In vitro Antiurolithiatic Activity of Carabao Grass (*Paspalum conjugatum*) Extracts

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ABSTRACT

The mineralization of calcium oxalate deposits in the kidneys results from the crystallization of minerals in concentrated urine. Over time, these deposits form into kidney stones. Their formation is a symptom of deteriorating renal function and condition, for which we currently do not have a satisfactory treatment regimen. This study explored the ability of extracts of carabao grass (Paspalum conjugatum) to dissolve calcium oxalate crystals in vitro without significantly increasing the pH of the resulting solution, which researchers used as a marker for measuring toxicity. Calcium oxalate stones were produced from an aggregation assay using standard nucleation protocols, the concentration of which was determined using ultraviolet analysis spectrophotometry at 660nm. Researchers prepared carabao grass extracts by subjecting dried samples to Soxhlet extraction in ethanol. The solvent was removed using a rotary evaporator. The extract was divided into four concentrations (25%, 50%, 75%, and 100%). The calcium oxalate crystals were exposed to the extracts and the negative control. The resulting crystals were then weighed. The pH of the resulting solution was also measured. Results showed that a solution of the extract above 75% was able to dissolve calcium oxalate crystals at a significantly higher rate than concentrations of 50% and below. Results also showed that only the pure extract did not significantly alter the pH of the resulting solution above the normal range of human blood. Therefore, Carabao grass extracts exhibit in vitro antiurolithiatic activity. Researchers recommend expanding the study to cover the confirmatory testing of phytochemicals and cytotoxic studies.

Keywords

Paspalum conjugatum, calcium oxalate crystals, Carabao grass extracts, absorbance, pH, kidney, dissolution assay, antiurolithiatic activity

INTRODUCTION

The formation of kidney stones, also called nephrolithiasis, is a global health issue with enormous financial ramifications. Kidney stone formation is on the rise worldwide, and the affected population is getting younger, while recurrence rates remain high (Barnela et al., 2012). The epidemiology of kidney stones shows that episodal recurrence of renal stones occurs constantly and disproportionately, affecting older populations and both males and females equally (Johnson et al., 1979, as cited in Wyatt, 2020).

This complication can be traced to a variety of diseases related to type 2 diabetes, dyslipidemia, hypertension, and other lifestyle diseases. However, medical science has shown that genetics may also influence its formation. Although genetics play a different role in kidney stone formation over lifestyle choices, kidney stones are structurally the same regardless of formation influence; they are insoluble urinary salts shaped by con-crystalline proteins that accumulate in the kidneys (Aggarwal et al., 2017). Kidney stones are one of the most widely known urinary system infections. Current medical management of the kidneys is costly, often disproportionally affecting the poor. Prevention is preferable as it reduces the cost of medical management, morbidity, the risk from procedures, potential future complications, and recurring infection (Parks & Coe, 1996).

The human diet influences the development of kidney stones. It is closely associated with how much calcium a person eats (Holmes & Assimos, 2004). The most common stone formers, idiopathic stone formers, create calcium oxalate deposits in Randall's plaque in the kidneys (Evan et al., 2014). The formation of plaque in other areas of the body involves reactive oxygen species that induce oxidative stress, which results in the mineralization of extracellular matrices over subepithelial deposits on the surfaces of the renal papillae (Khan, 2012). Although this is one explanation of the pathogenesis of kidney stone formation, the current medical understanding of kidney stone formation suggests that it is too complex to fully understand.

Moreover, medical scientists agree that dietary intake plays a major role in developing and managing nephrolithiasis (Coe et al., 2010). *In vitro* formation of calcium oxalate crystals is affected by the density, particle size, and protein content of solutions that mimic the environments of urine, contributing to a better understanding of particle interaction. In an analysis, oxalate promotes and retains the structural integrity of stones formed *in vitro* and, therefore, should be the focus of interaction separation in the dissolution of these crystals (Christmas et al., 2002).

The evaluation of renal stone disease includes the determination of typical symptoms like "colicky flank pain" that radiates toward the lower abdomen and is often associated with nausea and vomiting. The reduction or total absence of urine flow and increased crystal formation may manifest if the stone enters the ureter. On the other hand, clinicians prefer to manage the disease when it is detected earlier to reduce pain. Comorbidities of stone formation are also investigated through an analysis of complete medical and thorough family history relevant to anatomic and symptomatic concerns (Miller & Lingeman, 2007). The diagnosis of kidney stones is not a full diagnosis of the person's kidney health; rather, it suggests a broad spectrum of underlying illnesses related to their formation (Barnela et al., 2012).

Because renal stone formation is often an indication of more complications in the kidneys, comorbidities in people exhibiting renal stone formation often result in the development of kidney failure, which is managed with hemodialysis or treated temporarily with kidney replacement. Donations of kidneys are scarce and often costly, so many people opt to turn to hemodialysis at the beginning of renal diseases (Icks et al., 2009). In addition to the negative physical effects of hemodialysis treatments, which often include skin discoloration and complications brought by the attachment of arteriovenous connections embedded on the forearms, the constant replacement of single-use equipment for every hemodialysis session produces up to eight kilograms of potentially biohazardous waste. In total, they produce a high cost of waste disposal, contributing to the already high cost of treatment (Piccoli et al., 2015).

Eighty-six percent of incident hemodialysis patients in the Philippines started on hemodialysis after a diagnosis of end-stage renal disease determined from an initial assessment of kidney stone formation (Cruz et al., 2011). The overall costs of hemodialysis, coupled with equipment purchases and the frequency of blood transfusions, make hemodialysis a near-last option for most Filipinos who may need to be treated. The Philippine Health Insurance Corporation (PHIC) only covers 45 to 90 sessions of hemodialysis in one year and only approves Php 2,500 of coverage of the nearly average Php 10,000 cost of sessions inclusive of professional fees and facility use, notwithstanding the multiple sessions of hemodialysis a patient needs each week for the 52 weeks of one year (Philippine Health Insurance Corporation, 2015). Therefore, physicians prefer to manage kidney stone formation before it develops into more serious complications in the kidneys that require hemodialysis treatments.

Preventative and manageable measures against kidney stone formation, particularly in individuals who have exhibited an increased risk for it, are centered on using aggregation assays employing in vitro use of calcium oxalate crystals, particularly calcium oxalate monohydrate. A variety of chemicals such as pyrophosphate, disodium dichloromethylene diphosphonate, citrate, and disodium ethane-1-hydroxy-1,1-diphosphonate have been shown to exhibit inhibitory activity against renal calculi formation in vitro (Felix et al., 1977). The researchers hoped to use the same aggregation assay to produce a similar effect using plant-based material to reduce the environmental effects of medical production.

The current management of renal stones mostly focuses on reducing stone size and eventual extraction by surgical methods. However, more recent developments in treatment have allowed doctors to change management pathways for patients. Globally, there is a preference for less invasive techniques, with endoscopic procedures performed regularly for patients suffering from the disease, and procedures involving stone deformation using shock wave lithotripsy have become routinely used (Lee & Bariol, 2011).

Research on medical alternatives has increasingly focused on plants with phytochemical content and established properties. Carabao grass, scientifically known as *Paspalum conjugatum*, is widely available in the subtropics and the tropics, including Pacific nations like the Philippines. Carabao grass has a high concentration of proteins, particularly from pollen with no recorded or known allergens (Castor et al., 2016), making it highly valuable and available as a potential source of alternative treatment.

In the Philippines, the entirety of carabao grass is used ethnomedically in parts of Mindanao (Rubio & Naïve, 2018). It has a history of being used in traditional medicine as an agent for wound healing. Its extracts have been shown to exhibit antimicrobial properties against *Staphylococcus aureus* due to the presence of sterols, flavonoids, tannins, triterpenes, saponins, and glycosides (Garduque et al., 2019). These contents contribute to other potential activity for extracts of carabao grass, such as the control of lipid metabolism (Valitova et al., 2016), immune system support (Group, 2016), and a variety of properties such as antiviral, antifungal, anticancer, and antitumor (Xiao et al., 2015).

The most compelling factor in the pursuit of this study is the detection of flavonoids in carabao grass. Flavonoids have been found to have the ability to prevent the activity of some proteins from causing aggregation, particularly that of prostacyclin, to allow platelets to form a plug in primary hemostasis by inhibiting superoxide anions like oxalate that facilitate aggregation (Robak & Gryglewski, 1996). Having found no literature to support the exploration of the dissolution or prevention of kidney stone formation, the researchers sought to determine if carabao grass extracts could have antiurolithiatic activity *in vitro* using a calcium oxalate dissolution assay and pH testing.

Specifically, the researchers sought to determine if carabao grass extracts formulated to different concentrations in aqueous solutions (25%, 50%, and 75% volume-by-volume) or its pure form could reduce the mass of solid calcium oxalate crystals in a dissolution assay after treatments are applied to masses measured indirectly using spectroscopy. As an evaluation of the safety of carabao grass as a potential treatment for kidney stones, the researchers also measured the pH levels of solutions after treatments were applied before replicating each of them.

This study explores the potential activity of carabao grass to dissolve calcium oxalate crystals produced *in vitro* as a representative of kidney stones, referred to medically as renal calculi, and as a common human urolith. However, it does not look into the complete chemical process of dissolution or test the prevention of aggregation *in vivo*, as suggested by other studies. It also does not directly measure the mass of calcium oxalate crystals dissolved in the solutions after treatment but, instead, uses absorbance data from ultraviolet analysis spectrophotometry as an indirect indicator of antiurolithiatic activity. The researchers also did not presume pH to be a total measure of cytotoxicity. They used pH values to measure safety on the presumption of potential cytotoxicity from lower pH or stronger acidity. Furthermore, the researchers considered the suitability for human use in pursuit of this study.

METHODOLOGY

The researchers used a calcium oxalate aggregation and dissolution assay to demonstrate the ability of carabao grass extracts to dissolve calcium oxalate crystals. Calcium oxalate was used to represent kidney stones because of their identical chemical makeup with the assay's ability to demonstrate some degree of toxicity measurement by the use of solution pH (Christmas et al., 2002). Calcium oxalate is a highly insoluble salt of calcium and oxalic acid, or oxalate, that can be allowed to crystalize. The impact of calcium oxalate manifests as a deposition in the kidneys that leads to the formation of kidney stones and its primary component (Franceschi & Nakata, 2005). In place of actual kidney stones, which are available for purchase from sources not readily available or affordable to the researchers, calcium oxalate crystals were aggregated from standard protocols established by Atmani and Khan (2000).

Initially, 0.555 g of calcium chloride was dissolved in one liter of distilled water. The same amount of distilled water was used to dissolve 0.670 g of sodium chlorate. Three preparations for each solution were prepared and calibrated to 60° C in a water bath for one hour before being cooled to 37° C overnight. After which, 500 mL of each solution was combined, allowed to react for 60 minutes, and centrifuged at 1,500 rpm for five minutes. Calcium oxalate aggregates were collected by evaporating the solutions in a drying oven set to 80° C for two hours.

Preparations of the carabao grass before extraction were based on the procedures described by Ekwenye and Njoku (2006). The researchers gathered carabao grass samples from the grounds of the University of Negros Occidental – Recoletos, which were washed and kept in distilled water to prevent contamination and then air-dried overnight. The next day, the air-dried carabao grass was oven-dried at 40° C for 60 minutes to complete moisture removal. The grass was cut into coarse particles before being ground using an electric blender. Samples were kept in clean beakers covered with paraffin film and stored at 4° C until ready for use.

The researchers utilized Soxhlet extraction procedures from the procedures described by Markom et al. (2000). Researchers immersed 80 g of coarsely ground carabao grass in 150 mL of 99% ethanol for 24 hours. The resulting solution was filtered, and the immersed carabao grass filled the thimble of a Soxhlet extraction setup along with dried samples to fill the thimble to maximum capacity. Ethanol was placed inside the flask, which completed the setup. The solvent was heated to reflux, completing the Soxhlet cycle several times over 3 hours until the leaves in the thimble discolored. The process was repeated until all of the leaves were consumed.

The crude extract was placed into a rotary evaporator after cooling and run until all ethanol was removed from the solution. The removal of ethanol was confirmed using a flame test on the remaining extract. Aqueous solutions were prepared aside from the pure extract, preparing volume-by-volume concentrations of 25%, 50%, and 75% for the treatment. The pure extract was labeled 100%. The absorbance of each solution was measured using ultraviolet analysis spectroscopy at 660 nm, the wavelength for the measurement of the calcium oxalate in the solution.

For the dissolution assay, two grams of calcium oxalate crystals were exposed to each solution in three trials for 60 minutes without agitation. Researchers then separated the liquid into aliquots, the absorbance of which was also measured by a spectrophotometer at the same wavelength in the pre-test measurement.

The pH was chosen as the indicator for toxicity in this study due to its ability to measure potential corrosiveness and, therefore, its resultant safety for consumption. The determination of weak and strong acidity allowed researchers to determine that safety. The assumption of carabao grass extract as an organic chemical classifies it as a weak acid with a pH range of near 5 to just below 7 (Johnson, 2018), enabling the buffer system of the blood to adapt. Within these ranges, weak acids can be classified industrially as food acids that do not completely ionize in solution and will not produce irritation. It will also be classified as non-toxic (Virox, 2015).

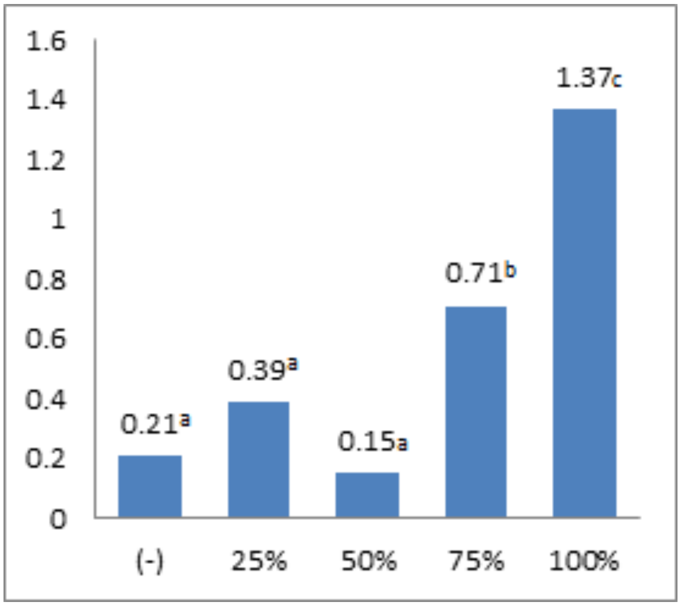
Data collected for the concentration of calcium oxalate pre-treatment was measured indirectly as absorbance was subtracted from the same measure post-treatment. The difference in these values was used as the indicator for dissolution activity. The measurement of pH was also recorded. Both pH and absorbance difference values were subjected to a One-Way Analysis of Variance (ANOVA) to determine if a significant difference existed between values. Duncan's Multiple Range Test was performed to differentiate those differences between variables in the study.

RESULTS, DISCUSSION, AND IMPLICATIONS

The dissolution assay revealed that the solutions of carabao grass at 25% and 75%, as well as the pure extract, were able to dissolve more particles of calcium oxalate from the aggregates into the solution. Available data also showed that, statistically, the pure extract outperformed all other treatments in the dissolution assay. The difference in absorbance between the pre-treatment and the post-treatment values revealed that the extracts, at the right concentrations, can dissolve calcium oxalate crystals, which were originally highly insoluble in water and should have been insoluble in aqueous solutions.

The deposition of calcium oxalate crystals in the kidneys may be attributed to diets rich in calcium and a genetic predisposition for such deposits in the kidneys, as well as the ionic activities of calcium and oxalic acid in the human body. The presence of flavonoids has already been proven to inhibit the deposition of urinary calcium oxalate crystals in animal models by reducing the production of enzymes that synthesize oxalic acid as a marker of crystal deposition in the kidneys (Soundararajan et al., 2006). The mechanism for preventative

Figure 1
Difference in Absorbance of Solutions after Dissolution Assay



activity may play a role in dissolution activity post-aggregation due to the possible inhibition of oxalate production, calcium activity, or the nature of chemical equilibrium in aqueous solutions of solid solutes.

Researchers can safely eliminate aggregate density (Sharma et al., 2019) and solubility action as a reason for the difference in results. As mentioned, a standard mass of the calcium oxalate aggregate was exposed to standard volumes of each treatment concentration at standardized times of exposure. It can be presumed that the amount of calcium oxalate dissolved into the treatments was a direct result of the properties of the contents of the carabao grass extract.

Flavonoids, which are present in carabao grass according to literature, include flavones, flavonol, and glycosides in plants. Of the flavonoids present in plants, kaempferol and luteolin have the greatest inhibitory activity against the deposition of calcium oxalate crystals that could lead to kidney stone formation (Sahabudin et al., 1999). More recent studies also showed the presence of quercetin, a flavonoid that is as ubiquitous as kaempferol in grasses and carabao grass, and that its concentration in carabao grass produces antioxidant activity (Garcia, 2018). Quercetin is proven to prevent the formation of calcium oxalate stones in animal models alongside the use of hyperoside (Zhu et al., 2014). It has also been proven that luteolin, which is also present in carabao grass (Da Silva Pinto et al., 2015), prevents the aggregation of calcium oxalate and is beneficial for patients who have recurrent stone formation (Orhan et al., 2015). Literature showing the presence of such phytochemicals in carabao grass supports the possible role that flavonoids have in the dissolution of calcium oxalate crystal aggregates and may help kidney stone patients remove stones non-invasively.

The pH of any solution can be determined by the minute changes in the concentration of hydrogen ions in the solution. Because of the logarithmic function of pH as a measurement, higher pH means lower hydrogen ion concentration. One of the central determinants of blood pH is the presence of strong electrolytes in the plasma, of which calcium is ionized (Kellum, 2000). The dissolution of calcium oxalate is not simply a removal of the aggregation factors that lead to crystallization because calcium oxalate itself is a crystal. Therefore, the dissolution of calcium oxalate results from the break of the bonds between calcium and oxalic acid, the latter reforming as hydrogen ions are reintegrated back into its structure. The increase in calcium ions in solution, but the subsequent decrease in hydrogen ion concentration, leads to an increase in the pH of the resulting solution.

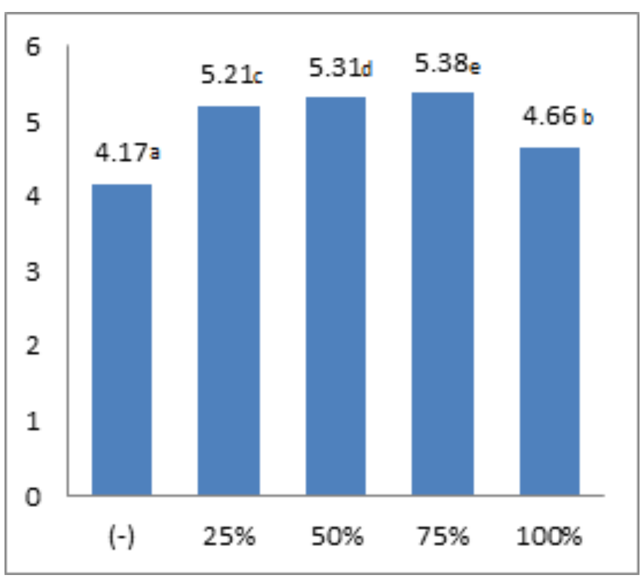
As seen in Figure 2, the pH measurement results showed that because extracts in concentrations were present in an aqueous solution, the change in pH was great despite relatively lower activity in absorbance. All of the pH values were within the active range of the pH buffer system of blood, which maintains blood pH between 7.35 and 7.45 (Nattie, 2013), given the sheer volume of blood in the body compared to the milliliters of potential treatment.

Results also showed that acidic development in the three diluted solutions of carabao grass extract was well within the pH of 5 to 7, which classifies it as a weak acid. The application of pure extract, although producing an acid with a pH of 4.66, shows that, even in pure form, the acidity is weak. Acids are produced from aqueous solutions, and any potential application of the extract may need aqueous delivery to be manageable in the human body.

Statistical analysis of pH level shows that the 75% extract, earlier only second to the pure extract in terms of its ability to dissolve calcium oxalate crystals, had the highest pH, significantly higher than the next concentration at 50%. It showed that the concentration may prove to be the ideal concentration for dissolving calcium oxalate crystals in aqueous solutions, of which urine is also one.

The potential for the treatment of kidney stones using carabao grass extract could prove useful in the medical field, given more *in vitro* analysis of the activities of phytochemical components of carabao grass and its use as a partner in the therapeutic management of kidney stones. The preferred management of renal calculi, currently shock wave

Figure 2
Post-test pH Levels of Solutions



lithotripsy monotherapy, is currently costly, with research showing that it is more effective when used in conjunction with other therapies that can reduce calculi size for better extraction (Chandhoke, 1996).

On the other hand, the limitations of this study, which include not addressing which phytochemicals are present in the carabao grass extract produced, do not give a clear understanding of how the dissolution of the calcium oxalate aggregates was possible *in vitro*. Literature shows the presence of flavonoids and other phytochemicals in carabao grass extracts (Esparagoza & Gallego, 2020). However, this study does not explore the direct activity of flavonoids on dissolution and pH levels. It is entirely possible that carabao grass collected at the University could contain highly charged ions that inhibit or compete with calcium and perform its disbanding from oxalic acid just as other highly charged anions inhibit calcium oxalate aggregation *in vitro* (Robertson et al., 1973). Due to this limitation, the researchers cannot determine with certainty the reason for the similar pH activity of the 50% extract concentration with other concentrations. However, its significantly lower dissolution activity was only statistically similar to the negative control, which was distilled water.

The researchers were also careful not to equate pH results with true cytotoxicity. Cytotoxic activity refers to the potential toxicity of a substance against cells, notably through deleterious effects that disrupt normal cell function and lead to cell death. Such activities may be preventable through cellular action (McGaw et al., 2014). The researchers understood that the methodology presented in the study limited the expression of toxicity only to acute toxicity, which was the ability of the carabao grass extract to be corrosive due to its inherent acidity. Therefore, researchers used pH only as a marker for cytotoxicity and as a screen for potential cytotoxicity, as acutely toxic substances are likely to be cytotoxic.

CONCLUSION AND RECOMMENDATIONS

This study showed that carabao grass (*Paspalum conjugatum*) extract exhibits antiurolithiatic activity *in vitro*, as supported by literature on its phytochemical contents and significant activity phytochemicals demonstrated in an experimental setup by dissolution assay and pH measurement. The researchers concluded that there was a significant difference between the differences of absorbance between different concentrations of the carabao grass extract and that the pure extract exhibited the highest activity based on the differences in absorbance. However, 25% and 75% aqueous extracts of carabao grass also exhibited significantly higher dissolution activity than the negative control and the 50% extract concentration, making them suitable for dissolution.

Researchers also conclude that the pH values of the aqueous solutions of carabao grass extract after the dissolution assay remained at levels that classify it as a weak acid, therefore also signifying its safety for consumption and its inability to cause a major disruption to the buffer system of human blood, minimizing damage and toxicity. The researchers also concluded that there existed a significant difference between the pH levels of resulting aqueous solutions after the dissolution assay, with the 75% carabao grass extract having the highest pH and, therefore, being the least acidic. However, the pure extract and the aqueous solutions of 25% and 50% carabao extract had pH levels that were within the range that can be accommodated by the buffer system of human blood and were, therefore, acceptable alongside the 75% extract.

The researchers recommend that patients and community members should explore alternative medicine as a legitimate reference for treatment, provided that competent professionals must issue it. Although the use of carabao grass extract as a treatment showed potential, this study only serves as an initial stage to launch a larger study that can verify its effects in vivo. This study showed that the use of such plants in traditional practice has scientific backing, and patients may find alternatives backed by reliable sources and professionals that could ease treatment with lesser costs and drawbacks.

The researchers recommend the cultivation of carabao grass for pharmaceutical purposes. Literature proves that carabao grass contains phytochemicals that can be used in a variety of activities, and this study showed that carabao grass has the potential to treat a very common ailment. It supports the cultural use of carabao grass as a treatment for common illnesses. Instead of being treated as a common weed, researchers recommend that carabao grass should be treated as a potential source of phytochemicals for medical use.

The researchers recommend that doctors and other health professionals should explore treatments for patients using alternative medicine, particularly through the use of plants in ubiquity, like carabao grass. This study showed that the potential for exploration is not limited to manufactured, synthetic treatment and that preliminary investigation should be expanded further by professionals in the field.

In terms of this study, it is recommended to explore the areas of this study that were not tackled due to the limitations previously expressed. Phytochemical analysis should be done to confirm whether the extraction method described in this study was able to extract the phytochemicals of interest- flavonoids. It is also recommended to experiment on the isolation of those flavonoids from carabao grass and demonstrate its direct ability to dissolve or prevent the formation of kidney stones *in vitro* and *in vivo* using animal models. It is also recommended that the study be expanded to include other activities that may contribute to the potential use of the extract as a treatment, such as cytotoxic studies and antioxidant activity.

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