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Polygonatum kingianum rhizome extract alleviates collagen antibody−induced arthritis by modulating proinflammatory cytokine production in mice

Huyen Do Thi Thanh[1,2], Phuong Ngo Thi[2], Hien Nguyen Thi Thu[3], Nga Nguyen Thi[4], Hung Le Ngoc[5], Thao Do Thi[1,4], Ha Le Minh[1,2]✉

[1]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam*

[2]*Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam*

[3]*Hanoi Open University101 Nguyen Hien str, Hai Ba Trung Dist., Hanoi, Vietnam*

[4]*Laboratory of Bioassay, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam*

[5]*Chemistry Lab Project, Center for Research and Technology transfer, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam*

ABSTRACT

Objective: To evaluate the anti-arthritic effects of *Polygonatum kingianum* rhizome extract using both *in vitro* and *in vivo* models.

Methods: Lipopolysaccharide-induced RAW 264.7 macrophages were treated with an ethanol extract of *Polygonatum kingianum* rhizomes at different concentrations to determine nitric oxide and prostaglandin E_2 (PGE_2) production. For *in vivo* study, *Polygonatum kingianum* ethanol extract was further investigated for its anti-inflammatory effect in a mouse model with collagen antibody-induced arthritis. Phytochemical study of *Polygonatum kingianum* ethanol extract was also performed.

Results: Saponins (142 mg/g total yield) was the main component in the *Polygonatum kingianum* ethanol extract. 5α,8α-ergosterol peroxide, (*E*,*E*)-9-oxooctadeca-10,12-dienoic acid and 3-(2′-hydroxy-4′-methoxy-benzyl)-5,7-dihydroxy-8-methyl-chroman-4-one were isolated from the extract. *Polygonatum kingianum* ethanol extract exhibited potential anti-inflammatory effects by inhibiting nitric oxide and PGE_2 production in RAW 264.7 cells in a dose-dependent manner. The level of arthritis in mice with collagen antibody-induced arthritis was significantly reduced (P<0.01) after treatment with *Polygonatum kingianum* ethanol extract, particularly at a dose of 1 000 mg/kg body weight. Besides, the extract demonstrated the regulatory effects on serum tumor necrosis factor-alpha, interleukin-6, and interleukin-10 in treated mice.

Conclusions: *Polygonatum kingianum* ethanol extract has beneficial effects on inflammatory cytokine regulation and PGE_2 inhibition in an experimental mouse model with collagen antibody-induced arthritis. The phytochemical screening reveals that the saponin, as the main component, and sterols (daucosterol and 5α,8α-ergosterol peroxide) from *Polygonatum kingianum* ethanol extract may contribute to its promising *in vitro* and *in vivo* anti-inflammatory activities.

KEYWORDS: Anti-inflammatory; Cytokines; *Polygonatum kingianum*; Rheumatoid arthritis

1. Introduction

Polygonatum kingianum (*P. kingianum*) Coll. & Hemsl. (Family: Liliaceae) is a plant distributed across the temperate and subtropical regions of Asia, mainly in China, Laos, and Vietnam[1]. The plant rhizomes have been used in Chinese traditional medicine as a tonic and remedy to treat lung diseases, upset stomachs, diabetes, hyperlipidaemia, related metabolic syndromes, and ringworm[2−4]. In Vietnam, the plant is a valuable herb for the treatment of tuberculosis, haemoptysis, angina, coronary artery disease, diabetes, hypotension, autonomic nervous system disorders, back pain, joint pain and arthritis including osteoarthritis[1]. Previous phytochemical investigations of this plant's rhizomes have reported the isolation of steroidal saponins, flavonoids, alkaloids, phenolics, fructose

✉To whom correspondence may be addressed. E-mail: halm2vn@gmail.com

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derivatives, and phytosterols[5,6]. However, very few biological studies on the plant have been published. To date, there are only two reports on the antidiabetic effects of total saponins and the antimicrobial activities of some compounds from *P. kingianum*[2,7]. Therefore, additional studies on the biological activities of compounds from this plant should be conducted to explore the usage of the plant in folk medicine. In Vietnamese traditional medicine, the rhizomes of *P. kingianum* are treated by steaming in hot water and drying before use to enhance their effects and prevent itchiness[1]. In the present paper, we aimed to evaluate the anti-inflammatory effects of an ethanol extract of *P. kingianum* rhizomes in both *in vitro* and *in vivo* models.

2. Materials and methods

2.1. Preparation of ethanolic extract of P. kingianum rhizomes

The fresh rhizomes of *P. kingianum* were collected in Ha Giang, Vietnam in September 2017. The scientific name was identified by Dr. Nguyen Van Du, Vietnam Academy of Science and Technology (VAST). The voucher samples (HTHĐ-09.2017) have been deposited at VAST. The fresh rhizomes of *P. kingianum* were washed, steamed in hot water, and then dried. The process was repeated 9 times until the rhizomes became soft and black according to the processing method used in traditional Vietnamese medicine[1]. The treated rhizomes were sliced and dried in an oven at 40 ℃. The obtained dried powder (1.0 kg) was soaked in 95% ethanol (each 4.5 litres, 3 times) at room temperature for 24 h. The extract was then filtered and concentrated under reduced pressure in a rotary evaporator to yield a residue of the ethanol extract (87.5 g, brown solid). The ethanol extract of *P. kingianum* rhizomes was stored at –20 ℃ for further phytochemical characterization and pharmacological tests.

The main phytochemical components contained in the ethanol extract of *P. kingianum* rhizomes were screened (test for terpenoids; sterols, flavonoids; saponins) using standard procedures[8,9].

2.2. Extraction and isolation of several main compounds from P. kingianum rhizome extract

The ethanol extract of *P. kingianum* rhizomes (29.4 g) was suspended in water, partitioned with *n*-hexane, ethyl acetate, *n*-butanol to obtain fractions: *n*-hexane (5.2 g), ethyl acetate (15.0 g) and *n*-butanol (7.0 g). The *n*-hexane extract (5.2 g) was applied to silica gel CC with *n*-hexane: ethyl acetate (50:1, v:v) to give five fractions (H1 to H5). Fraction H2 (0.87 g) was firstly subjected to silica gel CC with *n*-hexane: ethyl acetate (10:1; v:v) and then recrystallized in *n*-hexane: acetone (4:1; v:v) to yield compound (1) (255 mg). Compound (2) (18 mg) was obtained from fraction H3, silica gel CC, dichloromethane: ethyl acetate (40:1; v:v). The ethyl acetate extract (15.0 g) was subjected to silica gel CC with dichloromethane: ethyl acetate (10:1; v:v) to give five fractions (E1 to E5). Fraction E1 (3.6 g) was subsequently separated using YMC RP-18 CC with methanol: water (1:3, v:v) to yield compound (3) (48 mg). Compound (4) (52 mg) was obtained from fraction E3 by silica gel CC with chloroform: methanol (15:1; v:v). The *n*-butanol (7.0 g) was dissolved in distilled water (0.1 g/mL) and then purified with D101 macroporous resin. It was washed with distilled water (3 times) to remove polysaccharide and eluted with 85% ethanol eluent (5 times). The eluant was collected and condensed to give 4.2 g of total saponin, the yield was 142.8 mg/g of the ethanol extract of *P. kingianum* rhizomes.

2.3. Chemicals

Prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 commercial ELISA kits were purchased from Biovision Inc. (Chester Springs, PA, USA). Lipopolysaccharides (LPS) and all reagents for cell culture were obtained from Sigma-Aldrich Chem. Co. (St. Louis, MO., USA). The anti-collagen antibodies cocktail was provided by Modi Quest Research (Nijmegen, The Netherlands).

2.4. Cell culture and treatment

The RAW 264.7 macrophage cells (ATCC® TIB-71™) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% antibiotic (Anti-Anti, Sigma-Aldrich Chem. Co.) at 37 ℃, 5% CO_2 incubation. Cells were subcultured every 2-3 days. To assess nitric oxide (NO) or PGE_2 inhibitory activities, 1×10^5 RAW 264.7 cells in Dulbecco's Modified Eagle Medium were incubated in the 96-well plates overnight. Different concentrations of the ethanol extract of *P. kingianum* rhizomes were added into the pre-seeding wells before LPS stimulation for 24 h at 37 ℃ in a CO_2 incubator. The medium suspension was collected for NO and PGE_2 determination. The remained cells were used for examining cellular viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of routine[10]. The Griess assay was used for NO determination[11]. Meanwhile, the PGE_2 level in cell medium was measured by the PGE_2 kit (Biovision Inc., Chester Springs, PA, USA) following the manufacturer's guidance.

2.5. Anti–inflammatory evaluation using collagen antibody–induced arthritic mice (CAIA)

Healthy albino BALB/c mice (8–10 weeks old) were obtained from the Institute of Biotechnology, VAST (Hanoi, Vietnam). Mice were housed in a climate-controlled room (25 ± 2) ℃ with a 12-hour light/12-hour dark cycle. Standard food and water were provided.

To induce CAIA, 24 male BALB/c mice (randomly 10-week-old mice) were injected intraperitoneally (*i.p.*) with 3 mg of the cocktail of anti-collagen antibodies on day 0 and following with intravenous injection with 25 μg of LPS on day 3 and day 9 to boost arthritic development. Mice having initial symptoms of arthritis on day 4 were divided into 4 groups (6 mice/group). The negative control (group 1) received water daily for 10 d; the treatment groups (groups 2 and 3) were treated with the ethanol extract of *P. kingianum*

rhizomes at doses of 500 and 1 000 mg/kg b.w. daily by intra-gastric gavage for 10 d, respectively; the positive control (group 4) orally received dexamethasone at a dose of 0.5 mg/kg b.w. daily. Due to the results from a previous acute study, which proved that ethanol extracts of *P. kingianum* rhizomes had no effects on survival rate, body weight, food, or water consumption in mice up to 5 000 mg/kg b.w., the extract was administered at 500 and 1 000 mg/kg b.w. for the anti-arthritis test. The score of clinical arthritis was recorded depending on the degree of inflammation and swelling[12]. In CAIA, redness and swelling of joints are symptoms used to score arthritis; lower arthritis scores indicate lower arthritic pathogenesis. At the end of the study, the hind paws of 3 experimental mice per group were collected, fixed immediately in 10% neutral formalin for pathological analyses. After decalcifying, all tissues were embedded in paraffin. Finally, 3 mm thickness of joint sections were stained by hematoxylin and eosin (H&E) for histopathological analyses[13]. The production of IL-6, IL-10, and TNF-α in sera of all experimental mice was also measured by using Biovision ELISA kits following the manufacturer's protocols.

2.6. Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego CA). One-way analysis of variance (ANOVA) followed by Bonferroni post-test was applied to compare statistically the difference between the mean of sample and control while two-way ANOVA and Bonferroni post-test was used for comparing among independent groups at diference time points. In all comparisons, $P<0.05$ was considered statistically significant.

2.7. Ethical statement

The care and use of laboratory animals were executed in conformity with Vietnamese Ethical Laws, European Communities Council Directives of November 24, 1986 (86/609/EEC), guidelines and Approval from Scientific Council of Institute of Biotechnology, VAST dated on January 19, 2019.

3. Results

3.1. Phytochemical screening of P. kingianum ethanol extracts

Phytochemical screening of *P. kingianum* ethanol extracts showed that it contained mainly terpenoids, sterols, flavonoids; saponins compounds. Saponins were found to be the main component with a total saponin yield of 142 mg/g of the extract. From *P. kingianum* ethanol extracts, four compounds were isolated and determined to be 5α,8α-ergosterol peroxide (1); (*E*,*E*)-9-oxooctadeca-10,12-dienoic acid (2); 3-(2′-hydroxy-4′-methoxy-benzyl)-5,7-dihydroxy-8-methyl-chroman-4-one (3) and daucosterol (4). Their structures had been elucidated by detailed spectroscopic analyses, including 1D and 2D NMR techniques and along with references (data not shown)[14–17].

3.2. Effect on NO production

The *in vitro* anti-inflammatory effects of *P. kingianum* ethanol extracts on LPS-induced RAW 264.7 macrophages were investigated by measuring NO level. As shown in Table 1, the NO production was inhibited by *P. kingianum* ethanol extracts in a dose-dependent manner. The extract at 20 and 100 μg/mL exhibited potential anti-inflammatory properties by clearly inhibiting more than 30% NO production compared with non-treated cells. The extract presented 50% NO inhibition activity (IC_{50}) at (41.68 ± 4.65) μg/mL. However, little effect was observed at the lower concentrations. The ethanol extracts of *P. kingianum* rhizomes also had minimal impacts on RAW 264.7 cell viability; more than 80% of cells were alive after treatment of 100 μg/mL of the extract for 24 h.

3.3. Effect on PGE_2 production

Besides decreased NO production, PGE_2 levels were also significantly lower in LPS-induced cells treated with 20 and 100 μg/mL of ethanol extracts of *P. kingianum* rhizomes than that of non-treated cells ($P<0.01$) (Figure 1). However, at 4 μg/mL, the extract could not inhibit PGE_2 secretion.

Table 1. Inhibitory effects of ethanol extracts of *Polygonatum kingianum* rhizomes at different concentrations on NO production and cell viability.

Concentration (μg/mL)	NO inhibition (%)	Cell viability (%)
0.8	0.79 ± 1.57	98.53 ± 2.83
4	5.07 ± 3.14	99.53 ± 0.28
20	32.02 ± 0.90	95.73 ± 0.94
100	86.87 ± 0.45	82.27 ± 5.66

Data are expressed as mean ± SD. NO: Nitric oxide.

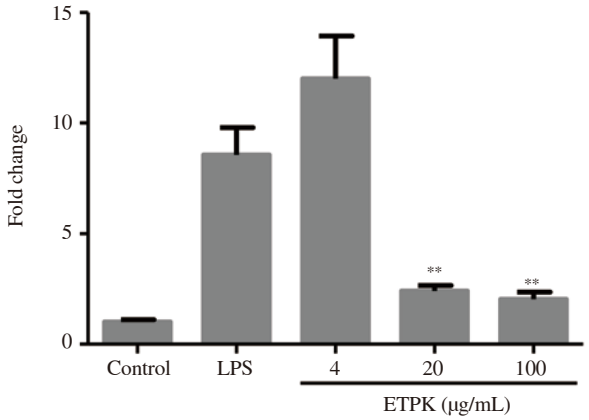

Figure 1. Effects of ethanol extracts of *Polygonatum kingianum* (*P. kingianum*) rhizomes on PGE_2 production. Data are expressed as mean ± SD of three independent experiments. One-way ANOVA followed by Bonferroni post-test was used for statistical comparison between the treatment and control groups. $^{**}P<0.01$. ETPK: ethanol extracts of *P. kingianum* rhizomes.

3.4. Effect on arthritis score

Based on the promising *in vitro* anti-inflammatory results, we decided to search for activities of ethanol extracts of *P. kingianum* rhizomes at an *in vivo* level using CAIA. In the CAIA model, the joints of mice in the placebo group showed serious inflammatory symptoms, such as red and swollen digits and paws. Arthritis score in this group was gradually increased with time, and at the highest level at day 10. In contrast, starting from day 6, mice exposed to 1 000 mg/kg b.w. of ethanol extracts of *P. kingianum* rhizomes exhibited a significant reduction in these symptoms and arthritis scores compared with the control (Supplementary Figure). The strong anti-rheumatic activities of the tested extract at this dosage prolonged till the end of the experiment. On the day 14, the arthritis scores in the group treated with ethanol extracts of *P. kingianum* rhizomes (1 000 mg/kg b.w.) were decreased by nearly 30% compared with the control group (Figure 2). However, mice in the group treated with 500 mg/kg b.w. of ethanol extracts of *P. kingianum* rhizomes could not effectively reduce the inflammatory symptoms and arthritic scores at all tested time points.

3.5. Histopathological results

The histopathology of the joints in the control group revealed strong oedema, inflammatory leukocytes, macrophages, and synovial hyperplasia (Figure 3). Inflammatory cell infiltration and oedema in the joints were reduced by administration of 1 000 mg/kg b.w. of ethanol extracts of *P. kingianum* rhizomes.

3.6. Effect of ethanol extracts of P. kingianum rhizomes on cytokine production

As displayed in Figure 4A, 1 000 mg/kg b.w. of ethanol extracts of *P. kingianum* rhizomes remarkably suppressed the production of TNF-α by almost 48% compared with the vehicle control ($P< 0.05$). Another proinflammatory cytokine related to arthritis, IL-6, was also evaluated. Administration of the extract repressed IL-6 production in a concentration-dependent manner (Figure 4B). Of the two

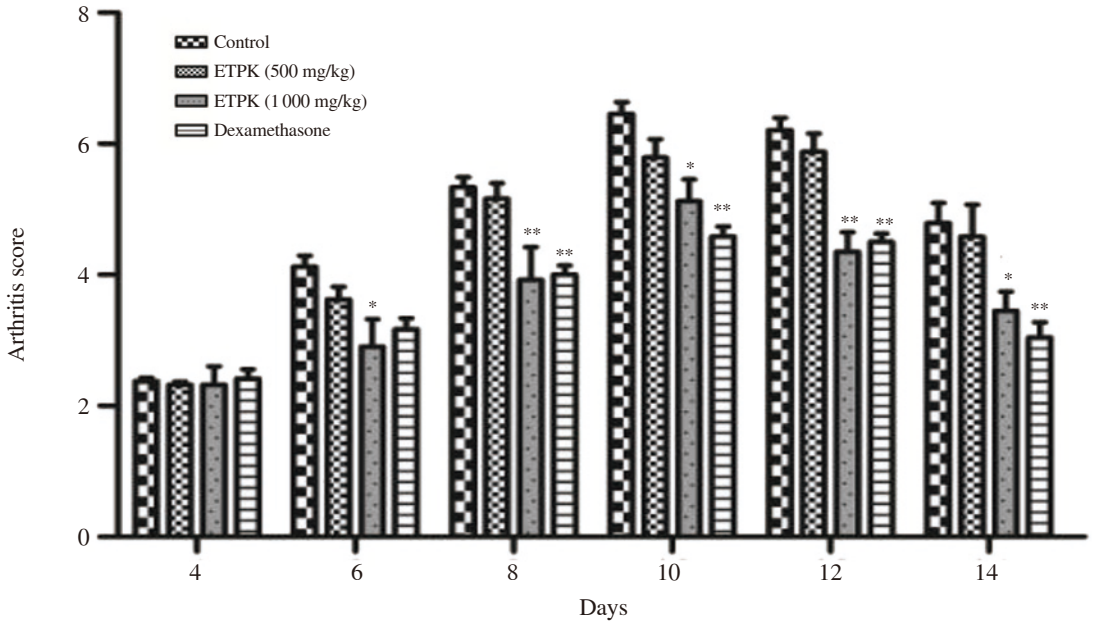

Figure 2. Effects of ethanol extracts of *P. kingianum* rhizomes on arthritis scores. Data are expressed as mean ± SD. Two-way ANOVA followed by Bonferroni post-test was used for statistical comparison between the treatment and control groups. $^{*}P<0.05$; $^{**}P<0.01$.

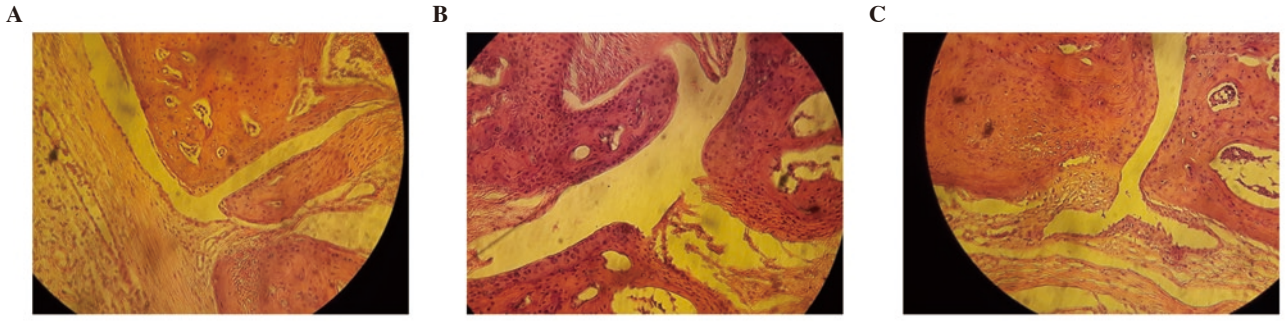

Figure 3. Histopathology of joint sections of experimental mice at ×200 magnification. (A) Negative control mice shows remarkably increased fibrin and infiltration of leukocytes; (B) the photomicrograph of joint of mice treated with 1 000 mg/kg b.w. of ethanol extract of *P. kingianum* rhizomes shows fewer inflammatory cells; (C) the histopathological image of joint of mice treated with dexamethasone shows much less obvious inflammation.

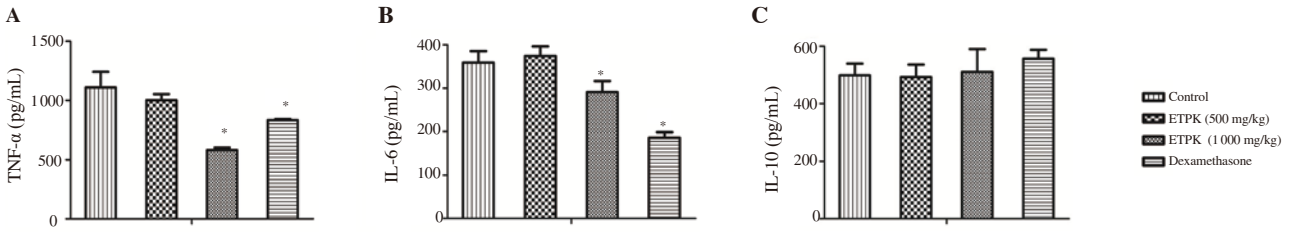

Figure 4. The effects of ethanol extracts of *P. kingianum* rhizomes on cytokine production. (A) TNF-α level; (B) IL-6 level; (C) IL-10 level. Data are expressed as mean ± SD (n=6). One-way ANOVA followed by Bonferroni post-test was used for statistical comparison between the treatment and control groups. $^{*}P<0.05$.

experimental doses, 1 000 mg/kg b.w. of the extract significantly and effectively reduced IL-6 secretion into mouse serum compared with the lower dose of 500 mg/kg b.w. Although the ethanol extracts of *P. kingianum* rhizomes inhibited the production of proinflammatory cytokines such as IL-6 and TNF-α, it had a mild effect on IL-10, an anti-inflammatory cytokine (Figure 4C) (P>0.05).

4. Discussion

The phytochemical study of ethanol extracts of *P. kingianum* rhizomes exhibited the presence of four compounds (1-4), of which compound (1) (5α,8α-ergosterol peroxide) was reported to have antibacterial, anti-inflammatory, antiviral, antioxidant, antitumor and immunosuppressive properties[18]. It is a known compound obtained from a variety of fungi, lichens, sponges, and marine organisms[19]. Some studies also have shown that compound (4) (daucosterol) exhibits anti-inflammatory and immunomodulating activities, promotes the proliferation of neural stem cells, as well as inhibits the proliferation of human breast and gastric cancer cells[20,21]. Besides, saponins are found to be the main component in this extract which was in consistent with the previous report[22]. The phytochemical screening results suggest that the saponin and sterols (daucosterol and 5α,8α-ergosterol peroxide) may contribute to anti-inflammatory activities of the ethanol extract of *P. kingianum* rhizomes[23].

In rheumatoid arthritis (RA), the imbalance between two kinds of cytokines, *i.e.*, proinflammatory and anti-inflammatory cytokines, exacerbates the pathogenesis. TNF-α is one of the key proinflammatory mediators involved in RA[24]. The inflammatory response in RA is mediated by the action of TNF-α, which is produced by macrophages, dendritic cells, lymphocytes, and fibroblasts. TNF-α also mediates other elements, such as PGE_2 and proinflammatory cytokines (IL-1 and IL-6), and induces production of granulocyte–monocyte colony-stimulating factor or endothelial leucocyte adhesion molecule-1 *via* both autocrine and paracrine mechanisms[25,26]. These elements continuously recruit more inflammatory cells and promote the articular pathology of RA (*e.g.*, bone destruction, cartilage erosion, or pannus formation)[26]. Likewise, IL-6 is a multifunctional cytokine that not only promotes inflammation and articular joint destruction, but also induces systemic symptoms, including inflammatory anaemia, coagulation, and thrombopoietin activation[27]. The levels of both IL-6 and TNF-α are high in the blood of patients with RA[28] and their inhibition has positive effects on RA treatment. Our results showed that the ethanol extracts of *P. kingianum* rhizomes inhibited IL-6 and TNF-α production, which may have resulted in reduced RA symptoms. Moreover, the extract inhibited *in vitro* NO production, which is considered a mediator of many inflammatory diseases, including arthritic disorders[29]. As reported elsewhere, NO at micromolar concentrations is associated with pathophysiological mechanisms[11,30]. Previous studies have suggested that overproduction of NO by inducible nitric oxide synthase is related to inflammatory pathologies, including RA. NO is also overproduced in the inflamed joints of RA patients, suggesting that suppression of NO overproduction may be a target for RA treatment[29]. This result is consistent with previous reports demonstrating NOS inhibition by *P. kingianum*[7]. Moreover, the ethanol extracts of *P. kingianum* rhizomes also remarkably inhibited PGE_2 production.

In RA pathogenesis, PGE_2 is produced during inflammatory processes *via* responding mediators such as TNF-α and interleukin-17. The presence of PGE_2 exacerbates the symptoms of RA, including osteoclastogenic suppression, pain, and inflammation[31]. According to McCoy *et al.*[32], PGE_2 and other prostanoids are therapeutic targets for RA. Previously, *P. kingianum* was studied mainly in terms of its effects on diabetic and related diseases, whereas few studies have focused on its anti-inflammatory effects. Thus, the result from our phytochemical study provides evidence to prove that the ethanol extract of *P. kingianum* rhizomes which contained the reported anti-inflammatory components, presents its anti-arthritic properties and effectiveness.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

HDTT, PNT, and NNT searched the literature and designed the study. Both HNTT and HLN did experiment work. HDTT, TDT, and HLM were responsible for data analysis. HDTT, PNT, TDT, and HLM did preparation, editing, and review of the manuscript.

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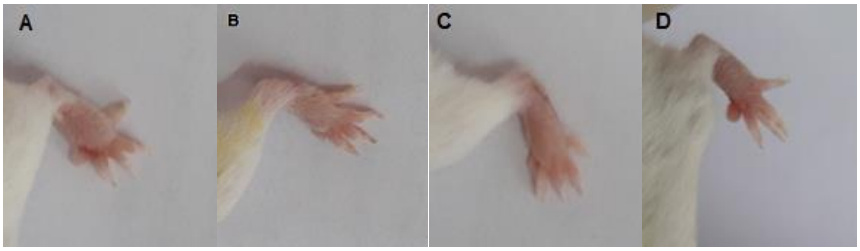

Supplementary Figure: Effects of **ETPK** on the morphological reprsentation of RA joints: (A) control group; (B) **ETPK** 1000 mg/kg b.w treatment; (C) **ETPK** 500 mg/kg b.w treatment; (D) Dexamethasone treatment.