**Original Paper**

Beneficial anti-inflammatory and anti-arthritic activities of Grape seed proanthocyanidin extract in Freund's Complete Adjuvant-induced Rheumatoid arthritis in rats

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disorder with massive suffering and permanent joint destruction. The Grape seed proanthocyanidin extract (GSPE) beneficial effects were evaluated in adjuvant-induced arthritis (AIA). RA was induced by 0.1 mL Freund's complete adjuvant (CFA) that was injected once into the rat's right hind leg footpads. Thirty male rats were equally divided into 5 groups. Group I (Normal control): Rats received no drugs, Group II (CFA- induced arthritis for treatment): Rats injected with 0.1 ml CFA on the 1st day and sacrificed after 29 days of CFA injection, Group III (CFA + GSPE treated): After 14 days of CFA injection, rats treated with GSPE (100 mg·kg⁻¹ b wt./day) for 15 sequential days. Group IV (CFA- induced arthritis for protection): Rats injected with 0.1 ml CFA on 15th day of the experiment and sacrificed 15 days later after arthritis induction. Group V (CFA + GSPE protected): Rats pretreated with GSPE (100 mg·kg⁻¹ b wt./day) one day before CFA injection and 14 days later after arthritis induction. A significant up-regulation in IL-1 β , MMP-3 and LOX-1 gene expressions were observed in the arthritic rats' synovial membrane. However, significant down-regulations in all previous parameters were observed in GSPE treated groups. Also, GSPE significantly activated Nrf2 gene in the liver with marked up-regulation of Lactoferrin (LF) gene in arthritic rats' synovial membrane. Interestingly, improvement of histopathological alterations in synovium and stifle joint capsule of arthritic rats supported the anti-arthritic effects of GSPE. Conclusively, GSPE ameliorates adjuvant induced arthritis via its anti-arthritic and joint protecting properties.

1. INTRODUCTION

Rheumatoid arthritis (RA) has both articular and systemic effects (McInnes and Schett, 2011), where cytokine imbalance is a pivotal factor in the pathogenesis and progression of this disease. Several experimental models were developed in rats to evaluate the antirheumatic drugs potential benefits (Snehalatha et al., 2013). The model of complete Freund's adjuvant (CFA)-induced rat arthritis is clinically, immunologically, and pathologically similar to human RA (Kim and Moudgil, 2009). It is marked with bone resorption and proliferation of periosteal bones (Ahmed et al., 2015). Li et al., (2018), recorded a marked increase in the level of tumor necrosis factor alpha (TNF- α), nuclear factor kappa B (NF- κ B), interleukin-1 β (IL-1 β) and nuclear factor erythroid 2-related factor 2 (Nrf2) in the arthritic rats. Also, a significant amplification in matrix metalloproteinase-3 (MMP-3) gene expression was detected by Aniss et al., (2020).

Currently, 80% of the world population utilizes the plant-based medications for successful RA pain relief (Sahoo et al., 2010). GSPE, which belongs to a group of flavonoids, is produced from *Vitis vinifera* (red grapes) that contains

95% of natural proanthocyanidin potent antioxidants (Wren et al., 2002; Wei et al., 2018). GSPE has an anti-arthritic effect in Adjuvant-Induced Arthritis (AIA) model by modifying T cell subsets (Ahmad et al., 2013). Moreover, it activates the Nrf2 pathway (Sun et al., 2016), while inhibiting proinflammatory mediators including TNF- α , IL-1 β (Rajput et al., 2019), transforming growth factor β 1 (TGF- β 1) (Zhou et al., 2015) and MMP-3 (Khaddam et al., 2014) and the proteolytic enzymes, supporting the GSPE suppressive effects in osteoclasts (Kwak et al., 2020). Accordingly, this study aims to evaluate the anti-arthritic properties and potential protective and therapeutic effect of Grape seed proanthocyanidin extract (GSPE) in Freund's Complete adjuvant-induced rheumatoid arthritis in rats through determination of the mRNA gene expression levels of IL-1 β , MMP-3, LOX-1, LF and Nrf2 in arthritic rats synovial membrane.

2. MATERIAL AND METHODS**2.1. Experimental animals:**

Thirty white albino male rats, 4–5 weeks old with average weight of 150–200 g were used in this study. Rats were

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handled according to the suggested National Ethical Guidelines for the care of Laboratory Animals, as the Animal Ethics Committee of Faculty of Veterinary Medicine, Benha University, Egypt. The animals were left for 2 weeks for acclimatization before the beginning of the experiment.

2.2. Chemicals and antioxidant agent:

The antioxidant and chemicals used in the present study were

2.2.1 Freund's complete adjuvant

Freund's complete adjuvant (CFA) was bought from SIGMA Chemical Company (USA) containing 10mg/ml of heat-killed *M. tuberculosis* (10mg/ml). It was injected once subcutaneously (0.1 mL CFA) into the right hind leg footpads of rats (Nasuti et al., 2019).

2.2.2 Grape Seed Proanthocyanidin Extract (GSPE):

Grape Seed Proanthocyanidin Extract was purchased from (Al Debeiky Pharma Company for Pharmaceutical industries, Al Obour, Cairo, Egypt). GSPE was dissolved in 100% Dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration by sterilized saline solution then administered orally in a dose of (100 mg kg⁻¹ b wt./day) (Ahmad et al., 2013).

2.3. Experimental design

The rats were randomly divided into five major equal groups of six rats each placed in individual cages and graded as follows:

Group I: Normal control group: Rats received no drugs, provided with ordinary diet for 29 successive days. They served as an untreated control group for all experimental groups.

Group II: CFA- induced arthritis for treatment: AIA was induced in rats on the first day by a single subcutaneous injection of 0.1 ml of CFA in right hind leg footpad of rats, which were sacrificed 29 days later after CFA injection.

Group III: (CFA + GSPE treated): Rats received GSPE (100 mg·kg⁻¹ b wt./day, orally) for 15 successive days, after 14 days of CFA injection and were then sacrificed.

Group IV: (CFA- induced arthritis for protection): Rats injected with 0.1 ml CFA in right hind leg footpad on the 15th day of the experiment and were sacrificed 15 days later after arthritis induction.

Group V: (CFA + GSPE protected): Rats were pretreated with GSPE (100 mg kg⁻¹ b wt./day) one day prior to CFA injection, on the 14th day, followed by 15 days later after arthritis induction, then were sacrificed at (30th day).

2.4. Sampling (Livers and stifle joints)

At the end of the trial, rats were anesthetized and sacrificed by cervical decapitation according to Animal Ethics Committees, stifle joints and livers tissues were collected once from all rat groups on 30th day of the experiment.

The livers and stifle joints of all rat groups were removed and cleaned by rinsing with cold saline, put in Eppendorf tubes and immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for the molecular analysis of nuclear factor erythroid 2-related factor 2 (Nrf2) gene expression in liver tissue and interleukin-1β (IL-1β), matrix metalloproteinase 3 (MMP-3), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and lactoferrin (LF) in synovial membrane of stifle joints by reverse transcription polymerase chain reaction (RT-PCR).

Moreover, rats stifle joint were taken and immediately fixed in 10% neutral formalin solution, the washed samples were dehydrated by using graded ascending concentrations of ethyl alcohol starting with 50% and ending with absolute alcohol, then blocked in hard paraffin and cut into sections of approximately 5 microns thick. Paraffin was removed from the sections that were subsequently stained with Harris hematoxylin and eosin for histopathology examination according to the technique described by (Bancroft and Gamble technique, 2008).

2.5. Molecular analysis

The mRNA expressions content of Nrf2 in rat livers and IL-1β, MMP-3, LOX-1 and LF in the synovial membrane of stifle joint of arthritic rats were determined using real time quantitative polymerase chain reaction (real-time qPCR). Samples were processed simultaneously, as previously described (Bush et al., 2001). β-actin was used as the load control. Total RNA was isolated using High Kit for pure RNA isolation (Thermo Scientific, Fermentas, #K0731). The produced cDNAs from the reverse transcribed template RNAs using Revert Aid™ H Minus Reverse transcriptase kit (#EP0451, Thermo Scientific, Fermentas, USA) were amplified on Faststart Universal SYBR Green Master (Roche, GER). The target genes were normalized with β-Actin by the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001).

Table 1 Forward and reverse primers sequence for real time PCR

Gene	Forward primer	Reverse primer
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTCCATGGTGAAGTCAAC
MMP-3	AGC AAG GAC CTC GTT TTC ATT	GTC AAT CCC TGG AAA GTC TTC A
LF	ACAAGCGGAGACTCGTTCAG	CGATCGCTACGACGTACTCC
Nrf2	CACATCCAGACAGACACCAGT	CTACAATGGGAATGTCTCTGC
LOX-1	CATGGGCCCTTAACTGGGA	GAGAGCCGTTCTCCATAGC
β-actin	AAGTCCTCACCTCCCAAAG	AAGCAATGCTGTCACCTTCCC

2.6. Statistical Analysis

Results were expressed as mean ± SE using SPSS (18.0 software, 2011). Using one-way ANOVA followed by Duncan's test, data were analyzed. Values were statistically significant at $p < 0.05$.

3. RESULTS

The data presented in table (2) revealed a significant up-regulation ($P \leq 0.05$) in IL-1β, MMP-3, and LOX-1 gene expression levels, and down-regulation in LF gene expression level in the synovium of the adjuvant injected rats (G2 and G4) compared with the normal controls (G1). The gene expression levels of IL-1β, MMP-3, and LOX-1 were significantly down-regulated following administration of GSPE either before (G5) or after (G3) induction of arthritis, with lowest expression in the protected group (G5). Moreover, administration of GSPE elevated LF gene expression level in both (G3) and (G5), with highest expression within the protected cluster (G5), in comparison to the arthritic control groups (G2 and G4).

The obtained results conferred in table (3) showed that Nrf2 gene expression level within the arthritic rat livers was significantly elevated following administration of GSPE either before (G5) or after (G3) induction of RA, with highest expression in the protected cluster (G5), as compared with the arthritic control groups (G2 and G4).

Table 2 Effect of GSPE administration on synovial membrane IL-1 β , MMP-3, LOX-1 and LF gene expression levels in Freund's Complete Adjuvant-induced Rheumatoid arthritis in rats.

Animal Groups	IL-1 β		MMP-3		LOX-1		LF	
	Fold change mean	SEM	Fold change mean	SEM	Fold change mean	SEM	Fold change mean	SEM
Group I: Normal control group	1.00 ^f	0	1.00 ^f	0	1.00 ^f	0	1.00 ^a	0
Group II: CFA- induced arthritis for treatment	4.76 ^b	0.25	7.57 ^b	0.38	4.96 ^b	0.29	0.16 ^c	0.02
Group III: CFA + GSPE treated	2.38 ^d	0.13	3.51 ^d	0.17	3.20 ^d	0.19	0.61 ^c	0.04
Group IV: CFA- induced arthritis for protection	6.02 ^a	0.31	10.48 ^a	0.48	6.06 ^a	0.37	0.08 ^e	0.01
Group V: CFA + GSPE protected	1.91 ^e	0.13	2.38 ^e	0.13	2.20 ^e	0.11	1.04 ^a	0.07

Data are presented as (Mean \pm SEM). SEM = Standard error mean. Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$).

Table 3 Effect of GSPE administration on liver tissue Nrf2 gene expression level in Freund's Complete Adjuvant-induced Rheumatoid arthritis in rats.

Animal Groups	Nuclear factor erythroid 2-related factor 2 (Nrf2)	
	Fold change mean	\pm SEM
Group I: Normal control group	1.00 ^f	0
Group II: CFA- induced arthritis for treatment	1.72 ^e	0.1
Group III: CFA + GSPE treated	4.23 ^c	0.24
Group IV: CFA- induced arthritis for protection	1.95 ^e	0.1
Group V: CFA + GSPE protected	8.06 ^a	0.41

Data are presented as (Mean \pm SEM). SEM = Standard error mean. Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$).

Microscopical examination of the stifle joint tissues obtained from normal control rats (Group I) revealed normal histological appearance of synovium and stifle joint capsule with an absence of inflammation; this joint preserved also normal architecture with intact articular cartilage and a well-defined joint space as shown in Figure (1).

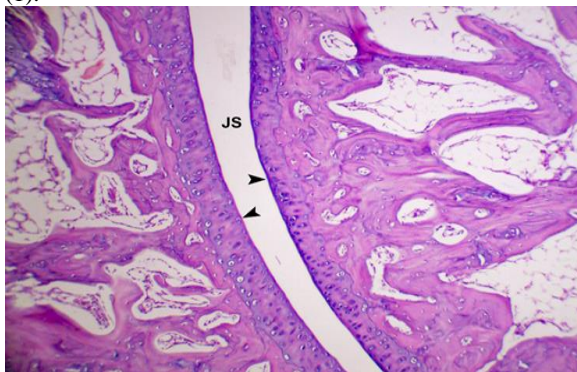


Fig. 1 Stifle joint of a control rat, group I, showing normal architecture with intact articular cartilage and a well-defined joint space (JS). H&E stain x 200

The examined synovium of the affected stifle joint obtained from CFA treated rats (Group II) revealed various histopathological alterations including pannus formation; the synovium was diffusely and markedly thickened and formed numerous wide papillary villar projections. Occasionally, hyperplastic synovial villi contain large multinucleated cells. The proliferated synovial cells and infiltrating pannus markedly eroded the subchondral bone and articular cartilage as highlighted in Figure (2a). Macrophages, lymphocytes and few plasma cells mainly infiltrated the fibrous joint capsule as seen in Figure (2b). Meanwhile, microscopical examination of stifle joint obtained from the treated group with GSPE (Group III), revealed moderate improvement in its histology. The synovium was mildly thickened without any signs of inflammation. The formation of synovial edema and few papillary villar projections was also observed Figure (3). Macrophages and lymphocytes mainly infiltrated the fibrous joint capsule.

The affected stifle joint obtained from CFA treated rats in (Group IV), showed similar histopathological alterations as Group II, with mild erosion of the articular cartilage at the joint margins by the infiltrating pannus as illustrated in Figure (4).

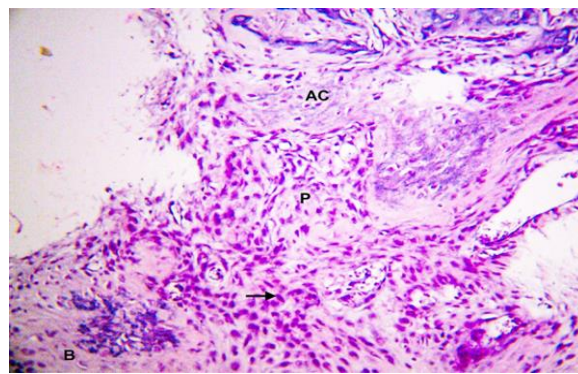


Fig.2a Stifle joint of rat, group II, showing proliferated synovial cells (arrow) and infiltrating pannus (P) eroded and replaced the articular cartilage (AC) and subchondral bone (B). H&E stain x 400.

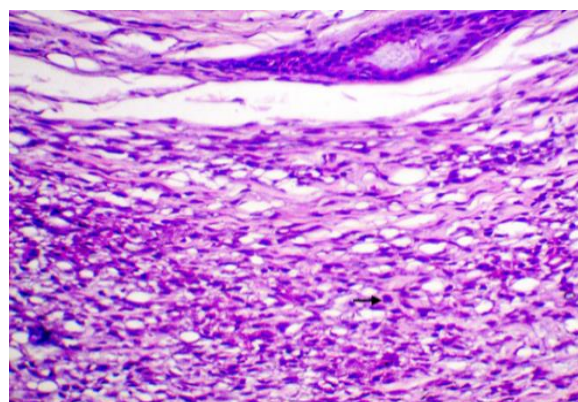


Fig. 2b Stifle joint of rat, group II, showing marked infiltration of fibrous joint capsule by mononuclear inflammatory cells (arrow). H&E stain x 400.



Fig. 3 Stifle joint of rat, group III, showing edema (E) of synovium and formation of few papillary villar projections (arrow). H&E stain x 200.

Histopathological examination of the stifle joint obtained from the protected group with GSPE (Group V) revealed edema of the synovium and formation of a few papillary villar projections lined by hypertrophied and hyperplastic synovial cells without signs of inflammation as demonstrated in Figure (5). Macrophages and lymphocytes mainly infiltrated the fibrous joint capsule.

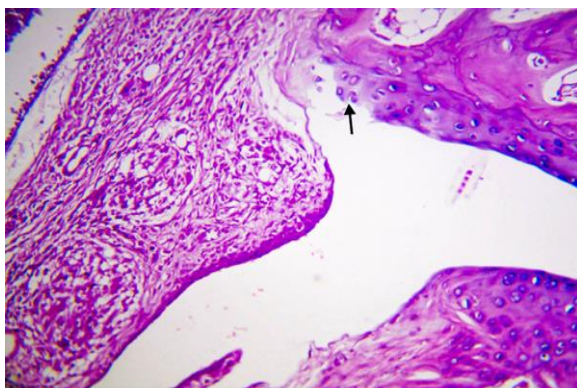


Fig. 4 Stifle joint of rat, group IV, showing mild erosion (arrow) of the articular cartilage at the joint margins by the infiltrating pannus. H&E stain x 400.

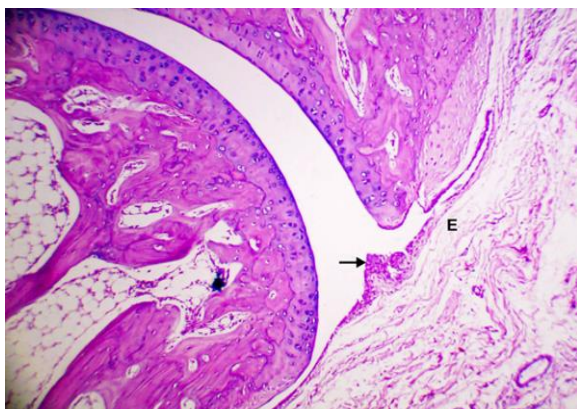


Fig. 5 Stifle joint of rat, group V, showing edema (E) of synovium and formation of few papillary villar projections lined by hypertrophied and hyperplastic synovial cells (arrow). H&E stain x 100.

4. DISCUSSION

The antigenic CFA induce the immune system by stimulating the production of cellular mediators and certain immunoglobulins (Mythilypriya et al., 2008). Generally, the immune-stimulatory efficiency of CFA is not comparable by any adjuvant, as it is considered the gold standard adjuvant (Fontes et al., 2017), with its simple administration. The CFA- induced arthritis severity causing permanent joint malformations like ankylosis. Therefore, a

CFA rats model was used in this study to evaluate the GSPE potency in treating and preventing RA.

The presented data revealed a significant up-regulation ($P \leq 0.05$) in IL-1 β , MMP-3, and LOX-1 gene expression levels, and down-regulation in LF gene expression level in the synovial membrane of the adjuvant injected rats (G2 and G4) as compared with the normal controls (G1). Similarly, Zeb et al., (2017) showed that injection of CFA subcutaneously in rat feet significantly induced the gene expression of TNF- α (317-fold) and IL-1 β (40.66- fold) in paw tissues. Moreover, a significant upregulation ($p < 0.05$) in MMP-3 gene expression and in serum levels of TNF- α and IL-1 β versus the normal control group were demonstrated in adult male rats injected with 0.1 CFA (Aniss et al., 2020).

TNF- α and IL-1 β enhance fibroblast proliferation, activate neutrophils, they additionally can increase macrophage infiltration into the inflamed sites with excessive auto-antibody production (Kaneko et al., 2001) and increase the other cytokines' expression. IL-1 β is 100 times more powerful than TNF α in agitating cartilage degradation in vivo. So IL-1 β was thought to be a pivotal link between cartilage destruction and synovium inflammation. While the external osteoid layer removal was mediated by MMP-3, allowing osteoclasts to be attached to the underlying bone that enables bone resorption (Cunnane et al., 2001). Moreover, MMP-3 induces the proteolytic activation of the pro-matrix metalloproteinase-9 (pro-MMP-9) that promotes recruitment of T cells, neutrophils, monocytes, and osteoclasts (Jones et al., 2008) and membrane-bound Vascular Endothelial Growth Factor (VEGF) releasing, which leads to angiogenesis and disease progression (Szekanecz and Koch, 2009).

Furthermore, Ox-LDL (oxidized low-density lipoprotein) and its receptor LOX-1 which were detected within the RA synovium and in its chondrocytes (Nakagawa et al., 2002) were resulting in increasing the mRNA production of monocyte chemoattractant protein-1 (MCP-1) (Akagi et al., 2009) and VEGF. Levels of MMPs including metalloproteinase-1 (MMP-1) as well as MMP-3 were elevated upon stimulation of fibroblast synovial cells with ox-LDL (Kakinuma et al., 2004), which occurred primarily via the NF- κ B signaling pathway. Toll-like receptor 4 (TLR4) of macrophages elevated the LOX-1 expression and the uptake of oxLDL Hossain et al., (2015), thus TGF- β , interleukin 6 (IL-6), and MCP-1 were released (Van Tits et al., 2011). These data supported the obtained results, which clarified that the LOX-1 gene expression level was significantly increased in the synovial membrane of AIA rats (G2 and G4).

Meanwhile, in the existing study gene expression levels of IL-1 β , MMP-3, and LOX-1 in the synovial membrane of AIA rats were significantly down-regulated following administration of GSPE either before (G5) or after (G3) induction of arthritis, with the lowest expression in the protected group (G5). These results are harmonized with Ahmad et al., (2014) who clarified the anti-inflammatory activity of GSPE since its administration increased the mRNA expressions of interleukin 10 (IL-10) and TGF- β , while reducing the IL-1 β and TNF- α levels. Also, Khaddam et al., (2014) revealed that GSPE inhibited MMP-3. Additionally, Shao et al., (2020) mentioned that treatment with proanthocyanidins, significantly down-regulated the mRNA expression level of LOX-1. Therefore, GSPE markedly suppressed inflammation and consequently

arthritis progression in the rat model, as confirmed by histopathological findings that showed GSPE suppressed soft tissue and synovial membrane inflammations, so considerably reduced bone erosion and joint degradation.

The current study revealed a significant ($P \leq 0.05$) down-regulation of LF in synovial membrane of CFA injected rats (G2 and G4) compared to the normal control group (G1). The major component within the secondary neutrophil granules lactoferrin (LF), exhibits antibacterial, antiviral, anti-fungal properties, inhibits several proinflammatory cytokines production, including IL-1 β , IL-6, interleukin 8(IL-8) and TNF- α . It also transfers to the nucleus, so prevents NF- κ B activation (Haversen et al., 2002). It can also promote anti-inflammatory mediators production including IL-10, interleukin4 (IL-4) (Zimecki et al., 2005).

Moreover, the administration of GSPE elevated LF gene expression level in both (G3) and (G5), with the highest expression within the protected cluster (G5), in comparison to the arthritic control groups (G2 and G4). Also, microscopic examination of the stifle joint obtained from the treated and protected rat groups with GSPE, revealed moderate improvement in its histology. The synovium was mildly thickened and formed a few papillary villar projections lined by hypertrophied and hyperplastic synovial cells without any signs of inflammation.

The transcription factor Nrf2 promotes the transcription of various anti-oxidant enzymes upon binding to the antioxidant response element (ARE) on the DNA, thereby reducing the pro-inflammatory pathways (Jung and Kwak, 2010). If any oxidative stress condition is present, such as ROS and/or inflammation, the Nrf2 / HO-1 antioxidant pathway can be activated (Kobayashi et al., 2013). A slightly increased HO-1 and Nrf2 expression levels are detected in patients with RA and in CIA mice (Wruck et al., 2011). The reduced Nrf2 level is a factor that can lead to RA, along the increased levels of TNF- α , IL-1 β and IL-17, as Nrf2 prevents the production of these proinflammatory cytokines. The relation between NF- κ B/IL-1 β and Nrf2 was suggested to be a promoter for the oxidative injury and joint destruction in animals (Vomhof-DeKrey and Picklo, 2012). Nrf2 also assessed to inhibit the inflammation by keeping the NF- κ B signaling via the up-regulation of HO-1 (Bellezza et al., 2012).

In the present study, CFA- treated rats showed significant up-regulation in Nrf2 gene in the arthritic rats' liver. Similar data were recorded by Wu et al., (2016) who reported that injecting rats with CFA induced RA, significantly elevated Nrf2 expression in rats' synovial tissues ($p < 0.001$) compared with the controls, however, its level markedly increased in the inflamed joints of treated groups from RA when compared with the control AIA-positive group. In the current study, the hepatic Nrf2 gene expression level in arthritic rats was significantly elevated following administration of GSPE either before (G5) or after (G3) induction of arthritis, when compared with the arthritic control groups (G2 and G4). Similarly, Liu et al., (2017) showed that the protein and the gene expression of Nrf2 in the rat livers were markedly up-regulated as a result of GSPE treatment. Also, Nazima et al., (2020) revealed that GSPE treatment (100 mg kg⁻¹ b wt. for 4 weeks) notably normalized the renal expression of Nrf2/Keap1 (Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1) and its downstream regulatory proteins. They suggested that GSP ameliorates oxidative stress through the activation of Nrf2 pathway.

Overall, the results of this study demonstrated that GSPE not only inhibits the key inflammatory factors in RA but also stimulates crucial anti-inflammatory mediators, which in turn support further evaluation for these antioxidant compounds as a treatment for AIA and RA. Thus, the GSPE had therapeutic effects against RA by inhibiting IL-1 β , MMP-3 and LOX-1 gene expression levels in the AIA rats' synovial membrane, while it up-regulated their LF gene expression levels. Moreover, it activates the gene expression level of Nrf2 in arthritic rats' livers.

5. CONCLUSIONS

It could be concluded that, GSPE as a natural compound may provide a safe promising alternative or complimentary anti-rheumatic substance that ameliorates RA in rat joints. Also, GSPE has a strong anti-inflammatory agent that ameliorates RA of rats joint through modulation of LF and inhibiting IL-1 β , MMP-3, and LOX-1 signaling pathways. So, these results confirm the strong anti-arthritic and joint protective effect of GSPE in arthritis.

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