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ORIGINAL RESEARCH

Anti-Nociceptive and Anti-Inflammatory Activities of the Ethyl Acetate Extract of *Belamcanda chinensis* (L.) Redouté in Raw 264.7 Cells in vitro and Mouse Model in vivo

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Purpose: Inflammation and accompanying pain is a common global health problem that seriously affects human quality of life worldwide. Here, we aimed to investigate the anti-nociceptive and anti-inflammatory activities of the ethyl acetate extract of *B. chinensis* (EAEBc) along with the underlying mechanisms of action.

Methods: The in vitro anti-inflammatory activity of EAEBc was explored using an LPS-induced RAW264.7 cell inflammatory model. Nitric oxide (NO) production, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 levels were evaluated. In vivo anti-nociceptive and anti-inflammatory activities of EAEBc were assessed with the aid of classical experimental mouse models. In addition, LPS-induced biomarker contents (TNF-α, IL-1β, IL-6, NO, iNOS, and PGE2) and formalin-induced serum inflammatory factors (NO, PGE2, 5-HT, β-EP, substance P, and NE) were determined in mice.

Results: In vitro, EAEBc significantly reduced LPS-induced NO generation and suppressed the production of TNF-α, IL-1β, and IL-6 in RAW264.7 cells in a concentration-dependent manner. In vivo, EAEBc downregulated serum TNF-α, IL-1β, IL-6, NO, iNOS, and PGE2 contents in mice with LPS-induced inflammation in a dose-dependent manner. EAEBc displayed anti-inflammatory activity in carrageenan-induced paw edema and xylene ear edema tests. Intragastric administration of EAEBc at test doses of 100 and 200 mg/kg led to inhibition of nociception and capillary permeability induced by acetic acid to varying degrees. Similarly, EAEBc exerted analgesic effects in the formalin and hot plate tests. In particular, the administration of EAEBc reversed the changes in the levels of inflammatory indicators NO, PGE2, 5-HT, β-EP, substance P, and NE in a mouse model of formalin-induced pain.

Conclusion: Our findings provide considerable evidence to support the extensive application of *B. chinensis* in traditional medicine and demonstrate the utility of this plant species as an effective candidate for prevention or treatment of various pain and inflammation-related conditions.

Keywords: medicinal plants, *Belamcanda chinensis*, anti-nociceptive activity, anti-inflammatory activity, inflammatory mediators

Introduction

Inflammation and pain involve a complex cascade of events incorporating numerous mediators including prostaglandin, pro-inflammatory cytokines, and chemokines, which seriously affect the physical and mental health of patients. Medicinal plants, in particular, traditional Chinese medicine and folk medicinal herbs with analgesic and anti-inflammatory activities, are an ongoing research hotspot due to their reliable curative effects, fewer adverse reactions, and extensive use over thousands of years of clinical practice.[1,2] *Belamcanda chinensis* (L.) is a large perennial herb (family Iridaceae) 60–120 cm in height, which is native to several countries in South-East Asia (China, Japan, Korea, Bhutan, Myanmar, Nepal, Philippines, Thailand, and Vietnam).[3–5] The herb has also been naturalized as an ornamental garden plant in some areas of Europe and North America. In East Asia, the dried rhizome of the plant has long been used to treat inflammation, throat symptoms, asthma, swollen liver and

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spleen, arrow poisoning, gonorrhea, and malaria. Notably, this herb is one of the main components of the traditional Chinese medicine prescription for lung disease.[6–9] In China, the plant grows on dry slopes, grasslands, valleys and beaches and is widely distributed across various provinces and regions. The crude extracts and main bioactive components of *B. chinensis* exhibit multiple biological activities. The dried rhizome is commonly used as a traditional Chinese medicine for detoxification, sore throat relief, clearing heat, eliminating sputum, reducing edema, and curing pain,[8–10] and the 2020 edition of the Chinese Pharmacopoeia recommends a root extract dose of 3–10 g.[8] In Thai and Vietnamese traditional medicine, *B. chinensis* is commonly applied to menstrual disorders, such as period irregularities, amenorrhea and dysmenorrhea,[11] and used to treat liver complaints and as an expectorant in Nepal.[12]

Phytochemical screening has successfully facilitated the isolation of >100 chemical constituents from different parts of *B. chinensis*. Isoflavonoids, flavonoids, and iridal-type triterpenoids are the three main components isolated from the *B. chinensis* rhizome.[3,13–15] Isoflavonoids, including tectorigenin, tectoridin, irigenin, irisflorentin and iristectorigenin A, are considered the most active compounds of *B. chinensis* rhizome. At the same time, flavonoids (irigenin, apigenin, hispidulin, luteolin, isorhamnetin, rhamnazin), xanthones (mangiferin, isomangiferin, neomangiferin) and iridal-type triterpenoids (iriditectoral, iridotectoral A, iridotectoral B, belamcandal) have been isolated from rhizomes, leaves, and seeds of *B. chinensis*. Among these, irisflorentin is one of the most abundant and bioactive constituents with multiple reported biological activities including anti-inflammatory and anti-tumor effects.[15] Irisflorentin has been documented in the Chinese Pharmacopoeia (Version 2020) as the major standard compound for evaluation and quality control of *B. chinensis* and its preparations. According to this source, the irisflorentin content in *B. chinensis* rhizome, analyzed via HPLC, should not be less than 0.1%.[8]

Medicinal herbs for the treatment of pain and inflammation-associated diseases have a history of long-term usage in humans and animals.[1] The considerable potential of natural products for inflammation and pain disorders is widely documented.[16–18] Development and analysis of plant-derived analgesic and anti-inflammatory drugs is therefore a research area of significant clinical value. Two thousand years ago, the rhizomes of *B. chinensis* were first listed in the Chinese Herbal list "Shen Nong Ben Cao Jing". Since then, *B. chinensis* extracts or decoctions have been of considerable interest to medical scholars. The substantial long-term interest in herbal products as a potential source of phytopharmaceuticals has highlighted the importance of gaining insights into their underlying mechanisms of action. To date, multiple beneficial biological activities of *B. chinensis* crude extracts and isolated compounds have been reported in vivo and in vitro, including antioxidative, antimutagenic, antitumor, anti-renal fibrosis, anti-inflammatory, antibacterial, antiviral, hypoglycemic, and anti-angiogenic properties.[3,14,15,19–22] A number of the above activities are known to contribute to the efficacy of *B. chinensis* in traditional medicine, but pharmacological studies so far have predominantly focused on monomeric compounds. Several recent studies suggest that isoflavoneirigenin, tectorigenin, and tectoridin have anti-inflammatory properties in vitro,[7,23–25] but in-depth and systematic in vivo animal studies on anti-nociceptive and anti-inflammatory effects of *B. chinensis* are yet to be conducted. Considering the traditional applications of *B. chinensis* in the treatment of lumbago, muscular pain, sore throat, tonsillitis and asthma, and amenorrhea, the present study was designed to evaluate the anti-nociceptive and anti-inflammatory activities of EAEBc, both in vitro and in vivo. In addition, the underlying mechanisms were explored, focusing specifically on the related cytokines.

Materials and methods

Plant Material and Extraction

Rhizomes of *B. chinensis* were collected in Shaanxi province, China, in 2018. Wei Guifang, the deputy director of the Traditional Chinese Pharmacy Department in Honghui Hospital, Xi'an Jiaotong University, identified the plants. The voucher specimen (No. YJ001) is stored in the Pharmacy Department. Dried rhizomes were pulverized using a mechanical grinder and 300 g powder macerated in 3 L of 70% ethanol with continuous reflux extraction for 3 h. The extract obtained was concentrated to dryness with a rotary evaporator (SENCO Technology Co., Ltd. Shanghai, China) and the EAEBc yield rate was 31.25% (w/w). Next, ethyl acetate was used as an extraction solvent to obtain EAEBc. The resulting extracts used for pharmacodynamic analyses were suspended in 1% Tween 80 at the required concentrations. Extensive HPLC analysis of EAEBc revealed the presence of common flavonoids, such as irisflorentin, iridin, irigenin, tectorigenin, tectoridin, quercetin and kampferol. In addition, ursolic acid and betulonic acid were detected.

In vitro Experiments

Cell Culture

The RAW264.7 macrophage cell line was acquired from the Chinese Academy of Sciences. RAW264.7 cells were rapidly removed from liquid nitrogen and resuscitated. Next, cells were incubated in DMEM containing fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) and cultured in 5% CO_2 at a constant temperature. Upon complete adherence of RAW264.7 cells to the wall (up to 80% growth), cells were digested with trypsin and sub-cultured at a ratio of 1:3. Culture was continued until complete adherence of cells to the wall (growth up to 80% density) for subsequent experiments.

Cell Grouping and Intervention

RAW264.7 macrophage cells were divided into blank, model (LPS, 1 μg/mL), and EAEBc treatment (32, 64, and 128 μg/mL) groups. In addition to the blank group, all cells were incubated with 1 μg/mL LPS. Briefly, cells were inoculated into 96-well plates at a density of 4×10^5 cells/well. After 24 h, the culture medium was discarded, followed by incubation with/without EAEBc solution (32, 64, and 128 μg/mL) and LPS (1 μg/mL) for 24 h. The culture medium was collected for subsequent experiments.

Cell Viability

The viability of RAW264.7 macrophage cells was measured using the MTT assay. Cells were inoculated into 96-well plates at a density of 1×10^4 cells/well. After 24 h, the medium was discarded, and cells were incubated with or without EAEBc solution at various concentrations (4, 8, 16, 32, 64, and 128 μg/mL) under the same conditions for 24 h. After incubation for a further 24 h, 10 μL MTT was added to each well and the culture continued for 4 h. After this time, the medium was discarded and 100 μL DMSO added to each well. Absorbance of each well at 450 nm was measured using an automatic microplate reader for determination of cell viability.

NO and Cytokine Production Assays

RAW264.7 cell inoculation and culture were conducted as described above. NO was measured using a nitrite assay kit (Elabscience Biotechnology Co., Ltd., Wuhan, China) and TNF-α, IL-6, and IL-1β levels assessed using the respective enzyme-linked immunosorbent assay kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) on a microplate reader at 450 nm, respectively. All the procedures were performed strictly following the manufacturer's instructions of.

In vivo Experiments

Experimental Animals

Young adult KunMing (KM) male and female mice (Scxk (Guangdong) 2020–0051, 22–24 g) obtained from BesTest Bio-Tech Co., Ltd. Zhuhai, China, were used. Animals were maintained at a controlled room temperature of 22 ± 2°C with free access to pellet food and water under a 12 h light/dark cycle. Experiments complied with the management regulations of Guangdong Medical Laboratory Animal Center (Guangdong, China) and were carried out in accordance with NIH guidelines.[26] All protocols were approved by the Animal Care Committee of Zunyi Medical University (ZYLS-[2020] No. 2–081).

Drugs and Reagents

Acetic acid (Taishan Xinning Pharmaceutical Co. Ltd., Taishan, China), xylene (Tianjin Fuyu Chemical Reagent Co. Ltd, Tianjin, China), morphine (The First Pharmaceutical Company of Shenyang, Northeast Pharmaceutical Group, Shenyang, China), carrageenan (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and formalin (Tianjin Tianli Chemical Reagent Company, Tianjin, China) were utilized in our experiments. EAEBc was dissolved in saline solution containing 1% Tween-80.

Anti-Inflammatory Assays

LPS-Induced Inflammation in Mice

Sixty mice were randomly divided into six groups: blank (normal) (0.9% NaCl containing 1% Tween 80), model, positive drug (indomethacin (Indo), 10 mg/kg), and different doses of EAEBc solution (50, 100, and 200 mg/kg,

respectively). Animals in the blank group were intragastrically administered normal saline, while those in the other groups received EAEBc or the positive drug Indo. Intragastric EAEBc and Indo (0.2 mL/10 g) were administered at the selected doses for 7 continuous days. At 30 min after the final administration, all mice (except the blank group) were intraperitoneally injected with LPS (150 μg/kg). After 1 h, blood was collected from the heart, packed in a blood collecting vessel, and centrifuged at 3000 g for 10 min for serum collection. The absorbance values (OD) of serum inflammatory factors (TNF-α, IL-6, IL-1β, NO, iNOS and PGE2) were measured according to instructions of the respective ELISA kits using a Thermo Scientific microplate reader. The levels of inflammatory factors in each mouse serum sample were evaluated.

Xylene-Induced Ear Swelling in Mice

Xylene-induced ear swelling in mice was carried out according to a previously reported protocol.[27] Fifty mice were randomly divided into model (0.9% NaCl), positive drug (Indo, 10 mg/kg), and three EAEBc treatment groups at doses of 50, 100, and 200 mg/kg (10 mice per group). Mice of the model group were intragastrically administered 0.9% NaCl, the positive drug group intragastrically administered Indo (10 mg/kg), and test groups intragastrically administered different doses of EAEBc once a day for 7 consecutive days. At 30 min after the final administration, each mouse received 30 μL xylene on the anterior and posterior surface of the right-ear lobe. The left ear of each mouse was used as the control. After 60 min, mice were sacrificed via cervical dislocation and circular sections with a diameter of 8 mm obtained from both ears with a cork borer and weighed. The degree of ear swelling was calculated based on the weight of the left ear without application of xylene in the same mouse.

Carrageenan-Induced Paw Edema in Mice

The anti-inflammatory effect of EAEBc was experimentally validated using the carrageenan-induced hind paw edema model.[27] Animal grouping and treatment administration followed the procedure detailed in "Xylene-Induced Ear Swelling in Mice". Before treatment with the test drugs, initial paw thickness was determined with an electronic digital caliper (Wuxi Xigong Measuring Co. Ltd., Wuxi, China). At 30 min after the final administration, 25 μL of 2% carrageenan solution in normal saline was injected subcutaneously into the right-hind paw. Paw thickness was measured with an electronic digital caliper at 1, 3, and 5 h after treatment with test drugs and the percentage of inhibition calculated.

Antinociceptive Assays

Acetic Acid-Induced Writhing Test

The acetic acid-induced writhing test in mice was conducted according to a previous report.[28] Animal grouping and treatment administration followed the procedure detailed in "Xylene-Induced Ear Swelling in Mice". At 30 min after the final administration, 0.7% acetic acid (0.1 mL/10 g body weight) was injected intraperitoneally into individual mice. The number of abdominal constrictions and stretching over a period of 0–30 min displayed by each mouse was counted and recorded.

Acetic Acid-Induced Leukocyte Migration Test

The effects of EAEBc and Indo on leukocyte migration in acetic acid-treated mice were evaluated according to a previously described method.[29] Animal grouping and administration of drugs followed the protocol detailed in "Xylene-Induced Ear Swelling in Mice". At 60 min after the final administration, 0.5% Evans blue solution (0.1 mL/10 g) was injected via the intravenous route into a caudal vein, followed by intraperitoneal injection of 0.7% acetic acid into each mouse. After 20 min, mice were killed immediately and the abdominal cavity opened. Next, the abdominal cavity was rinsed with normal saline solution three times (3 mL each time) and peritoneal fluid collected in a vessel, followed by centrifugation at 3000 g for 10 min, and the supernatant was collected. Absorbance values were recorded using a UV-Vis spectrophotometer at a wavelength of 590 nm and the plasma protein content that bound to Evans blue was calculated to determine capillary permeability.

Formalin-Induced Paw Licking Test

Formalin-induced tonic pain experiments were conducted according to the method of Wu et al.[28] Animal grouping and treatment administration followed the protocol detailed in "Xylene-Induced Ear Swelling in Mice". At 60 min after the final administration, 2.0% formalin solution (20 μL) was subcutaneously injected into the right sub-plantar surface of the hind paw. The time spent by mice licking or biting their paws was recorded during the 1st phase (0–5 min) and 2nd phase (15–30 min) of the test. Next, the average times of licking or biting the footpad in each group were compared. Levels of serum inflammatory transmitters (NO, PGE2, 5-hydroxytryptamine (5-HT), β-endorphin (β-EP), substance P (SP), and norepinephrine (NE)) in mice were additionally determined. Briefly, 1 h after the final administration, a subcutaneous injection of 2.0% formalin solution (20 μL) was administered into the right sub-plantar surface of mice treated with EAEBc and Indo (excepting the blank group). After 1 h, blood was obtained through the eyeball vein centrifuged at 3500 g for 15 min, and the serum collected for determination of NO, PGE2, 5-HT β-EP, SP and NE levels.

Hot Plate Test

Experiments were carried out according to a previously reported protocol.[29] Animal grouping and treatment administration followed the procedure detailed in "Xylene-Induced Ear Swelling in Mice". At 30, 60, 90, and 120 min after treatment, mice were individually placed on a heated plate maintained at 50 ± 1.0°C for a maximum time of 40 s. The time for forepaw licking or jumping was taken as the latency time and the percentage of inhibition evaluated. Prior to initiation of experiments, mice were screened by placing individually on the hot plate set at 50 ± 1.0°C, and those failing to lick their hind paw or jump (nociceptive responses) within 5 s or longer than 30 s excluded.

Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA (GraphPad Prism 5), followed by Student's two-tailed *t*-test for comparison between test and control groups. Dunnett's test was conducted when experiments involved three or more groups. The Chi-square test was used to determine significant differences. The level of significance for all the tests was set at $p < 0.05$.

Results

Anti-Inflammatory Activity in vitro

The MTT assay was used to investigate the viability of RAW264.7 cells pretreated with different concentrations of EAEBc. Compared with the blank control group, EAEBc (8, 16, 32, 64 and 128 μg/mL) did not significantly inhibit RAW264.7 cell growth ($p = 0.662$) (Figure 1A), indicative of no toxicity of the selected concentrations of EAEBc to RAW264.7 cells. As shown in Figure 1B, compared with the blank control group, stimulation with 1 μg/mL LPS for 24 h significantly promoted TNF-α release ($p < 0.001$), which was suppressed upon EAEBc treatment in a dose-dependent manner. Compared with the LPS group, TNF-α release was remarkably reduced in the presence of 64 and 128 μg/mL EAEBc.

Similarly, stimulation with 1 μg/mL LPS for 24 h induced a significant increase in IL-6, IL-1β, and NO levels, which was suppressed by EAEBc in a dose-dependent manner in LPS-treated RAW264.7 cells. In particular, EAEBc at a concentration of 128 μg/mL significantly inhibited IL-1β, IL-6, and NO release ($p < 0.001$) in LPS-induced RAW264.7 cells (Figure 1C–E). Our results suggest that EAEBc exerts an effective anti-inflammatory effect that is concentration-dependent.

Anti-Inflammatory Activity in vivo

Effect of EAEBc on LPS-Induced Inflammation in Mice

As shown in Figure 2A–F, compared with the blank group, mice treated with LPS displayed increased TNF-α, IL-6, IL-1β, NO, iNOS, and PGE2 contents in blood plasma. This increase was reversed to varying degrees upon administration of different doses of EAEBc (Figure 2A–F). Compared with levels in LPS-treated mice, EAEBc at a dose of 200 mg/kg significantly inhibited TNF-α, IL-6, IL-1β, NO, iNOS, and PGE2 contents in blood plasma. Moreover, the levels of TNF-α, IL-6, NO, iNOS and PGE2 were significantly decreased in mice treated with 100 mg/kg EAEBc. The PGE2 level was

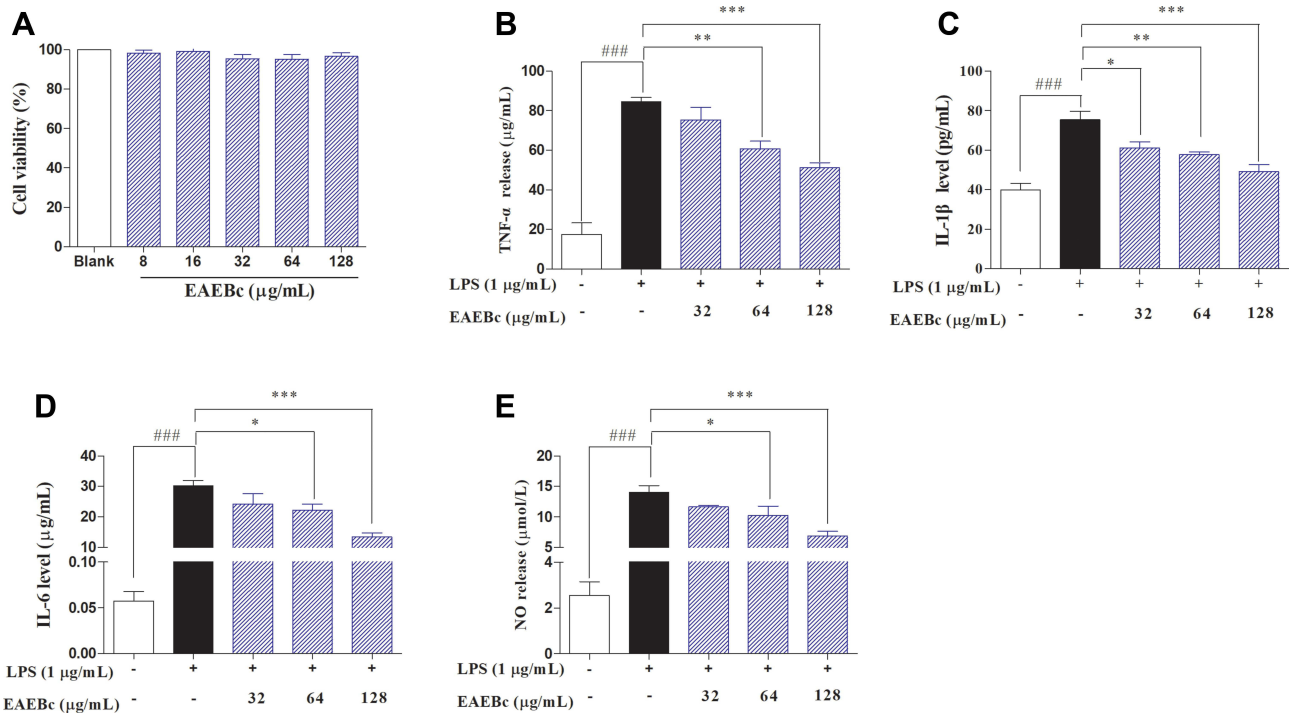

Figure 1 In vitro effects of EAEBc on LPS-induced inflammation in RAW264.7 cells.

Notes: (**A**) Effects of EAEBc (8, 16, 32, 64, and 128 μg/mL) on viability of RAW264.7 cells. (**B–D**) Effects of EAEBc (32, 64, and 128 μg/mL) on TNF-α, IL-1β, and IL-6 release in LPS-induced RAW264.7 cells. (**E**) Effects of EAEBc (32, 64, and 128 μg/mL) on NO release in LPS-induced RAW264.7 cells. Values for each group represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the LPS group. ###p < 0.001 compared with the normal (blank) group.

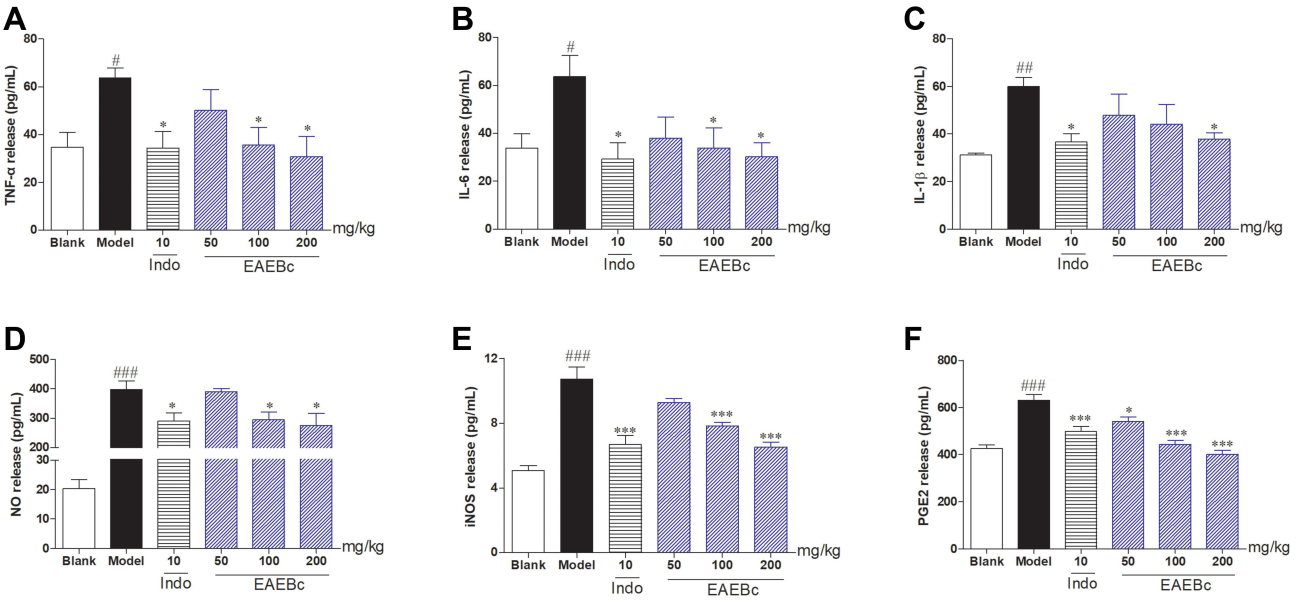

Figure 2 In vivo effects of EAEBc on LPS-induced inflammatory model mice.

Notes: (**A–C**) Effects of EAEBc (50, 100, and 200 mg/kg) on cytokine (TNF-α, IL-6 and IL-1β) levels in serum of LPS-induced inflammatory model mice. (**D**) Effects of EAEBc (50, 100, and 200 mg/kg) on NO in serum of LPS-induced inflammatory model mice. (**E–F**) Effects of EAEBc (50, 100, and 200 mg/kg) on iNOS and PGE2 contents in serum of LPS induced inflammatory model mice. Values for each group represent mean ± SEM. *p < 0.05, ***p < 0.001 compared with the LPS group. #p < 0.05, ##p < 0.01, ###p < 0.001 compared with the blank group.

markedly lower in mice treated with 50 mg/kg EAEBc ($p < 0.05$). In particular, EAEBc at concentrations of 100 and 200 mg/kg induced a notable decrease in LPS-mediated production of iNOS and PGE2 in a dose-dependent manner ($p < 0.001$).

Effect of EAEBc on Xylene-Induced Ear Swelling in Mice

As shown in Figure 3A, 30 μL xylene promoted a significant increase in the weight of the ear piece, which was markedly reduced by EAEBc and Indo treatment. Briefly, oral treatment with EAEBc at doses of 50, 100, and 200 mg/kg ip suppressed xylene-induced ear swelling in mice with inhibition rates of 19.62%, 37.5%, and 41.3%, respectively, compared to vehicle. The standard drug Indo (10 mg/kg, ip) reduced ear edema triggered by xylene by up to 72.16% compared to vehicle.

Effect of EAEBc on Carrageenan-Induced Paw Edema in Mice

Administration of a range of doses of EAEBc (50, 100, and 200 mg/kg) at 1, 3, and 5 h reduced paw edema induced by carrageenan (1%, 25 μL) to varying degrees in a dose-dependent manner (Figure 3B). Briefly, carrageenan-induced hind paw edema in mice was initiated at 0.5 h. Maximal inflammation was detected at ~3 h, after which a gradual decline was observed. Our results showed that treatment with EAEBc at doses of 100 and 200 mg/kg for 1, 3, and 5 h significantly reduced swelling induced by carrageenan. In particular, in the presence of 200 mg/kg EAEBc, paw volume was markedly reduced from the observation time, with respective inhibition rates of 47.02%, 37.02%, and 50.34% at 1 h, 3 h, and 5 h, respectively. The reference drug, Indo (10 mg/kg), also significantly relieved the degree of paw edema in test mice at 1 h, 3 h, and 5 h. EAEBc at a dose of 50 mg/kg alleviated the increase in swelling, but this reduction was not significantly different compared with the model group.

Anti-Nociceptive Activity in vivo

Effect of EAEBc on Acetic Acid-Induced Writhing in Mice

Oral treatment with EAEBc (50, 100, and 200 mg/kg) produced a marked dose-dependent reduction in abdominal stretching induced by intraperitoneal injection of 0.7% acetic acid in mice with inhibition rates of 19.94%, 40.32%, and 52.42%, respectively, compared to the model group. The reference drug, Indo, also significantly inhibited writhing activity induced by acetic acid, with superior (55.24% inhibition) activity at a dose of 10 mg/kg (Figure 4A). In addition, compared with the model group, EAEBc (100 and 200 mg/kg) exerted significant inhibitory effects on permeability caused by acetic acid, as displayed in Figure 4B ($p < 0.05$).

Effect of EAEBc on Formalin-Induced Paw-Licking in Mice

In the formalin test, intraperitoneal injection of EAEBc reduced formalin-induced nociceptive responses during both first (0–5 min, neurogenic) and second (15–30 min, inflammatory) phases (Figure 4C). Nevertheless, EAEBc was more

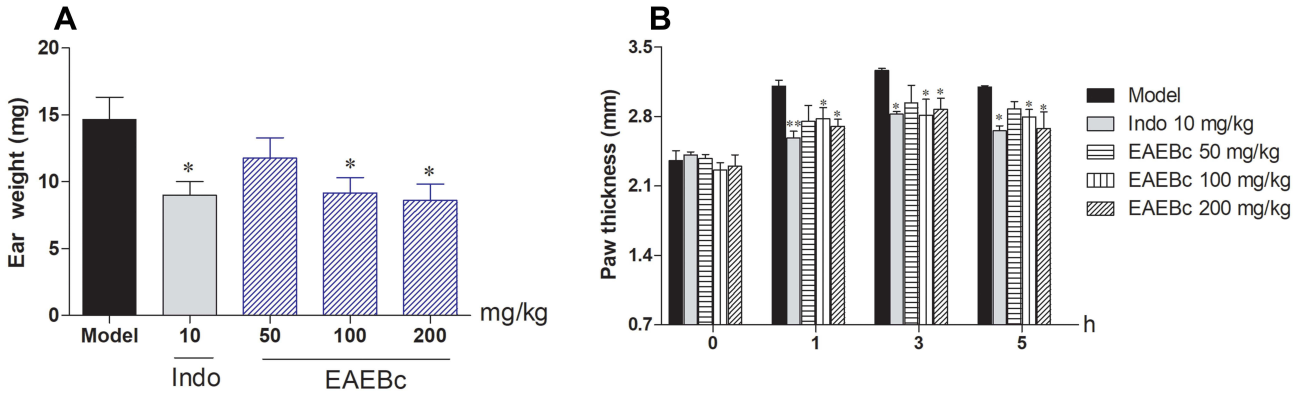

Figure 3 Anti-inflammatory activity of EAEBc in vivo.
Notes: (**A**) Effects of EAEBc and Indo (10 mg/kg) on xylene-induced ear swelling in mice. Ear weight (mg) = left-ear weight–right-ear weight. (**B**) Effects of EAEBc and Indo (10 mg/kg) on carrageenan-induced paw edema. Each value represents mean ± SEM. *p < 0.05, **p < 0.01 compared with the model group.

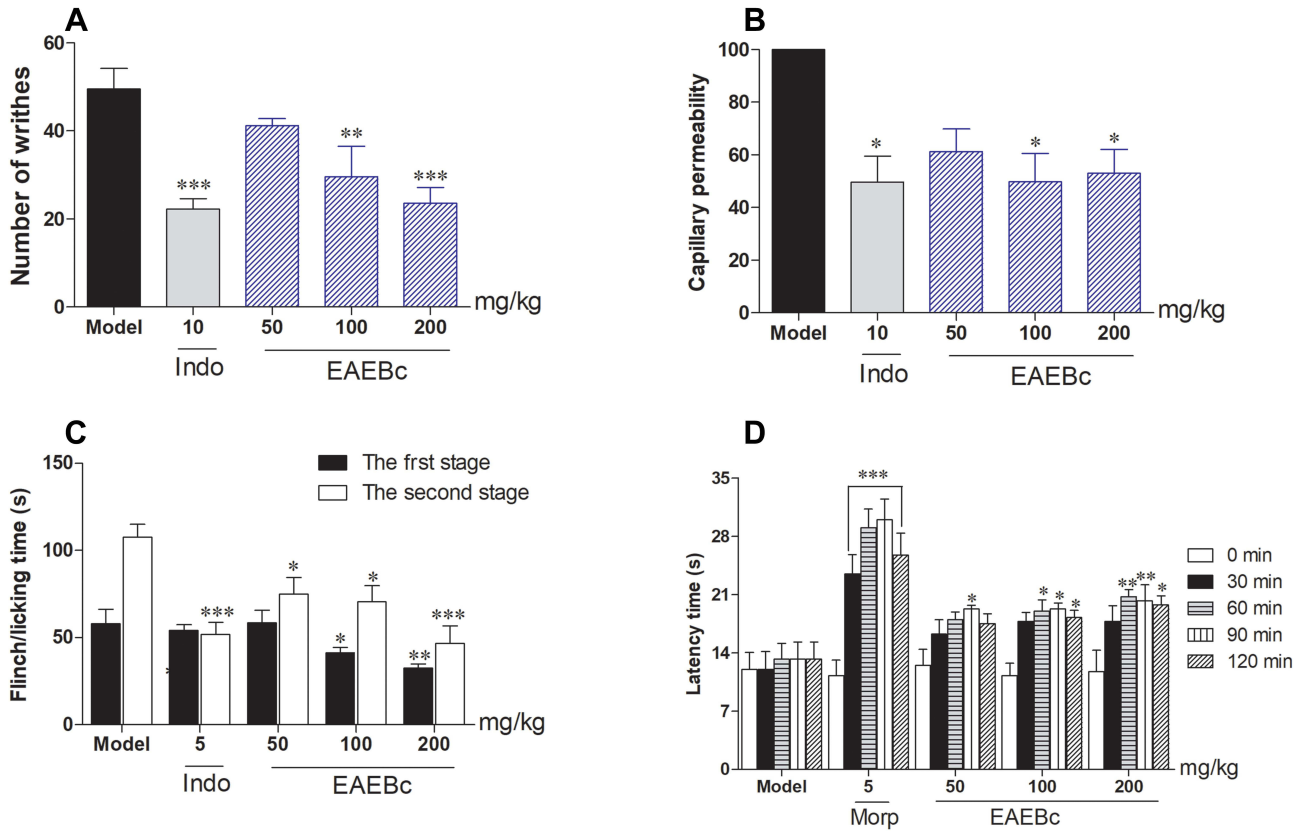

Figure 4 EAEBc activity in antinociceptive tests in mouse models.

Notes: (**A**) Effects of EAEBc and Indo on acetic acid-induced writhing in mice. (**B**) Effects of EAEBc and Indo on leukocyte migration in acetic acid-induced mice. (**C**) Effects of EAEBc and Indo on formalin-induced paw licking/biting time in mice. First stage: 0–5 min (neurogenic pain); Second stage: 15–30 min (inflammatory pain). (**D**) Effects of EAEBc and morphine (Morp) in the hot plate test. Each value represents mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the model group.

effective in reducing pain in the second (inflammatory) phase. In the neurogenic (first) phase, only the medium and high doses of EAEBc (100 and 200 mg/kg) effectively reduced the formalin-induced nociceptive effect with inhibition rates of 28.88% and 43.97%, respectively. At the second stage, 50, 100, and 200 mg/kg EAEBc induced marked inhibition of formalin-mediated inflammation with inhibition rates of 30.23%, 34.42%, and 56.51%, respectively. Indo (10 mg/kg) caused a significant reduction in the duration of nociceptive activity in the second phase (inhibition rate of 51.86%).

Effects of EAEBc on Serum Inflammatory Factors in the Formalin Test

Compared with the blank control group, serum NO, PGE2, 5-HT, substance P, and NE contents were significantly increased, while β-EP was significantly decreased in formalin-treated mice ($p < 0.05$), as shown in Table 1. Compared to model control mice, administration of EAEBc (200 mg/kg) significantly inhibited substance P release in serum, similar to results obtained with Indo. Meanwhile, the administration of EAEBc (100 and 200 mg/kg) induced a marked increase in the serum β-EP content in formalin-treated mice. Furthermore, pretreatment with EAEBc inhibited serum PGE2, NO, 5-HT, and NE production in a dose-dependent manner, while these factors were significantly increased in mice treated with formalin and normal saline.

Effect of EAEBc on Pain in the Hot Plate Test in Mice

As shown in Figure 4D, treatment with EAEBc (50, 100, and 200 mg/kg) elicited a significant increase in latency reaction in the thermal nociceptive test. After 30 min pretreatment with EAEBc (50, 100, and 200 mg/kg), a gradual increase in response latency in the thermal stimuli test was observed. This effect, achieved with 100 and 200 mg/kg EAEBc, remained significant after 60, 90, and 120 min of EAEBc administration. EAEBc at a dose of 200 mg/kg induced an increase in threshold of pain of mice by ~51.06%, 76.59%, 72.34%, and 68.09% at 30, 60, 90, and 120 min,

Table 1 Effect of EAEBc on Serum Inflammatory Factors in Formalin Test (X ± SEM, n = 6)

Group	Dose (mg/kg/d)	β-EP (pg/mL)	SP (pg/mL)	5-HT (pg/mL)	NE (ng/mL)	PGE2 (pg/mL)	NO (pg/mL)
Blank	–	4.57±1.35	21.25±6.70	36.82±5.29	2.41±0.25	0.43±0.05	89.82±9.01
Model	–	2.64±1.08$^{\#}$	41.4±11.72$^{\#}$	51.43±9.51$^{\#}$	4.85±0.94$^{\#\#\#}$	0.63±0.05$^{\#\#}$	207.1±18.98$^{\#\#\#}$
Indo	10	4.36±0.99*	28.52±11.40	39.28±5.13*	4.03±0.11*	0.42±0.08**	176.5±11.88*
EAEBc	50	2.75±0.80	41.28±14.11	50.14±6.98	4.47±0.4	0.57±0.07	194.9±13.52
	100	3.54±0.57	38.23±3.67	46.91±5.09	4.24±0.13	0.48±0.08*	179.1±16.55*
	200	4.37±1.15*	28.69±6.44	39.15±5.57*	4.02±0.27*	0.44±0.10**	181.0±10.88*

Notes: Compared with model group, *$p < 0.05$, **$p < 0.01$; Compared with blank group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

respectively. Pretreatment with the standard drug morphine (5 mg/kg) significantly induced an analgesic effect at all times in mice ($p < 0.001$) on the hot plate test.

Discussion

The rhizome of *B. chinensis* is traditionally used for the treatment of inflammation and pain-related diseases in East Asia phototherapy systems. However, existing reports on the specific activities of rhizomes that contribute to their therapeutic effects have not been compared to date. In the present study, we evaluated the anti-nociceptive and anti-inflammatory activities of the ethyl acetate extract of *B. chinensis* using thermal and chemical stimuli to assess its potential as a central and/or peripheral (inflammatory site) analgesic with the aid of both in vitro and in vivo inflammatory models, with particular focus on the underlying mechanisms. Our results clearly suggest that this common herb not only has promising anti-inflammatory properties but is also able to exert anti-nociceptive effects through both the peripheral and central nervous systems. The anti-nociceptive and anti-inflammatory effects of EAEBc may be dependent on inhibition of inflammatory cytokines and mediators, such as TNF-α, IL-6, IL-1β, as well as iNOS and PGE2. The present study aimed to systematically and comprehensively explore the anti-inflammatory properties of EAEBc in vivo and in vitro. The anti-nociceptive activity of EAEBc was additionally validated in vivo using a mouse model.

Inflammation plays a crucial role in tissue defense responses to pathogens, exogenous substances, irradiation, and even damaged cells.[30,31] LPS is a known pathogen-derived molecule that activates multiple inflammatory signals. Inflammatory injury caused by LPS in RAW264.7 macrophages is widely used as a classical model for anti-inflammatory drug screening in vitro. LPS-induced RAW264.7 macrophages secrete a variety of inflammatory mediators or pro-inflammatory factors, including NO, PGE2, TNF- α, IL-6, IL-12, IL-23, IL-1β, and COX-2. Increased levels of these inflammatory mediators and cytokines are closely associated with the occurrence and development of inflammatory diseases.[32–34] Inflammation-related mediators also serve as critical targets for LPS-induced inflammatory diseases. Changes in these cytokines are often used as important indicators to evaluate the efficacy of candidate anti-inflammatory drugs. Therefore, crude extracts or compounds that can inhibit the production or release of inflammatory mediators and cytokines have potential for development as therapeutic candidates for inflammation and related diseases.

Based on the traditional use of plant material for treatment of a range of conditions, such as muscle pain, sore throat, tonsillitis, asthma, and dysmenorrhea, we investigated the actions of EAEBc in the LPS-induced RAW264.7 cell inflammatory model in vitro as well as LPS-induced mouse inflammatory model in vivo. EAEBc exerted effective inhibitory effects on NO generation as well as IL-6, IL-1β, and TNF-α secretion in LPS-induced RAW264.7 cells. Consistently, data from our in vivo experiments show that EAEBc downregulates serum contents of TNF-α, IL-1β, IL-6, NO, iNOS, and PGE2 in a dose-dependent manner in LPS-treated mice. Experimental models of chemical-induced inflammatory responses include the carrageenan-induced paw edema test and xylene-induced ear swelling model. Carrageenan-induced inflammation is a commonly used acute and highly reproducible inflammatory test, while xylene-induced edema leads to vasodilation and edematous changes and also serves as an acute neurogenic inflammatory model. Here, the carrageenan-induced paw edema test was employed to further establish the anti-inflammatory activity of EAEBc. Consistently, pretreatment of mice with EAEBc suppressed carrageenan-induced paw edema in mice from the first hour, with significant reduction of paw thickness at 3 h. Experiments on the neurogenic inflammatory model of xylene-induced ear swelling showed that EAEBc at

doses of 50, 100, 200 mg/kg suppressed ear swelling in mice in a dose-dependent manner (19.62%, 37.5%, and 41.3% inhibition relative to the group treated with vehicle (normal saline), respectively). Experimental data from the formalin test validated the effects of EAEBc in the inflammatory phase, supporting the conclusion that *B. chinensis* has significant anti-inflammatory activity. Future studies should focus on clarifying the molecular mechanisms of action of *B. chinensis* and identifying the active constituents responsible for its effects.

In general, anti-inflammatory drugs also have anti-nociceptive activity. Accordingly, we employed classical analgesia experimental models, including abdominal writhing, hot plate and formalin, to evaluate the potential peripheral analgesic effect of *B. chinensis*. Acetic acid-induced writhing is related to increased peritoneal fluid levels of COX and PGE[35] and is commonly used to examine the analgesic effects of drugs. The formalin test, a valid and reliable model of nociception, has been applied to various classes of analgesic drugs. This model comprises two distinct phases: early phase (neurogenic pain) and late phase (inflammatory pain).[27] Notably, several inflammatory transmitters (PGE2, NO, 5-HT, β-EP, substance P, and NE) are produced and released at the second stage of formalin-induced pain in mice.

The hot plate test, known to involve activation of supraspinal structures, is used in basic heat-induced pain research to select narcotic analgesics.[35] In our experiments, EAEBc induced an extension of pain latency in a dose-dependent manner with significant anti-nociceptive activity against both acetic acid-induced writhing and a remarkable increase in latency in the nociceptive response induced by heat, especially at a high dose of 200 mg/kg. Taking into account the mechanisms of action of the inducing agents, we further examined the effects of EAEBc on formalin-induced nociception in mice, which showed significant antinociceptive effects of EAEBc administered intraperitoneally at doses of 50, 100 and 200 mg/kg at both neurogenic and inflammatory phases. In the neurogenic phase, inhibition was 6.90%, 28.88%, and 43.97% while in the inflammatory phase, inhibition was 30.23%, 34.42%, and 56.51%, respectively, compared to the vehicle group. In view of the collective findings, we propose that the anti-nociceptive effect of EAEBc is mediated via both peripheral and central mechanisms. Additionally, EAEBc may exert a good analgesic effect against inflammatory pain by inhibiting inflammatory mediators associated with the nociceptive responses, such as PGs, COX, TNF-α, interleukin and substance P. EAEBc inhibited nociception in both acetic acid-induced abdominal writhing and formalin tests, but was more effective against inflammatory pain.

Previous chemical and pharmacological investigations have revealed contributory roles of isoflavonoids and flavonoids in the bioactivities of medicinal plants. In this study, EAEBc was identified as an excellent source of flavonoids, including irisflorentin, iridin, irigenin, tectorigenin, tectoridin, quercetin and kampferol, which could underlie its antinociceptive and anti-inflammatory effects. Limited studies have demonstrated the presence of the *B. chinensis*-like isoflavonoids (irisflorentin, tectorigenin and tectoridin) in plants, which exert anti-inflammatory effects by suppressing prostaglandin E_2, COX-2, NO, tumor necrosis factor α, and interleukin-6 production in vitro.[7,36] Moreover, the anti-inflammatory activity of irisflorentin may be attributed to the inhibition of NO, TNF-α, IL-1β, and IL-6 mediated by the inactivation of ERK1/2- and p38-mediated activator protein-1 pathways.[37,38] Lim et al[25] reported that tectorigenin exerts anti-inflammatory effects through down-regulation of the inflammatory mediators iNOS, COX-2, TNF-α, and IL-6 by suppressing NF-kB/ERK/JNK-related signaling pathways. Recent experiments by Kim and co-workers (2021) showed that triterpenoids, such as isoiridogermanal and iridobelamal A, isolated from *B. chinensis* exert anti-inflammatory effects through the NF-κB pathway.[14] Based on these reports, we propose that *B. chinensis* could exert an anti-inflammatory effect through regulation of ERK1/2- and p38-mediated activator protein-1 signaling and the NF-κB pathway. Several patents have been issued for the development of ursolic acid as an anti-inflammatory agent. Combined in vitro and in vivo data support the theory that more than one of these isoflavonoids, flavonoids, and triterpenoids potentially contribute to the anti-nociceptive and anti-inflammatory activities of *B. chinensis* in experimental rodent models. Thus, the anti-nociceptive and anti-inflammatory activities of these compounds require comprehensive investigation. Data from the present study data provide a framework for further research on mechanisms of action, active compounds and structure–activity relationships, which should aid in the future development of novel, improved therapeutic agents derived from *B. chinensis*.

Conclusion

Our collective in vitro and in vivo findings clearly demonstrate anti-nociceptive and anti-inflammatory activities of *B. chinensis* extract, providing a theoretical basis for further pharmaceutical research and supporting traditional claims of its

medicinal value in China. However, the active constituents and precise anti-nociceptive and anti-inflammatory pathways of *B. chinensis* require further investigation. Additionally, detailed exploration of the biochemical and preclinical toxicity, bioavailability and pharmacokinetics, as well as active molecules of the extract is warranted to facilitate functional application of this herb in modern phototherapy.

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Disclosure

The authors declare that there are no conflicts of interest in this work.

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