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Anti-inflammatory Activity of *Pseudobombax ellipticum* (Kunth) Dugand Leaves on Complete Freund's Adjuvant-Induced Arthritis in Rats

Ahmed S. Mohamed[1], Rabab H. Sayed[2], Ali M. El Shamy[1], Fatma S. El Sakhawy[1], Rania A. El Gedaily[1]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr Al-Aini St., Cairo 11562, Egypt*
[2] *Pharmacology and Toxicology Department, Faculty of Pharmacy, Cairo University, Kasr Al-Aini St., Cairo 11562, Egypt*

Abstract

Rheumatoid arthritis (RA) is a chronic progressive disabling autoimmune disease characterized by systemic inflammation of joints leading to damage of cartilage and bone around the joints. The adverse effects of many synthetic drugs, used as standard treatment for rheumatoid arthritis, compromise the therapeutic treatment. There is a prompt need for drugs with improved efficacy and safety profiles. The effect of *Pseudobombax ellipticum* (Kunth) Dugand leaves total extract and its fractions on complete Freund's adjuvant (CFA)-induced arthritis model in rats was investigated. Thirteen groups of 5 rats each were used. CFA-induced arthritis group was compared to 10 groups treated with the plant extracts relative to standard diclofenac treated group and normal control group. The plant extracts attenuated the increase in knee joint diameter and reversed the decrease in body weight as compared to CFA-injected rats. CFA-injected rats displayed a marked elevation in serum tumor necrosis factor (TNF)- α, interleukin (IL)-1β, prostaglandin (PG) E2, and nuclear factor kappa-B (NF-kBp65), while rats treated with plant extracts displayed lowered serum levels of these pro-inflammatory cytokines. Also, the complete destruction and roughness of articular surface, shown in sections from CFA-injected rats, was markedly improved in sections from rats treated with the plant extracts. It is clear that, the picture of the disease was improved morphologically, biochemically and histologically by the plant extracts. Furthermore, 4 compounds were isolated for the first time with previously reported anti-inflammatory activity. These results may reveal the potential role for *P. ellipticum* (Kunth) Dugand leaves extracts as possible anti-inflammatory agent in rheumatoid arthritis management.

Keywords: Rheumatoid arthritis, *Pseudobombax ellipticum,* anti-inflammatory, CFA, rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease which can lead to permanent disability and has a significantly elevated mortality rate[1]. The disease is associated with a chronic inflammatory process, which can damage not only joints but also extra-articular organs, including the lung, heart, kidney, nervous system, eye, skin and digestive system[2]. Recent studies reported that NF-kBp65 is one of the main inflammatory transcription factors in RA, which stimulates the production of various pro-inflammatory cytokines such as (PG) E2, (TNF)- α and (IL)-1β, which are the key players in initiation and progression of RA[3].

Non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs (DMARDs) and biological agents were used for symptomatic relief and to help maintain joint function in RA patients, but these medications exhibit severe adverse effects such as hypertension, gastric ulcers, renal abnormalities and hepatotoxicity[3]. Further research is needed to discover new agents for

*Corresponding author e-mail: ahmed.abobakr@pharma.cu.edu.eg.(Ahmed S. Mohamed).
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management of RA with improved efficacy and safety profiles[2].

Members of the family Bombacaceae were used traditionally in many countries due to their analgesic, antipyretic and anti-inflammatory activities[4]. Members of the genus *Pseudobombax* exhibited antibacterial, antifungal, cancer chemopreventive, anti-Alzheimer's and HIV- 1 reverse transcriptase inhibitory activities and they were reported to be rich in phenolic acids, procyanidins, flavonoids, tannins and terpenoids[5][6]. *Pseudobombax marginatum* Robyns bark hydro-alcoholic extract exhibited potent anti-inflammatory and antinociceptive effects, it is therapeutically indicated for spinal inflammation and its tea (infusion) is used for pelvic inflammatory disease[7]. Few reports were found, concerning the chemical composition and biological activities of *P. ellipticum* (Kunth) Dugand. The triterpene β-lupeol was isolated from its stem bark and showed potent gastro-protective effect[8]. The anthocyanin cyanidin-3,5-diglucoside was isolated from it's flowers[4]. Recently it's flowers exhibited potent antisickling activity against sickle cell anemia and eight anthocyanins were identified for the first time using liquid chromatography mass spectrometry (LC/MS)[9]. Also, from *P. ellipticum* (Kunth) Dugand and cultivar alba (Hort.) leaves, bark and flowers, 89 compounds of different classes like simple phenols, flavonoids, fatty alcohols, terpenoids, and fatty acids were identified using ultra high performance LCMS-based molecular networking in negative and positive modes with promising anti-Alzheimer activity[6].

Investigating the anti-inflammatory activity of *P. ellipticum* (Kunth) Dugand leaves on CFA-induced arthritis model in rats with isolation and identification of its major phytoconstituents is the aim of this study.

2. Material and methods

2.1. Plant material

P. ellipticum (Kunth) Dugand leaves were collected from Mostafa El Abed botanical garden, during September 2019 at the pre flowering stage. Dr. Mohamed El Gibali, Senior Botanist & Consultant at Orman Botanic Garden, Giza, Egypt, kindly carried out the plant identification. A vouchered specimen of the plant,with serial number (20.09.2022) was kept at the Pharmacognosy Department, Faculty of Pharmacy, Cairo University. The collected leaves (large palmately compound with five elliptical rounded dark-green leaflets) were dried in shade, finely powdered and the powder was used for further analysis.

2.2. Extraction, fractionation and isolation

The 3 weeks air dried powdered leaves (5 kg) were extracted with 70% ethanol (20 L) for three times in a closed cylindrical glass container (20*50 cm) at room temperature at one week intervals. The total hydro-ethanolic extract (THEE) was evaporated under reduced pressure in a rotary evaporator (Büchi, Switzerland) at temperature of 60 °C, yielding a dark blackish-green residue (400 g)[10].

THEE (200 g) was suspended in distilled water (500 mL) and successively partitioned between n-hexane (500 mL, 3 times), methylene chloride (MC) (500 mL, 3 times), ethyl acetate (EtOAc) (500 mL, 3 times) and water-saturated n-butanol (500 mL, 3 times). Under reduced pressure, each fraction was concentrated to give n-hexane (40 g), MC (8 g), EtOAc (18 g) and water-saturated n-butanol (10 g), respectively[11].

A part of n-hexane fraction (20 g) was chromatographed on vaccum liquid chromatography (VLC) column (10 cm diameter) packed with silica H (130 g) using petroleum ether–acetone gradients (10 : 0, 9 : 1, 8 : 2, 6.5 : 3.5 and 5 : 5, v/v) collecting 5 fractions (1.5 L each) to afford **compound 1** (16 mg) and **compound 2** (14 mg) as pure crystals in fractions 2 and 3, respectively.

Also, a part of MC fraction (7 g) was chromatographed on VLC column (5 cm diameter) packed with silica H (70 g) using methylene chloride –methanol gradients (10 : 0, 9.8 : 0.2, 9.5 : 0.5, 9 : 1, 8.5 :0.5, 8 : 2, 7 : 3 and 6 : 4, v/v) collecting 56 fractions (250 ml each). Fractions 27-29 were collected together (0.45 g) and subjected to further fractionation in column packed with silica gel G (25 g) using methylene chloride –methanol gradients (10 : 0, 9.9 : 0.1, 9.7 : 0.3, 9.5 : 0.5, 9 : 1and 8.5 :0.5, v/v) collecting 13 sub fractions (20 ml each). Residue in sub fractions 11 and 12 was purified by hexane to afford **compound3** (10 mg).

Furthermore, a part of EtOAc fraction (10 g) was also chromatographed on VLC column (5 cm diameter) packed with silica H (80 g) starting with ethyl acetate-methanol gradient (10 : 0 v/v) collecting

fractions from 1-7 (0.5 L each) followed by gradients (9.5 : 0.5, 9 : 1, 8 : 2 and 7 : 3, v/v) collecting fractions from 8-11 (0.5 L each). Fractions 3 and 4 were collected together (0.5 g) and subjected to further fractionation in column packed with silica gel G (25 g) starting with ethyl acetate-methanol gradient (10: 0 v/v) collecting sub fractions from 1-17 (20 ml each) followed by gradient (9.9: 0.1v/v) collecting fractions from 18-24 (20 ml each).The sub fraction 23 (40 mg) was purified using sephadex column to afford **compound4** (25 mg).

2.3. ^{1}H-NMR, ^{13}C NMR, TLC and TLC-MS analysis

The ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H-NMR and ^{13}C NMR) spectra were recorded on a Bruker High Performance Digital FT-NMR Avance III 400 MHz spectrometer. Each isolated molecule (1.5–2 mg) was dissolved in 0.7 ml of deuterated solvent in centrifugation tube followed by sonication for 5 min and finally transfered into 5 mm diameter NMR tubes. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F254 TLC Plate Merck in normal phase. The reagent used for visualizing TLCs plates was *p*-Anisaldehyde. Thin-layer chromatography coupled to mass spectrometry (TLC-MS) was also made on 60 F254 (Merck) silica gel plates in normal phase coupled to Advion compact mass spectrometer (CMS) NY | USA[12].

2.4. Animals

Male adult Wistar rats weighing 150-200 g were obtained from the Egyptian Drug Authority, Cairo, Egypt. Rats were kept under controlled environmental conditions of constant temperature (25 ◦C ± 2 ◦C), humidity (60% ± 10%) and a 12/12-h light/ dark cycle. Rats were allowed to acclimatize for one week before the experiment. A standard chow diet and water were provided ad libitum. The experiment was approved by the Ethics research committee of Faculty of Pharmacy, Cairo University [Approval number: MP (3122)], and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised 2011).

2.5. Experimental design

Sub-plantar injection of 100 µL of CFA in the right hind paw was used to induce arthritis in rats[13], while the left paw served as control and received 100 µL of saline. 100 µL of CFA was injected in the same right hind paw after 7 days to enhance secondary arthritis. After 14 days, rats were randomly divided into 13 groups of 5 rats each (group size was based on a power analysis (power = 0.8, α = 0.05) using effect sizes previously determined[14]), and treated as follows: 1) CFA-induced arthritis (CFA), 2) CFA-induced arthritis treated with diclofenac (3 mg/kg, p.o) (DIC)[15], 3)CFA-induced arthritis treated with THEE (100 mg/kg, p.o) (TE 100), 4)CFA-induced arthritis treated with THEE (200 mg/kg, p.o) (TE 200), 5)CFA-induced arthritis treated with hexane fraction (100 mg/kg, p.o) (HF 100), 6)CFA-induced arthritis treated with hexane fraction (200 mg/kg, p.o) (HF 200), 7)CFA-induced arthritis treated with MC fraction (100 mg/kg, p.o) (MCF 100), 8)CFA-induced arthritis treated with MC fraction (200 mg/kg, p.o) (MCF 200), 9)CFA-induced arthritis treated with EtOAc fraction (100 mg/kg, p.o) (EF 100), 10)CFA-induced arthritis treated with EtOAc fraction (200 mg/kg, p.o) (EF 200), 11)CFA-induced arthritis treated with butanol fraction (100 mg/kg, p.o) (BF 100), 12)CFA-induced arthritis treated with butanol fraction (200 mg/kg, p.o) (BF 200). Oral administration of either diclofenac or plant extracts was started on day 14 and continued for till day 28. A separate group of animals was run concurrently as a normal control and received 0.1% tween 80 the vehicle of the extracts.

2.6. Morphological, biochemical and histopathological analysis

The anti-inflammatory activity was assessed by measuring knee joint diameter on days 0, 14, 17, 20, 23, 24 and 28 following CFA injection using digital electronic caliper. The body weight of rats was also measured on days 0, 14, 17, 20, 23, 24 and 28. After assessing the anti-inflammatory activity on day 28, blood samples were withdrawn from the retro-orbital plexus under light anesthesia and sera were separated to be used in measuring inflammatory markers.

Animals were then euthanized by cervical dislocation; hind paws were removed and fixed in 10% formalin to be used in histopathological investigation. Specimens were decalcified in formic acid 10%, trimmed, washed in water, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embeded in paraffin. Thin sections (4-6 µm) were processed and stained with Hematoxylin & Eosin stain(H&E)[16].

The serum levels of pro-inflammatory cytokines (TNF-α, IL-1β, PGE2, and NF-kBp65) were assessed using rat ELISA kits obtained from MyBiosource

(San Diego, USA) according to the manufacturer's instructions.

2.7. Statistical analysis

As mean ± S.E, data are presented and using one-way ANOVA followed by Tukey's multiple comparison, results were analyzed. To carry out the statistical analysis and to create the graphical presentations, the GraphPad Prism software (version 8; GraphPad Software, Inc., San Diego, CA, USA) was used. A probability level of <0.05 was accepted as statistically significant in all statistical tests.

3. Results and Discussion

3.1. Isolation of the main active constituents of *P. ellipticum* (Kunth) Dugand leaves

Four compounds were isolated for the first time from the plant under study. The triterpene, lupeol acetate (C1) and the sterol, *β*-sitosterol (C2) were isolated from hexane fraction, while its glycoside, *β*-sitosterol-glucoside (C3) was isolated from chloroform fraction. Finally, the flavonoid glycoside, quercetin-3-*O*-rhamnoside (C4) was isolated from ethyl acetate fraction. The presence of these compounds could be linked to the observed anti-inflammatory properties of the plant. **Compound** 1 was isolated as a white crystalline solid. It showed a single spot on TLC with Rf 0.8 (hexane / ethyl acetate, 9.5: 0.5). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) and ^{13}C NMR (CDCl3, 100 MHz) were agreed to previously reported data[17]. From these spectroscopic data (Fig. 1), the isolated compoundwas identified as lupeol acetate.

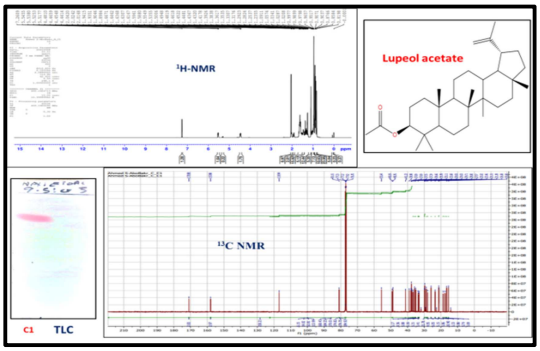

Fig. 1: The ^{1}H-NMR and ^{13}C NMR spectrums, structure and TLC of compound 1(Lupeol acetate).

Compound 2 was isolated also as a white crystalline solid. It showed a single spot on TLC with Rf 0.47 (hexane / ethyl acetate, 8: 2). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) was similar to previously reported data[18]. From the ^{1}H-NMR spectrum and Co-TLC with authentic sample (Fig. 2), the isolated compound was identified as *β*-sitosterol.

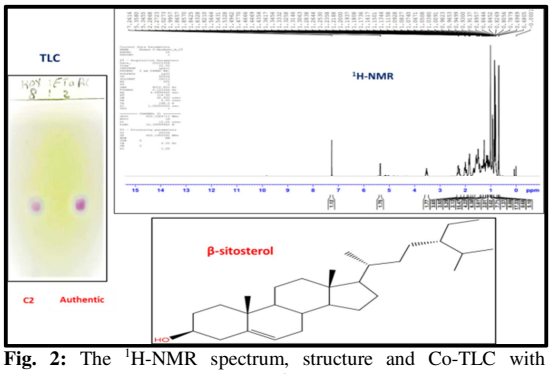

Fig. 2: The ^{1}H-NMR spectrum, structure and Co-TLC with authentic sample of compound 2 (*β*-sitosterol).

Compound 3 was isolated as a white powder. It showed a single spot on TLC with Rf 0.8 (chloroform / methanol, 8: 2). The ^{1}H-NMR spectrum (400 MHz, CDCl3) was in accordance with the previously reported data[19]. From the ^{1}H-NMR spectrum and Co-TLC with authentic sample (Fig. 3), the isolated compound was identified as *β*-sitosterol-glucoside.

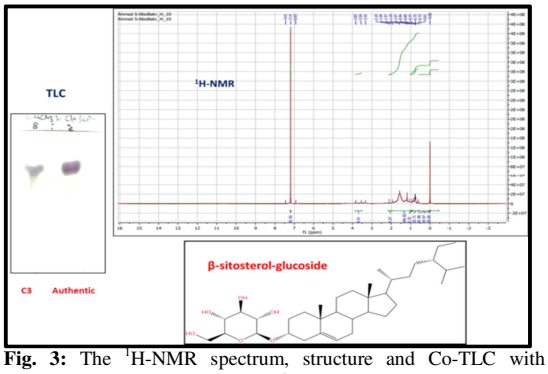

Fig. 3: The ^{1}H-NMR spectrum, structure and Co-TLC with authentic sample of compound 3 (*β*-sitosterol-glucoside).

Compound 4 was isolated as a yellow crystalline solid. It showed a single spot on TLC with Rf 0.6 (ethyl acetate / methanol / water, 100: 13.5: 10). Its TLC-MS had a molecular ion base peak [M-H] at m/z = 447 corresponding to the molecular formula $C_{21}H_{19}O_{11}$. The ^{1}H-NMR spectrum (400 MHz, CH3OH) was similar to the previously reported data[20]. From these spectroscopic data (Fig. 4), the isolated compound was identified as quercetrin (quercetin-3-*O*-rhamnoside).

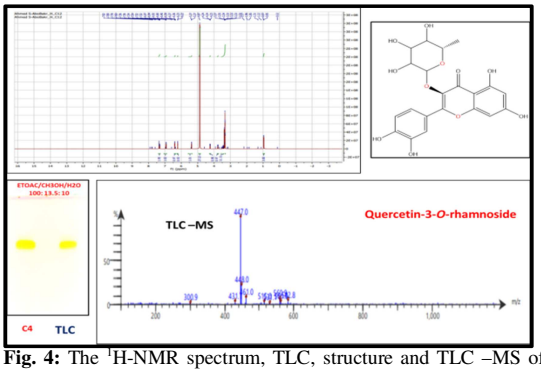

Fig. 4: The ^{1}H-NMR spectrum, TLC, structure and TLC –MS of compound 4 (Quercetrin).

3.2. Effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced changes in body weight and joint diameter

Body weight and knee joint diameter were measured to assess the progression of rheumatoid arthritis. As shown in fig. 5A, body weight of rats started to decrease after 14 days post-CFA injection as compared to control rats. This decrease was opposed starting from day 17 post-CFA injection by HF 200 treatment only as compared to CFA group. However, the body weight decrease was reversed by DIC, TE 200, HF 100, MCF 200, EF 200, BF 100 and BF 200 treatment starting from day 20 post-CFA injection. Likewise, knee joint diameter of rats started to significantly increase 14 days post-CFA injection (Fig. 5B). Treatment with DIC, TE 200, HF 100, HF 200, MCF 200, EF 200, BF 100 and BF 200 started to attenuate the increase in knee joint diameter from day 20 post-CFA injection as compared to CFA-injected rats.

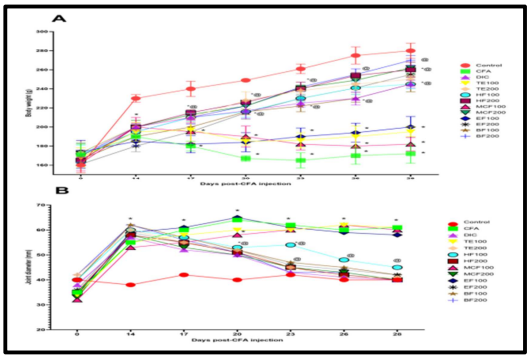

Fig. 5: Effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced changes in (A) Body weight and (B) knee joint diameter. Values are expressed as mean ± S.E. (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. * *vs* Control, @ *vs* CFA at p<0.05.

3.3. Effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced changes in serum inflammatory biomarkers

The characteristic inflammation of RA occurs due to the abundance of the pro-inflammatory cytokines. CFA-injected rats revealed a marked elevation in serum TNF-α, IL-1β, PGE2 and NF-κB p65 to reach 7.8-, 4-, 2-, and 3-folds the control group values, respectively. However, rats treated with DIC, TE 200, HF 100, HF 200, MCF 200, EF 200, BF 100, and BF 200 revealed lowered serum TNF-α levels to reach 34.3%, 38.8%, 50.7%, 36.5%, 40.7%, 50.4%, 51.9%, and 36.3% the CFA-group values, respectively (Fig. 6A). Besides, treatment with DIC, TE 200, HF 200, MCF 200, EF 200, BF 100, and BF 200 decreased serum IL-1β levels to reach 40.6%, 62.3%, 53.1%, 66.3%, 70.9%, 73% and 44.4%, respectively as compared to CFA-treated rats (Fig. 6B). Treatment with DIC, TE 200, HF 100, HF 200, MCF 200, EF 200, BF 100, and BF 200 also succeeded to reduce serum PGE2 levels to reach 65.8%, 74.3%, 83.6%, 73.8%, 75%, 74.5%, 71%, and 66.1% (Fig. 6C). Finally, rats treated with DIC, TE 200, HF 100, HF 200, MCF 200, EF 200, BF 100, and BF 200 exhibited lowered serum levels of NF-κB p65 to reach 38.7%, 52.6%, 79.5%, 58.3%, 59.2%, 52.9%, 70.7%, and 41.6% the CFA-group values, respectively (Fig. 6D).

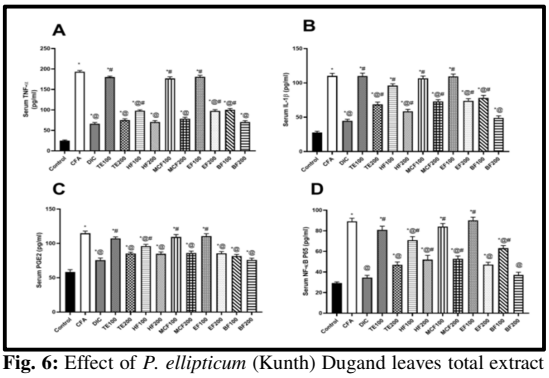

Fig. 6: Effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced changes in serum inflammatory markers: (A) TNF-α, (B) IL-1β, (C) PGE2, and (D) NF-κB p65. Values are expressed as mean ± S.E. (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. * *vs* Control, @ *vs* CFA, # *vs* DIC at p<0.05.

3.4. Effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced histopathological changes

Microscopic examination of joint sections from the control group revealed normal histological structure (Fig. 7A). In contrast, sections from CFA-injected

rats showed complete destruction and roughness of articular surface as well as substitution by fibrous connective tissue (Fig. 7B). Sections from rats treated with DIC, HF 200, BF 100, and BF 200 demonstrated marked improvement in the histological structure, where the articular surface was apparently normal (Fig. 7C, G, L and M, respectively). However, section from rats treated with TE 200, HF 100, MCF 200, and EF 200 showed partial improvement in the histological structure with partial roughness of the articular surface as well as partial replacement with fibrous connective tissue (Fig. 7E, F, I, and K, respectively). Sections from rats treated with TE 100, MCF 100, and EF 100 showed no improvement in the histological structure as compared to CFA group (Fig. 7D, H, and J, respectively).

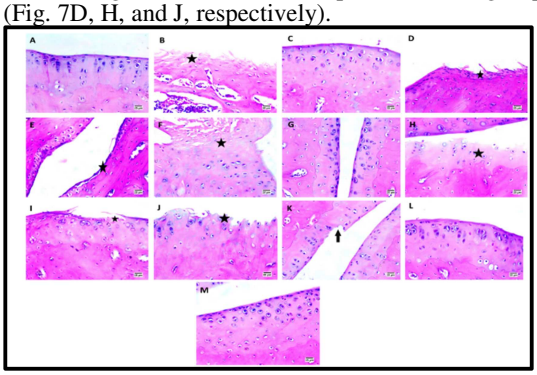

Fig. 7: Effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced histopathological changes. Representative photomicrographs for sections of the rat hind paw stained with H&E. **A**: Control group showing normal histological structure of articular surface; **B:** CFA group showing complete destruction of articular surface and substitution by fibrous connective tissue (star); **C:** DIC group showing apparently normal articular surface; **D:** TE 100 group showing complete destruction of articular surface as well as substitution by fibrous connective tissue (star); **E:** TE 200 group showing partial destruction of articular surface (star); **F:** HF 100 group showing partial destruction of articular surface (star); **G:** HF 200 group showing apparently normal articular surface; H: CF 100 group showing complete destruction of articular surface (star); I: CF 200 group showing partial destruction of articular surface (star); J: EF 100 group showing complete destruction of articular surface and roughness (star); K: EF 200 group showing partial destruction of articular surface (star), and L and M: BF 100 and 200 groups showing normal histological structure of articular surface.

4. Discussion

CFA-induced arthritis in rats has been employed widely as a model for chronic systemic inflammation as it possesses many features in common with human RA[13]. Pro-inflammatory cytokines play an important role in RA. NF-kB p65 is one of the main inflammatory transcription factors in RA, which stimulates the production of various pro-inflammatory cytokines such as PGE2, TNF-α and IL-1β that are the key players in initiation and progression of RA. A great improvement of the CFA-induced arthritis in rats upon treatment with *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions was achieved relevant to the standard diclofenac. The improvement was at three levels, morphologically, biochemically and histologically. Morphologically, the plant extracts attenuated the increase in knee joint diameter and reversed the decrease in body weight as compared to CFA-injected rats. Biochemically, the serum levels of the highly important inflammatory mediators TNF-α, IL-1β, PGE2 and NF-κB p65 were markedly decreased in rats treated by the plant extracts as compared to CFA-injected rats. Finally, histologically, the complete destruction and roughness of articular surface showed in CFA-injected rats was improved and appeared apparently normal in rats treated by the plant extracts. Furthermore, the major constituents of *P. ellipticum* (Kunth) Dugand leaves were isolated in an attempt to discover the constituents responsible for the anti-inflammatory effect. All of the isolated compounds have been reported to have both *in-vivo* and *in-vitro* anti-inflammatory activity. Lupeol acetate isolated from *Himatanthus drasticus* (Mart.) Plumel exhibited *in-vivo* and *in-vitro* anti-inflammatory activity in several models of inflammation[21]. Also, lupeol acetate isolated from *Balanophora spicata* Possessed antinociceptive and anti-inflammatory activities *in vivo* and *in vitro*[22]. β -sitosterol isolated from *Nyctanthes arbortristis*leaves exhibited *in-vivo* analgesic and anti-inflammatory activity[18]. β -sitosterol and β-sitosterol-glucoside were isolated as analgesic constituents from the leaves of *Mentha cordifolia* Opiz[19]. β-sitosterol-glucoside isolated from *Trachelospermum jasminoides* (Apocynaceae) exhibited anti-inflammatory effects in lipopolysaccharide-stimulated RAW 264.7 murine macrophages[23]. Finally, quercetin-3-*O*-rhamnoside isolated from Burdock (*Arctium lappa* L.) leaf ameliorated lipopolysaccharide- induced inflammation and oxidative stress in RAW264.7 cells[24].

5. Conclusion

The effect of *P. ellipticum* (Kunth) Dugand leaves total extract and its fractions on CFA-induced arthritis in rats was investigated for the first time. All extracts exhibited a promising anti-inflammatory

activity relevant to the standard diclofenac. Furthermore, 4 compounds were isolated for the first time with previously reported anti-inflammatory activity. Further research is required for the identification and the isolation of more active constituents and to measure the probability of the plant in the management of several health problems.

6. Authorship contribution statement

Ahmed S. Mohamed: Methodology, Software, Investigation, Writing- Original draft **RababH. Sayed**: Methodology, Software and Investigation **Ali M. El Shamy**: Conceptualization, Supervision, Writing - Reviewing and Editing **Fatma S. El Sakhawy**: Conceptualization, Supervision, Writing - Reviewing and Editing **Rania A. El Gedaily**: Conceptualization, Validation, Supervision, Writing - Reviewing and Editing.

7. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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