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Ethanolic extract of *Pereskia aculeata* induces Anti-Inflammatory Responses through P38/MK2/TTP-mediated signaling pathway

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Abstract: *Pereskia aculeata* Miller, a member of the Cactaceae family, is a plant with pharmacological potential due to its containing compounds with various biological activities, which include anti-inflammatory, anti-cancer and analgesic activities. In this study, we evaluated the anti-inflammatory effects of an ethanolic extract of *P. aculeata* Miller (EEPA) and the signalling pathways by which it exerts these effects. *In vitro*, EEPA inhibited the secretion of inflammatory factors NO, IL-6 and PGE_2 in ipopolysaccharide-stimulated RAW264.7 macrophages (P<0.05). Treatment of RAW264.7 cells with EEPA also significantly decreased the levels of P-P38 and P-MK2, while upregulating the expression of TTP (P<0.05). *In vivo* anti-inflammatory activity assays revealed that EEPA reduced the degree of foot and joint swelling, the splenic index and the serum concentrations of TNF-α and IL-6 in in adjuvant-induced arthritis rats (P<0.05). Similarly, EEPA treatment of mice inhibited the acetic acid-induced exudation of Evans blue dye from peritoneal capillaries and significantly prolonged heat-stimulated pain response time (P<0.05). Taken together, these results suggest that EEPA exerts anti-inflammatory effects *in vitro* and *in vivo*. Thus, this study provides experimental and technical support for the development of a novel anti-inflammatory treatment based on *P. aculeata* Miller.

Keywords: *Pereskia aculeata* miller; ethanolic extract; anti-inflammatory; P38MAPK.

INTRODUCTION

Inflammation is a complex form of biological defence characterised by a response involving vascular damage and an integrated pathological process of injury and repair. Recent increases in the rate of death due to inflammatory diseases, such as arthritis, cardiovascular disease, gastrointestinal diseases and neurodegenerative diseases, have raised concern worldwide (Cioffi *et al*. 2021). In patients with inflammatory diseases, the main signs of a pathological response are pain, swelling and other immune responses, which are caused by various pro-inflammatory cytokines and other mediators. Presently, pain and other inflammation-related diseases usually treated using non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoid analogues (GCs) (González-Ponce *et al*. 2018; Al-Sayed *et al*. 2018; Bindu, Mazumder and Bandyopadhyay 2020). However, these drugs have serious adverse side effects, which has led to a search for drugs that can effectively treat inflammation with few side effects (Schjerning, McGettigan and Gislason 2020; Reichardt *et al*. 2021). Recently, researchers have identified herbs and natural products with excellent biological and pharmacological activities and few side effects; accordingly, plant extracts are expected to become an indispensable source of pharmacological agents for the treatment of various human diseases (Sharma, Chaubey and Suvarna 2021).

Pereskia aculeata Miller, a member of the Cactaceae family of plants, contains substantial concentrations of protein, minerals trace elements and low concentrations of lipids (Kazama *et al*. 2012; Pinto Nde and Scio 2014). *P. aculeata* Miller is also known as the Barbados gooseberry, is easy to grow, bears edible fruit and has leaves that can be used as a vegetable (Souza *et al*. 2016). A methanolic extract of *Pereskia aculeata* Miller significantly inhibits ear swelling, vasodilation and leukocyte infiltration in mice, with no systemic toxicity (Pinto Nde, Machado, *et al*. 2015; Pinto Nde, Duque, *et al*. 2015; Pinto *et al*. 2016), indicating that *Pereskia aculeata* Miller has anti-inflammatory and analgesic effects on animals with few side effects. Furthermore, *Pereskia aculeata* Miller has been used to treat inflammatory diseases, such as rheumatoid arthritis, in traditional Chinese medicine clinical practice.

The aim of this study was to assess the anti-inflammatory and analgesic effects of an ethanolic extract of *P. aculeata* Miller (EEPA). An *in vitro* model of inflammation was established by treating the RAW264.7 macrophage cell line with lipopolysaccharide (LPS) and the intracellular concentrations of NO, PGE_2 and IL-6 levels and P38, P-P38, P-MK2 and TTP protein expression levels, in order to explore the regulation of anti-inflammatory-related pathways *in vitro*. *In vivo* experiments were conducted to establish a rheumatoid arthritis (AIA) model in SD rats

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induced by Freund's complete adjuvant (FCA). Subsequently, the rats' body weights, degree of foot and joint swelling, splenic index and serum concentrations of inflammatory factors were measured to evaluate the anti-AIA activity of EEPA. In addition, the effectiveness of EEPA for treating acute inflammation and pain in mice was assessed using a hot plate assay and peritoneal capillary permeability assay. These experiments provided experimental data and technical support for the further development of *P. aculeata* Miller as a pharmacological resource.

MATERIALS AND METHODS

Biological materials and chemicals

Roswell Park Memorial Institute 1640 medium and foetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). An NO assay kit and enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, TNF-α and IL-6 were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Primary antibodies specific for β-actin, P38, P-P38, P-MK2 and TTP were purchased from Cell Signaling Technology (Danvers, MA, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), a protein phosphatase inhibitor mixture, radioimmunoprecipitation assay (RIPA) lysate (tissue/cell), BCA protein assay kit and bovine serum albumin fraction V (BSA) were obtained from Solarbio Biotechnology Co., Ltd. (Beijing, China). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Common chemicals and solvents were of analytical reagent grade, unless indicated otherwise.

Ethanolic extract of Pereskia aculeata Miller

Plant material were collected from Hezhou City, Guangxi Zhuang Autonomous Region, China (23°39′0″-25°09′0″ N; 111°05′0″-112°03′0″ E) in July 2017. The sample were taxonomically identified and authenticated by Huang Deqing (Teacher, Guilin Medical University Pharmacognosy teaching and research section). Fresh *Pereskia aculeata* Miller plants (4.3kg) were crushed and soaked three times in 75% ethanol (10L), after which the extracts were combined and concentrated using a vacuum rotary evaporator until essentially alcohol-free. The resulting EEPA (1.07g/mL) was dispensed into containers and stored at -20°C for anti-inflammatory experiment *in vivo*. The crude extract was collected and passed through quantitative analytical filter paper to remove impurities. The resulting filtrate was stored overnight at -80°C and then dried in a vacuum freeze dryer. After lyophilization, it was placed in sterile EP tubes for cellular experiments. Then the dose of EEPA in rats was determined by referring to the dose of EEPA in folk medicine.

Anti-inflammatory experiments in vitro

Cell culture and viability assay

MTT assay was used to determine the viability of RAW264.7 cells treated with various concentrations of EEPA (Chang, Guo, *et al*. 2021). RAW264.7 cells were obtained from the pharmacology laboratory of Guilin Medical University and then inoculated into 96-well plates at a density of 5×10^3 cells/well. The plates were then placed in an incubator (37ºC, 5 % CO_2) for 24h. Next, the cells were treated with various concentrations of EEPA (0, 50, 100, 150, 200, 250, 300, 400, 500, 600, 800 and 1000μg/mL) for 24h. After treatment, 20μL of MTT solution were added to each well and the plates were then incubated for 4h incubation. Subsequently, the absorbances of cells were measured at 490nm using a microplate reader.

Measurement of NO, PGE_2 and IL-6

RAW264.7 cells (1×10^6 cells/mL) were cultured in an incubator for 24 h. After incubation, EEPA (0, 200, 400 and 800μg/mL), dexamethasone (DXM, 10^{-5} M, positive control) and a normal control dish were incubated for 30 min and then stimulated with lipopolysaccharide (1μg/mL) for 24h. After treatment, the supernatants were collected from the cultures and placed in EP tubes for subsequent experiments. The NO assay kit was used to measure NO concentrations in the supernatants. This assay is based on the principle that NO, oxygen and water aggregate to produce nitrate and nitrite, which react with a colour developer to produce a red azo compound. Thus, the concentration of NO in each culture's supernatant was calculated by measuring the absorbance at 550nm. The concentrations of PGE_2 and IL-6 in the culture supernatants were quantified using the required ELISA kits, according to the manufacturer's instructions.

Western blot assay

Western blot analysis was performed using previously reported methods (Chang, Zhang, *et al*. 2021; Gong *et al*. 2021). After collecting the supernatant, cells were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. After lysis, the cells were centrifuged at 15,000rpm for 15 min at 4°C and then the protein concentrations were measured using a BCA protein assay kit. The proteins in each lysate (50μg protein/lane) were separated via 10% SDS-PAGE and then electroblotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1h and then incubated with primary antibodies (1:1000 dilution) overnight at 4ºC. The next day, the membranes were washed with TBST and then incubated with HRP-conjugated secondary antibodies (1:2000 dilution) and gently shaken at room temperature for 1.5h. Subsequently, the membranes were thoroughly washed with TBST and the antibody-reactive protein bands were then visualised using enhanced chemiluminescence (ECL) and quantified using image analysis software.

Anti-inflammatory experiments in vivo

Animals

All of the animals were obtained from the Experimental Animal Centre of Guilin Medical University. The animal research protocols were approved by the Guilin Medical University Animal Committee. The experimental animals

were divided into three batches: SD Rats (8-10 weeks old; body weight 200±20g) for use in the AIA model experiments; KM mice (4-6 weeks old; body weight 20±3g) for use in the vascular permeability experiment; KM mice (4-6 weeks old; body weight 20±3g) for use in the hot plate experiment. The animals were maintained in a controlled environment with a temperature of 25±2ºC, a relative humidity of 50±15% and a 12h light/12h dark cycle. The animals were allowed to acclimatise in an environment with adequate food and water for 1 week before the start of the experiments. All of the animals were treated humanely and experiments were conducted in accordance with the International Association for the Study of Pain guidelines (Li *et al*. 2009).

Freund's complete adjuvant induced arthritis in SD rats-induction, treatment and evaluation

Arthritis was induced as described in the literature (Bashir *et al*. 2021; Puppala *et al*. 2022). SD rats were randomly divided into six groups: a normal control group (no treatment); a model control (0.1mL FCA); a positive control group (0.1mL FCA+5mg/kg·day DXM); an H-EEPA group (0.1mL FCA+10g/kg·day EEPA); an M-EEPA group (0.1mL FCA+5g/kg·day EEPA); and an L-EEPA group (0.1mL FCA+2.5g/kg·day EEPA).

In all of the rats except those in the normal control group, AIA was induced by a single intradermal injection of 0.1 mL FCA suspension (1 mg/mL heat killed *Mycobacterium tuberculosis* in paraffin oil and mannitol monooleate) into the right hind paw. Treatments as described in the above group descriptions were administered by gavage from day 14 to day 21. The paw thickness (cm), joint diameter (cm) and body weight were then measured on days 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 and the arthritis scores were assessed on days 13, 15, 17, 19 and 21 using the following scoring criteria (Mbiantcha *et al*. 2017). Ears were scored as 0 for no nodular redness, 1 for single-ear nodular redness, 2 for double-ear nodular redness. The nose was scored as 0 for no nasal connective tissue erythema, 1 for marked nasal connective tissue erythema. The tail was scored as 0 for no nodules, red with tail or 1 for marked nodules, red with tail. The paws were scored as 0 for no redness or swollen paws, 1 for a single red and swollen paw, 2 for two red and swollen paws, 3 for three red and swollen paws or 4 for four red and swollen paws. The above-mentioned scores were summed to yield a global arthritis assessment, with a maximum possible score of 8. The measurements and recordings for each animal were performed by different researchers throughout the experiment.

Sample preparation for spleen index

The rats were sacrificed by cervical dislocation at the end of treatment administration and the spleens were collected, weighed and used to calculate the splenic index (spleen weight/rat body weight) (Guo *et al*. 2021).

Cytokines measurement by ELISA

The rats were sacrificed on day 22 post-FCA inoculation (Chen *et al*. 2022). Orbital blood was collected at room temperature and centrifuged at 3500 rpm for 15 min. The sera were collected in EP tubes and stored at -80°C. Serum concentrations of TNF-α and IL-6 were detected using ELISA. The optical density was determined spectrophotometrically at 450nm.

Apillary permeability of KM mice abdomen induced by acetic acid

The effect of EEPA on the acute inflammatory response in KM mice was tested using the peritoneal capillary permeability assay (Wang *et al*. 2015; Cruz *et al*. 2016). The animals were randomly divided into six groups: A normal control group (no treatment); a positive control group (Evans blue+acetic acid+10mg/kg·day DXM); a negative control group (Evans blue+acetic acid); an H-EEPA group (Evans blue+acetic acid+20g/kg·day EEPA); an M-EEPA group (Evans blue+acetic acid+10g/kg·day EEPA); and an L-EEPA group (Evans blue+acetic acid+ 5g/kg·day EEPA).

All of the mice were weighed and EEPA and DXM were administered by gavage at a dose of 0.2 mL/10 g for 3 days. After gavage, all of the mice except those in the normal control group were injected with 0.2 mL Evans blue dye (0.25% in normal saline) via the caudal vein. Thirty minutes later, the mice treated with Evans blue dye were injected with a 0.6% acetic acid solution at a dose of 1 mL/100 g. After thirty minutes, all mice were sacrificed by cervical dislocation and each peritoneal cavity was washed with stroke-physiological saline solution and collected into EP tube. After centrifugation, the dye content in the supernatant was measured at 610nm using a spectrophotometer.

Hot plate test for analgesic study

The experiment was conducted with reference to the literature (Chandran, George and Abrahamse 2020; Wahid *et al*. 2020; Abdallah *et al*. 2021). Mice were randomly divided into the following five groups: a normal control group (no treatment); a positive control group (150mg/kg·day aspirin); an H-EEPA group (20g/kg·day EEPA); an M-EEPA group (10g/kg·day EEPA); and an L-EEPA group (5g/kg•day EEPA).

For the hot plate test, the mice were placed on a hot plate set to 50 ± 0.5°C and screened for latency in the range of 10-60 s. After screening, mice were randomly divided into five groups; the basal pain threshold of each mouse was measured before administration (0h) and the pain response threshold was measured at 1, 2 and 3h after gavage aspirin and EEPA for 3 days.

STATISTICAL ANALYSIS

All of the data are presented as means±standard deviations. One-way ANOVA was used for comparison between

groups. The tests were performed using SPSS 19.0 (SPSS Inc., USA). $P<0.05$ was considered to indicate statistical significance. Graphs were generated using GraphPad Prism (vison 6.0, GraphPad Software Inc, La Jolla, CA).

RESULTS

Anti-inflammatory effects *in vitro*

Effects of EEPA on viability of RAW264.7

MTT assays were used to evaluate the cytotoxicity of EEPA on RAW264.7. As shown in fig. 1, compared with control group, EEPA (100, 150, 200, 250, 300, 400, 500, 600 and 800μg/mL) had no significant effect on cell growth ($P>0.05$). Therefore, EEPA was used at concentrations of 800, 400 and 200μg/mL in subsequent experiments to prevent marked cytotoxicity.

Effects of EEPA on the secretion of NO, PGE_2 and IL-6 in LPS-stimulated RAW264.7 cells

The effects of EEPA (800, 400 and 200μg/mL) on the secretion of NO, PGE_2 and IL-6 were measured to investigate its potential anti-inflammatory activity. Compared with the control group, LPS stimulation significantly increased the secretion of NO, PGE_2 and IL-6 by RAW264.7 cells ($P<0.001$), indicating that the inflammation model *in vitro* was successfully established. As shown in fig. 2A, RAW264.7 cells in the H-EEPA (800 μg/mL), M-EEPA (400 μg/mL) and L-EEPA (200 μg/mL) groups exhibited significant reductions in NO secretion (46.34%, 24.04% and 12.89%, respectively) compared with the negative control ($P<0.05$). Fig. 2 B shows that two doses of H-EEPA and M-EEPA significantly reduced LPS-induced PGE_2 production in RAW264.7 cells ($P<0.05$) by 76.92% and 34.12%, respectively. As shown in to fig. 2 C, LPS-stimulated IL-6 production by RAW264.7 cells in the H-EEPA and M-EEPA groups was inhibited by 52.57% and 35.13%, respectively ($P<0.05$).

Effects of EEPA on the expression of protein in the P38-MK2-TTP-regulated signalling pathway in LPS-stimulated RAW264.7 cells

To investigate whether EEPA exerts its anti-inflammatory activities by P38-MK2-mediated regulation of TTP activity, we examined the phosphorylation of proteins in the MAPK pathway. As shown in fig. 3A and 3B, P-P38 and P-MK2 protein expression was significantly increased in LPS-stimulated RAW264.7 cells compared to normal controls and the level of P-P38 and P-MK2 was significantly lower after H-EEAP and M-EEPA treatment compared to negative controls ($P<0.05$). Meanwhile, Tristetraprolin (TTP) could be significantly accumulated in RAW264.7 cells under the effect of EEPA, indicating that TTP plays an important anti-inflammatory role in LPS-stimulated macrophages (Cao and Sethumadhavan 2020; Ma *et al*. 2021). These results indicate that EEPA exerts anti-inflammatory effects in LPS-stimulated RAW264.7 cells by inhibiting P38-MK2-TTP-mediated protein stabilisatio.

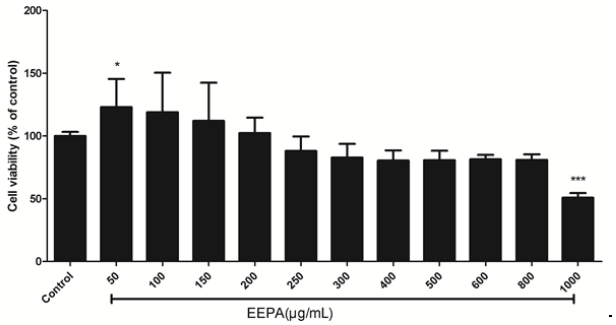


Fig. 1: Effects of different concentrations of EEPA on RAW264.7 cell viability. Data are shown as the mean ± SD of three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the control group.

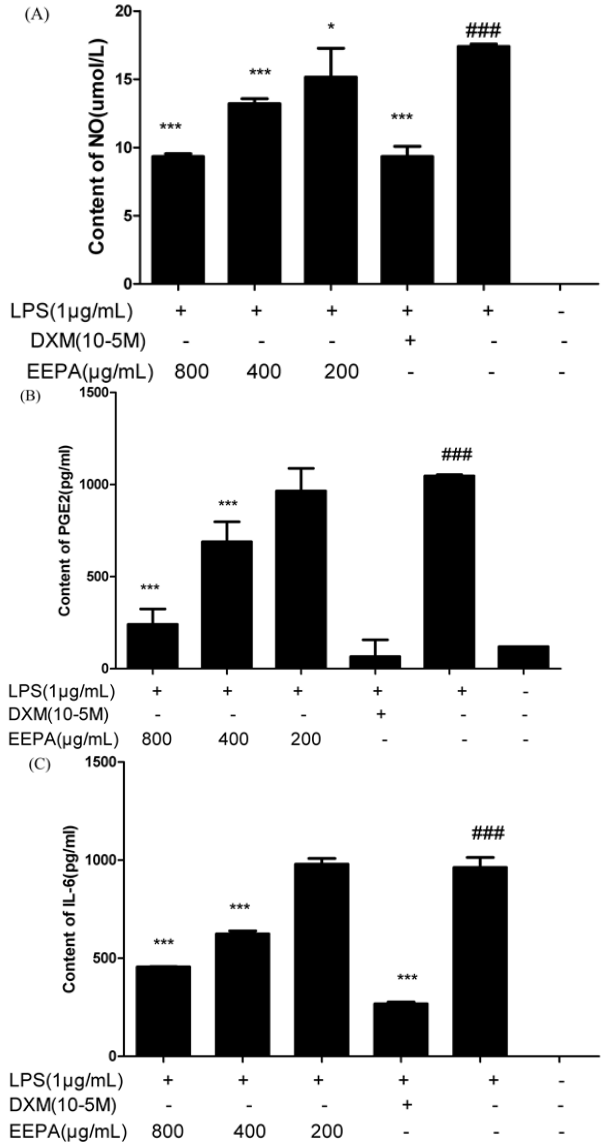


Fig. 2: Effects of EEPA on the production and expression of NO, PGE_2 and IL-6 in LPS-stimulated RAW264.7 cells. (A) Effect of EEPA on the content of NO induced by LPS in RAW264.7. (B) Effect of EEPA on the content of PGE_2

induced by LPS in RAW264.7. (C) Effect of EEPA on the content of IL-6 induced by LPS in RAW264.7. Data are shown as the mean ± SD of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the negative control group, $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01, $^{\#\#\#}$*P*<0.001 compared with the normal control group.

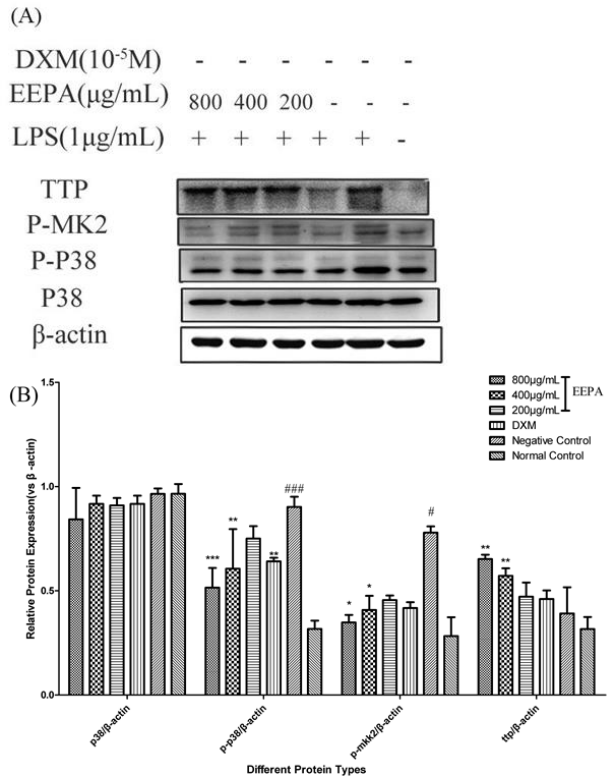


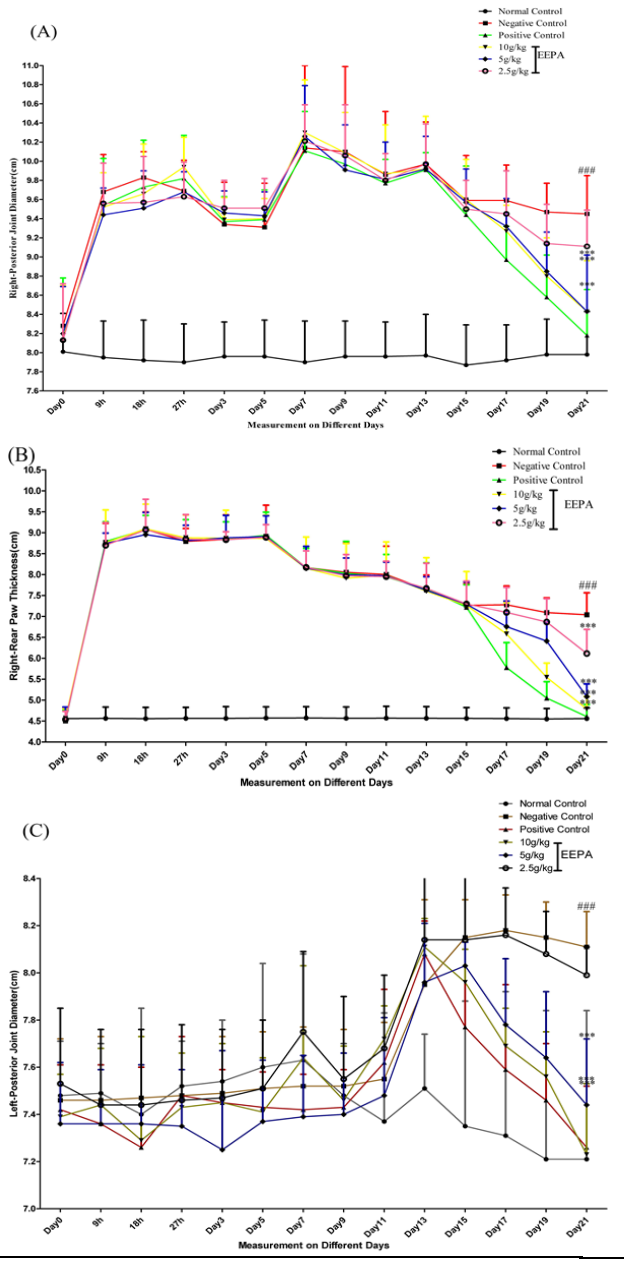
Fig. 3: Effect of EEPA on the content of NO induced by LPS in RAW264.7.Effects of EEPA on the expression of P-P38, P38, P-mk2 and TTP expressions in LPS stimulated RAW264.7. (A) Western blot analysis of P-P38, P38, P-mk2 and TTP and β-actin in LPS stimulated RAW264.7. (B) Quantitative analysis of P-P38/β-actin, P-mk2/β-actin and TTP/β-actin. Data are shown as the mean ±SD of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the negative control group, $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01, $^{\#\#\#}$*P*<0.001 compared with the normal control group.

Anti-inflammatory effects in vivo

Effect of EEPA on rear paw thickness, posterior joint diameter, arthritic score, body weight and spleen index in AIA rats

As shown in the fig. 4, AIA rats exhibited significant inflammatory responses, characterised by paw swelling, weight loss and splenomegaly. All of these inflammatory symptoms were improved to various extents by EEPA treatment. As seen in fig. 4A, H-EEPA, M-EEPA and L-EEPA dose groups significantly inhibited the swelling of the right joint (reductions of 10.85%, 8.26% and 1.48%, respectively) compared to the negative control group (*P*<0.05). As shown in fig. 4B, H-EEPA, M-EEPA and L-EEPA significantly inhibited the swelling of the right paw (reductions of 10.37%, 5.89% and 2.85%, respectively) compared with the negative control group (*P*<0.05). Fig. 4C shows that the H-EEPA and M-EEPA groups had similar rates of inhibition of left joint swelling compared with the negative control group (reductions of 10.79%; *P*<0.05). fig. 4D shows that in the H-EEPA, M-EEPA and L-EEPA groups, swelling of the left paw was inhibited by 31.95%, 27.82% and 13.16%, respectively, compared with the normal control group (*P*<0.05).

Changes in the body weights of the rats are shown in fig. 4E. Mental atrophy, loss of appetite and weight loss occurred in rats after AIA induction. After EEPA administration, the weight data showed a slowly rebounding trend.



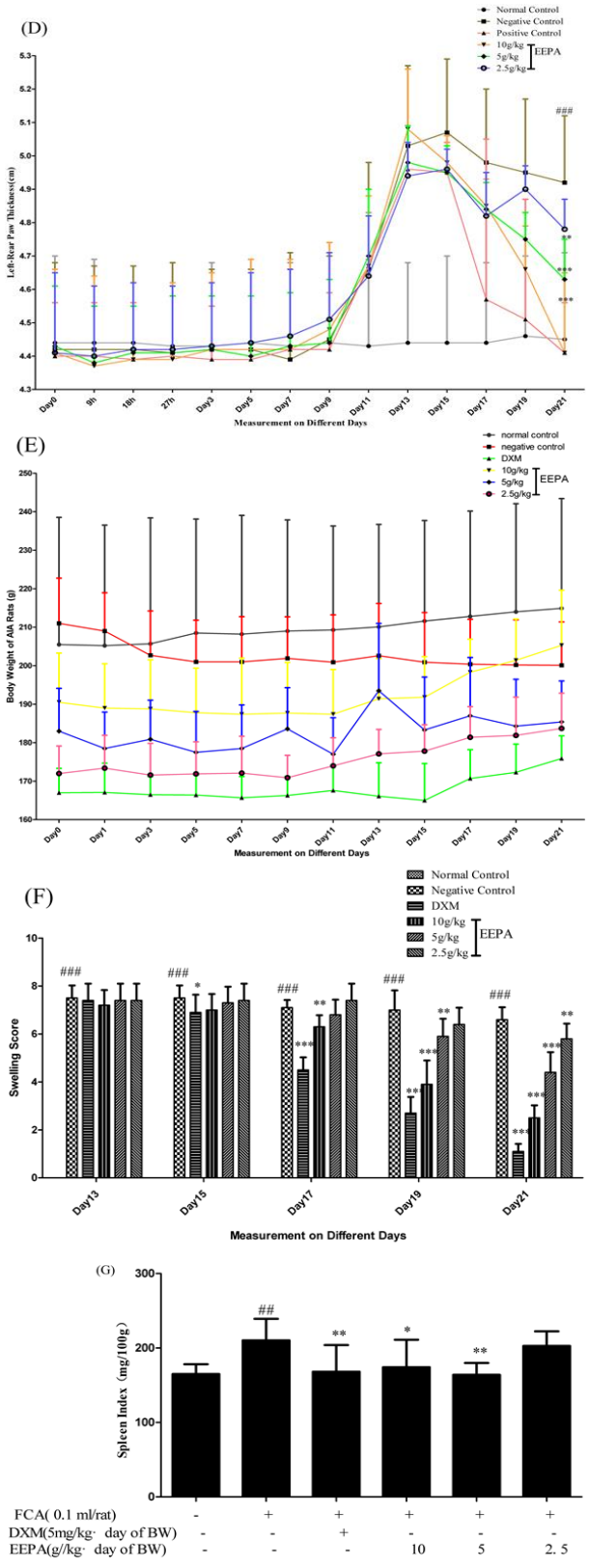


Fig. 4: Effect of EEPA on rear paw thickness, posterior joint diameter, arthritic score, body weight and spleen index in AIA rats. (A) Effects of EEPA on swelling of right posterior joint in AIA rats. (B) Effects of EEPA on swelling of right rear paw in AIA rats. (C) Effects of EEPA on swelling of left posterior joint in AIA rats. (D) Effects of EEPA on swelling of left rear paw in AIA rats. (E) Effects of EEPA on body weight in AIA rats. (F) Swelling scores of AIA rats in different groups. (G) The spleen index of AIA rats. Data are shown as the mean ± SD of ten independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the negative control group, #*P*<0.05, ##*P*<0.01, ###*P*<0.001 compared with the normal control group.

Fig. 4G shows that rats in the H-EEPA and M-EEPA groups had a significantly lower splenic index than those in the negative control group (*P*<0.05). M-EEPA was the most effective treatment, reducing the splenic index by 22.00% relative to the negative control, while H-EEPA reduced the splenic index by 17.15%.

Effect of EEPA on the production of TNF-α and IL-6 in AIA rats

As shown in fig. 5A, the concentrations of the inflammatory cytokines TNF-α and IL-6 were significantly increased in the negative control group compared with the normal control group (*P*<0.05) and the concentrations of both cytokines were significantly reduced after EEPA administration compared with the negative control group (*P*<0.05).

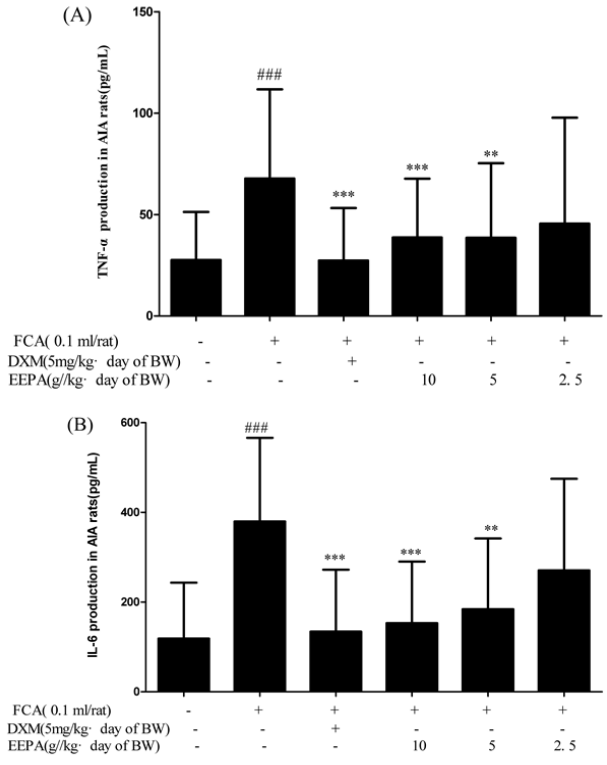


Fig. 5: Effects of EEPA on the expression of inflammatory cytokines in AIA rats. (A) The levels of TNF-α were measured by ELISA kit. (B) The levels of IL-6 were measured by ELISA kit. Data are shown as the mean ± SD

of eight independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the negative control group, #*P*<0.05, ##*P*<0.01, ###*P*<0.001 compared with the normal control group.

Effect of EEPA on peritoneal capillary permeability in mice

As shown in fig. 6, H-EEPA and M-EEPA administration groups significantly inhibited the Evans blue solution in the peritoneal capillaries after acetic acid induction compared to the negative control group (*P*<0.05) with 38.49% and 14.88% inhibition, respectively. This indicates that H-EEPA and M-EEPA had significant effects on the vascular response in mice.

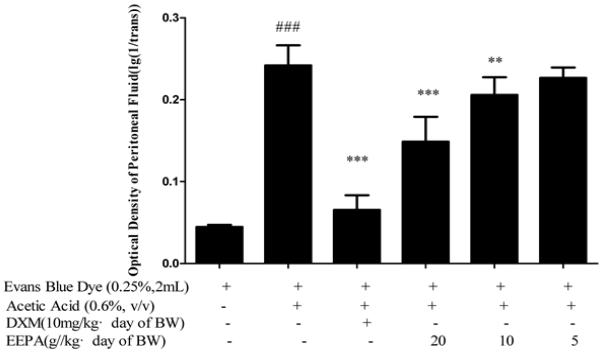


Fig. 6: Effects of EEPA on acetic acid-induced peritoneal capillary permeability response in mice. Data are shown as the mean ± SD of eight independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the negative control group, #*P*<0.05, ##*P*<0.01, ###*P*<0.001 compared with the normal control group.

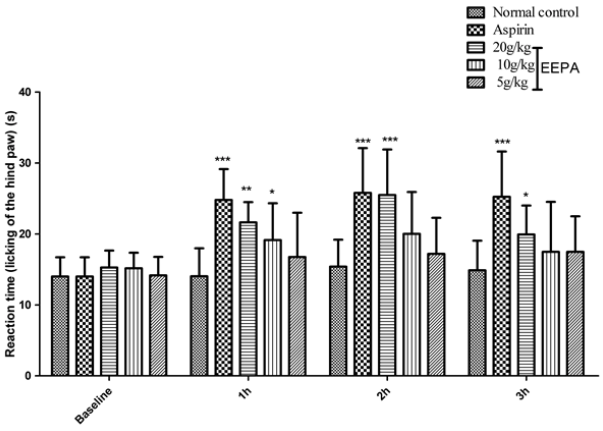


Fig. 7: Analgesic effect of EEPA on the hot plate test. Data are shown as the mean ±SD of ten independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the normal control group.

Analgesic effect of EEPA during the hot plate test

Mice treated with various concentrations of EEPA (20, 10 or 5g/kg·day) showed differences in analgesic activity compared with the normal control group in the three phases of the hot plate test (1h, 2h and 3h). As shown in fig. 7, compared with the basic pain threshold, H-EEPA and M-EEPA exerted significant analgesic effects at 1h after administration (*P*<0.05) and at the stage of test(2h, 3h) only H-EEPA exerted the best analgesic effect (*P*<0.001), suggesting that EEPA has effective analgesic activity.

DISCUSSION

Inflammatory pathological processes include metaplasia, exudation and hyperplasia and the stages of inflammation exhibited different characteristics, with tissue degeneration and fluid exudation predominating in the early stages, followed by hyperplasia and significant pain in the later stages (Obiri and Osafo 2013). Although common anti-inflammatory and analgesic drugs such as NSAIDs can relieve pain, they also adversely affect the quality of life of most patients (Wöhrl 2018; MacDonald *et al*. 2018). *P. aculeata* Miller was reported to be a safe and effective treatment for local chronic and acute inflammatory conditions (Pinto Nde, Machado, *et al*. 2015). Moreover, *P. aculeata* Miller has been used in traditional medicine contexts to treat RA due to its lack of without significant adverse effects, indicating the value of studying its anti-inflammatory effects and mechanisms. However, it was unable to fully investigate the pharmacological activity and mechanism of action of *P. aculeata* Miller.

Accordingly, in the current study, various animal models were used to evaluate the anti-inflammatory and analgesic effects of EEPA *in vivo*. Considering the efficacy of traditional medicinal treatment of RA, combined with the AIA rat model which is highly compatible with human RA, there is a great advantage in using the AIA model to explore the role of EEPA in the study of chronic immune inflammation. The results showed that EEPA significantly inhibited joint and paw swelling in AIA rats and affected the splenic index, which is similar to the folk treatment of RA. However, there was no significant change in the results under three different doses administered is because we refer to the most suitable dose in folk medicine for the transformation. As TNF-α and IL-6 are important biochemical indicators of the development of inflammation and indicators of treatment-related diseases (Ahsan *et al*. 2021), it is valid to determine the concentrations of these cytokines to confirm the pharmacological activity of a drug. In this study, treatment of AIA rats with H-EEPA and M-EEPA significantly reduced serum concentrations of TNF-α and IL-6 (*P*<0.05). Vascular changes and pain are important indicators of the acute inflammatory response (Fullerton and Gilroy 2016) and the peritoneal capillary permeability assay is a classic method for studying acute, early inflammation in mice, which is characterised by local vasodilation and increased capillary permeability. The results showed that two treatment doses of H-EEPA and M-EEPA significantly inhibited the amount of Evans blue exudation into peritoneal capillaries (*P*<0.05). Pain induced by inflammation is a key cause of intense

discomfort in patients, so the hot plate test was used to verify the analgesic effect of EEPA in mice. The results show that EEPA significantly prolonged the pain response time in mice after heat stimulation ($P<0.05$), indicating that EEPA reduces pain. Taken together, the results show that EEPA exerts strong analgesic and anti-inflammatory effects *in vivo*.

This paper show that treatment of LPS-stimulated RAW264.7 cells with EEPA significantly reduced their secretion of NO, PGE_2 and IL-6 and altered their levels of P-P38, P-MK2 and TTP protein. NO is an important endogenous regulator and PGE_2 and IL-6 play central roles in amplifying the inflammatory cascade, which is also mediated by TTP (Hill *et al*. 2010; Joe *et al*. 2011; Kyriakis and Avruch 2012). TTP is a substrate of MK2, which is activated by p38 and is negatively regulated by P-P38 and P-MK2. In addition, P-P38, P-MK2 and TTP are key determinants of the inflammatory response and targets of anti-inflammatory therapies. This study suggests that there is no significant toxic effect of EEPA at certain doses, which confirms the safety of this medicinal plant. In addition, treatment of LPS-induced RAW264.7 cells with H-EEPA and M-EEPA decreased the phosphorylation of P38 and MK2 and increased the level of TTP ($P<0.05$), confirming that EEPA may exert its anti-inflammatory effects via the P38-MAPK signalling pathway.

CONCLUSION

In summary, EEPA has some inhibitory effects on acute and chronic inflammation. Its mechanism of action involve regulating the P38 MAPK signalling pathway to suppress the secretion of PGE_2 and IL-6, which ultimately inhibits the progression of inflammation. This study is the first to elucidate the pharmacological activity of an extract of *P. aculeata* Miller and its mechanism, which is consistent with previous related studies supporting the pharmacological activity of this plant. However, further studies are needed to investigate its pharmacological activity in depth. Therefore, developing suitable animal models to validate its efficacy and mechanism is promising to improve it as a drug.

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