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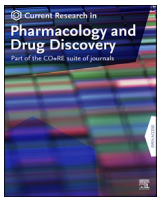

Amelioration of experimentally induced inflammatory arthritis by intra-articular injection of visnagin

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A B S T R A C T

Background: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial hyperplasia, cartilage destruction and bone erosion. Visnagin (VIS) is a proven anti-inflammatory agent and in this study, we aimed to evaluate the anti-arthritic activity of VIS when administered via intra-articular (I.A.) route of administration.

Materials and methods: RAW 264.7 cells were stimulated with lipopolysaccharide (LPS) (1 μg/mL) and treated with VIS at concentrations of 12.5 and 25 μM. Arthritis was induced in Sprague Dawley rats by administering Complete Freund's Adjuvant (CFA) (1 mg/mL) through (I.A.) route and treated with VIS via (I.A.) route at doses of 3 and 10 mg/kg twice a week for 3 weeks. Protective effects were assessed by arthritic score, behavioral studies for pain evaluation, radiological assessment, histopathological examination and molecular studies.

Results: Our results indicated that VIS significantly reduced LPS induced inflammation in RAW 264.7 cells. While in arthritic rats, VIS reduced the disease scorings with improvement towards pain. Pathological examination demonstrated that VIS reduced knee joint inflammation and cartilage destruction. Radiographic analysis and molecular studies also supported the protective effects of VIS.

Conclusion: The results of the study imply that VIS exerted potential anti-inflammatory and anti-arthritic activity in *in vitro* and *in vivo* models of RA.

1. Introduction

Despite the wide range of available treatment procedures, intra-articular (I.A.) administration of pharmacological agents are now being increasingly accepted to suppress the joint pain associated with arthritic diseases (Jones et al., 2019; Sayed Aly, 2008). A number of previous studies where animal models of joint inflammation treated with small-molecules, biologic therapies, devices and gene therapies had reported high therapeutic efficacy when administered via (I.A.) route (Chen et al., 2015; Brandt et al., 2001; Kang and Im, 2014). The reason for the wide acceptance of (I.A.) administration is because of its high therapeutic effectiveness by improving the delivery and retention of drug compounds at the site of action with simultaneous reduction in systemic exposure and drug related side effects. However clinical translation of this concept in clinics remained static for only analgesics, glucocorticoids, hyaluronic acid (HA) since two decades (Jones et al., 2019). Biological therapies though well progressed in this area, systemic complications and economic burden are the main drawbacks which make them difficult to outreach for socially weak groups. Hence it is warranted to increase screening of small molecules against arthritis for (I.A.) administration (Pucino et al., 2006).

Natural compounds exhibit potent anti-inflammatory and antioxidant properties, due to which they are considered as a first line of intervention in most of the ailments (Schumacher et al., 2011). To overcome the side effects of the present day therapy of RA, emphasis on plant based molecules for screening against RA is widely ongoing among the RA research groups (Little and Parsons, 2000; Choubey et al., 2013). Though the exact pathophysiology of RA is unknown till date, agents which can exhibit potent anti-inflammatory and antioxidant properties can be beneficial against RA therapy (Ishibashi, 2013). Visnagin (VIS) a 4-methoxy-7-methyl-5H-furo [3,2-g] benzopyran-5-one, is furanochromone extracted from the fruit of *Ammi visnaga*. VIS containing khella seeds are found majorly in Middle East countries such as Egypt and Turkey and also in Northern African countries (Duarte et al., 1995). VIS and related compounds, including khellin and visnagin, are traditionally used to treat angina pectoris as they exhibit peripheral and coronary vasodilatation activity via inhibiting Ca2+ channels to prevent calcium influx into the cell. Pre-clinical studies proved VIS to be useful in protecting

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oxalate-induced cell death in renal epithelial cells and doxorubicin induced cardiomyopathy inhibition of mitochondrial malate dehydrogenase 2 (MDH2) and cytochrome P450 (CYP)1A enzyme (Liu et al., 2014; Duarte et al., 2000). It was identified that VIS has anti-inflammatory effects when investigated on LPS induced inflammation in BV-2 microgilal cells (Lee et al., 2010). Recent study of Pasari*et al* proved that VIS is beneficial in cerulean induced pancreatitis via modulating Nuclear factor erythroid 2-related factor (Nrf-2)/nuclear transcription factor (Nf-κB) pathway with inhibition of nitric oxide (NO) production, furthermore it also reduced the expression and production of pro-inflammatory cytokines like Interleukin (IL)-1β, IL-6 and Tumor Necrosis Factor alpha (TNF-α) (Pasari et al., 2019). With the aforementioned evidences with respect to biological activities of VIS, the present study was designed to investigate the anti-inflammatory activity of VIS in *in vivo* model of CFA induced inflammatory arthritis.

2. Materials and Methods

2.1.Chemicals and reagents: VIS (Cat No: T341649), CFA (Cat No: F5881), incomplete FA (ICFA, Cat No: F5506), Glacial acetic acid (Cat No: PHR1748), Dibutylphthalate polystyrene xylene (DPX) mounting agent (Cat No: 06522), Hydrochloric acid (HCL)(Cat No: 320331), bovine serum albumin (BSA) (Cat No:A2153), sodium dodecyl sulfate (SDS) (Cat No: L3771), dimethyl sulphoxide (DMSO)(Cat No:D8418), bichinchonic acid (BCA) (Cat No: 71285) kit etc. used for the study were purchased from Sigma Aldrich, USA. Antibodies-anti-phospho nuclear factor kappa B (pNF-κB) (Ser 536/p65) (Cat No: 3033S), anti-p p38 mitogen-activated protein kinase (MAPK) (Thr 180/Tyr 182) (Cat No: 4511T) anti-p p42/44 MAPK (Thr202/Tyr 204) (Cat No: 4695T) were procured from Cell Signaling Technology (USA). Anti-cyclooxygenase (COX-2) (Cat No: sc-376861) anti-nuclear factor erythroid 2-related factor 2 (Nrf-2) – (Cat No: sc-13032) anti-matrix metalloproteinase-2 (MMP-2) – (Cat No: sc-13594) and were obtained from Santa Cruz Biotechnology (USA). All other chemicals were of analytical grade and were obtained commercially. IL-1β, IL-22, and IL-17 ELISA kits were purchased from eBioscience, USA.

2.1. Evaluating VIS in in-vitro model of inflammatory arthritis

2.1.1. Cell culture and treatment

RAW 264.7 macrophage cells were procured from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM with high glucose medium (Hyclone laboratories) (Cat No: SH30262.01) and supplemented with 10% fetal bovine serum and 1% antibiotic solution (Sigma Aldrich, USA) (Cat No: P4083). Cells were grown at 37 °C maintained in 5% CO_2 incubator. Lipopolysaccharide (LPS) (Sigma Aldrich, USA) (Cat No: L4391), a component of outer membrane of Gram negative bacteria was used to stimulate immune cells and investigated the innate immune response at a concentration of 1 μg/mL in RAW 264.7 macrophages. VIS was dissolved in DMSO and made a stock concentration of 10 mM, stored at −20 °C and diluted with respective media at required concentrations before use.

2.1.2. Cell viability assay

To evaluate the effects of VIS on cell viability, mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yr)-2, 5-diphenyl tetrazolium bromide (MTT) to formazan based assay was performed on RAW 264.7 macrophages. Here 5*103 cells per well were seeded in complete medium (100 μL) in each well of 96-well microculture plates and incubated at 37 °C in a CO2 incubator. Next day when all the cells adhered, VIS was added to the wells with 7 serial dilutions starting from 300 μM. After 24 h of incubation with VIS, cells were washed twice with PBS (50 mM, pH 7.4) and incubated with 0.5 mg/mL of MTT taken in 100 μL of culture medium at 37 °C for 4 h. Post incubation, supernatant was aspirated carefully and formazan crystals developed were dissolved in DMSO (200 μL). Optical density at 570 nm was measured using a microplate reader (SpectraMax, Molecular Devices) (Jacobs and LJJJoBC, 2001). IC_{50} values were derived by linear regression method: % cell inhibition (from control absorption) versus different concentrations of VIS (μM). All the values were expressed as mean ± SEM of three independent experiments.

2.1.3. Anti-inflammatory activity of VIS on RAW 264.7 macrophages by nitric oxide (NO) assay

Anti-inflammatory activity of VIS was evaluated by performing by nitric oxide scavenging assay. This assay was performed in RAW 264.7 cells grown in 12 well plates. Then cells were stimulated with LPS (1 μg/mL) and concomitantly treated with 5 serial dilutions of VIS based on the IC_{50} value (4 concentrations were selected below IC_{50} value). RAW 264.7 cells, control cells were culture in culture medium without any stimulus and treatment, while LPS control cells had only LPS stimulation without any treatment and maintained similarly. After 24 h of incubation period, equal amount of culture medium (supernatant) and Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride and 1% sulfanilamide in 5% H_3PO_4) was mixed and incubated at room temperature for 10 min. The optical density at 590 nm was measured using a microplate reader (SpectraMax, Molecular Devices) and values were compared with a standard curve prepared with NaNO2 (1–200 μM) (Jacobs and LJJJoBC, 2001). Final results were expressed as μM.

2.1.4. DPPH assay

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was performed to evaluate the free radical scavenging activity of VIS according to the previously reported method (Khurana et al., 2019). Briefly, 1 part of 0.1 mM solution of DPPH in ethanol was reacted with 3 parts of VIS concentration which were also prepared in ethanol in different concentrations. The reaction was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using microplate reader. Ascorbic acid was used as the reference standard. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = (A0 - A1)/A0)*100$$

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of VIS and reference. All the tests were performed in triplicates and the results were averaged.

2.1.5. Western blot analysis

RAW 264.7 cells were scraped from the petri plates in ice-cold lysis buffer followed by sonication and centrifuged at 5000 g for 10 min. While synovial tissues were homogenized (2500 rpm) thrice for 10 s with 30 s interval gap by placing the homogenizer tube on ice. The homogenization was followed by sonication and centrifugation as mentioned above. In both the experiments, supernatants were retrieved and protein concentrations were measured with the BCA protein assay kit. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoretically transferred to 0.22 μm pore size nitrocellulose membrane (Biorad, USA). The membrane was blocked with 3% bovine serum albumin (BSA) and incubated with specific primary antibodies diluted in tris buffer saline-tween 20 (TBST) at 4 °C overnight. Next day primary antibodies were removed and rinsed three times with TBST, and then membranes were incubated with corresponding secondary antibodies. Then protein bands were detected by chemiluminescene reagent. Protein bands densities were measured using Image-J software (Goud et al., 2019).

2.2. Evaluating VIS in in-vivo model of rheumatoid arthritis

2.2.1. Experimental animals

Sprague-Dawley rats (200–250g), purchased from Palamur Biosciences Private Limited, Mahabubnagar, India were housed in polypropylene cages at a room temperature of 21 ± 2 °C with 12 h light/12 h dark cycles and had free access to standard pellet diet and water. All experimental procedures were performed in according with the regulations specified by committee for the purpose of control and supervision of experiments on animals (CPCSEA). The protocol of this study was approved by the Institutional Animal Ethical Committee (IAEC), NIPER-Hyderabad.

2.2.2. Experimental design

Experimental animals with n = 8 in each group were included and study was conducted for 21 days. Animals were divided into 5 groups: **Group 1** - Sham control; **Group 2** - CFA + Vehicle (0.1 ml of 1 mg/ml CFA); **Group 3** - CFA + VIS low dose (3 mg/kg/twice a week); **Group 4** - CFA + VIS High dose (10 mg/kg/twice a week) and **Group 5-** MTX (2.8 ng/kg/twice a week**); Group 6**–Perse (VIS alone (10 mg/kg/twice a week)).All the protective treatments were administered via (I.A.) route by dissolving in 25 μl of DMSO as vehicle. Equal volume of DMSO was given in CFA control animals to eliminate the vehicle biasness. Sham control animals were given an empty injection prick at the (I.A.) region so as to nullify the pain effect with the (I.A.) injection.

2.2.3. Disease induction by CFA

Rats were injected by CFA into knee joints on day 0 followed by booster dose with ICFA on day 7. All (I.A.) administrations were performed to animals after anesthetizing them with isoflurane. All the treatments were started the day after the induction of arthritis. All behavioral parameters were performed on 0, 7, 14 and 21 day of the study period (Pearson, 1956). Then rats were sacrificed under sodium phenobarbital anesthesia (50 mg/kg I.P.), synovial tissues and knee joints were collected and fixed in 10% formalin solution for performing staining procedures. Synovial tissues were collected and stored at −80 °C to elucidate the molecular mechanisms involved in protective effect of VIS. All efforts were made to minimize animals suffering.

2.2.4. Knee joint diameter

Edema of the knee joint of animals was measured as the symbol of disease index using digital Vernier Caliper (Bär et al., 2004). Briefly, hairs on the knee joints of the animals were shaved before experimentation and width of the knee was measured on days 0, 7, 14, 21 of the study period.

2.2.5. Foot print analysis

According to previously reported methods, the functional recovery of animals from the pain and discomfort was assessed by the walking pattern of animals. Here animals were acclimatized in experimentation area several times and allowed to walk on ink absorbing paper, with their hind paws dipped in blue ink. The corresponding prints on the paper were scanned and reported (Barbosa et al., 2019).

2.2.6. Joint hyperalgesia and joint stiffness

For measuring the joint hyperalgesia on days 0, 7, 14, 21 the body of rats were gently taken into left palm and held from the back and with the aid of right fingers ipsilateral knee of the animals were subjected to bending and extension as mentioned. Briefly, one time motion of forward and other in backward which were repeated for at least 5 times within its limits of range of motion. The ease in the moment was scored accordingly: Score 2: restrictions of full range of movement of the knee in both bending and extension; Score 1: restriction of full range of movement of the knee in bending or extension; Score 0: No restriction. The total number of vocalizations during this procedure was recorded for each knee (the maximum score was 10 for each paw) for the joint hyperalgesia measurement while restriction in movement of the joint was recorded for joint stiffness (Lima-Garcia et al., 2011; Radhakrishnan et al., 2003).

2.2.7. Thermal hyperalgesia

To measure the abnormal increased sensitivity to pain during the study period, thermal hyperalgesia was performed using modified method of Hargreaves et al. (1988). Briefly, animals were acclimatized to an apparatus consisting of individual perspex boxes on an elevated glass table. Later animals were subjected to mobile radiant heat source on the hind paw, and the paw withdrawal latencies were measured as the time taken by the animals to remove its hind paw from the heat source. The cut-off point was set for maximum of 15 s to prevent any tissue damage.

2.2.8. X-ray analysis

On 21st day of the study, animals were anesthetized using isoflurane and were placed on X-ray plates, the projections of the arthritic and treated knee joint of animals were taken. X-ray was taken at the knee joints for the confirmation and evaluation of the severity of arthritis in CFA induced rats (Blackham et al., 1977).Images were taken at Ankura Diagnostics, Hyderabad (Agfa DX-D 300 DR System). Characteristic disease features including, periosteal reaction/hypertrophy, muscle swelling, articular space and the marginal bone erosions were evaluated as on the severity scale point of 0–3 for radiological scoring. The scores from each animal were summed and results were expressed as the median of each group (Anchi et al., 2022).

2.2.9. Estimation of inflammatory cytokines levels by ELISA

Pro-inflammatory cytokines such as IL-17, IL-22 and IL-1β were estimated in the synovial tissue of rats by using kit based method (eBioscience, USA) as per our previous methods. Results were expressed as pg/mg of protein in synovial tissue homogenates (Bale et al., 2018).

2.2.10. Histological examination

Synovial tissues and Knee joints were fixed in 10% neutral buffered formalin solution. Fresh formalin solution was replaced after 24 h of fixation. Knee tissues were subjected to decalcification with 10% EDTA-PBS (pH 7.4) solution for 10 days (Williams et al., 1996). Later tissues were processed using gradient alcohols and xylene, subjected to paraffin infiltration followed by embedding of the tissue. Embedded tissues were made into 5 μm thickness sections by using microtome, mounted on slides and stained with respective staining techniques such as hematoxylin& eosin (H&E), 0.01% safranin-O/0.2% Fast green, 0.4% toluidine blue/0.02% fast green. The intensity of cartilage degeneration was evaluated upon comparison with sham control animals (Getzy et al., 1982; Anchi et al., 2021). Histopathology scoring was done on severity scale of 0–3 by considering the characteristic features like inflammatory infiltration, synovial hyperplasia, pannus formation, cartilage thinning, synovial vascularity, cartilage erosions. The scores from knee joint and synovial tissues of each animal were summed and results were expressed as the median of each group.

2.2.11. Immunohistochemistry

The synovial sections were deparaffinised, cleared and dehydrated initially. Following this antigen retrieval was performed using citrate buffer (pH 6). Endogenous peroxides were later masked with 3% H_2O_2 and blocked for non-specific areas with 3% BSA. Sections were then probed with anti-p65Nf-κB primary antibodies overnight at 4 °C in a humidity chamber. Next day, further procedure was performed with PolyExcel HRP/DAB Detection system kit (PathnSitu Biotechnologies, USA) according to manufacturer's instructions. The protein positivity was visualized by adding the DAB (3,3′-diaminobenzidine tetrachloride) detection system with hematoxylin counter stain. Images were captured under light microscope (Olympus, India) at 400X magnification (Pulivendala et al., 2020).

2.2.12. Statistical analysis

All results were expressed as mean ± SEM. Statistical analysis was performed by ANOVA followed by Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism Version 6 software. Probability values less than 0.05 levels were considered as statistically significant.

3. Results

3.1. Effect of VIS on cell cytotoxicity

To investigate the concentrations of VIS required for the cell culture experiments, we initially evaluated the cytotoxicity of VIS. The results of the MTT assay demonstrated that VIS did not show any significant cytotoxicity on RAW 264.7 cells (Fig. 1a) when 5 concentrations below 300 μM was studied. However, significant cytotoxicity was observed only at higher concentrations of VIS (≥100 μM). Therefore, we selected concentrations of 12.5 and 25 μM as the sub maximal concentration to study the activity of VIS for further experiments.

3.2. VIS reduced NO production during inflammatory conditions

The external bacterial toxins like LPS and pro-inflammatory cytokines (IL-1β, IL-17 and TNF-α) have been widely used to study inflammatory responses *in vitro*. Stimulation with LPS showed accumulated nitrite production from the cells when compared to the normal cells. From Fig. 1b it is observed that treatment with VIS showed significance decrease in nitrite production in macrophages ($P < 0.0001$).

3.3. Antioxidant activity of VIS by DPPH assay

Nitrogen-centered free radical DPPH when dissolved in ethanol produces violet/purple color and upon reactivity towards antioxidants develops fade to shades of yellow color. In our study also we also observed

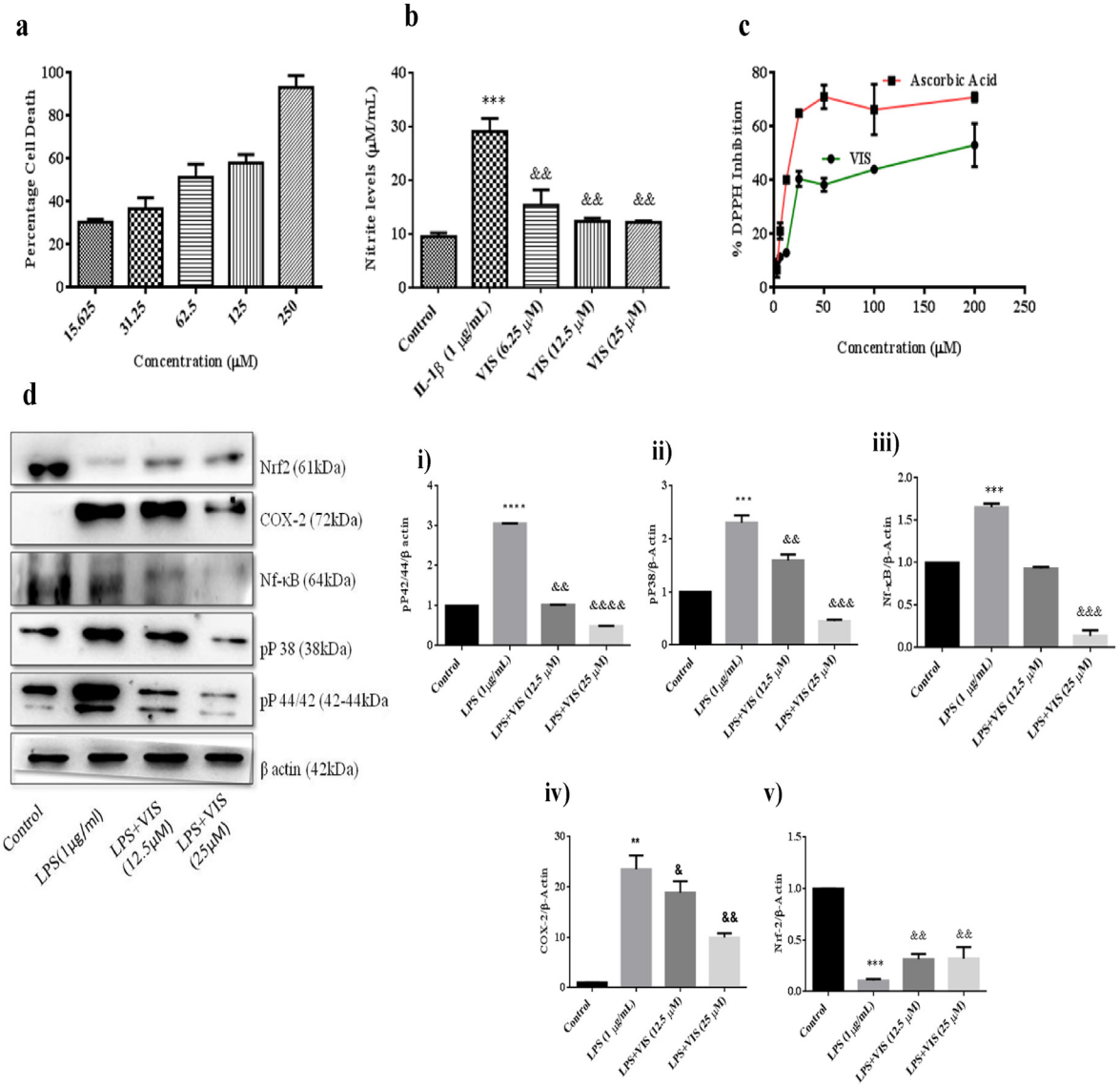

Fig. 1. *In-vitro* anti-inflammatory and antioxidant activity of VIS: a) Cytotoxicity evaluation of VIS on RAW 264.7 macrophage cells. b) Estimation of nitrite levels in LPS (1 μg/mL) stimulated 264.7 macrophage cells with different concentrations of VIS treatment. c) Antioxidant activity of VIS (cell-free assay) evaluated by DPPH assay with ascorbic acid as positive control. d) Representative immunoblot images of anti-inflammatory effects of VIS in LPS (1 μg/mL) stimulated 264.7 macrophage cells (i–v) Densitometry analysis of corresponding immunoblot images with respect to β-actin as the internal control. Here data represented as mean ± SEM, ****$p < 0.0001$,***$p < 0.001$,**$P < 0.01$ vs Control; &&&$p < 0.001$, &&$p < 0.01$, &$p < 0.05$ vs LPS control ($n = 3$). Data was analysed by one way ANOVA followed by Tukey's multiple comparison.

the reduction of DPPH color with the VIS addition in the dose dependent manner. This reduction was calculated in terms of % and compared with the control (DPPH + ethanol without any treatment). Here ascorbic acid was used as the standard (Fig. 1c).

3.4. VIS treatment decreased the expression of inflammatory mediators in RAW 264.7 macrophages

We subsequently investigated the ability of VIS to modulate the expression of p65NF-κB, pP-38, p P44/42, COX-2 which are the key markers of inflammation. The results showed that LPS stimulation markedly upregulated the expression of above mentioned markers as shown in (Fig. 1d). Impressively, VIS treatment decreased the expression of these markers owing to its protection against inflammation. Nrf2 is a transcription factor known to maintain the redox homeostasis which was observed to be reduced with LPS stimulation and VIS treatment concentration dependently maintained the expression exhibiting the protective effects of VIS against RA.

3.5. VIS exhibited anti-arthritic activity with improvement in knee swelling and pain severity in CFA challenged rats

To evaluate the pain amelioration by VIS, functional assessment was performed using foot print analysis. Individual walking patterns were

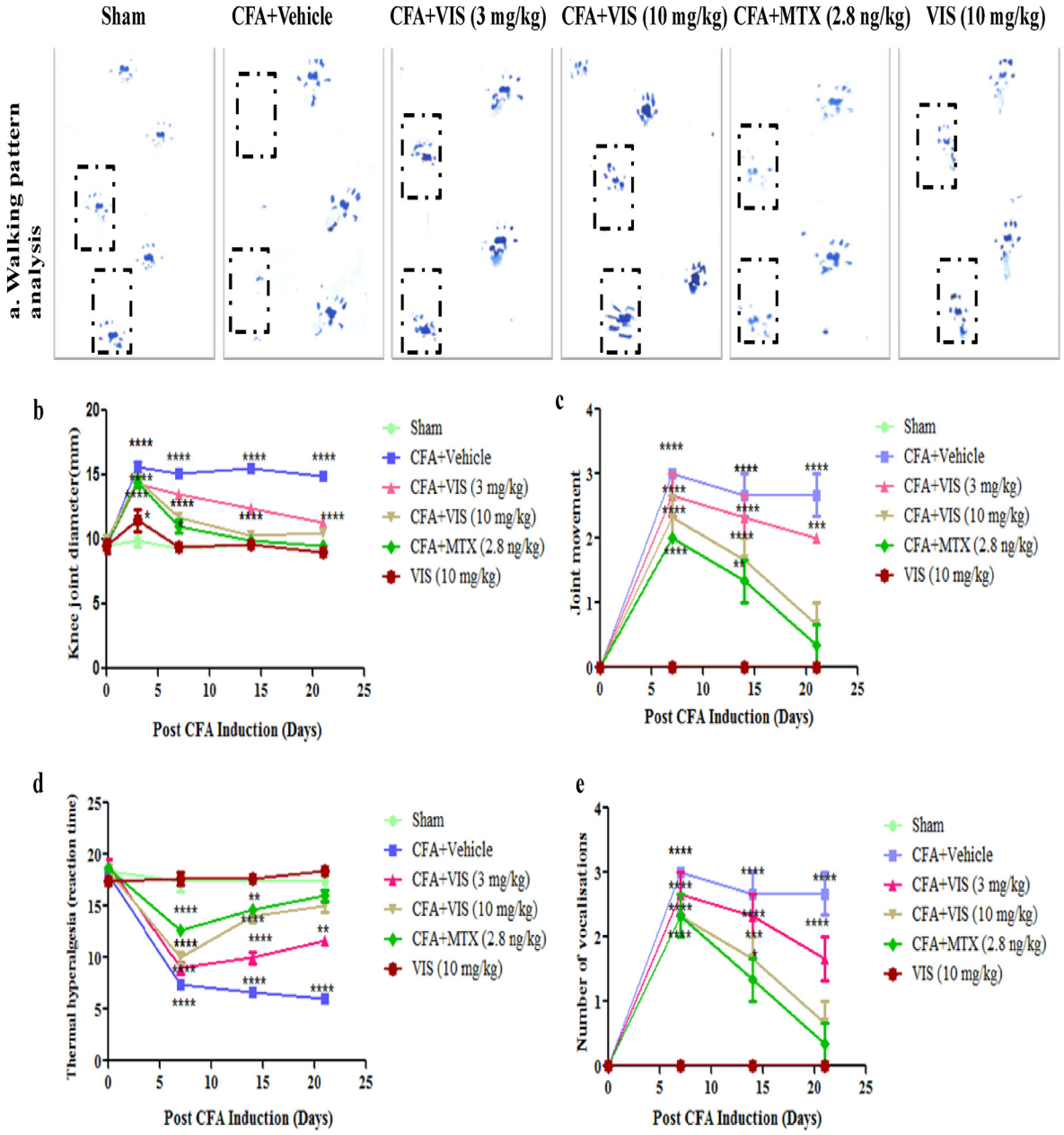

Fig. 2. Evaluation of *in vivo* anti-arthritic activity of VIS through different behavioral studies. a) Representative foot prints of different groups of rats on the walking track analysis.Here boxes indicates the ipsilateral foot prints with respect to the treatment animals. b) Representation of time dependent changes observed in Knee swelling, when measured with Vernier calipers in different groups of animals.c) Mechanical hyperalgesia which was observed to be increase with CFA induction was dose dependently reduced with intra-articular (I.A.) treatment of VIS at both doses (3 and 10 mg/kg) d) Simultaneously the grief that was produced during mechanical hyperalgesia corresponding to the pain was recorded in terms of vocalizations and the same was represented in the graph e) Thermal hyperalgesia corresponding to the pain parameter was evaluated by Hargreaves instrument which depicted reduced latency time of CFA animals with improved latency indicating the animals resistance/reduced pain in animals when treated with (I.A.) VIS in dose dependent manner. Here n = 3 animals from each group were analysed.Here ****$p < 0.0001$ vs sham; &&&&$p < 0.0001$, &&&$p < 0.01$, &$p < 0.05$ vs CFA control. Data was analysed by two-way ANOVA followed by Tukey's multiple comparison.

observed and compared with the sham control and arthritic control animals. Here arthritic rats showed very little or no stamping of the ipsilateral knee because of the pain retention in the animals. While VIS treatment showed dose dependent improvement in the walking patterns of the animals with complete spread of toes while walking. This indicates the reduction in pain associated symptoms in animals treated with VIS. VIS alone treatment also showed similar pattern in comparison to sham control animals, which exhibits its safety towards (I.A.) administration (Fig. 2a). After 3 days of CFA administration, features of RA were evident in rats with significant increase in the knee joints. Due to this, animals were observed to have hindrance in joint flexibility with joint stiffness. When observed in Fig. 2a average knee diameter of CFA control group animals was increased time dependently from 8 to 15 mm from day 0–21. While VIS treated animals exhibited improvement in swelling as the final measurement on day 21 was around 10 mm. VIS alone did not show any significance knee swelling proving its safety after (I.A.) administration. With increase in knee swelling, CFA control animals showed restriction in movement as there was hindrance in joint flexibility. The joints of normal rats showed no restriction throughout the observation period. When stiffness was calculated as one of the arthritic parameters, results indicated that score in the ipsilateral knee in the CFA treated rats increased progressively. Treatment with VIS via (I.A.) route decreased the scores progressively in dose dependent manner (3 and 10 mg/kg) (Fig. 2c). As edema formed is enriched with the inflammatory cytokines and arachnoids family members, are majorly responsible for pain stimulations. To study the effect of VIS on reduction of pain, thermal hyperalgesia experiments were performed. It was clear that CFA control animals suffered with high pain based on the obtained reflexes (more vocalizations in hyperalgesia/less response time in thermalgesia) recorded in comparison to sham control animals. VIS treatment results also depicted that VIS could reduce this pain from moderate to appreciable levels in dose dependence manner (Fig. 2d &e).

3.6. VIS ameliorated the bone damage incurred by CFA

The radiographic images of the knee joints of all study groups of rats are shown in Fig. 3. It is evident from the radiographic images that CFA induced rats developed multiple bone erosions, irregular joint spaces, soft tissue swelling. These are also the clinical symptoms in the RA patients. When examined for the treatment efficacy of VIS in improving this condition, it was evident from Fig. 3a that 3 mg/kg dose of VIS treatment resulted with persistence of marginal erosions and joint space reduction, while improvement in soft bone swelling was observed. While 10 mg/kg treated rats showed improvement in joint space narrowing and soft bone swelling, though marginal erosions existed were considered mild in comparison to CFA control animals. MTX treated animals also showed similar results with reversion in joint space reduction and diminished bone erosions. These results thus establish the anti-arthritic activity of VIS with the supportive radiological scoring displayed in Fig. 3b.

3.7. VIS restored the histological changes and cartilage destruction in rats challenged with CFA

To further confirm whether VIS exerts anti-arthritic effects by modulating histological changes, we performed H&E staining in synovial tissue and knee joints to observe key morphological changes produced by CFA, while toluidine blue/fast green, safranin-O-staining were performed to evaluate the cartilage damage in the knee tissues. From Fig. 4a, it can be observed that CFA treated animals displayed the destruction of articular cartilage thinning and pannus formation; distortion of spongy bone and bone marrow tissue when compared with sham control animals. These alterations were improved slightly with 3 mg/kg treated VIS animals in terms of decreased infiltrations and recovered articular thinning. While 10 mg/kg VIS treated animals exhibited good anti-arthritic activity with reconstructed cartilage and decreased infiltration signs. These observations were equally observed with MTX treated animals. Similar therapeutic changes were observed in the H&E stained synovial tissues. It can be observed that H&E stained CFA challenged synovial tissues showed markedly increased synovial vasculature and infiltrations with musculature of dense tissue. These observations were done in comparison to sham control animals which showed normal architecture of the synovial tissue (Fig. 4). While treatment with VIS (3 mg/kg) did not show any significant reduction in these observations, while mild reduction in the infiltrations can be observed. Better therapeutic effect

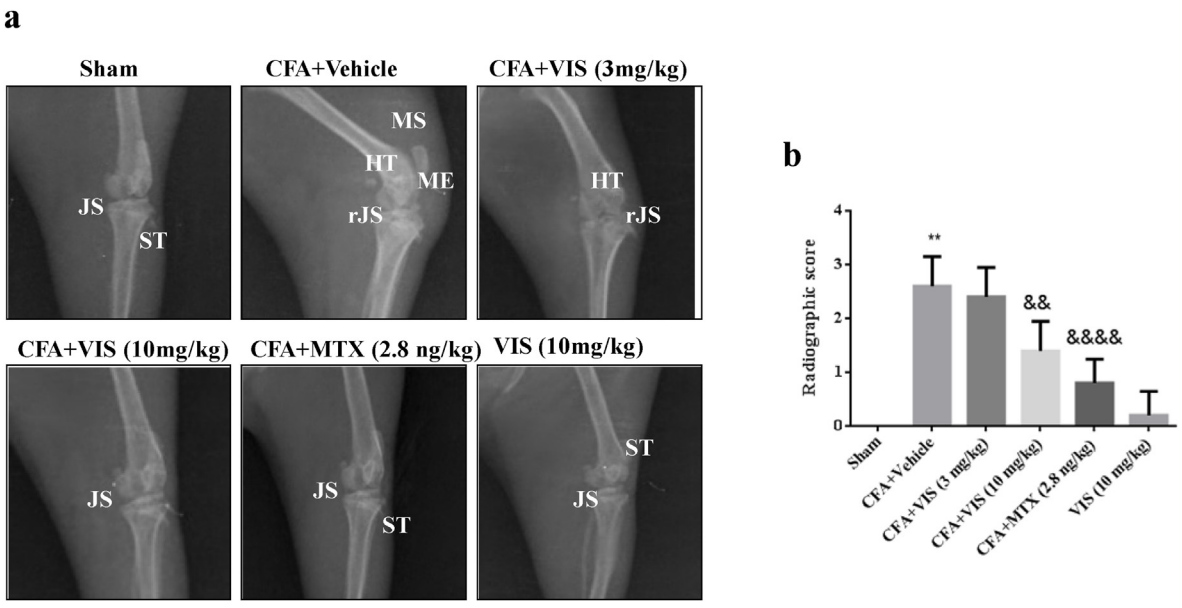

Fig. 3. a) Radiographical analysis of knee joints from different groups of rats. i) Sham animals showing normal joint space with healthy cartilage at knee joint (JS) periarticular soft tissue; CFA animal showing periosteal reaction/hypertrophy (HT), soft narrowed JS (rJS), muscle swelling (MS) and marginal erosions (ME); (I.A.) VIS (3 mg/kg) treated rats showing still rJS, HT; while VIS (10 mg/kg) treated animals showing normal JS with minimal ST and HT; VIS alone (10 mg/kg) treated animals did not exhibit any significant morphological changes and was observed to be similar to sham control animals. b) Radiological scoring performed on 0–3 scale based on range of severity. Values are expressed as mean ± SEM. Here ****$p < 0.0001$ vs Sham; &&&&$p < 0.0001$ &&$p < 0.01$ vs CFA control. Data was analysed by one way ANOVA followed by Tukey's multiple comparison and $n = 3$ animals from each group were analysed.

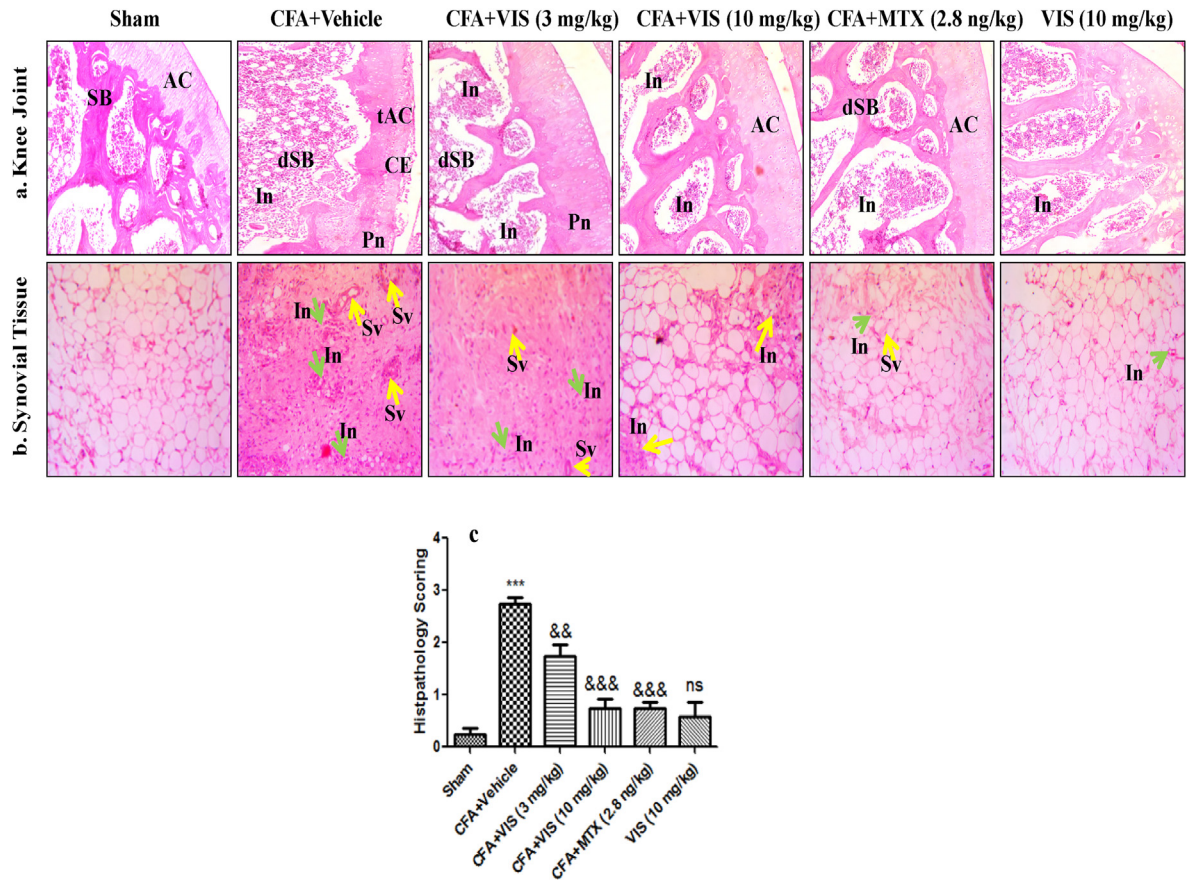

Fig. 4. H&E stained histopathological analysis of knee joints and synovial tissues. Histopathological images of knee joints were taken at (200X) and synovial tissues were taken at (400X) magnification. a) Knee joints: Sham group representing healthy articular cartilage (AC), spongy bone (SB); CFA induced RA indicating damaged and thinning of Ac (tAC) with distorted and inflamed SB (dSB). Here when the CFA challenged rats when treated with VIS (I.A.) treatment exhibited ameliorated architectural damage with improved AC and SB. While treatment of CFA induced rat with VIS at 3 mg/kg showed persistence of pannus in the cartilage, while at 10 mg/kg animals did not show any signs of pannus formation in the knee joints. b) Synovial tissue of normal control animals showed areolar subintima, while CFA induced rat showed, synovial hyperplasia (Sh), synovial vasculature (Sv) filled with stroma and inflammatory cells (In). VIS treatment (3 mg/kg) also exhibited infiltrations with stroma. Whereas VIS (10 mg/kg) treated animals showed reduced infiltrations with restored areolar subintima. However no significant changes were observed with VIS alone treated animals. c)Represents histological scoring performed between different treatment groups.Values are expressed as mean ± SEM. Here ***$p < 0.001$ vs Sham; &&&$p < 0.0001$ &&$p < 0.01$ vs CFA control. Data was analysed by one way ANOVA followed by Tukey's multiple comparison and $n = 3$ sections from each group were analysed.

was observed with VIS 10 mg/kg treatment, which showed reduced infiltration with slight remnants of disease pathological observations. Standard drug MTX treated animals also showed similar good improvement in decreased synovial hyperplasia and infiltrations in protection towards arthritis. While supporting the restored histological changes of VIS mediated anti-arthritic effect was further confirmed by cartilage specific staining by Safranin-O-staining and toluidine blue/fast green staining (Fig. 5a and b), where complete reconstruction of cartilage was seen with VIS 10 mg/kg treated and MTX treated animals than in 3 mg/kg treated animals. Moreover, VIS 10 mg/kg alone treated animals did not exhibit any significant damaging features of either knee joint or synovial tissue thus inferring safe. These stainings illustrated that articular cartilage (AC) destruction was prevented upon VIS treatment.

3.8. VIS reduced the pro-inflammatory cytokines in the synovial tissues of CFA induced arthritic rats

To validate the anti-inflammatory effect of VIS, we here evaluated the levels of various pro-inflammatory cytokines like IL-17, IL-22 and IL-1β in the synovial tissues by ELISA (Fig. 6a–c. As CFA significantly increased these pro-inflammatory cytokines, treatment with VIS (10 mg/kg) ameliorated the levels, exhibiting its therapeutic efficacy in treatment of RA (Fig. 6d). As Nf-κB plays important role in the inflammatory diseases like RA we performed IHC analysis, which showed remarkable immunostaining corresponding to intense brown color in the synovial tissue of CFA challenged group. While VIS treatment (10 mg/kg) reduced this intensity of staining in comparison to CFA animals owing to its protective activity. Thus, these results confirm the anti-inflammatory potential of VIS towards RA (Fig. 6e). Additionally, VIS treatment dose dependently reduced the phosphorylated mitogen-activated protein kinase (MAPKinases) p42/44 levels with simultaneous decrease in expression of MMP-2 and COX-2 for exhibiting the anti-arthritic activity.

4. Discussion

RA is one of the most common inflammatory autoimmune diseases affecting one among ten individuals globally (Pulivendala et al., 2020). Present day treatment approaches includes non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs, immuno suppressants and anti-cytokines (Pulivendala et al., 2020; Bachmeier et al., 2005; Tayal and Kalra, 2008). In spite of their considerable therapeutic activities, long term utility result in extra-articular manifestations like potential toxic effects to vital organs such as liver, intestine, kidney and heart leading to immunodeficiency with poor efficacy (Singh et al., 1991). With the culminating data, these manifestations are considered as predators in mortality of RA. In order to overcome

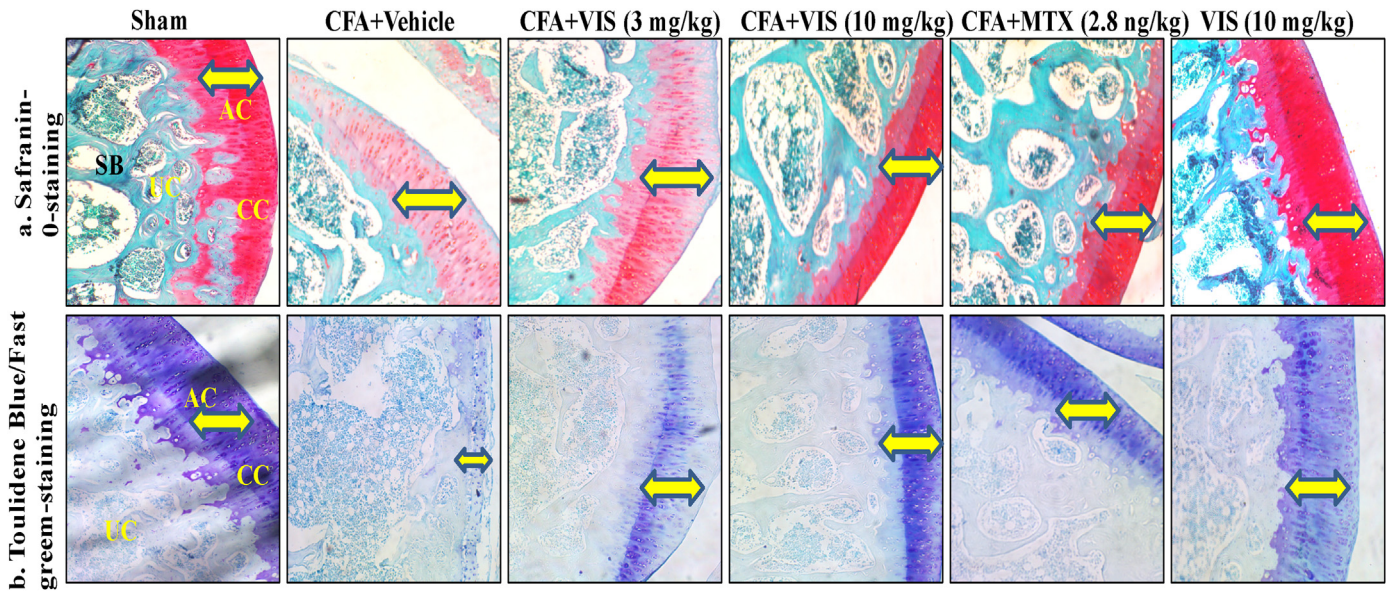

Fig. 5. a) Effect of VIS treatment on articular cartilage erosion of knee joint was studied by Safranin-O-staining and toulidene blue/fast green staining. Here in both the staining Sham animals exhibiting normal knee-joint histology with normal uncalcified cartilage (UC), calcified cartilage (CC), and subchondral bone (SB) with richly staining articular structure (AC)This staining was represented by yellow color double sided arrow. CFA induced RA group showing cartilage degradation with minimal or no staining in the articular region, treatment with VIS to the CFA induced rats were observed to preserve the cartilage destruction in a dose dependent manner. However no significant destruction was observed with VIS alone treatment group. Here n = 3 sections from each group were analysed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

these, site specific treatment approaches like (I.A.) delivery evolved have been developed and many are already in clinical set-ups (Evans et al., 2014). These include (I.A.) shots of steroids, hyaluronic acid, platelet-rich plasma (PRP), biologicals (TNF-α and JAK inhibitors). However, the main draw back with these is that there should be large gap between each injection due of their side-effects (Šenolt et al., 2009). Additionally, this approach has not been economical while making it unreachable to the lower socio economic groups (Kunkel and Chensue, 1985). These all factors necessities for the development of therapeutically active molecules derived from natural source. Though there is a sustained development in this area of research, clinical translation is hampering due to unclear mechanism of actions and controversial efficacies. Hence improvement in (I.A.) treatment leaves a room for further rigorous and scientific development of natural molecules in treating joint disorders. In this study, in order to achieve effective treatment approach, we selected two strategies: i) to choose a natural compound with anti-inflammatory properties and ii) to deliver the compound site specifically (so as to minimize the extra-articular manifestations).

So the present study was designed initially to screen the anti-inflammatory effect of VIS on LPS induced inflammation in RAW 264.7 macrophages. LPS is commonly used to screen the anti-inflammatory potency of the molecules in the macrophages, where it involves in the metabolism of arachidonic acid (Doerfler et al., 1994). Impairment to its metabolism releases PGE2 and COX-2 which are the mediators for the release of inflammatory mediators and pro inflammatory cytokines, MAPKinases etc. and contribute to various chronic diseases including arthritis (Rasheed and Haqqi, 2012). Here similar results were obtained, where LPS stimulation resulted in increased expression of COX-2, pP42/44, pP38, which are markedly reduced with VIS treatment in a concentration dependent manner. This result indicates that anti-inflammatory effects of VIS maybe due to inhibition of COX-2 expression. Previous studies also reported that LPS is involved in synthesis of nitrogen species responsible for the inflammation (Jacobs and Ignarro, 2001). NO formed is also observed in various diseases and hence was evaluated for its production with or without VIS treatment. Here we observed remarkable increase in NO production with LPS stimulation and VIS treatment reduced the production of NO, while confirming its anti-inflammatory activity.

In recent years, there has been a substantial growth of interest in systems of molecules/drugs derived from medicinal plants with therapeutic effects in both developing and developed countries. This is due to their origin, minimal side effects, economic with their wide spread availability and ease of acceptance along with their effectiveness in simple to complex mechanisms of diseases. Even in treatment of RA like conditions several molecules or active constituents of plants have been evaluated for their efficacy in bringing down the disease pathological conditions. This is the first experimental study of the anti-arthritic effect of VIS in an animal model. Despite of several advantages, translation of these natural pharmacological agents is always challenging due to their poor pharmacokinetics. Hence, to overcome this concerns, site specific studies are now encouraged to achieve maximum therapeutic activity of pharmacological agents with minimal side effects and dosing frequency. Following this, studies were conducted in this direction and when polyphenols like epigallocatechin gallate, tannic acid when I.A administered to CFA induced rats, they effectively prevented the cartilage destruction and inflammation (Natarajan et al., 2015).In the present study, (I.A.) injections of VIS were studied to repair the developed characteristic disease features induced by CFA in rats with its previously demonstrated anti-inflammatory activities. CFA induced RA in rat model of arthritis was used because of its widely preferred pre-clinical model. The role of VIS in ameliorating the pro inflammatory cytokines, subsiding the pain and cartilage destruction with recovery as a therapeutic agent in RA was demonstrated in the present study. (I.A.) route of administration of VIS was preferred because of its high *in vivo* therapeutic doses (100 mg/kg) which may produce systemic effects with minimal exposure at the site of inflammation. Hence this condition requisites local targeted approach rather than systemic exposure. Very few studies have been conducted on the anti-inflammatory activities of VIS and its derivatives. In the present study, treatment with 10 mg/kg VIS (I.A.) injected rats produced a greater anti-RA effect than the 3 mg/kg treated groups with high dose treated joints exhibited less cartilage destruction, pannus formation, erosion of cartilage and bone. While synovium tissue was also

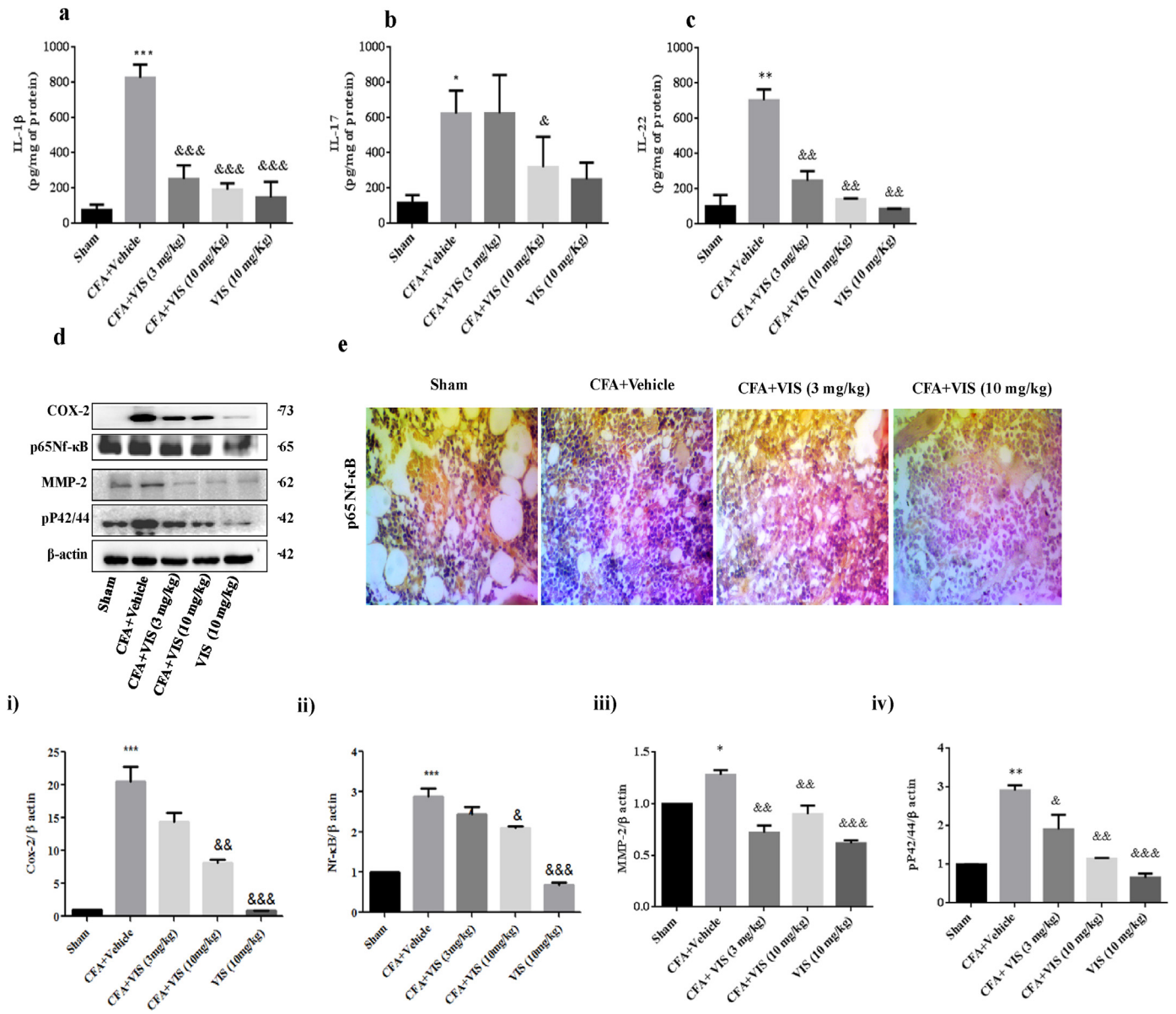

Fig. 6. (a–c) Effect of VIS on various pro-inflammatory cytokines (IL-1β, IL-17, IL-22) in synovial tissue produced by CFA in RA model of rat. d) Immunoblotting analysis for evaluation of protein expression in different treatment groups e) Immunostaining of Nf-κB expression in the synovial tissues, represents the intensity of activation of receptor with corresponding to the positive DAB staining. Here n = 12–18 sections from each group were analysed. i-iv) Densitometry analysis of immunoblots with respect to β-actin as internal control. Here data represented ***p < 0.001, **p < 0.01,*p < 0.05 vs Sham; &&& p < 0.001, && p < 0.01, &p < 0.05 vs CFA control. Data was analysed by one way ANOVA followed by Tukey's multiple comparison.

deprived of synovial atrophy and inflammation in VIS treated animals. Correlating this effect, less joint destruction and bone erosion was evident in the radiographs of the VIS treated joints.

The AC of the knee is one of the highly organized connective tissues, which majorly aids in frictionless movement between the articulating joint surfaces and regulates the transmission of loads with a low frictional coefficient (Wong and Carter, 2003). Destruction of the AC, results in reduced synthesis of matrix components with further destruction by disintegrin and metalloproteinases (Caterson et al., 2000). Due to deficiency of blood vessels and lymphatic supply, it exhibits a low range of intrinsic healing and repair, this makes it challenging for repair and restoration in any condition of injury (James et al., 2008). Hence, here we considered that restoration of AC as a prognostic feature of any anti-arthritic compound and hence we evaluated the same for VIS treated animal model. Impressive results were obtained when we evaluated the health of cartilage by two different staining i.e safranin-o staining and toulidine blue/fast green staining which indicated anti-arthritic property of VIS.

Pro-inflammatory cytokines, such as IL-1β, IL-6, IL-17, IL-22 and TNF-α are reported to play a major role in the physiopathology of RA. Elevated levels of IL-17 (Th 17) usually promote recruitment of neutrophils and macrophages (infiltration) resulting in synovial joint tissue damage leading to pathogenesis of RA. Similarly, CFA also increases IL-1β, IL-6, IL-17, and TNF-α levels in serum and synovial tissues with simultaneous reduction of anti-inflammatory cytokine IL-10. Here we observed a significant reduction of IL-1β, IL-17 and IL-22 cytokine levels in the inflamed synovial tissue of CFA challenged rats, confirming its anti-inflammatory property, VIS significantly decreased these cytokine levels. While correlating to the statement made by Xu et al., that any drug that has a potential to suppress the Nf-κB activation can be an anti-arthritic drug, VIS was evaluated for its expression via immunostaining analysis (He et al., 2008). Here CFA treated group exhibited strong

staining for the Nf-κB protein, VIS attenuated Nf-κB expression with moderate to mild staining at 3 mg/kg and 10 mg/kg treated groups. Another important observation of the present study is very clear cut safety profile of multiple doses of VIS upon (I.A.) administration did not produce any abnormal biochemical or physiological effects. However, this study lacks conformational safety studies on organ toxicities which need to be performed in future. Also, efficacy of VIS in present study will be debatable due to absence of (I.A.) pharmacokinetics data and presence of this can strengthen the outcome of the study results. Further, development of suitable drug delivery systems will enhance the activity and efficacy of VIS for treatment of RA in clinics.

5. Conclusion

To the best of our knowledge, this is the first study to demonstrate the local inflammatory effect of (I.A.) injection of VIS for treatment of RA in reducing the synovial inflammation. In summary, VIS may be a potential lead for the treatment of RA via (I.A.) route. VIS treatment attenuated production of pro-inflammatory cytokines like IL-17, IL-22, TNF-α and inflammatory mediators like Nf-κB and MAP Kinases, which are responsible for disease aggravation. Additionally, VIS treatment also protected from the cartilage destruction amid the milieu of inflamed joints which is a prime requisite for anti-arthritic agent. However, further molecular studies are required to investigate its effectiveness as an anti-arthritic agent.

*Authors contribution

Conceptualization, study Design and funds collections was done by Dr. Chandraiah Godugu, experimental work, Data collection was done by Sowmyasree Gurram, Pratibha Anchi, Biswajit Panda, Sayali Santosh Tekalkar, Ravindra Bapu Mahajan. Statistical analysis and data interpretation was performed by Sowmyasree Gurram, Pratibha Anchi. Pratibha Anchi wrote the manuscript. Pratibha Anchi and Biswajit Panda revised the manuscript. Chandraiah Godugu revised and edited the final revision of current manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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