Antinociceptive and Anti-Urolithiatic Effects of Ensete glaucum (Roxb.) Cheesman Seed Aqueous Extract in Mice

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Abstract

Ensete glaucum seeds, being known as one of traditional medicine, are commonly used in folk medicine to treat urinary stones, edema, and osteoarthritis-related problems. However, no scientific evidence has been reported to support these uses. This study focused on investigating the antinociceptive and antiurolithiatic activities of E. glaucum seed aqueous extract (EGE). The antinociceptive effect of EGE was evaluated in mice using thermal (hot plate test) and chemical (acetic acid and formalin-induced nociception test) pain models at various doses (50, 100, 200, 400 mg/kg; p.o.). The anti-urolithiatic activity of the EGE (200, 400 mg/kg; p.o.) was assessed in the sodium glyoxylate-induced urolithiasis in mice and in vitro nucleation and aggregation assays. The analgesic effect of EGE was detected at several doses via peripheral and central antinociceptive mechanisms in the acetic acid-induced writhing (50-400 mg/kg), hot plate (100-200 mg/kg), and formalin-induced licking (200-400 mg/kg) tests. EGE had potential against urolithiasis through its ability to modify several serum and urine biochemical parameters on glyoxylate-induced nephrolithiasis. The extract at the dose of 400 mg/kg significantly improved the inflammatory cells, kidney tissue structure, and renal calcification. The extract also exhibited significant anti-urolithiatic, anti-inflammatory, and antioxidant activities in some in vitro models. These outcomes suggest that E. glaucum aqueous seed extract possesses antinociceptive activity and may aid in the prevention of urinary stones. Further studies are needed to elucidate the effectiveness of E. glaucum seeds in the analgesic activity and management of urolithiasis disease.

I. Introduction

Pain is an unpleasant sensation simulated externally or internally, giving the actual or potential warning signal associated with tissue damage [1]. Pain causes negative physical or mental effects such as insomnia, fatigue, dizziness, anxiety, and heart palpitations,... Pain is often the cause leading to the medical examination of many diseases such as headache, osteoarthritis, urinary stones, and stomach ulcers, ... [2]. In medicine, two common groups of drugs are prescribed for analgesia: opioids and nonopioids. Opioid-related drugs are derived from opium or synthesized while nonopioid drugs are divided into two subclasses, acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs) [3]. Although these kinds of conventional medications take advantage of rapid effects, they can cause several side effects in long-term use for patients. In addition to common side effects such as nausea, vomiting, respiratory depression, and constipation, the opioid group is known for its potential for addiction, hallucination, or neurotoxicity in prolonged usage, or at high dosages. Nonopioid use can cause gastrointestinal bleeding, peptic ulcer disease, or severe renal impairment in some cases [2, 3]. These drawbacks are also a reason for the trend in researching medicinal material in therapeutics to reduce side effects and drug tolerance. In particular, as pain is considered a signal for many diseases or pathologies inside the body, investigating the analgesic activity in medicinal materials will open a potential therapy in supporting treatment but also reduce adverse effects, toxicity, and hypersensitivity in long-term use [4].

Urolithiasis is a common urologic disease in Vietnam and around the world with an increasing incidence [5]. Urolithiasis is a major challenge for the medical industry because of its high rate of incidence and lifetime recurrence [6]. This is a silent disease. Initially, stone formation does not cause any symptoms. Later, signs and symptoms of stone disease consisted of intense cramping pain, pain in the back side, hematuria, urinary tract infections, blockage of urine flow, and hydronephrosis [5]. Recently, synthetic drugs and some interventional procedures such as extracorporeal shock wave lithotripsy, and ureteroscopy have been utilized to treat urolithiasis. Nonetheless, there are no effective medicines to use in clinical therapy properly because stone removal cannot be completed, or stone recurrence is still a possibility. In addition, exposure to shock waves in therapeutic doses leads to acute renal damage, renal impairment, a reduction in renal function, and an increase in stone recurrence [7]. Due to the many side effects of urologic stone treatment, phytotherapeutic agents could be useful as either an alternative or an adjunctive therapy in the management of urolithiasis [7].

Ensete glaucum (Roxb.) Cheesman, also known as snow bananas, are widely distributed in South and Southeast Asia are listed as Myanmar, India, China, Indonesia, Lao, Vietnam, Thailand, and Philippines [8]. *E. glaucum* is a medicinal plant that has been newly exploited in recent years and was proven to contain saponins, flavonoids, and alkaloids, which are potential bioactive compounds in many plants belonging to the Musaceae family [9]. Due to its special reproduction, which is different from other plants belonging to the *Ensete* genus, they grow and develop singly and by seedling. As the plant matures and fruits, all the nutrients will support its fruits and seeds, so there are many expectations that the pharmaceutical activity of *E. glaucum* seeds might be higher. Vietnamese people traditionally use the dried seed of *E. glaucum* for diuretic treatment, anti-inflammation (edema), kidney stone treatment, constipation in children, and diabetes treatment support.

Until now, the use of *E. glaucum* seeds in the treatment of urinary stones has been through experience and word of mouth, and the antiurolithiatic and analgesic activities have not been proven effective by scientific experiments. Therefore, this study was performed to evaluate the potential of the aqueous extract of *E. glaucum* seeds in the management of pain and urolithiasis. The antinociceptive effect and antiurolithiatic potential of the *E. glaucum* seed aqueous extract in mice were detected. The extract also had antiurolithiatic, anti-inflammatory and antioxidant effects through different mechanisms.

II. Materials and methods

2.1 Plant materials and extraction

Ensete glaucum seeds taken from ripe fruits were collected from Bac Ai District, Ninh Thuan Province, Vietnam in September 2020. The samples were identified and authenticated by MSc. Le Duc Thanh (Research Center of Ginseng and Medicinal Materials Ho Chi Minh City, Vietnam) and a voucher specimen (TNDL-EGS-2020) were deposited for *Ensete glaucum* (Roxb.) Cheesman. Bad seeds were removed, and then the sample was washed with tap water and distilled water. This was followed by drying and grinding into powder for study.

The dried powdered material was extracted with distilled water by the decoction method for 60 minutes (total ratio is 1: 20 *w/v*) to obtain liquid extracts. The liquid extracts were collected by filtering and then concentrated using a rotary evaporator at 80 °C under reduced pressure to obtain crude extracts. The crude extract was stored at -15 °C and dissolved in a suitable solvent to yield a stock solution.

2.2 Experimental animals

All healthy *Swiss albino* mice of both genders weighing from 20 ± 2 g (from the Institute of Vaccines and Medical Biologicals in Nha Trang City, Vietnam) were stabilized for 7 days before testing, and raised in a room at a temperature of 25 ^{0}C, humidity at 50-65%, and on a 12 light/12 dark cycle. They were raised in PP-plastic cages (33 × 21 × 15 cm) with adequate food and water. The oral administration volume was 10 mL/kg body weight, and the oral administration time in the experiment was between 8 and 9 am. Experimental studies on mice followed the guidelines of the Guidelines for the Care and Use of Laboratory Animals and Ministry of Health-Vietnam (No. 141/QĐ-K2ĐT).

2.3 Acute oral toxicity test

Acute oral toxicity of the polyextract was investigated according to the Organization for Economic Cooperation and Development (OECD) guideline number 423 and the Vietnam Ministry of Health [10, 11]. The test was conducted in two phases (mice were starved for 12 hours before testing): Phase 1 (primary), 6 mice (3 males, 3 females) were given the sample at the highest concentration that could be injected via a needle (50 mL/kg volume), general movements, behavioral manifestations, hair state, feeding, urination and number of dead mice were monitored and recorded in 4-72 hours. After 72 hours, the mice showed no signs of abnormality or death, and continued monitoring for 7 days and 14 days. In the case of dead mice, the dose was lowered to find the LD_{50} (lethal dose, 50%). Mice were dissected to observe the macroscopic and microscopic organs (if there was death).

2.4 Antinociceptive tests

2.4.1 Acetic acid-induced writhing test

The writhing test was performed as described previously with some slight modifications [12]. Male mice were divided randomly into 6 groups (n = 9): (I) Negative control group (distilled water, per os, *p. o.*); (II) Positive control group (aspirin pH 8 100 mg/kg, *p. o.*); (III-VI) Experimental groups with 4 doses of the extract (50, 100, 200, 400 mg/kg, *p. o.*). Two hours after oral administration of the drug and extract, mice were intraperitoneally injected with 0.6% (*v/v*) acetic acid (Merck Co., Darmstadt, Germany) to induce pain. The pain manifestation was measured after 10 minutes of injection over a period of 30 min. Analgesic activity was determined based on the reduction in the number of abdominal writhing frequencies.

2.4.2 Hot plate test

The method was described previously with some minor modifications [13]. Male mice were divided randomly into 4 groups (*n* = 8): (I) Negative control group (distilled water, *p. o.*); (II) Positive control group (tatanol codeine 177 mg/kg, *p. o.*); (III-IV) Experimental groups with 2 doses of the extract (100 mg/kg and 200 mg/kg, *p. o.*). The drug and extract were administered via the oral route. The hot-plate temperature was set at 55 ± 0.5 ^{0}C. Mice showing an initial nociceptive response between 8 and 30 seconds were selected for the experiment. To avoid tissue damage, the cut-off time was set at 60 seconds. The latency time recorded was from the moment the animal was placed on the hot plate until it licked the hind paw or jumped. The estimation was determined before administration, and then 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min, and 240 min after extract/drug administration.

2.4.3 Formalin-induced licking test

The formalin-induced pain method was carried out previously described with minor modifications [14]. Male mice were divided randomly into 4 groups (*n* = 8): (I) Negative control group (distilled water, *p. o.*); (II) Positive control group (Tatanol codeine 177 mg/kg, *p. o.*); (III-IV) Experimental groups with 2 doses of the extract (200 mg/kg and 400 mg/kg, *p. o.*). Two hours after oral administration of the drug and extract, mice received an intraplanter injection with 20 µL of 2.5% (*v/v*) formalin (Merck Co., Darmstadt, Germany) into the left hind paw. Then, the animal was placed individually in the flat, translucent chamber (15 × 14 × 20 cm) and observed for 30 minutes. After the injection, two distinct periods or phases of licking/biting behaviour occur in phase I (0-5 min) and phase II (15-30 min). The analgesic activity was evaluated based on the total duration of paw licking in 2 phases, and on scoring the behavior in phase II. The rodent stood and walked firmly on the injected paw equal to 0, the injected paw was not fully lifted equal to 1; the injected paw completely lifted off the floor equal to 2; the injected paw was licked or chewed – which was the expression of the most painful sensation, equal to 3.

2.5 Experimental design of sodium glyoxylate-induced urolithiasis in mice

The animal model was carried out within 14 days. After being housed for one week, 56 mice were equally divided into 7 groups of 8 mice. To induce renal calculi CaO_x, sodium glyoxylate (Sigma Co., St. Louis, MO, USA) at a dose of 100 mg/kg was administered by successive intraperitoneal injections for 7 consecutive days (from day 1 to day 7). In the normal group, 0.9% saline NaCl was intraperitoneally injected from day 1 to day 7. Groups that received the treatment with the extract would drink the extract for 14 days, while the cystone-treated group only drank from day 7 to day 14 [15].

2.5.1 Serum analysis

On the 7th and 14th days, blood from mouse tails was collected, centrifuged to obtain the serum, and analysed for the content of creatinine, urea nitrogen, uric acid, calcium, and phosphate by biochemical kits.

2.5.2 Urine analysis

On the 7th and 14th days, animals were kept in individual metabolic cages and 24 h urine samples were collected. Animals had free access to drinking water during the urine collection period. The urine samples were analysed for the levels of phosphate, calcium, and magnesium with the help of diagnostic biochemical kits.

2.5.3 Histopathological examination

Kidney tissues were collected and fixed in 10% Neutral Buffered Formalin, processed routinely, and embedded in paraffin. Three-micron-thick sections were prepared and stained with hematoxylin and eosin dye for microscopic investigation. The stained sections were examined and photographed under a light microscope (Nikon Eclipse Ts2R-FL).

2.6 Nucleation and aggregation assays

2.6.1 Nucleation assay

First, the solution of 10 mM calcium chloride and 1 mM sodium oxalate was prepared in crystallization buffer containing 10 mM Tris–HCl and 90 mM NaCl (pH 7.4). The experiment was performed in triplicate to a 24-well plate. As described briefly, 190 µL of 10 mM $CaCl_2$ was added into 24 well-plate before adding 20 µL of the tested extract at various concentrations. Cystone was used as the positive control group, and it was dissolved in distilled water to give tested concentrations. In addition, for the control of each time, an equal volume of the basic buffer was added into the well. To induce the crystallization reaction, 190 µL of 1 mM $Na_2C_2O_4$ was added into each well. Thereafter, the mixture was incubated at 37 °C in a water bath for 30 minutes [16]. Crystal images were captured randomly from 9 high-power fields (HPFs) with 400× magnification under Nikon inverted phase-contrast light microscope ECLIPSE Ts2 (Nikon Eclipse Ts2R-FL). Crystal sizes and the number of crystals were measured using NIS Element D software (Nikon), whereas crystal mass was calculated from total crystal areas of 9 HPFs per well using the following equation:

$$Crystal\,areas\left(\frac{\mu m^2}{HPF}\right) = Average\,crystal\,area\,in\,each\,field\left(\mu m^2\right) \times Number\,of\,crystal\,in\,each\,field\left(1/HPF\right)$$

2.6.2 Aggregation

First, individual COM (calcium oxalate monohydrate) crystals were prepared by mixing 10 mM calcium chloride and1 mM sodium oxalate in buffer at a ratio of 1: 1 (*v/v*). Then the solution was incubated at 25 °C for at least one hour. The supernatant was discarded by centrifugation, whereas COM crystals were harvested and washed three times with methanol. After another centrifugation, methanol was discarded, and the crystals were air-dried by evaporation overnight at room temperature. The COM crystals were resuspended in Tris-buffer saline at a concentration of 800 µg/mL. Briefly, 150 µL of COM crystal solution was added to 50 µL of the extract at different concentrations and as the same procedure for positive control cystone. Besides, for the control of each time, an equal volume of the basic buffer was added into

the well. Then the plate was continuously shaken on a rotary shaking machine at 25 °C for one hour [16, 17]. Thereafter, images of the formation of CaO_x crystal aggregates were observed under the Nikon inverted phase-contrast light microscope ECLIPSE Ts2. Several CaO_x crystal aggregates were counted from 3 randomized HPFs per well. The experiment was performed in triplicate in 96-well plate.

2.7 Anti-inflammatory assays

The anti-inflammatory activity of the aqueous extract of *E. glaucum* seeds was examined by protein denaturation, heat-induced hemolysis, and hypotonicity-induced hemolysis assays according to the protocol previously described [18].

2.8 Antioxidant assays

The antioxidant activity of the extract was evaluated through the DPPH radical scavenging assay, ABTS radical cation decolorization assay, and reducing power assay [19].

In the DPPH assay, 25 µL of the extract at different concentrations was incubated with 25 µL of 0.6 mM DPPH solution (Sigma Co., St. Louis, MO, USA) in methanol to reach a total volume of 200 µL in total volume. The mixture was kept in the dark for 30 min at room temperature. The absorbance was measured at 515 nm.

In the ABTS assay, 5 µL of the extract at different concentrations was mixed with 145 µL of ABTS (Sigma Co., St. Louis, MO, USA) solution, the reactions were incubated for 6 min at room temperature and the absorbance was measured at 734 nm.

In the reducing power assay, the reactive mixture, 40 µL of the extract at various concentrations, 100 µL of 200 mM sodium phosphate buffer (pH 6.6), and 100 µL potassium ferricyanide 1%, were incubated at 50 °C for 30 min. Then 100 µL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 min. Next, 100 µL of the supernatant was mixed with an equal volume of double-distilled water and 20 µL of 0.1% ferric chloride. The absorbance was measured at 700 nm. The reducing power activity was subsequently analysed via optical density and EC_{50} values. The lower the optical density value, the weaker the reduction activity of the sample. The EC_{50} value is the concentration of effective antioxidants for the absorbance to reach 0.5, which was calculated through the equation illustrating the correlation between the concentration of the sample and its optical density.

Ascorbic acid (Sigma Co., St. Louis, MO, USA) was used as a positive control. All measurements were performed in triplicate using a plate reader (Biotek, USA), and an average of each sample was calculated. The percent DPPH/ABTS inhibition was calculated by the equation:

$$Inhibition\ (\%) = \frac{(A_c - A_{0c}) - (A_t - A_{0t})}{(A_c - A_{0c})} \times 100$$, where A_c, A_{0c}, A_t and A_{0t} are the absorbance of the control (with DPPH/ABTS solution and without test extract), the blank (without DPPH/ABTS solution and test

extract), the sample (with DPPH/ABTS solution and test extract), and the control sample (without DPPH/ABTS solution and with test extract), respectively.

2.9 Phytochemical screening

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites present in *E. glaucum* seeds. Screening was performed according to the method of Cuilei [20] with alkaloids, flavonoids, tannins, triterpenoids, saponins, coumarins, anthraquinones, anthocyanosides, proanthocyanidins, lipids, volatile oils, carotenoids, reducing agents, and organic acids. The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemicals.

2.10 Determination of total polyphenol and flavonoid contents

The total polyphenol content was estimated by Folin-Ciocalteu's method. Briefly, a 5 µL test sample was mixed with 10 µL of Folin-Ciocalteu's reagent (Merck Co., Darmstadt, Germany) and 165 µL of double-distilled water. Then 30 µL of Na_2CO_3 (20% *w/v*) was poured into this mixture immediately 5 min. The reaction was kept in less-exposure-to light condition for 2 hours at room temperature. The absorbance was measured at 758 nm using a plate reader (Biotek, USA) and all determinations were made in triplicate. The total polyphenol content was calculated from the calibration plot of the gallic acid standard (Sigma Co., St. Louis, MO, USA) and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g d. w.) [19].

The flavonoid content was estimated based on the aluminum chloride colorimetric method. Briefly, 20 µL of 2% *w/v* $AlCl_3$ (Merck Co., Darmstadt, Germany) was added to 30 mL diluted extract, and 150 µL methanol was then added to the mixture. After that, the solution was mixed and incubated for 15 min at room temperature. The absorbance of the reaction mixtures was calibrated at 433 nm by a plate reader (Biotek, USA). The tests were carried out in triplicate. The total flavonoid content was estimated from the calibration plot of quercetin standard (Sigma Co., St. Louis, MO, USA) and expressed as milligrams of quercetin equivalents per gram of dry weight (mg QE/g d. w.) [19].

2.11 Statistical analysis

The results are expressed as mean ± standard error of mean (mean ± SEM). Data analysis was performed using R software, version 4.2.2. Analysis of variance with ANOVA and subsequent multiple comparisons of means according to Tukey's test were performed. Shapiro Wilk's test was used to test the normal distribution of data. If the normal distribution was not given, a Kruskal-Wallis test on ranks was performed. Differences were considered statistically significant if the *P* values were less than 0.05. For regression analysis, dose-response relationships and IC_{50} were calculated using R-package "drc" (Analysis of Dose-Response Curves).

III. Results

3.1 Antinociceptive effects of E. glaucum aqueous seed extract

The results illustrated the analgesic effect of *E. glaucum* aqueous seed extract (50–400 mg/kg). The extract at doses of 50, 100, 200, and 400 mg/kg (EGE50-400) expressed a significant reduction in the average number of writhing at approximately 32.78, 26.78, 25.44, 34.56 writhes, equivalent to 59.14%, 66.62%, 68.28%, and 56.91% inhibition of abdominal constriction, respectively, compared to the control group (Vehicle). The 100 mg/kg aspirin group had a 67.87% inhibition of writhing, equivalent to approximately 25.78 writhes, a significant difference compared to the control group. There was no convincing difference between EGE and aspirin (Fig. 1).

The antinociceptive effect of the treatment groups could be estimated as compared to the control group (Vehicle) at each time period throughout the experiment (Fig. 2). The EGE 100 mg/kg expressed a compelling effect at 30 mins with 41.52 secs ($p < 0.01$), with a %MPA (percentage maximal possible analgesia) approximately of 53.86% compared to the control group, while Tatanol Codein exhibited a significant effect at 60 mins with 39.5 secs ($p < 0.05$), with %MPA of approximately 47.90%. The antinociceptive effect of EGE 100 mg/kg and Tatanol Codein was maintained throughout the experiment. For instance, at 180 mins, the EGE 100 mg/kg group had a latency time of 43.83 secs, which %MPA was relatively 60.25%, while Tatanol Codein was at 37.32 secs, which %MPA was around 44.28%. Both were significantly different compared to the control group. Furthermore, there was no significant difference between the 100 mg/kg EGE and the Tatanol Codein. In contrast, although 200 mg/kg EGE displayed a change in latency time, the effect was not convincing compared to the control group at every point of time in the experiment and exhibited a downtrend from 90 mins. Moreover, significant differences between EGE 100 mg/kg and EGE 200 mg/kg at 30 mins and 180 mins were observed.

The formalin-induced hind paw-licking test showed the analgesic effect of the extract at both phases. While the late phase expressed a compelling anti-inflammatory analgesic effect, the periphery antinociceptive effect shown in the early phase was not obscurely different (Fig. 3). In the late phase 2 (Fig. 3a), all treatment groups (EGE 200 mg/kg, EGE 400 mg/kg, and Tatanol Codein 177 mg/kg) exhibited a significant reduction in the duration of paw licking at 48.71 secs, 33.93 secs, and 33.62 secs, respectively, compared to the control group (104.05 secs). There was no significant difference between the EGE group and the reference drug group. The percentage of inhibition of paw licking measured in 200 mg/kg EGE, 400 mg/kg EGE, and Tatanol Codein groups was 53.19%, 67.39%, and 67.69%, respectively.

In addtion, behavioral scores were also recorded to estimate the inflammatory analgesic activity of *E. glaucum* seed extract in the late phase (phase 2) (Fig. 3b). The pain behavior scores of the Tatanol Codein group compared with the control group were reduced throughout the observation period. Meanwhile, the mean pain behavior scores of the mouse group treated with the extract at the 2 doses were lower than those of the control group up to 25 minutes and appeared to be similar at later times. In general, the EGE groups had an average behavioral score in the late phase in an approximate range of 3.4–3.7 but it was insignificant as compared with the control group.

3.2 Antiurolithiatic effects of E. glaucum aqueous seed extract in mice

The results in Fig. 4 showed that injection of 100 mg/kg sodium glyoxylate (SG) for 7 days did not significantly change serum calcium, phosphorus, creatinine, and uric acid levels compared with those between groups. Nevertheless, SG-injected mice, which received the extract dose of 400 mg/kg, had lower values of serum phosphorus and creatinine than untreated SG-injected mice. SG-injected mice had a lower serum uric acid level than normal mice, and the data were not statistically significant. There was a decreasing trend in serum uric acid among groups of SG-injected mice receiving the extract doses of 200 mg/kg and 400 mg/kg compared with the untreated SG-injected mouse group. Intraperitoneal injection of sodium glyoxylate at a dose of 100 mg/kg for 7 consecutive days caused an increase in serum urea in SG-injected mice compared to normal mice. The serum urea of SG-injected mice receiving the extract at 200 mg/kg was not quite different from the untreated SG-injected group, while SG-injected mice receiving a dose of 400 mg/kg had a lower serum urea concentration than untreated SG-injected mice.

As shown in Fig. 5, the serum calcium of SG-injected mice treated with 200 mg/kg extract and 750 mg/kg cystone was not quite different from tha of the untreated SG-injected group, while SG-injected mice that received a dose of 400 mg/kg had lower serum calcium concentration than the untreated SG-injected mice. The extract of 400 mg/kg reduced calcium level better than cystone. Besides, normal mice received the extract at 400 mg/kg had significant reduce in serum calcium levels compared to normal mice. The SG-injected mice treated with the extract dose of 400 mg/kg got a lower serum phosphorous, urea, creatinine, and uric acid levels than untreated SG-injected group, but the difference was not statistically significant. In particular, the extract at 400 mg/kg gave a better reduction than cystone. SG-injected mice that had a lower level of uric acid in serum compared to normal mice and the data were statistically significant. There was a slight increase in serum uric acid in the SG-injected mice received the extract dose of 200 mg/kg compared with untreated SG-injected group. Only SG-injected mice that received the extract at 400 mg/kg had reduced the levels of uric acid compared to the untreated SG-injected mice. This was similar in the normal mouse group that received the extract at a dose of 400 mg/kg.

On the other hand, the results showed that the administration of sodium glyoxylate (100 mg/kg, intraperitoneal injection, *i. p.*) for 7 days increased the concentration output of urine phosphorous, magnesium, and calcium in comparison between SG-injected and normal groups, but these differences were not statistically significant. The extract was able to reduce these parameters after 7 days of treatment (Fig. $6a_7$-c_7). While the calcium level in the SG-injected group remained higher than that in the normal group, the magnesium level in the SG-injected group decreased compared with that in the normal group after 14 days of testing. The extract at a dose of 200 mg/kg was able to reduce calcium concentration while at a dose of 400 mg/kg it increased magnesium level. There was no significant change in urine phosphorous levels between the SG-injected groups treated with the extract for 14 days and the untreated SG-injected group (Fig. $6a_{14}$-c_{14}).

At day 7 and 14, SG-injected mice treated with the extract 200 mg/kg showed an upward trend in both drinking water and excreted urine compared to untreated SG-injected group, but it did not gained statistic significant. The SG-injected mice treated with the extract 400 mg/kg had a lower volume in drinking water and excreted urine than pathological mice, it also was non-significant difference. Besides, normal mice

received the extract 200 mg/kg was higher in both water drink and urine excretion compared to normal mice, but normal mice received the extract 400 mg/kg was contrast (Online resource Fig. 7).

Although the experimental results did not show significant changes in serum and urine biochemical parameters in mice injected with sodium glyoxylate (100 mg/kg, *i. p.*) for 7 days compared with normal mice as well as mouse group treated with the extract and cystone compared with SG-injected mouse group, however, significant renal histopathological changes were observed after 14 days of testing. The SG-injected mouse groups treated with the extract 400 mg/kg and cystone 750 mg/kg had a normal structure in the renal tubules and glomeruli, no crystal deposition and no other abnormalities were observed, similar to those observed in normal mice. The SG-injected mouse group received the extract 200 mg/kg observed the presence of inflammatory cells and glomerular enlargement. Meanwhile, SG-injected mouse group showed enlarged tubule and glomeruli, inflammation, scattered damaged renal tubule, and small stone or calcification in the renal tubules in the renal pyramidal region (Fig. 8).

3.3 In vitro antilithiatic activity of E. glaucum aqueous seed extract on calcium oxalate crystallization

The calcium oxalate crystal area (μm^2) was significantly reduced by the extract or the cystone at different concentrations (Fig. 9a, a'). The average crystals size of the control (Fig. 9a) was 13.28 μm^2, while at the concentration 62.5 µg/mL of the extract, the crystal size was 6.19 μm^2 (> 50% reduction), equivalent to similar to cystone. The results also indicated that the extract had ability to reduce crystal sizes according to concentration increased. Simultaneously, the extract and cystone broke the stone into smaller fragments, thus increasing the number of crystals and crystal areas (μm^2/HPF) as shown in the Fig. 9b, b' and 9c, c', respectively, making it easier to avoid the development of urinary stones by causing the secretion of small particles by the kidneys and reducing the chances of retention in the urinary tract.

The results showed that the number of crystal aggregation was reduced in a concentration-dependent manner of the extract and cystone (Fig. 9d, d'). The control group of the extract got an average number of crystal aggregation was 6.89, it was just 2.89 aggregations at the concentration 1.25 mg/mL (significantly reduced by 58.01%) (Fig. 9d). The extract at the remaining concentration still had ability to inhibit crystal to adhere together, but these number was non-significant compared to control. Meanwhile, the control group of cystone had approximately 6.22 number of aggregations, by comparing that value with 2.22 crystal aggregation at concentration of 1.25 mg/mL (Fig. 9d'), it was significantly dropped by 64.31%. The results indicated that the extract and cystone had ability to inhibit crystal aggregation according to concentration increased.

To demonstrate the ability in inhibition of CaO_x crystallization of the extract and cystone, images of COM, COD, and aggregated COM crystals were taken under the microscope at 40 × magnification. It was easily to see the trends of the extract and cystone was crystal morphology transformation and crystals area reduction (Online resource Fig. 10, 11). Also, the extract and cystone worked effectively to prohibit the chance for crystal to aggregate (Online resource Fig. 12, 13). These effectiveness was clearer when the concentration increased.

3.4 In vitro anti-inflammatory and antioxidant activities of E. glaucum aqueous seed extract

Preliminary analysis results revealed the presence of phytochemicals such as lipids, triterpenoids, alkaloids, coumarins, flavonoids, proanthocyanidins, anthocyanosides, tannins, and saponins in *E. glaucum* seeds (Online resource Table 1**).** Total polyphenol content and total flavonoid content of EGE were also estimated at 47.19 ± 0.75 (mg GAE/g d. w.) and 18.00 ± 2.43 (mg QE/g d. w.), respectively.

The results in Table 2 and Fig. 14 (Online resource) also denoted that the *E. glaucum* seed aqueous extract has anti-inflammatory and antioxidant activities. The anti-inflammatory effect of the extract was through its ability to inhibit protein denaturation and stabilize cell membranes, while the antioxidant effect was through the DPPH free radical scavenging, $ABTS^{\bullet+}$ radical cation quenching, and reducing power mechanisms, but the effect was lower than that of the control drug.

Table 2
In vitro anti-inflammatory and antioxidant activities of the EGE

Assay	EGE	Positive control
Protein denaturation (IC_{50}, µg/mL)	310.37 ± 15.76	141.53 ± 9.05
Heat-induced hemolysis (IC_{50}, µg/mL)	226.45 ± 16.54	51.15 ± 2.91
Hypotonicity-induced hemolysis (IC_{50}, µg/mL)	69.57 ± 2.38	54.24 ± 1.79
DPPH (IC_{50}, µg/mL)	128.53 ± 5.44	1.39 ± 0.06
ABTS (IC_{50}, µg/mL)	51.39 ± 3.47	1.03 ± 0.05
Reducing power (EC_{50}, µg/mL)	263.07 ± 43.01	3.22 ± 0.56

IV. Discussion

Medicinal plants are believed to be an important source of novel active ingredients with therapeutic effects [21]. Many plants and their preparations have been documented for medicinal and health care purposes. Therefore, research on the pharmacological effects of medicinal plants is the current trend [22]. This study investigated the analgesic and urinary stone preventive effects of *E. glaucum* seeds.

Acetic acid is used as a chemical factor that stimulate the secretion of cyclooxygenase (COX) and lipoxygenase (LOX), which mediates the generation of endogenous nociceptive mediators like prostaglandin and histamine [23]. The *E. glaucum* aqueous seed extract of tested doses (50–400 mg/kg) showed antinociceptive activity in this model. Hot plate test is considered as one of the sensitive methods to estimate the central analgesic activity via antinociceptive mechanism, which is sensitive with analgesics belong to opioid group [24]. The results showed that *E. glaucum* aqueous seed extract at both doses (100 mg/kg and 200 mg/kg) exhibited the central analgesic effect and thereby inactivating the pain pathway at the supra-spinal level. In the formalin-induced hind paw licking model, despite the fact

that the early phase did not show a compelling pain release at all groups in this study, the late phase, the extract groups at dose of 200 mg/kg and 400 mg/kg illustrated a decrease in the hind paw licking, especially, the extract dose at 400 mg/kg inhibited the paw licking over 50% as compared to the control group. This model is a combination of the peripheral analgesic activity, and the inflammatory analgesic activity that function in the central nervous system. The early phase represents the stimulation of C fiber afferent at the periphery, while the late phase indicates the prolonged pain, classified as an inflammatory pain since it also generating COX and LOX, mediating the release of histamine, serotonin, prostaglandins, bradykinin with the activation of the dorsal horns of the spinal cord [25, 26]. As a results, the *E. glaucum* seed extract exhibited a significant anti-inflammatory analgesic effect in the late phase of this model. Hence, the *E. glaucum* seeds probably possess a potential compound as an inhibitor of inflammatory mediators. In conclusion, the present study demonstrated antinociceptive in both chemical (acetic acid and formalin)-induced and thermal (hot plate)-induced nociception test models of *E. glaucum* seed extract. Overall, these findings indicated that the antinociceptive effect of the *E. glaucum* seed extract is mediated through the central and peripheral mechanisms. Therefore, antinociceptive mechanism of *E. glaucum* seeds could be studied further, for example, activation of opioid receptors and modulation of the L-arginine/NO-dependent/cGMP-independent pathway [27, 28].

Furthermore, this study also provided preliminary scientific evidence about the *E. glaucum* seeds in anti-urolithiatic activity *in vitro* and *in vivo*. Calcium oxalate is the main component in kidney stone. Key events involving in the pathological biomineralization include crystal nucleation, growth, and aggregation [29]. Because nucleation is an important first step for the initiation of crystals formation, thus the inhibition of CaO_x nuclei will be very helpful in preventing crystal formation. Crystals bind to others throughout process known as aggregation. Adhered crystals are held in place and cannot be easily separated, crystals will increase in size and aggregate within the urine of the tubules, these aggregates enlarge and block urine outflow which cause renal damage [30]. The aqueous extract from *E. glaucum* seeds had potential in inhibiting the calcium oxalate crystal nucleation and aggregation. In clinical urolithiasis, COM (calcium oxalate monohydrate) is more frequently observed than COD (calcium oxalate dihydrate). COM crystals are the most important factors contribute to the urologic stone formation. Because COM crystals are the most thermodynamically stable stones, and they have a greatest adsorptive capability, therefore it can bind to macromolecules like proteins, glycoproteins on renal tubular epithelial cell surfaces of urinary tract. Additionally, individual COM crystals can aggregate to create the agglomeration, hence COM plays the most important role in kidney stone formation. Meanwhile, COD crystals are less stable than COM crystals and easily excreted [5]. Obviously, there were two common trends showing the inhibit activity of the *E. glaucum* seed extract which the crystal sizes were decreased, and the form of crystals were changed from COM into COD. Also, the extract showed potential to decrease the number of crystal aggregations. Less number of crystal aggregations, the more effective in the management of urolithiasis, because when the crystals tend to bind together, its size become larger, and it is hard to be excreted out.

In this study, intraperitoneal injection of sodium glyoxylate induced nephrotoxicity [31]. Urolithiasis mice got higher level of both serum urea and serum creatinine compared to normal control. In non-protein nitrogenous substances such as calcium, uric acid accumulates in the blood. Pathological mice received the treatment with the extract had a low concentration of uric acid, and calcium in serum. This result showed effectiveness of the extract in the management kidney stone disease. About the urine, there are many inorganic and organic inhibitors of crystallization, and magnesium is one such well-known inhibitors [32]. Low level of magnesium was also encountered in stone formation. Mice with urolithiasis caused by sodium glyoxylate had considerably less magnesium in their urine. However, urolithiasis mice with a treatment with 400 mg/kg had higher amount of magnesium in its serum. Therefore, this dose was kind of potential to control the kidney stone formation. Because magnesium is responsible to make complexes with oxalate and reduce the calcium oxalate supersaturation, this lowers the nucleation and growth rate of calcium oxalate crystals as a result [33]. It was an increasing trend in urinary phosphorous of urolithiasis mice at day 7, but after consuming the extract for the 7 next days, the level of phosphorous was decreased significantly. On the other hand, the results of microscopic examination of the mice kidneys showed that the extract also had renoprotective effect against sodium glyoxylate-induced damages. In our experimental conditions, sodium glyoxylate can promote CaO_x stone formation but the formation may not be maximal, biochemical parameters in serum and urine did not seem to change clearly. Therefore, it is necessary to re-investigate the pathogenicity dose and time of sodium glyoxylate in accordance with the laboratory conditions and our laboratory mouse strains.

On the other hand, crystal deposition inside the urinary tract can cause inflammation and cell damage. The results showed that the extract also has *in vitro* anti-inflammatory activity through the mechanism of inhibiting protein denaturation and stabilizing cell membranes. In urolithiasis, oxalate is the major stone-forming component and has been reported to cause lipid peroxidation and tissue damage [34]. In this study, the antioxidant activity of the extract from *E. glaucum* seeds was demonstrated by free radical scavenging mechanism (DPPH, ABTS) and reducing power.

Phytochemical study of *E. glacum* seeds showed the presence of triterpenoids, alkaloids, coumarins, flavonoids, proanthocyanidins, anthocyanosides, tannins, and saponins. The amount of total polyphenol, flavonoid and antioxidant activity of *E. glaucum* seed extract was considerable. Analgesic effect is also influenced by the free radicals and inflammation [35]. Therefore, pain inhibition action of the extract could be partially credited to the action of the extract against free radicals and anti-inflammation. Plant containing flavonoids have been reported to antioxidant and anti-inflammatory activities [36]. Several analgesic mechanism of flavonoids were reported such as reducing the release of NO, PGE2, or proinflammatory cytokines, blocking the central calcium channel, and inhibiting the synthesis of prostaglandin [37]. Besides, terpenes and its main three subtype such as monoterpenes and sesquiterpenes, diterpenes, and triterpenes all possesses an potential anti-inflammatory analgesic activity [38]. Tannins had been proved to have a significant central acting and periphery analgesic effects [39]. Saponins were also studied for the inhibition of PGI and other proinflammatory interleukins [40]. Previous studies had proved the analgesic activity of several species in the Musaceae family, for example, *Musa*

acuminata stems in “Study of Anti-inflammatory and Analgesic Activity of Musa spp. Peel.” (Lee S, Singaram N and Hassan H, 2016, preprint), *Musa sapientum* leaves [41], peel and leaves of *Musa parasidiaca* [42, 43]. Polyphenols and flavonoids have also been shown to be active against urolithiasis through a number of mechanisms involving oxalate resistance, antioxidant, anti-inflammatory, angiotensin inhibition, and diuretic effects [44]. Protective roles of polyphenol- and flavonoid-rich plant extracts as well as polyphenol and flavonoid compounds against urolithiasis were reported [44, 45]. Saponin rich fraction from plants inhibited *in vitro* calcium oxalate crystal nucleation and aggregation, and against chemical-induced urolithiasis in rats [46]. Besides, some previous studies had also showed the anti-urolithiatic activity of several species in the Musaceae family, for instance, *M. balbisiana* fruits [47], *M. paradisiaca* pseudostems [48], *Ensete superbum* (Roxb.) Cheesman pseudostems [49].

In summary, this is the first study that has demonstrated the effects of the *E. glaucum* seed aqueous extract including analgesic, anti-inflammatory, anti-urolithiatic, antioxidant activities. These effects could positively support the prevention and treatment of urinary stones of *E. glaucum* seeds.

V. Conclusion

Ensete glaucum (Roxb). Cheesman aqueous seed extract had the analgesic effect via antinociceptive mechanism on peripheral, central and anti-inflammatory models. The extract had prophylactic effects against urolithiasis *in vivo* and *in vitro* by reducing the crystal size nuclei, ability for crystal to aggregate and improving histopathology. The extract also had *in vitro* anti-inflammatory and antioxidant activities. The analgesic and urinary stone-preventing activities of *E. glaucum* seed extract provided scientific support for the traditional use of *E. glaucum* seeds in the treatment of diseases. Hence, further study should be conducted to elucidate the effects of this plant material.

Abbreviations

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

COD: Calcium oxalate dihydrate

COM: Calcium oxalate monohydrate

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EC_{50}: Effective concentration, 50%

EGE: *Ensete glaucum* seed aqueous extract

GAE: Gallic acid equivalent

HPFs: High-power fields

IC_{50}: Inhibitory concentration, 50%

$LD_{50:}$ Lethal dose, 50%

MPA: Maximal possible analgesia

NSAIDs: Nonsteroidal anti-inflammatory drugs

QE: Quercetin equivalent

SEM: Standard error of mean

SG: Sodium glyoxylate

Declarations

Animal ethical approval

The animal studies were conducted at the Research Center of Ginseng and Medicinal Materials Ho Chi Minh City, National Institute of Medicinal Materials, Ministry of Health - Vietnam. All animal experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The animals were maintained, controlled and studied according to approved guidelines and regulations (141/QĐ-K2ĐT).

Data availability

All the data generated during this study were statistically analysed and are presented in the figures and tables. The dataset could be available upon reasonable request from the corresponding author.

Authors' contribution

LTKO: performed the experiments, analysed the data, and participated in manuscript writing; LHT: conceptualized, designed, conducted the experiments, analysed the data, and wrote the paper; LVM: supervised the work, edited and reviewed the manuscript. All the authors have read the final manuscript and approved the submission.

[#]Equal distribution as co-first authors

Conflict of interest statement

The authors declare that they have no competing interests.

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Figures

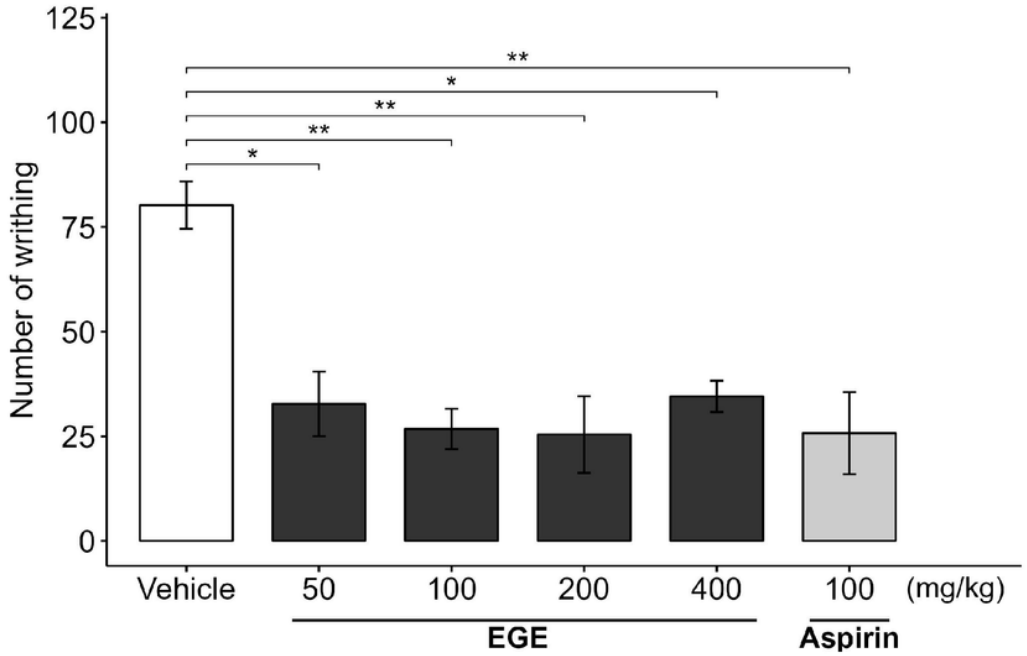

Fig. 1

Figure 1

Antinociceptive effect of EGE in acetic acid-induced writhing test. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 9), $^{*}p < 0.05$ and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

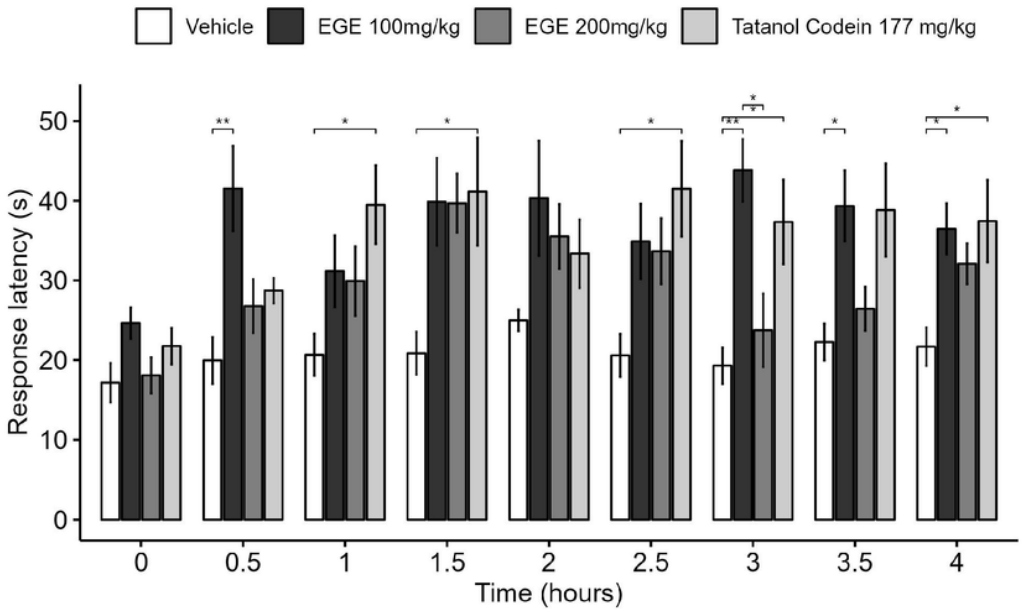

Fig. 2

Figure 2

Antinociceptive effect of EGE in hot plate test. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{*}p < 0.05$ and $^{**}p < 0.01$: significantly different compared to the control group (Kruskal–Wallis test and Dunn´s multiple comparison test)

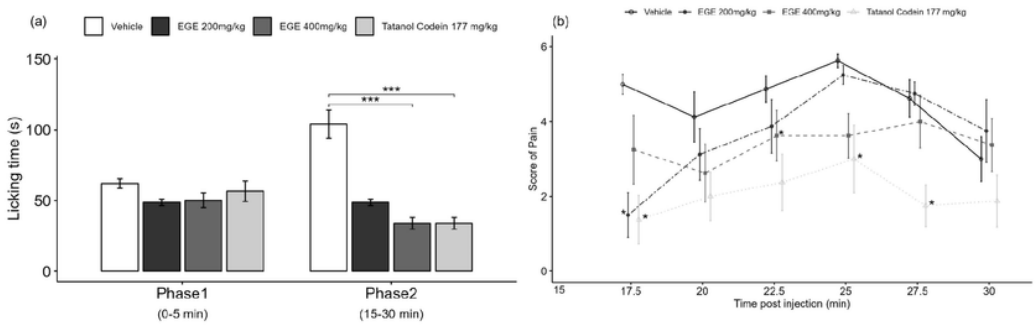

Fig. 3

Figure 3

Antinociceptive effect of EGE in formalin-induced paw licking test. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{*}p < 0.05$ and $^{***}p < 0.001$: significantly different compared to the control group (Kruskal–Wallis test and Dunn´s multiple comparison test)

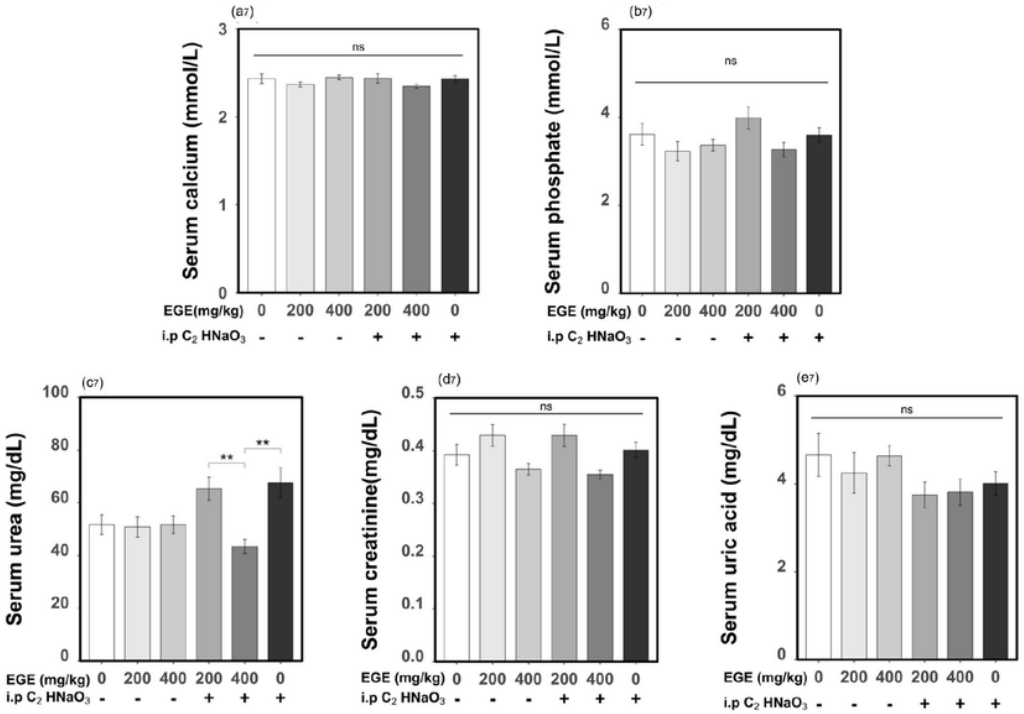

Fig. 4

Figure 4

Effect of EGE on serum parameters in sodium glyoxylate-induced urolithiasis in mice after 7 days of treatment. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

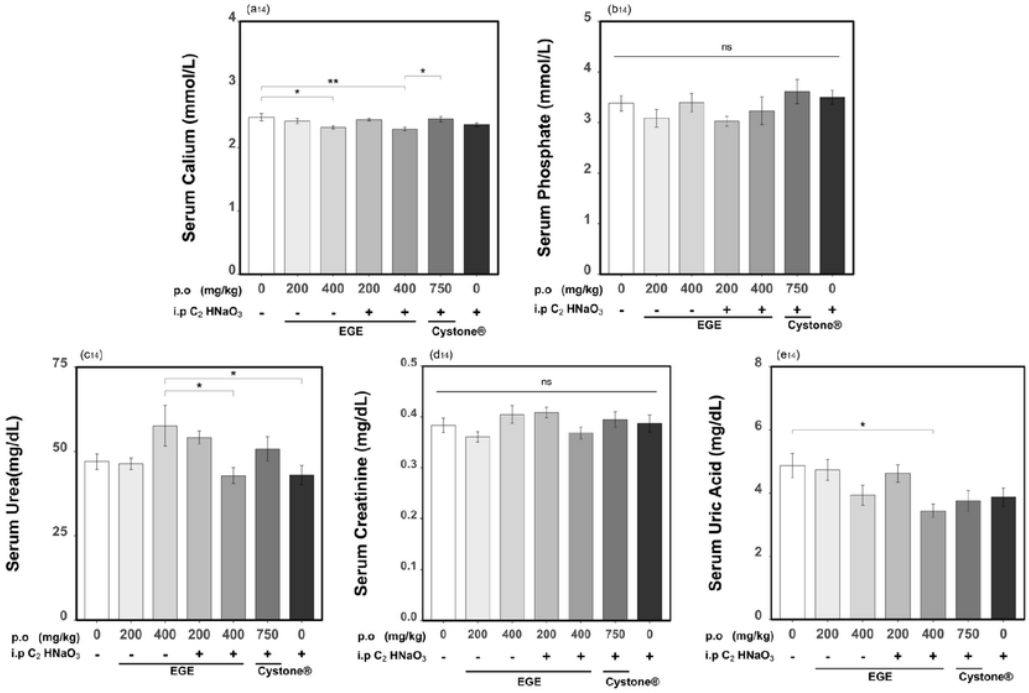

Fig. 5

Figure 5

Effect of EGE on serum parameters in sodium glyoxylate-induced urolithiasis in mice after 14 days of treatment. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different, $^{*}p < 0.05$ and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

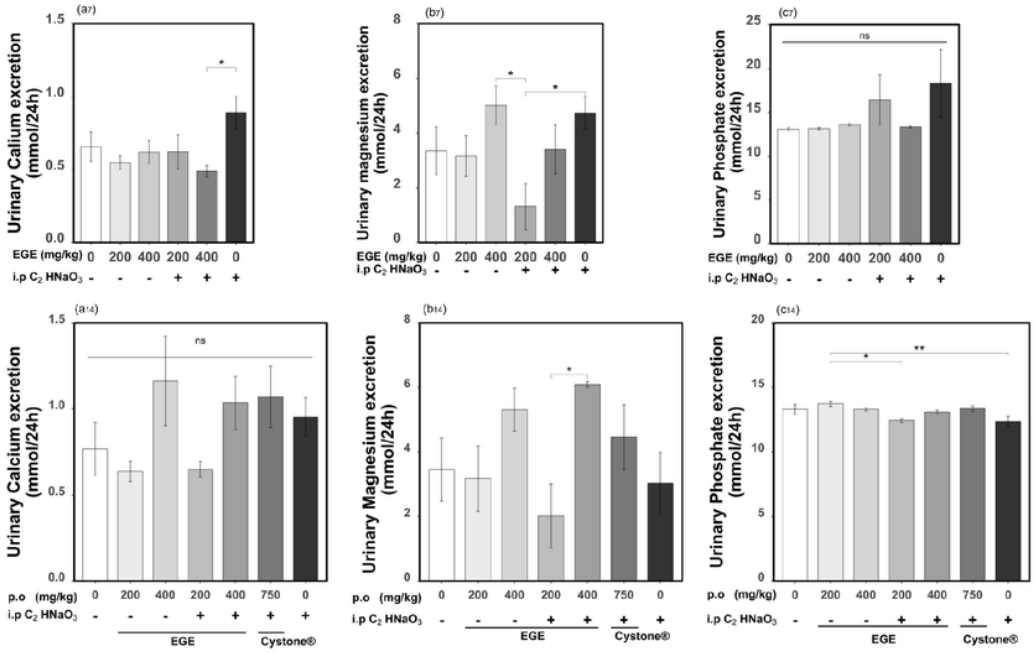

Fig. 6

Figure 6

Effect of EGE on urine parameters in sodium glyoxylate-induced urolithiasis in mice after 7 (a_7-c_7) and 14 (a_{14}-c_{14}) days of treatment. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different, $^{*}p < 0.05$ and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

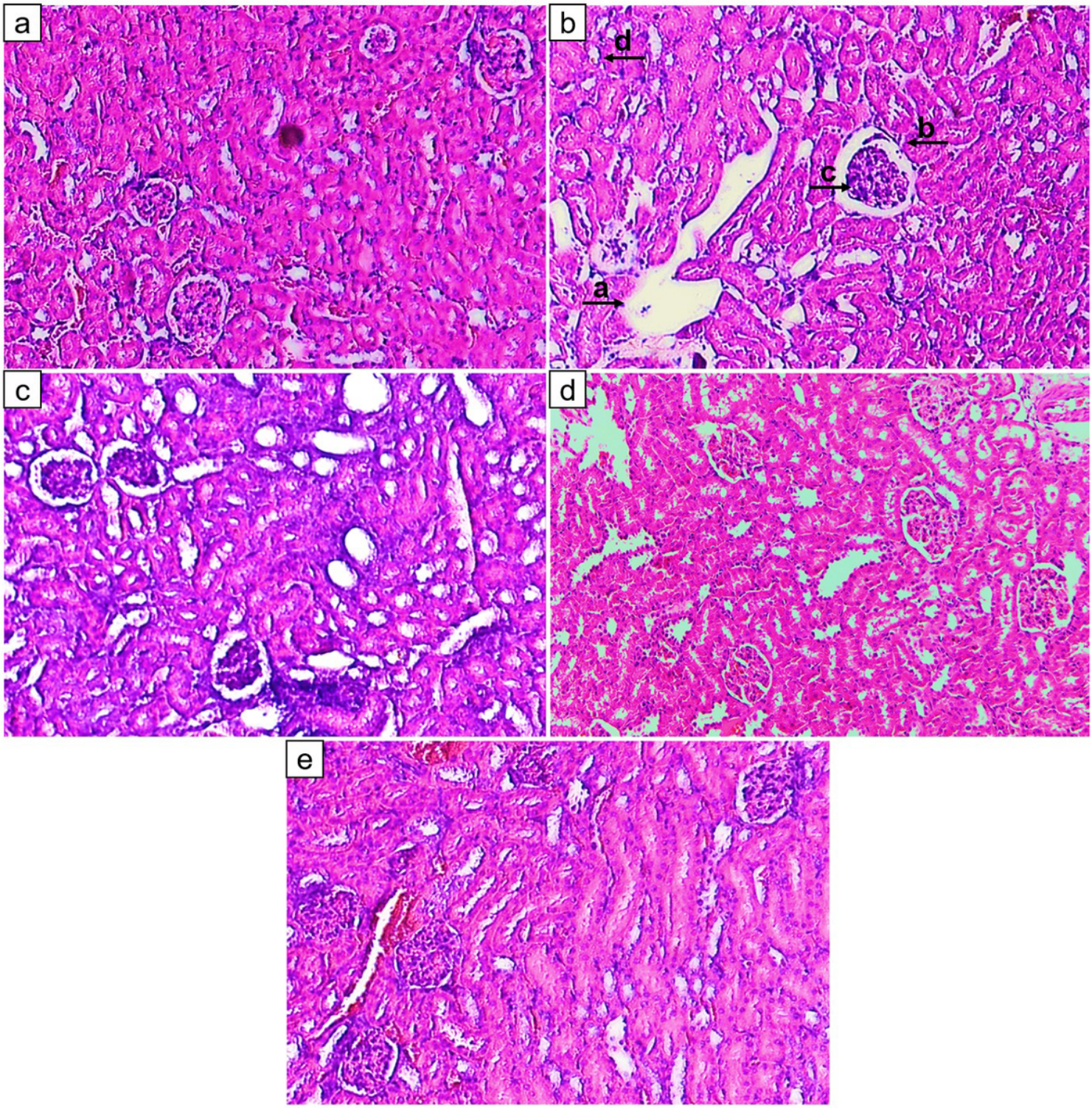

Figure 7

Representative images of renal histopathology after 14 days of experiment. a: Physiological group; b: Pathological group, showing histological changes including tubular (a) and glomerular (b) enlargement, lymphocytic infiltration (c) and calcium oxalate crystal deposition (d); c and d: Prophylactic treatment with EGE at the dose of 200 and 400 mg/kg; e: Treatment with Cystone at the dose of 750 mg/kg. Microscopic magnification: 20×

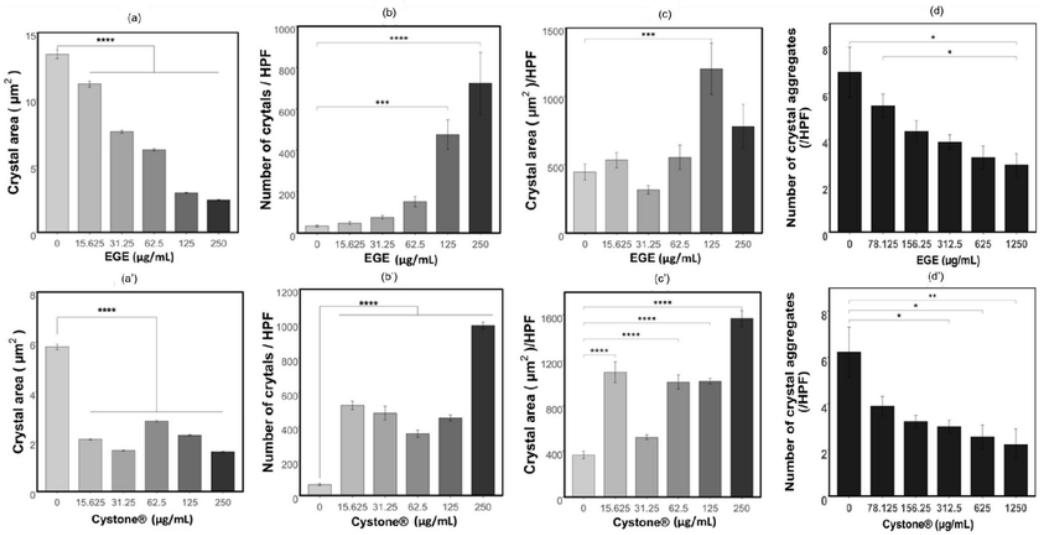

Fig. 9

Figure 8

EGE inhibited the formation and aggregation of calcium oxalate crystals. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$: significant difference (One-Way Anova and Tukey test). (a-b) Effect of EGE in nucleation assay, (a'-b') Effect of cystone in nucleation assay, (d) Effect of EGE in aggregation assay, and (d') Effect of cystone in aggregation assay

Supplementary Files

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