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Research Article

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

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ABSTRACT

Ensete glaucum (Roxb.) Cheesman (*E. glaucum*) seeds are commonly used in Vietnamese folk medicine to treat urinary stones, edema, and osteoarthritis-related problems. Nevertheless, no reported scientific evidence has been found to substantiate these traditional practices. This study focused on investigating the potential antinociceptive and anti-urolithiatic properties 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. 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 *in vitro* anti-urolithiatic, anti-inflammatory, and antioxidant activities. These outcomes suggest that E. glaucum aqueous seed extract possesses antinociceptive activity and may aid in preventing urinary stones. Further studies are needed to elucidate the effectiveness of *E. glaucum* seeds in the analgesic activity and management of urolithiasis disease.

Keywords:
Ensete glaucum (Roxb.) Cheesman seeds; Antinociception; Anti-urolithiasis; Anti-inflammation; Antioxidant

1. 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 materials 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].

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Urolithiasis is a common urologic disease in Vietnam and worldwide 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. 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].

Urolithiasis due to CaO_x crystal deposition can lead to excessive synthesis of proinflammatory molecules, causing inflammation, renal cell damage and loss, and even chronic renal failure[7,8]. Therefore, in addition to analgesics, muscle relaxants, and invasive procedures to remove stones, anti-inflammatory drugs are also used in the treatment of urinary tract stones. On the other hand, oxalate is the main stone-forming component and has been reported to cause lipid peroxidation and tissue damage by reacting with unsaturated fatty acids on cell membranes. Oxalate-induced membrane damage is mediated by lipid and protein peroxidation through reactive oxygen species with altered biochemical reactions, including the impairment of antioxidant defenses, inflammatory promotion, the failure of calcium transport channels, and mitochondrial dysfunction. Calcium and oxalate accumulate, then precipitate and form stones[8,9]. Thus, anti-inflammatory and antioxidant agents may play an important role in the adjuvant treatment of urinary stones, for which plants are a potential candidate[7].

Ensete glaucum (Roxb.) Cheesman, also known as snow bananas, is widely distributed in South and Southeast Asia are listed as Myanmar, India, China, Indonesia, Lao, Vietnam, Thailand, and Philippines[10]. *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[11]. 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 Cystone was used as a positive control in antiurolithiatic test. Cystone is an herbal product for urinary stone disease developed based on Ayurvedic medicine. Cystone has been reported to have the ability to prevent stone formation, possess antibacterial and anti-inflammatory properties, and provide antispasmodic effects[12]. It's often used as a standard drug in studies of new herbal medicines. However, the clinical evidence supporting its effectiveness is not yet robust[13]. 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.

2. MATERIALS AND METHODS

2.1. Plant materials and extraction

E. 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 °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 extract was investigated according to the Organization for Economic Cooperation and Development (OECD) guideline number 423 and the Vietnam Ministry of Health[14,15]. 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 orally 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%). In phase 2 (determination), the experiment was repeated with 10 male and 10 female mice. Dead mice (if any) have their organs histologically dissected.

2.4. Antinociceptive tests

2.4.1. Acetic acid-induced writhing test

The writhing test was performed as described previously with some slight modifications[16]. Male mice were divided randomly into 6 groups (n = 9): (I) Vehicle group (distilled water, *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[17]. 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 °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[18]. 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 behavior 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. Sodium glyoxylate (C_2HNaO_3) (Sigma Co., St. Louis, MO, USA) at a dose of 100 mg/kg was administered by intraperitoneal (*i. p.)* injections for 7 consecutive days (from day 1 to day 7) to induce renal calculi CaO_x.

Mice were equally divided into 7 groups of 8 mice. Group I: Physiological control (0.9% NaCl *i. p.* day 1 to day 7 + distilled water *p. o.* day 1 to day 14); Group II: 200 mg/kg extract-administered normal mice (0.9% NaCl *i. p.* day 1 to day 7 + 200 mg/kg extract *p.o.* day 1 to day 14); Group III: 400 mg/kg extract-administered

normal mice (0.9% NaCl *i. p.* day 1 to day 7 + 400 mg/kg extract *p.o.* day 1 to day 14); Group IV: 200 mg/kg extract-administered pathological mice (100 mg/kg C_2HNaO_3 *i. p.* day 1 to day 7 + 200 mg/kg extract *p.o.* day 1 to day 14); Group V: 400 mg/kg extract-administered pathological mice (100 mg/kg C_2HNaO_3 *i. p.* day 1 to day 7 + 400 mg/kg extract *p.o.* day 1 to day 14); Group VI: 750 mg/kg cystone-administered pathological mice (100 mg/kg C_2HNaO_3 *i. p.* day 1 to day 7 + 750 mg/kg cystone *p.o.* day 7 to day 14); Group VII: Pathological control (100 mg/kg C_2HNaO_3 *i. p.* day 1 to day 7 + distilled water *p. o.* day 1 to day 14)[19].

2.5.1. Serum analysis

On the 7th and 14th days, blood from mouse tails was collected, centrifuged to obtain the serum, and analyzed for the content of creatinine, urea nitrogen, uric acid, calcium, and phosphate by biochemical kits (Alinity Abbott, Abbott Laboratories, IL, USA).

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 analyzed for the levels of phosphate, calcium, and magnesium with the help of diagnostic biochemical kits (Alinity Abbott, Abbott Laboratories, IL, USA).

2.5.3. Histopathological examination

At the end of the experiment, 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 a crystallization buffer containing 10 mM Tris–HCl and 90 mM NaCl (pH 7.4). The experiment was performed in triplicate on 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[20]. 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 area 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:

$$Crystal\ mass\ (\mu m^2/HPF) = Average\ crystal\ area\ in\ each\ field\ (\mu m^2) \times Number\ of\ crystal\ in\ each\ field\ (1/HPF)$$

2.6.2. Aggregation assay

First, individual COM (calcium oxalate monohydrate) crystals were prepared by mixing 10 mM calcium chloride and 1 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[20,21]. 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[22].

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 according to the protocol previously described[23].

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[24] 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.

Total polyphenol and flavonoid contents of *E. glaucum* seed extract was estimated by Folin-Ciocalteu's method and aluminum chloride colorimetric method, respectively, based on the previously described[23].

2.10. 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).

3. RESULTS

3.1. Acute oral toxicity test of *E. glaucum* aqueous seed extract

In the preliminary stage, 3 males and 3 females were received a maximum dose possible through the needle the extract (21.57 g/kg). After 6 and 12 hours, all animals did not exhibit any abnormal behaviors such as heavy breath, overactive, or biting. All rodents behaved well with surrounding agents and had normal appearance, alike as before administration, food was also consumed as normal. Within 72 hours, no lethal case was reported, and 100% mice were all alive in 14 days. Similar results were obtained in phase 2 with no lethal case reported.

Since there was no lethal case reported, D_{max} is 21.57 g/kg of body weight. According to D_{max} and traditional usage, doses of 20, 10, 5, 2.5 g of dried materials were used for pharmacological testing, which equivalent to approximately 1/50, 1/100, 1/200 and 1/400 D_{max}. To be in detailed, dose at 200 mg/kg (10 g of dried materials) was first investigated. If the effect was demonstrated, the dose was then lowered to 100 or 50 mg/kg (5 or 2.5 g of dried materials); if not, the dose was increased to 400 mg/kg (20 g of dried materials).

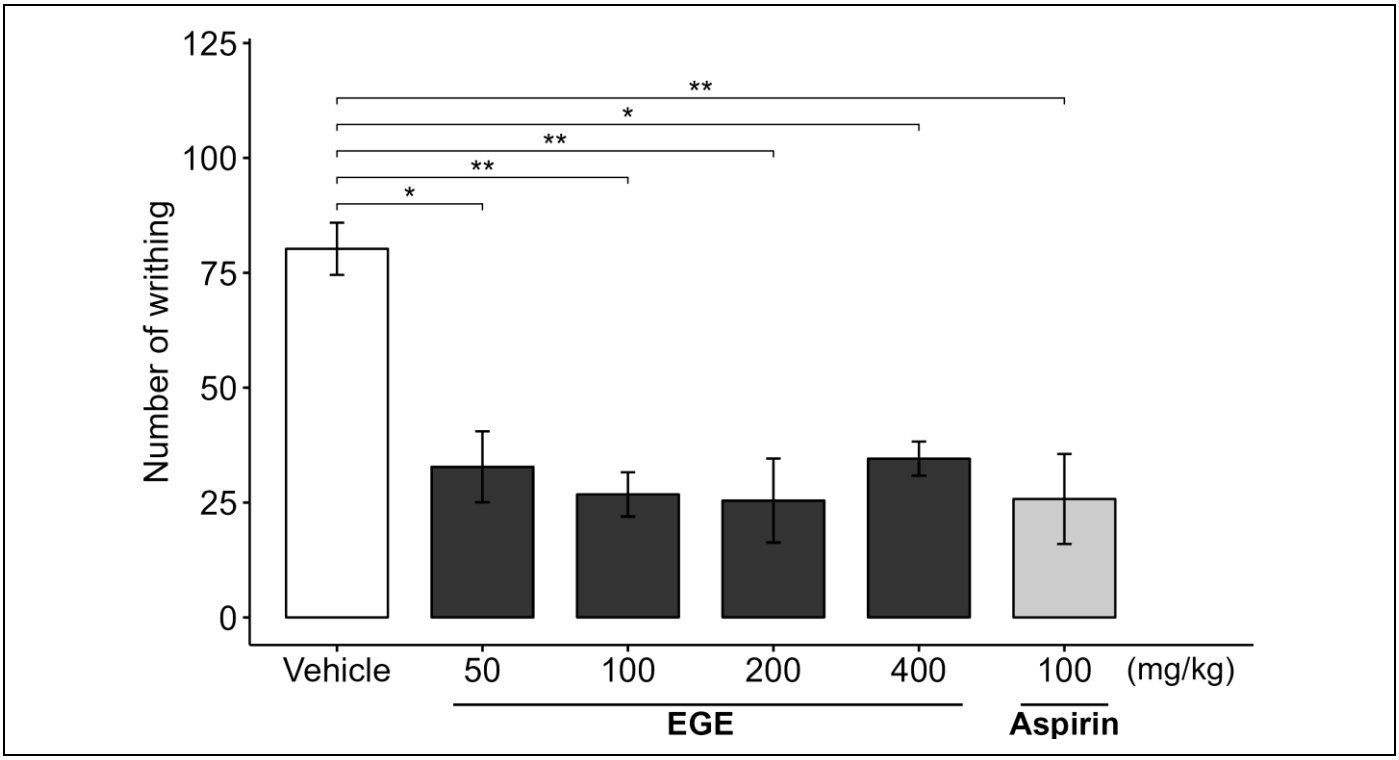

Figure 1 Antinociceptive effect of EGE in acetic acid-induced writhing test. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 9), $^{*}p < 0.05$ and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

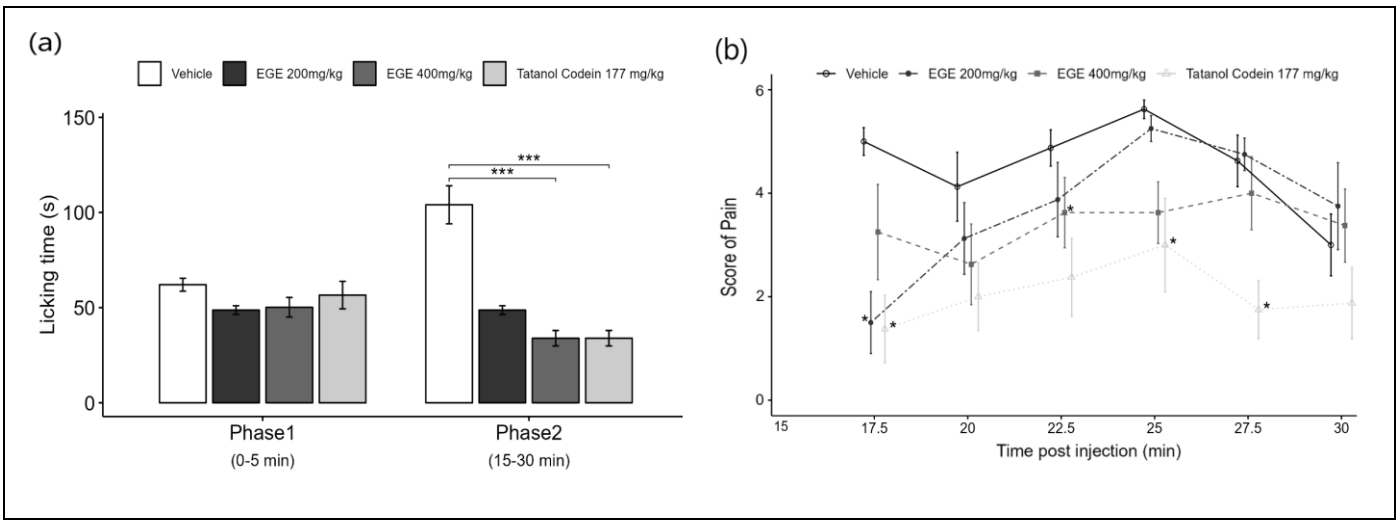

Figure 2 Antinociceptive effect of EGE in formalin-induced paw licking test. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{*}p < 0.05$ and $^{***}p < 0.001$: significantly different compared to the control group (Kruskal–Wallis test and Dunn´s multiple comparison test)

3.2. Antinociceptive effects of *E. glaucum* aqueous seed extract

The antinociceptive effect of *E. glaucum* aqueous seed extract (EGE) was investigated using three models of nociceptive behavior in mice induced by chemicals (acetic acid, formalin) and thermal stimulation.

The *E. glaucum* aqueous seed extract at doses of 50, 100, 200, and 400 mg/kg (EGE 50-400 mg/kg) significantly reduced the average number of acetic acid-induced writhing in 20 minutes of monitoring to approximately 32.78 ± 7.72, 26.78 ± 4.81, 25.44 ± 9.14, 34.56 ± 3.72 writhes. This equates to a reduction of 59.14%, 66.62%, 68.28%, and 56.91% of writhes in comparison to the vehicle (80.22 ± 5.68). The aspirin group (100 mg/kg) exhibited a 67.87% reduction of writhing 25.78 ± 9.79 writhes, a significant difference compared to the vehicle group. There was no convincing difference between EGE groups and aspirin group (Figure 1).

The biphasic configuration with short (10 min) periods of minimal nociceptive behavioral responses was observed after formalin injection into the hind paw. There was no statistical significance between groups in phase 1 (0-5 min). Pretreatment 2 hours with EGE 400mg/kg significantly inhibited hind paw-licking time in phase 2 (15-30 min) (Figure 2a). The cumulative licking time of EGE 400 mg/kg and Tatanol Codein 177 mg/kg (33.93 ± 4.01 and 33. ± 4.06 secs, respectively) was significantly shorter compared to vehicle (104.05 ± 9.99 secs) (p<0.001). EGE 200 mg/kg also reduced 53.19% licking time in phase 2 compared to the vehicle, but no significant difference exists. In addition, behavioral scores were also recorded to estimate the inflammatory analgesic activity of *E. glaucum* seed extract in the late phase (phase 2) (Figure 2b). Overall, the Tatanol Codein group was assessed to have significantly lower pain scores (p<0.05). Meanwhile, the EGE group demonstrated less severe levels of pain-liked behavior, but it was insignificant as compared with the vehicle.

The antinociceptive effect was also observed in hot-plate model through an increase in detecting heat pain threshold, as expressed by response latency (Figure 3). The EGE 100 mg/kg and Tatanol Codein 177 mg/kg significantly increased response latency compared to the vehicle after 30 and 60 minutes of oral administration, respectively. The effect of the two groups lasts throughout the following period of time although it is not maintained continuously. In contrast, the higher-dose EGE 200mg/kg has an unclear increase in latency time, the effect was not convincing when compared to the vehicle at any point of time and it exhibited a downtrend after 90 minutes.

3.3. Anti-urolithiatic effects of *E. glaucum* aqueous seed extract in mice

The results in Figure 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

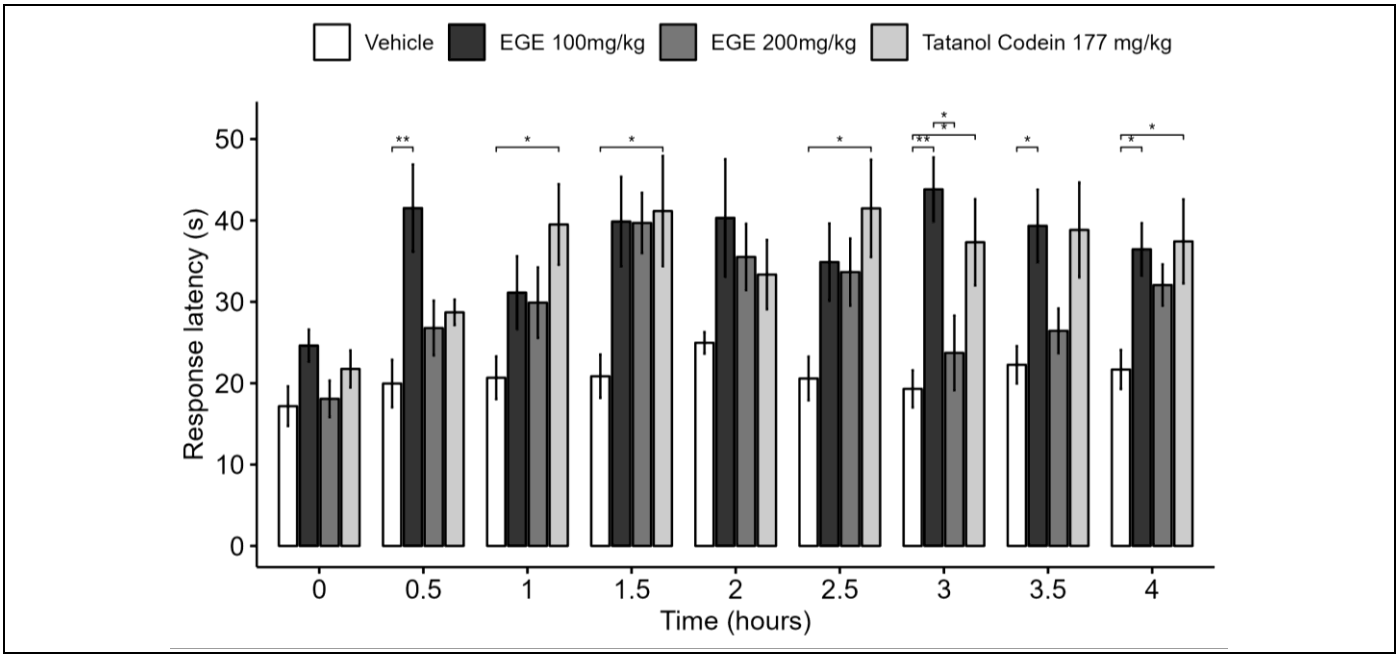

Figure 3 Antinociceptive effect of EGE in hot plate test. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{*}p < 0.05$ and $^{**}p < 0.01$: significantly different compared to the control group (Kruskal–Wallis test and Dunn´s multiple comparison test)

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.

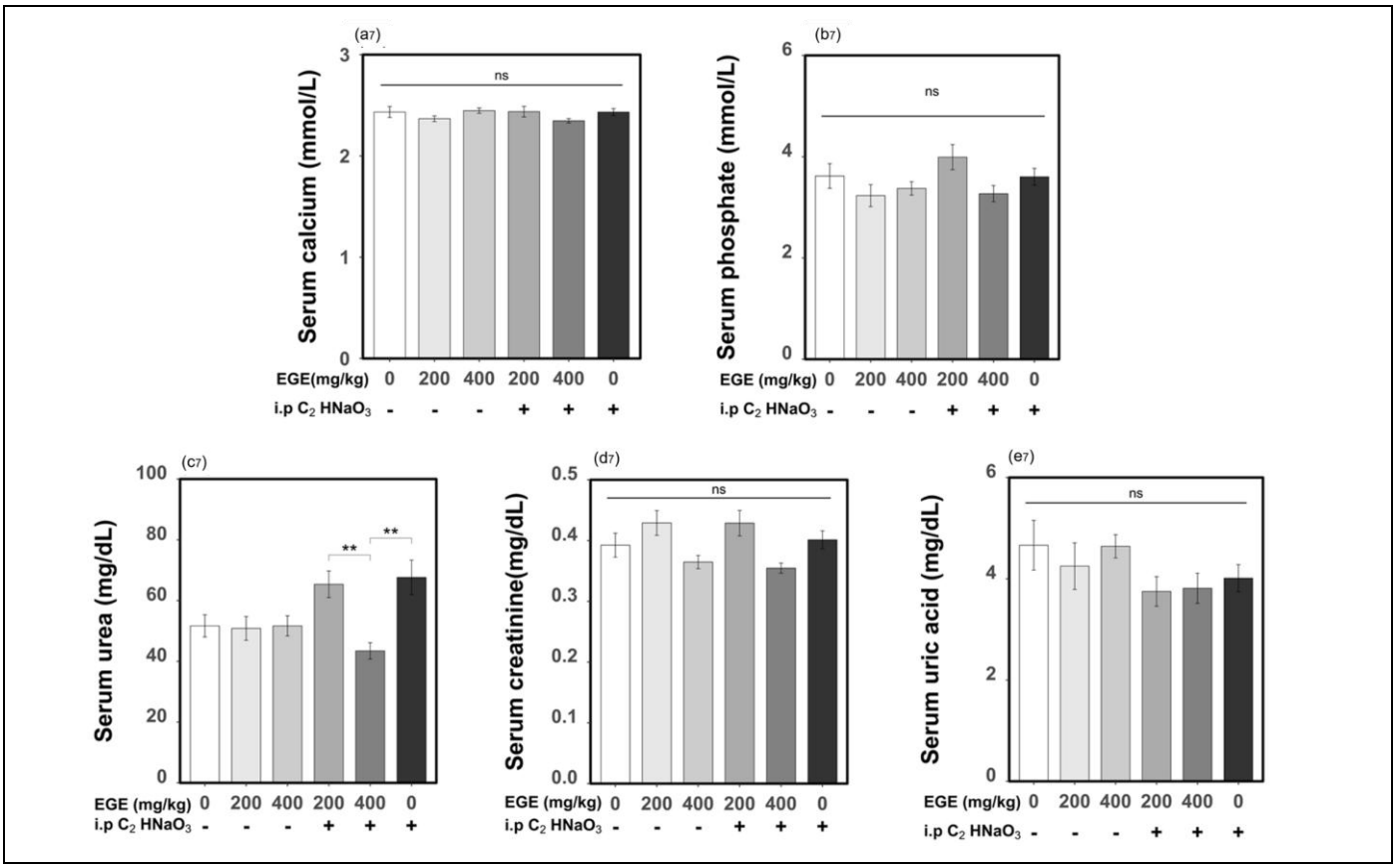

Figure 4 Effect of EGE on serum parameters in sodium glyoxylate-induced urolithiasis in mice after 7 days of treatment. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

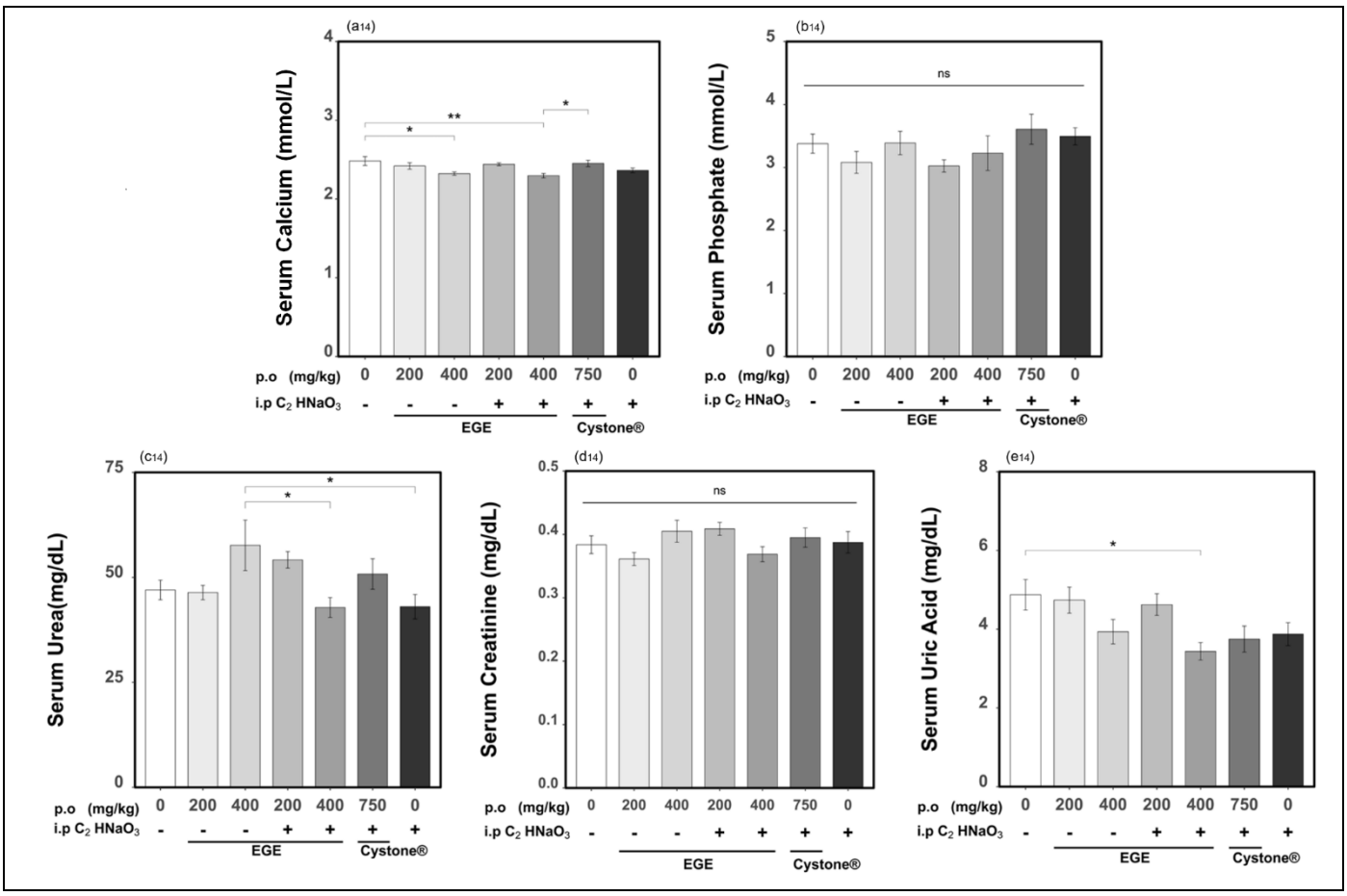

Figure 5 Effect of EGE on serum parameters in sodium glyoxylate-induced urolithiasis in mice after 14 days of treatment. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different, $^{*}p < 0.05$ and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

As shown in Figure 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 a significant reduction in serum calcium levels compared to normal mice. The SG-injected mice treated with the extract dose of 400 mg/kg got lower serum phosphorous, urea, creatinine, and uric acid levels than the untreated SG-injected group, the difference was not statistically significant. In particular, the extract at 400 mg/kg gave a better reduction than cystone. SG-injected mice 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 the 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 (Figure 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 (Figure 6a_{14}-c_{14}).

On days 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 the untreated SG-injected group, but it did not gain statistical significance. The SG-injected mice treated with the extract 400 mg/kg had a lower volume in drinking water and excreted urine than pathological mice, there also was

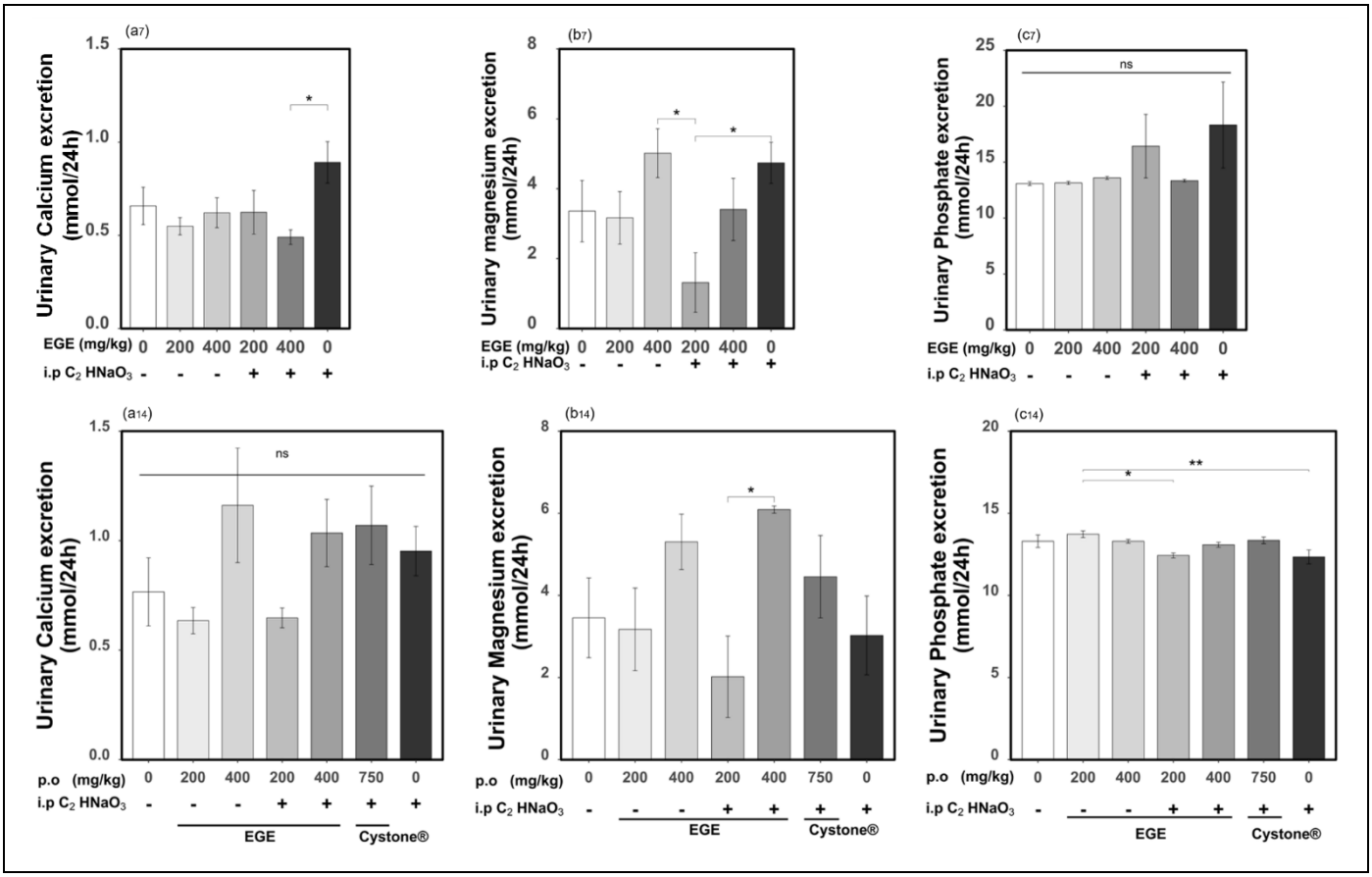

Figure 6 Effect of EGE on urine parameters in sodium glyoxylate-induced urolithiasis in mice after 7 (a_7-c_7) and 14 (a_{14}-c_{14}) days of treatment. EGE: *E. glaucum* aqueous seed extract, data was expressed as mean ± SEM (n = 8), $^{ns}p > 0.05$: not significantly different, $^{*}p < 0.05$ and $^{**}p < 0.01$: significant difference (Kruskal–Wallis test and Dunn´s multiple comparison test)

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 (Figure S1).

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 (3/6 samples) observed the presence of inflammatory cells and glomerular enlargement, while the remained stayed normal (Table S1). Meanwhile, SG-injected mouse group (5/6 samples) showed enlarged tubule and glomeruli, inflammation, scattered damaged renal tubule, and small stone or calcification in the renal tubules in the renal pyramidal region (Figure 7, and Table S1).

3.4. *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 (Figure S2-a, a'). The average crystal area of the control (Figure S2-a) was 13.28 ± 0.33 μm^2, while at the concentration 62.5 μg/mL of the extract, the crystal area was 6.19 ± 0.08 μm^2 (>50% reduction), equivalent to similar to cystone. The results also indicated that the extract had ability to reduce crystal areas according to concentration increase. Simultaneously, the test revealed an increase in calcium oxalate crystal formation as measured by an increase in the number and crystal mass (μm^2/HPF) of both EGE and cystone (Figure S2-b, b' and S2-c, c', respectively)

In aggregation test, the results showed that the number of crystal aggregation was reduced in a concentration-dependent manner of the extract and cystone (Figure S2-d, d'). The control group of the extract got an average number of crystal aggregation was 6.89 ± 1.08 aggregation/HPF, it was just 2.89 ± 0.48 aggregation/HPF at the concentration 5 mg/mL (significantly reduced by 58.05%) (Figure S2-d).

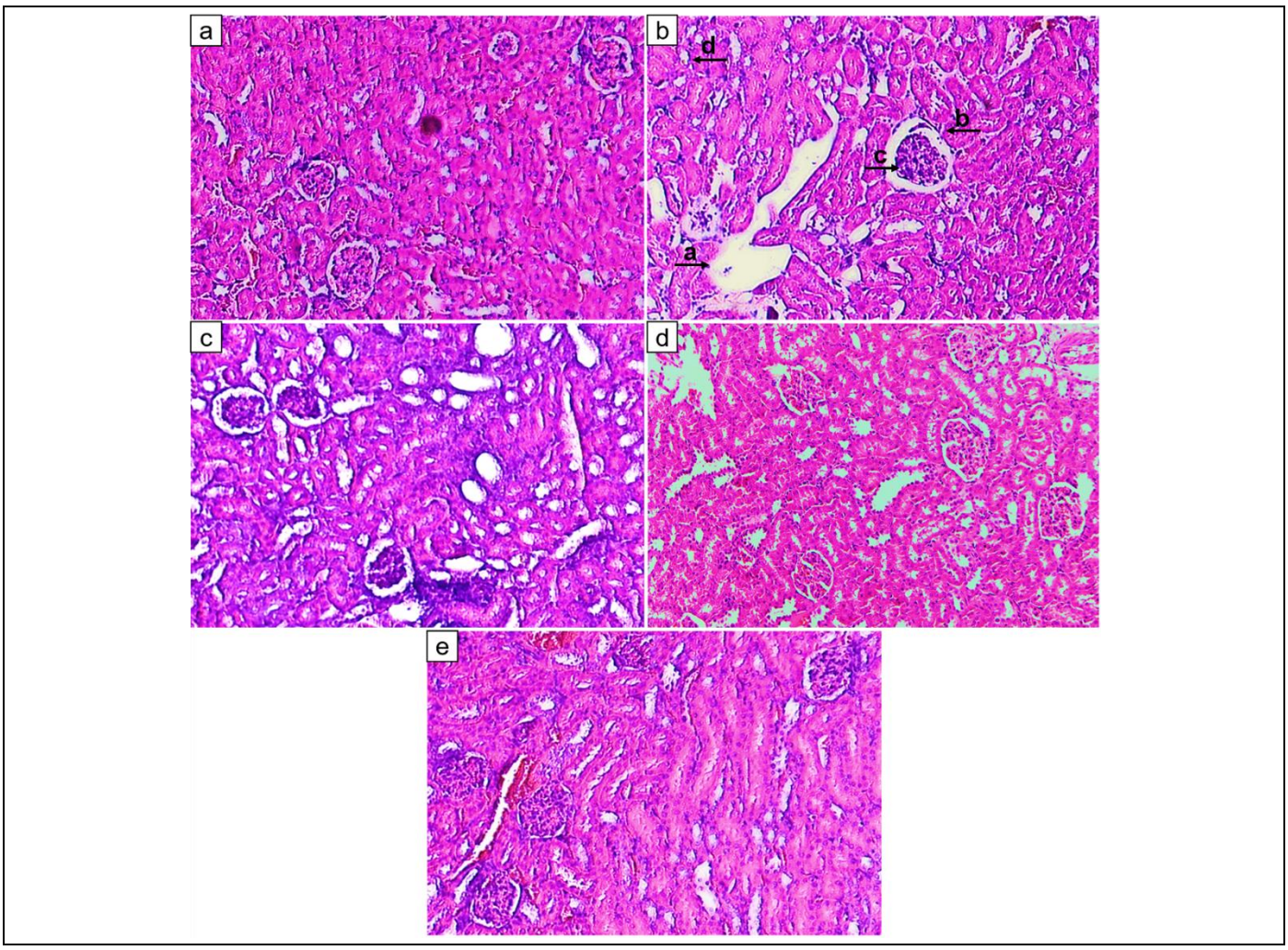

Figure 7 Representative images of renal histopathology after 14 days of experiment. a: Physiological group; b: Pathological group, showing histological changes including tubular (a) and glomerular (b) enlargement, lymphocytic infiltration (c) and calcium oxalate crystal deposition (d); c and d: Prophylactic 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×

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 ± 1.08 number of aggregations, by comparing that value with 3.00 ± 0.29 crystal aggregation at concentration of 1.25 mg/mL (Figure S2-d'), it was significantly dropped by 51.18%.

The trend of crystal type (COM, COD, and aggregated COM crystals) in the nucleation test was preliminarily evaluated through 40 × magnification images. Cystone clearly shows the concentration-dependent tendency to make crystals smaller and convert crystals from COM to COD form (Figure S3). EGE also exhibits a tendency to decrease crystal area when increasing concentration, however, no crystal shape transformation is observed (Figure S4). Images of EGE and cystone in the aggregation test are also provided (Figure S5, S6).

3.5. *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 (Supplementary Table 2). 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 1 and Figure S7 also denoted that the *E. glaucum* seed aqueous extract has anti-inflammatory and antioxidant activities. The *in vitro* anti-inflammatory effect of the EGE was through its ability to inhibit albumin denaturation and stabilize mice blood 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 positive control (Vitamin C).

Table 1 *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

4. DISCUSSION

This study has demonstrated that the *E. glaucum* seed extract has no evident acute oral toxicity even at a high dose (21.57 g/kg). So, 50–400 mg/kg dosage is safe for *in vivo* studies.

Acute renal colic is severe pain caused by stone migration in the urinary tract. Vietnamese ethnic groups believe that *E. glaucum* can improve renal colic. To investigate the analgesic properties of EGE, we performed screening on three basic mice models involving peripheral, inflammatory, and central nociceptive activity.

The abdominal writhing test is a very sensitive and well recommended model for screening the potency analgesic agents. Acetic acid is used as a chemical factor that stimulates the secretion of cyclooxygenase (COX) and lipoxygenase (LOX), which mediates the generation of endogenous nociceptive mediators like prostaglandin and histamine[25]. The abdominal writhing easily prevented by nonsteroidal anti-inflammatory and opioid drugs (although muscle relaxants and other non-analgesic drugs also work). The *E. glaucum* aqueous seed extract showed significant antinociceptive activity in this model even at a low dose (50 mg/kg).

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[26]. The results showed that *E. glaucum* aqueous seed extract at 100 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 in all groups in this study, the late phase, the extract groups at the dose of 400 mg/kg illustrated a decrease in the hind paw licking. 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 prolonged pain, classified as an inflammatory pain since it also generates COX and LOX, mediating the release of histamine, serotonin, prostaglandins, bradykinin with the activation of the dorsal horns of the spinal cord[27,28]. 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[29,30].

Furthermore, this study also provided preliminary scientific evidence about the *in vitro* and *in vivo* anti-urolithiatic activity of *E. glaucum* seeds. Calcium oxalate is the main component in kidney stone. Key events involving in the pathological biomineralization include crystal nucleation, growth, and aggregation[31]. Because nucleation is an important first step for the initiation of crystal formation, thus the inhibition of CaO_x nuclei will be very helpful in preventing crystal formation. Crystals bind to others throughout a 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 causes renal damage[32]. 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 that contribute to urologic stone formation. COM crystals are the most thermodynamically stable stones, and they have the greatest adsorptive capability, therefore it more difficult to excrete than COD crystals[5]. EGE also tends to reduce crystal area and inhibit crystal aggregation *in vitro* similar to cystone. However, EGE does not show a potential to convert from COM to COD.

In *in vivo*, intraperitoneal injection of glyoxylate was used to induce nephrotoxicity[33]. Urolithiatic mice got higher levels 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 the effectiveness of the extract in the management of kidney stone disease. About the urine, there are many inorganic and organic inhibitors of crystallization, and magnesium is one such well-known inhibitor[34]. A 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 treated with 400 mg/kg had higher amounts of magnesium in serum. Therefore, this dose has the potential to control kidney stone formation. Because magnesium is responsible for making complexes with oxalate and reducing the calcium oxalate supersaturation, this lowers the nucleation and growth rate of calcium oxalate crystals as a result[35]. 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. Besides, the effect of the extract on some biochemical parameters evaluated on day 7 tended to be better than on day 14 of the experiment. This may be due to some objective reasons such as the response of the experimental animals and the dosage of the pathological model agent, for example, the serum urea of the untreated pathological group on day 7 was about >60 mg/dL but on day 14 it decreased to about <50 mg/dL, leading to an unclear difference gap with the treated groups; the standard deviation of some parameters was still relatively high while the sample size was still relatively small, for example, the parameters of urinary calcium and magnesium. However, the results of microscopic examination of the mice kidneys showed that the kidneys of the untreated mouse group were damaged and the extract also had renoprotective effect against sodium glyoxylate-induced damages. This is an initial study of the effects of the *E. glaucum* seed extract on a sodium glyoxylate-induced urinary stone mouse model. In our experimental conditions, sodium glyoxylate can promote CaO_x stone formation but the formation may not be maximal, and 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[7,8]. 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[36]. 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[37]. 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[38]. Besides, terpenes and its main three subtype such as monoterpenes and sesquiterpenes, diterpenes, and triterpenes all possesses an potential anti-inflammatory analgesic activity[39]. Tannins had been proved to have a significant central acting and periphery analgesic effects[40]. Saponins were also studied for the inhibition of PGI and other proinflammatory interleukins[41]. Previous studies had proved the analgesic activity of several species in the Musaceae family, for example, *Musa balbisiana* peels[42], *Musa sapientum* leaves[43], peel and leaves of *Musa parasidiaca*[44,45]. 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[46]. Protective roles of polyphenol- and flavonoid-rich plant extracts as well as polyphenol and flavonoid compounds against urolithiasis were reported[46,47]. Saponin rich fraction from plants inhibited *in vitro* calcium oxalate crystal nucleation and aggregation, and against chemical-induced urolithiasis in rats[48]. Besides, some previous studies had also showed the anti-urolithiatic activity of several species in the Musaceae family, for instance, *M. balbisiana* fruits[49], *M. paradisiaca* pseudostems[50], *Ensete superbum* (Roxb.) Cheesman pseudostems[51].

In summary, this is the first study that verificatted the effects of the *E. glaucum* seed aqueous extract including analgesic, anti-inflammatory, anti-urolithiatic, antioxidant activities. The extract at 400 mg/kg may be considered as a potential dose for further studies.

5. CONCLUSION

Ensete glaucum (Roxb). Cheesman aqueous seed extract had the antinociceptive effect in the *in vivo* peripheral and central models. The extract had prophylactic effect against urolithiasis *in vivo* and *in vitro* by reducing the crystal area 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 at the potential dose of 400 mg/kg provided scientific support for the traditional use of *E. glaucum* seeds in the treatment of diseases. These results could appear promising for studying prevention and treatment of urinary stones of *E. glaucum* seeds.

Animal ethics 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.

Conflict of interest

The authors declare that they have no competing interests.

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