animals were provided with standard rodent chow and water *ad libitum*. All animal experiments were approved by the Institutional Animal Ethics Committee (CPCSEA Registration No. 544/PO/c/02/CPCSEA).

**Cell culture**

A mouse monocyte-macrophage cell line, RAW264.7 obtained from American Type Culture Collection (Manassas, VA, USA) was maintained in RPMI 1640 medium (pH 7.2) supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 μg/ml) and amphotericin-B (1 μg/ml) at 37°C, 5% CO₂. Cells were sub-cultured every 72 h, using 1x10⁶ cells as the initial inoculum. The absence of mycoplasma contamination was confirmed by the MycoAlert™ PLUS assay kit.

**Establishment of an LPS accelerated model of collagen induced arthritis (CIA)**

Arthritis was induced in rats using an emulsion of CII and complete Freund’s adjuvant (CFA) as previously described (Brand et al., 2007) with modifications (Fig. 1A). Briefly, to induce CIA, the emulsion prepared by drop wise addition of pre-chilled CII (2 mg/ml) to an equal volume of pre-chilled
CFA (2 mg/ml) and was administered on day 0 at the base of tail (200 µl/animal, i.d.), followed by LPS on day 14 [1 mg/ml in phosphate buffered saline (PBS, 0.02 M, pH 7.2)] 100 µl per animal, i.p.]. On day 15, a booster dose of CII in CFA emulsion (100 µl, i.d. Fig. 1A) was injected at the base of the tail.

The development of arthritis was quantified by measuring the paw volume plethysmometrically twice weekly, and an arthritic score as described previously (Brand et al., 2007): 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to metatarsal joints; 4, erythema and severe swelling of entire paw or ankylosis of the limb. To minimize the error, the volume of each hind paw was measured thrice and the average considered.

The control group (Group I) was randomly reserved before the first immunization. On day 11, the CII immunized animals that showed clinical symptoms were randomly allocated to five groups (n = 6), wherein Group II, the CIA control group received the vehicle (1% CMC w/v in water) from days 11-27. Groups III, IV and V received APC 5, 10 and 20 mg/kg b.w., p.o., in 1% CMC respectively from days 11-27, whereas Group VI received methotrexate (MTX, 1.5 mg/kg b.w., i.p./week in two divided doses). All animals were sacrificed 24 h after the last dose, i.e., day 28 wherein heparinized blood was collected from the retro-orbital plexus and centrifuged (2,000 g for 10 minutes), and the plasma was stored at -80°C. Animals were then sacrificed, the hind paws were excised and washed with ice cold 0.9% NaCl. One paw was immediately fixed in 10% neutral buffered formalin, while another hind paw was stored at -80°C for biochemical assays.

Changes in body weight were monitored and calculated by subtracting body weight of day 0 from day 28. On day 28, the spleens were removed, weighed immediately, after which the splenic index was calculated for each rat as the ratio of the spleen: body weight (Gowayed et al., 2015).

**Histopathology and radiological evaluation of bony architecture**

Radiographs of the hind paws were taken with a 55 kVp exposure for 6.4 mAs (Siemens Multiphos 15, Mumbai, Maharashtra, India). Post CII challenge, animals were euthanized, and their limbs fixed in 10%
neutral buffered formalin for 48 h followed by decalcification in 10% formic acid for one week. They were then transferred to 20% sucrose and kept overnight at 4°C. Finally, they were embedded in optimal cutting temperature compound and after being sectioned (5 μm), were stained with hematoxylin-eosin (https://csr.mgh.harvard.edu/data/wiki_pages/11/embedding_and_sectioning_of_decalcified_frozen_sections.pdf; https://www.urmc.rochester.edu/MedicalLibraries/URMCMedia/musculoskeletal-research/core-services/histology/documents/HBMICenterMeetingPresentation.pdf). Stained sections were evaluated microscopically (EVOS FL Auto Cell Imaging System, Life Technologies, Carlsbad, CA, US) at 40× and 100× magnification. To evaluate the cartilage and bone damage, morphometric measurements of joint space were quantified using an ocular micrometer. A comprehensive histological scoring system as described by Pan et al., 2009 was applied wherein cellular infiltration, and cartilage/bone erosion were scored on a scale of 0-3, 0 representing no pathological changes; 1, mild; 2, moderate; and 3, severe and the sum of the two scores was calculated.

**Estimation of myeloperoxidase (MPO) activity**

The paw tissues submerged in liquid nitrogen were pulverized using a Bessman tissue pulverizer and then homogenized (10% w/v) in 50 mM potassium phosphate buffer, pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide. After sonication and three freeze-thaw pulses in liquid nitrogen, the homogenates were centrifuged (12,000 g, 15 minutes, 4°C). The resultant supernatant (100 µl) was added to a reaction mixture containing o-dianisidine (0.167%) and H₂O₂ (0.005%) in 50 mM phosphate buffer (pH 6.0), final volume being 3 ml. Following a one h incubation at 37°C, absorbances were measured at 450 nm and MPO activity was expressed as U/minute/mg protein (Kumar et al., 2002).

**Measurement of reactive oxygen species (ROS)**

The generation of ROS was measured in paw tissues using 2′, 7′-dichlorodihydrofluorescein diacetate (H₂DCFDA). The pulverized paw tissues were homogenized in Locke’s buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM dextrose, pH 7.4) to yield a 5% (w/v) homogenate. The reaction mixture (1 ml) containing homogenate (50 µl) in Locke’s buffer, was incubated with H₂DCF-DA (5 µM) for 45 minutes in the dark at 37°C and analyzed for fluorescence (Excitation

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nm/Emission530 nm) using a spectrofluorimeter (Spectramax M2e, Molecular Devices, Sunnyvale, CA, USA). The values were expressed as relative fluorescence units (RFU)/mg of protein (De et al., 2015).

**Estimation of lipid peroxidation (LPO)**

Lipid peroxidation was measured in terms of generation of thiobarbituric acid reactive substances (TBARS, De et al., 2015). Briefly, the pulverized paw tissues were homogenized (10% w/v) in PBS and after centrifugation (12,000 g for 15 minutes), the supernatant (300 µl) was mixed with a solution (600 µl) of TCA (15%), TBA (0.375%) and HCl (0.25 N). It was agitated at 100°C for 30 minutes in a water bath, then cooled and the resultant pink colored complex [TBA-malondialdehyde (MDA) complex] was extracted by butanol and pyridine (15:1; v/v) and absorbances of the upper organic layer were measured at 532 nm. The concentration of TBARS was calculated using 1.56×10^5 M−1cm−1 as the molar extinction coefficient of MDA.

**Estimation of glutathione (GSH)**

Tissue lysates (10% w/v) were prepared by homogenizing the pulverized tissues in an extraction buffer [0.1% Triton X-100 and 0.6% sulphosalicylic acid in 0.1 M potassium phosphate buffer containing 5 mM EDTA (KPE buffer)]. The lysates (20 µl) were incubated at room temperature in the dark for 30 seconds with a reaction mixture containing DTNB (60 µl, 1 mg/1.5 ml of KPE) and glutathione reductase (60 µl, 10 U/3 ml of KPE), followed by addition of β-NADPH (60 µl, 1 mg/1.5 ml of KPE, Manna et al., 2015). Absorbances of the resultant 2-nitro-5-thiobenzoic acid were measured every 30 seconds for 2 minutes at 412 nm. The concentration of GSH was extrapolated from a standard curve and expressed as nM of GSH/mg of protein.

**Western blotting**

RAW247.7 cells were pre-treated with APC (0-5 µg/ml) followed by LPS (1 µg/ml), after which cytoplasmic or nuclear extracts were prepared for western blotting and stored at -80°C (Manna et al., 2015). Cellular proteins (50 µg per lane) were resolved on 10% SDS-PAGE and then transferred to polyvinyl difluoride membranes, the non-specific binding sites were blocked with 20 mM Tris-HCl, pH 7.4 containing 2% BSA. Following an overnight incubation at 4°C with antibodies (1:1000) against
phosphorylated and total ERK1/2, SAPK/JNK, p38, STAT1 and STAT3 along with Nrf2, HO-1, α-Tubulin and Histone H3, their bindings were detected using HRP-conjugated secondary antibodies (1:2000) and a chemiluminescent substrate, followed by analysis using DNR chemiluminescent imaging (DNR Bio-Imaging Systems Ltd. Jerusalem, Israel). Densitometric analysis was performed, and phosphorylated proteins were normalized with total protein, whereas Nrf2 and HO-1 were normalized to Histone H3 and α-tubulin respectively.

**Determination of nitric oxide (NO)**

The presence of nitrite, a stable oxidation product of NO was measured as an indicator of NO production using the Griess assay (Sarkar et al., 2008). Briefly, RAW264.7 cells (1×10⁶/ml/well) in 6 well tissue culture plates were incubated at 37°C, 5% CO₂ for 1-3 h, after which they were treated with LPS (1µg/ml) in the presence of APC (0-5 µg/ml) for an additional 48 h. Equal volumes of the culture supernatant and Griess reagent (equal volume of 0.1% NEDD in water and 1% sulphanilamide in 5% phosphoric acid) were mixed, incubated in the dark at room temperature for 10 minutes and absorbances measured spectrophotometrically at 546 nm. The levels of nitrite in the culture supernatants were assayed using a standard curve generated using sodium nitrite (0-100 µM).

**Measurement of cytokines**

Plasma levels of pro-inflammatory cytokines TNF-α and IL-6 and levels of TNF-α, IL-6 and IL-12p40 in culture supernatants derived from RAW264.7 cells were quantified by ELISA as per manufacturer’s instructions.

**Statistical analysis**

All data were expressed as mean ± standard error of mean (SEM). Comparisons among multiple groups were carried out by Kruskal-Wallis or one-way ANOVA followed by Dunnets or Tukey’s Multiple comparison test for nonparametric and parametric data respectively. Two-way ANOVA followed by Bonferroni post tests was performed to analyze interaction between the two independent variables on the dependent variable. Results were considered as statistically significant if $P < 0.05$. All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).
Results

APC attenuated the progression of arthritis

The development of arthritis was evaluated twice weekly by plethysmometric measurement of paw edema and visual scoring (Figs. 1B-D). In CIA rats, substantial paw edema was evident from day 15 onwards (Figs. 1B and D, Group II). APC (5 mg/kg b.w., p.o., Group III) caused a significant decrease only on day 15, whereas with the higher concentrations of APC (10 and 20 mg/kg b.w., p.o., Group IV), paw edema was attenuated from day 18 onwards (Figs. 1B and D), and was comparable with the MTX-treated group (Group VI, Figs. 1B and D). Similarly, APC (20 mg/kg b.w.) reduced the arthritic score from day 15 onwards to levels comparable with MTX (Fig. 1C).

In patients with RA, cachexia and attenuation of gain in body weight are associated with disease chronicity and a poor treatment outcome, whereas disease control is generally accompanied with weight gain (Baker et al., 2015). In the CIA group (Group II), there was a significant reduction in the gain in body weight ($P < 0.001$), which was prevented by APC (20 mg/kg b.w., $P < 0.001$, Table 1). However, in the MTX-treated group, there was no gain in body weight (Table 1).

The increased proliferation of autoreactive B cells in RA is accompanied by an increased production of immunoglobulin and splenomegaly (Nishiya et al., 2000; Lazaro and Morel, 2015), measurable in terms of a ‘splenic index’. In the CIA model (Group II), there was a 1.90-fold increase in the splenic index as compared with normal rats (Table 1, $P < 0.001$). This was prevented by APC (10 and 20 mg/kg b.w.), evident by the 1.36-fold ($P < 0.01$) and 1.73-fold ($P < 0.001$) decrease respectively (Table 1). However, MTX failed to impact on the splenic index (Table 1).

APC reduced severity of tissue damage

In the CIA model, radiological analysis of the paw tissues revealed conspicuous soft tissue swelling and bony erosion, that resulted in destruction of the bony architecture (Fig. 2A, Group II). APC (10 and 20 mg/kg b.w., Groups IV and V) dose-dependently prevented the soft tissue swelling and bony erosion (Fig. 2A). Importantly, the radiological improvement in Group V was comparable with the MTX-treated group (Fig. 2A, Group VI).
Histological evaluation of H & E stained sections of the hind paw in the CIA group (Group II) confirmed the development of severe arthritis, as evidenced by marked cellular infiltration, erosion, and narrowing of the joint space (Figs. 2B & C). APC (20 mg/kg b.w., Group V) and MTX (Group VI) prevented the cellular infiltration, cartilage erosion, and narrowing of the joint space (Figs. 2B & C).

In patients with RA, raised levels of TNF-α and IL-6 are strongly associated with disease severity, and a similar scenario was evident in the CIA model of arthritis, with plasma levels of TNF-α being 2.50-fold higher than normal rats (691.30 ± 52.71 vs. 275.70 ± 31.25 pg/ml, P < 0.001, Fig. 3A). APC (10 and 20 mg/kg b.w., Groups IV and V) decreased TNF-α by 1.60-fold (432.60 ± 21.76 pg/ml, P < 0.01) and 2.05-fold (336.90 ± 20.69 pg/ml, P < 0.001) respectively (Fig. 3A). Similarly, the levels of IL-6 were 2.38-fold higher than control rats (1048.00 ± 93.90 vs. 440.20 ± 44.26, P < 0.001, Fig. 3B) and pre-treatment with APC (10 and 20 mg/kg b.w.) significantly decreased its levels by 1.51-fold (695.70 ± 58.30 pg/ml, P < 0.05) and 1.62-fold (648.30 ± 60.02 pg/ml, P < 0.05) respectively (Fig. 3B). Similarly, MTX (Group VI) significantly lowered the levels of TNF-α and IL-6 (Figs. 3A & B).

**APC prevented generation of a redox imbalance**

The generation of a redox imbalance i.e. oxidative stress is a major contributory factor in the etiopathogenesis of RA (Kundu et al., 2012), and was validated in the CIA model of arthritis. As the cellular infiltrate in the joints is primarily composed of neutrophils, MPO activity was measured in the paw tissue. Group II demonstrated a significant 4-fold elevation (16.76 ± 2.78 vs. 4.19 ± 0.96 U/minute/mg protein, P < 0.001, Fig. 4A). APC (20 mg/kg b.w. Group V) and MTX (Group VI) significantly reduced the MPO activity to 5.39 ± 1.10 (P < 0.001) and 2.49 ± 0.41 U/minute/mg protein (P < 0.001) respectively (Fig. 4A).

The generation of ROS in paw tissues was estimated fluorometrically using H₂DCFDA, wherein the fluorescence of DCF is directly proportional to the generation of ROS (De et al., 2015). In the CIA model (Group II), the levels of ROS were significantly elevated by 4.84-fold (1295.00 ± 102.40 vs. 267.50 ± 20.03 RFU/mg protein, P < 0.001, Fig. 4B). The higher doses of APC (Groups IV and V), significantly attenuated the generation of ROS by 2.13 (609.1 ± 57.5 RFU/mg protein, P < 0.05) and 2.49-fold (520.60
± 59.54 RFU/mg protein, \(P < 0.01\) respectively, whereas MTX failed to restrict the enhanced generation of ROS (Fig. 4B).

Oxidative damage is associated with lipid peroxidation, following the interaction of ROS with membrane polyunsaturated fatty acids, initiating a self-propagating chain reaction, which culminates in membrane degradation (Datta et al., 2014). In the CIA model (Group II), the levels of LPO were elevated by 5.86-fold (1.70 ± 0.15 vs. 0.29 ± 0.25 nM/mg protein, \(P < 0.001\), Fig. 4C). Pre-treatment with APC (5, 10, and 20 mg/kg, b.w.) caused a significant dose dependent reduction to 1.19 ± 0.05 (\(P < 0.05\)), 0.64 ± 0.03 (\(P < 0.01\)) and 0.41 ± 0.01 (\(P < 0.001\)) nM/mg protein respectively (Fig. 4C). MTX failed to prevent the CIA induced lipid peroxidation (1.78 ± 0.11 nM/mg protein, Fig. 4C).

Glutathione (GSH) is the major cellular nonenzymatic anti-oxidant critical for maintaining the redox homeostasis (De et al., 2015). In the CIA model (Group II), the levels of GSH were significantly depleted by 2.50-fold (63.84 ± 1.46 vs. 159.10 ± 12.06 nM/mg protein, \(P < 0.001\), Fig. 4D). The highest dose of APC (20 mg/kg b.w. Group V) effectively prevented the depletion of GSH (112 ± 9.0 nM/mg protein, \(P < 0.01\), Fig. 4D), whereas it persisted with MTX (Fig. 4D).

**Effect of APC on release of inflammatory mediators in macrophages**

To evaluate the effect of APC on the activation status of macrophages, mediators of inflammation were quantified. LPS induced a dramatic increase in the release of NO, TNF-\(\alpha\), IL-6 and IL-12 (Table 2). With the highest concentration of APC, a significant decrease in the levels of NO and pro-inflammatory cytokines (TNF-\(\alpha\), IL-6, IL-12) was demonstrated (Table 2).

**Effect of APC on signaling pathways in macrophages**

Synovial macrophages sourced from patients with RA demonstrated an increased expression of toll like receptors (TLR) 2 and 4 (Huang and Pope, 2009). This activation of TLRs translated into an upregulation of pro-inflammatory signaling pathways, as evident by the LPS induced time-dependent phosphorylation of ERK1/2 and SAPK/JNK at 15-30 minutes (Fig. 5A). Accordingly, the effect of APC (1.25-5.0 \(\mu\)g/ml) on phosphorylation of ERK1/2 and SAPK/JNK was examined at 15 minutes of treatment with LPS.
wherein it reduced the phosphorylation of ERK1/2 and SAPK/JNK (Fig. 5B). Owing to the inability of LPS to activate p38, it was replaced by anisomycin (0-60 minutes Fig. 5C), and a time dependent enhancement was evident, that peaked at 15 minutes (Fig. 5C). Pretreatment with APC (1.25-5.0 μg/ml) failed to alter the anisomycin induced phosphorylation of p38 (Fig. 5D).

Another key regulator of macrophage activation and function is the Janus kinase (JAK)-signal transduction and activator of transcription (STAT) pathway whose phosphorylation status was examined. LPS induced the activation of STAT1 from 1h onwards (Fig. 6A), and the addition of APC (1.25-5.0 μg/ml) translated into a dose dependent attenuation (Fig. 6B). The activation status of macrophages is also regulated by IL-10 via activation of STAT3, by virtue of its inhibition of STAT1 (El Kasmi et al., 2006; Hutchins et al., 2013). Accordingly, the impact of APC on the STAT1/STAT3 balance was studied, wherein APC (2.5 μg/ml) caused a time dependent increase in the phosphorylation of STAT3, which was maximal at 30 minutes (Fig. 6C), but was not concentration dependent (Fig. 6D).

**APC induced Nrf2 translocation in macrophages**

Nrf2 is a key transcriptional factor responsible for upregulation of the anti-oxidant response element pathway, that translates into an enhanced expression of several anti-oxidant and phase II detoxifying enzymes (Ma and He, 2012, Basu et al., 2016). Under homeostatic conditions, Nrf2 is present in the cytoplasm attached to a cytosolic protein Keap1 (Keum and Choi, 2014). The addition of APC (1.25-5.0 μg/ml) led to its nuclear translocation, which was maximal at 30 minutes (Fig. 7A) but remained unchanged irrespective of the concentration of APC (Fig. 7A). Similarly, APC (1.25-5.00 μg/ml) raised the expression of hemeoxygenase-1 (HO-1), another cytoprotective downstream enzyme, but was not a dose dependent effect (Fig. 7B).

**Discussion**

The etiology of RA is consistently associated with an inappropriate activation of the inflammatory pathways along with a pivotal contributory role for oxidative stress (Filippin et al., 2008; Hitchon and El-Gabalawy, 2004). This has been substantiated by epidemiological studies, wherein the dietary intake of anti-oxidants and their serum levels inversely correlated with the incidence of RA (Bae et al., 2003; 2009;
Comstock et al., 1997). This is further endorsed by the beneficial impact of anti-oxidants, such as vitamin E, tempol, α-lipoic acid, N-acetylcysteine, the polyphenolic fraction of green tea, and (-)epigallocatechin-3-gallate in a CIA model of arthritis (De Bandt et al., 2002; Cuzzocrea et al., 2000; Lee et al., 2007; Kroger et al., 1997; Haqqi et al., 1999; Morinobu et al., 2008). DBA/1 mice or Lewis rats are considered as an excellent model of arthritis. However, they are difficult to maintain, necessitating development of alternative approaches. Sprague-Dawley or Wistar rats develop arthritis but tend to demonstrate variability, with regard to initiation and duration of disease (Song et al., 2015; Wagner et al., 2008). This was overcome by augmenting the B-cell activation using LPS (Thornton and Strait, 2016; Yoshino and Ohsawa, 2000) and from day 15 onwards, the rats developed severe arthritis as evident by paw edema and joint immobility (Figs. 1B-D). Prophylactic administration of APC (20 mg/kg p.o.) translated into a reduction in paw edema and arthritis score; importantly, the degree of improvement was comparable with MTX (Figs. 1B-D). Furthermore, the catabolism associated with the CIA model evident in their failure to gain body weight (Challal et al., 2016) was effectively prevented by APC (20 mg/kg p.o., Table 1). However, MTX despite its therapeutic effectiveness failed to demonstrate the same, and could be attributed to its anti-cancer activity.

The splenomegaly associated with autoimmune diseases occurs secondary to B-cell proliferation and enhanced sequestration of neutrophils (Wang et al., 2011; Lazaro and Morel, 2015) which was reflected in the CIA model (Table 1). APC (20 mg/kg b.w.) reduced the splenic index which MTX failed to ameliorate, possibly due to the associated neutropenia (Lazaroa and Morel, 2015). The radiological changes reported in the CIA model include periostitis, bony erosion, joint mal-alignment and cartilaginous deterioration (Al-Abd et al., 2014), and these features were evident in the CIA group (Fig. 2A). The effectiveness of APC in limiting the cellular infiltration, erosion of cartilage, and loss of joint space validated its anti-arthritic activity (Figs. 2B-C).

A consistent component of the pathology of RA is the enhanced presence of pro-inflammatory cytokines TNF-α and IL-6 (Wang and Zhong, 2015; Sharma et al., 2011). They are responsible for the enhanced osteoclastogenesis and expression of matrix metalloproteinases, that culminates in cartilaginous degradation (Thummuri et al., 2015). In agreement with previous studies, the levels of TNF-α and IL-6
were elevated in the CIA rats and its decrease by APC (Figs. 3A-B), corroborated that the efficacy of
APC was partly mediated via mitigation of the pro-inflammatory milieu. Similar effects have been
demonstrated by the polyphenolic fraction from green tea, coumarin derivatives, celestrol, etc. (Haqqi et
al., 1999; Hemshekhar et al., 2013; Nanjundaiah et al., 2012).

Neutrophils sourced from the synovial fluid of patients with RA generated large amounts of ROS, which
correlated with their disease activity score 28 and markers of oxidative damage (Kundu et al., 2011, 2012,
Datta et al., 2014). Similarly, in the CIA model, the inflamed synovial tissue and cartilage demonstrated
raised levels of ROS and products of oxidative damage (Wang and Zhong, 2015). Additionally,
myeloperoxidase, present in polymorphonuclear leukocytes augmented the cytotoxicity of H₂O₂ and O₂⁻
leading to cellular damage (Fietz et al., 2008). The increased MPO activity in the paw tissues of the CIA
group was effectively decreased by APC (Fig. 4A), and was comparable with previous studies (Viji et al.,
2010; Al-Abd et al., 2014). Alongside, APC prevented the enhanced generation of ROS in inflamed paw
tissues (Fig. 4B). Furthermore, evidence of oxidative damage, in terms of an enhanced degree of lipid
peroxidation (LPO, Fig. 4C) was decreased by APC, and corroborated with its free radical scavenging
activity (Srimani et al., 2009; Mitra et al., 2016). Other natural polyphenols like Isorhamnetin, Coumarin,
Triphala and Vitamin-E also demonstrated enhanced free radical scavenging activity (Wang and Zhong,
2015; Hemshekhar et al., 2013; Kalaiselvan et al., 2015; Rossato et al., 2015).

The effectiveness of MTX was evident in terms of reduction in disease severity and joint destruction (Fig. 1A-C), but its inability to ameliorate the reduction in body weight, splenomegaly, oxidative stress and
associated oxidative damage (Fig. 4, and Table 1) limited its overall efficacy. MTX, an anti-metabolite
inhibits dihydrofolate reductase and causes folate deficiency, which then triggers a redox imbalance
(Pravenc et al., 2013). Additionally, MTX upon hepatic metabolism is converted to 7-hydroxy-MTX and
is subsequently oxidized to a polyglutamated derivative. This polyglutamated MTX causes oxidative
stress and accounts for the MTX-induced hepatotoxicity (Cronstein, 2005). This inherent pro-oxidant
activity of MTX (De et al., 2015; Cronstein, 2005) accounted for its inability to reduce the enhanced
generation of ROS, LPO, and depletion of GSH. It may be proposed that compounds like APC with anti-

oxidant and anti-arthritic activity should be considered as an add-on with MTX to improve the overall management of RA.

The intimal lining of the synovium is composed of macrophage-like synoviocytes (type A) and fibroblast-like synoviocytes (FLS, type B). Macrophages play a role in the disease pathogenesis (Kinne et al., 2007 and references therein) and facilitated generation of the arthritic synovium, secondary to secretion of pro-inflammatory cytokines (e.g. IL-1β, IL-6, and TNF-α) via activation and proliferation of FLS (Kinne et al., 2007 and references therein). Clinical studies have demonstrated an increase in the number of macrophages in the inflamed synovium, that was directly linked to severity of RA (Kinne et al., 2007 and references therein). Accordingly, we undertook the studies in a RAW264.7 murine macrophage cell line, wherein APC attenuated the levels of LPS induced pro-inflammatory cytokines (Table 2), and reduced the production of NO (Table 2), thereby endorsing its potential to regulate the inflammatory status of macrophages (Sarkar et al., 2008).

Mitogen activated protein kinases or MAPKs including p38, ERK and JNK are activated in the rheumatoid synovium, and contribute towards the associated destructive and inflammatory mechanisms operational in RA (Paunovic and Harnett, 2013; Schett et al., 2000). Experiments with selective inhibitors of ERK and JNK have corroborated their role in osteoclastogenesis, secretion of pro-inflammatory cytokines (IL-6, IL-12, IL-23 and TNF-α), collagenase production, mast cell degranulation and migration of macrophages, indicating that modulation of these signalling pathways are promising therapeutic targets for RA (Guma et al., 2010; Ohori et al., 2007; David et al., 2002; Schepetkin et al., 2015). Studies with Resveratrol, Geniposide, Pyrroloquinoline Quinone, Thymoquinone (Zhong et al., 2012; Li et al., 2016; Liu et al., 2016; Thummuri et al., 2015) have endorsed the same. Pre-treatment with APC diminished the LPS-induced stimulation of ERK1/2 and SAPK/JNK (Fig. 5B), but failed to impact on the phosphorylation of p38 (Fig. 5D).

In RA, the hematopoietin family of pro-inflammatory cytokines (e.g. IL-6, IL-2, IL-12, etc.) are triggers for the JAK/STAT signaling pathway. Studies of JAK/STAT expression in human RA have demonstrated IL-6 to be the key trigger along with an increased activation of STAT1 in the RA synovium (Walker and
Smith, 2005 and references therein). Given the raised plasma levels of IL-6 (Fig. 3B), the impact of APC on this pathway was examined. LPS caused a time dependent activation of STAT1 which was prevented by APC (Figs. 6A-B). IL-10 has been proposed to exert its anti-inflammatory effect on macrophages, via signaling through STAT3 which suppresses the pro-inflammatory effects of STAT1 (Hu et al., 2008; El Kasmi et al., 2006). The ability of APC to activate STAT3 in a time dependent manner endorsed its anti-inflammatory potential (Figs. 6C-D).

Another approach towards generating an anti-inflammatory environment in the rheumatoid synovium is via upregulation of the anti-oxidant pathways. APC caused an up-regulation of anti-oxidants, both enzymatic (catalase, superoxide dismutase, Sarkar et al., 2013) and non-enzymatic (GSH, Fig. 4D). In a pro-oxidant milieu, rebound activation of the transcription factor Nrf2 which is normally sequestered in the cytoplasm by an inhibitory protein Kelch-like ECH-associated protein 1 (Keap1) occurs. It undergoes nuclear translocation and its subsequent binding to the anti-oxidant/electrophile response elements translates into an anti-inflammatory environment. Akin to APC, several synthetic and naturally occurring oxidizable polyphenols also triggered the nuclear translocation and activation of Nrf2 (Stefanson and Bakovic, 2014; Keum and Choi, 2014) as also an enhanced expression of HO-1, a key cytoprotective enzyme that is encoded by Nrf2 (Figs. 7A-B).

Taken together, this study has established the potent anti-arthritic activity of APC, which was mediated through inhibition of pro-inflammatory cytokines, amelioration of oxidative stress and mitigation of leukocyte infiltration. This anti-inflammatory activity was secondary to modulation of the pro-inflammatory MAPK and JAK/STAT signaling pathways, concomitant with induction of the cytoprotective Nrf2/HO-1 pathway. Hence, APC via its modulation of the inflammatory signaling pathways may be considered as a viable alternative for improved management of RA. However, studies should be performed to evaluate the effect of APC on FLS to further delineate its action.

Authorship Contributions
Participated in research design: De, M. Chatterjee
Conducted experiments: De, Manna, Kundu, De Sarkar
Contributed new reagents or analytic tools: Chattopadhyay, U. Chatterjee

Performed data analysis: De, M. Chatterjee, U. Chatterjee

Wrote or contributed to the writing of the manuscript: De, Manna, De Sarkar, M. Chatterjee
References


induced macrophages is mediated by suppression of iNOS and COX-2 via the NF-kappa B pathway.

*Int Immunopharmacol* 8:1264-1271.


Thymoquinone prevents RANKL-induced osteoclastogenesis activation and osteolysis in an in vivo

Viji V, Kavitha SK, and Helen A (2010) Bacopa monniera (L.) wettst inhibits type II collagen-induced

Protoc Pharmacol 5.51.1-5.51.8.

1653.


Woolf AD and Pfleger B (2003) Burden of major musculoskeletal conditions. Special Theme-Bone and

2016.

Yadav SK, Adhikary B, Bandyopadhyay SK, and Chattopadhyay S (2013) Inhibition of TNF-α, and NF-
xB and JNK pathways accounts for the prophylactic action of the natural phenolic, allylpyrocatechol
against indomethacin gastropathy. Biochim Biophys Acta 1830:3776-3786.

Yoshino S and Ohsawa M (2000) The role of lipopolysaccharide injected systemically in the reactivation

Resveratrol inhibits LPS-induced MAPKs activation via activation of the phosphatidylinositol 3-
Footnotes

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Figure legends

Figure 1: Effect of APC in a CIA model
A: Study design for induction of arthritis in an animal model. Sprague Dawley rats were immunized with CII emulsion on days 0 and 15, along with LPS (100 μg, i.p.) on day 14. Normal rats (Group I) were reserved before immunization, while the immunized rats were divided into five groups, and received vehicle (Group II), APC (5, 10 or 20 mg/kg b.w., p.o., Groups III-V) or MTX (1.5 mg/kg/week i.p., Group VI) from days 11-27. All rats were sacrificed on day 28.

B and C: Edema volume (B) and arthritic scores (C) of CIA rats were recorded twice weekly after the 2nd CII immunization as described in Materials and methods. All data are expressed as mean ± SEM (n=6).

D: Representative images of the hind paws of rats of different experimental groups.

Figure 2: Radiological and histological evaluation of joint damage in a CIA model
A: Representative radiographs of paws from normal (Group I), CIA control (Group II), APC treated CIA rats (Groups III-V) and MTX treated CIA rats (Group VI) as described in Materials and methods. F: Front paw; H: Hind paw.

B: Representative micrographs of H&E stained sections of hind paw joints (magnification, 40× and 100×), from normal (Group I), CIA control (Group II), APC treated (Groups III-V) and MTX treated (Group VI) CIA rats as described in Materials and methods. Double headed arrow indicates the joint space, white arrow indicates synovial membrane, white triangles indicate cellular infiltration, C-indicates cartilage.

C: Semiquantative assessment of tissue damage (infiltration and erosion), and joint space of H&E stained sections of hind paw as described in Materials and methods. All data are expressed as mean ± SEM (n=6).

Figure 3: Effect of APC on pro-inflammatory cytokines TNF-α and IL-6
Plasma levels of TNF-α (A) and IL-6 (B) were estimated by ELISA collected on day 28 from normal rats (Group I), vehicle treated CIA (Group II), APC treated (5, 10 or 20 mg/kg b.w., p.o., Groups III-V) and MTX (1.5 mg/kg b.w., i.p. per week, Group VI) treated CIA rats as described in Materials and methods. All data are expressed as mean ± SEM (n=6).
Figure 4: Effect of APC on the redox status

Paw tissue excised from control rats (Group I), vehicle treated CIA (Group II), APC (5, 10 or 20 mg/kg b.w., p.o., Groups III-V) treated CIA rats, and MTX (1.5 mg/kg b.w., i.p. per week, Group VI) treated CIA rats were processed for estimation of MPO activity (A), generation of ROS (B), TBARS (C) and GSH (D) as described in Materials and methods. All data are expressed as mean ± SEM (n=6).

Figure 5: LPS mediated activation of MAPkinase signalling pathway in RAW264.7 cell line

A: Representative western blot of RAW264.7 cells treated with LPS (1 µg/ml, 0-120 minutes) using antibodies against ERK1/2 or SAPK/JNK, its phosphorylated counterpart and α-tubulin as described in Materials and methods. The results are representative of three experiments.

B: Representative western blot showing the effect of APC on phosphorylation of ERK1/2, SAPK/JNK in RAW264.7 cells (1×10^5/ml, 1) that were incubated with LPS (1 µg/ml, 15 minutes, 2) in the presence of APC [1.25 (3), 2.5 µg/ml (4) or 5.0 µg/ml, (5) for 1 h] using antibodies against ERK1/2, SAPK/JNK, its phosphorylated counterpart and α-tubulin as described in Materials and methods. The results are representative of three experiments.

C: Representative western blot of RAW264.7 cells treated with anisomycin (0.5 µg/ml, 0-120 minutes) using antibodies against p38, its phosphorylated counterpart and α-tubulin as described in Materials and methods. The results are representative of three experiments.

D: Representative western blot showing the effect of APC on phosphorylation of p38 in RAW264.7 cells (1×10^5/ml, 1) that were incubated with anisomycin (0.5 µg/ml, 15 minutes, 2) in the presence of APC [1.25 (3), 2.5 µg/ml (4) or 5.0 µg/ml, (5) for 1 h] as described in Materials and methods using antibodies against p38, its phosphorylated counterpart and α-tubulin. The results are representative of three experiments.

Figure 6: Effect of APC on activation of STAT1/STAT3 pathway in RAW264.7 cell line

A: Representative western blot of cells treated with LPS (1 µg/ml) for 0-120 minutes, using antibodies against STAT1, phosphorylated STAT1 and α-tubulin as described in Materials and methods. The results are representative of three experiments.
B: Representative western blot of cells (1x10⁶/ml/well, 1) incubated with LPS (1 μg/ml, 1h, 2) in the presence of APC [1.25 (3), 2.5 μg/ml, (4) or 5.0 μg/ml, (5)] using antibodies against STAT1, its phosphorylated counterpart and α-tubulin as described in Materials and methods. The results are representative of three experiments.

C: Representative western blot of cells treated with APC (2.5 μg/ml, 0-120 minutes) using antibodies against STAT3, its phosphorylated counterpart and α-tubulin as described in Materials and methods. The results are representative of three experiments.

D: Representative western blot of cells treated with APC (1.25-5.0 μg/ml, 30 minutes) using antibodies against STAT3, its phosphorylated counterpart and α-tubulin as described in Materials and methods. The results are representative of three experiments.

**Figure 7: Effect of APC on the Nrf2 signaling pathway in RAW264.7 cell line**

A and B: Representative western blot of cells treated with APC (2.5 μg/ml, 120 minutes) or APC (1.25-5.0 μg/ml) using antibodies against Nrf2 (A) and HO-1 (B) as described in Materials and methods.
Table 1: Changes in body weight and splenic index in a CIA model of arthritis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Change in body weight (g)</th>
<th>Splenic Index</th>
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</thead>
<tbody>
<tr>
<td>Group I</td>
<td>74.90 ± 8.05</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Group II</td>
<td>14.93 ± 7.16*</td>
<td>0.38 ± 0.01*</td>
</tr>
<tr>
<td>Group III</td>
<td>40.33 ± 17.10</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Group IV</td>
<td>46.33 ± 1.96</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Group V</td>
<td>50.72 ± 8.33*</td>
<td>0.22 ± 0.02**</td>
</tr>
<tr>
<td>Group VI</td>
<td>15.25 ± 1.89</td>
<td>0.41 ± 0.03</td>
</tr>
</tbody>
</table>

Changes in body weight and splenic index at day 28 in naive (Group I), CIA (Group II), APC treated CIA rats (5, 10 and 20 mg/kg b.w., p.o., respectively, Groups III-V) and MTX treated CIA rats (Group VI) as described in Materials and methods. All data were expressed as mean ± SEM (n=6). *P < 0.001 as compared to Group I; †P < 0.01 and ‡P < 0.001 as compared to Group II.
Table 2: Effect of APC on mediators of inflammation in RAW 264.7 cell line

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrite (µM)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.22 ± 0.02</td>
<td>205.9 ± 24.84</td>
<td>14.91 ± 7.07</td>
<td>97.91 ± 1.77</td>
</tr>
<tr>
<td>LPS</td>
<td>5.40 ± 0.35*</td>
<td>1417.00 ± 116.10*</td>
<td>5870.00 ± 763.5*</td>
<td>878.7 ± 84.36*</td>
</tr>
<tr>
<td>LPS + APC (1.25 µg/ml)</td>
<td>3.69 ± 0.32</td>
<td>1269.00 ± 45.70</td>
<td>3717.00 ± 149.90</td>
<td>569.70 ± 158.10</td>
</tr>
<tr>
<td>LPS + APC (2.5 µg/ml)</td>
<td>3.10 ± 0.36</td>
<td>981.00 ± 37.41</td>
<td>2542.00 ± 84.31*</td>
<td>593.40 ± 172.20</td>
</tr>
<tr>
<td>LPS + APC (5.0 µg/ml)</td>
<td>1.85 ± 0.24*</td>
<td>700.30 ± 45.65**</td>
<td>1258.00 ± 66.04**</td>
<td>308.70 ± 31.91**</td>
</tr>
</tbody>
</table>

Cells (1×10⁶/ml) were incubated with LPS (1 µg/ml, 16 h), in the presence of APC (1.25-5.0 µg/ml) and levels of pro-inflammatory cytokines were measured in the culture supernatants as described in Materials and methods. All data were expressed as mean ± SEM. Experiment was repeated thrice in duplicates. *P < 0.001 as compared to control; *P < 0.01 and **P < 0.001 as compared to LPS treated cells.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7