Research Square

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Formulation of Calendula officinalis, Glycine max, and Aloe vera extract topical gel ointment attenuates rheumatoid arthritis in rats via inhibition of pro-inflammatory cytokines

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Research Article

Keywords: Calendula officinalis, Glycine max, Aloe vera, arthritis, anti-inflammatory, gel ointment

Posted Date: December 8th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2266658/v1

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Additional Declarations: No competing interests reported.

Abstract

Background: Several pharmacological effects have been reported on *Calendula officinalis* (C), *Aloe vera* (A) and *Glycine max* (G) scientific validation of its rheumatoid arthritis property of the combination of these plants in topical gel ointment has not been done so far. Due to its promising therapeutic effects, to improve its efficacy, we designed combined these plants in gel ointment (CAG/gel). This study aimed to investigate special participation of a new topical herbal formulation containing CAG in gel ointment in vitro and in vivo anti-inflammatory models.

Methods: HPLC analysis were performed to investigate the chemical composition of herbal formulation. The efficacy of CAG/gel as potential treatment was supported in CFA-induced-arthritis rats in lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 studies and proliferation of B and T lymphocytes.

Result: In the phytochemical profile, seventeen flavonoids were identified. Results indicated that topical administration of CAG/gel to CFA-induced-arthritis rats showed a significant reduction of paw swelling like those of the diclofenac used as reference. Arthritic joints produce the remission of disease and recovery of the joint structure and regulates the levels of proinflammatory mediators. CAG/gel modulates adjuvant-induced rheumatoid arthritis by regulating inflammatory cytokines. CAG/gel treatment showed that the polyphenolic-rich extract no cytotoxic effect in RAW 264.7 cells and also exhibited significantly inhibition production of tumor necrosis factor (TNF)-α, interleukin 1β (IL-1β), interleukin (IL)-6, nitric oxide (NO),and inducible nitric oxide synthase (iNOS) in LPS-induced inflammation in macrophages RAW 264.7. In addition, CAG/gel significantly decreased the abnormal proliferation of B and T lymphocytes.

Conclusion: Findings demonstrated that treatment with CAG/gel provides satisfactory rheumatoid arthritis property to those in the standard diclofenac. The improved rheumatoid arthritis, especially in the 10% CAG/gel groups, can be attributed to phytochemical content which reveals the presence of anti-inflammatory and antioxidant as the major active components. The mechanism of anti-rheumatoid arthritis associated to decrease of inflammatory mediators and pro-inflammatory cytokines to its immune function. The CAG/gel exhibited a significant anti-inflammatory activity with a synergistic effect between the flavonoids contained in the three plants. Thus, CAG/gel ointment is a reasonable alternative to the topical formulation for the treatment of rheumatoid arthritis conditions.

Background

Trauma, autoimmune damage, and toxic infections are responsible for the inflammation organisms react to inflammation through the down-regulation of protein expression and pro-inflammatory; when are increased there are modifications in the vascular system, they assist to recruit the immune cells [1]. Rheumatoid arthritis (RA) is a chronic autoimmune disorder related to joint disturbance causing anti-bodies to select the self-neoepitopes to activate defense cells and macrophages in an autoimmune response related to chronic arthritic attacks. Self-epitopes are biomarkers that decrease unbearable pain,

muscle strength, inflammation, joint stiffness, and loss of mobility difficulty in daily activities. Similarly, as in processes with inflammation, pro-inflammatory mediators (cytokine: interleukin-6, interleukin-1, and TNF-α) play an essential role in the pathogenesis of RA [2]. Currently being used glucocorticoids, non-steroidal anti-inflammatory drugs, and disease-modifying anti-rheumatic drugs can reduce arthritis progression and ameliorate the symptoms but they don't cure RA, frequent administration even high doses are required to obtain acceptable effectiveness causing untoward side-effects [3]. Also decreasing the cytokines level and preventing joint destruction have been developed, as biological agents but the benefits have not been as expected causing serious infections including tuberculosis reactivation [4]. (Therefore, novel therapeutic strategies for RA treatment are still urgently warranted.

Dermal application to those in the conventional oral delivery for drugs offers several advantages including decreases the maximum plasma concentration of the medicine (Cmax), continuous medicine delivery, ameliorate the tolerability profile and allow reach a high local drug concentrations without systemic side effects [5].

Medicinal plants contain pharmacodynamic bioactive compounds whose additive and synergistic therapeutic effect is beneficial in the management of metabolic disorders. Most pharmaceutical drugs are developed from medicinal plants based on local communities' knowledge and the subsequent isolation of the main active ingredients. However, there is a scarcity of scientifically accurate reviews and experimental evidence on the efficacy and safety of medicinal plants. The systematic study of medicinal plants and the investigation of their biologically active phytochemical compounds for the management of metabolic disorders have emerged as a key development in modern medicine [6].

A great variety of medicinal plants contain phytoconstituents with anti-inflammatory properties, which are effective alternatives focusing on synthetic drugs for their toxic effects. Many medicinal plants have been studied to improve inflammation and related diseases. Among them *C. officinalis* flower extract has been used to prepare several topical pharmaceutical formulations due to its rich content of phytochemicals, since it contains a wide variety of bioactive compounds like carotenoids which possesses anti-inflammatory effects [7]. *C. officinalis* flower also included flavonoids as quercetin that is an excellent antioxidant, it also has an anti-inflammatory effect on signaling pathways, such as MAPK, NFκB, and STAT1 [8], inhibit the production of prostaglandin synthesis and cytokines such as COX-2 enzyme [9]. *Glycine max* and its bioactive compounds including isoflavonoids such as daidzein, genistein, and a higher flavonoid and total phenolic content (79 flavonoid compounds have been identified) have cosmeceutical and dermatological properties such as antioxidative, anti-inflammatory, and collagen stimulating among other [10]. However, soybean extracts play an important role in immune modulation into severe diseases including arthritis through its phytochemicals [11]. Arthritis is alleviated with avocado/soybean unsaponifiables blocking inflammatory processes related with the stimulation of connective tissue proliferation [12]. Recent studies on *Aloe vera* were focused on anti-inflammatory properties of phytochemical content like aloin is able to inhibit ROS production, cytokines, and

JAK1-STAT1/3 signaling pathway [13] and Barbaloin inhibiting the ROS-mediated PI3K/AKT/NF-κB

pathway [14]. Aloe-emodin and rhein sulfates/glucuronides inhibited MAPKs phosphorylation, iNOS expression, nitric oxide production, and pro-inflammatory cytokines [15]. While acemannan increased NF-κB/DNA binding, IL-8 and IL-6 expression in human gingival fibroblast via a toll-like receptor signaling pathway [16]. *A. vera* decrease IL-8 generation by inhibiting extracellular signal-regulated kinases pathway and p38 [17]. The purpose of this investigation was to prepare topical gel ointment containing *C. officinalis*, *G. max*, and *A. vera*, extracts to reach a synergistic anti-inflammatory effect of phytochemicals of the three plants in order to achieve alleviate rheumatoid arthritis through modulation of immune system.

Materials And Methods

Plants and preparation of extract

Voucher specimens of the above three plants *C. officinalis* (T-2356), *G. max* (T-2267), *A. vera* (T-2512), was deposited in the Herbarium of the Universidad Autónoma Metropolitan (of the CDMX (México). The plants were ground to powder and mixed in a ratio1:1:1, extracted with a hydroalcoholic solution at a concentration of 60% (v/v); treatment time was 10 min, at a frequency of 40 kHz and temperature of 30 °C in an ultrasonic bath, for the extraction of functional metabolites from polyherbal formulation (CAG).

Total polyphenolic content (TPC)

Total polyphenolic content was evaluated using the Folin–Ciocalteu assay, according to the European Pharmacopoeia [18]. Two milliliters of CAG extract were diluted 25 times with distilled water and mixed with Folin–Ciocalteu reagent (1.0 mL), which was diluted to 25.0 mL with a solution of sodium carbonate (Na_2CO_3; 290 g/L). Absorbance was measured after 30 min at 760 nm, and the results were shown as mg of gallic acid equivalent (GAE)/g dried plant material obtained from a calibration curve of gallic acid (R^2 = 0.996).

Identification of phytochemicals

LC-MS/MS was used to analyze the extracts of the plants, *C. officinalis*, *G. max, A. vera,* and *C. Longa* that was carried out on Agilent series 1290 UHPLC system consisting of a quaternary pump, autosampler, and a thermostatic column compartment (Agilent Technologies, Santa Clara, CA, USA). Phenolic profiles were achieved using a C18 analytical column (2.1 mm × 100 mm, 3.5 µm). Elution was carried out with water-formic acid (0.1%) (A) and water-acetonitrile (B) as mobile phase, starting with a gradient of B 10%–18% (0-4 min); 18%–20% B (4–9 min); and 20%–20% B (9–10 min). Chromatograms were registered at 280 nm and were established by their retention time compared with those of reference standards. MS spectra were conducted on an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Corporation, Santa Clara, CA, USA). The full scan mass covered the range from 100 up to 1500 m/z [19].

Preparation of topical Gels with CCAG/NPs

Carbopol 974 (CP) was dispersed in ethanol/water. Under stirring, it was homogenized, for 30 min. Propylene glycol and CAG extract at concentrations of 5% and 10%, were incorporated into it. Result in a good viscosity and translucent appearance then was Incorporated triethanolamine in Milli Q water and let rest for 2 hours at room temperature till completed the Carbopol hydration procedure. Paraben was used as conservative [20].

Animals

Forty-two healthy Wistar rats males 190 ± 10 g, with 7-week-old, were used in this investigation, maintained under standard controlled conditions at 60% + 5 RH, 12:12h dark and light cycle, and 24°C. They were granted free access to drinking water and fed the standard diet. The National School of Biological Sciences provided the animals for this study, were acclimatized for one week. At the beginning of the study, all rats were weighed, and blood was collected (0 days before induction of adjuvant arthritis) and analyzed in order to verify that all animals have these similar parameters. Then rats were randomly divided into 6 groups with 7 animals each. The number of animals in each group was used in order to obtain significant statistical data. In an individual standard rat cage each animal was housed. The negative group (control -) consisted of healthy rats; positive group (Control+) consisted of untreated animals. procedures in animals followed the guidelines stated in “Principles of Laboratory Animal Care” (NIH publication 85-23, revised 1985 and the Mexican Official Normativity (NOM-062-Z00-1999). The authors declare that all of the experiments comply with the guidelines stated in "Principles of Laboratory Animal Care" (NIH publications 85-23, revised 1985) and Mexican Official Normativity (NOM -062-Z00-1999). This study was approved by the Ethical Review Board for Animal Research and Ethics Committee of National School of Biological Sciences (National Polytechnic Institute). Approval number: ENCB/CEI/046/2022. All the methods used in this study are incompliance with ARRIVE guideline

Adjuvant arthritis (AA)

Arthritis was induced in Wistar rats by a single injection of 100 µl Freund’s Adjuvant complete (CFA) (Sigma-Aldrich, St. Lois MA.USA) containing heat-killed *Mycobacterium tuberculosis* (10 mg/ml), 0.15ml mannitol, and monooleate in 0.85ml liquid paraffin was injected in the sub plantar region of the left hind paw to each animal. Injection of oil-based adjuvant on hind paw of the rats trigger redness and swelling in joints and paw in induced arthritis animal development inflammation. Severe arthritis initiates in the interval of 12 to 14 days and persists until 20 to 23 days [21]. Then grouping and classifying rats in agreement to their weight, each animal in the tail was marked with a number and located in an individual cage with a number recognizing the cages. By this research, 42 male rats were assigned into 7 groups (6 animals each). Group I- Negative control (control -), Group II-AA untreated animals (control +); Group III-AA + BAS (ointment base); Group IV- AA + CAG /gel 5%); Group V- AA + CAG /gel 10%); Group VI- AA +diclofenac sodium (20mg/kg; standard control). From day 0 to 12, AA rodents were treated once a day, just like the normal and CAG /gel group at the same time. The paw volume was measure with a Plethysmometer (SD Instruments, San Diego, CA. USA). Hind paws oedema index was evaluated after sacrifice using anesthesia (pentobarbital sodium, 40 mg/kg, i.p.) which indicated the degree of disease

recovery. The hind paw of each rat was dissected and weighed. The arthritis Index was reported as the ratio (mg/g) of hind paw weight versus body weight [22]. The arthritis severity was evaluated every four days by visual observation, using a score of 0–4 according to the following scale: Grade 0 = No arthritis in the paw; Grade 1 = Swelling and redness in the paw; Grade 2 = Malformation of the paw; Grade 3 = Incapacity to use the paw; Grade 4 = Distortion of the joint, dysfunction or bursting of the skin [23]. On the 21st day, animals were sacrificed using anesthesia (pentobarbital sodium, 40 mg/kg, i.p.) and blood was collected from the posterior vena cava through a laparotomy incision and centrifuged after of collection at 4000 rpm for 15 minutes at 4°C. Serum was frozen at -40°C before proceeding to the analysis. IL-6, TNF-α, and NF-κB, levels were assayed using enzyme-linked immunosorbent assays (ELISA, Invitrogen Corporation, CA, USA) according to the manufacturer's instructions.

Preparation of cell-free extract of the knee joints

Knee joints were removed from arthritic and non-arthritic rats and homogenized in 5 vol of 50 mM Tris HCl buffer, pH 7.4. The homogenate was centrifuged at 3000× g for 5 min, and the resulting supernatant was used for the estimation by immune markers. NF-κB, was assessed by rat ELISA-based kit (ELISA, Invitrogen Corporation, CA, USA). The test was performed as per the instructions of the manufacturer.

Cells

Murine macrophage cells line RAW264.7 were provided by ATCC The cells were cultured in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% FBS, streptomycin (50 U/mL) and 1% penicillin (50 U/mL) in a 5% CO2 atmosphere at 37 ◦C.

Cell Viability

In 96-well plates RAW 264.7 cells were seeded at a cellular density of 4×10^4 cells/well in DMEM, then were incubated for 48 h till 80% confluency. Then, the cells were treated with various concentrations of CAG/gel for 24 h, at a concentration of 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg/mL. DMSO (0.1%) served as a negative control, and cells without treatment were used as the blank control. After cells were incubated with 5 mg/mL tetrazolium 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) solution for 4 h at 37 °C and 5% CO2. After the formation of the purple formazan crystals, DMEM was replaced by 200 µL of dimethyl sulfoxide (DMSO) to dissolve crystals, and the absorbance was determined at 500 nm using an ELISA reader (TECAN Infinite 200 Pro, Switzerland) [23].

In vitro anti-inflammatory effect

RAW 264.7 cells were seeded a cellular density of 4×10^4 cells/well in 96 well plates in DMEM and were incubated until 80% confluency for 48 h. Then, was added to induce inflammation, LPS at 0.1 µg/mL, and plates were incubated another 3 h. After examples were added at a concentration of 100 µg/mL, evaluated by colorimetric assay MTT and incubated for 24 h with the treatments, cell supernatants were collected. Cells were detached and counted. Production of NO, iNOS, PGE2 and inflammatory cytokines

TNFα , IL-1β, iL-6 were quantified using assay kit (BD Biosciences, USA) according to the manufacturer's instructions [24].

T and B lymphocytes proliferation test

CD3+ T cells and CD19+ B cells were separated from C57BL/6 mice spleen cell suspension by magnetic beads [20]. In 96-well plates was inoculated CD3+ T lymphocytes and after were treated with various concentrations of CAG/gel of 0.1, 0.3, and 1.0 c/(mg·mL^{-1}) in a medium. The control group was treated only with DMSO at 0.5% V/V. After to promote T cell receptors (TCRs) to stimulate the proliferation of T lymphocytes was administered CD3/28 antibodies (2 μg·mL^{-1}, Sigma-Aldrich, St Louis MA. USA). Then 5 μL of CCK-8 solution (Beyotime Biotechnology) was added after 48 h in culture to each well and incubate at 37 °C/5% CO2 for 4 h. Methotrexate (0.1, 0.3, and 1.0 mg·mL^{-1}) was used as positive control. The absorbance was measured at 450 nm. All values were corrected by cell-free controls [25].

B lymphocytes proliferation was determined with the same experiment used in the T lymphocytes studies. To stimulate CD19+ B lymphocytes proliferation was used lipopolysaccharide (LPS) at a final concentration of 1 μg·mL^{-1}.

Statistical Analysis

The data were expressed as Mean+ SD. One-way ANOVA followed by either Sidak's or Dunnett's test was used to determine statistical significance between means. The criteria for significance were $p < 0.05$. The results of the arthritis index and cytokines levels were statistically analyzed using SPSS 15.0 and compared among groups.

Results

Phytochemicals

The results of LC-MS/MS analysis used to identify phenolic compounds are shown in Fig 1 and through comparison with reference compounds and determining UV/vis spectrum these were identified. In investigating the phytochemical composition of the three plants studied contains the following phenolic compounds: Chlorogenic acid (1), Protocatechuic acid (2), Genistein (3) Cinnamic acid (4), Isovitexin (5), Vanillic acid (6), p-coumaric acid (7), Syringic acid (8), 5-feruloylquinic acid (9), Caffeic acid (10), Glycitin (11), Isoquercetrin (12), Glycitein (13), Ferulic acid (14), Daidzin (15), Cinnamaldehyde (16), Daidzein (17) (Table 1). The results of TPC shows highest values 510.28 mg GAE/g (Table 1).

Table 1. Identification and characterization of the polyphenols from the combination of *Calendula officinalis*, *Glycine max, and Aloe vera*

Total phenol content 510.28 mg GAE 100 g-1 DW			
Characterization			
Compound	RT min	λmax (nm)	$[M+H]^+$ *m/z* (ESI-MS: positive ion)
Caffeic acid	26.5	210, 240, 325	181, 163, 145, 135
Cinnamic acid	14.1	203, 215, 273	147, 125, 109
Cinnamaldehyde	40.3	241, 300	133, 132, 104, 103, 77, 61
Chlorogenic acid	5.8	219, 241, 331	353, 191, 161
p-coumaric acid	20.4	212, 283	165, 147, 133, 119, 91
Daidzein	42.6	248,303, 327	255, 227, 153, 137, 119, 91
Daidzin	34.1	250, 302	417, 254, 223, 195
5-feruloylquinic acid	20.9	302, 325	367, 191, 135
Ferulic acid	33.5	235,322	193, 177, 148, 133
Genistein	14.9	259, 327	272, 271, 243, 215, 153
Glycitein	30.6	280, 319	285, 266, 245
Glycitin	28.1	256, 321	447, 382, 340, 322, 307, 200
Isoquercetrin	29.3	262, 358	465, 305, 304, 303, 85
Isovitexin	15.8	270, 334	431, 341, 311
Protocatechuic acid	7.8	242, 294	154, 138, 109, 81
Syringic acid	19.2	275	198, 183, 127
Vanillic acid	17.6	259, 252	312, 297, 282, 223, 193, 166, 151, 125, 107

Effect of CAG/gel on paw swelling and TNF-α, IL-6, NF-κB, TGF-β levels in serum

Exposition to CFA, induced primary arthritis in the left hind paw of diabetic animals with a significant swelling in the paw and remains for 21 days compared to control. Table 2 indicated the secondary lesions of arthritis studied in the right hind paw, although it was not significant as secondary arthritis. As shown in Table 2, animals treated with CAG/gel showed a significant increase in arthritis index as compared to the control. From the 12th day to the 21nd day, the CAG/gel group and the diclofenac group showed statistically significant differences (p<0.05) as compared to the model group. Administration of

CAG/gel markedly inhibits paw swelling from 5th to 21st day when compared to the arthritis control group. Results were like those of the diclofenac used as a drug reference.

Table 2. Effect of CAG/gel on arthritis Index in CFA induced arthritis in rats

Number	days	Control	AA control	CAG/gel	Diclofenac
0	0	0	0	0	
1	0	2.5±0.32	2.0±0.24	2.1±0.30	
5	0	2.7±0.44	1.8±0.12	1.9±0.27	
12	0	2.8±0.51	1.3±020*	1.2±0.22*	
16	0	2.2±0.29	1.1±0.31*	1.0±0.24*	
21	0	1.9±0.46	1.0±0.35*	1.0±0.0.33*	

In each group (means ± SD), Arthritis index (AI) from the 1st day to the 21nd days. On the 21nd day,

the CAG/gel group showed a significant difference (*$p < 0.05$) as compared with the AA group.

Treatment of CAG/gel, a strong inhibition of paw volume was observed in CFA-induced-arthritis model (Table 3). According to results the therapeutic effect of CAG/gel was to those in the diclofenac used as standard control. Findings indicated that CAG/gel could enhance AA.

Table 3. Effect of CAG/gel on paw volume (mm^3) in CFA induced arthritis in rats

Number	days	Control	AA control	CAG/gel	Diclofenac
0	1100	0	0	0	
4	1100	1900±21.5	1600±18.9	1650±26.7	
8	1100	2400±19.4	1400± 23.8*	1500±24.5**	
12	1100	2700±32.1	1300 ±19.7**	1450±35.6 **	
16	1100	2450±29.9	1200±32.9*	1300±0.24*	
21	1100	2400± 34.6	1150±31.6**	1200±0.0.33*	

Paw volume for each group is presented as the means ± SD, from the 1st day to the 21nd days. The significance of differences *p < 0.05; **p < 0.01 as compared with the AA group.

The ELISA data shown in Fig 2A-D, TNF-α, IL-6, NF-κB, TGF-β levels in the model group were significantly higher than those in the control group. After administration of CAG, CAG/gel and diclofenac on 21nd day,

had lower levels compared to the disease control (p <0.05). However, in the group III with gel treatment had no significant effect on TNF-α, IL-6, NF-κB, TGF-β levels compared to the disease control.

Effect of CAG, CAG/gel, and diclofenac on cytokine in joint

IL-6, IL-1β, IL-10, NF-κB, and TNF-α have an important role in inflammation and rheumatoid arthritis (Fig 3A-E). Immunization with CFA significantly (p<0.05) increased the cytokine inflammatory IL-6, IL-1β, NF-κB, and TNF-α and reduce cytokine anti-inflammatory IL-10 (Fig 3C). However, administration of CAG, CAG/gel, and diclofenac used as positive control significantly reduced these levels in the IV, V, and VI groups to those in the disease control The treatment restored the levels of cytokine anti-inflammatory IL-10 in CAG, CAG/gel, and diclofenac treatment in IV, V, and VI groups to those in the disease control. Thus, group III rats did not show a significant Inhibition in these cytokines compared to the disease control.

Effects of CAG, CAG/gel, on cell viability in RAW 264.7 cells

By the MTT assay were analyzed the effects of CAG, CAG/gel on cell viability in RAW 264.7 without LPS and incubating cells for 24 h. As shown in Fig 4A-B at the concentrations of CAG, CAG/gel of 0.625–40 μg/mL, the RAW 264.7 cells viability were not decreased within 24 of incubation. Results indicated that CAG, and CAG/gel had no cytotoxic effect on RAW 264.7 cells at the concentrations of 0.625–40 μg/mL

Effect of CAG/gel on NO, iNOS, and PGE2 production in LPS-stimulated RAW264.7 cells

The amounts of nitrite, a stable metabolite of NO, and iNOS, and PGE2 production are showed in Fig 4C-E in LPS-activated RAW 264.7. Cells NO, iNOS and PGE2 production were increased significantly after LPS treatment. Exposure to CAG/gel at concentrations of 5-40 μg/mL inhibited NO, iNOS, and PGE2 expressions.

Effect of CAG/gel on cytokines TNFα , IL-1β, and iL-6

Our experimental evidence showed that, after LP treatment, the expression of TNF-α, IL-1β, and IL-6 levels were significantly increased in the LPS-treated cells those in the untreated cells (Figure 5 A-C). Nevertheless, the levels of TNF-α, IL-1β, and IL-6 were significantly inhibited in the cells exposed to CAG/gel in a dose-dependent manner (Fig 5 A-C). Findings indicated that CAG/gel significantly reduced the production of TNF-α, IL-1β, and IL-6 levels.

T and B cell proliferation

By the he stimulation of CD3/28 antibodies assay were analyzed the effects of CAG, CAG/gel on T and B cell proliferation. As indicated the Fig 6 A-B, CAG, and CAG/gel inhibited significantly (p<0.05) T/B lymphocyte proliferation to those of the positive control (methotrexate).

Discussion

The anti-inflammatory effects of these plants can be attributed to the content of phenolic compounds since, in numerous studies, they have been investigated for their anti-inflammatory potential, including Chlorogenic acid is able to inhibit the LPS triggered IL-1β and TNF-α generation [26]. Protocatechuic acid reduced invasion, migration, and cell proliferation this effect was mediated by the inhibition of NF-jB and Akt/mTOR signalling pathways [27]. Genistein possesses anti-angiogenesis, anti-inflammatory, antiproliferative, and joint protection properties [28]. Cinnamic acid is a lipoxygenase LO inhibitor and interferes in the formation of leukotrienes [29]. Isovitexin enhances the regulation of TLR4/MyD88/NF-jB inflammatory response signal transduction pathway [30]. Vanillic acid affecting I-κBα phosphorylation in the NF-κB signaling pathway reducing inflammation showed an increasing non-collagenous and collagenous matrix for regeneration of cartilage [31]. *p*-coumaric acid exerts the anti-inflammatory properties such as inhibition of inflammatory cell infiltration, levels of TNF-α and IL-6 by via p-CA, reducing the symptoms of arthritis in rats [32]. Caffeic acid inhibit anti-angiogenic, inflammatory, anti-apoptotic effect for the amelioration of Adjuvant-induced arthritis [33]. Glycitin has an anti-inflammatory effect involving the NF-κB signaling pathway that improved joint degenerative diseases [34]. Ferulic acid improved rheumatoid factor (RF), C-reactive protein (CRP), reduce the secretion of TNF- α, and TGF-β levels increased JAK2 levels [35]. Cinnamaldehyde has anti-inflammatory properties via suppressing proliferation and metastasis of RA-FLSs by blockage of PI3K/AKT signaling pathway [36]. Daidzein suppressed MMP-13 and TNF-α, IL-1β, in both knee-joints and serum [37]. Total polyphenolic content was 510.28 mg GAE 100 g-1 DW (Table 1). The present study confirmed the anti-arthritis properties of the phytochemicals identified in the CAG extract.

In the present investigation, *C. officinalis, A. vera* and *G. max* combination was evaluated for their preventive and benefices properties in CFA -induced rheumatoid arthritis in rats, thought of inflammatory and oxidative processes. Enhance in the swelling of the paw is associated with the anti-arthritic effect of several drugs. The monitoring of swelling in the paw is easy and a reliable test for determining the degree of inflammation. The rheumatoid arthritis is considered a chronic inflammatory state, as a consequence of joint capsule and ligament swelling generating an accumulation of monocytes and granulocytes increased activation of macrophages, producing several cytokines, including TNF-α, IL-6, NF-κB, and TGF-β associated with rheumatoid arthritis [38].

The treated group with CFA showed a significant increase in arthritis index compared to the control with a maximum value on the 5th day followed by a gradual reduction. Treatment with CAG, CAG/gel and diclofenac significantly reduced the arthritis index compared to the arthritis control rats. The use of commercial anti-rheumatic drugs produces a systemic distribution that requires higher doses to keep an effective drug level at the joint site, and it is related to serious side effects [39], for this we are developing a topical application of gel for the treatment of rheumatoid arthritis herbal formulation was used containing the combination of C. *officinalis*, *G. max,* and *A. vera,* extract. Gel can reach drug delivery at the site of inflammation and increase the drug level at the lesion, improving the therapeutic effect.

CFA-induced paw edema inflammatory model is mostly used for obtaining chronic 21-day inflammation and, as a factor of evaluating inflammation is paw swelling, widely used for determining the anti-arthritic

activity of CAG. The inflammatory process in the 5th to 21st day includes an increase in a reduction in motility, paw swelling, and arthritis index, representing the post-acute stage of inflammation. These parameters became normal after 21 days, indicating the pass to the late phase of inflammation. In this study, CAG/gel induce a marked inhibition in paw swelling and the arthritis index from the 5th day to the 21st day of administration as compared to the arthritis control group. Results confirm that CAG/gel inhibit TNF-α, IL-6, NF-κB, and TGF-β levels Thus, CAG, and CAG/gel reduce paw swelling, inhibit the inflammatory cascade, decrease arthritic edema in the lateral paw of the rats, and prevents the generalization of the inflammation from the lateral paw to the contralateral, indicating the protective effect of the NPs in reducing systemic generalization.

The treatment with CAG and CAG/gel exhibited a reduction in joints by interfering with the inflammatory cytokines, indicating its anti-inflammatory effect in CFA-induced rheumatoid arthritis. Elevated levels of NF-κB, were suppressed by CAG, and CAG/gel inhibiting the joint inflammation due to its ability to impede its binding to DNA and inhibit the transfer of NF-κB, and suppressed the stimulation of other inflammatory cytokines including COX, IL-6, TNF-α, enzyme nitric oxide synthase IL-1β. Activation of Tumor necrosis factor-alpha (TNF-α), activates the generation of other inflammatory markers (IL-6, TGF-β, and IL-1β) facilitating vasodilatation and infiltration of leukocytes at the site of disease [40] mobilize monocytes and neutrophils toward the joint through stimulation of chemokines and activate matrix metalloproteinase (MMPs) [41], IL-10 is the immune-regulatory cytokine, that avoids damage to cartilage and bone in rheumatoid arthritis by regulating the stimulation of NF-κB, TNF-α, and IL-1 [42]. Finding demonstrated that CAG, and CAG/gel exhibited a significant decrease in the levels of IL-1β TNF-α, IL-6 and prevents NF-κB activation and increase IL-10 level improving the integrity of joint in the rheumatoid arthritis.

Macrophages are central cells implicated in the inflammatory process, participating in the antigen presentation, phagocytosis secretion and initiates the generation of inflammatory mediators in macrophages regulating the inflammatory response. LPS can activate macrophages producing a big number of inflammatory cytokines [43].

In addition, in this investigation determined the potential anti-inflammatory of CAG and CAG/gel in LPS-activated RAW 264.7 cells as model system. Findings, showed that the polyphenolic-rich extract

no cytotoxic effect in RAW 264.7 cells. CAG/gel showed significant anti-inflammatory effect by reducing the generation of inflammatory mediators in LPS-activated RAW 264.7 cells.

Excessive NO trigger detrimental results including carcinogenesis and chronic inflammation. However, NO has an important role in the development of inflammation [44]. Activated macrophages transcriptionally express iNOS, are liable for the excessive and prolonged generation of NO [45]. Experimental evidence showed that CAG/gel is responsible of inhibition of iNOS protein, consequently, might inhibited the synthesis of NO in LPS-activated RAW264.7 cells then CAG/gel treatment decreasing the NO production showing a very good anti-inflammatory effect. PGE2 is an inflammatory cytokine involved in inflammatory responses [46]. LPS significantly increase the production of PGE2, thus CAG/gel can reduce

PGE2 release induced by LPS, in dose-dependent. PGE2 is an essential mediator of inflammatory process formed through the activity of the COX-2 enzyme.

Macrophages, stimulated by LPS, could release a large number of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, involved in several immunological processes including [47]. The pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, has important role in the inflammatory response, such as induction of the acute-phase response and activation of the leukocytes [48]. Consequently, these pro-inflammatory cytokines have been used as targets to evaluate anti-inflammatory treatment. Finding indicated that CAG/gel showed significant anti-inflammatory effect in LPS-stimulated RAW264.7 macrophages by reducing the generation of LPS-induced pro-inflammatory mediators such as NO, iNOS, PGE2, TNF-α and IL-6. According to our results inhibition of iNOS protein levels indicated that CAG/gel could regulate inflammatory effects through inhibiting the iNOS pathway A combination of *Calendula officinalis*, *Glycine max, Aloe vera* extract (CAG), was developed. CAG/gel were investigated on CFA, induced rheumatoid arthritis, and knee joints. The encapsulated CAG regulates the levels of proinflammatory cytokines and inflammatory mediators. Results reveal the potent effect of CAG/gel to reduce inflammation and joint destruction simultaneously. Therefore, this gel ointment may attenuate inflammation and cartilage degeneration in rheumatoid arthritis.

Considering that abnormal proliferation of T/B lymphocytes plays an important role in the pathogenesis of rheumatoid arthritis [49]. (investigated for CAG and CAG/gel ability to inhibit proliferation of T/B lymphocytes. Finding of CAG/gel on T/B lymphocyte proliferation assay together with the experiments of serum cytokine test suggested the mechanism of anti-rheumatoid arthritis activities of CAG/gel may be associated to the inhibition of pro-inflammatory cytokines and consequently its immune effect [50].

Conclusions

Our Findings demonstrated that CAG/gel had an anti-inflammatory effect in CFA, induced rheumatoid arthritis, and knee joints in rats regulating the levels of proinflammatory cytokines and inflammatory mediators such as COX, TNF-α, IL-1, IL-6, NF-κB, TGF-β, MMPs and IL-10 levels. Therefore, this herbal formulation may attenuate inflammation and cartilage degeneration in rheumatoid arthritis. CAG/gel ointment downregulated inflammatory mediators in LPS-stimulated RAW 264.7 cells by attenuating the generation of NO, iNOS, PGE2, TNF-α and IL-6 thus modulating the pathway. CAG/gel inhibit T/B lymphocyte proliferation assay indicated that the mechanism of anti-rheumatoid arthritis associated to decrease of inflammatory mediators and pro-inflammatory cytokines to its immune function. Nevertheless, findings indicate that CAG/gel ointment could be an effective agent for the therapy of inflammatory conditions including rheumatoid arthritis.

Abbreviations

CAG herbal formulation

CAG/gel herbal formulation in gel

CAT catalase

DMEM Dulbecco's modified Eagle's

GPx glutathione peroxidase

HPLC high performance liquid chromatography

iNOS inducible nitric oxide synthase

IL-6 interleukin-6

IL-8 interleukin-8

IL-1β interleukin 1β

NO nitric oxide

NF-κB nuclear factor-kappa B

ROS reactive oxygen species

(TNF)-α tumor necrosis factor

Declarations

Acknowledgements

The authors would like to thank Biol. Aurora Chimal of department of Botany of National School of Biological Sciences for identification of plant.

Authors' contributions

R.M.P.G.: Conceptualization, Supervision, Reviewing and Editing and J.T.P.: Original draft preparation, Methodology, Investigation, Writing. The author(s) read and approved the final manuscript.

Funding

This article was financially supported by National Polytechnic Institute (grant ID = 20220199). The funder had no role in the design of the study, data collection, analysis, interpretation of the data, and writing of the manuscript.

Availability of data and materials

All the data supporting the results of this investigation are available within the article

Ethics approval and consent to participate

The authors declare that all of the experiments comply with the guidelines stated in "Principles of Laboratory Animal Care" (NIH publications 85-23, revised 1985) and Mexican Official Normativity (NOM -062-Z00-1999). This study was approved by the Ethical Review Board for Animal Research and Ethics Committee of National School of Biological Sciences (National Polytechnic Institute). Approval number: ENCB/CEI/046/2022. All the methods used in this study are incompliance with ARRIVE guideline

Consent for publication

Not applicable

Competing interests

All authors declared no conflicting interests and the budget for the investigation was managed from the usual department research provided by the National Polytechnic Institute of México

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Figures

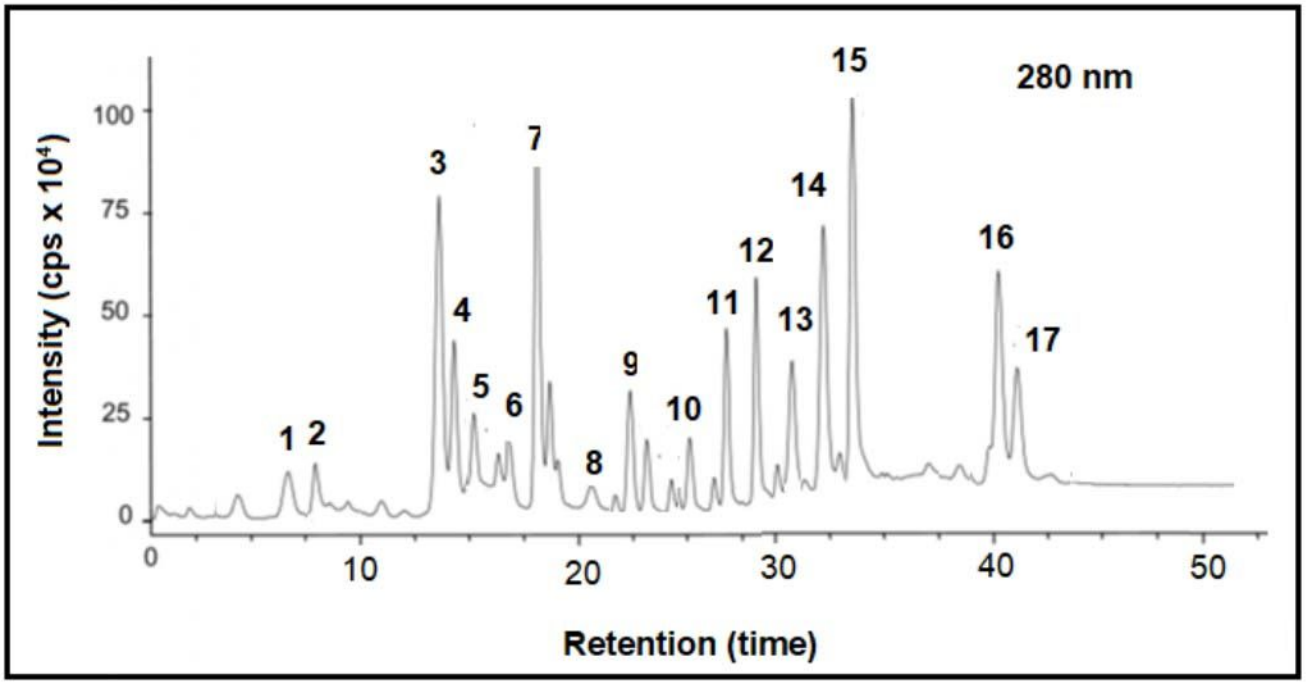


Figure 1

HPLC chromatogram of Phenolic compounds of the hydroalcoholic extract: Chlorogenic acid (1), Protocatechuic acid (2), Genistein (3) Cinnamic acid (4), Isovitexin (5), Vanillic acid (6), p-coumaric acid (7), Syringic acid (8), 5-feruloylquinic acid (9), Caffeic acid (10), Glycitin (11), Isoquercetrin (12), Glycitein (13), Ferulic acid (14), Daidzin (15), Cinnamaldehyde (16), Daidzein (17)

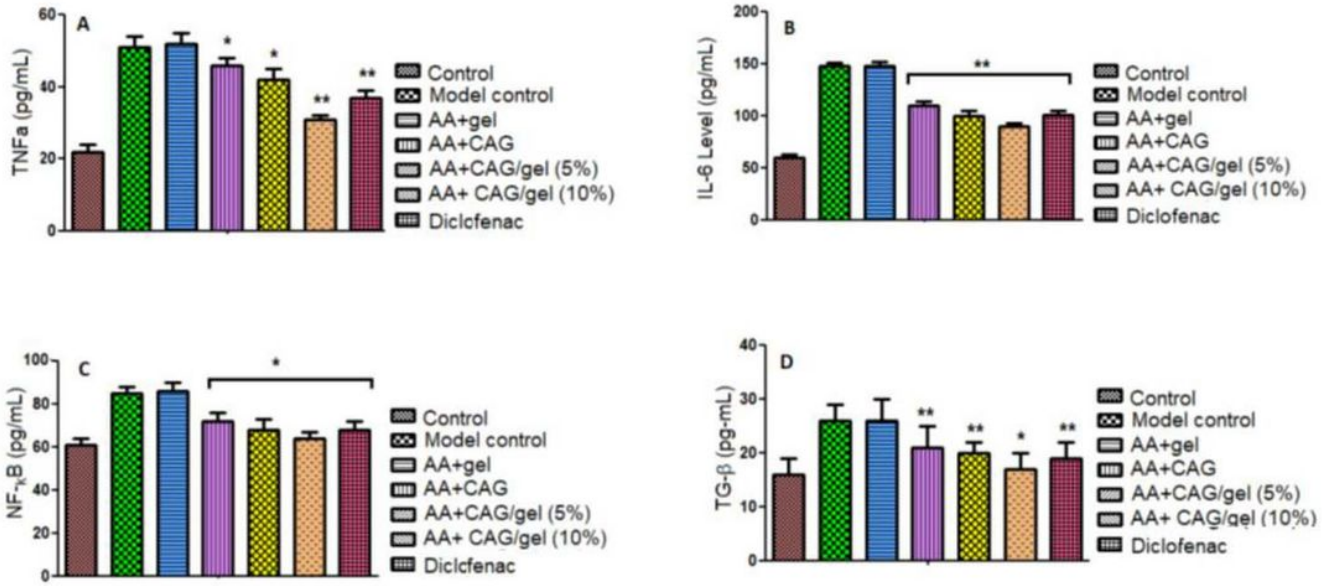


Figure 2

Serum levels of TNF-α (A); IL-6 (B); NF-κB (C); TGF-β (D). Values are presented as mean ± SD; animals in each group (n = 5) on the 21th day. Statistically significant differences (*p < 0.05, **p < 0.01) as compared with the model group. IL-6, NF-κB, TGF-β and TNF-α were measured as pg/g protein.

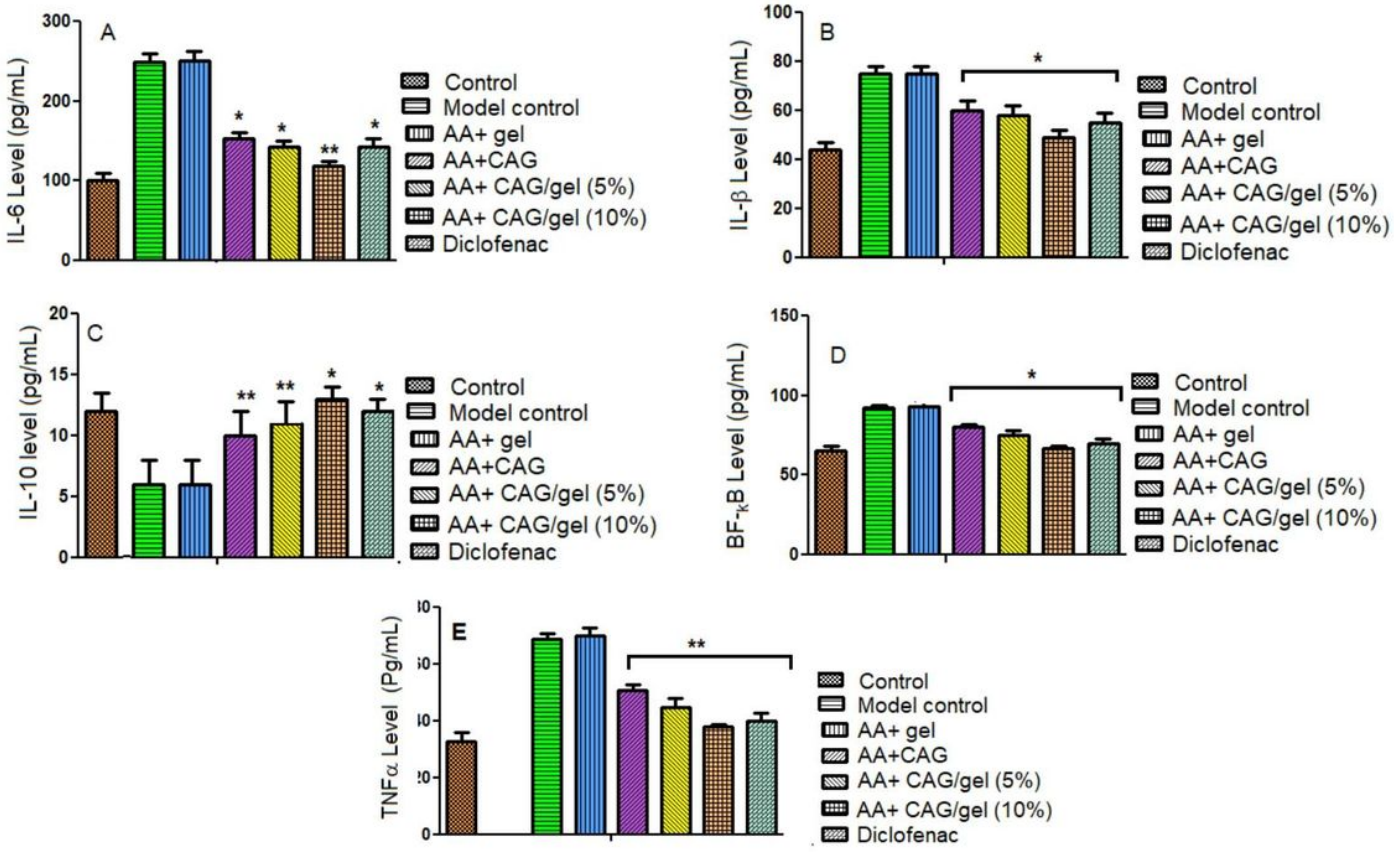


Figure 3

Effect of CAG, and CAG/gel on cytokine in joint. Values are presented as mean ± SD; animals in each group (n = 5). IL-6 (A); IL-1β (B); IL-10 (C); NF-κB (D); TNFα Level (E). *p < 0.05, **p < 0.01, compared with the LPS group, # p < 0.001, compared with the control group.

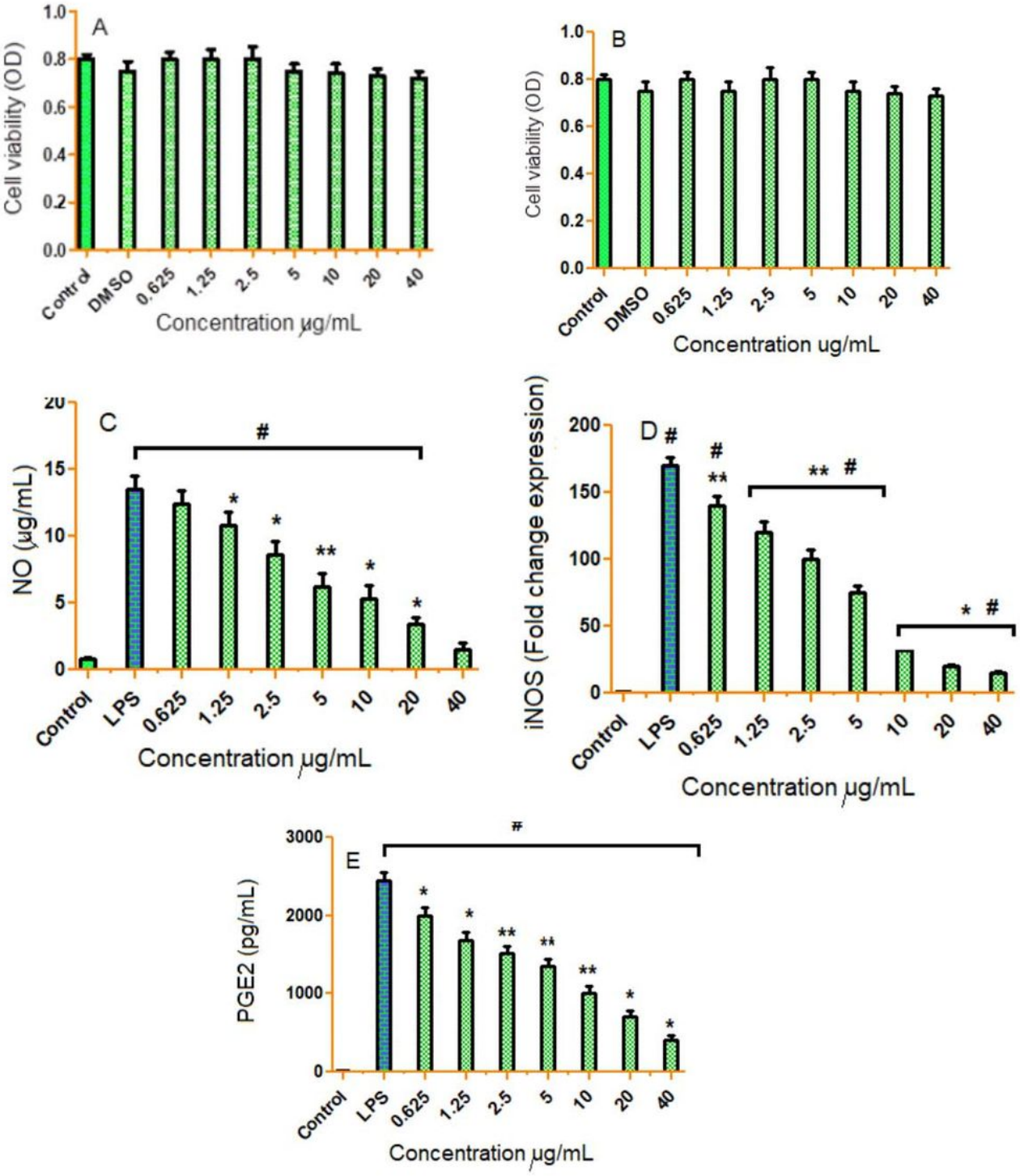


Figure 4

The results are expressed as the means ± SD (n = 3). (A) cells viability of CAG; (B) cells viability of CAG/gel were determined by MTT assay; Effect of CAG/gel on NO production (C); INOS expression (D); PGE2 (E) production in LPS-activated RAW 264.7 cells. *p < 0.05, **p < 0.01, compared with the LPS group, # p < 0.001, compared with the control group.

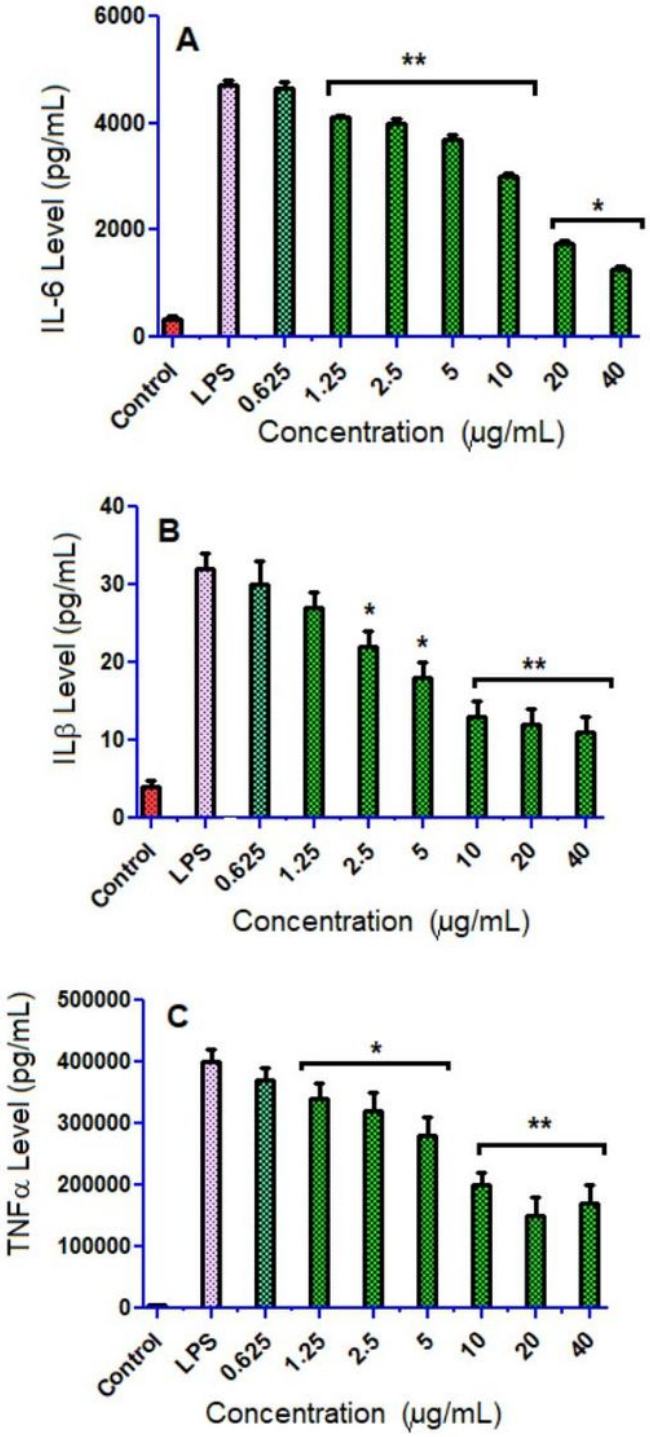


Figure 5

Effects of CAG/gel in in LPS-induced RAW 264.7 cells. (A) interleukin- (IL-) 6; IL-1β (B); TNF-α (C); Three independent assays were carried out , and the data are presented as the mean ± DS. ∗P < 0:05 and ∗∗P < 0:01 compared to the cells treated with LPS alone.

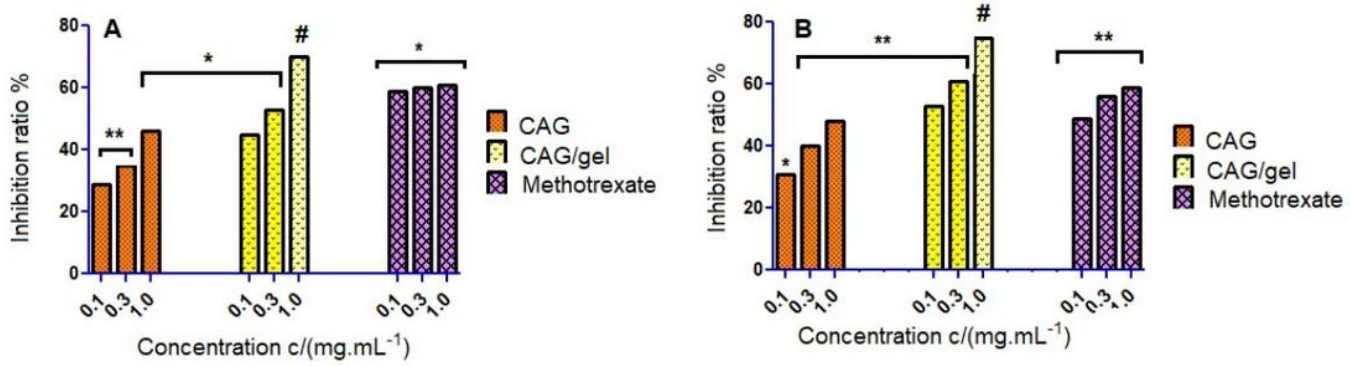


Figure 6

T/B lymphocytes proliferation assay. T lymphocytes proliferation; GAG extract; CAG/gel; and methotrexate (A). B lymphocytes proliferation; GAG extract; CAG/gel; and methotrexate (B).Statistically significant differences (*p < 0.05, **p < 0.01, # p < 0.001) as compared with CD3/28 antibodies induced proliferation.