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The Effectiveness of Lime Juice (Citrus aurantifolia) in Preventing Calcium Oxalate Crystal Formation: An *In Vitro* Model

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ABSTRACT

Background: Calcium oxalate (CaOx) stones are the most prevalent type of urolithiasis and are associated with high recurrence rates. Preventive approaches targeting early crystallization stages, particularly nucleation and aggregation, are therefore crucial. Citrate is a recognized inhibitor of CaOx crystallization; however, pharmacological citrate therapy may face limitations in long-term use. Lime juice (Citrus aurantifolia), a natural source of citrate, may serve as an alternative preventive agent.

Objective: To evaluate the inhibitory effect of lime juice (Citrus aurantifolia) on calcium oxalate crystal formation in vitro.

Methods: A true experimental study with a post-test only control group design was conducted using a synthetic urine model. Five groups were assessed: negative control, positive control (potassium citrate 0.6%), and lime juice at concentrations of 2.5%, 5%, and 10% (v/v). Crystallization was monitored turbidimetrically at 620 nm for 60 minutes. Nucleation slope (SN), aggregation slope (SA), time to maximum absorbance (Tmax), and inhibition percentages were analyzed using one-way ANOVA and Tukey's HSD test.

Results: All lime juice concentrations significantly reduced aggregation and prolonged Tmax compared with the negative control. The 5% concentration showed the strongest nucleation inhibition among natural treatments, while 2.5% demonstrated the highest aggregation inhibition.

Conclusion: Lime juice effectively inhibits CaOx crystallization in vitro and shows potential as a natural, accessible preventive strategy.

KEYWORDS: Kidney stones, calcium oxalate, lime juice, citrate, crystallization, nucleation inhibition, aggregation inhibition

INTRODUCTION

Kidney stones represent a growing global nephrological disorder with a substantial clinical and economic burden, particularly among individuals of productive age.[1] In 2021, an estimated 106 million incident cases of urolithiasis were reported worldwide, reflecting a persistent upward trend over the past two decades.[2] In Southeast Asia, the incidence is estimated at approximately 941 cases per 100,000 population, indicating a significant regional burden.[2] In Indonesia, data from the National Basic Health Research (Riskesdas) reported a national prevalence of 0.6% in 2013, with a higher prevalence observed in East Nusa Tenggara (0.7%), exceeding the national average.[3] At the local level, surgical reports from Kupang have shown that urinary tract stones constitute a major proportion of urological procedures, with bladder and ureteral stones dominating operative cases.[4]

Calcium-based stones account for approximately 80–85% of urinary tract calculi, with calcium oxalate (CaOx) being the most prevalent composition.[1,5] The formation of CaOx stones is initiated when urine becomes supersaturated with calcium and oxalate ions, triggering crystal nucleation within the renal tubules or interstitium.[5] If this early crystallization process is not adequately inhibited, crystals undergo growth and aggregation, eventually forming clinically significant stones that may lead to urinary obstruction,

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infection, and progressive renal injury.[5,6] The recurrence rate of calcium oxalate stones remains high, reaching up to 50% within ten years, underscoring the limitations of purely curative approaches.[1,6]

Given this high recurrence risk, preventive strategies targeting the early stages of crystallization are increasingly emphasized. Current European Association of Urology (EAU) guidelines highlight the importance of reducing urinary supersaturation and enhancing endogenous inhibitors of crystallization, particularly citrate, as a cornerstone of stone prevention.[7] Citrate acts as a natural inhibitor by complexing with free calcium ions, thereby reducing calcium oxalate supersaturation and interfering with nucleation and crystal growth pathways.[7]

Natural dietary sources rich in citrate have therefore attracted growing interest as adjunctive or alternative preventive strategies. Citrus fruits are known to contain high concentrations of citric acid and various bioactive compounds with potential anti-crystallization properties.[8,9] In particular, lime (Citrus aurantifolia) contains substantial amounts of citric acid as well as flavonoids such as hesperidin and eriocitrin, which may contribute to its inhibitory effects on crystal formation.[10] Experimental studies have demonstrated that plant-derived inhibitors can effectively suppress calcium oxalate crystallization in vitro by interfering with nucleation and aggregation processes.[11] At the molecular level, citrate has been shown to stabilize pre-nucleation ion associates and amorphous calcium oxalate clusters, thereby increasing the energetic barrier required for crystal nucleation and delaying the transition to stable crystalline phases.[12] This non-classical crystallization mechanism provides a strong theoretical basis for investigating citrate-rich natural products as inhibitors of calcium oxalate stone formation.

Although potassium citrate is widely recommended as the pharmacological standard for calcium oxalate stone prevention, its long-term use may be limited by gastrointestinal side effects, patient adherence, and cost considerations.[13] Consequently, interest has grown in identifying accessible dietary alternatives that may provide comparable inhibitory effects. While citrus fruits such as lemon have been relatively well studied, data specifically evaluating the effects of lime juice (Citrus aurantifolia) on distinct phases of calcium oxalate crystallization remain limited.[14] Therefore, this study aimed to evaluate the effectiveness of lime juice (Citrus aurantifolia) in inhibiting calcium oxalate crystal formation using an in vitro synthetic urine model. By assessing nucleation rate, aggregation rate, and induction time of crystallization, this study seeks to provide mechanistic insight into the potential role of lime juice as a natural, accessible, and cost-effective non-pharmacological strategy for calcium oxalate stone prevention.

METHODS

This study employed a true experimental design with a post-test only control group layout. The experiment was conducted at the Biochemistry–Chemistry Laboratory, Faculty of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, in September 2025.

The materials used in this study consisted of synthetic urine components, crystallization induction solutions, and crystallization inhibitors. Synthetic urine was prepared by dissolving calcium chloride (CaCl2, 8 mM), sodium chloride (NaCl, 200 mM), sodium acetate (10 mM), and sodium oxalate (Na2C2O4, 1 mM) in deionized water. The pH of the solution was adjusted to 5.7 ± 0.1 using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH), and the solution was equilibrated at 37 °C prior to use to simulate physiological conditions. Crystallization was induced using freshly prepared calcium chloride (CaCl2, 8 mM) and sodium oxalate (Na2C2O4, 1 mM) solutions.

Fresh lime juice (Citrus aurantifolia) was used as the test inhibitor. The juice was filtered through a 0.45 µm syringe filter membrane to obtain a clear, particle-free filtrate. Potassium citrate at a concentration of 0.6% (6 g/L) served as the positive control inhibitor. The experiment consisted of five groups: a negative control (synthetic urine without inhibitor), a positive control (synthetic urine supplemented with potassium citrate 0.6%), and three experimental groups treated with lime juice at concentrations of 2.5%, 5%, and 10% (v/v). Each group was performed in quadruplicate, yielding a total of 20 experimental units.

Calcium oxalate crystallization was monitored using a UV–Vis spectrophotometer (Thermo Scientific Multiskan GO) at a wavelength of 620 nm. The assay was conducted in 96-well microplates with a total reaction volume of 200 µL per well. For each reaction, 50 µL of calcium chloride solution was mixed with 50 µL of the respective inhibitor solution (lime juice or potassium citrate). A baseline absorbance reading was obtained prior to crystallization induction. The reaction was initiated by adding 100 µL of sodium oxalate solution. Turbidity, expressed as optical density (OD), was recorded at 60-second intervals over a 60-minute observation period.

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The dynamics of crystal formation were evaluated using several parameters. Time to maximum absorbance (Tmax) was defined as the time required to reach peak optical density, indicating the completion of the nucleation phase. The nucleation slope (SN) was calculated from the initial ascending portion of the absorbance–time curve and represented the rate of crystal nucleus formation. The aggregation slope (SA) was determined from the descending portion of the curve following Tmax and represented the rate of crystal aggregation. The percentage of inhibition was calculated by comparing SN or SA values of the treatment groups with those of the negative control.

Statistical analysis was performed using the Shapiro–Wilk test to assess data normality and Levene's test to evaluate homogeneity of variances. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Normality testing using the Shapiro–Wilk test demonstrated that the nucleation slope (SN) and aggregation slope (SA) data were normally distributed across all groups, with p-values greater than 0.05. Homogeneity of variance was confirmed using Levene's test, yielding p-values of 0.285 for SN and 0.221 for SA. These results indicated that the assumptions required for parametric analysis were fulfilled.

Table 1. Normality and homogeneity test results

Parameter	Test	p-value	Interpretation
SN	Shapiro–Wilk	> 0.05	Normal distribution
SA	Shapiro–Wilk	> 0.05	Normal distribution
SN	Levene's test	0.285	Homogeneous variance
SA	Levene's test	0.221	Homogeneous variance

Descriptive analysis of crystallization parameters showed that the negative control group exhibited the highest nucleation and aggregation rates, reflecting the fastest calcium oxalate crystal formation. All lime juice concentrations reduced both SN and SA values compared with the negative control. Among the natural treatment groups, the 5% lime juice concentration produced the lowest mean SN value, approaching the inhibitory effect observed in the potassium citrate group. In addition, all treatment groups prolonged the time to maximum absorbance (Tmax), indicating a delay in crystal formation dynamics.

Table 2. Descriptive statistics of crystallization parameters

Inhibitor Concentration	SN (Mean)	SA (Mean)	Nucleation Inhibition (%)	Aggregation Inhibition (%)	Tmax (minutes)
Negative Control (0%)	0.000461	0.001587	0.00	0.00	30
Lime Juice 2.5%	0.000322	0.000496	30.09	68.70	50
Lime Juice 5%	0.000291	0.000626	36.79	60.52	56
Lime Juice 10%	0.000314	0.000625	31.85	60.63	58
Potassium Citrate 0.6%	0.000185	0.000320	59.87	79.79	54

One-way analysis of variance revealed statistically significant differences among groups for both nucleation slope ($p = 0.003$) and aggregation slope ($p < 0.001$), indicating that inhibitor type and concentration significantly influenced calcium oxalate crystallization.

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Table 3. One-way ANOVA results

Parameter	p-value	Interpretation
SN	0.003	Statistically significant
SA	< 0.001	Statistically significant

Post hoc comparison using Tukey's HSD test demonstrated that, during the nucleation phase, only lime juice at a concentration of 5% and potassium citrate 0.6% differed significantly from the negative control. In contrast, during the aggregation phase, all lime juice concentrations as well as potassium citrate showed highly significant inhibitory effects compared with the control.

Table 4. Tukey HSD post hoc analysis compared with negative control

Parameter	Treatment Group	p-value
SN	Lime Juice 2.5%	> 0.05
	Lime Juice 5%	0.046
	Lime Juice 10%	> 0.05
	Potassium Citrate 0.6%	0.001
SA	Lime Juice 2.5%	≤ 0.001
	Lime Juice 5%	≤ 0.001
	Lime Juice 10%	≤ 0.001
	Potassium Citrate 0.6%	≤ 0.001

Overall, these results indicate that lime juice effectively inhibits calcium oxalate crystal aggregation at all tested concentrations, while its inhibitory effect on nucleation is concentration-dependent, with the strongest effect observed at 5%. The prolongation of Tmax across treatment groups further supports the role of lime juice in delaying crystal formation.

DISCUSSION

The results of this study demonstrate that lime juice (Citrus aurantifolia) possesses significant inhibitory effects on calcium oxalate (CaOx) crystallization. This is evidenced by a reduction in both the nucleation rate (SN) and aggregation rate (SA), as well as a delay in the induction time for crystal formation, as reflected by an increase in Tmax. These findings indicate that lime juice can act as a natural inhibitor across key stages of CaOx crystallization.[5,6]

The most plausible mechanism underlying these findings is related to the citric acid content of lime juice. Citrate is a well-recognized inhibitor of calcium-containing kidney stones because it complexes with free calcium ions, thereby reducing calcium oxalate supersaturation and interfering with crystallization pathways.[7,15] Phillips et al. emphasized that citrate salts play a key role in the prevention and treatment of calcium-containing kidney stones by decreasing free calcium availability and disrupting crystal formation processes.[15] In addition, citrate has been shown to stabilize pre-nucleation ion associates and amorphous calcium oxalate particles, increasing the free-energy barrier for crystallization and effectively delaying the formation of stable crystal nuclei.[12,16]

In the nucleation phase, this study found that the 5% lime juice concentration produced the most optimal inhibitory effect among the tested natural concentrations, with an SN value that approached the positive control. This suggests an "effective concentration window" in which citrate-mediated stabilization of pre-nucleation species is maximized.[12,16] Notably, the 10% concentration did not further improve nucleation inhibition, indicating a plateau effect. This may reflect saturation of calcium-binding interactions or changes in ionic strength and ion activity that can modulate crystallization kinetics in solution-based models.[12,13]

In contrast, the aggregation phase demonstrated robust inhibition across all lime juice concentrations tested (2.5%, 5%, and 10%), with highly significant differences compared with the negative control ($p \leq 0.001$). This indicates that lime juice is particularly

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effective at preventing small CaOx crystals from aggregating into larger particles, which are more likely to be retained within the urinary tract and subsequently develop into clinically relevant stones.[5,6] The strong anti-aggregation effect observed even at 2.5% suggests that relatively low concentrations of lime juice may be sufficient to disrupt crystal–crystal interactions that contribute to stone growth.[5,6]

The delay in crystallization kinetics was also clearly reflected in Tmax, which shifted from 30 minutes in the negative control to 50–58 minutes in the lime juice-treated groups. A prolonged induction time is generally interpreted as a protective effect, as delayed crystallization reduces the likelihood of stable crystal formation and subsequent retention.[5,6] These findings are consistent with clinical observations reported by Nagappan et al., who demonstrated that lime juice can serve as a dietary alternative to potassium citrate for urine alkalinisation and may increase urinary citrate availability, thereby lowering calcium oxalate stone risk.[14,17] Zomorodian and Moe further reinforced that citrate is a central protective factor against calcium stones, acting through inhibition of nucleation, growth, and aggregation processes.[18]

Despite these promising findings, several limitations should be acknowledged. First, the standardized synthetic urine model, while useful for experimental control and reproducibility, cannot fully represent the complex physiological milieu of human urine, including endogenous macromolecules and trace ions that may either inhibit or promote crystallization.[13] Second, pH was adjusted prior to the assay but was not re-measured after the addition of lime juice; changes in pH may have contributed to the observed inhibition, given the known influence of pH and ionic composition on CaOx crystallization behavior.[7,13] Future research should therefore incorporate in vivo validation and detailed morphological assessment of CaOx crystals, for example using scanning electron microscopy (SEM), to clarify how lime juice affects crystal size, shape, and aggregation patterns beyond turbidity-based kinetics.[13]

CONCLUSION

This study demonstrates that lime juice (Citrus aurantifolia) is effective in inhibiting calcium oxalate crystal formation in vitro by acting on multiple stages of the crystallization process. Lime juice showed a strong inhibitory effect on crystal aggregation, with aggregation inhibition percentages of 68.70%, 60.52%, and 60.63% at concentrations of 2.5%, 5%, and 10%, respectively. Among these, the 2.5% concentration exhibited the highest numerical effectiveness in preventing crystal aggregation, indicating that relatively low concentrations are sufficient to disrupt the merging of calcium oxalate crystals into larger aggregates.

In addition to its effect on aggregation, lime juice was also able to inhibit the nucleation phase of calcium oxalate crystallization. The nucleation inhibition percentages observed at concentrations of 2.5%, 5%, and 10% were 30.09%, 36.79%, and 31.85%, respectively. The 5% concentration produced the most optimal inhibitory effect on nucleation, suggesting the presence of an effective concentration range at which lime juice maximally interferes with initial crystal core formation.

Furthermore, lime juice significantly delayed the induction time of crystal formation, as reflected by the postponement of the time to maximum absorbance (Tmax). While the negative control reached Tmax at 30 minutes, treatment with lime juice shifted Tmax to 50, 56, and 58 minutes at concentrations of 2.5%, 5%, and 10%, respectively. This delay indicates that lime juice slows the overall crystallization kinetics and prolongs the time required for crystals to reach maximal formation.

Overall, these findings indicate that lime juice (Citrus aurantifolia) effectively inhibits both nucleation and aggregation of calcium oxalate crystals and delays crystal formation kinetics in vitro. Lime juice therefore has potential as a natural, accessible, and cost-effective non-pharmacological agent for the prevention of calcium oxalate kidney stone formation.

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