ASSESSING THE INHIBITORY PROPERTY OF *Ruellia tuberosa* L. EXTRACT ON CALCIUM OXALATE CRYSTALLIZATION IN VITRO

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ABSTRACT

This study evaluated the inhibition and dissolution of calcium oxalate crystals that cause kidney stones using extracts from *Ruellia tuberosa* L. Leaf, stem and root parts were extracted by 70% ethanol maceration. The root extract gave the highest yield of 30.58% and contained flavonoid, terpenoid and tannin compounds. The ability to inhibit nucleus formation was assessed by spectrophotometric method at 620 nm to determine the absorbance of the crystal nucleus. The leaf extract showed the strongest activity with IC50 of 5.13 mg/mL. The inhibition of crystal growth was monitored by spectrophotometry at 214 nm for 10 minutes. The leaf extract also gave the best results with IC50 of 7.15 mg/mL. The inhibition of crystal aggregation was measured at 620 nm for 6 hours, the stem extract showed the highest activity with IC50 of 7.1 mg/mL. The ability to dissolve crystals was evaluated by titration method, the stem extract achieved 51.48% dissolution at 20 mg/mL. The results showed that *R. tuberosa* extracts can inhibit kidney stone formation, with the leaf extract effectively inhibiting the early stages and the stem extract inhibiting the later stages and dissolving crystals. This could be a natural therapy for kidney stones. Further research should isolate the bioactive compounds responsible for this activity.

ĐÁNH GIÁ KHẢ NĂNG ỨC CHẾ SỰ HÌNH THÀNH TINH THỂ CALCIUM OXALATE TRONG ĐIỀU KIỆN INVITRO CỦA CAO CHIẾT CÂY TRÁI NỔ *Ruellia tuberosa* L.

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TỪ KHÓA

Calcium oxalate
Ức chế sỏi
Tan sỏi
Sỏi thận
Ruellia tuberosa

TÓM TẮT

Nghiên cứu này đánh giá khả năng ức chế và hòa tan tinh thể calcium oxalate gây sỏi thận của các chiết xuất từ cây *Ruellia tuberosa L.* Các bộ phận của cây (lá, thân và rễ) được chiết xuất bằng phương pháp ngâm trong ethanol 70%. Cao chiết từ rễ cho hiệu suất cao nhất 30,58% và có chứa các hợp chất flavonoid, terpenoid, tanin. Khả năng ức chế sự hình thành mầm tinh thể được đánh giá bằng phương pháp đo độ hấp thụ ở 620 nm. Cao chiết lá cho hoạt tính ức chế mạnh nhất ở giai đoạn hình thành mầm tinh thể với IC50 5,13 mg/mL. Đánh giá khả năng ức chế sự phát triển tinh thể được thực hiện và theo dõi bằng phương pháp đo độ hấp thụ ở bước sóng 214 nm trong 10 phút. Chiết xuất lá cũng cho kết quả ức chế phát triển tốt nhất với IC50 7,15 mg/mL. Khả năng ức chế sự kết tinh của tinh thể được thực hiện bằng phương pháp đo độ hấp thụ ở 620 nm trong 6 giờ. Cao chiết thân cho hoạt tính ức chế cao nhất với IC50 7,1 mg/mL. Khả năng hòa tan tinh thể được đánh giá bằng phương pháp chuẩn độ, cao chiết thân cho hiệu quả hòa tan cao 51,48% với hàm lượng 20 mg/mL. Kết quả cho thấy các chiết xuất *R. tuberosa* có khả năng ức chế quá trình hình thành sỏi thận, với chiết xuất lá ức chế hiệu quả giai đoạn đầu, chiết xuất thân ức chế giai đoạn sau và hòa tan tinh thể. Đây có thể là liệu pháp tự nhiên cho bệnh sỏi thận.

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1. Introduction

Kidney stones, also known as urolithiasis, are hard masses formed from crystals in the kidneys [1]. There are four main types of kidney stones: calcium oxalate (CaOx), uric acid, struvite, and cystine [2]. CaOx stones are the most common, accounting for over 60-80% of kidney stones [2], [3]. These stones exist in two main forms: calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD). COM stones are more common clinically and have a greater affinity for renal tubular cells, leading to stone formation [4].

Kidney stones form when urine becomes saturated with stone-forming salts like calcium oxalate [5]. This leads to nucleation, where crystals coalesce into clusters. The clusters grow and aggregate into larger particles that can get retained in the kidney [6].

Current clinical treatments like lithotripsy, percutaneous nephrolithotomy, and endoscopic lithotripsy have limits like tissue damage, residual fragments, infections, and renal dysfunction [7]. Therefore, there is interest in using bioactive compounds from natural sources as alternative treatments since they can inhibit and dissolve stones with fewer side effects.

Ruellia tuberosa L. belongs to the Acanthaceae family in the order Lamiales. R. tuberosa is categorized in the Magnoliophyta division, Magnoliopsida class, and Ruellia genus [8]. Two synonym names for this species are Ruellia picta and Ruellia clandestina. This perennial herbaceous plant is native to tropical and subtropical regions of several continents, including Asia and the Americas. *R. tuberosa* has long been used in traditional medicine systems such as Ayurveda due to properties like antipyretic, anti-inflammatory, and analgesic effects [8]. Further pharmacological research is warranted on this species to investigate other potential therapeutic applications. *Ruellia tuberosa* L. (Acanthaceae) is a traditional Asian folk medicine used for pain relief, antimicrobial, and antioxidant effects [9], [10]. It contains flavonoids and terpenoids that can effectively inhibit CaOx crystallization [6].

This study aims to assess the inhibitory properties of *R. tuberosa* extracts on CaOx crystallization *in vitro*, with the goal of developing natural medicinal products for preventing and treating kidney stones.

2. Materials and methods

2.1. Plant Material Collection and Extraction

Fresh leaves, stems and roots of *Ruellia tuberosa* L. were collected from Can Tho city, Vietnam. The plant materials were cleaned, shade dried, and damaged parts were removed. The dried materials were extracted by maceration in 70% ethanol [11], [12].

Moisture content was determined by drying 1 g fresh samples at 105°C until constant weight, then calculating moisture percentage using the formula:

$$\text{Moisture}(\%) = \left(1 - \frac{\text{M2}}{\text{M1}}\right) \times 100 \quad (1)$$

Where M1 is the weight of the fresh sample before drying (g) and M2 is the final constant weight of the dried sample (g).

2.2. Phytochemical Screening

The screening of bioactive compounds in the extracts was conducted to determine their potential for inhibiting kidney stone crystallization. Preliminary phytochemical screening for plants under study was carried out. It was a qualitative analytical study that aimed to find phytoconstituents present in the three plants. Extracts were prepared according to the respective test methods. The extracts were subjected to a qualitative analysis to detect major classes of alkaloids, tannins, terpenoids, flavonoids and saponins, as described by Jha, et al. [13] and Rajanna, et al. [14].

2.3. Nucleation Assay for Calcium Oxalate Crystals

A spectrophotometric assay was performed to determine the appropriate extract concentration for inhibiting calcium oxalate crystal nucleation [15], [16]. The extracts and sodium citrate (positive control) were dissolved in water at 0.5-10 mg/mL. Crystallization was initiated by adding 3 mM $CaCl_2$ and 5 mM $Na_2C_2O_4$ to artificial urine containing 0.05 M TRIS and 0.15 M NaCl buffer (pH 6.5), with or without inhibitors.

For the assay, 950 µL $CaCl_2$ was mixed with 100 µL inhibitors or blank, then 950 µL $Na_2C_2O_4$ was added and shaken for 3 minutes. Inhibition was measured at 620 nm. The percentage inhibition was calculated using:

$$\% \text{ Inhibition } = \left(1 - \frac{S}{C}\right) \times 100 \quad (2)$$

Where C is the absorbance of the sample without inhibitors and S is the absorbance of the sample with inhibitors.

2.4. Growth Assay for Calcium Oxalate Stone Formation

The growth assay followed the method of Chaudhary, et al. [17] to determine the appropriate extract concentration for inhibiting calcium oxalate crystal growth. The extracts and sodium citrate were dissolved in water at 1.25-10 mg/mL. The reaction began by combining 1 mL of 4 mM $CaCl_2$ and 1 mL of 4 mM $Na_2C_2O_4$ into a 1.5 mL buffer solution (containing 90 mM NaCl, 10 mM TRIS, pH 7.2). Following this, 30 µL of a 1.5 mg/mL slurry of calcium oxalate monohydrate (COM) crystals in 50 mM sodium acetate (pH 5.7) was introduced into the mixture.

Oxalate consumption was monitored by measuring the decrease in absorbance at 214 nm for 10 minutes, with and without inhibitors. Readings were taken every minute.

The relative inhibitory activity was calculated as:

$$\% \text{ Relative inhibition } = \left(1 - \frac{S}{C}\right) \times 100 \quad (3)$$

Where C is the slope of the trendline without inhibitors and S is the slope of the trendline with inhibitors.

2.5. Aggregation Assay for Inhibiting Calcium Oxalate Crystal Aggregation

The aggregation assay followed Saha and Verma's method to evaluate the appropriate extract concentration for inhibiting calcium oxalate crystal aggregation [6]. The extracts and sodium citrate were prepared at 1.25-10 mg/mL in water. CaOx crystals were prepared by mixing 50 mM $CaCl_2$ and 50 mM $Na_2C_2O_4$, equilibrating at 60°C for 1 hour, then cooling overnight at 37°C. The crystals were harvested by centrifuging at 10,000 rpm and evaporating at 37°C.

COM crystals were resuspended at 1 mg/mL in 0.05 M TRIS and 0.15 M NaCl buffer (pH 6.5). The experiments were conducted at a temperature of 37°C, comparing samples treated with inhibitors against those without inhibitors.

Absorbance was measured at 620 nm at 30-360 minute intervals. Inhibition was calculated using:

$$\% \text{ Inhibition } = \left(1 - \frac{Si}{Sc}\right) \times 100 \quad (4)$$

Where Si is the slope of the trendline with inhibitors and Sc is the slope of the trendline without inhibitors.

2.6. Evaluation of Calcium Oxalate Crystal Dissolution via Titration

CaOx crystals were prepared by reacting equal volumes of 0.1 M $CaCl_2$ and 0.1 M $Na_2C_2O_4$ for 30 minutes at room temperature [18]. The crystals were harvested by centrifugation at 10,000 rpm, washing, and drying at 70°C for 3 hours before weighing.

Semi-permeable membranes were prepared by removing the calcium shell from the membrane-shell interface using overnight soaking in 2M HCl [16]. After washing, the inner content was removed and the membranes stored refrigerated in ammonia solution at pH 7-7.4.

For crystal dissolution analysis [19], 2 mg $Ca_2C_2O_4$ and inhibitor were packed into the semi-permeable membranes. These were suspended in 100 mL 0.1 M TRIS buffer at 37°C for 7-8 hours. Afterwards, the contents were titrated with 0.01 N $KMnO_4$ in 2N H_2SO_4 until a light pink endpoint [20]. The calcium content was calculated based on 1 mL 0.01 N $KMnO_4$ equivalent to 0.2 mg calcium [20].

2.7. Statistical Analysis

Microsoft Excel 2019 and R software were used for data collection and analysis. Data were analyzed using analysis of variance (ANOVA) with significance level of 5%.

3. Result and Discussion

3.1. Optimization of Extraction Process and Yield Analysis

The extraction procedure involved the use of 70% ethanol as the solvent, a critical choice explained in the methods section. This selection was crucial due to the potential risks associated with using water alone, which could introduce unwanted contaminants such as organic acids, glucose, and proteins, negatively affecting the bioactive components. Conversely, the use of 96% ethanol as the sole solvent was found to reduce the extraction yield. As a result, 70% ethanol emerged as the preferred option, offering a high extraction yield while effectively eliminating bacterial contaminants, as corroborated by prior research [21].

The initial fresh weights of the plant parts were as follows: 793 g for leaves, 1,100 g for stems, and 1,930 g for roots. After the removal of moisture through the drying process, the dry weights were determined to be 153.6 g for leaves, 304.3 g for stems, and 356.3 g for roots. The moisture content, representing the percentage of water within the plant parts, was measured at 81.65% in leaves, 75.35% in stems, and 73.38% in roots. The extracted compound weights were ascertained as follows: 13.11 g per 100 g of dried leaves, 28.04 g per 200 g of dried stems, and 61.17 g per 200 g of dried roots.

In summary, the extraction efficiency, serving as a metric for assessing the extraction process's effectiveness relative to the initial material, yielded efficiency values of 13.11% for leaves, 14.02% for stems, and notably higher efficiency at 30.58% for roots.

3.2. Bioactive Compound Presence in Plant Extracts

Table 1 displays the presence of various bioactive compounds in extracts from different parts of *R. tuberosa*, including leaves, stems, and roots. Alkaloids were absent in extracts from all plant parts tested. In contrast, tannins, terpenoids, and flavonoids were present in leaf, stem, and root extracts, suggesting these compounds are prevalent throughout the plant. Saponins showed a differential distribution, being present in leaf and root extracts but absent from stem extracts.

Table 1. *Result of the bioactive compound presence in the Extract*

Bioactive compounds	Results		
	Leaf extract	Stem extract	Root extract
Alkaloids	-	-	-
Tanins	+	+	+
Terpenoids	+	+	+
Flavonoids	+	+	+
Saponins	+	-	-

Note: "+" : Positive result; "-" : Negative results

In summary, tannins, terpenoids, and flavonoids appear widespread in *R. tuberosa*, while saponins exhibit a more localized distribution, being found in leaves and roots but not stems.

These findings align with prior research investigating hydroethanolic extracts of this species, which corroborated the existence of tannins, terpenoids, and flavonoids [22]. The variation in saponin content highlights the complexity of bioactive compound localization within the plant. The widespread presence of tannins, terpenoids, and flavonoids may have implications for further research into the medicinal properties and applications of *R. tuberosa*.

3.3. Calcium Oxalate Nucleation Inhibition

Figure 1 shows the concentration-dependent inhibition of calcium oxalate nucleation by sodium citrate, leaf extract, stem extract, and root extract. The leaf extract exhibited the highest inhibition, reaching 99.2% at 10 mg/mL. This potent inhibition indicates that it can effectively prevent calcium oxalate crystallization. The stem extract also showed increased inhibition with higher concentrations, plateauing at 51.2% inhibition at 10 mg/mL, though declining slightly at 8 mg/mL. The root extract demonstrated the lowest inhibition, peaking at 24.3% at 10 mg/mL. The one-way ANOVA test indicates a statistically significant difference ($p < 0.05$).

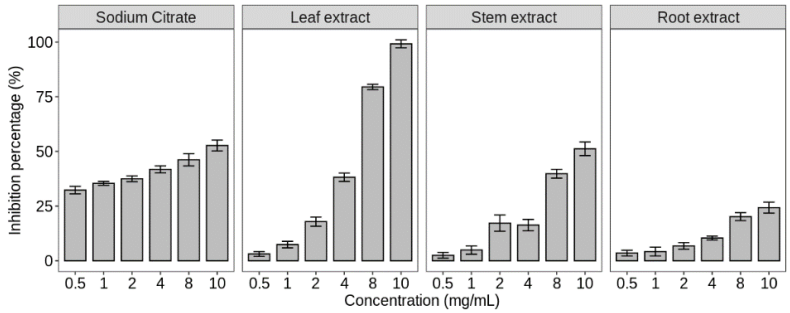

Figure 1. *Results of calcium oxlate nucleation inhibition*

Sodium citrate displayed increasing inhibition up to 52.7% at 10 mg/mL, likely due to citrate binding calcium and enhancing urine macromolecule inhibition [23], [24]. The leaf extract's lower IC50 of 5.13 mg/mL compared to sodium citrate's 9.18 mg/mL may be due to direct interactions of bioactives with calcium ions [24].

In summary, the leaf extract showed the highest inhibition, outperforming sodium citrate. The stem extract also showed promise, while the root extract was less effective. These results suggest that *R. tuberosa* leaf extract has potential for preventing calcium oxalate crystallization and kidney stones.

3.4. Calcium Oxalate Growth Inhibition

Figure 2 shows the concentration-dependent inhibition of calcium oxalate crystal growth by sodium citrate, leaf extract, stem extract, and root extract. Sodium citrate demonstrated the highest inhibition, reaching 86.89% at 10 mg/mL, indicating it strongly prevents crystal growth. The leaf extract also exhibited significant inhibition, peaking at 66.46% at 10 mg/mL, demonstrating its potency for inhibiting growth. The stem extract showed increased inhibition up to 49.34% at 10 mg/mL. The root extract exhibited the least inhibition, reaching a maximum of 30.31% at 10 mg/mL concentration. The one-way ANOVA test indicates a statistically significant difference ($p < 0.05$).

The leaf extract's IC50 value of 7.15 mg/mL, in contrast to sodium citrate's 3.47 mg/mL, indicates that sodium citrate potentially inhibits growth by binding to calcium ions [24]. The extract might function by forming a coating on crystal surfaces and interacting with calcium or oxalate ions [25]. Enhanced inhibition observed at 10 mg/mL in all samples could stem from saponins and flavonoids converting COM to COD, potentially facilitating more straightforward passage through renal tubules [26], [27].

In summary, sodium citrate and leaf extract strongly inhibited calcium oxalate crystal growth, with sodium citrate being most effective. The stem extract also showed good inhibition, while the

root extract was weaker. These results indicate *R. tuberosa* leaf extract could help prevent kidney stones by inhibiting crystal growth.

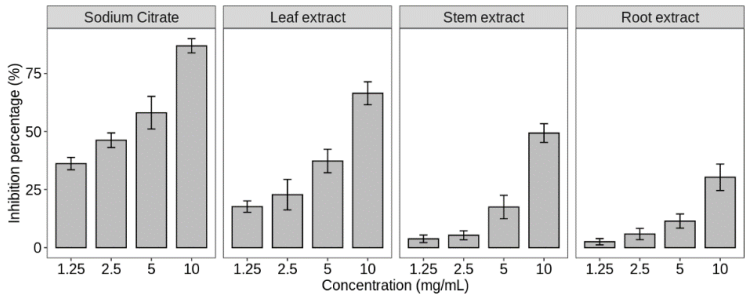

Figure 2. *Results of calcium oxlate growth inhibition.*

3.5. Calcium Oxalate Aggregation Inhibition

Figure 3 shows the concentration-dependent inhibition of calcium oxalate crystal aggregation by sodium citrate, leaf extract, stem extract, and root extract. The leaf extract also exhibited increased inhibition up to 53.54% at 10 mg/mL, suggesting it can inhibit aggregation. The stem extract showed maximum inhibition of 66.9% at 10 mg/mL. The root extract demonstrated the lowest inhibition overall, peaking at 42.57% at 10 mg/mL. Sodium citrate displayed the highest inhibition, reaching 96.21% at 10 mg/mL, indicating it strongly prevents aggregation. The one-way ANOVA test indicates a statistically significant difference ($p < 0.05$).

The greater IC50 value observed for the stem extract, at 7.1 mg/mL in contrast to sodium citrate's 5.04 mg/mL, implies that sodium citrate disrupts aggregation by binding to calcium ions [24]. As aggregation is a crucial step impeding crystal enlargement [3], [4], impeding this process may more effectively hinder kidney stone formation using sodium citrate than with *R. tuberosa* extract.

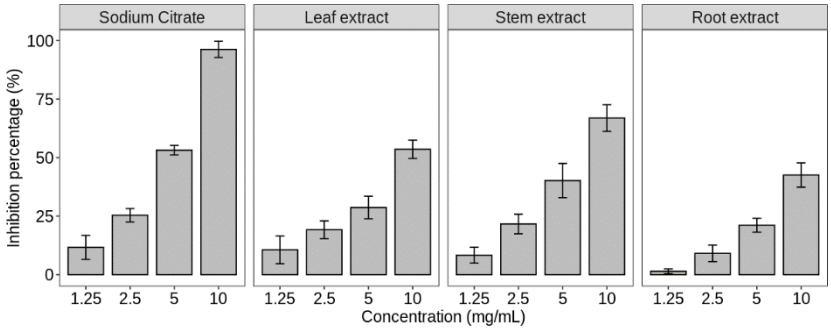

Figure 3. *Results of calcium oxlate aggregation inhibition*

In summary, sodium citrate showed the highest aggregation inhibition. The stem extract also displayed strong inhibition while the root extract was weakest. These results suggest the extracts may reduce kidney stones by inhibiting calcium oxalate aggregation.

3.6. Calcium Oxalate Crystal Dissolution

Figure 4 shows the concentration-dependent dissolution of CaOx crystals by sodium citrate, leaf extract, stem extract, and root extract. The leaf extract exhibited lower but consistent increases in dissolution, reaching 28.67% at 20 mg/mL, suggesting some dissolution capacity. The stem extract showed the highest dissolution, maximizing at 51.48% at 20 mg/mL, demonstrating its strong crystal dissolution ability. The root extract achieved 39.36% dissolution at 20 mg/mL, but was less effective than the stem extract. Sodium citrate displayed increasing dissolution up to 40.49% at 20 mg/mL, indicating it can effectively dissolve crystals. The one-way ANOVA test indicates a statistically significant difference ($p < 0.05$).

The stem and sodium citrate were most effective for crystal dissolution, with the stem extract having the highest percentage. The root extract was moderately effective, while the leaf extract had the lowest capacity. The extracts' dissolution abilities may be attributed to terpenoids and flavonoids [19], [28]. The weight method was preferred for its accuracy, simplicity and lower cost versus titration [18]. These results indicate *R. tuberosa* stem extract could help eliminate kidney stones through its crystal dissolution ability.

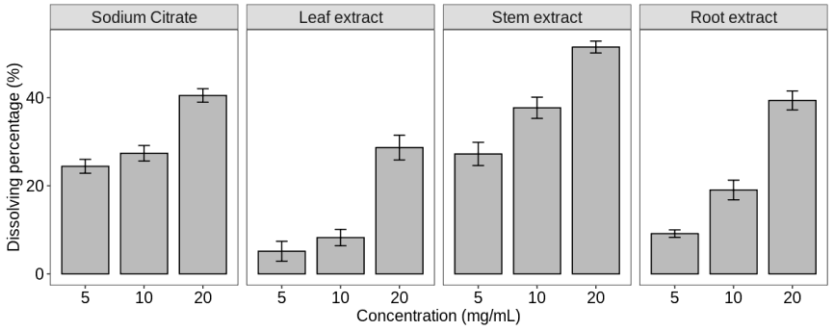

Figure 4. *Calcium oxalate crystals dissolving percentage by titration method.*

4. Conclusion

The study explored the application of *Ruellia tuberosa* L. extract in dissolving and inhibiting the formation of CaOx crystals, spanning the nucleation, growth, and aggregation phases. Extracts from the leaves, stems, and roots, obtained through maceration with 70% ethanol, demonstrated promising inhibitory properties. The research findings revealed that the leaf extracts possessed the highest potential for inhibiting calcium oxalate nucleation, as evidenced by their low IC50 value of 5.13 mg/mL. Additionally, the leaf extract exhibited remarkable effectiveness in inhibiting the growth of CaOx crystals, with an IC50 of 7.15 mg/mL. Meanwhile, the stem extract showcased the highest inhibition of calcium oxalate crystal aggregation, boasting an IC50 of 7.1 mg/mL. Notably, the stem extract excelled in dissolving CaOx crystals, achieving a dissolution rate of 51.48% at 20 mg/mL when assessed by titration. These findings underline the importance of future research aimed at isolating and identifying the specific bioactive compounds within the plant extracts. Such endeavors may pave the way for the development of novel therapeutic applications in the prevention and management of kidney stones and calcium oxalate-related disorders.

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