In-vitro and *In-vivo* Antiarthritic Activity of the Ethanol Extracts of Leaves of *Astragalus glycyphyllos* in CFA-induced Arthritis in Rats

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The effectiveness of the ethanolic extract of *A. glycyphyllos* leaves was evaluated for treating arthritis. This was done by examining its ability to protect chondrocytes (cartilage cells) from inflammation caused by IL-1β stimulation, as well as its ability to alleviate arthritis generated by CFA in rats. Rats in the CFA control group exhibited increased paw inflammation on days 1, 7, 14, 21, and 28. Inflammation was greatly reduced when treated with *A. glycyphyllos* extract at doses of 100 and 200 mg/kg. There was a positive correlation between the extract dosage and the rats' body weight. The extract alleviated inflammatory and arthritic symptoms in animals with CFA, leading to an improvement in the arthritic index. Animals afflicted with arthritis exhibited elevated levels of inflammatory markers (TNF-α, IL-1β, and IL-6) and a decrease in their ability to move. The administration of the extract significantly improved the locomotor score and strongly suppressed the activation of inflammatory markers. The extract effectively reduced the production of NO, PGE2, MMP1, and MMP13 in IL-1β-stimulated PRCs, with the extent of reduction depending on the dosage. This investigation showcased the therapeutic properties of the *A. glycyphyllos* extract in treating arthritis.

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INTRODUCTION

The severe, incurable disease known as rheumatoid arthritis (RA) is characterized by edema, pain, with tightness in the synovial joints. The exact etiology of this awful sickness is unknown. However, it shares a tight relationship with an autoimmune reaction triggered by many genetic plus environmental factors. The economy and society are greatly impacted by chronic disease and the deaths that follow. The pathophysiological signs of RA include significant synovial cartilage or bone deterioration, pannus formation, and leukocyte infiltration, with chronic inflammation. Nevertheless, the real trigger of RA is yet unidentified (Alamgeer *et al.* 2017). Clinical RA indications can vary from small discomfort as well as swelling to serious symptoms including entirely or partial joint inactivity as well as muscle weakness, such as contractions, depending on the individual.

On the other hand, as the only cellular component of articular cartilage, chondrocytes are in charge of maintaining tissue homeostasis and synthesizing the substantial turnover volume of extracellular matrix (ECM) components. Degenerative disorders, including osteoarthritis (OA), are associated with chondrocyte function impairment (Eccleston 2023). OA is the most common musculoskeletal illness, accounting for 15% of all cartilage problems and affecting around 100 million adults over the age of 45 worldwide. The prevalence of arthritis is one percent worldwide, and the ratio of affected women to affected men is three to one (Bennell *et al.* 2010).

Inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), or IL-6 affect inflammation and joint damage as arthritis progresses. The stimulation of chondrocyte hypertrophy by interlukin-1β (IL-1β) results in the production of hypertrophic markers such as MMP-13, ColX, and Runx2. In addition, this stimulation causes the breakdown of the collagen network and the death of articular chondrocytes by increasing the production of neoepitopes, inflammatory mediators, and neuropeptides. The production of inflammatory sensitizers such as neuropeptides and cytokines is associated with this process, which compromises the integrity of the cartilage. The breakdown of the collagenous framework includes the production of proteolytic enzymes such as MMP-1 and MMP-13 and sequential signaling cascades. IL-1β-induced inflammatory cytokines unregulated TNF-α, IL-6, PGE, nitric oxide, and iNOS. Moreover, IL-1β rises NO generation, intensifies COX-2 activity, and up-regulates MMP expression—all of which are connected to the deterioration of cartilage and extracellular matrix (Thudium *et al.* 2019; Mei *et al.* 2021).

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) as a first line of treatment for patients with rheumatoid arthritis (RA) is well-established. However, long-term use of NSAIDs has been connected with certain potential side effects, such as gastroduodenal issues and a dysfunctional kidney, which are likely caused by a decreased level of cyclo-oxygenase for a fall in prostaglandin content (Guo *et al.* 2018; Bullock *et al.* 2019; Radu and Bungau 2021).

Because of its emollient, diuretic, and invigorating qualities, *Astragalus glycyphyllos* is a plant that is utilized in several European nations. The leaves and seeds are used as laxatives and to treat a variety of illnesses in the Caucasus area, such as urolithiasis, oliguria, scrofulosis, and dermatitis. Aerial parts are utilized in Belarus to treat gastrointestinal ailments, leucorrhoea, and uteroptosis. The herb is utilized as a laxative, diuretic, and mucoactive agent in Ukraine to treat dermatitis, rheumatism, and STDs. The herb grows along the Volga River and is used to treat disorders of the central nervous system. The plant is used as a diuretic, expectorant, and in gynecology to induce childbirth and hasten placenta separation in the Carpathian area.

A. glycyphyllos has been shown in Russian clinical research to possess hypotensive, anticoagulant, and diuretic properties. The plant is used in Bulgarian traditional medicine to treat tachycardia, renal inflammation, cardiac insufficiency, calculosis, elevated blood pressure, and inflammation of the kidneys. It also functions as an antihypertensive and diuretic (Lysiuk and Darmohray 2016). This plant is used traditionally to reduce inflammation and rheumatism. Therefore, in this study, an attempt was made to evaluate the potency of the ethanolic leaves extract of *A. glycyphyllos* in experimentally induced arthritis *in vivo* and *in vitro* models.

EXPERIMENTAL

Chemicals

Complete Freund's adjuvant (CFA), type IV collagenase, interleukin-1β (IL-1β), and enzyme-linked immunosorbent assay (ELISA) kits for nitric oxide (NO), prostaglandin E2 (PGE2), matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-13 (MMP-13), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Pronase was purchased from Roche (Basel, Switzerland). Fetal bovine serum and Dulbecco's Modified Eagle's Medium were purchased from Gibco Inc. (Billings, MT, USA), and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Thermo Fisher Scientific (Waltham, MA, USA). The cytotoxicity test and assessment of inflammatory markers were conducted on a microplate reader (Thermo Fisher Scientific). The other substances employed in this investigation were AR grade.

Plant Materials

In February 2023, an *A. glycyphyllos* plant was gathered from the vicinity of the university campus. After the plant was identified and verified, a sample voucher was kept for future use and given the reference number AG/Feb-2023/001.

Preparation of Extract

The leaves were collected and thoroughly washed with distilled water. Subsequently, they were dried at room temperature under shaded conditions to avoid exposure to direct sunlight. The dried leaves were then ground into a fine powder and defatted with petroleum ether (60 to 80 °C) for 72 hours. After filtration to remove the petroleum ether, the powder was subjected to ethanol extraction for 72 hours with periodic shaking. The resulting filtrate was collected, and ethanol was recovered by distillation. The remaining residue was heated at a low temperature of 40 °C to remove residual alcohol. A viscous extract was obtained, yielding 8.25% w/w, which was subsequently stored at 4 °C (Firdous and Koneri 2012).

Experimental Animals

In this investigation, male Albino Wistar rats weighing 180 to 200 g were employed. The animals were kept in polypropylene cages at a temperature of 22±1°C and a 12-h day/night cycle. Before the trial, they were given a regular pellet diet, allowed to drink water whenever they pleased, and given a week to acclimate. The study was approved by Yunan Zhongkai Animal Experiments Medical Board (Approval Number: YN9981).

Grouping and Induction of Arthritis

The rats were divided into five groups (N=6), as follows.

Group I: For 28 days, the animals were given 0.30 % w/v CMC (1 ml/kg; p.o.) and were used as a typical control group.

Group II: For 28 days, the animals were fed CFA control (0.1 mL of CFA at the left hind paw on day 1 + 0.30 % w/v CMC (1 ml/kg; p.o.).

Group III: For 28 days, the animals had 20 mg/kg of intraperitoneal diclofenac sodium in addition to 0.1 mL of CFA at the left hind paw on day 1.

Group IV: For 28 days, the animals had 100 mg/kg of *A. glycyphyllos* extract p.o. and 0.1 mL of CFA at the left hind paw on day 1.

Group V: For 28 days, the animals had 200 mg/kg of *A. glycyphyllos* extract p.o. and 0.1 mL of CFA at the left hind paw on day 1.

CFA was used to develop experimental immunological arthritis in Wistar rats with a few minor group categorization adjustments. All groups—aside from group I—had their hind limbs clipped and cleaned with 70% (v/v) alcohol. This was followed by a 0.1 mL CFA treatment that contained 10 mg/mL of heat-killed *Mycobacterium tuberculosis*. Before CFA was administered into the subplantar region of the left hind paw, each animal had a mild diethyl ether anesthetic injection. Day 1 denotes the adjuvant administration period. Paw volume was recorded from day 1 to day 21 following oral delivery of the vehicle, extract, and diclofenac (Cortés *et al*. 2022; Nickels *et al*. 2023).

Assessments of Arthritis

Paw volume

A digital plethysmometer (Ugo Basile, 37140) was used to measure the swelling of both hind paws on days 1, 7, 14, 21, and 28 after the CFA injection.

Body weight

The body weights were measured after the CFA injection starting on day three and again on days 7, 14, 21, and 28. The weight change was computed as follows,

$$\% \text{ body weight change} = \frac{W_t - W_1}{W_t} \times 100 \quad (1)$$

where W_1 is the animal's bodyweight on day 1 and W_t is the bodyweight at time t.

Arthritic index

To gauge the severity and course of induced arthritis, clinical symptoms and indicators were graded visually for each leg on a range of 0 to 4. A score of zero denotes no change, a score of one denotes some edema and erythema in the limb, a score of two denotes mild swelling and erythema, a score of three denotes major swelling and erythema, and a score of four denotes severe limb deformity and immobility. A score of more than one indicates the presence of arthritis in either of the two rear limbs. However, the highest possible score for arthritis is 8. Following the CFA injection, the measurement was initiated on day 3 and was repeated on days 7, 14, and 21.

Locomotion

Pain reduces an animal's ability to move when they have arthritis. An actophotometer was used to measure the locomotor activity of the arthritic animals in each group for 5 min throughout the investigation. The apparatus was filled with each animal and switched on to measure the movement scores. An animal may go around a square arena with this equipment. It is powered by photoelectric cells connected to a circuit counter. A count is taken after the animal breaks the light beam striking the photocell. In addition to six integrated photo-sensors, it features a 4-digit digital counter that indicates the movement activity. The locomotion activity was measured on days 1, 7, 14, 21, and 28.

Cytokines measurement

Blood was drawn from the retro-orbital sinus and put into Eppendorf tubes at 24 h following the last dosage. After allowing the blood to coagulate for 20 min at room temperature, it was centrifuged for 10 min at 4 °C at 4000 rpm. Serum was separated and

cooled to -20 °C for biochemical examination. Following the manufacturer's instructions, pro-inflammatory cytokines (TNF-α, IL1-β, and IL-6) in the blood were measured using readily accessible ELISA kits (Sigma-Aldrich, USA).

Complete blood count

On day 21, all groups had heart punctures to obtain blood, which was then collected in the proper blood collection containers while under a mild dosage of diethyl ether anesthesia. A programmed Blood Cell Analyzer (Mindray, USA) was used to measure the hematological variables immediately after the blood sample was collected. These included hemoglobin content (HGB), packed cell volume (% PCV), total red blood count (RBC), total white blood cell count (WBC), and erythrocyte sedimentation rate (ESR).

Chondrocytes Culture

The tissues were enzymatically digested with 10 g/L of pronase and 1g/L of type IV collagenase for 30 min and 6 h, respectively, at 37 °C, after primary rat chondrocytes (PRCs) were extracted from the knee joints of 4-week-old Sprague-Dawley rats (Nickels *et al*. 2023). The process of filtration was used to exclude debris and undissociated cells from the chondrocyte single-cell suspension (SCS). After that, the obtained SCS was centrifuged for 10 min at 3600 rpm. Following centrifugation, chondrocytes were resuspended in "Dulbecco's modified Eagle's medium" (DMEM). The cells were seeded in culture flasks with 10% Fetal Bovine Serum (FBS) and antibiotic media with penicillin (100 U/mL) and streptomycin (100 µg/mL). The monolayer cultures were established at a concentration of 6×10^6 cells/mL. The cells were then incubated at 37 °C in a humidified incubator with 5% CO2. Every three days, the culture media were changed (Li *et al*. 2020). The PRCs were pre-treated with 0.1% dimethyl sulfoxide (DMSO, control) or different doses of *A. glycyphyllos* leaves extract for 1 h, and then stimulated with IL-1β (10 ng/mL) for 24 h in order to examine the chondroprotective impact of *Astragalus glycyphyllos*.

Cell Viability Assay

The isolated chondrocytes were planted at a density of 5×10^3 cells per well onto a microplate with 96 wells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, or MTT test, was used to measure cell viability and gauge the cytotoxic effects of *A. glycyphyllos* leaves extract following pre-treatment with *A. glycyphyllos* leaves extract and IL-1β stimulation. By the manufacturer's protocol, this was completed. 100 µL of MTT was added to each well, and the mixture was incubated for 4 h. Supernatants were supplied with 150 µL DMSO to dissolve the crystals, and then agitated for 10 min. Absorbance at 490 nm was measured using a microplate reader. Percent viability was estimated using the formula: % viability (Survival) = OD_{test} /$OD_{control}$, where OD indicates optical density (Srivastava *et al.* 2017).

NO Assay

Using Griess reagent (GR) to quantify the nitrite concentrations in the supernatant cell culture in accordance with the manufacturer's instructions, NO generation was assessed spectrophotometrically. After receiving a 2-h pretreatment with *A. glycyphyllos* leaves extract , chondrocytes were stimulated with 10 ηg/mL of IL-1β. Following a 24-h period, the supernatants were extracted. Each supernatant was then combined with an equivalent volume of GR (150 µL) and allowed to incubate for five min at room temperature. Then, at 540 nm, absorbance was measured (Liu *et al.* 2017).

ELISA

Chondrocytes pre-treated with *A. glycyphyllos* extract and stimulated with IL-β (10 ng/mL) had their cell supernatant collected. The tests were conducted in accordance with the manufacturer's protocol Sigma-Aldrich, USA, and sandwich ELISAs were used to validate the levels of PGE2, MMP-1, and MMP-13 released by the chondrocytes.

Statistical Analysis

Every group's outcome was presented as mean ± SE. The groups were compared using a one-way analysis of variance (ANOVA), and P values of less than 0.05 were considered statistically significant. The "Tukey Multiple Comparison Test" was then conducted with version 8.1 of Graph Pad Prism.

RESULTS

Effect of *A. glycyphyllos* Extract on Inflammation in Arthritic Animals

Figure 1 illustrates the impact of an ethanolic extract of *A. glycyphyllos* leaves on paw inflammation in rats given a CFA challenge.

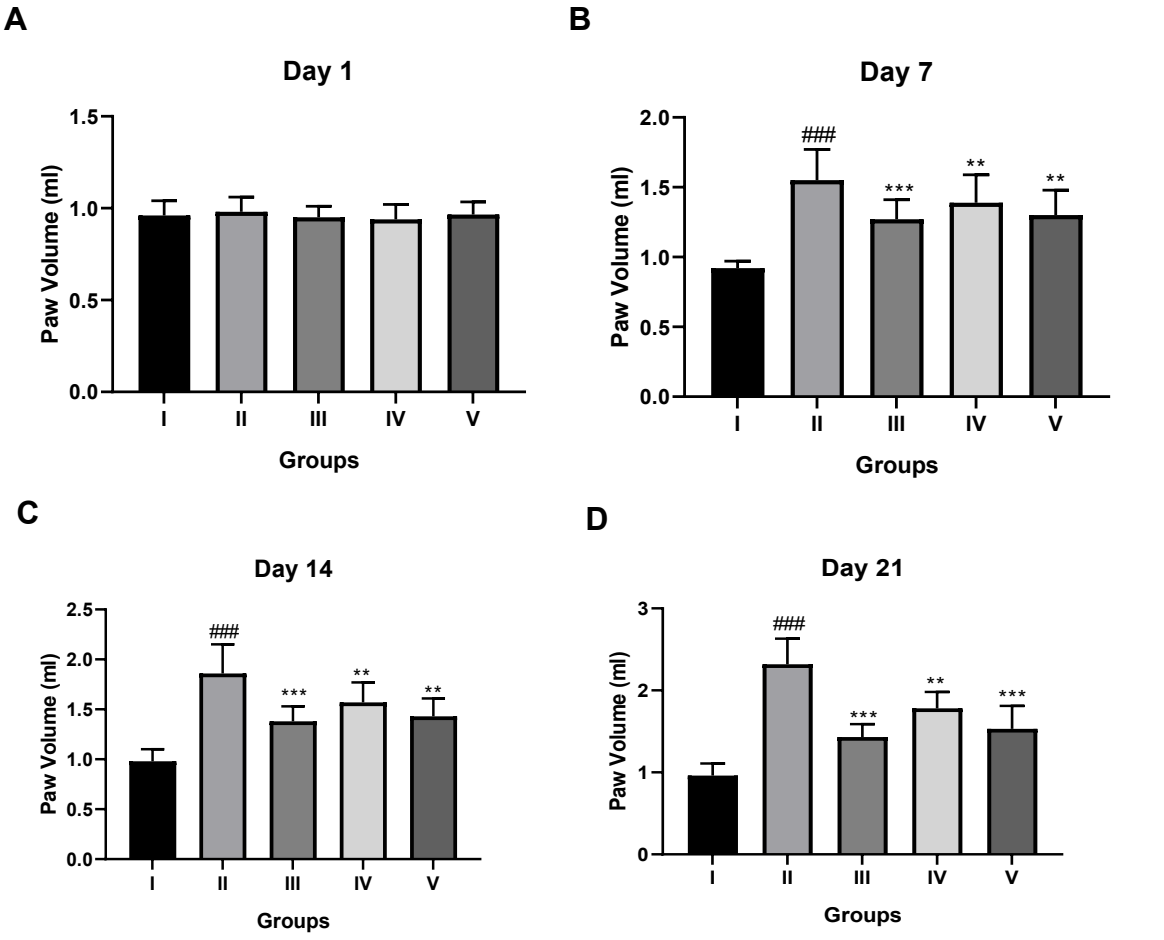

Fig. 1 (A-D). Effect of *A. glycyphyllos* leaves extract on paw inflammation in CFA challenged arthritic animals. (A) Day 3,(B) Day 7, (C) Day 14, (D) Day 21, (E) Day 28. Mean ±SEM (n= 6). ###P<0.001 considered statistically noteworthy as judged against normal control group; **P<0.01 as well as ***P<0.001 considered statistically noteworthy as judged against CFA control group.

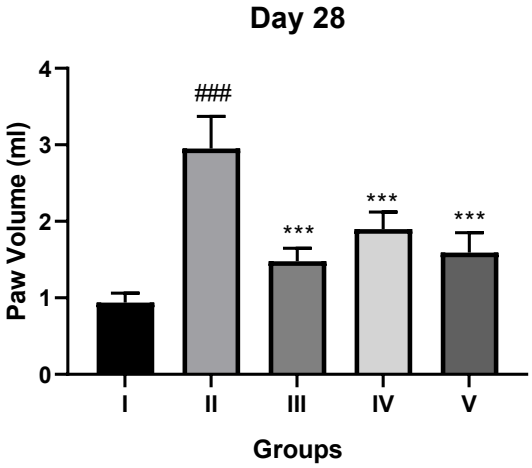

Fig. 1 (E). Effect of *A. glycyphyllos* leaves extract on paw inflammation in CFA challenged arthritic animals. (A) Day 3,(B) Day 7, (C) Day 14, (D) Day 21, (E) Day 28. Data are represented as mean ±SEM (n= 6). ###P<0.001 considered statistically noteworthy as judged against normal control group; **P<0.01 as well as ***P<0.001 considered statistically noteworthy as judged against CFA control group.

When comparing the CFA-challenged rats treated with *A. glycyphyllos* extract (100 and 200 mg/kg) to the animals in the CFA control group at days 7, 14, 21, and 28, a progressive decrease in paw inflammation was observed. When comparing day 1 to day 7, it was observed that the mice in the CFA control group had 1.5 times more paw inflammation on day 7.

On the other hand, the extract (100 and 200 mg/kg) was found to significantly ($p<0.01$) reduce inflammation when compared to the rats in the CFA control group. On days 14, 21, and 28, it was noted that the extract (at 100 and 200 mg/kg) had the same general pattern of impact on paw inflammation in arthritic animals. The period between days 14 and 28 had an increase in inflammation.

When comparing day 1 to day 28, the animals in the CFA control group had an increase in inflammation of more than twice as much. In contrast, compared to the animals in the CFA control group, the animals that were given the extract (at doses of 100 and 200 mg/kg) and were CFA-challenged showed a substantial ($p<0.01$) reduction in inflammation.

Body Weight Changes in Arthritic Animals after *A. glycyphyllos* Treatment

The effects of *A. glycyphyllos* extract (100 and 200 mg/kg) on the body weight of arthritic animals with CFA challenges are shown in Table 1. Days 1 and 28 were used to measure the body weight. On day 28, it was discovered that the body weight of the normal control mice had increased (13.5%). When compared to normal control animals, the CFA control group's body weight had significantly ($p<0.001$) decreased. The body weight of the CFA animals decreased significantly. However, arthritic animals treated with the *A. glycyphyllos* extract (100 and 200 mg/kg) showed a significant ($p<0.01$) increase in body weight.

Table 1. Following *A. glycyphyllos* Therapy Change in the Body Weight of Arthritic Animals

Groups	Treatment	Body weight (gm)		% changes
		Day 1	Day 28	
I	Normal control	195.92±5.03	326.48±5.22	13.49
II	CFA control	193.76±4.63	252.60±8.51$^{###}$	-26.97
III	Diclofenac sodium (10 mg/kg; i.p.)	192.55±4.98	301.64±4.16***	4.50
IV	*A. glycyphyllos* extract (100 mg/kg; p.o.)	196.02±4.61	306.82±3.62***	5.22
V	*A. glycyphyllos* extract (200 mg/kg; p.o.)	195.75±5.16	316.50±4.02	9.58

Data are represented as mean ±SEM (n= 6). $^{###}$P<0.001 considered statistically noteworthy as judged against normal control group; *P<0.05 considered statistically noteworthy as judged against CFA control group.

Table 2. Effect of *A. glycyphyllos* Leaves Extract on Locomotor Activity of Arthritic Animals

Groups	Treatment	Locomotor score				
		Day 1	Day 7	Day 14	Day 21	Day 28
I	Normal control	462.27±10.78	484.08±10.17	471.29±9.91	486.21±8.74	479.50±8.92
II	CFA control	454.83±13.23	201.45±6.28$^{###}$	188.04±5.44$^{###}$	151.38±3.92$^{###}$	137.06±3.40***
III	Diclofenac sodium (10 mg/kg; i.p.)	470.24±11.66	314.33±7.55***	318.63±5.01***	352.60±3.65***	369.63±4.78***
IV	*A. glycyphyllos* extract (100 mg/kg; p.o.)	468.16±8.54	302.93±5.15***	325.95±5.51***	345.12±4.65***	360.38±4.40***
V	*A. glycyphyllos* extract (200 mg/kg; p.o.)	458.28±9.32	358.28±5.49***	352.18±4.11***	370.26±4.85***	392.46±5.17***

Data are represented as mean ±SEM (n= 6). $^{###}$P<0.001 considered statistically noteworthy as judged against normal control group; ***P<0.001 considered statistically noteworthy as judged against CFA control group.

Effect of *A. glycyphyllos* Extract on Arthritic Index in Arthritic Animals

The arthritic index was determined on the third day after the CFA injection by measuring the thickness of the hind paw, the swelling and redness of the paw phalanges, and the signs of inflammation on the nose, mouth, ears, tail, and eyes. The arthritic index significantly ($p<0.01$) decreased in the diclofenac and extract-treated groups (100 and 200 mg/kg) compared to the CFA-control group (Fig. 2). The animal groups with arthritis that were given *A. glycyphyllos* extract (200 mg/kg) showed fewer clinical signs of arthritis than the animal groups that were given diclofenac (100 mg/kg). On day 28, the CFA control animals had the highest arthritic index.

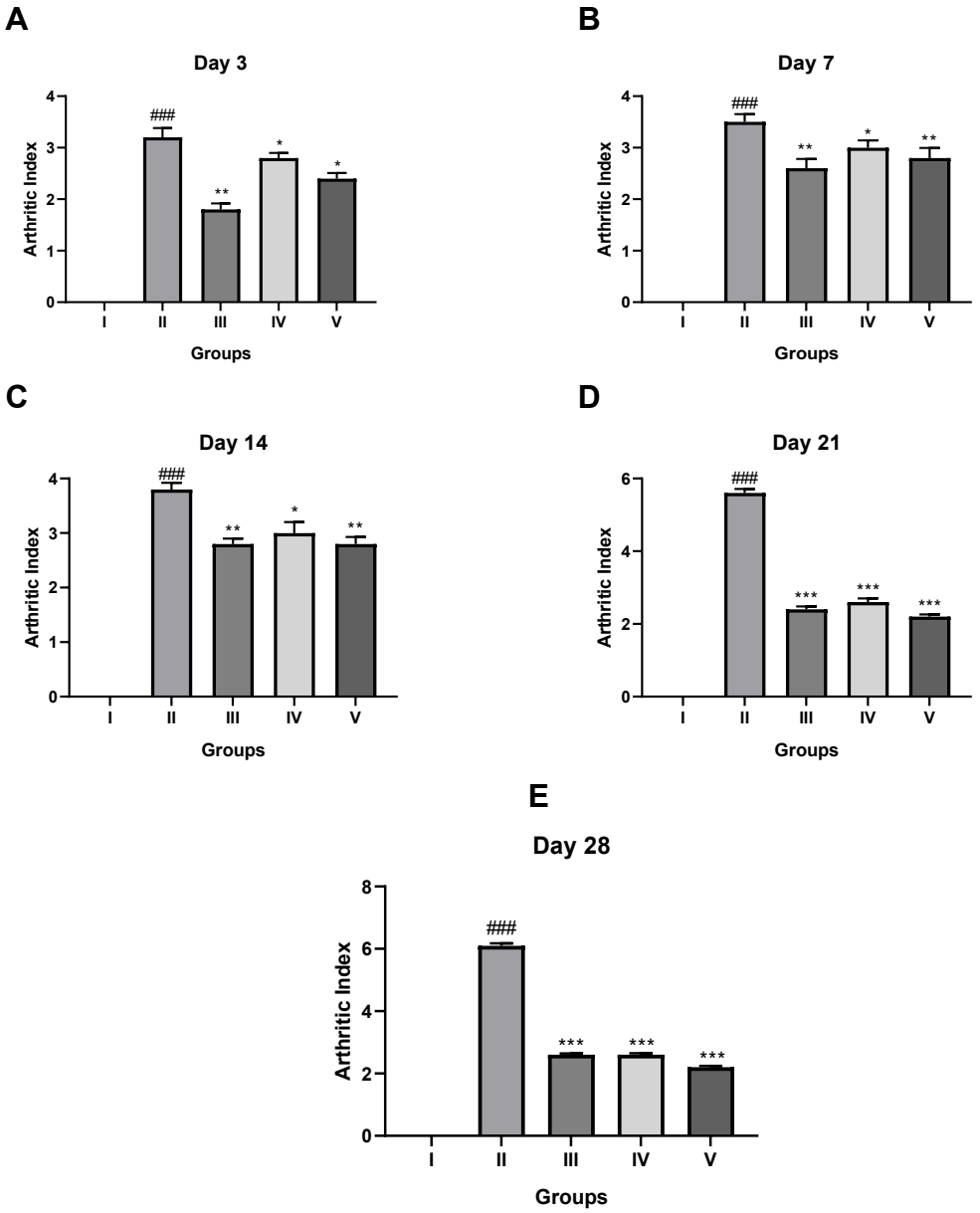

Fig. 2. Effect of *A. glycyphyllos* leaves extract on arthritic index in CFA challenged arthritic animals on days (A) 3, (B) 7, (C) 14, (D) 21, and (E) 28. Data are represented as mean ±SEM (n= 6). ###P<0.001 considered statistically significant *versus* normal control group; *P<0.05, **P<0.01 as well as ***P<0.001 considered statistically significant *versus* CFA control group.

A. glycyphyllos Leaves Extract Restructure the Locomotor Activity of Arthritic Animals

Table 2 shows the locomotor scores of the animals treated with *A. glycyphyllos* extract (100 and 200 mg/kg). Compared with the animals in the CFA control group, there were significant improvements in the locomotion scores of the animals challenged with CFA. The CFA control group's animals had severe movement impairment, which may have been brought on by discomfort, and their locomotion was drastically reduced by day 28. Spontaneous motility significantly increased with both dosages of *A. glycyphyllos* extract.

A. glycyphyllos Leaves Extract Ameliorate the Serum Inflammatory Cytokines in Arthritic Animals

Early RA may become more inflammatory due to the increase in plasma TNF-α. Furthermore, the induction of inflammation and the transition from acute to chronic inflammation depend on IL-1β and IL-6. In this experiment, the rats in the CFA control group had significantly higher levels of the inflammatory markers listed above (Fig. 3). When compared to rats in the CFA control group, animals treated with *A. glycyphyllos* extract (100 and 200 mg/kg) exhibited a significant ($p<0.01$) reduction in TNF-α, IL-1β, and IL-6. In CFA-challenged arthritic mice, the greatest dose of the extract demonstrated a stronger potential impact against the rise in inflammatory markers.

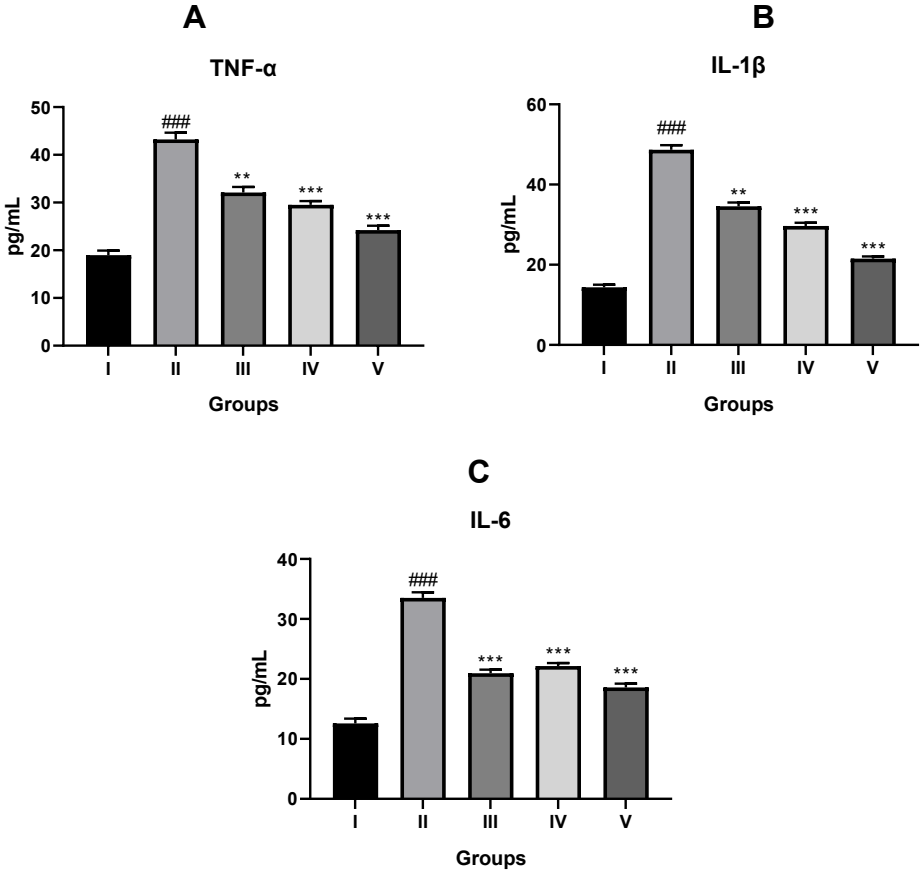

Fig. 3. Effect of *A. glycyphyllos* leaves extract on inflammatory cytokines (A) TNF-α,(B) IL-1β, (C) IL-6 in CFA challenged arthritic animals. Data are represented as mean ±SEM (n= 6). ###P<0.001 considered statistically noteworthy as judged against normal control group; **P<0.01 as well as ***P<0.001 considered statistically noteworthy as judged against CFA control group.

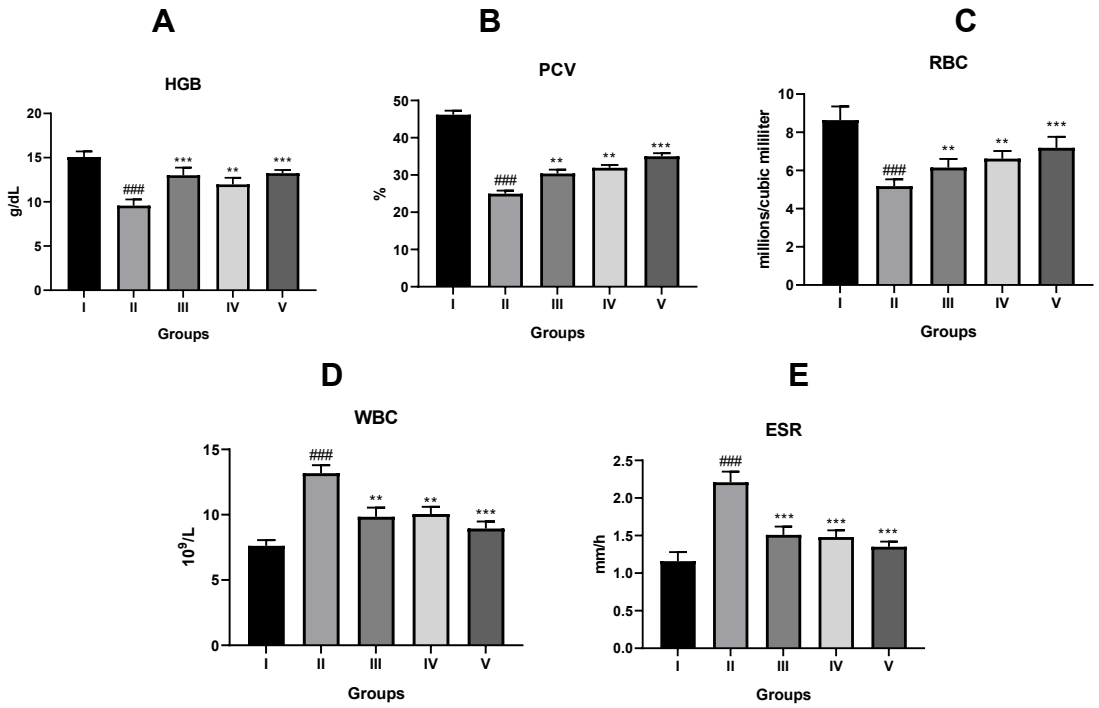

Fig. 4. Effect of *A. glycyphyllos* leaves extract on hematological parameters (A) HGB, (B) PCV, (C) RBC, (D) WBC, (E) ESR in CFA challenged arthritic animals. Data are mean ±SEM (n= 6). ###P<0.001 considered statistically noteworthy as judged against normal control group; **P<0.01 as well as ***P<0.001 considered statistically noteworthy as judged against CFA control group.

Effect of *A. glycyphyllos* Leaves Extract on Hematological Parameters in Arthritic Animals

Figure 4 displays the hematological parameters evaluated in animals challenged with CFA. Comparing the CFA control group to the normal control group, there was a large rise in WBC and ESR and a significant decrease in HBG, PVC, and RBC. Nonetheless, the HBG level, PVC percentage, and RBC count all significantly ($p<0.01$) improved after receiving therapy with *A. glycyphyllos* extract (100 and 200 mg/kg). Moreover, both dosages markedly reduced the ESR and WBC count. In arthritic animals with CFA challenges, the greatest dose of the extract had a more pronounced benefit against the hematological artifact.

Effect of *A. glycyphyllos* Extract on IL-1β-stimulated PRCs Cell Viability

The effects of *A. glycyphyllos* extract at different doses (50, 100, 200, 300, 500, 1000, and 2000 µg/mL) in PRCs were determined using the MTT test (Table 3).

Table 3. Outcome of *A. glycyphyllos* Leaves Extract on Viability of PRCs

***A. glycyphyllos* Leaves Extract (µg/mL)**	**% of viable PRCs**
50	99.97±0.13
100	99.93±0.15
200	94.32±0.17
300	89.65±0.20
500	70.09±0.23
1000	51.33±0.21
2000	24.54±0.19

Data are shown in a triplicate manner (mean±SEM)

It was found that chondrocyte viability was unaffected by *A. glycyphyllos* extract at concentrations of 50 and 100 µg/mL. Thus, 50 and 100 µg/mL doses were selected for more research.

Extract from *A. glycyphyllos* Leaves Reduces the Levels of NO, PGE2, and Enzymes that Break Down the Matrix in PRCs when Exposed to IL-1β

In PRCs, IL-1β induction results in an inflammatory response that is both excessive and long-lasting. Chondrocyte destruction may be induced by the rise in NO and PGE_2 levels brought on by IL-1β; however, the destruction may be prevented by inhibiting the activation of these inflammatory mediators (Aury-Landas *et al*. 2017). Thus, it was determined whether the extract might lower the high levels of inflammatory mediators NO and PGE_2 by examining its influence on the IL-1β-induced inflammatory response in PRCs. The Griess reagent and the sandwiched ELISA method were used, respectively, to evaluate these. The findings show that expression of nitric oxide (NO) and prostaglandin E2 (PGE2) was significantly elevated in the IL-1β-treated group. Nevertheless, the increase of NO and PGE2 was reduced in a dose-dependent manner by pre-treatment with *A. glycyphyllos* leaves extract (Table 4). Furthermore, it is well known that iNOS is the primary catalyst for NO production (Liu *et al*. 2017); as a result, the downregulation of iNOS by the extract may be the cause of the decrease in NO levels in PRCs.

The release of MMPs and ADAMTSs (short for a disintegrin and MMP with thrombospondin shapes or motifs) is triggered by inflammatory mediators, NO, and PGE2. This process further destroys collagen and aggrecan, which are crucial components of the cartilage matrix (Aury-Landas *et al.* 2017). Consequently, ELISA assays were used to determine the impact of the extract on the MMP-1 and MMP-13 levels of IL-1β-challenged PRCs. The outcomes showed that following IL-1β therapy, MMP-1 and MMP-13 levels dramatically rose. On the other hand, extract pre-treatment dose-dependently reduced MMP-1 and MMP-13 levels caused by IL-1β (Table 4).

Table 4. Effect of *A. glycyphyllos* Leaves Extract on NO, PGE_2, MMP-1 and MMP-13 Levels in PRCs

Treatment	NO (µmol)	PGE_2 (ρg/mL)	MMP-1 (ηg/mL)	MMP-13 (ηg/mL)
Control (IL-1β)	90.62±2.59	985.21±14.70	254.10±2.90	219.27±2.45
A. glycyphyllos (50 µg/mL)	62.58±1.81**	702.26±15.28*	116.36±2.15***	90.36±1.47***
A. glycyphyllos (100 µg/mL)	40.72±1.05***	412.37±16.30***	76.27±1.22***	60.82±0.81***

Data are represented as mean ±SEM (n= 6). $^{###}$P<0.001 considered statistically noteworthy as judged against normal control group; *P<0.05, **P<0.01 as well as ***P<0.001 considered statistically noteworthy as judged against control group.

Discussion

Multifactorial autoimmune arthritis is characterized by persistent swelling in several joints accompanied by inflammatory cells. NSAIDs are commonly used as monotherapy or in conjunction with other therapies to treat arthritis. However, their severe side effects make finding more effective and secure treatments difficult (Khurana and Berney 2005; Ferreira *et al.* 2016). Clinical trials have employed medicinal plants to treat

arthralgia, which is more convenient and well-tolerated for individuals with arthritis (Kaur *et al.* 2012).

In rats with CFA exposure, the anti-arthritic properties of *A. glycyphyllos* leaf extract (100 and 200 mg/kg) were examined in this work. The results showed that the extract from *A. glycyphyllos* prevented inflammatory paw edema in both the acute and chronic phases of arthritis caused by CFA. The CFA-induced RA model was used to evaluate the anti-arthritic efficacy as well as the physiological or therapeutic modulation of systems connected to inflammation. After CFA induction, there was localized inflammation for the first two to four days, followed by chronic inflammation that lasts for many weeks.

Multiple immunological, clinical, and chronic features of CFA have been connected to arthritis in humans (Gupta *et al.* 2020; Weng *et al.* 2021). The arthritic grade, a gauge of joint inflammation, indicates the severity of arthritis. Increased inflammatory processes, the formation of arthritic nodules in the tail and ear, the continuation of swelling on the opposing side, and a protracted secondary reaction were among the CFA's early reactions. In the present investigation, at days 7, 14, 21, and 28, the extract from *A. glycyphyllos* leaves (100 and 200 mg/kg) significantly reduced paw inflammation. Furthermore, the arthritic index values were dramatically lowered by both *A. glycyphyllos* extract dosages.

In comparison to the animal group that supplemented with diclofenac, the group that received *A. glycyphyllos* extract at a dosage of 200 mg/kg experienced an increase in body weight, indicating its safety, moderate toxic effects, or reduced side effects. Due to the ulcerative or gastrointestinal disturbance side effects of diclofenac, it's possible that the animal group that took the medication had a modest increase in body weight. In the CFA control group, there was a noticeable decrease in body weight. However, *A. glycyphyllos* extract at a dosage of 200 mg/kg showed a better percentage of body weight growth than *A. glycyphyllos* extract (100 mg/kg) or the diclofenac group.

The results of the locomotion scoring demonstrate how the CFA-control group's chronic RA progressed and how it impaired mobility. On the other hand, the animal species that received *A. glycyphyllos* extract showed a noticeable increase in motility. Consequently, it was shown that, in comparison to the CFA control group animals, rats with CFA challenges who were given *A. glycyphyllos* extract (at doses of 100 and 200 mg/kg) between days 7 and 21 had a noteworthy enhancement in their locomotor score. This implies that the *A. glycyphyllos* extract helped delay the start of RA or, at a minimum, reduced the severity of the illness.

There is no denying the substantial role inflammation plays in RA, and proinflammatory cytokines are believed to be key participants in the pathogenesis of the illness. According to an overwhelming amount of evidence, these cytokines can cause immune cell infiltration. This is followed by the release of matrix metalloproteinases, which are mostly in charge of breaking down cartilage in disorders like osteoarthritis and arthritic conditions (Stambolov *et al.* 2023). Moreover, they can trigger the NF-κB pathway, which amplifies the inflammatory cascade and raises proinflammatory cytokine levels even more. The inflammatory response in RA can be made worse by macrophages, immune cells, and T cells, thereby producing more TNF-α and IL-6. The results showed that *A. glycyphyllos* extract reduced TNF-α, IL-1β, and IL-6 blood levels in the treated RA rats, suggesting that the anti-inflammatory qualities of *A. glycyphyllos* extract may be useful in lowering RA.

Iron deficiency anemia is one hematological indication of RA. Changes in hematological and biochemical indicators were also observed throughout this experiment. Usually, low Hb and RBC levels cause anemia. This results from an aberrant bone marrow, erythropoietin insufficiency, and erythrocyte loss triggered by elevated IL-1β in mice with arthritis (Alamgeer *et al.* 2017). It was found that while WBC and ESR levels were raised, the HBG, PVC, and RBC levels in the CFA control group were considerably lower than those in the normal control group. However, following the consumption of *A. glycyphyllos* extract (100 and 200 mg/kg), the HBG level, PVC percentage, and RBC count considerably recovered. Both doses resulted in significant drops in the WBC count and ESR. At the maximum dosage, the extract's effect against the hematological abnormality in CFA-challenged arthritic rats was more noticeable.

One of the main factors thought to be responsible for the PRCs' destruction and OA development is IL-1β. Under normal circumstances, chondrocytes, which are components of cellular cartilage, balance anabolic and catabolic metabolism and maintain the extracellular matrix (ECM) (Zhao *et al.* 2023). Regarding the articular cartilage along with other joint components, IL-1β, both alone and in concert with specific mediators, induces inflammatory and catabolic responses.

The action of IL-1β blocks chondrocytes in the context of ECM component synthesis and interferes with the synthesis of main structural proteins, such as Type-II collagen and aggrecan. Inflammatory cytokines primarily harm articular cartilage (Wojdasiewicz *et al.* 2014). Furthermore, the function of chondrocytes in the synthesis of MMPs, particularly interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and collagenase 3 (MMP-13), is influenced by IL-1β. These MMPs have a significant effect on cartilage components (Vincenti and Brinckerhoff 2002). Therefore, a novel treatment agent for OA may be an agent that prevents the production of MMPs and inflammatory cytokines. The results of this study showed that extract therapy decreased the elevated levels of MMP-1 and MMP-13.

When there is an inflammatory response, arachidonic acid stimulates COX-2 to create PGE_2. NO is produced from the amino acid L-arginine by the enzymatic action of iNOS, which is triggered by inflammatory stimuli. This suggests that proinflammatory genes, such as COX-2 and iNOS, are upregulated in chondrocytes and are in charge of initiating the destruction of PRCs stimulated by IL-1β (Choy and Panayi 2001; Mohammed 2003; Sweeney and Firestein 2004). Thus, the impact of extract on NO and PGE_2 secretion from PRCs in the presence of IL-1β was studied. The extract was discovered to drastically lower inflammatory mediators like NO and PGE2 in PRCs, which was likely caused by a decline in the expression of iNOS and COX-2 (Hochberg 2002; Yoon *et al.* 2013; Fan *et al.* 2015).

Flavonoids, the most common kind of natural polyphenols, contain several pharmacological properties, most notably anti-inflammatory properties. Several studies demonstrate that naturally occurring flavonoids stop the onset of RA by blocking the NF-κB signaling cascade and reducing the release of inflammatory cytokines (Behl *et al.* 2021). The inflammatory processes that result in arthritis depend on the NF-κB signaling system. The transcriptional targets of the NF-κB pathway include TNF-α and IL-6, two inflammatory cytokines. Since the pathogenic changes of arthritis are associated with enhanced NF-κB pathway activation, targeting this pathway has been demonstrated to be an effective therapy technique. In addition, there is mounting data that natural flavonoids, through their inhibition of the NF-κB signaling pathway, have protective effects against the pathological alterations associated with OA (Jin *et al.* 2010; Panche *et al.* 2016; Yadav

et al. 2020). Natural flavonoids have the potential to inhibit inflammatory reactions mediated by NF-κB activation, degradation of the extracellular matrix, and death of chondrocytes. The variably substituted groups on the structures may be related to the distinct biological activities of natural flavonoids on the NF-κB signaling pathway in OA chondrocytes. In this study, several researchers who focus on the NF-κB signaling pathway examine the effectiveness and mechanism of action of natural flavonoids against the development of OA. Flavonoids have the potential to be effective NF-κB signaling cascade inhibitors for the treatment of OA (Ye and Zhou 2023).

The leaves of *A. glycyphyllos* have a considerable quantity of flavonoids and polyphenols (Tsiklauri *et al.* 2021). The results of this study suggest that the presence of polyphenols and flavonoids may be the cause of the anti-arthritic effect of *A. glycyphyllos* extract. Additionally, the present study has some limitations, including the use of only one extract concentration and a limited number of experimental animals. Additionally, the study focused on the acute phase of arthritis, and the long-term effects of the extract remain unknown. Future studies could investigate the dose-dependent effects of *A. glycyphyllos* extract and explore its therapeutic potential in models of chronic arthritis. Elucidating the exact molecular mechanisms underlying the extract's anti-arthritic effects would also be a valuable direction for future research. Furthermore, clinical trials could be conducted to assess the safety and efficacy of *A. glycyphyllos* extract in human patients with arthritis.

CONCLUSIONS

1. The results of this study showed that the extract of *A. glycyphyllos* has an anti-arthritic effect by the reduction in paw inflammation, inflammatory markers, arthritic score, and paw inflammation in complete Freund's adjuvant (CFA)-challenged rats, as well as the improvement in locomotion and hematological parameters.
2. Furthermore, the extract lowered the increased levels of NO, PGE2, MMP-1, and MMP-13 in primary rat chondrocytes produced by IL-1β.
3. The anti-inflammatory activities of *A. glycyphyllos* extract are responsible for its benefits against CFA-induced arthritis (*in vivo*) and IL-1β-induced ~~PCRs~~ Primary rat chondrocytes(*in vitro*).

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