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Original Article

Navel orange peel ethanolic extract and naringin ameliorate CFA-induced arthritis in Wistar rats through their modulatory effects on Th1/Th2/Th17 cytokines and oxidative stress

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Abstract: Rheumatoid arthritis (RA) is an autoimmune illness affecting joint articulations, leading to a disability state. Currently, there is no satisfying optimal therapy except for immunosuppressants, which have variable and bad effects after long-term use. Hence, researchers have attempted to develop other alternative, safer, and more effective natural treatment agents that are effective and without undesirable effects. The objective of this research is to assess the antiarthritic properties of navel orange peel ethanolic extract (NOPEE) and naringin (NAR) in experimentally induced RA in male Wistar rats. RA was induced *via* two successive subcutaneous injections of 0.1 mL complete Freund's adjuvant (CFA) into a footpad of the right hind leg. The arthritic rats were orally treated with 100 mg/kg body weight (b.w.)/day of NOPEE or with 25 mg/kg b.w./day of NAR for 14 days. Results showed that treatment with NOPEE or NAR obviously counteracted the increased ankle joint circumference, inflammatory cell infiltration, pannus development, cartilage degradation, and synovial hyperplasia that developed in CFA-induced arthritic rats. Additionally, the elevation of serum rheumatoid factor (RF), prostaglandin E2 (PGE-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-17 (IL-17) were significantly declined in parallel to enhanced level of serum interleukin-4 (IL-4). Furthermore, NOPEE and NAR supplementation, reversed the negative oxidative effects of lipid peroxidation (LPO), nitric oxide (NO), as well as improved the antioxidant glutathione level (GSH), glutathione reductase (GR) and superoxide dismutase (SOD) activities. Overall, the anti-arthritic effects of NOPEE and NAR may be mediated through their modulatory effects on T helper (Th)1/Th2/Th17 cytokines, oxidative stress, and the antioxidant defense system.

Keywords: Rheumatoid arthritis, navel orange peel ethanolic extract, naringin, Wistar rats

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes stiffness, musculoskeletal discomfort, swelling, degradation of the joints and bones, with an increased risk of developing osteoporosis, and deformity that leads to progressive disability [1-4]. It affects between 0.3 and 1% of the population and is more common in women and in developed economies and countries [5]. The cytokine network's imbalance is important in the pathophysiology of RA. The etiology of RA is based on the patient's body reacting to autoantigens, such as citrullinated peptides, or a foreign peptide, such as a viral or bacterial peptide. The synovial membrane in joints is then penetrated by activated T and B lymphocytes and monocytes, which generate cytokines and encourage leukocyte migration as well as the expansion of synovial fbroblast- and macrophage-like cells [6]. In rheumatoid joints, a disproportion between proinflammatory and anti-inflammatory cytokines favors the induction of autoimmu-

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nity, chronic inflammation and thereby joint destruction [7-9]. The regulation of the two key proinflammatory cytokines in RA, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), is of crucial importance in RA [5, 10-12]. In arthritis and osteoclastogenesis pathology, the investigation of the roles of these cytokines and other cytokines, such as interleukin-17 (IL-17), interleukin-18 (IL-18), and receptor activator of nuclear factor κB (RANK) ligand, the receptor activator of nuclear factor-kappa B (NF-κB), will help us better understand the pathophysiology of chronic arthritis and may help to improve present treatments [13]. Moreover, interleukin-4 (IL-4) also interleukin-10 (IL-10) are cytokines that have anti-inflammatory properties and thought to be promising modulators in the treatment of RA [5, 11, 14]. Although prostaglandin E2 (PGE-2) is also produced in response to proinflammatory cytokines, IL-1β, and TNF-α, it was found to have both pro- and anti-inflammatory effects, dependent on the receptor subtype, cell population, activation environment, and tissue receptor gene expression [15-17]. As a result, cytokines are regarded as potentially powerful indicators of RA, with roles expected to expand in the future, and a thorough understanding of the cytokine balance in RA may aid both the diagnostic and therapeutic processes [9, 18-20].

Adjuvant-induced arthritis (AIA) in rats [11, 21] or mice [22, 23] is a chronic inflammatory illness marked by synovial membrane invasion and joint destruction [2, 24, 25]. It is more like human RA clinically, pathologically, immunologically, and histologically [5, 11, 12, 21]. Not only does AIA assist in the knowledge of RA etiology in people, but it also aids in the development of new RA therapeutics [21]. Therefore, this research used complete Freund's adjuvant (CFA)-induced arthritis as an investigational animal model to assess the ameliorative effects of various tested agents.

Alternative, safer, and more effective natural product-based medications are required due to the side effects and toxicity of widely used anti-arthritic pharmaceuticals [11, 26].

Citrus flavonoids, which make up a large group of flavonoids, have been shown to have anti-inflammatory properties [27, 28] and have the ability to decline lipid peroxidation (LPO) and increase the antioxidant defence system's potency [29]. The antioxidant properties of the peel (flavedo and albedo) and juice of some commercially grown citrus fruits (Rutaceae), including grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), lime (*Citrus aurantiifolia*), and sweet orange (*Citrus sinensis*), were assessed *in vitro* and it was found that the volatile and polar fractions of citrus peels as well as crude and polar fraction of citrus fruit juices revealed their free radicals (FRs) scavenging capacity and LPO inhibiting efficiency [30]. Citrus flavonoids, and naringin (NAR), which is one of the important components, have been shown to have potent anti-inflammatory and antioxidant properties in metabolic disorders [27, 29, 31]. Moreover, the therapeutic effects and underlying mechanisms of NAR, a flavanone glycoside of naringenin, against RA were clarified using network pharmacology research and *in vitro* clinical confirmation. NAR has been shown to decrease the intensity of clinical symptoms and to reduce the generation of inflammatory mediators and proinflammatory cytokines both *in vitro* and *in vivo* [30].

However, the effects of NOPEE and NAR on RA have not been studied. As a result, the goal of this study is to evaluate the effect of NOPEE and NAR on arthritic rats and discover their likely action mechanisms.

Materials and methods

Experimental animals

Forty-healthy male Wistar rats (divided into 4 categories, each with ten animals) with an average weight of 120±10 gm were obtained from the Egyptian holding company for biological products and vaccines (VACSERA) Animal Facilities (Helwan Station, Cairo, Egypt) and utilized as a mammalian animal model in this experiment. The rats were overseen for about two weeks before starting experimental work to eradicate any infections. In the animal house, the animals were kept in polypropylene cages with well-aerated stainless-steel lids (Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt) at normal range of temperature (22±2°C) and relative humidity (55±5%). The rats were given an adequate amount of standard food pelleted diet and water ad libitum and weighed weekly during the experimental periods. All techniques follow the

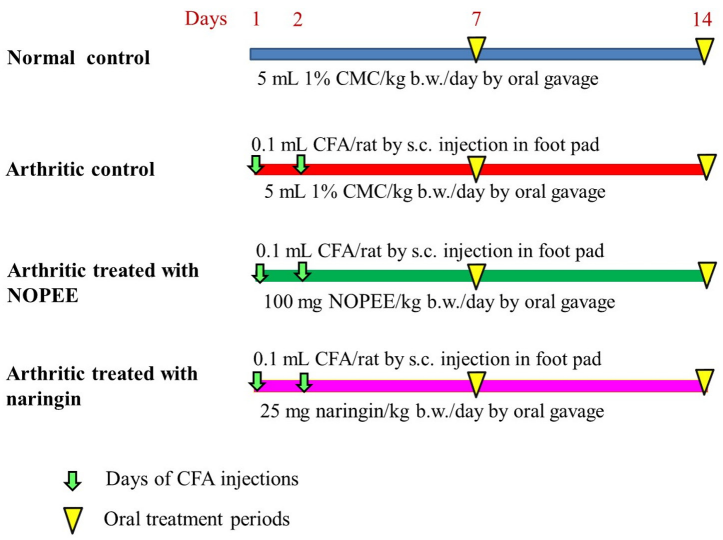

Figure 1. Experimental design and animal grouping. CMC, carboxymethylcellulose; s.c., subcutaneous; kg. b.w., kilogram body weight; CFA, complete Freund's Adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin.

requirements of the Faculty of Science's Experimental Animal Ethics Commission, Beni-Suef University, Egypt (Ethical Approval Number: BSU/FS/2015/5).

Chemicals

CFA, a heat-destroyed Mycobacterium tuberculosis mineral oil-based suspension, was purchased from Sigma Chemical Company (MO, USA). NAR was obtained from Sigma-Aldrich Chemie GmbH, Eschenstr. 582024 Taufkirchen, Germany. The rest of the chemicals are of analytical reagent grade.

Induction of RA

The male Wistar rats were given CFA to induce RA. For more extensive severity the disease, arthritis was induced by a double subcutaneous (s.c.) injection of 0.1 mL CFA into a footpad of a rat's right hind leg for two days [32, 33].

Preparation of NOPEE

The navel orange fruits were obtained from the local market. The fruits were peeled by hand and rinsed under running water until they were clean. After that, the peels were air-dried. An electric grinder was used to grind the dried peels. For 72 hours, the powder (500 g) was steeped in 100% ethanol. To remove the peel particles, the mixture was filtered through a Whatman No. 2 filter paper. To guarantee full extraction, the residue was extracted twice under the same conditions. A rotary evaporator was used to filter the extract and evaporate it to semi-solid bulk. The extract was stored in dark jars in the refrigerator at 4°C until it was needed.

Dose preparation of NOPEE and NAR

NOPEE as well as NAR were given to arthritic rats *via* oral gavage at doses of 100 mg/kg b.w./day [34] and 25 mg/kg b.w./day [35] for 14 days. These doses were added and dissolved in a volume of 5 mL carboxymethylcellulose (CMC) and at a concentration of 1% w/v.

Animal grouping

The animals were placed into 4 categories, each with ten animals (**Figure 1**): 1) Normal control group: Rats of this group were provided the corresponding volume of 1% CMC *via* oral gavage for 14 days. 2) Arthritic control group: The arthritic rats were given the corresponding volume of 1% CMC (5 mL/kg b.w./day) *via* oral gavage for 14 days. 3) Arthritic group treated with NOPEE: The rats of this group were treated daily with 100 mg/kg b.w. of NOPEE (dissolved in 5 mL 1% CMC) *via* oral gavage for 14 days. 4) Arthritic group treated with NAR: The rats were treated daily with 25 mg/kg b.w. of NAR (dissolved in 5 mL 1% CMC) *via* oral gavage for 14 days.

By the end of experiment, the ankle circumference of the rats' right hind leg was measured. Blood was drawn from the jugular vein after the rats were anaesthetized by inhaling ether to reduce pain, suffering and distress. The serum was separated by centrifugation at 3,000 rpm for 15 minutes after the blood had clotted. The pure, non-hemolyzed supernatant sera were rapidly collected, split into three separate portions for each animal, and stored at temperature 30°C until use. The animals were ethically euthanized. Each rat's hind right ankle was detached and treated in 10% neutralized formalin buffer for histopathology. A half gram of

Table 1. Effects of NOPEE and NAR on right hind leg circumference at the paw region in CFA-induced arthritic rats

Parameter / Groups	Right hind leg circumference at paw region (cm)			
	1 W	% change	2 W	% change
Normal	2.20±0.04[c]		2.45±0.04[c]	
Arthritic control	2.83±0.20[b]	28.60	3.28±0.14[a]	33.90
Arthritic rats treated with NOPEE	2.00±0.70[b]	2.80	2.83±0.09[b]	-13.70
Arthritic rats treated with NAR	2.95±0.10[b]	4.20	2.93±0.09[b]	-10.70
F-probability	P<0.001			
LSD at the 5% level	0.33			
LSD at the 1% level	0.44			

Data are expressed as mean ± SE. Number of animals in each group is six. Means, which have different symbols a, b, c and d are significantly different at *P*<0.05. % changes were calculated by comparing arthritic controls with normal and arthritic treated groups with arthritic control. CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; cm, centimeter; 1 W, 1 week; 2 W, 2 weeks; LSD, Least Significant Difference.

liver was homogenized using a Teflon homogenizer (Glas-Col, Terre Haute, USA) in 5 mL cold ice, 0.9% NaCl for the determination of antioxidants and oxidative stress parameters. The homogenate was gained and saved in the freezer at -30°C.

Detection of markers related to inflammation and oxidative stress

Serum PGE-2, RF, IL-1β, TNF-α, IL-4 and IL-17 levels in the serum were assayed following the manufacturer's instructions using kits of enzyme-linked immunosorbent assay (ELISA) technique bought from R&D Systems (Minneapolis, MN, USA). Moreover, the liver LPO and NO as well as reduced GSH levels were assayed according to manufacturer's methods [36-38], respectively. The GR and SOD was measured dependent on the procedure of Goldberg & Spooner [39] as well as Marklund and Marklund [40].

Histopathological investigation

The articular bones in the right leg ankle were detached, cleaned in 0.9% NaCl, and preserved in 10% neutralized formalin buffer. The fixed ankles were decalcified for 2-3 weeks at 4°C with a 10% ethylenediamine tetra-acetic acid (EDTA) solution in distilled water with a pH of 7.4 and EDTA renewal every week [41]. The samples were regularly processed, implanted in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) in the Histopathology Department, Faculty of Veterinary Medicine, Beni-Suef University [42]. Histological lesions and alterations were studied in stained sections put on glass slides.

Statistical analysis

The statistics were examined using one-way and two-way analysis of variance (ANOVA) via PC-STAT (Version IA (C), University of Georgia, USA), followed by Least Significant Difference (LSD) analysis, to determine the major effects and compare the various groups [43]. The number of samples in each group is six (n=6). The general effect between groups is expressed by the F-probability for each marker. The mean and standard error were used to express the data. For LSD, statistically non-significant differences were defined as P>0.05, whereas statistically significant differences were defined as P<0.05 and P<0.01 respectively.

Results

Effect on paw circumference of the right hind leg

The circumference of the right hind leg in the paw region was used as an indicator of paw edema, it significantly (P<0.01; LSD) increased during the 1st and 2nd weeks with percentage changes of 28.60 and 33.90, respectively, comparing with the corresponding normal controls. The administration of arthritic rats with NOPEE and NAR induced a non-significant effect (P>0.05; LSD) during the 1st week and a highly significant effect (P<0.01; LSD) during the 2nd week (**Table 1**).

Effect on serum RF and PGE-2 levels

The serum RF level (P<0.01; LSD) increased significantly in arthritic rats during the 1st and 2nd weeks with percentage changes of 47.90 and 132.07, respectively, compared to the corresponding normal controls. The arthritic rats treated with NOPEE and NAR produced a non-significant action during the first week, although they developed a highly significant effect during the 2nd week (**Table 2**).

The serum PGE-2 level was significantly (P<0.01; LSD) increased in CFA-induced arthritic rats during the 1st and 2nd weeks with percentage changes of 40.17 and 45.18, respectively, compared to the corresponding normal controls. The arthritic rats treated with NOPEE produced a non-significant (P>0.05; LSD) decrease in the PGE-2 level, while the administration of NAR induced a highly significant decrease (P<0.01; LSD).

Effect on the IL-1β, TNF-α, and IL-17 levels

Shown in **Table 3**, there was a remarkably significant elevation (P<0.01; LSD) in the levels of serum IL-1β, TNF-α, and IL-17 in the CFA group during the 1st and 2nd weeks in comparison to control rats with recorded percentage changes of 316.80, 119.79, and 96.59 during the 1st week and 107.89, 137.33, and 34.95 during the 2nd week, respectively.

The treatment of arthritic rats with NOPEE induced a highly significant reduction (P<0.01; LSD) in the higher serum IL-1β level during the 1st (-32.18%) and 2nd (-27.33%) weeks, while the arthritic group treated with NAR resulted in a significant enhancement (P<0.01; LSD) only during the 1st week (-84.97%). The raised serum TNF-α level in arthritic animals significantly improved due to treatment with NAR during the 1st week and because of treatments with both NOPEE and NAR during the 2nd week. On the other hand, the elevated serum IL-17 level in arthritic rats was significaltly ameliorated due to treatments with NOPEE and NAR during the 1st week as well as treatment with NAR only during the 2nd week (**Table 3**).

Effect on serum IL-4 level

The level of IL-4 in serum was remarkably reduced in CFA-rats during 1st and 2nd weeks with percentage changes of -10.50 and -17.89, respectively, compared to the equivalent values of the control group. However, the effect was significant (P<0.01; LSD) during the 2nd week and non-significant (P>0.01; LSD) during the 1st week after CFA injection. The arthritic rats treated with the NOPEE induced a highly significant decrease (P<0.01; LSD) during the 1st and 2nd weeks, while the treatment with NAR showed no significant effect (P>0.05; LSD) at both tested periods (**Table 4**). The IL-4 concentration increased detectably due to NAR treatment for 2 weeks with a recorded percentage change of 9.84 as compared with the control group (**Table 4**).

Effect on liver LPO and NO level

The levels of the LPO and NO were significantly increased (P<0.01; LSD) in the CFA group during the 1st and 2nd weeks compared with the control group with recorded percentage changes of 42.35 and 115.47 during the 1st week and 49.23 and 323.34 during the 2nd week, respectively, as compared with the respective values of the control group. The treatment of arthritic rats with NOPEE induced a highly significant effect (P<0.01; LSD) in the levels of LPO and NO during the 1st and 2nd weeks. The treatment of arthritic rats with NAR induced a highly significant effect (P<0.01; LSD) in the level of NO during the 1st and 2nd weeks and a significant effect (P<0.05; LSD) on LPO during the 1st week and a non-significant effect (P>0.05; LSD) during the 2nd week (**Table 5**).

Effect on liver GSH content and GR and SOD activities

GSH content showed a highly significant (P<0.01; LSD) reduction in liver of CFA-induced arthritic rats during the 1st and 2nd weeks with recorded percentage changes of -54.83 and -50.57, respectively, compared to those of control rats' group. The arthritic rats given NOPEE and NAR, had a significant (P<0.05; LSD) and highly significant (P<0.01; LSD) respectively, decrease in GSH content during the 1st week with recorded percentage changes of 32.65 and 43.04 as compared to control group. As the period extended to 2 weeks, the NOPEE induced a significant change in the lowered GSH content, while NAR treatment showed no significant effect (P>0.05; LSD).

Table 2. Effects of NOPEE and NAR on serum RF and PGE-2 levels in CFA-induced arthritic rats

Parameter / Groups	RF (μ/mL)				PGE-2 (pg/mL)			
	1 W	% change	2 W	% change	1 W	% change	2 W	% change
Normal	13.03±0.57^{d}		13.50±1.00^{d}		137.9±7.00^{c}		133.01±5.49^{c}	
Arthritic control	19.28±0.95^{b,c}	47.90	31.33±1.20^{a}	132.07	193.3±5.97^{a}	40.17	193.10±1.90^{a}	45.18
Arthritic rats treated with NOPEE	16.16±1.18^{c,d}	-16.20	19.58±0.92^{b}	-37.50	181.00±4.14^{a}	-6.21	187.70±6.71^{a}	-2.80
Arthritic rats treated with NAR	17.93±0.88^{b,c}	-7.00	20.96±1.51^{b}	-33.09	156.43±7.58^{b}	-19.30	142.43±1.38^{b}	-26.24
F-probability	P<0.001				P<0.001			
LSD at the 5% level	3.15				15.81			
LSD at the 1% level	4.24				21.30			

Data are expressed as mean ± SE. Number of animals in each group is six. Means, which have different symbols a, b, c and d are significantly different at $P<0.05$. % Changes were calculated by comparing arthritic control with normal and arthritic treated groups with arthritic control one. CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; RF, rheumatoid factor; ACCP, anti-cyclic citrullinated peptide; 1 W, 1 week; 2 W, 2 weeks; LSD, Least Significant Difference.

Table 3. Effects of NOPEE and NAR on serum IL-1β, TNF-α and IL-17 levels in CFA-induced arthritic rats

Parameter / Group	IL-1β (pg/mL)				TNF-α (pg/mL)				IL-17 (pg/mL)			
	1 W	% change	2 W	% change	1 W	% change	2 W	% change	1 W	% change	2 W	% change
Normal	32.81±1.24^{e}		38.01±2.50^{e}		31.80±1.86^{f}		36.00±0.2.75^{e,f}		40.80±1.81^{d}		50.02±3.01^{c,d}	
Arthritic control	136.7±3.13^{a}	316.8	79.02±5.51^{b,c}	107.89	69.74±2.64^{b}	119.79	85.44±3.30^{a}	137.33	80.21±6.12^{a}	96.59	67.50±10.00^{a,b}	34.95
Arthritic rats treated with NOPEE	92.7±244^{b}	-32.18	57.50±3.76^{d}	-27.33	65.16±0.85^{b,c}	-6.56	60.43±4.85^{c,d}	-29.27	53.8±3.71^{c,d}	-32.92	73.46±2.11^{a}	8.83
Arthritic rats treated with NAR	73.9±11.01^{c}	-84.97	78.43±4.65^{b,c}	-0.75	52.73±2.42^{d}	-24.39	41.43±1.43^{e}	-51.50	57.6±2.50^{b,c}	-28.18	44.00±2.59^{c,d}	-34.81
F-probability	P<0.001				P<0.001				P<0.001			
LSD at the 5% level	14.72				8.00				13.64			
LSD at the 1% level	19.83				10.78				18.37			

Data are expressed as mean ± SE. Number of animals in each group is six. Means, which have different symbols a, b, c, d, e and f are significantly different at $P<0.05$. % Changes were calculated by comparing arthritic control with normal and arthritic treated groups with arthritic control one. CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; 1 W, 1 week; 2 W, 2 weeks; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; IL-17, interleukin 17; LSD, least significant difference.

Table 4. Effects of NOPEE and NAR on serum IL-4 level in CFA-induced arthritic rats

Parameter / Groups	IL-4 (pg/mL)			
	1 W	% change	2 W	% change
Normal	116.83±7.70^{a,b}		126.00±2.35^{a}	
Arthritic control	104.5±2.13^{b,c,d}	-10.50	103.45±0.60^{c,d}	-17.89
Arthritic rats treated with NOPEE	83.36±2.49^{e}	-20.22	83.36±6.02^{e}	-19.42
Arthritic rats treated with NAR	98.53±1.55^{d}	-5.70	113.63±6.43^{b,c}	9.84
F-probability	P<0.001			
LSD at the 5% level	12.81			
LSD at the 1% level	17.25			

Data are expressed as mean ± SE. Number of animals in each group is six. Means, which have different symbols a, b, c, d and e are significantly different at *P*<0.05. % Changes were calculated by comparing arthritic control with normal and arthritic treated groups with arthritic control one. CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; IL-4, interleukin 4; 1 W, 1 week; 2 W, 2 weeks; LSD, Least Significant Difference.

Table 5. Effects of NOPEE and NAR on liver LPO and NO level in CFA-induced arthritic rats

Parameter / Groups	LPO (nmole MDA/100 mg tissue/hr)				NO (nmole/100 mg tissue)			
	1 W	% change	2 W	% change	1 W	% change	2 W	% change
Normal	12.82±0.36^{c}		13.00±0.30^{c}		7.10±0.16^{c}		7.11±0.18^{c}	
Arthritic control	18.25±0.12^{a}	42.35	19.40±0.72^{a}	49.23	15.32±0.80^{b}	115.47	30.10±3.40^{a}	323.34
Arthritic rats treated with NOPEE	14.82±0.19^{b,c}	-18.79	9.06±0.37^{d}	-53.29	4.93±0.37^{c}	-67.81	7.27±0.40^{c}	-75.84
Arthritic rats treated with NAR	8.76±0.37^{d}	-52.00	17.30±0.10^{a,b}	-10.82	7.41±0.55^{c}	-51.63	6.45±0.33^{c}	-79.17
F-probability	P<0.001				P<0.001			
LSD at the 5% level	2.65				3.63			
LSD at the 1% level	3.57				4.89			

Data are expressed as mean ± SE. Number of animals in each group is six. Means, which have different symbols a, b, c and d are significantly different at *P*<0.05. % Changes were calculated by comparing arthritic control with normal and arthritic treated groups with arthritic control one. CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; LPO, lipid peroxidation; NO, nitric oxide; 1 W, 1 week; 2 W, 2 weeks; LSD, Least Significant Difference.

The GR activity was highly significantly diminished in the liver of CFA-induced rats during the 1st and 2nd week with recorded percentage changes of -23.68 and -40.50, respectively, compared to the consistent control group. The treatment with NOPEE produced a significant decrease (P<0.05; LSD) during the 1st week, while it induced a significant increase (P<0.01; LSD) during the 2nd week. Conversely, the treatment with NAR induced a highly significant and a significant decrease in GR activity during the 1st and 2nd weeks with recorded percentage decreases of 26.39 and 22.73, respectively, compared to the arthritic control group.

The SOD activity was significantly declined (P<0.01; LSD) during the 1st week compared to the control group, while it improved significantly during the 2nd week. The treatment of rats with NOPEE and NAR triggered a significant increase (P<0.01; LSD) in SOD activity, while it produced a non-significant result during the 2nd week (**Table 6**).

In **Tables 7** and **8**, the data of one-way besides two-way ANOVA analysis were represented. One-way test of ANOVA for all detected parameters revealed that the overall effect among groups was very highly significant (P<0.001). A two-way test of ANOVA depicted that the efficacy of treatment-time interaction was significant on most of the tested parameters.

Histopathological effects

Figures 2 and **3** show the histological modifications to the right ankle joint in the tested groups. The bone surfaces in the synovium, the joint cavity between two movable bones, are enclosed by articular cartilage, which deficiencies a perichondrium. A two-part articular capsule surrounds and connects the ends of the two bones. The outer portion is a fibrous capsule that surrounds each bone's articular cartilage and extends far beyond it. The inner part is called the synovial membrane. As a result, either the synovial membrane or articular cartilage lines the whole joint cavity. A thin sheet of

Table 6. Effects of NOEE and NAR on liver GSH content and GR and SOD activities in CFA-induced arthritic rats

Parameter / Groups	Total GSH (nmole/100 mg tissue)				Liver GR (mU/100 mg tissue)				SOD (U/gm tissue)			
	1 W	% change	2 W	% change	1 W	% change	2 W	% change	1 W	% change	2 W	% change
Normal	54.58±30[a]		56.65±2.00[a]		83.86±4.46[a]		76.23±7.43[a]		10.66±1.08[c,d]		9.33±1.28[d,e]	
Arthritic control	24.65±1.54[e]	-54.83	28.00±2.48[d,e]	-50.57	64.00±1.40[b]	-23.68	49.00±4.44[c]	-40.50	7.51±0.37[e]	-29.54	13.83±0.73[b]	48.23
Arthritic rats treated with NOPEE	32.7±1.22[c,d]	32.65	48.05±1.75[b]	71.61	50.38±2.31[c]	-21.28	77.01±1.90[a]	57.16	14.10±1.69[b]	87.75	14.10±0.41[b]	01.95
Arthritic rats treated with NAR	35.26±0.65[c]	43.04	25.98±3.12[e]	-7.21	47.11±0.68[c,d]	-26.39	37.86±2.17[d]	-22.73	16.62±0.18[a]	121.30	12.46±0.35[b,c]	-9.90
F-probability	P<0.001				P<0.001				P<0.001			
LSD at the 5% level	6.16				10.76				2.13			
LSD at the 1% level	8.29				14.5				2.87			

Data are expressed as mean ± SE. Number of animals in each group is six. Means, which have different symbols a, b, c, d and e are significantly different at P<0.05. % Changes were calculated by comparing arthritic control with normal and arthritic treated groups with arthritic control one. CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; GSH, glutathione; GR, glutathione reductase; SOD, superoxide dismutase; 1 W, 1 week; 2 W, 2 weeks; LSD, Least Significant Difference.

Table 7. Analyses of variance of the effects of NOPEE and NAR on right hind leg circumference, RF, PGE-2, IL-1β, TNF-α, IL-17 and IL-4 in CFA-induced arthritic rats at various periods of time

Source of variation		Right hind paw circumference	RF	PGE-2	IL-1β	TNF-α	IL-17	IL-4
One-way ANOVA	General effect							
	In between groups			P<0.001				
Two-way ANOVA	Normal-arthritic effect							
	Treatment			P>0.05				
	Time			P>0.05				
	Treatment-time	P>0.05	P<0.001	P<0.05	P<0.001	P<0.05	P<0.05	
	Arthritic-NOPEE effect							
	Treatment			P>0.05				P<0.05
	Time			P>0.05				
	Treatment-time	P>0.05	P<0.001	P>0.05	P<0.01		P<0.05	P>0.05
	Arthritis-NAR effect							
	Treatment		P>0.05				P>0.05	P>0.05
	Time		P>0.05				P<0.05	P>0.05
	Treatment-time	P>0.05	P<0.001	P>0.05	P<0.001		P>0.05	P<0.05

CFA, Complete Freund's Adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; RF, rheumatoid factor; ACCP, anti-cyclic citrullinated peptide; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; IL-17, interleukin 17; IL-4, interleukin 4; ANOVA, analysis of variance.

Table 8. Analyses of variance of the effects of NOPEE and NAR on various oxidant and antioxidant markers in CFA-induced arthritic rats at various periods of time

Source of variation		LPO	NO	GSH	GR	SOD
One-way ANOVA	General effect					
	In between groups			P<0.001		
Two-way ANOVA	Normal-arthritic effect					
	Treatment			P>0.05		
	Time			P>0.05		
	Treatment-time	P>0.05	P<0.001	P<0.05	P>0.05	P<0.001
	Arthritic-NOPEE effect					
	Treatment			P>0.05		
	Time			P>0.05		
	Treatment-time		P<0.01		P<0.001	
	Arthritis-NAR effect					
	Treatment			P>0.05		
	Time			P>0.05		
	Treatment-time	P<0.001		P<0.01	P>0.05	P<0.001

CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; LPO, lipid peroxidation; NO, nitric oxide; GSH, glutathione; GR, glutathione reductase; SOD, superoxide dismutase; ANOVA, Analysis of variance.

connective tissue with many blood and lymphatic vessels makes up the synovial membrane. The surface facing the joint cavity is coated with epithelioid cells, which secrete hyaluronic acid and phagocytize debris. They are not epithelial because they do not rest on a basement membrane. The viscous synovial fluid, which lubricates the joints, is made up of hyaluronic acid and a dialysate of plasma from the blood vessels of the membrane (**Figures 2A**, **2B**, **3A** and **3B**).

The characteristics of CFA rats' joints are synovial hyperplasia, with excessive synovial growth resulting in pannus and cell exudation into the joint space. Erosion of bone and cartilage during the 1st week (**Figure 2C** and **2D**) and 2nd week (**Figure 3C-F**) after CFA injection. CFA rats had a higher incidence of synovial hyperplasia, a huge influx of inflammatory cells, and an accumulation of many monomorphonuclear and polymorphonuclear cells in the joint area was observed compared with the normal group. In contrast, the NOPEE-treated arthritic rats showed a considerable decrease in joint damage and/or inflammation as indicated during the 1st week (**Figure 2E** and **2F**) and 2nd week (**Figure 3G** and **3H**). The synovial membrane in the joints became almost like normal synovium. The joint histology was almost identical with that of the normal group. On the other hand, less decrease in arthritis severity was detected in the rats treated with NAR at the 1st week (**Figure 2G** and **2H**) and 2nd week (**Figure 3I** and **3J**). Despite the absence of obvious joint injury, like bone and cartilage erosions, synovial hyperplasia and the invasion of inflammatory cells are present.

Discussion

RA is a progressive autoimmune disease that affects roughly 1% of people and its prevalence rises with age. It is primarily related to increased inflammatory responses within the synovial joints of articular joints like bone, ligaments, hyaline cartilage, tendons, and synovial membrane and affects mostly the foot, hand and wrist, but it can also affect large joints [44, 45].

This study uses the Wistar rat CFA-induced arthritic model because it shares characteristics with human RA, including the invasion of inflammatory cells, synovial membrane proliferation, and joint damage [46]. Most researchers agree that inhibiting arthritis induced in rats via CFA is one of the best test methods to identify drugs that have anti-arthritic action since it strongly matches human arthritis [21, 47, 48].

In our study, the increased paw circumference of arthritic rats of the right leg was significantly decreased during the 2nd week because of

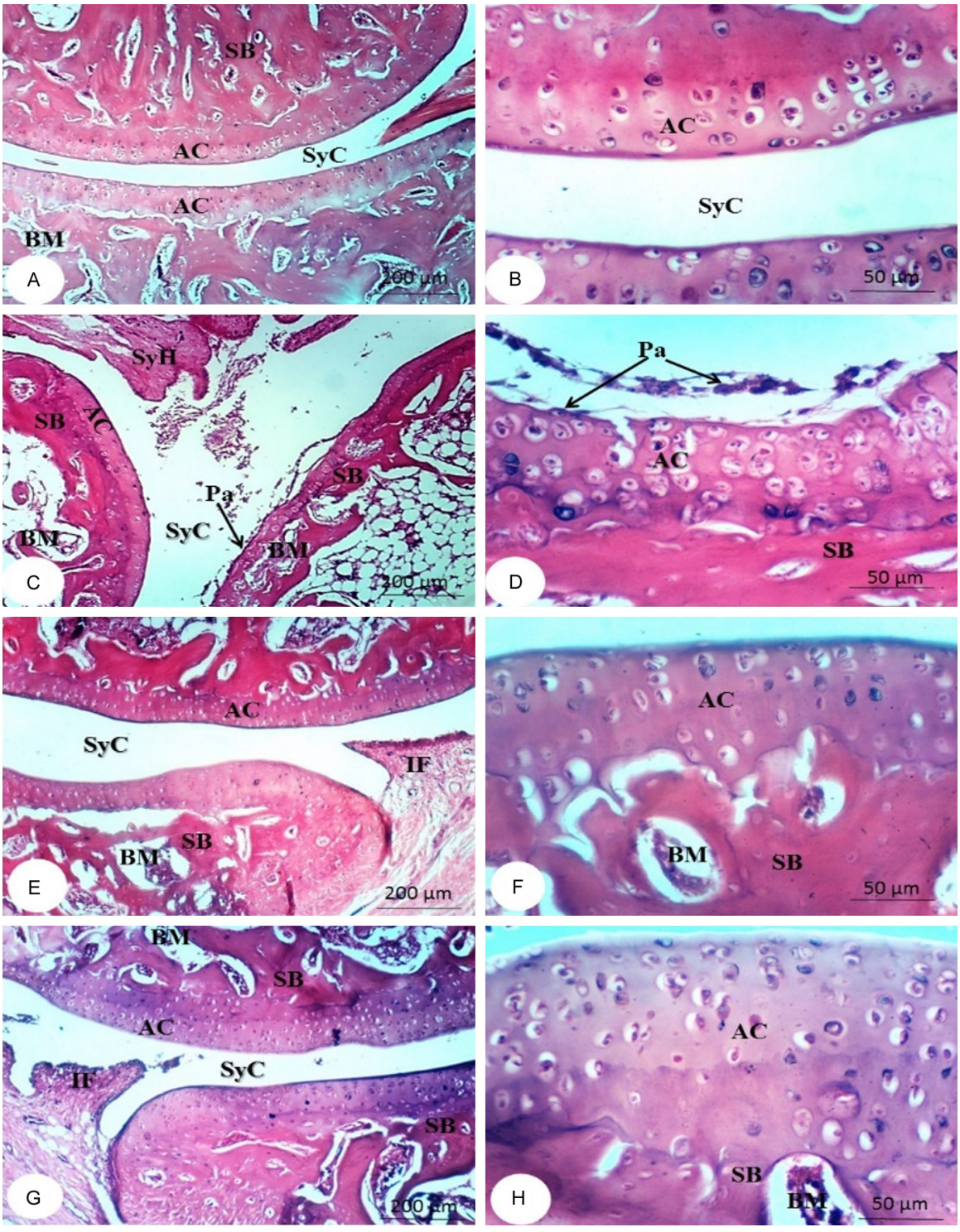

Figure 2. Photomicrographs of sections in ankles of rats from normal group (A and B), arthritic control (C and D) and arthritic groups treated with NOPEE (E and F) and NAR (G and H) at the end of the 1st week showing articulating cartilage (AC), synovial cavity (SyC), sponge bone (SB), bone marrow (BM), skeletal muscle (SkM), synovial hyperplasia (SyH), pannus formation (Pa), and eroded articulating cartilage (EAC). CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin.

treating them with NOPEE and NAR. The decrease in the right leg circumference due to treatment with NOPEE and NAR reflects the reduction in the rate of swelling, that could be

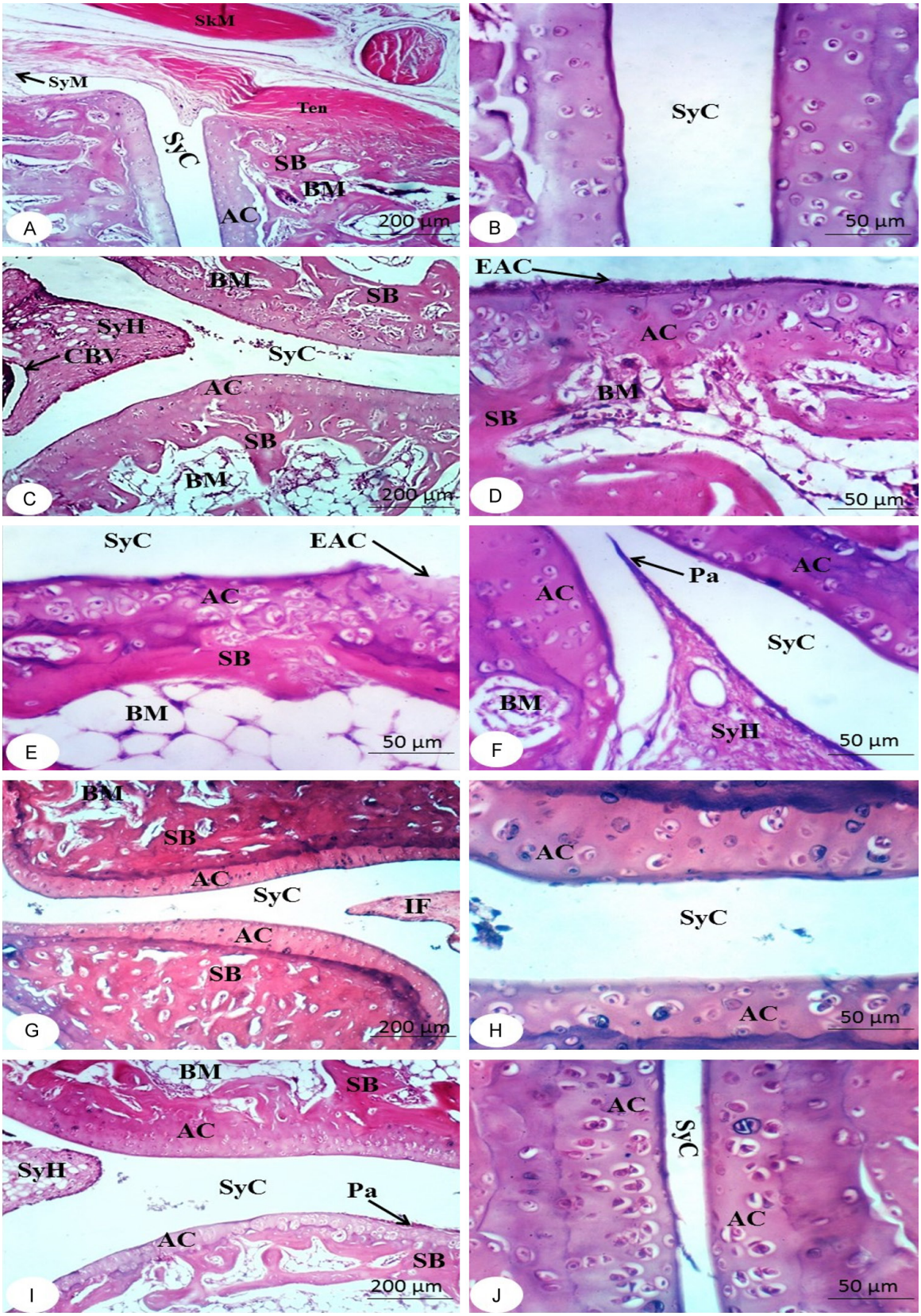

Figure 3. Photomicrographs of sections in ankles of rats from rats from normal group (A and B), arthritic control (C-F) and arthritic groups treated with NOPEE (G and H) and NAR (I and J) at the end of the 2nd week showing cartilage

(AC), sponge bone (SB), bone marrow (BM), synovial cavity (SyC), synovial membrane (SyM), congested blood vessel (CBV), tendon (Ten), skeletal muscle (SkM), erosin in articular cartilage (EAC), synovial hyperplasia (SyH) and pannus formation (Pa). CFA, complete Freund's adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin.

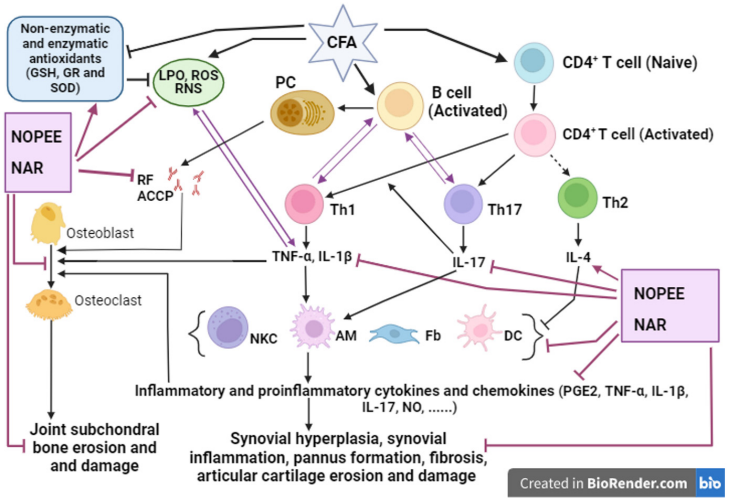

Figure 4. Schematic diagram showing the immunomodulatory effect of NOPEE and NAR in CFA-induced arthritis. Activation, →; inhibition, ⊤; CFA, Complete Freund's Adjuvant; NOPEE, navel orange peel ethanolic extract; NAR, naringin; PC, plasma cell; ROS, reactive oxygen species; LPO, lipid peroxidation; RNS, reactive nitrogen species; RF, rheumatoid factor; ACCP, anti-cyclic citrullinated peptide; CD, Cluster Differentiation; Th, T helper cell; NKC, natural killer cells; AM, activated macrophages; DC, dendritic cells; Fb, fibroblast; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor-α; IL, interleukin; NO, nitric oxide; GSH, glutathione; GR, glutathione reductase; SOD, superoxide dismutase.

attributed to the reduction in edema, diminution of inflammation, and a decrease in synovial tissue hyperplasia as described by the histology data of the present study and stated also by other previous studies [11, 49]. Such macroscopic investigation was done through microscopic and histologic examination of the joint sections. The CFA rats showed an enormous inflammatory response, synovial hyperplasia, and an aggregation of many monomorphonuclear and polymorphonuclear cells in the joint space compared with the control group. Conversely, rats treated with NOPEE demonstrated a clear reduction in joint damage or inflammation as indicated during the 1st and 2nd weeks. Moreover, the arthritic animals treated with NAR showed no signs of harm or damage to the joint although the inflammation and synovium proliferation were somewhat pronounced. Therefore, the preceding findings illustrated the suppressed arthritis-related paw edema and inflammatory responses in both treatments. RF is the main circulating immunoglobulin G antibody and an important serum test for the diagnosis of RA [50, 51]. B lymphocytes are the source of RF and anticitrullinated protein antibodies (ACCP), which support the development of immunological complexes in the joint (**Figure 4**) [5, 6]. Moreover, they respond and produce cytokines and chemokines which promote angiogenesis, leukocyte infiltration, and synovial hyperplasia [52].

In RA, TNF-α is the major mediator of the inflammatory response [53]. TNF-α is released by macrophages during the erosive process of cartilage and bone, and it causes the creation of other cytokines like IL-1β [54]. Additionally, IL-17 is able to promote inflammation and it is very important in the etiology of RA by triggering a number of proinflammatory mediators, such as chemokines, cytokines, macrophages, monocytes, chondrocytes and other mediators of the breakdown of bone and cartilage in synovial fibroblasts (**Figure 4**) [55]. On the other hand, the anti-inflammatory mediator, IL-4, is a cytokine synthesized by CD4+ Th2 lymphocytes, which is a characteristic for this subset of lymphocytes. In addition, IL-4 is also secreted by mast cells and basophils [56, 57]. In the present study, the CFA-induced arthritic rats displayed a significant increase in the serum proinflammatory cytokines IL-1β, TNF-α and IL-17, while it showed an obvious decrease in the anti-inflammatory cytokine IL-4 in parallel with the increase of serum RF after 1 and 2 weeks of CFA administration. Overall, in CFA-induced arthritis, there is a Th1 and Th17 dominant response (**Figure 4**).

The PGE-2 pathway is active in the rheumatoid joint, which also exhibits high amounts of this mediator in the synovial fluid and robust expression of its synthesis-related enzymes in the synovium like cyclooxygenase (COX)-1 and 2 as

well as microsomal PGE-2 synthase-1 (mPGES-1) [58-61]. PGE-2 promotes the growth of autoaggressive T helper 17 (Th17) cells, which maintains inflammatory pathways [62, 63]. This is confirmed by the results of the present study, which indicated that the elevation in the serum PGE-2 level in arthritic rats was concomitant with the increase in the serum IL-17 level during the 1st and 2nd weeks.

In the present study, the increased serum RF was considerably decreased by treatment with NOPEE and NAR. The decrease in the RF level by treatment with NOPEE and NAR indicated a decrease in the inflammatory cells. This finding is consistent with other research [64] which revealed that many members of the Citrus genus are famous for their therapeutic, physiological, and pharmacological properties, which include antibacterial, antioxidant, and anti-inflammatory actions [65]. In the same way, it was indicated that flavonoids are strong anti-inflammatory compounds [27, 66].

To research the potential anti-inflammatory mechanisms of action of NOPEE and NAR in the present study, the serum concentrations of RF, PGE-2, TNF-α, IL-1β, IL-17, and IL-4 were determined. The serum RF, PGE-2 (acute inflammatory cytokines), TNF-α, IL-1β, and IL-17 (proinflammatory cytokines) were pointedly elevated in the arthritic control rats. The anti-inflammatory IL-4 serum level was depleted. These changes in the cytokines ensure that in experimental CFA-induced arthritis, a predominance of Th1 and Th17 on Th2 can be observed (**Figure 1**). Numerous earlier authors have supported this finding [11, 44, 67]. Unbalanced Th1/Th2/Th17 lymphocyte cytokine production undoubtedly contributes significantly to the development of RA. The Th2 cytokine, IL-4, stimulates antibody-mediated immune responses while the Th17 cytokine, IL-17, is principally in charge of eliminating extracellular as well fungal pathogens [7, 68-70].

In the current study, the NOPEE treatment caused a significant drop in the high serum level of RF, IL-1β, and TNF-α levels during the 1st and 2nd weeks while IL-17 levels changed during the 1st week. In addition, the IL-4 concentration clearly decreased during the 1st week of NOPEE treatment. These effects could be explained by the lowered levels of proinflammatory markers [IL-6, monocyte chemotactic protein 1 (MCP-1), IFN-γ, and TNF-α], and higher levels of adiponectin and IL-10 in the plasma or liver as indicated by [71].

NAR therapy led to reduction in the increased serum RF, PGE-2, IL-1β, TNF-α, and IL-17 levels, although it caused a non-significant change in the decreased serum IL-4 level in arthritic rats. Thus, this flavonoid may induce anti-inflammatory activities by decreasing the proinflammatory and inflammatory cytokines. These results are consistent with the study of Jain and Parmar [72] that suggested that the anti-inflammatory effects could be attributed to the increase in adiponectin and IL-10 in addition to the decrease in the proinflammatory cytokines in the plasma and liver.

The cause of RA is mainly joint inflammation initiated by oxidative stress [73]. Oxidative stress that is produced within an inflamed joint can produce connective tissue destruction and autoimmune phenomena in rheumatoid synovitis [74]. Moreover, oxidative stress also has a vital role in the pathology of autoimmune diseases by breaking down the immunological tolerance, enhancing the inflammation, and inducing apoptotic cell death [75].

Reactive oxygen species (ROS) and LPO generation that is stimulated suggests that an imbalance between ROS production and clearance occurs, resulting in oxidative stress [76] that in turn contributes to tissue damage [77]. Cell membrane polyunsaturated fatty lipids undergo oxidation, which results in the production of lipid peroxyl radicals. These radicals then promote oxidation and cause cell membrane damage [78]. Additionally, elevated NO production is important to the pathophysiology of RA (**Figure 4**). Treatment for chronic autoimmune illnesses may involve a novel therapeutic strategy called NOS inhibition [79, 80].

In the current study, the LPO and NO level significantly increased in the liver of CFA-administered rats compared with the normal group [7]. The therapy of arthritic rats with NOPEE significantly improved the elevated LPO during the 1st and 2nd weeks, while the treatment with NAR produces a significant decrease only during the 1st week. Both NOPEE and NAR made a significant reduction in the high NO in arthritic rats at both tested periods. Similarly, other reports revealed that NAR has antioxi-

dant, metal chelating, and FR scavenging properties and offers some protection against mutagenesis and LPO [81, 82] and largely, citrus and its flavonoids have antioxidant properties [83].

Tripeptide γ-glutamylcysteinylglycine or GSH is a significant non-enzymatic modulator of intracellular redox homeostasis and is present at millimolar concentrations in all cell types. GSH is an important FR scavenging compound, which catalyzes the conversion of ROS into a less reactive radical. Moreover, in a process known as glutathionylation, GSH can bind to proteins covalently and function as an enzyme cofactor for a variety of defense-related enzymes. Thus, glutathione can actively intercept free radicals (FRs) or serve as a substrate for GPx and GST when lipid hydroperoxides, hydrogen peroxides, and electrophilic chemicals are detoxified [84]. Furthermore, GSH is a crucial tripeptide and an antioxidant that is not enzymatically produced inside of cells, which participates in the removal of FRs, such as superoxide anions, H_2O_2, and alkoxy radicals, and the management of membrane protein thiols and serves as a substrate for GPx and GR [85]. Similarly, GSH, an amino acid derivative with a sulfhydryl group, is a highly unique compound with several crucial functions, including acting as a sulfhydryl buffer to protect cells from oxidative damage. It also has the capacity to prevent oxidative stress, preserve the usual reduced state within the cell, and act as a peroxide scavenger to make up for catalase's diminished role in antioxidant defense [86]. Thus, glutathione plays a fundamental role in combating oxidative stress [87].

In the present findings, a decrease in the content of GSH and GR activity in the liver of arthritic rats was observed. As a result, these decreased GSH levels may be related to the use of GSH with GPx and GST as its substrates [88]. However, treating the arthritic rats with NOPEE significantly increased their liver GSH content during the 1st and 2nd weeks of CFA injection, though the treatment with NAR significantly increased liver GSH content only during the 1st week. These results agreed with the reports of Jagetia and Reddy [72] who stated that NAR as a flavonoid can increase the GSH level and also, Abdel Moneim [89] reported that the citrus peel has a scavenging ability in rats after exposure to oxidative stress.

In the present study, the liver GR activity of the arthritic rats was significantly decreased as a result of treatment with NOPEE and NAR for one week. As the period continued to two weeks, GR activity significantly increased as a result of treatment with NOPEE, while it decreased as a result of NAR treatment. Such discrepancy in the effect of NOPEE and NAR may be due to the suggestion that peel extracts are extremely complicated combinations of many components with varying activity [90, 91].

SOD is crucial to the development and treatment of RA since the extracellular SOD serves as the main catalytic antioxidant in the joint fluid [92]. In the present study, the lowered SOD activity in the arthritic rats during the 1st week was greatly enhanced because of NOPEE and NAR treatment which produced a remarkable but a non-significant effect during the 2nd week. These findings demonstrated in a straightforward manner the efficiency of NOPEE and NAR as anti-arthritic and anti-inflammatory medications in preventing the onset and spread of RA.

TNF-α and ROS commonly influence each other in a positive feedback loop [93] as shown in **Figure 4**. Accordingly, the therapeutic inhibition of TNF-α signaling efficiently inhibits ROS production and reduces joint inflammation in RA [94]. Therefore, it can be suggested that the link between TNF-α and ROS production may have an important role in the development of CFA-induced RA and inhibition of TNF-α and ROS production have crucial role in the therapy of the disease (**Figure 4**).

Conclusion

The NOPEE as well as NAR exhibit strong anti-inflammatory and antioxidant effects in rat RA induced with CFA. Both treatments effectively inhibit the damaging actions in the articular ankle joints. Thus, these therapeutic agents could be applied in the treatment of RA. However, further studies are needed to determine their negative effects on human health.

Disclosure of conflict of interest

None.

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