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Antinociceptive efficacy of *Clerodendrum petasites* S. Moore, a Thai medicinal plant, and its CNS safety profiles

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ABSTRACT

Background: *Clerodendrum petasites*, an herbal plant in Thailand, has been used for many years in folk medicine. However, scientific evidence regarding CNS safety pharmacology and antinociceptive activity of *C. petasites* (CP) has not yet been well characterized.
Purpose: The present study aimed to assess the CNS safety pharmacology and antinociceptive and antiinflammatory effects of CP extract.
Methods: The effect of CP extract on CNS safety pharmacology was assessed using LABORAS automated home cage monitoring and rotarod test. Its pharmacological activity was evaluated both in-vitro, and in-vivo using hot-plate, acetic acid-induced writhing, formalin, and carrageenan-induced paw edema models.
Results and conclusion: CP extract significantly improved thermal and chemical nociceptive behaviors and acute inflammatory pain at all doses: 300, 600, and 1200 mg/kg, p.o. The antiinflammatory effect of CP extract in inflammatory pain models was comparable to the effect of positive control: indomethacin 10 mg/kg at all dose levels tested. Further, the CP extract at 600 mg/kg dose significantly inhibited 82.3% of carrageenan-induced total edema. In-vitro, CP extract at 12.5, 25, and 50 μg/mL concentrations significantly reduced the expression of LPS-induced nitric oxide, IL-6, and TNF-α expression in both RAW 264.7 macrophage and BV-2 microglial cell lines. In addition, CP extract did not show any potential effects on the CNS, indicated by no significant effects on motor coordination, spontaneous locomotor activity, general behaviors, and well-being compared to vehicle-treated mice ($p > 0.05$). Overall, the present study evidences the potential antinociceptive, antiinflammatory efficacies of CP extract with a favorable CNS safety profile.

1. Introduction

Pain is a leading cause of disability worldwide, and its incidence is steadily increasing due to increasing pain-related diseases.[1] Nociceptive pain is an unpleasant symptom characterized by the progressive activation of the nociceptors by noxious and non-noxious stimuli.[2] In addition, the activation of nociceptors can also be mediated by inflammatory mediators known as inflammatory pain. Physiologically, inflammation is a defensive response

Abbreviations: CNS, central nervous system; NSAIDs, nonsteroidal anti-inflammatory drugs; CP extract, *Clerodendrum petasites* S. Moore ethanolic extract; IACUC, Institutional Animal Care and Use Committee; %MPE, % maximum possible effect.

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from the human body. However, excessive inflammation causes severe symptoms such as heat, swelling, redness, and pain.[3] Steroidal, nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids have been widely prescribed for pain relief. These available drugs have a wide range of side effects, such as the increased risk of osteoporosis, renal damage, thrombosis, gastrointestinal bleeding, and increased blood pressure.[4] Certain side effects of analgesics have also been linked to their effects on the central nervous system (CNS).[5–7] It is, therefore, necessary to develop new drugs that have fewer side effects but are more effective in the treatment of pain. In this case, the study of herbal extracts with better properties will be an alternative approach to address the issues mentioned above.

Evidence indicates that undesirable CNS side effects contribute to the withdrawal of drugs from the market.[8] Furthermore, several analgesic drug candidates that have been proven to be pharmacologically effective in preclinical studies have failed in clinical trials. One of the reasons for this failure is safety concerns due to unwanted side effects.[9] Only 11% of analgesic drug candidates tested in clinical trials passed to the market.[10] Hence, the assessment of CNS safety pharmacology of herbal extracts has attracted recent attention, in which it has been evaluated in *Alstonia scholaris* (L.) R. Br leaves extract,[11] *Cassia occidentalis* Linn. stem extract,[12] and *Plinia cauliflora* fruit extract[13] Further, the abundance of pharmacologically active compounds in herbal extracts is linked topotential adverse effects.[14] Therefore, it is crucial to determine the safety pharmacology of herb extracts, including their CNS safety pharmacology.

It is well known that medicinal plants are commonly used by the population without access to essential medicines and by ethnic groups and villagers in rural areas of Thailand.[15] At present, the use of traditional Thai plants is well preserved. *Clerodendrum petasites* S. Moore, a Thai medicinal plant belonging to the Lamiaceae family, has been used for many years as a folk medicine in many regions of Thailand for its anti-inflammatory and antipyretic effects.[16] *C. petasites* S. Moore is widespread in several other countries, including China, India, Malaysia, Sri Lanka, and Vietnam.[17] In Thailand, the plant is commonly prepared as a multi-herbal recipe known as 'Ha-Rak' and has been approved as herbal medicine by the Thai FDA. Scientific evidence has shown that *C. petasites* S. Moore has a wide range of biological and pharmacological activities in inflammation, rheumatism, fever, cough, and vomiting.[18,19] The phytochemical components of *C. petasites* S. Moore have been extensively studied. Vanillic acid, verbascoside, 4-coumaric acid, cirsimaritin, ferulic acid, nepetin, luteolin, apigenin, naringenin, hispidulin, hesperetin, and chrysin were found in the aerial parts, leaves, or roots of the plant,[18] in which some studies have shown that these active compounds act effectively as potential analgesics.[20–26]

Although *C. petasites* S. Moore has been effective in traditional use and scientifically reported to have pharmacological activities, little is known about its safety pharmacology in the CNS to the best of our knowledge. The potential analgesic efficacy of medicinal plants should be in line with theirsafety pharmacology in the CNS. Thus, considering the medicinal use of this plant, this study aimed to investigate the CNS safety pharmacology and antinociceptive and antiinflammatory properties of ethanolic extract of *C. petasites* S. Moore in mice.

2. Materials and methods

2.1. Drugs and chemicals

Hispidulin (ChemFaces, Wuhan, China), tween 80 (Srichansahasoth Co., Thailand), acetic acid (Merck, Darmstadt, Germany), formaldehyde (Merck), indomethacin (IND; Sigma), morphine sulfate (MRP; Thai FDA), and sterile water (SW; General Hospital Products Public Co.) were used in this study. HPLC grade acetonitrile and methanol (MeOH) were purchased from RCI Labscan (Bangkok, Thailand). Ultrapure water was obtained from Barnstead™ MicroPure™ Water Purification System (Thermo Fisher Scientific, Massachusetts, USA). Glacial acetic acid was purchased from Merck KGaA (Darmstadt, Germany).

2.2. Plant material

Whole plants of *C. petasites* S. Moore (CP, 500 g) were harvested from Rayong Province, Thailand, and authenticated by Associate Professor Surattana Amnuoypol at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Herbarium specimens with assigned vouchers as SS-SA-2015-191015 were deposited in the Museum of Natural Medicines, Chulalongkorn University.

2.3. Plant extraction

The plants were macerated with 2 L of 95% ethanol for 5 days at room temperature and then filtered. The filtrate was evaporated to dry under vacuum conditions. The yield of the extract was 6.85%. The crude extract was kept at −20°C until used.

2.4. Standardization of ***C. petasites*** *S. Moore extract*

Hispidulin is one of the most common bioactive compounds in CP extract[27] and is commonly used for standardization purposes.[28] Hence, in this study, hispidulin was used to standardize CP extract before its subsequent use in therapeutic efficacy evaluations. Quantitative analysis of hispidulin in *C. petasites* extract was performed using Agilent 1290 Infinity II UHPLC system combined with Purospher® STAR RP-18 endcapped reversed-phase column (250 x 4.6; 5 μm, Merck, Darmstadt, Germany) and C-18 guard column. The mobile phase was acetonitrile (**A**) and 20% acetonitrile in 0.1% aqueous acetic acid (**B**). A sample solution of *C. petasites* was prepared by dissolving 1 mg of *C. petasites* extract in 1 mL of MeOH, followed by filtration through a 0.22 μm PTFE membrane syringe filter. Hispidulin stock solution was prepared by dissolving 2 mg of standard hispidulin in 2 mL of MeOH. The solution was filtered through a 0.22 μm PTFE membrane syringe filter. Working solutions were obtained by serial dilution of stock solution with MeOH to 100, 80, 60, 40, 20, and 10 μg/mL, respectively. All solutions were kept at 5°C before analysis. The gradient system was 0–25% **A** for 15 min, 25% **A** for 5 min, 25–94% **A** for 2.5 min, 94% **A** for 2.5 min, and 0% **A** for 10 min. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. The experiments were carried out at 35°C and monitored by a diode array detector at 337 nm. A calibration curve was constructed by plotting the concentration versus peak area of hispidulin.

2.5. Animals

Male ICR mice obtained from Nomura Siam International Co. Ltd. and Mahidol University, Bangkok, Thailand, were used in these experiments. Mice weighing 28–35 g (5–7 weeks old) were maintained in a controlled room with a temperature of 22±2°C and humidity of 50%–60% on a 12/12 h light/dark period. Mice were allowed to have access to food and water *ad libitum*. All procedures performed in this study were compliant with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Pharmaceutical Sciences (Protocol Number 2133007), Chulalongkorn University.

2.6. Drug administration

A high dose of CP extract exhibiting pharmacological activity (1200 mg/kg body weight) was selected to examine the safety pharmacology as previously reported in a study.[29] The CP extract was dissolved in 2% (w/v) Tween 80, and the positive control, chlorpromazine (5 mg/kg), was dissolved in normal saline and orally administered to mice with a constant volume of 10 ml/kg. Furthermore, the CP extract at doses of 300, 600, and 1200 mg/kg was used to assess its antinociceptive activity along with morphine (10 mg/kg) and indomethacin (10 mg/kg) as positive controls.

2.7. Determination of antinociceptive and antiinflammatory effects of CP extract

2.7.1. Hot-plate test

Acute thermal nociception was evaluated with the hot-plate test (Harvard Apparatus). Mice were individually placed in a transparent plastic cage cylinder and recorded until pain-like behaviors such as licking and jumping were achieved. The temperature was set at 55 ± 0.5°C, and measurements were taken at different time points at 15, 30, 45, 60-, 90-, 120-, and 240-min post-treatment. Morphine 10 mg/kg was given orally as a positive control. A 45-s cut-off was imposed to avoid tissue damage.

The antinociceptive activity was expressed as the mean % maximum possible effect (%MPE):

$$\%MPE = \frac{(Post - drug\ latency) - (Pre - drug\ latency)}{(Cut - off\ time) - (Pre - drug\ latency)} \times 100$$

After obtaining %MPE, the area of analgesia was assessed by measuring the area under the curve (%MPE x minutes-post drug administration).

2.7.2. Acetic acid-induced writhing test

Visceral nociception was induced by intraperitoneal administration of acetic acid (0.6% in sterile water, 10 mL/kg) to the abdomen of mice, and the number of abdominal writhes was counted. To test the activity of the extracts, mice were administered orally with the extract 1 h before acetic acid injection. Animal behavior was recorded, and the number of writhes was counted for 30 min. Indomethacin at the dose of 10 mg/kg was given orally as a positive control.

2.7.3. Formalin test

Nociceptive and inflammatory pain-like behaviors were evaluated with the formalin test. Briefly, mice were treated with the extract 1 h before the intraplantar injection of formalin (20 μL, 2.5% in sterile water) and placed in a plexiglass cylinder and observed. Immediately after formalin administration, pain responses such as licking or biting the hind paw were observed, and the time spent on those behaviors was recorded and analyzed. Pain-like behaviors were recorded after formalin injection at 0–5 min (first or neurogenic phase) and at 15–30 min (second or inflammatory phase). Indomethacin10 mg/kg was given orally as a positive control.

2.7.4. Carrageenan-induced paw edema test

The mouse model of carrageenan-induced paw edema was employed to determine the anti-inflammatory effects of CP extract. One hour after treatment, mice were administered 1% carrageenan in normal saline intraplantar to the left hind paw (50 μL/paw). The volumes of hind paws were determined at 0 h (baseline before carrageenan administration, V_0) and every hour for 6 h after carrageenan administration (V_t) using a plethysmometer (UGO Basile, VA, Italy). Then the percentage change in hind paw volume was calculated using the equation below and presented in a time course curve.

$$\%change\ in\ paw\ volume = \frac{V_t - V_0}{V_0} \times 100$$

The area under the time course curve was calculated and used to determine the % inhibition of paw edema by each intervention.

$$\%inhibition\ of\ paw\ edema = \frac{AUC_{Control} - AUC_{Treatment}}{AUC_{Control}} \times 100$$

2.8. In-vitro assessments of CP extract

2.8.1. Cell culture

RAW 264.7 (ATCC, Manassas, VA, USA) and BV-2 cells (AcceGen Biotechnology, New Jersey, USA) were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were kept in an incubator at 37°C under 5% CO_2.

2.8.2. MTT assay

MTT assay was performed to assess the cell viability after being treated with the extracts. Briefly, cells were seeded in 96 wells at the concentration of 2×10^4 cells/well. After 24 h, cells were treated with various concentrations of the extract (6.25, 12.5.25, 50, 100 μg/mL) for another 24 h. The media was removed, followed by the addition of MTT solution (0.5 mg/mL), and incubated for 3 h. The formazan crystals were dissolved with DMSO, and the absorbance was measured under a microplate reader at 570 nm (CLARIOstar®, BMG Labtech, Ortenberg, Germany).

2.8.3. Cell culture treatment and LPS stimulation

The cells were seeded in 24-well plates with a concentration of 2×10^4 cells/well for 24 h. The cells were then pretreated with the vehicle, positive control, and the CP extract for 1 h followed by coincubation with LPS 1 μg/mL. The duration of LPS incubation for RAW 264.7 and BV-2 cells was 12 h and 24 h, respectively. After incubation, the media was collected to measure the inflammatory mediator levels. The nitrite level of media was measured using nitrite assay (Griess), and cytokine levels (TNF-α and IL-6) were measured using ELISA kits.

2.8.4. Nitrite assay (Griess) and ELISA assay

Nitrite Assay (Griess) and ELISA kits were purchased from Sigma-Aldrich, St. Louis, MO, USA, and BioLegend, San Diego, CA, USA, respectively. The assays were performed in accordance with the manufacturer's instructions.

2.9. Behavioral tests to determine the CNS side effects of the CP extract

2.9.1. Effects on spontaneous locomotor activity, general behavior, and well-being

The effects of CP extract on spontaneous locomotor activity were assessed using the automated behavioral analysis LABORAS (Metris, Hoofddorp, Netherlands). The behaviors included were climbing, locomotion (walking, running, jumping), rearing, and immobility, and undefined behaviors were excluded from the analysis. In brief, mice were orally administered with CP extract 1200 mg/kg, chlorpromazine 5 mg/kg (positive control), or vehicle (2% Tween 80). After 1 h, each mouse was placed in the LABORAS cage (22 × 16 × 14 cm), and behaviors were recorded for 30 min.

General behaviors and well-being of mice were determined

using the LABORAS. The behavioral changes were assessed day and night (24 h), such as mobile behaviors (climbing, locomotion, and rearing), immobility, food and water intake, and body weight changes. On the experimental day, mice were orally administered with CP extract, chlorpromazine, or vehicle, and subsequently, each mouse was placed in the LABORAS cage for 24 h.

2.9.2. Rotarod test

Motor coordination was evaluated in mice receiving a high therapeutic dose of CP extract (1200 mg/kg, p.o.) using the rotarod apparatus (Ugo Basile, Italy). The speed of the rotating rod was adjusted to 17 rpm (constant speed). Mice were placed on the rotating rod, and the duration of mice remained on the rod until they fell was considered as rotarod latency. The cut-off was set to be 180 s. Before the testing day, mice were first trained for two consecutive days to remain on the rotating rod for 180 s. Mice were administered with CP extract followed by assessments of rotarod latency at 0, 30, 60-, 90-, 120-, and 240-min post-administrations.

2.10. Statistical analysis

All data are shown as mean ± SEM. 6–10 mice per group were used in each experiment. The number of mice/groups used for each experiment was stated in the figure legends. Statistical significance was determined using GraphPad Prism 9.1, analyzed by one-way or two-way ANOVA followed by *post hoc* Bonferonni. $P < 0.05$ was considered significant. The levels of significance are indicated by: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. Results

3.1. Standardization of C. petasite extract by hispidulin content

In this study, The UHPLC method was developed to obtain a chemical fingerprint of *C. petasites* and quantify the amount of hispidulin in *C. petasites* extract. The method was modified from a previous report for optimal separation.[28] The UHPLC chromatogram of *C. petasites* ethanolic extract demonstrated sharp, symmetric, and well-separated peaks with two major peaks. One of the major peaks at a respective retention time of 19.6 min represents hispidulin (Fig. 1A). This peak was further confirmed by comparing it with the chromatogram of the hispidulin standard, which also revealed a peak of hispidulin at the same retention time of 19.6 min (Fig. 1B and C). The calibration curve of hispidulin was established by plotting the respective peak area of different concentrations of standard hispidulin ranging from 10 to 100 μg/mL with a coefficient of determination (R^2) of 0.9967 (Fig. 1D). The limit of detection (LoD) and limit of quantitation (LoQ) were calculated to be 5.81 and 17.6 μg/mL, respectively. From the calibration curve, the amount of hispidulin in *C. petasites* extract was quantified to be 2.02% w/w of extract.

3.2. Effect of C. petasite extract on acute thermal nociception

The effect of CP extract on thermal nociception was determined using the hot-plate test. The results demonstrated that CP extract at all doses could improve thermal nociception, as indicated by a significantly higher area of analgesia compared to that of the control group (2% tween 80). All doses showed comparable results compared to that of positive control, morphine 10 mg/kg (Fig. 2A).

3.3. Effect of C. petasite extract on acute visceral nociception

The effect of CP extract on visceral nociception was assessed using the acetic acid-induced writhing test. All doses of the extract significantly inhibited the writhing response. Furthermore, the effect of the extract was comparable with the positive control (indomethacin 10 mg/kg) at all the doses tested (Fig. 2B).

3.4. Effect of CP extract on acute inflammatory pain

The effect of CP extract on acute inflammatory pain was assessed using the formalin test. The results demonstrated that the extract at all doses exhibited significant effects inphase I (Fig. 2C) and phase II (Fig. 2D) of the formalin test. All doses of CP extract showed comparable antiinflammatory activity to 10 mg/kg indomethacin in phase II. These results indicate the ability of the extract to improve nociceptive and inflammatory pain in the formalin test.

3.5. Effect of CP extract on carrageenan-induced inflammation

The time course curve indicates the change in paw edema volume at each hour for 6 h after carrageenan administration and treatment interventions (Fig. 3A). As shown in the figure, carrageen administration progressively increased the volume of the injected paw. The paw volume of vehicle-treated mice peaked at 2 h and then gradually reduced. Treatment with indomethacin and CP extract substantially reduced the paw volume, where the peak effect was observed at 2 h and lasted for 6 h. Further, to compare the efficacy of each intervention, the area under the time course curve was determined and expressed as total edema (Fig. 3B). CP extract significantly reduced paw edema at all doses, and the effect is comparable to indomethacin 10 mg/kg. As shown in Fig. 3C, CP extract at 600 mg/kg dose exhibited the highest efficacy, inhibiting 82.3% of carrageenan-induced paw edema.

3.6. Effect of CP extract on inflammatory releases in LPS-stimulated RAW 264.7 and BV-2 microglial cells

To assess the effects of CP extracts on inflammatory mediator release at the cellular level, RAW 264.7 and BV-2 microglial cells stimulated with LPS were used. Firstly, the cytotoxicity profile of the CP extract was determined to obtain a non-toxic concentration of the extract, which was further used for subsequent tests. As shown in Fig. 4, concentrations of less than 50 μg/ml demonstrated no toxicity to the cells, whereas 100 μg/ml of the extracts elicited toxicity to RAW 264.7 and BV-2 microglial cells, indicated by a significantly reduced cell viability compared to that of untreated cells. Hence, the concentration of 50 μg/ml and below were used in NO and cytokine assays. The results showed that CP extract concentration dependently suppresses inflammatory mediator releases (NO, TNF-α and IL-6) in LPS-stimulated RAW 264.7 and BV-2 cells (Fig. 5). A high concentration of the extracts (50 μg/ml) demonstrated comparable effects with the positive control, either dexamethasone 50 μg/ml or minocycline 50 μg/ml.

3.7. CNS side effects of the CP extract

3.7.1. The effect of C. Petasite extract on spontaneous locomotor activity

The effect of CP extract on spontaneous locomotor activity was assessed in the LABORAS automated behavioral analysis. Fig. 6A shows the position distribution of mice in the LABORAS cage during 30 min observation period. As shown in the figure vehicle-treated mouse explored the entire cage so did the CP-treated mouse. The position distribution of mice treated with chlorpromazine was exaggerated at one spot, indicating immobility. These results were further confirmed by quantitative analysis of different locomotive behaviors indicating no significant changes in the locomotor activity between the control and CP extract groups, except in rearing

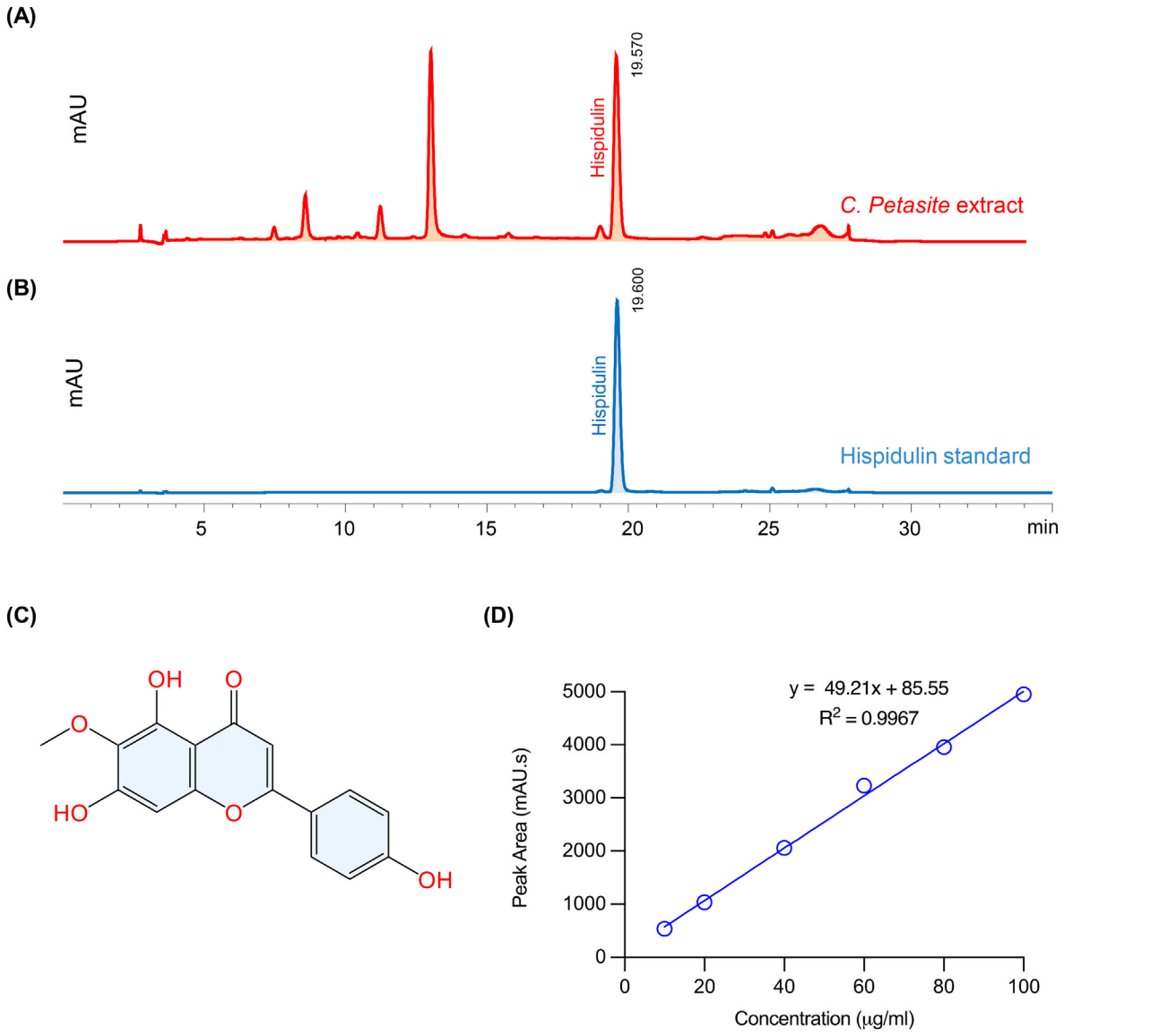


Fig. 1. UHPLC standardization of *C. petasite* extract. (**A**) Chromatogram of *C. petasites* EtOH extract and (**B**) Chromatogram of hispidulin standard. (**C**) Chemical structure of hispidulin, and (**D**) Calibration curve of standard hispidulin. All chromatograms were recorded at 337 nm.

behavior. Moreover, there was no statistically significant difference in mobile behaviors (climbing, locomotion, immobility, average speed, and distance traveled). Conversely, the CNS depressant chlorpromazine significantly reduced mobile behaviors and increased immobility compared to the vehicle-treated mice (Fig. 6). Overall, these results demonstrated no substantial effects of *C. petasite* extract in spontaneous locomotor activity.

3.7.2. The effect of C. Petasite extract on general behaviors and well-being of mice

Automated home cage monitoring LABORAS was also used to assess the general behaviors and well-being of mice. The assessments include long-term locomotor activity in the diurnal time of mice together with bodyweight changes, and food and water intake. As expected, mice exhibited more home cage behaviors in the nighttime than daytime since mice are nocturnal animals. Mice treated with chlorpromazine showed a significant reduction in mobile behaviors and increased immobility at nighttime compared to vehicle-treated mice, yet the difference is less evident in the daytime. Furthermore, there were no significant differences in long-term locomotor activity between the CP extract and the vehicle-treated groups, indicated by no significant difference in all behaviors tested, such as climbing, locomotion, rearing, immobility, average speed, and distance traveled (Fig. 7). The same results were observed in the weight changes, food, and water intakes, where the result demonstrated no substantial difference between control- and CP extract-treated groups (Fig. 8).

3.7.3. The effect of C. Petasite extract on motor coordination

The results demonstrated no significant differences in the rotarod latency between the control and CP extract groups, indicating no effect of CP extract on motor coordination at the highest dose of CP extract tested (Fig. 9).

4. Discussion

Although several lines of evidence demonstrate the ability of *C. petasite* extract to elicit multiple pharmacological activities, the

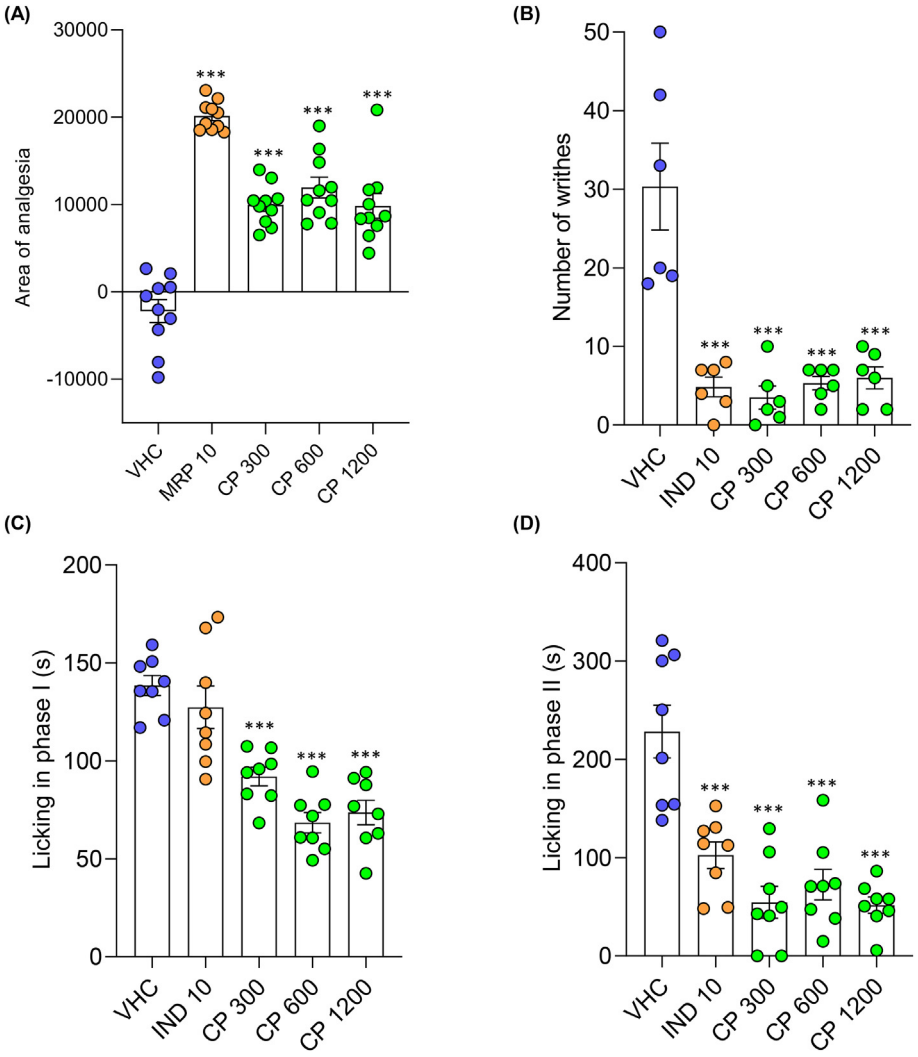


Fig. 2. Effect of CP extract on thermal nociception (A), visceral nociception (B), and acute nociceptive and inflammatory pain in mice (C and D), assessed by the hot-plate test, acetic acid-induced writhing test, and formalin test, respectively. Data are expressed as mean ± SEM (n = 10 for the hot-plate test, n = 6 for the acetic acid test, and n = 8 for the formalin test). The differences between groups were analyzed by One-way ANOVA followed by the Bonferonni *post hoc* test. ***p < 0.001. VHC, 2% tween 80; CP extract, *C. petasite* extract (300, 600, 1200 mg/kg, p.o.); MRP 10, morphine 10 mg/kg, p.o.; IND 10, indomethacin 10 mg/kg.

CNS safety profiles and its antinociceptive activity have not been thoroughly investigated. This study found that CP extract elicited antinociceptive and anti-inflammatory activities without showing any potential side effects on the CNS. In the CNS safety pharmacology testing, we found no potential effects of CP extract on motor coordination, spontaneous locomotor activity, and general behaviors of mice. Furthermore, in the nociceptive pain tests, the results demonstrated that CP extract decreased pain-like behaviors, such as thermal nociceptive pain, abdominal constriction, neurogenic and inflammatory pain.

Of interest, *Clerodendrum petasites* S. Moore exerts multiple pharmacological activities, including spasmolytic and antipyretic, and anti-inflammatory activities in rodents.[18,19] Here we found that CP extract was also effective for nociceptive pain treatments. CP extract improved nociceptive pain induced by thermal and chemical stimuli, which was evaluated using the hot-plate and acetic acid tests, respectively. The effects of CP extract were further evaluated in an acute model of inflammatory pain using the formalin test. CP extract inhibited both phases of responses in the formalin test, indicating CP extract can inhibit nociceptive and inflammatory pain. Phase I (early phase) is considered as direct activation nociceptors by formalin, whereas phase II (late phase) is an inflammatory response that leads to enhanced pain transmission. Our present study demonstrated that CP extract showed antinociceptive activity in several nociceptive pain models. Notably, the antinociceptive activity of that CP extract showed comparable results with standard drugs like morphine and indomethacin. This finding has a substantial value not only because it provides scientific evidence for the traditional use of CP extract in pain management but also because it lays a platform to discover potential bioactive compounds with greater analgesic efficacy and lesser side effects. Consequently, several studies have been done to assess the preclinical analgesic and anti-inflammatory effects of medicinal plants used in traditional medicine, including *Nymphaea lotus* leaves,[30] *Beridelia retusa* bark,[31] *Euadenia trifoliolata* leaves,[32] *Chlorophytum alismifolium* tubers,[33], and *Piper longum* fruits,.[34]

The pharmacologically active ingredients in the herb extract might be responsible for the effect of the CP extracts.[18] Interestingly, the bioactive compounds in *C. petasites* ethanolic extract have previously been characterized in a study conducted in Thailand. The researchers identified several flavonoids and phenolic acid derivatives, including hispidulin > nepetin > verbascoside > vanillic

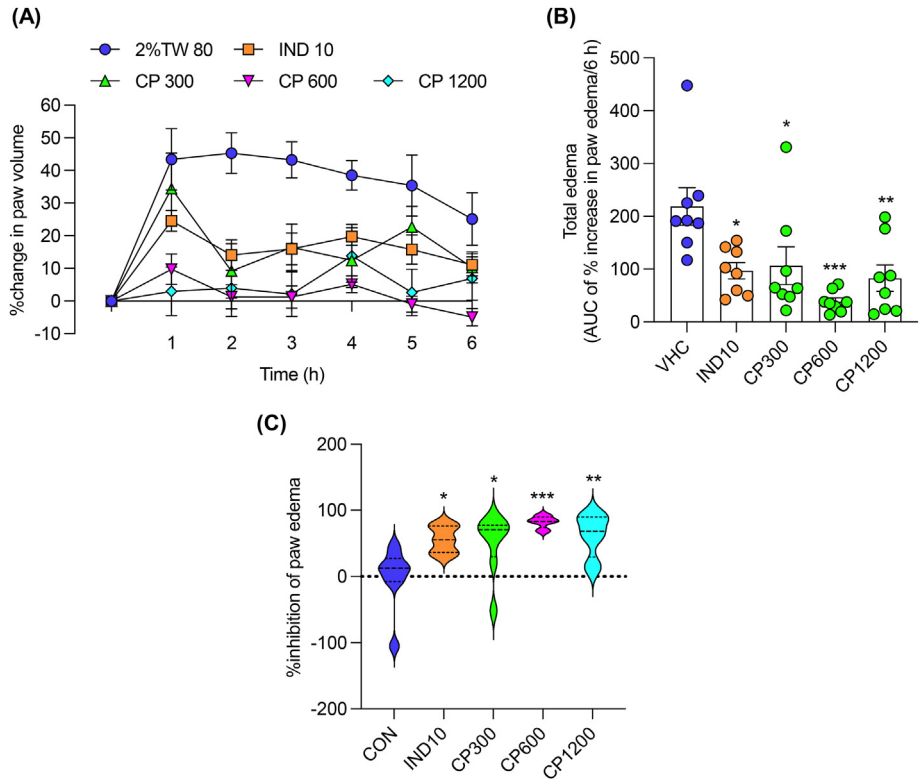


Fig. 3. Effect of CP extract on carrageen-induced inflammation. (A) Time course curve of %change in paw volume over 6 h. (B) Effect of CP extract on total edema, calculated using the area under the time course curves. (C) % inhibition of paw edema by each intervention. Data are expressed as mean ± SEM (n = 8 mice/group). The differences between groups were analyzed by One-way ANOVA followed by the Bonferonni *post hoc* test. *p < 0.05, **p < 0.01 and ***p < 0.001, compared to vehicle-treated group. VHC, 2% tween 80; CP extract, *C. petasite* extract (300, 600, 1200 mg/kg, p.o.); IND 10, indomethacin 10 mg/kg.

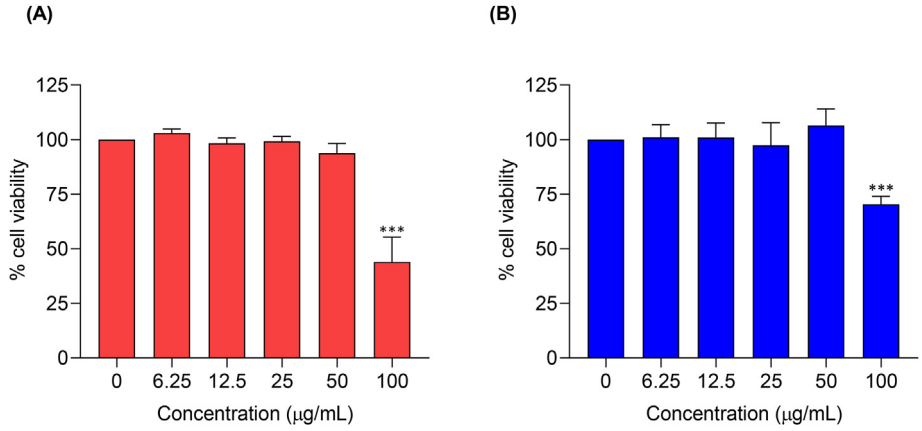


Fig. 4. Cytotoxicity profiles of CP extract in RAW 264.7 (A) and BV-2 microglial cells (B). Values are expressed as mean ± SD (n = 4). ***p < 0.001, the groups treated with extracts vs. vehicle (0.5% DMSO). The differences were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test.

acid > and apigenin, arranged in order of %yield.[27] Apart from that, several other compounds: ferulic acid, 4-coumaric acid, luteolin, naringenin, hesperetin, and chrysin, have been identified in CP extract with lower yield. Moreover, the analgesic potential of several of these bioactive compounds in CP extract has previously been reported, such as apigenin, ferulic acid, naringenin, luteolin, hesperetin, vanillic acid, verbascoside demonstrated potential analgesic activities.[20–26] Specifically, hispidulin, a main active compound of CP extract, was previously reported to exert anti-nociceptive and anti-inflammatory efficacy in rodents.[35] Hence, these bioactive agents may have contributed to the potential antinociceptive and anti-inflammatory effects observed in this study.

Peripheral and central inflammation are the major hallmarks of pain. The inflammation during pain was driven by the activation of the immune cells, including T cells, macrophages, monocytes, microglia, and astrocytes.[36] The activation of the immune cells is manifested by robust releases of inflammatory markers such as NO, IL-6, TNF-α, bradykinin, prostaglandin, and many others.[37] Therefore, the reduction of the inflammatory markers contributes to the improvement of the pain condition. In the present study, the effects of CP extract on the activated macrophage and microglia cells were

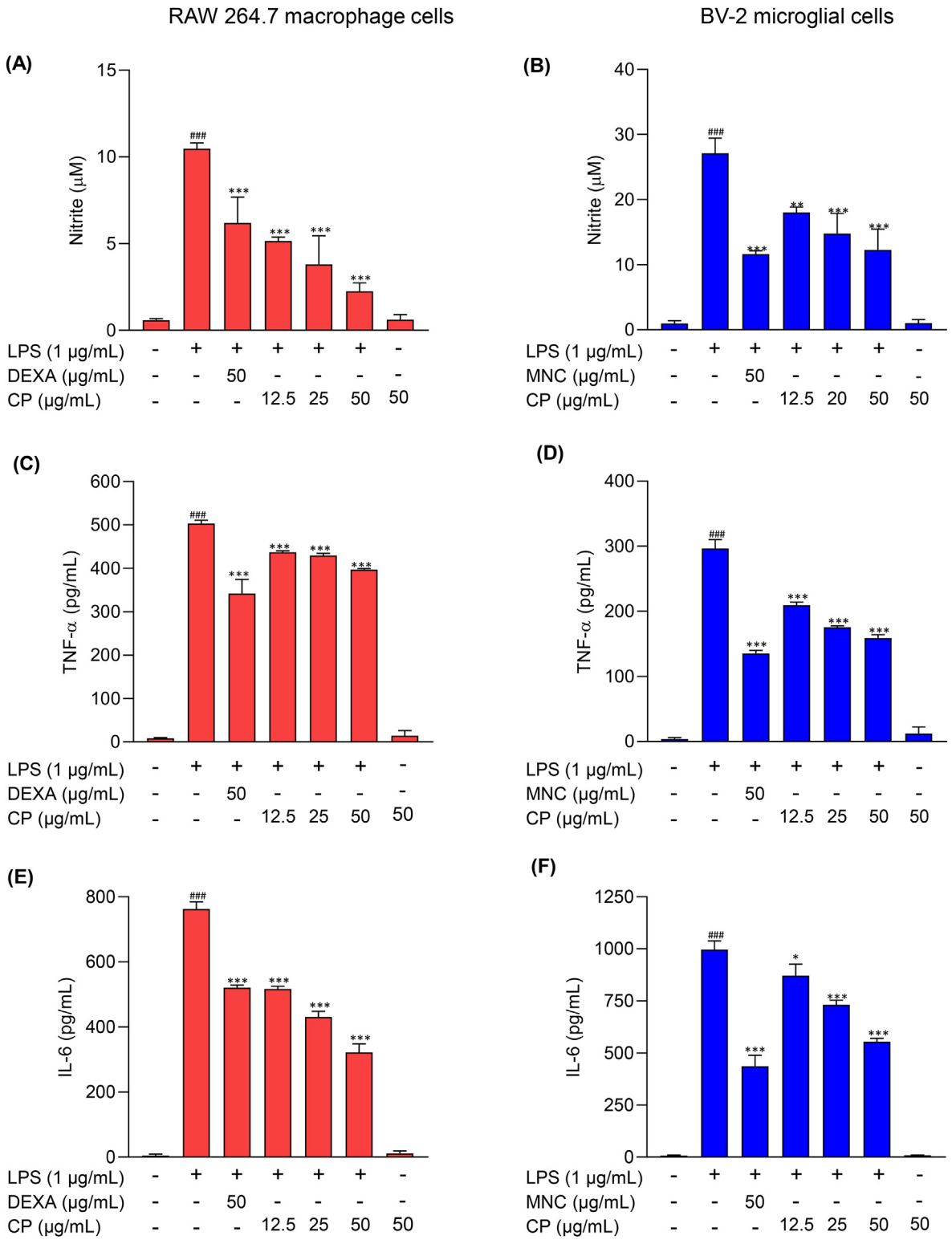


Fig. 5. CP extract reduces inflammatory mediators (NO, TNF-α, and IL-6) in LPS-stimulated RAW 264.7 and BV-2 cells. Values are expressed as mean ± SD (n = 3). $^{###p} < 0.001$, vehicle vs. LPS group. *p < 0.05, **p < 0.01, ***p < 0.001, the groups treated with positive control or extract vs. LPS-treated groups. (0.5% DMSO). The differences were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test. CP, *C. petasite* extract; DEXA, dexamethasone; MNC, minocycline.

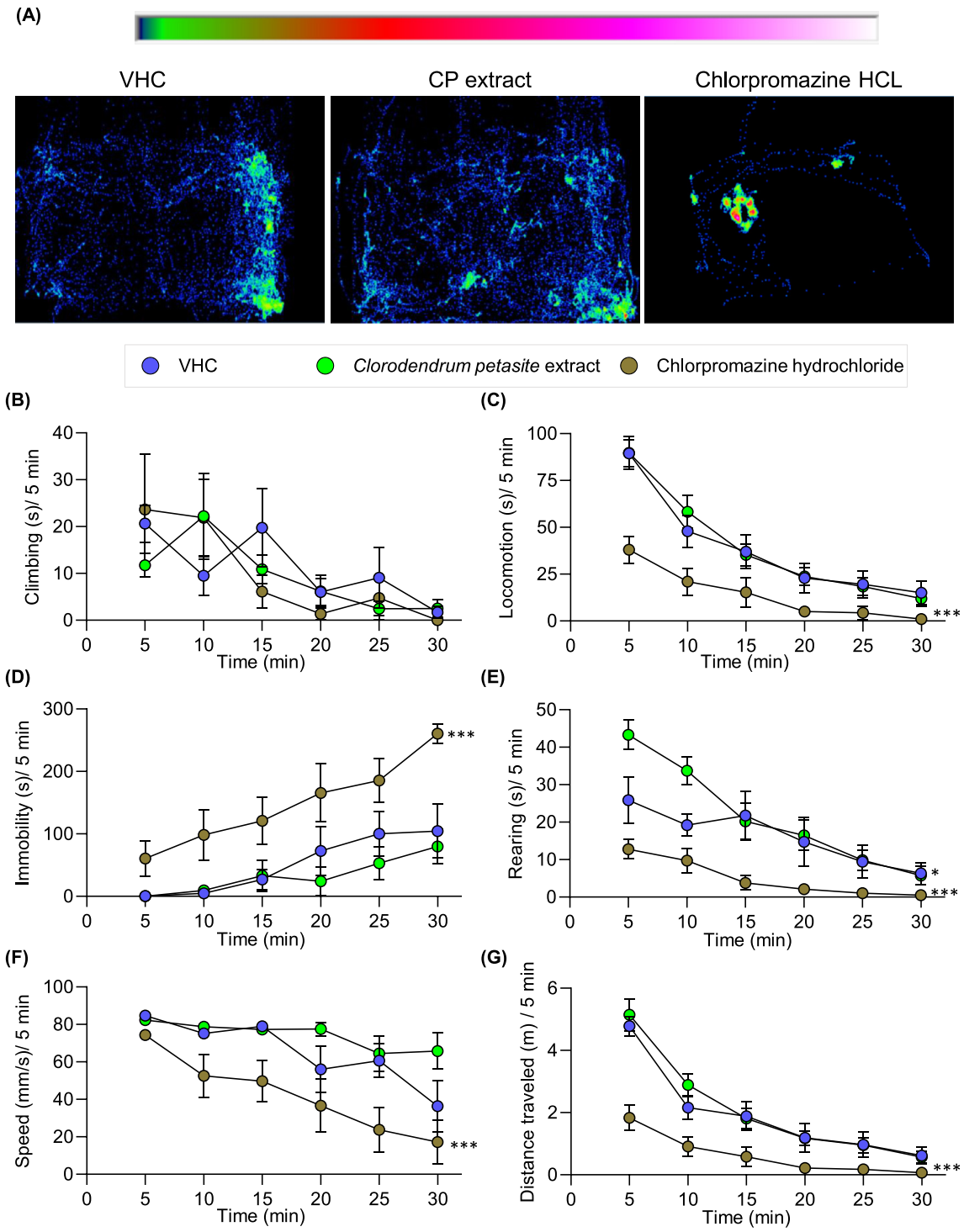
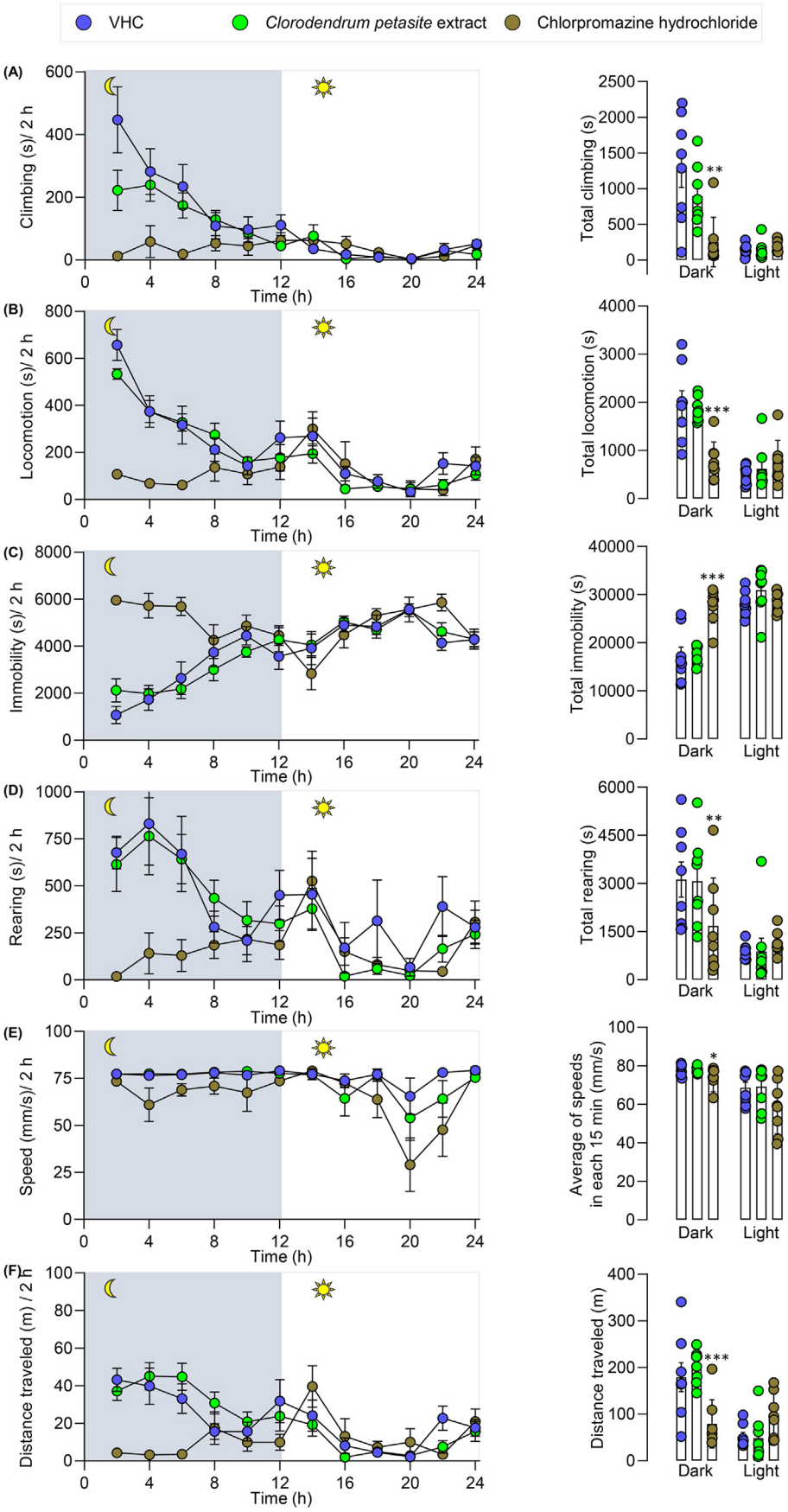


Fig. 6. Spontaneous locomotor activity of mice after CP extract administration. Data are presented as the mean ± SEM (n = 8), and differences between control and CP extracts were analyzed by two-way ANOVA followed by the Bonferonni *post hoc* test. *p < 0.05; ***p < 0.001. VHC, 2% tween 80; CP extract, *C. petasite* extract 1200 mg/kg.

investigated. The results demonstrated the significant effects of CP extract in reducing NO, TNF-α, and IL-6 in both cells, which indicates the potential role of CP extract in modulating immune cells. The ability of the CP extract to suppress inflammatory responses in the LPS -stimulated cells could be attributed due to the presence of hispiludin in the extract, as previous studies reported that hispiludin suppresses activated peripheral and central immune cells.[35,38] The ability of the CP extract to suppressproinflammatory mediators could be the major contributing factor in reducing pain-like behaviors in inflammatory pain models.

Several adverse events of analgesic drugs linked with their effects on the central nervous system have been reported.[5–7] NSAIDs


VHC
Clorodendrum petasite extract
Chlorpromazine hydrochloride
(A)
Climbing (s)/ 2 h
Time (h)
Total climbing (s)
(B)
Locomotion (s)/ 2 h
Total locomotion (s)
(C)
Immobility (s)/ 2 h
Total immobility (s)
(D)
Rearing (s)/ 2 h
Total rearing (s)
(E)
Speed (mm/s)/ 2 h
Average of speeds in each 15 min (mm/s)
(F)
Distance traveled (m) / 2 h
Distance traveled (m)
Dark
Light

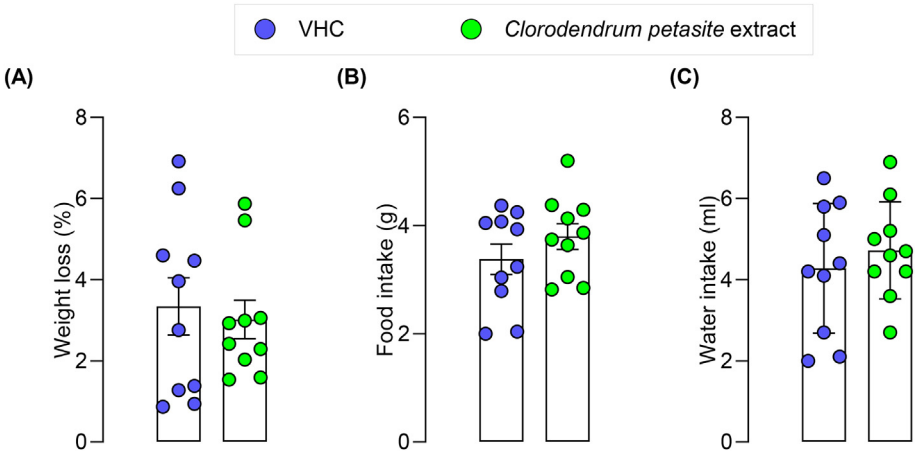


Fig. 8. Profiles of the well-being of mice after CP extract administration. The changes in body weight, food, and water intake were measured. Data are presented as the mean ± SEM (n = 8). VHC, 2% tween 80; CP extract, *C. petasite* extract 1200 mg/kg.

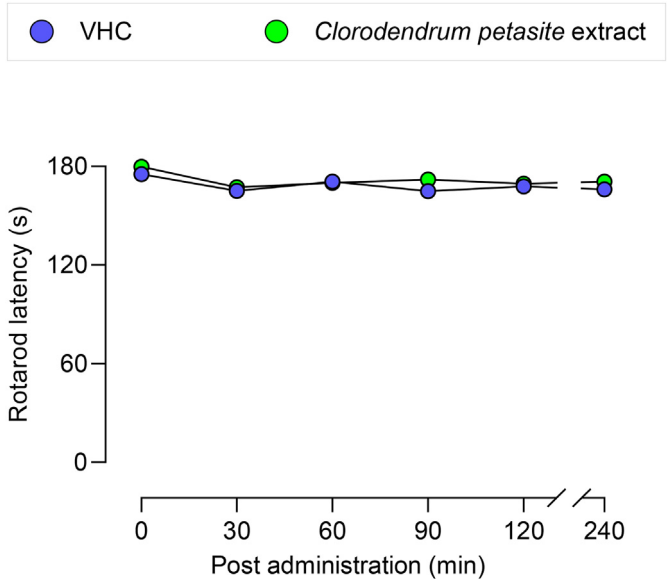


Fig. 9. Motor coordination of mice after CP extract administration. Data are presented as the mean ± SEM (n = 8). No significant differences were observed between 2% TW 80-treated and CP extract-treated mice. VHC, 2% tween 80; CP extract, *C. petasite* extract.

cause ataxia, disorientation, dizziness, dementia, recurrent falls, seizures, and vertigo.[7] Opioids promote miosis, nausea, rigidity, and vomiting.[6] Gabapentinoids, including gabapentin and pregabalin, cause sedation and dizziness.[5] Therefore, it is important to investigate not only the analgesic potency but also the potential side effects of candidate analgesic agents on the CNS. Several studies evaluated the CNS safety pharmacology of herbal extracts, including *Alstonia scholaris* (L.) R. Br leaves extract,[11] *Cassia occidentalis* Linn. Stem extract,[12] and *Plinia cauliflora* fruit extract[13], using the highest therapeutic doses of the extract for the testing. Here, we also evaluated the CNS safety pharmacology of CP extract using the highest therapeutic dose in automated home cage behavioral analysis LABORAS and rotarod test. Generally, assessments of safety pharmacology in the CNS were performed by measuring general behavior, spontaneous locomotor activity, and motor coordination.[39] The use of the LABORAS for CNS safety pharmacology has been validated and introduced previously.[40] The advantages of using LABORAS for the assessment of CNS safety pharmacology are its ability to detect behaviors such as climbing, rearing, walking, and immobility. Some of these behaviors are useful to indicate CNS side effects in the clinical setting.[41] In this study, the spontaneous and general behaviors of mice were assessed to characterize the sedative and CNS stimulative effects of CP extract. Overall, the results demonstrated no significant effects of the CP extract on spontaneous locomotor activity, general behaviors, well-being, and motor coordination of mice, indicating no potential side effects of the CP extract on the CNS.

5. Conclusions

In conclusion, our findings demonstrate that CP extract exhibited antinociceptive and anti-inflammatory activities without any potential CNS side effects. Although no potential CNS side effects of the extract were observed with a single administration of the CP extract, it is required to prove the CNS safety profile of CP extract with chronic administration. In addition, future studies are required to investigate the safety pharmacology of *Clerodendrum petasites* S. Moore extract on cardiovascular and respiratory systems. The potential side effects at the cellular and mechanistic levels are worthy of being investigated. Furthermore, assessing the effects of the active ingredients in the CP extracts in numerous animal models of pain, as well as its potential interactions (additive or synergistic interactions), is warranted.

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Declaration of competing interest

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

Fig. 7. Duration of home-cage behaviors of mice in automated home cage monitoring after CP extract administration. The home cage behaviors were indicated by duration of the climbing, locomotion, immobility, and rearing throughout the day and night. Data are presented as the mean ± SEM (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001. VHC, 2% tween 80; CP extract, *C. petasite* extract 1200 mg/kg.

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References

1. Mills SEE, Nicolson KP, Smith BH. Chronic pain: a review of its epidemiology and associated factors in population-based studies. *Br J Anaesth*. 2019;123(2): e273–e283. https://doi.org/10.1016/j.bja.2019.03.023.
2. Prescott SA, Ratté S. Somatosensation and pain. In: *In: Conn PMBT-CTN, ed. Conn's Translational Neuroscience. Academic Press*. 2017:517–539. https://doi.org/10.1016/B978-0-12-802381-5.00037-3.
3. Gallo J, Raska M, Kriegova E, Goodman SB. Inflammation and its resolution and the musculoskeletal system. *J Orthop Transl*. 2017;10:52–67. https://doi.org/10.1016/j.jot.2017.05.007.
4. Mathew E, Kim E, Zempsky W. Pharmacologic treatment of pain. *Semin Pediatr Neurol*. 2016;23(3):209–219. https://doi.org/10.1016/j.spen.2016.10.004.
5. Chincholkar M. Analgesic mechanisms of gabapentinoids and effects in experimental pain models: a narrative review. *Br J Anaesth*. 2018;120(6): 1315–1334. https://doi.org/10.1016/j.bja.2018.02.066.
6. Schug SA, Zech D, Grond S. Adverse effects of systemic opioid analgesics. *Drug Saf*. 1992;7(3):200–213. https://doi.org/10.2165/00002018-199207030-00005.
7. Auriel E, Regev K, Korczyn AD. Nonsteroidal anti-inflammatory drugs exposure and the central nervous system. *Handb Clin Neurol*. 2014;119:577–584. https://doi.org/10.1016/B978-0-7020-4086-3.00038-2.
8. Fung M, Thornton A, Mybeck K, Wu JHH, Hornbuckle K, Muniz E. Evaluation of the characteristics of safety withdrawal of prescription drugs from worldwide pharmaceutical markets-1960 to 1999. *Ther Innov Regul Sci*. 2001;35(1): 293–317. https://doi.org/10.1177/009286150103500134.
9. Yekkirala AS, Roberson DP, Bean BP, Woolf CJ. Breaking barriers to novel analgesic drug development. *Nat Rev Drug Discov*. 2017;16(8):545–564. https://doi.org/10.1038/nrd.2017.87.
10. Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J. Clinical development success rates for investigational drugs. *Nat Biotechnol*. 2014;32(1): 40–51. https://doi.org/10.1038/nbt.2786.
11. Zhao Y-L, Su M, Shang J-H, et al. Genotoxicity and safety pharmacology studies of indole alkaloids extract from leaves of Alstonia scholaris (L.) R. Br. *Nat Products Bioprospect*. 2020;10(3):119–129. https://doi.org/10.1007/s13659-020-00242-4.
12. Mugale MN, Shukla S, Chourasia MK, et al. Regulatory safety pharmacology and toxicity assessments of a standardized stem extract of Cassia occidentalis Linn. in rodents. *Regul Toxicol Pharmacol*. 2021;123, 104960. https://doi.org/10.1016/j.yrtph.2021.104960.
13. Calloi Palozi Rhanany Alan, Pires Guarnier Lucas, Moreira Romão Paulo Vitor, et al. Pharmacological safety of *Plinia cauliflora* (Mart.) Kausel in rabbits. *Toxicology Reports*. 2019;6:616–624. https://doi.org/10.1016/j.toxrep.2019.06.017.
14. Posadzki P, Watson LK, Ernst E. Adverse effects of herbal medicines: an overview of systematic reviews. *Clin Med*. 2013;13(1):7–12. https://doi.org/10.7861/clinmedicine.13-1-7.
15. Phumthum M, Balslev H, Kantasrila R, Kaewsangsai S, Inta A. Ethnomedicinal plant knowledge of the Karen in Thailand. *Plants*. 2020;9(7):1–9. https://doi.org/10.3390/plants9070813.
16. Pichaensoonthon C, Chaowalit M, Jirawong W. The Explanation of Traditional Recipes: Osot-Phra-Na-Rai. Bangkok, Thail Amarin Print Publ. Published online 2005.
17. Shrivastava A, Patel T. Clerodendrum and heathcare: an overview. *Med Aromat Plant Sci Biotechnol*. 2007;1(1):142–150.
18. Brimson JM, Onlamoon N, Tencomnao T, Thitilertdecha P. Clerodendrum petasites S. Moore: the therapeutic potential of phytochemicals, hispidulin, vanillic acid, verbascoside, and apigenin. *Biomed Pharmacother*. 2019;118, 109319. https://doi.org/10.1016/j.biopha.2019.109319.
19. Wang J-H, Luan F, He X-D, Wang Y, Li M-X. Traditional uses and pharmacological properties of Clerodendrum phytochemicals. *J Tradit Complement Med*. 2018;8(1):24–38. https://doi.org/10.1016/j.jtcme.2017.04.001.
20. Calixto-Campos C, Carvalho TT, Hohmann MSN, et al. Vanillic acid inhibits inflammatory pain by inhibiting neutrophil recruitment, oxidative stress, cytokine production, and NFκB activation in mice. *J Nat Prod*. 2015;78(8): 1799–1808. https://doi.org/10.1021/acs.jnatprod.5b00246.
21. Amin B, Poureshagh E, Hosseinzadeh H. The effect of verbascoside in neuropathic pain induced by chronic constriction injury in rats. *Phytother Res*. 2016;30(1):128–135. https://doi.org/10.1002/ptr.5512.
22. Xu Y, Lin D, Yu X, et al. The antinociceptive effects of ferulic acid on neuropathic pain: involvement of descending monoaminergic system and opioid receptors. *Oncotarget*. 2016;7(15):20455–20468. https://doi.org/10.18632/oncotarget.7973.
23. Fan X, Du K, Li N, et al. Evaluation of anti-nociceptive and anti-inflammatory effect of luteolin in mice. *J Environ Pathol Toxicol Oncol Off organ Int Soc Environ Toxicol Cancer*. 2018;37(4):351–364. https://doi.org/10.1615/JEnvironPatholToxicolOncol.2018027666.
24. Pinheiro MMG, Boylan F, Fernandes PD. Antinociceptive effect of the Orbignya speciosa Mart. (Babassu) leaves: evidence for the involvement of apigenin. *Life Sci*. 2012;91(9-10):293–300. https://doi.org/10.1016/j.lfs.2012.06.013.
25. Pinho-Ribeiro FA, Zarpelon AC, Fattori V, et al. Naringenin reduces inflammatory pain in mice. *Neuropharmacology*. 2016;105:508–519. https://doi.org/10.1016/j.neuropharm.2016.02.019.
26. Aswar M, Kute P, Mahajan S, Mahajan U, Nerurkar G, Aswar U. Protective effect of hesperetin in rat model of partial sciatic nerve ligation induced painful neuropathic pain: an evidence of anti-inflammatory and anti-oxidative activity. *Pharmacol Biochem Behav*. 2014;124:101–107. https://doi.org/10.1016/j.pbb.2014.05.013.
27. Thitilertdecha P, Guy RH, Rowan MG. Characterisation of polyphenolic compounds in Clerodendrum petasites S. Moore and their potential for topical delivery through the skin. *J Ethnopharmacol*. 2014;154(2):400–407. https://doi.org/10.1016/j.jep.2014.04.021.
28. Tangsongcharoen T, Issaravanich S, Palanuvej C, Ruangrungsi N. Quantitative analysis of hispidulin content in clerodendrum petasites roots distributed in Thailand. *Pharm J*. 2019;11(5):1093–1099. https://doi.org/10.5530/pj.2019.11.171.
29. Panthong A, Kanjanapothi D, Taesotikul T, Wongcome T, Reutrakul V. Anti-inflammatory and antipyretic properties of Clerodendrum petasites S. Moore. *J Ethnopharmacol*. 2003;85(1):151–156. https://doi.org/10.1016/S0378-8741(02)00368-9.
30. Rege MG, Ayanwuyi LO, Zezi AU, Odoma S. Anti-nociceptive, anti-inflammatory and possible mechanism of anti-nociceptive action of methanol leaf extract of Nymphaea lotus Linn (Nymphaeceae). *J Tradit Complement Med*. 2021;11(2): 123–129. https://doi.org/10.1016/j.jtcme.2020.02.010.
31. Tatiya AU, Saluja AK, Kalaskar MG, Surana SJ, Patil PH. Evaluation of analgesic and anti-inflammatory activity of Bridelia retusa (Spreng) bark. *J Tradit Complement Med*. 2017;7(4):441–451. https://doi.org/10.1016/j.jtcme.2016.12.009.
32. Sofidiya MO, Oloruntola OM, Sofola I, Fageyinbo MS. Antinociceptive activity of Euadenia trifoliolata (Schum. & Thonn.) Oliv. leaves and roots in mice. *J Tradit Complement Med*. 2016;6(3):289–293. https://doi.org/10.1016/j.jtcme.2015.07.006.
33. Abubakar A, Nazifi AB, Odoma S, Shehu S, Danjuma NM. Antinociceptive activity of methanol extract of Chlorophytum alismifolium tubers in murine model of pain: possible involvement of α2-adrenergic receptor and KATP channels. *J Tradit Complement Med*. 2020;10(1):1–6. https://doi.org/10.1016/j.jtcme.2019.03.005.
34. Yadav V, Chatterjee SS, Majeed M, Kumar V. Preventive potentials of piperlongumine and a Piper longum extract against stress responses and pain. *J Tradit Complement Med*. 2016;6(4):413–423. https://doi.org/10.1016/j.jtcme.2015.11.001.
35. Kavimani S, Mounissamy VM, Gunasegaran R. Analgesic and antiinflammatory activities of hispidulin isolated from helichrysum bracteatum. *Indian Drugs*. 2000;37:582–584.
36. Ji R-R, Chamessian A, Zhang Y-Q. Pain regulation by non-neuronal cells and inflammation, 80 *Science*. 2016;354(6312):572–577.
37. Ji RR, Xu ZZ, Gao YJ. Emerging targets in neuroinflammation-driven chronic pain. *Nat Rev Drug Discov*. 2014;13(7):533–548. https://doi.org/10.1038/nrd4334.
38. Thitilertdecha P, Tantithavorn V, Poungpairoj P, Onlamoon N. Determination of suppressive effect on human T-cell activation by hispidulin, nepetin, and vanillic acid. *Immunopharmacol Immunotoxicol*. 2019;41(6):591–598. https://doi.org/10.1080/08923973.2019.1675165.
39. Porsolt RD, Lemaire M, Dürmüller N, Roux S. New perspectives in CNS safety pharmacology. *Fundam Clin Pharmacol*. 2002;16(3):197–207. https://doi.org/10.1046/j.1472-8206.2002.00061.x.
40. Lynch JJ, Castagné V, Moser PC, Mittelstadt SW. Comparison of methods for the assessment of locomotor activity in rodent safety pharmacology studies. *J Pharmacol Toxicol Methods*. 2011;64(1):74–80. https://doi.org/10.1016/j.vascn.2011.03.003.
41. Mead AN, Amouzadeh HR, Chapman K, et al. Assessing the predictive value of the rodent neurofunctional assessment for commonly reported adverse events in phase I clinical trials. *Regul Toxicol Pharmacol*. 2016;80:348–357. https://doi.org/10.1016/j.yrtph.2016.05.002.