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, 2006). 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; Choube 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...
oxalate-induced cell death in renal epithelial cells and doxorubicin
cardiomyopathy inhibition of mitochondrial malate dehydro-
genase 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 inflamma-
tion in BV-2 microglial 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-kB) 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 aforemen-
tioned 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), Dibutyolphosphate polystrene yylene (DPX) mounting
agent (Cat No: 06522), Hydrochloric acid (HCL) (Cat No: 320331),
bovine serum albumin (BSA) (Cat No:2A153), sodium dodecyl sulfate
(SDS) (Cat L3771), dimethyl sulfoxphol (DMO) (Cat No:D8418),
bichinonic acid (BCA) (Cat No: 71285) kit etc. used for the study were
purchased from eBioscience, USA. IL-1β, IL-22, and IL-17 ELISA kits 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 sup-
plemented with 10% fetal bovine serum and 1% antibiotic solution
(Sigma Aldrich, USA) (Cat No: P4083). Cells were grown at 37 °C
maintained in 5% CO2 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 concentra-
tion 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 to
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). IC50 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 in-
dependent experiments.

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

Anti-inflammatory activity of VIS was evaluated by performing by
nitrice 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
IC50 value (4 concentrations were selected below IC50 value). RAW
264.7 cells, control cells were culture in culture medium without any
stimulus and treatment, while LPS control cells had only LPS stimulus
without any treatment and maintained similarly. After 24 h of incubation
period, equal amount of culture medium (supernatant) and Griess re-
agent (1:1 mixture of 0.1% N-(1-naphthyl) ethyl-
ediaminedihydrochloride and 1% sulfinilamide in 5% H3PO4) was
mixed and incubated at room temperature for 10 min. The optical density
at 590 nm was measured using a microplate reader (SpectraMax, Mo-
olecular Devices) and values were compared with a standard curve pre-
bred 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 mL
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 tempera-
ture 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.

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 homoge-
nization 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 sulfa-
te–polyacrylamide gel electrophoresis (SDS-PAGE) gel and electropho-
retically transferred to 0.22 μm pore size nitrocellulose membrane
(Biorad, USA). The membrane was blocked with 3% bovine serum al-
bumin (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 accordance 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 mg/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 ICF 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 (Afga DX-D 300 DR System). Characteristic disease features including, periosteal reaction/hypertrophy, muscle swellings, 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 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% H₂O₂ 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 (PathSitu 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 concentrations of 12.5 and 25 μM as the sub maximal concentration to study the activity of VIS for further experiments.

![Image](55x55 to 435x538)

**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...
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.
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 NFκB 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 (MAPKs) 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
expression. Previous studies also reported that LPS is involved in syn-
thesis 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 therapeu-
tic 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-arthritis 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 speci-
fic 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 (Senolt 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 therapeuti-
cally 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 effi-
cacies. 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 spe-
cifically (so as to minimize the extra-articular manifestations).

So the present study was designed initially to screen the anti-
flammatory 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-
flammatory effects of VIS maybe due to inhibition of COX-2 expression. Previous studies also reported that LPS is involved in syn-
thesis of nitrogen species responsible for the inflammation (Jacobs and
Ignarro, 2001).
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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