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Isoliquiritigenin, Liquiritigenin Rich Root Extract of Glycyrrhiza glabra Attenuates Inflammation in Macrophages and Collagen-Induced Arthritis in Rats

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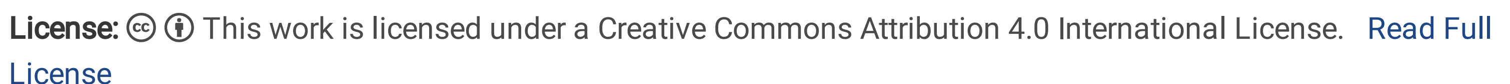

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Abstract

The aim of this study was to evaluate the potential effect of root extract of *Glycyrrhiza glabra* (*IVT-21*), isoliquiritigenin (ISL), and liquiritigenin (LTG) present in *G. glabra* root extract in *in-vitro* anti-inflammatory activity and we also investigate the effects of *IVT-21* in collagen-induced arthritis (CIA) rats. Primary peritoneal macrophage cells were used for check the anti-inflammatory effect of *IVT-21*. Apart from this Collagen-induced arthritis (CIA) was developed in Wistar rats. Animals were orally treated with *IVT-21* at dose rate of 30,100 and 300 mg /kg for 21 days. The chemical signature of *IVT-21* using HPLC analysis showed the presence of ISL and LTG as the main active ingredients. Treatment of *IVT-21*, ISL and LTG were able to reduce the production of pro-inflammatory cytokines (TNF-α, IL-6) in LPS-induced inflammation in primary peritoneal macrophages. *In-vivo* experimental pharmacology profile of *IVT-21* against rheumatoid arthritis revealed that oral administration of *IVT-21* significantly reduced the arthritis index, arthritis score, inflammatory mediators level in CIA rat's serum, and also reduced the NFκB-p65 expression as evidence of immunohistochemistry in knee joint tissue of CIA rats, reduce the inflammatory mediator's gene expression in a dose-dependent manner in paw tissue of CIA rats. Further, in *in-vivo* safety studies of *IVT-21* was found to be safe in experimental animals up to 2,000 mg/kg dose. The result of this study suggests the suitability of *IVT-21* as a drug-like candidate for further investigation in the management of inflammation and rheumatoid arthritis.

Introduction

Glycyrrhiza glabra L. (Leguminosae) is an approximately one-meter tall perennial herb commonly known as sweet wood and mulethi, and native in Western Asia, Eurasia (Fiore et al., 2005; Shah et al., 2018). The roots and stolen parts of the glycyrrhiza species are commonly known as licorice. Mulaithi root has numerous pharmacological properties like anti-viral, anti-cancer, anti-diabetic, anti-microbial and anti-inflammatory properties in addition to cardioprotective, hepatoprotective and immunomodulatory effects and its stem is used for the treatment of tuberculosis (Arseculeratne et al., 1985; Asl and Hosseinzadeh, 2008). It is used for the treatment of mouth ulcers, alimentary tract disorders, aphrodisiac and also used to cough-relieving treatment (Anil and Jyotsna, 2012; Saxena, 2005; Sedighinia and Afshar, 2012). Licorice mainly contains pentacyclic triterpenoid saponins and phenolics. This includes glycyrrhizin, glycyrrhetinic acid, flavonoids, isoflavonoids, chalcones, coumarins, stilbenoids, etc. Some major phenolics present in licorice are liquiritin, liquiritigenin, isoliquiritin, isoliquiritigenin, glabridin, formononetin, glabrene, hispaglabridin A, hispaglabridin B, glabrol etc (Chin et al., 2007; Mamedov and Egamberdieva, 2019; Nomura et al., 2002). Liquiritigenin (LTG) and its structurally related bioprecursor, Isoliquiritigenin (ISL), are two majorly explored bioactive secondary metabolites that possess diverse pharmacological activities like anti-inflammatory, anti-oxidant, anti-cancer, hepatoprotective and cardioprotective activities (Peng et al., 2015; Ramalingam et al., 2018). Isoliquiritigenin reduced osteoclastogenesis by reducing the expression of RANKL-RANK-TRAF6 pathways, thereby suppressing abnormal bone formation (Ji et al., 2018). Besides, it suppressed the activation of NF-κB and the production of MMPs induced by IL-1β in chondrocytes (Zhang et al., 2018). The earlier reports suggest that phenolics present in *G. glabra* showed significant anti-inflammatory potential; therefore present study aims to develop phenolics (ISL & LTG) rich extract of *G. glabra* coded as *IVT-21* and explore their anti-arthritic activity in the collagen-induced arthritis model.

Inflammation can be produced by numerous different causes, like a blood clots, physical injury, infection, immune system disorder, chemical exposure etc. and macrophages play an important role in the inflammatory process. Inflammations can have far-reaching medical consequences, because chronic inflammation is linked to several human diseases like rheumatoid arthritis, cancer, septic shock, allergy, gastro-enteritis, etc. (Roe, 2021). Rheumatoid arthritis (RA) is a progressive autoimmune joint disease caused by environmental and genetic factors (Meyer et al., 2019). The yearly occurrence of RA is approximately three in ten thousand people, increasing with age and peaking between 35 and 50 years (Gabriel, 2001). Inflammation, cartilage destruction, synovial tissue hyperplasia and vasculitis are the common characteristics of RA. While the mechanism of RA is unknown, evidence from biological, genetic and histopathological studies both in clinical research and *in-vivo* models shows that inflammation and joint destruction are linked with stromal tissue dysregulation and immune-mediated etiologies (McInnes and Schett, 2007). Inflammatory markers (TNF-α and IL-6) play a crucial role in RA diseases, so it is important to maintain a balance between the anti-inflammatory and pro-inflammatory markers (Alam et al., 2017). Production of TNF-α, induces several inflammatory markers like chemokines, cytokines,matrix metalloproteinases (MMPs), which finally leads to synovial inflammation and joint destruction (McInnes and Schett, 2007). Nonsteroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMRADs), immunosuppressive, and hormone-based drugs are used for the treatment of rheumatoid arthritis but regular and long time used these drugs have several side effects in RA patients. So, new medicines for RA management with a low occurrence of side effects and high effectiveness are urgently required (Kun et al., 2020). Medicinal and aromatic plants are being used for thousands of years in the traditional systems of medicine for the beneficial of numerous diseases. Natural molecules are much safer than synthetic molecules. Flavonoids, steroids, lignans, polyphenols, coumarins, terpenes and alkaloids are all these natural compounds that are scientifically verified to alleviate inflammation, pain and fever (Conforti et al., 2009; Singh et al., 2014).

Materials And Methods

Chemicals

Collagen, Complete Freund's Adjuvant, Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma-Aldrich. Dexamethasone was procured from Qualigens Fine Chemicals, India. C-RP kit (ERBA diagnostics kit), IL-17A ELISA kit (KRISHGEN Biosystem), Rat-specific MMP-1 and MMP-9 ELISA kits (Elabscience) Mouse-specific (TNF-α, IL-6) and rat-specific (TNF-α, IL-6) ELISA Kit was procured from BD Biosciences, USA. NFkB-p65 primary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA), N-(1-Naphthyl) ethylene-diamine dihydrochloride (NED) SD fine chem limited, Secondary antibody (Jackson immune).

Plant Material

G. glabra roots were obtained from Dabur India and authenticated by Dr. S. C. Singh, taxonomist (superannuated) of our institute.

Extract Preparation

The 2 Kg of *G. glabra* roots were cut into small parts, dried under a shaded environment, and powdered with a mechanical grinder. The powder roots were extracted with 4 litres of the ethanol-water mixture (1:1) at room temperature for 6 hours. This process was repeated for three times (4 litres X 3 times). After filtration, the combined hydroalcoholic solution was evaporated on the rota evaporator, giving 376 g of hydroalcoholic extract. This extract was subjected to acid hydrolysis in the presence of 5% HCl solution, and the resulting reaction mixture was extracted with ethylacetate which gave 81.6 g of ethyl acetate extract on concentration. The ethyl acetate extract was fractionated on silica gel (60–120 mesh size) chromatography using a mixture of ethyl acetate and chloroform solvent system as an eluent. The solvent system changes as a gradient mode from 100% $CHCl_3$ to 25% Ethyl acetate: $CHCl_3$. First, elution of column from $CHCl_3$ and 5% ethyl acetate: $CHCl_3$ solvent systems provided fractions containing an oily mixture. After that, other non-polar compounds came out at around 8–10% ethyl acetate: $CHCl_3$ solvent system. Then isoliquiritigenin followed by liquiritigenin came out around 12–15% ethyl acetate: $CHCl_3$ solvent system. Other polar compounds were eluted from 20–25% ethyl acetate: $CHCl_3$ solvent system. Discarding the first oily fraction and last polar compounds containing fractions, all the middle fractions eluted between 8–15% ethyl acetate:$CHCl_3$ solvent systems were combined and concentrated on a rota evaporator to provide 29.1 g of ISL and LTG enrich final extract, which was coded as *IVT-21*. The schematic representation of enrich extract preparation is given in Fig. 1.

Chemical characterization of IVT-21

Authentic Standard Compounds (ISL and LTG) were procured from Sigma-Aldrich. The HPLC analyses were carried out on Waters Alliance 2695 separation module equipped with a 2998 photodiode array detector (Waters, Milford, MA, USA). Separation was carried out using a Phenomenex C_{18} Luna® column (5 um; 250 x 4.6 mm). The TLC analyses were performed on silica gel TLC plates of Merck, India, with the fluorescent indicator (F_{254}), and TLC plates were visualized by ultraviolet light, and spots were developed by vanillin–sulphuric acid spraying reagent. Column Chromatography was performed on 60–120 mesh size silica gel (Merck, India).

In-vitro **study**

Primary Peritoneal Macrophages Isolation From Experimental Mice

Swiss albino mice (24 ± 2) were used for the primary macrophage cells isolation Institutional Animal Ethics Committee (IAEC) approved protocols (CIMAP/IAEC/2020-23/10). 1.0 mL peptone (1%) in saline was injected in peritoneal cavity of animals. Cells were isolated by phosphate buffer saline (PBS), pH-7.4 from mice in intraperitoneal region. Cell viability was checked by trypan blue exclusion and the viable macrophages at the concentration of 1.0× 10^6 live cells/mL were used for the experimentation.

Anti-inflammatory profile of IVT-21 **and ISL, LTG present in** IVT-21

For the analysis of anti-inflammatory profile macrophages were seeded in 48 cell culture plate (0.5 million cells /well) in DMEM in 5% CO2 incubator at 37°C. Cultured primary macrophages were treated with *IVT-21*, LTG and ISL at dose of 3, 10 and 30 µg/mL and dexamethasone (used as a standard drug) at a dose of 1 µg/ml. After 30 minutes of treatment, primary macrophages were stimulated with LPS at dose of 0.1µg/mL. After six hours

LPS stimulation, supernatants were collected and kept at -80ºC for the quantification of pro-inflammatory markers like TNF-α and IL-6 through ELISA (BD Biosciences, USA). Percentage (%) inhibition of pro-inflammatory markers was calculated as given equation:

$$\% \text{ inhibition} = \frac{(\text{Concentration of vehicle control} - \text{Concentration of test treatment})}{\text{Concentration of vehicle control}} \times 100$$

Cell Toxicity

Mice peritoneal primary macrophages were seeded in 96 cell culture plate (0.2 million cells /well) in DMEM (Dulbecco modified Eagle medium, Sigma) supplemented with 10% fetal bovine serum with 1× stabilized antibiotic–antimycotic solution (Sigma) in 5% CO_2 incubator at 37°C. Cell viability assay of *IVT-21* was studied by MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium) test. Briefly, after culture of peritoneal macrophages, cells were treated with *IVT-21* (10, 30 & 100 μg/mL) for 24 hours. Macrophages were incubated with MTT dye for four hours. After that the media was discarded and add 100μL DMSO and kept for 10 minutes at room temperature and measurement of absorbance taken at 550 nm (Spectramax; Molecular Devices, USA). Percentage (%) of survival = (average experimental absorbance / average control absorbance) X 100.

In-vivo **study**

Wister's rats (180–200 gram) were used in this study. All rats were obtained from institute animal house and acclimatized to experimental room for one week before experiment. All animals were ad libitum drinking water and feed with pellet animal feed and maintained under standard environmental temperature (22–24°C) and a 12 hours light/12 hours dark cycle circumstance. Animal experiments were carried out as per the appropriate procedure by the institutional animal ethics committee (IAEC) approved protocols (CIMAP/IAEC/2020-23/08) followed by the committee for the purpose of control and supervision of experimental animals (CPCSEA), Government of India (Registration no: 400/01/AB/CPCSEA).

Induction Of Rheumatoid Arthritis In Rats

Healthy and acclimatized animals were separated into 6 groups [n = 6 (male = 3 and female = 3)]. One of the groups served as normal control whereas, the other 5 groups were subjected to CIA induction. CIA was induced by the subcutaneous injection of 0.2 mL mixer of collagen and Freund's complete adjuvant (1:1 ratio) at the base of tail (Except normal group) on days 1. Collagen was dissolved in 0.05M acetic acid (4 mg/mL) and emulsified with Freund's complete adjuvant in equal volume. Booster dose was inserted in to the base of the tail (subcutaneous) avoiding the primary injection sites after 7 days of the primary immunization. The development of arthritis symptoms was detected after the 7–10 days of second immunization, paw and knee joint inflammation is the main visible sign of arthritis. After 3 weeks of the primary immunization animals were treated with test extract and standard drug for 21 days. Group- 1 (Normal control), group- 2 (CIA + Vehicle control) treated with 1 mL 0.7% CMC, group 3–5 orally treated with *IVT-21* at dose of 30,100,300 mg /kg respectably and group 6 received dexamethasone (0.1 mg/kg) used as a standard drug.

Behavioral Assessment Of Arthritis Index And Arthritis Score

Thickness of the joint and paw was measured by screw gauge on day 0,21,28,35 and 42 and arthritis index (AI) was calculated by given equation: AI (%) = [(Thickness of paw and joints on day x - Thickness of paw and joints on day 0)/ Thickness of paw and joints on day 0] X100. Visual monitoring of the arthritis score were performed to measure the severity of arthritis in all animals. The Arthritis scores ranged from 0 to 4 per paw, and were noted once a week for 21 days after the treatment. Scores were added and highest arthritis score was 16 per rat, with the highest arthritis score of 4 for each paw. Score 0 = no sign of arthritis, 1 = a low inflammation and/or redness, 2 = moderate inflammation and redness in joint and paw, 3 = extraordinary edema with limited joint activity, and 4 = excessive edema with difficulty in walking.

Mechanical Pain Thresholds Measurement

PAM instrument (pressure application measurement) (ugobasile, Italy; Model No.-38500) was used for the measurement of knee joint withdrawal threshold in gram force. It has a force transducer, fitted on thumb and placed on rat's knee joint. Mechanical force (300 gram force/second) was gradually increased and applied by the operator. To avoid the tissue damage mechanical force was applied only for 5 seconds. An attempt to withdraw the joint from operator's thumb and forefinger was taken an indication of the test end point. The limb withdrawal threshold (LWT) measurement was repeated for 3 times and mean LWT was calculated (Barton et al., 2007).

Quantification Of Inflammatory Markers

Six weeks after the first immunization, blood was collected by orbital plexus of experimental animals, and separated the serum for quantifying the inflammatory markers TNF-α, IL-6, IL-17A, MMP-1, MMP-9, C-RP, NO as per manufacture's instruction.

Histopathology And Immunohistochemistry (Ihc)

On day 42 (end of experiment day) animals were anesthetized and sacrificed, the knee joints were cut carefully, removed the skin and fixed in 10% formalin buffer. After the fixation and decalcification, the samples were processed and sectioned into 4 μm and stained with hematoxylin and eosin (H and E) staining (Suvarna et al., 2018).

Immunohistochemistry was used to measure NFκB-p65 in the knee joint tissue of experimental rats. Knee joint tissue section of five mm thickness were cut and taken on the poly- l- lysine coated slides. Tissue section were dewaxed and dehydrated then antigen retrieval and quenching step by EDTA and PBS + H_2O_2 (4:1) respectably. After that section were blocked with 5% BSA for 10 min and then incubated with NFκB-p65 primary antibodies (Santa Cruz, CA, USA) for 1.5 hours at room temperature. After primary antibody incubation tissue section were washed with PBS and HRP-conjugated secondary antibody (Jackson immune) was overlaid and incubated for 30 minutes at room temperature. Tissue section washed with PBS and overlaid with DAB (3́3 - diaminobenzidine) to detect antibody binding. Sections were counterstained using hematoxylin solution. The

stained sections were dehydrated, mounted and microscopic analysis were carried out (Sereika et al., 2018). Staining intensity was assessed on a semi quantitative five-point scale (0 = no expression, 1 = very low expression, 2 = low expression, 3 = high expression, and 4 = very high expression) by percentage of stained cells and synovial membrane damaged area (Choy et al., 2002). Immunohistochemical/ NFkB-p65 protein expression assessment was evaluated in a blinded manner by Olympus CX21*i*LED light microscope.

Rna Isolation And Rt-pcr Analysis

RNA isolation and RT-PCR analysis
After anesthetized and survival dislocation hind paw of experimental rats were cut, claws and skin were dissected, then paw tissues were directly frozen in liquid nitrogen then stored at -80˚C. Paw tissues were used for quantitative RT- PCR analysis. Approximately 45-50mg tissue was cut from frozen paw and homogenized in 1mL triazole regent by tissue homogenizer and isolate the total RNA with TRI-reagent (Sigma). Purity of RNA was checked by ration of A 260 /A 280 nm using Nano Drop (Thermo Scientific). The RNA sample showing A_{260}/A_{280} ratio 1.95–2.05 and A_{260}/A_{230} ratio > 1.5 were used for synthesis of cDNA using commercially available cDNA synthesis kit (Applied Biosystems) according to manufacturer's protocol. The Real time PCR reaction was performed with 12.5 µL of Power SYBR Green PCR master mix (Applied Biosystems) 0.8 µL of forward and reverse primers and 1.0 µL of sample cDNA. PCR reaction efficiency was optimized, and the final concentration of each primer was 0.5 µM. Real time PCR was carried out in Applied Biosystems QuantStudio™ 5 System using PCR master mix (SYBER green) the relative expression of TNFα, IL-6, IL-1 β, MMP-9, IL-17A mRNA was normalized to the amount of GADPH in the same cDNA using the relative quantification 2 − ΔΔCt method. The fold change in target gene cDNA relative to the GADPH internal control was determined using the following formula: Fold change = $2^{-\Delta\Delta Ct}$, where [ΔΔ Ct = ($Ct_{,target\ gene}$ – $Ct_{,GAPDH}$) treatment − ($Ct_{,target\ gene}$ – $Ct_{,GAPDH}$) Control]. Primer sequences for analysis of TNFα, IL-6, IL-1 β, COX-2, MMP-9, IL-17A and GAPDH are described in Table 1.

Acute Toxicity Study In Swiss Albino Mice

Safety evaluation of *IVT-21* was done as per reported method (Babu et al., 2021) at single acute oral doses at 2000 mg/kg.

Statistical Analysis

Results are expressed as the means ± standard error of mean (SEM) and compared the statistical significance of vehicle verses treatment groups by ANOVA. Differences with a P value < 0.05 were considered significant.

Results

HPLC quantitative analysis

The HPLC analysis suggested that the enrich extract (*IVT-21*) contained 6.65% of LTG and 3.13% of ISL (Fig. 2).

Chromatographic method

Samples and the mobile phase were filtered through a 0.22 μm PTFE membrane and HPLC column thermostated at 35°C. The optimum separation obtained with a gradient mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Gradient conditions were 0–27 min, 15–45% B; 27–37 min, 45–80% B; the column was kept at 80% B till 50 min. Column reconditioning was achieved with 15% B for 10 min by using equilibration between sample runs. The flow rate is set at 1.0 mL/min. System control and chromatographic data processing were performed by Waters Empower 3 Chromatography Software. Data acquisition in the range of 200–400 nm done to monitor possible interference of co-elution/neighbouring peaks of the sample matrix. The detector was set at 254 nm wavelength for simultaneous quantitation because of the optimum chromatographic signal response of the targeted phytochemicals.

In-vitro **study**

Anti-inflammatory activity of IVT-21 **and LTG, ISL present in** IVT-21

IVT-21, LTG and ISL compounds were assessed for their anti-inflammatory activity against the production of pro-inflammatory markers (TNF-α and IL-6) using the ELISA method in LPS-induced inflammation in primary macrophages at the concentrations 3,10 and 30 µg/mL. Pro-inflammatory markers production was significantly ($p < 0.05$) increased in LPS-stimulated macrophages when compared with normal macrophages. Cells treated with *IVT-21*, LTG and ISL significantly ($p < 0.05$) reduce the production of pro-inflammatory markers (TNF-α and IL-6) in a dose-dependent manner (Table-2). The percent inhibition of inflammatory cytokines production by the treatment of *IVT-21*, ISL and LTG an active constituent in a dose-dependent manner, is compared and depicted in Fig. 3 (A-C).

Cytotoxicity profile of IVT-21

To examine the direct effect of *IVT-21* on the cells, we performed the cell viability test using the MTT assay as shown in Fig. 4. *IVT-21* at the doses of 10, 30 and 100 µg/mL did not affect the cells' viability, suggesting these doses of *IVT-21* do not have a direct toxic effect on the cells when compared with non-treated cells.

In-vivo **study**

Effect of IVT-21 **on arthritis score and arthritis index in CIA animals**

Development of CIA was noticed with the swelling and redness of the joints after 2 weeks of the primary immunization. A plateau of the peak of CIA response was maintained from day 21–42. Oral treatment of *IVT-21* at a dose of (100,300 mg/kg) and dexamethasone (0.1 mg/kg) from day 21–42 significantly ($P < 0.05$) decrease the arthritis index of knee, elbow joint and paw in CIA animals (Fig. 5A-C). Figure 5E showed the arthritis score of all groups of experimental rats. Arthritis score was decreasing during the treatment period (days 21–42) in a dose-dependent manner. Arthritis score was 11 ± 0.68, 9.66 ± 0.80 and 9 ± 0.68 in animals treated with *IVT-21* at a dose of 30, 100 ,300 mg/kg respectively, animals treated with dexamethasone at a dose of 0.1 mg/kg show the arthritis score 8.66 ± 0.42 and non-treated CIA + vehicle group animals show the arthritis score was 14.33 ± 0.80.

Effect of IVT-21 **on mechanical pain threshold in CIA animals**

To determine the effect of *IVT-21* on pain induced by mechanical force, the limb withdrawal threshold (LWT) in gram force was measured on days 21, 28, 35, and 42 using PAM (pressure application measurement) apparatus. LWT was significantly ($P < 0.05$) increased in *IVT-21* treated animals when compared with the CIA + vehicle group of animals in a dose-dependent manner (Fig. 5D).

Effect of IVT-21 **on pro-inflammatory markers in CIA animals**

The serum concentrations of inflammatory markers (TNF-α, IL-6, IL-17A, MMP-1, MMP-9, C-RP, NO) level were significantly increased in CIA animals when compared with normal group animals. The serum concentration of above mentions inflammatory markers were significantly ($P < 0.05$) decreased in animals treated with *IVT-21* at a dose-dependent manner when compared with non-treated CIA + vehicle group animals (Fig. 6A-G).

Effect of IVT-21 **on histopathology and immunohistochemistry (IHC) of ankle joint after CIA injection**

On day 42 after primary immunization, the histological features were observed. The knee joint of CIA animals showed the typical signs of arthritis with inflammatory cells and inflammatory materials infiltration, cartilage degradation and synovial membrane disruption. In contrast, animals treated with *IVT-21* and dexamethasone all of the above mention's symptoms were alleviated in a dose-dependent manner. In the case of a normal control group of animals show that, synovial membrane was a smooth and had regular cellular arrangement, maintain the synovial space, without any inflammatory cell/ material infiltration, and no any erosion and disruption of cartilage tissue and synovial membrane when compared with CIA group of rats (Fig. 7). To further validate the NFκB-p65 protein expression by immunohistochemistry staining of the knee joint tissue of experimental animals. The results of immunohistochemistry show that the expression (brown color indicates) of NFκB-p65 protein was significantly ($P < 0.05$) increased in the non-treated vehicle group of animals compared to a normal control group of rats. In contrast, animals treated with *IVT-21* and dexamethasone significantly reduce the expression of NFκB-p65 protein in a dose-dependent manner compare with non-treated vehicle group of animals. whereas the normal group shows no or very low NF-kB p65 protein expression in the synovial joint tissue (Fig. 8).

Effect of IVT-21 **on mRNA level of inflammatory cytokines in CIA rat hind paws**

We measured pro-inflammatory cytokines gene expression in CIA group animals at the end of the treatment period (day 42). Paw tissue of CIA rats exhibited, significantly higher TNF-α, IL-6, IL-1 β, COX-2, MMP-9 and IL-17A gene expression compared with the normal group of rats. Oral treatment of *IVT-21*, significantly reduced the mRNA gene expression in a dose- dependent manner compare to CIA + vehicle group of rats (Fig. 9).

In-vivo **safety studies of** IVT-21

The result of this study exhibited that one-time oral treatment of *IVT-21* (2000 mg/kg) did not produce any mortality, behavioral changes in treated animals compared to non-treated animals. Similarly, no any significant changes were observed in biochemical as well as hematological parameters of the treated animals when compared with control group animals (Table 3).

Discusion

The chemical sign of G. glabra root extracts *(IVT-21*) using HPLC analysis presented the presence of isoliquiritigenin and liquiritigenin as the main active ingredients. Plant-based natural molecules play a significant role in the development of anti-inflammatory drugs in the pharmaceutical industry which can serve as good lead molecules suitable for further modification during the drug development process (Saxena et al., 2016). In the present study, we examined the anti-inflammatory profile of *G. glabra* root extracts, ISL, and LTG present in *G. glabra* root extracts against the LPS-induced inflammation in macrophages, ISL and LTG-rich *G. glabra* root extracts (*IVT-21*) was further evaluated in the *in-vivo* condition against collagen-induced arthritis model in rats. Several biomarkers of inflammation are implicated in chronic disease like cytokines (TNF-α, IL-1, IL-6, IL-8) and other proteins like cyclooxygenase-2 (COX-2), C-reactive proteins, matrix metalloproteinases (MMP), etc (Prasad et al., 2016). *In-vitro* anti-inflammatory studies of *IVT-21*, ISL, and LTG significantly reduce the pro-inflammatory cytokine (TNF-α, IL-6, IL-1 β) level without any cytotoxic effects in a dose-dependent manner. *G. glabra* and its natural compounds have shown anti-inflammatory activities (Yang et al., 2017). Inflammation response plays a key role in several diseases like lung disease (Yang et al., 2013b), rheumatoid arthritis (Yang et al., 2013a), periodontitis (Farhad et al., 2013) and colon linked inflammatory disease (Takhshid et al., 2012). Liquiritigenin inhibit the NF-κB activation in macrophages thereby reducing the pro-inflammatory markers and iNOS production (Kim et al., 2008). Isoliquiritigenin decreases the inflammation and fibrosis in the kidney of the unilateral ureteral obstruction model and inhibited inflammatory and fibrotic responses in BMDM by blocking Mincle/Syk/NF-kappa B signaling pathway (Liao et al., 2020). We have further evaluated the therapeutic effect of *IVT-21* in the *in-vivo* system using the collagen-induced arthritis model in rats. *In-vivo* study exhibit that, oral administration of *IVT-21* shows anti-arthritis effects in a dose-dependent manner. Collagen-induced arthritis (CIA) model is the most commonly used animal model for rheumatoid arthritis (RA) because of similar pathological and arthritis appearances between RA patients and CIA animal model (Liu et al., 2018). RA is an autoimmune and chronic disease that eventually leads to joint inflammation, damage, and bone destruction. In the case of a chronic condition, RA has brought a vast economic load to society. For the treatment of RA disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory agents (NSAIDs) and biologics have found some successes in improving the life quality of patients. But these drugs have several severe side effects and the high price of biologics, several people would favor choosing traditional herbal medicines as their long-term drug treatment approaches (Linghu et al., 2020). *In-vivo* study shows that oral administration of *IVT-21* significantly decreased the arthritis index of elbow and knee joint, paw, arthritis score, and significantly increase the limb withdrawal threshold (gf) of experimental rats using PAM apparatus in a dose-dependent manner, which detects the mechanical hypersensitivity of chronic inflammatory joint pain (Barton et al., 2007). In this experiment, we also found that the oral administration of *IVT-21* significantly decreased the production of TNF-α, IL-6, IL-17A, MMP-1, MMP-9, Nitric oxide, and C-RP levels in a dose-dependent manner in CIA rat's serum. Pro-inflammatory markers play important roles in RA development and are also important targets for the treatment of RA. Pro-inflammatory inhibitors are generally used for RA treatment (Chinese Rheumatology, 2018). Histological estimation of the knee joint, CIA animals shows arthritis features like inflammatory cell infiltration, decrease synovial space, cartilage destruction, and disruption of the synovial membrane. Oral treatment of *IVT-21* significantly alleviates inflammatory cell infiltration, reduces cartilage damage, and decreases the hyperplasia of the synovial membrane. Immunohistochemistry of the knee joint demonstrates that NFκB-p65 protein expression was significantly reduced in animals treated with *IVT-21* and dexamethasone in a dose-dependent manner compared with a non-treated group of CIA rats. NFκB-p65 is an important transcriptional factor involved in the production of pro-

inflammatory cytokines, protein, and adhesion molecules (Bureau et al., 2000). Oral treatment of *IVT-21*, significantly decrease mRNA gene transcription levels in the hind paws of CIA rats compare to the non-treated vehicle group of rats. mRNA levels of TNF-α, IL-6, IL-1 β, MMP-9, and IL-17A genes were significantly increased in the non-treated vehicle group of rats compared to the normal group of rats. Phenolics are present in several fruits, vegetables, herbs, spices and beverage which was used by human for food and traditional medicine (Yoon and Baek, 2005). It may affect the gut microbiota, which catabolized phenolic compounds for good absorption and may have useful for health at the intestinal level (Tomás-Barberán et al., 2016). Phenolic compounds have exhibited efficacy against several chronic diseases including RA because of their ability to control pro-inflammatory and pro-oxidant pathways and can control the production and action of inflammatory markers by modulating the action of other molecules involved in RA pathogenesis (Rosillo et al., 2016). Isoliquiritigenin is used for the treatment of osteoarthritis and reduced the IL-1β-induced MMPs production and NF-κB activation in *in-vitro* and *in-vivo* conditions (Zhang et al., 2018). ISL improved cisplatin-induced acute kidney injury by reducing Formyl peptide receptors 2 involved in macrophagic inflammation (Rui-Zhi et al., 2022), and it has a protective effect on chronic obstructive pulmonary disease induced by Cigarette smoke (Yu et al., 2018). liquiritigenin protects against hepatotoxicity induced by arsenic trioxide due to its antioxidant and anti-inflammatory activities (Zhang et al., 2021), and it reduce the IL-1β-induced inflammation and cartilage matrix degradation in rat chondrocytes (Tu et al., 2019). *IVT-21* was safe and well tolerated by Swiss albino mice up to the 2,000 mg/kg dose in acute oral toxicity. Safety is an essential component in the drug development process and is nowadays referred to as 'Pharmacovigilance'. This finding resembles a previous report that the pharmacological effect of plant-derived leads without any toxic effect at higher doses (Saxena et al., 2016).

Conclusion

In conclusion, the result of this study shows that *IVT-21*, ISL, and LTG were able to decrease the level of the pro-inflammatory cytokine in LPS-induced peritoneal microphases. Oral treatment of *IVT-21* can decrease the rheumatoid arthritis symptoms, reduce the inflammatory mediators in CIA rat's serum and also reduce the mRNA gene expression in paw tissue of CIA rats. This finding confirms the suitability of *IVT-21* as a drug-like candidate for further investigation in the management of rheumatoid arthritis.

Abbreviations

AI, Arthritis index; CIA, Collagen-induced arthritis; CMC, Carboxymethyl cellulose; COX-2, Cyclooxygenase-2; C-RP, C-reactive protein; Dexa, Dexamethasone; DMARDs, Disease modifying anti-rheumatic drugs; DMEM, Dulbecco's modified eagle's medium; *G. glabra, Glycyrrhiza glabra;* GF, Gram force; HRP, Horseradish peroxidase; IHC, Immunohistochemistry; IL, Interleukin; ISL, Isoliquiritigenin; LPS, Lipopolysaccharides; LTG, Liquiritigenin ; LWT, Limb withdrawal threshold; MMPs, Matrix metalloproteinases; NO, Nitric oxide; NSAIDs, Non-steroidal anti-inflammatory drugs; PAM, Pressure application measurement; PBS, Phosphate-buffered saline ; RA, Rheumatoid arthritis; RT- PCR, Reverse transcription–polymerase chain reaction; TNF-α, Tumor necrosis factor α.

Declarations

Acknowledgments

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Conflict of interest: Authors declare no conflicts of interest.

Data availability statement

All data generated during this study are included in this article and the pri[1]mary data for this study are available from the author on direct request.

Author contributions

DUB: cotributed to planning, manuscript preparation and supervised the experiment. VB: cotributed to performed the biological experiments and manuscript preparation. DSK and RSB: cotributed to planning the enrich extract development and manuscript preparation. MB: cotributed to help *in-vivo* experiment. KS and MS: cotributed to HPLC method development and quality standardization. ST: cotributed to Pilot plant extraction. MNM: cotributed to help in histopathology and Immunohistochemistry study. NK: cotributed to Collection and identification of plant material.

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Tables

Table 1: Real-time PCR primers used in the experiment.

Gene		Sequence 5' to 3'
GAPDH	Forward	CATGCCGGTCTGGCTAAATT
	Reverse	TACTTCGGCCACCCTATCCA
TNF-α	Forward	GTCAGAGCGGTGATTCAAAGG
	Reverse	CCACGGAGAGAGCCCAAAG
IL-6	Forward	CCAAGTGGGTTGGCTAGCA
	Reverse	GGAAAGGGAAAGAAGCAAATGA
IL-1β	Forward	CCGTGCGTGCTTAGATGTTG
	Reverse	AACGCCGAAGAGCAGATTGT
IL-17A	Forward	CTTTCCGGGTGGAGAAGATG
	Reverse	GGCGGACAATAGAGGAAACG
MMP-9	Forward	GGTGGTGACCCAAAAGACTCTCT
	Reverse	TGAGCTCTGTGAAGATATGGACTGA

Table 2: Dose dependent response of *IVT-21*,LTG and ISL on production of pro-inflammatory cytokines (TNF-α, IL-6,IL-1β) from LPS-activated primary macrophages. Data are expressed as mean ± SEM: *$p < 0.05$; vehicle vs treatment; # vehicle vs normal; (Tukey's multiple comparison test); n =3. NA-Not Applicable.

Compound	LPS (0.1μg/mL)	Dose (μg/mL)	TNF-α (pg/mL)	TNF-α% inhibition	IL-6 (pg/mL)	IL-6% inhibition
Normal	-	-	422±5.29	NA	678.9±37.23	NA
Vehicle		-	2500±198.1#	00	9274±495.59#	00
IVT-21		3	2222±94.49	11.13±3.85	9100±92.06	2.37±0.48
		10	1874±73.67*	25.05±2.94	7885±701.92	14.97±7.56
		30	1031±118*	58.78±4.72	3540±205.81*	61.83±2.21
LTG		3	1830±154.1	26.81±6.16	5485±415.56*	40.85±4.48
		10	1472±112.1*	41.11±4.4	4888±343.22*	47.29±3.70
		30	884.8±82.28*	64.6±3.29	4580±149.38*	50.61±1.61
ISL		3	1694±127*	32.22±5.08	3527±273.70*	61.97±2.95
		10	1282±95.07*	48.69±3.8	3155±83.47*	65.98±0.90
		30	866±71.78*	65.35±2.87	2624±270.85*	71.70±2.92
Dexamethasone		1	672.3±57.13*	73.1±2.28	2630±67.35*	71.64±0.72

Table 3. Effect of *IVT-21* on acute toxicity at 2000 mg/kg as a single oral dose in Swiss albino mice. Data are expressed as mean ± SEM, n = 6.

Parameters studied	Day 7th	
	Normal Control	
		IVT-21 (2000 mg/kg)
SGOT (U/L)	28.96±3.03	26.54±7.77
SGPT (U/L)	29.18±4.91	28.26±4.55
Creatinine (mg/dL)	0.45±0.06	0.48±0.03
Triglycerides (mg/dL)	77.7±8.01	71.8±11.07
Cholesterol (mg/dL)	51.5±4.83	46±2.27
Haemoglobin (g/dL)	14.93±0.51	15.17±0.22
RBC (million/mm3)	7.71±1.03	7.81±0.53
WBC (thousands/mm3)	8.1±0.29	8.35±0.42

Figures

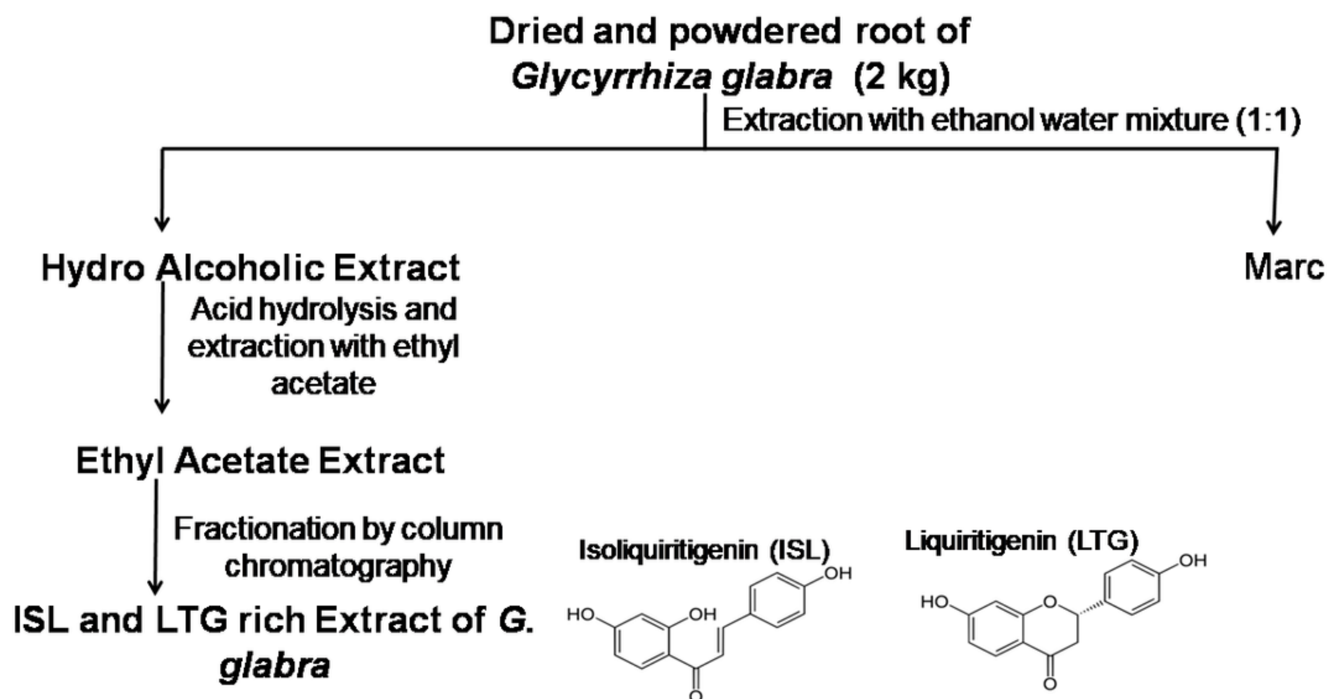

Figure 1

Schematic presentation of the preparation of standardized enrich extract.

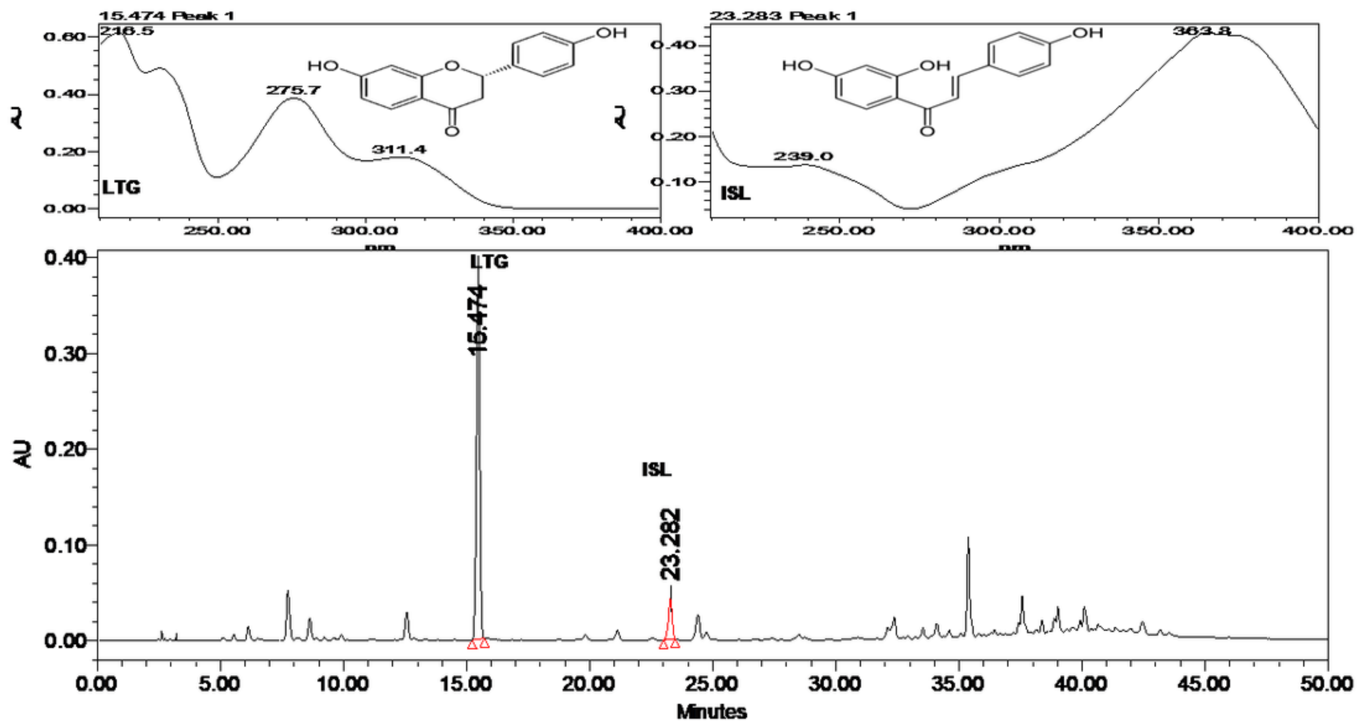

Figure 2

HPLC Chromatograms of *IVT-21.*

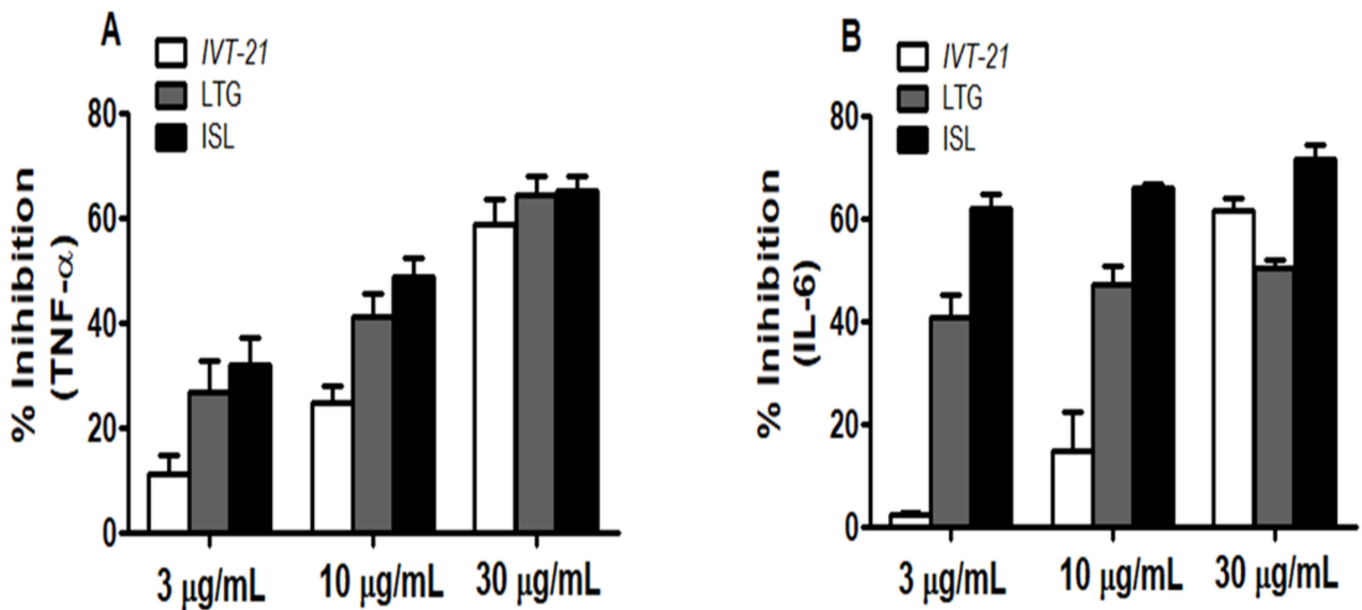

Figure 3

Effect of *IVT-21*, LTG and ISL on percent (%) inhibition of pro-inflammatory cytokine production in activated primary peritoneal macrophages. (A) TNF-α, (B) IL-6; n=3.

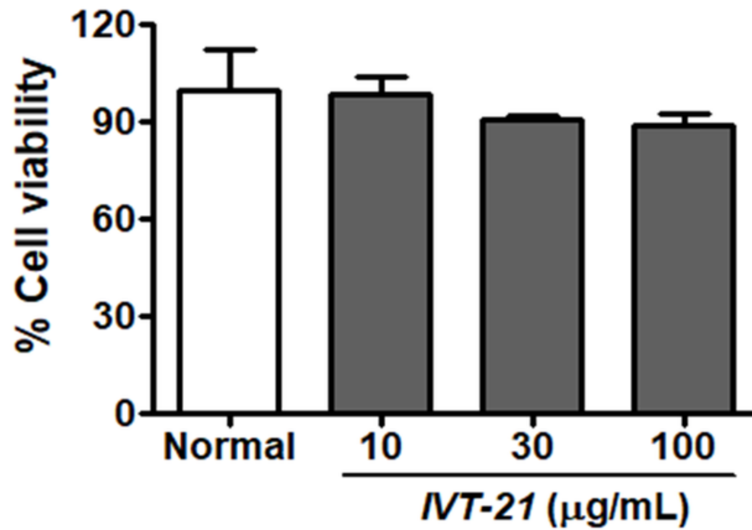

Figure 4

Cell viability assay of *IVT-21* at different concentrations in primary peritoneal macrophages, n = 3.

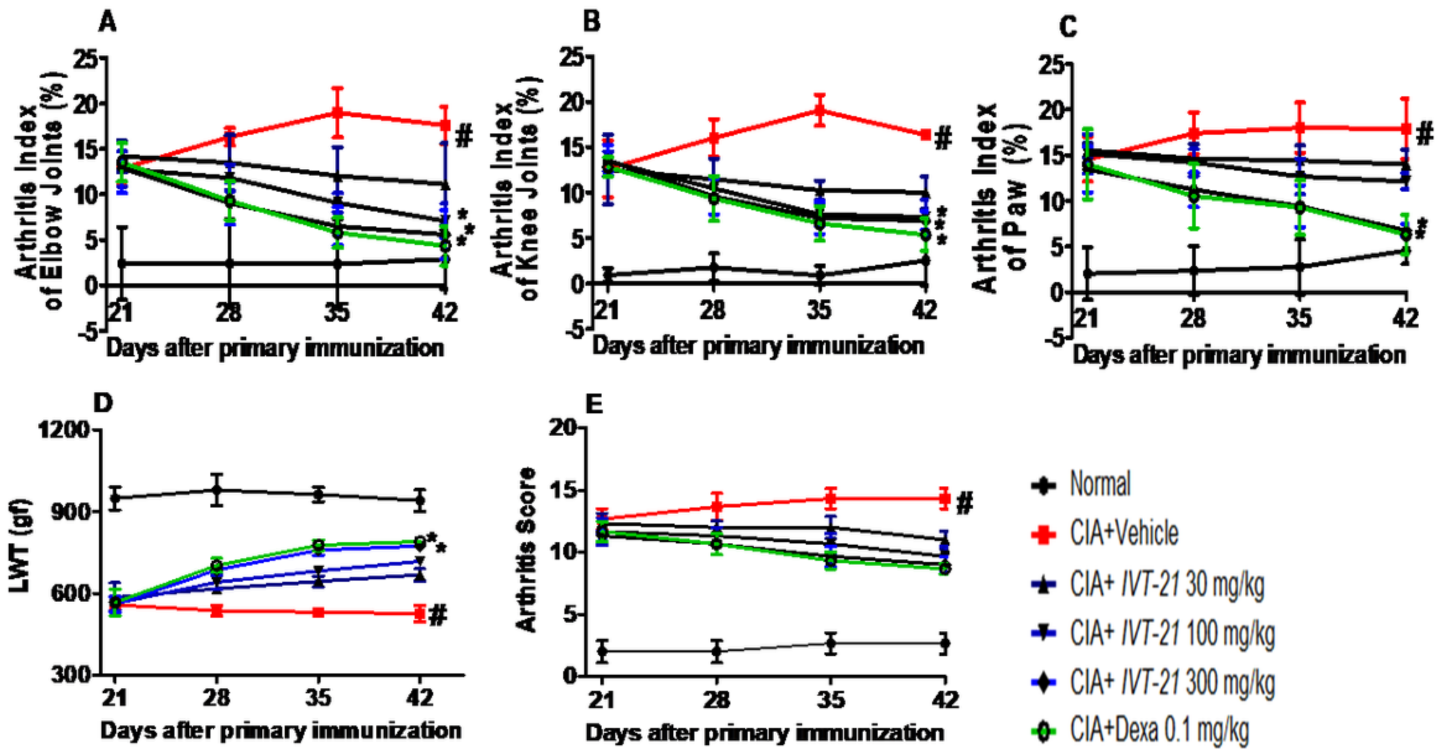

Figure 5

(A,B,C) Arthritis Index of Elbow, Knee Joint and Paw respectively. (D) Limb withdrawal threshold (LWT) measured by Pressure Application Measurement (PAM) in gram force. Data are expressed as mean ±SEM: *p<0.05; vehicle vs treatment; # vehicle vs normal; (Tukey's multiple comparison test); n=6. (E) Arthritis Score, Data are expressed as mean ±SEM: p<0.05; # normal vs vehicle; using one-way ANOVA followed by a non-parametric Kruskal-Wallis test; n=6.

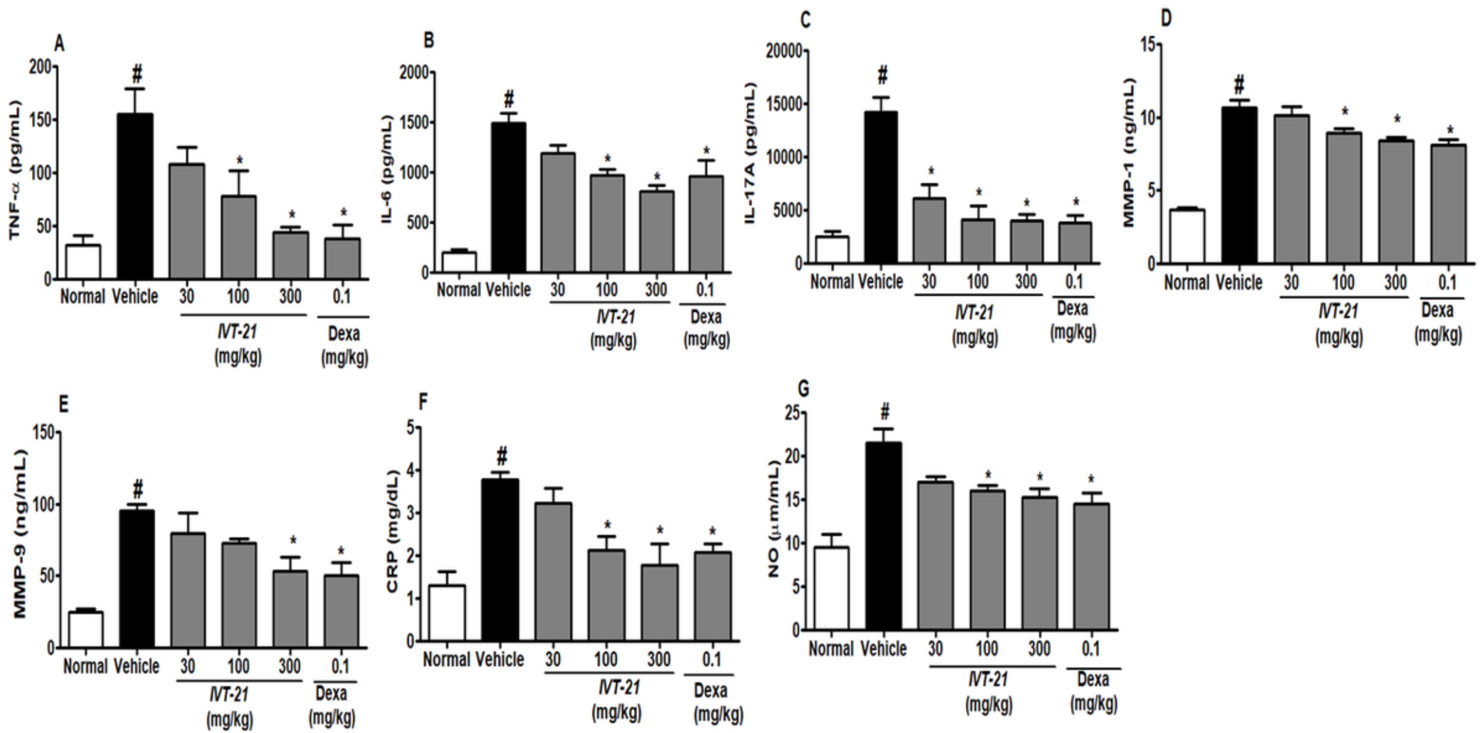

Figure 6

Effect of *IVT-21* treatment on the Inflammatory markers production in the serum of CIA rats. Fig. ((A) TNF-α (B) IL-6 (C) IL-17A, (D) MMP-1, (E) MMP-9, (F) CRP, (G) Nitric oxide. Data are expressed as mean ± SEM: *$p < 0.05$; vehicle vs treatment; # vehicle vs normal; (Tukey's multiple comparison test); n = 6.

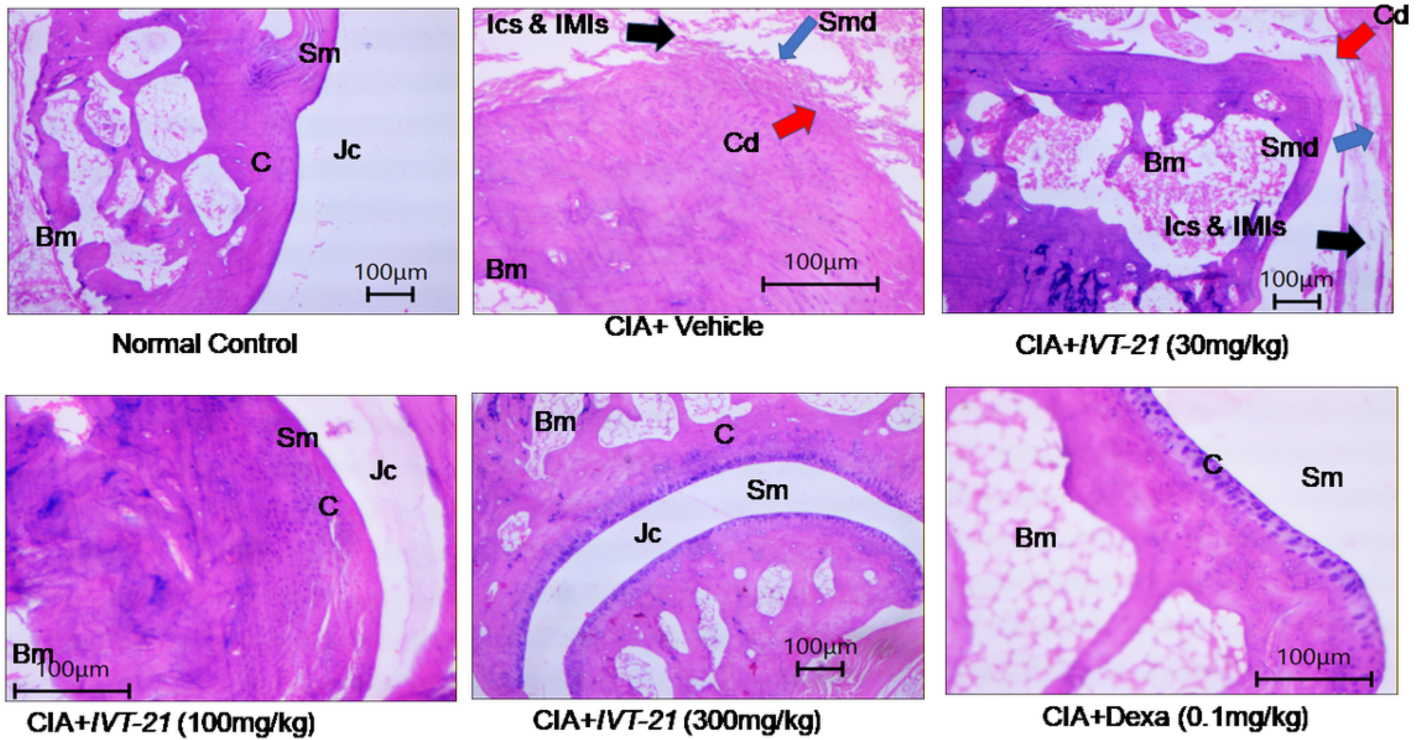

Figure 7

Rat: Knee joint: Representative histopathology images: Normal control group of rats showed no erosion and disruption of synovial membrane (Sm), no cartilage (C) damage, maintained joint cavity (Jc) and bone marrow (Bm). In vehicle treated rats, indicate the synovial membrane disruption/erosion (smd) (blue arrow), cartilage damage (cd) (red arrow), and inflammatory cells (ICs) and inflammatory material infiltration(s) (IMIs) (black arrow) which indicate the presence of arthritis, Animals treated with *GHEE-248* at dose of 100 and 300 mg/kg and dexamethasone at dose of 0.1 mg/kg, decrease the inflammation, maintain the synovial membrane and no erosion/damage of cartilage tissue as compare to vehicle group - H & E staining- 200 X.

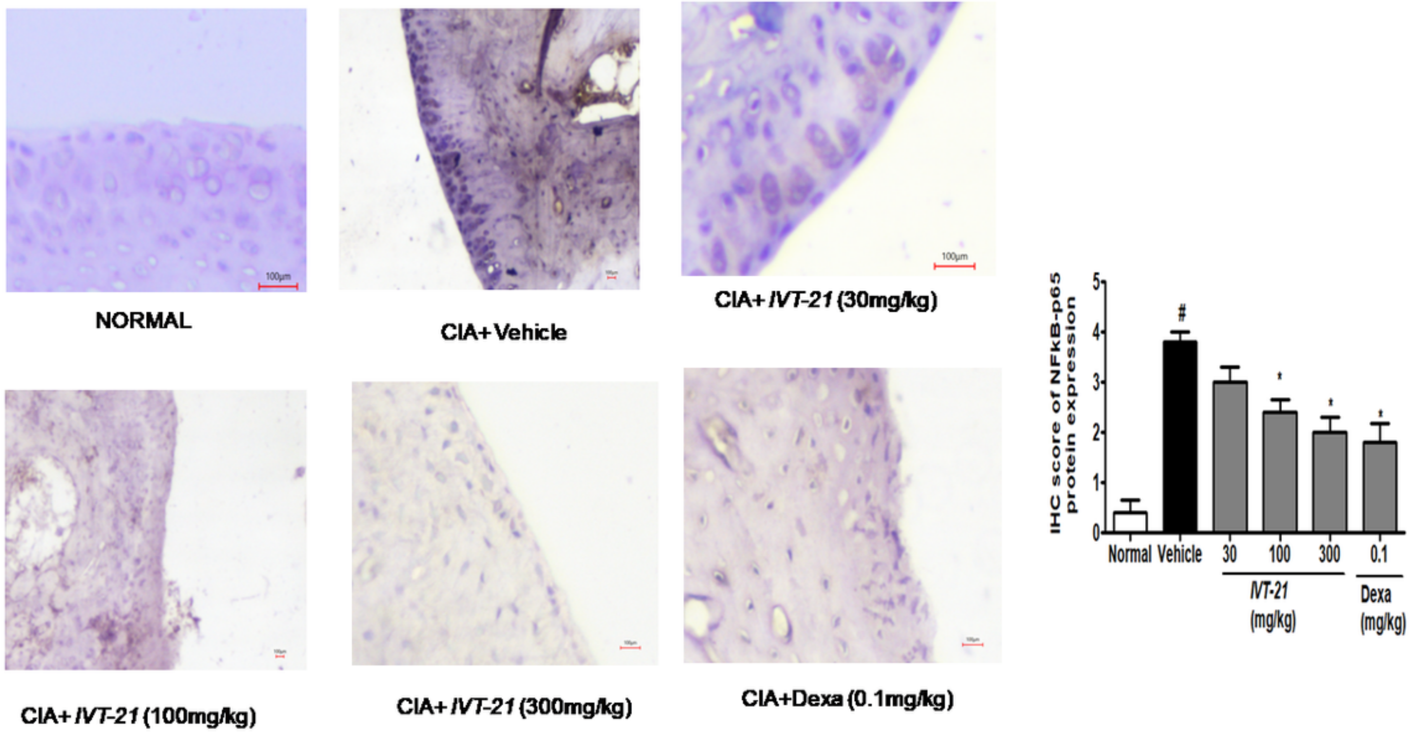

Figure 8

Effect of *IVT-21* on the expression level of NFkB-p65 proteins was detected by immunohistochemistry. Representative photomicrographs of IHC staining demonstrating the expression of NFkB-p65 on the knee joint surface and synovial membrane of the experimental rats – IHC- 200 X. Data are expressed as mean±SEM: *P<0.05; Vehicle vs treatment; # Vehicle vs normal ;n=5.

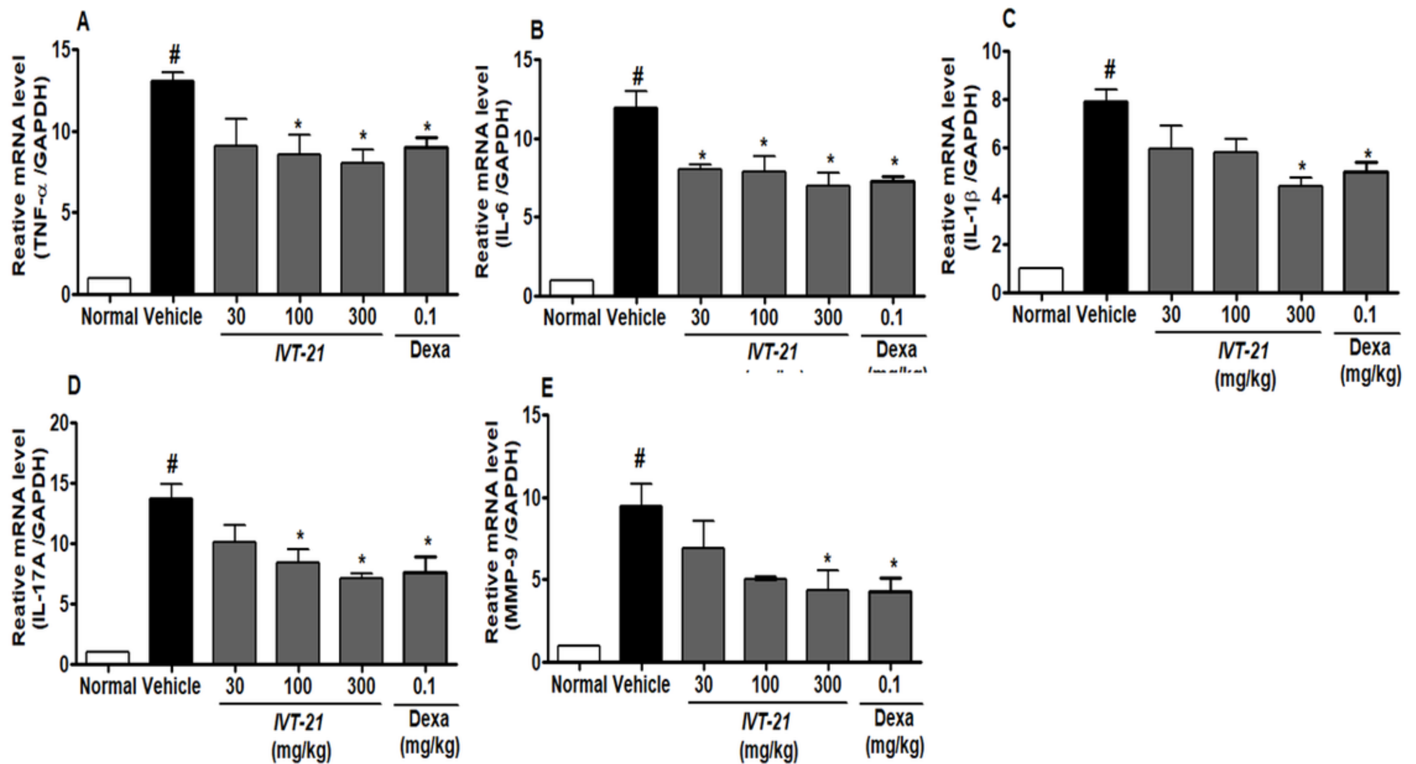

Figure 9

Effect of *IVT-21* on mRNA expression of inflammatory mediators (TNF-α, IL-6, IL-1 β,IL-17A,MMP-9) in paw tissue isolated from CIA rats. Data are mean ± SEM; n = 3. *Vehicle versus treatment, #normal versus vehicle (ANOVA; Tukey test), $p < 0.05$.

Supplementary Files

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