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Anti-nephrolithiatic activity of *Garcinia lanceifolia* Roxb: An *in vitro* Study

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Abstract
Urolithiasis is a chronic kidney disease characterized by the development of calculi inside the kidneys in a complicated sequence of supersaturation, nucleation, aggregation, crystal development, and retention. The purpose of the current study was to screen the *in vitro* anti-nephrolithiatic effects of *Garcinia lanceifolia* Roxb. var. *Oxyphylla* fruit extract (GLE), which is a potent medicinal plant, against calcium oxalate crystallization. To determine the effect of GLE to prevent calcium oxalate nucleation and aggregation, *in vitro* studies were carried out. The results demonstrated that GLE may be used as a therapeutic agent for urolithiasis since it significantly ($P < 0.001$) inhibited the nucleation and aggregation of calcium oxalate crystal. Phytochemical examination indicated GLE has a high amount of total phenolics and flavonoids and also contain reported bioactive compounds like hydroxycitric acid in the fruit of *G. lanceifolia*. This combined approach may explain the reported anti-nephrolithiatic effects. The findings support the traditional use of *G. lanceifolia* and the need for additional *in vivo* studies to determine its therapeutic usefulness and safety.

Keywords: *G. lanceifolia*, urolithiasis, nucleation, aggregation, flavonoids

INTRODUCTION

Kidney stones are a typical urological problem, which has become one of the most difficult issues in the human population over the past few years [1]. It is estimated that 10 percent to 12 percent of individuals in developed countries, of which 10 percent of the males and 3 percent of the females, are likely to develop urinary stones at some point in their lives. It is a condition that is caused by various factors, such as nutrition, heredity, and physical inactivity [2]. The fact that crystalline deposits like calcium, magnesium ammonium phosphates, uric acid, and cystine accumulate, grow in size, and happen to be the major cause of kidney stones, although there is no known clear mechanism of how they form [3, 4]. The high load of oxalate in the urine leads to oxidative kidney damage. Calcium oxalate (CaOx) is the most prevalent form of kidney stone crystal [5]. A multitude of different pharmacological therapies, such as diuretics and urinary alkalizers such as citrate, are widely utilized in the treatment of uroliths as well as various surgical interventions, and these treatments are not always effective [6, 7]. Due to its complex pathophysiology and etiology, urolithiasis currently lacks an effective treatment. Since ancient times, the ethnic people have been utilizing various medical herbs, and the majority of them appear to be efficient in terms of urolithic issue treatment. Generally, it is believed that medicinal plants are a cheap and harmless alternative. The action of several plants as anti-urolithiatics has recently been assessed [8, 9]. The effectiveness of the Clusiaceae family has been well documented in the treatment of various ailments including urinary problems [10]. On these grounds, the choice of the *Garcinia lanceifolia* Roxb. var. *Oxyphylla* to be used in the current study has been made. *G. lanceifolia*, known as Rupahi-thekera in Assamese, is an

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evergreen and unexplored medicinal plant mostly found in Assam, Meghalaya, and southern Bangladesh. Several ethnicities in northeastern India consume the fruit of the plant to cure a wide range of diseases and conditions, including stomachic, diarrhoea, dyspepsia, jaundice, and urinary problems. Polyphenols and other mineral fruits possess a high anti-obesity, antihyperlipidemic, and antioxidant effect [11, 13]. It is a very good source of bioactive secondary metabolites such as xanthones, benzophenones, garcinol, and hydroxycitric acid, which are organic acids [14]. The present research was carried out to evaluate the *in vitro* antilithic activity of *G. lanceifolia* against the crystallization of calcium oxalate, as although it is traditional claimed for urinary problems, no screening has yet been performed to determine the anti-nephrolithiatic activity of *G. lanceifolia*.

MATERIALS AND METHODS

Collection and identification of plant material

G. lanceifolia fresh fruits were gathered in Ashrang village, Dimahasao district, Assam, India. Identification and authentication of the plant was carried out by a Taxonomist at Central Ayurveda Research Institute, Guwahati, India, and a voucher specimen was deposited in the departmental herbarium section as a future reference.

Preparation of extract

G. lanceifolia fresh fruits were cut, air-dried, and powdered. To extract it, 350 g of the powdered material was macerated using 70% ethyl alcohol (3 L, four times), and filtration was performed with the help of filter paper. This process was repeated, and all the filtrates were pooled together. The pooled filtrate was evaporated at 40°C under low pressure on a rotavapor (Bucchi, New Castle), followed by lyophilization to get a hydroalcoholic extract of *G. lanceifolia* (GLE).

Preliminary phytochemical screening and HPTLC analysis

To investigate the current existence of the key phytochemicals in GLE, the phytochemical examination of extracts was conducted using the traditional approaches. GLE fingerprint profile with the help of different solvent systems was created using the HPTLC technique. Elution of the HTLC plates was done using various mobile phases, and the best separation was obtained using chloroform and methanol (8:2). After the process of elution, TLC plates were scanned at the wavelength of 254 nm.

Estimation of total phenolic content (TPC)

GLE was analyzed using the Folin-Ciocalteu procedure to determine the total amount of phenolic content [15]. Folin-Ciocalteu phenolic reagent (0.25 ml of 0.2 N) and GLE (0.5 ml of 1 mg/ml) were added and incubated in the course of five minutes, followed by the addition of 2 ml of sodium carbonate (75 g/l). Thereafter, they were incubated at 37°C, for 2 hours. In a Shimadzu spectrophotometer, the absorbance at 760 nm was recorded. The amount of phenol in the GLE was expressed in the form of milligrams of gallic acid (GA) equivalent per gram of extract.

2.7 Estimation of total flavonoid content (TFC)

Colorimetrically, the amount of flavonoids in GLE was determined [16]. In brief, 0.5 ml GLE (1 mg/ml) was added to the solution of sodium nitrate (0.15 ml, 5%). This was followed by the gradual addition of sodium hydroxide (2 ml, 4 percent) and aluminum chloride solution (0.15 ml, 10 percent). The water was added to the sample up to 5 ml; it was stirred and left to stand for an additional 15 minutes. The absorbance of the mixture at the wavelength of 510 nm was recorded. TFC of the extracts was expressed as rutin equivalent in mg/g of extract.

In vitro screening of anti-lithiatic activity

Evaluation of CaOx crystallization

In order to evaluate the inhibiting capacity of GLE on Ca-oxalate crystal formation, it was determined by an *in vitro* method, which entails crystallization with and without inhibitors. This *in vitro* experiment was evaluated using nucleation and aggregation assays [17,18].

Nucleation Assay

To assess whether calcium oxalate (CaOx) crystals were formed in the presence of the GLE, the nucleation assay was performed. Sodium oxalate ($Na_2C_2O_4$) 7.5 mmol/l and calcium chloride ($CaCl_2$) 5 mmol/l solutions in Tris HCl (0.05 mol/l) and NaCl (0.15 mol/l) were prepared at pH 6.5. Distilled water was used to dilute GLE in the concentration range of 100-1000 µg/ml. One milliliter of each range of plant extract was mixed with three milliliters of $CaCl_2$ solution, then three milliliters of $Na_2C_2O_4$ were added. The incubation of final combinations was performed at 37ºC in 30 minutes. The optical density (OD) of the mixtures at 620 nm was recorded thereafter. To determine the percent suppression of the nucleation by the plant extract, the following formula was taken and compared to the standard polyherbal drug, Cystone.

% Inhibition = $(1- OD_{Test}/ OD_{Control} \times 100)$.

Aggregation assay

Aggregation assay was used to determine the effect of GLE on CaOx crystal aggregation. An aqueous solution of $CaCl_2$ and $Na_2C_2O_4$ (50 mmol/l) in water was mixed and then heated to 60 ºC in a water bath and left to crystallize the CaOx crystals overnight at 37ºC. Following desiccation, CaOx crystal (0.8 mg/ml) was mixed with 0.05 Mol/l Tris-HCl and 0.15 Mol/l NaCl buffer (pH 6.5). Three milliliters of CaOx solution were combined with one milliliter of GLE (100-1000 µg/ml) and vortexed, then allowed to incubate for thirty minutes at 37ºC. The optical density of the final blends at 620 nm was measured, and the percent of inhibition of aggregation of the final blends was calculated as in the nucleation assay.

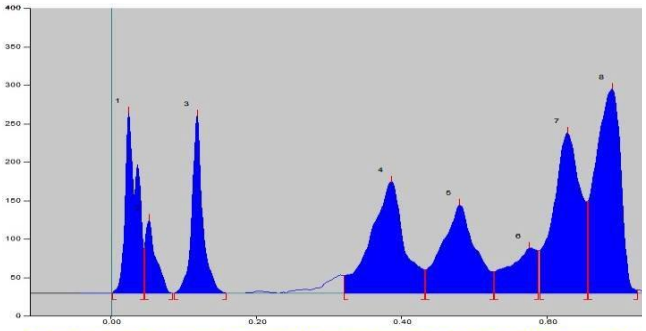
Statistical analysis

All the experiments were performed in triplicate, and the quantitative data were represented by a mean ± SEM (Standard Error of Mean). GraphPad Prism 8.0.2 was utilized to perform the statistical computations using

one-way analysis of variance (ANOVA) followed by a multiple comparison test. P values were deemed significant if they were less than 0.05.

Results

Percent yield of GLE was 7.42%. Based on qualitative phytochemical screening of GLE, terpenoids, tannins, saponins, sterols, and polyphenolic compounds, including flavonoids, were found to be the key constituents. HPTLC analysis of GLE using a solvent mixture of chloroform and methanol (8:2) gave nine distinct bands with different retention factors (Rf) (0.05, 0.10, 0.18, 0.50, 0.61, 0.68, 0.76, 0.84, 0.97) at a wavelength (λ) of 254 nm. These bands may be used as a marker of identification (Figure 1). The total phenol and flavonoid content of the gram of dried extract of *G. lanceifolia* was found to be 56.31±0.73 mg gallic acid and 44.23±1.02 mg rutin, respectively.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Ar
1	0.00 Rf	0.8 AU	0.03 Rf	34.9 AU	7.10 %	0.05 Rf	6.7 AU	156.3 AU	9.5
2	0.05 Rf	61.0 AU	0.06 Rf	95.3 AU	6.94 %	0.10 Rf	0.0 AU	110.2 AU	3.3
3	0.10 Rf	0.1 AU	0.14 Rf	30.8 AU	6.80 %	0.18 Rf	0.1 AU	762.4 AU	8.3
4	0.37 Rf	22.9 AU	0.45 Rf	44.8 AU	0.54 %	0.50 Rf	0.2 AU	487.2 AU	6.6
5	0.50 Rf	30.2 AU	0.56 Rf	14.8 AU	8.35 %	0.61 Rf	7.7 AU	319.3 AU	3.0
6	0.61 Rf	27.8 AU	0.67 Rf	58.8 AU	4.28 %	0.68 Rf	5.3 AU	925.2 AU	5.7
7	0.69 Rf	55.6 AU	0.73 Rf	108.2 AU	5.16 %	0.76 Rf	8.3 AU	452.8 AU	9.4
8	0.76 Rf	19.5 AU	0.80 Rf	165.2 AU	9.31 %	0.84 Rf	4.2 AU	513.7 AU	12.6
9	0.91 Rf	0.8 AU	0.96 Rf	20.8 AU	1.51 %	0.97 Rf	8.9 AU	504.8 AU	1.5

Fig.1 HPTLC chromatogram of *G. lanceifolia* fruit extract

When $Na_2C_2O_4$ solution was added to the reaction mixture consisting of $CaCl_2$ during the nucleation assay, many CaOx crystals were formed. As the GLE concentration increases, the OD decreases, indicating a decrease in the production of CaOx particles. The OD was highest (0.671±0.0074) in the positive control i.e., in the absence of GLE, and it was lowest (0.366±0.0051) at the highest concentration of GLE (1000 µg/ml). When 1000 µg/ml of GLE was added to the reaction mixture, the nucleation was significantly reduced (P < 0.001) to 45.05±0.57%, which was comparable to that produced by Cystone (53.66±0.58%) (Figure 2).

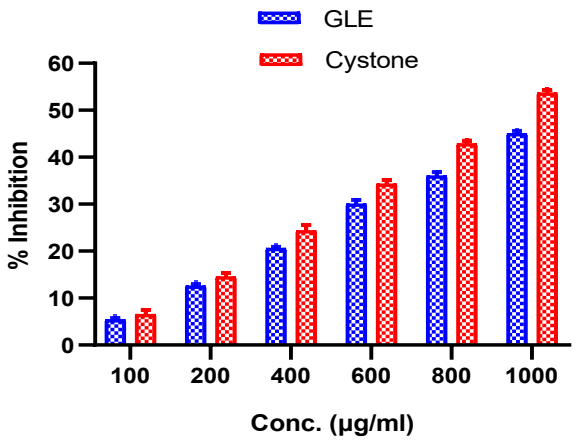


Fig. 2 Effect of GLE and Cystone on nucleation of CaOx crystals

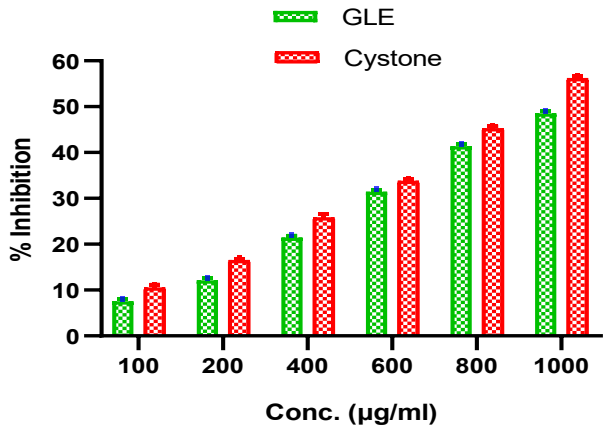


Fig. 3 Effect of GLE and Cystone on aggregation of CaOx crystals

In the aggregation assay, GLE significantly reduced the aggregation of preformed CaOx crystals (P < 0.001). At 1000 µg/ml, the GLE was found to cause a reduction in aggregation of 48.57±0.397%, which was comparable to the percentage reduction in aggregation produced by Cystone (56.15±0.46%) at the same concentration (Figure 3).

DISCUSSION

Calcium oxalate (CaOx) is the most prevalent crystalline element of kidney stones, and elevated levels of urinary oxalate are a significant component in oxidative infliction to the kidney [19,20]. Kidney stones are produced in many biochemical pathways, which include supersaturation, nucleation, growth, and crystal aggregation [3]. Inhibition of any of these phases can reduce the stone formation significantly. The present findings indicated that the effect of fruit extracts of *G. lanceifolia* was quite significant in reducing the *in-vitro* calcium oxalate crystal nucleation and aggregation.

The observed anti-nephrolithiatic effect of GLE might be associated with the presence of high content of total flavonoid, phenolic content, and hydroxycitric acid (HCA) in *G. lanceifolia* fruit. Since it is well reported that these elements possess chelating, anti-inflammatory, and antioxidant effects that are capable

of deterring the formation of crystals and protecting the renal epithelial cells [21,22]. The fruit of *G. lanceifolia* contains the highest concentration of hydroxycitric acid (239.25 mg/g), a derivative of citric acid [23]. As per previously reported studies, HCA efficiently inhibits the deposition of calcium oxalate (CaOx) crystals, reduces crystal adhesion, and mitigates oxidative damage. The pronounced anti-crystallization activity of GLE may be attributed to its high HCA content, which enables it to chelate free calcium and oxalate ions and thereby prevent the formation of CaOx complexes.

CaOx polymorphism is one such well known phenomena that has got a major role to play in urolithiasis. The stones frequently have crystals of calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) [24]. Among these, COM is more adhesive and aggregative and thermodynamically stable. Consequently, COM crystals adhere strongly to renal epithelial cells, cause tissue injury, and form large aggregates, thereby promoting crystal retention and accelerating stone formation relative to COD [25]. Hence, a shift from COM toward COD formation is considered a key strategy for preventing calculus development.

In the present study, GLE significantly reduced CaOx crystal nucleation and growth, demonstrating strong anti-urolithic potential. This effect may be plausibly attributable to the existence of HCA, one of the significant phytoconstituents that are known to disrupt the crystal formation pathways. According to earlier studies, HCA can prevent up to 60% of COM crystal formation by suppressing nucleation and slowing down the pace of crystal growth [26]. Therefore, it is likely that the HCA-induced interference of the crystallization of the COM can mediate the crystal-modulatory action shown in the current investigation, which supporting that GLE has the potential to be employed as a natural medicinal drug in the treatment of urolithiasis. Furthermore, the production of tiny crystals in the presence of GLE supports the inhibitory effect of GLE on crystal development.

Phytochemical screening of GLE revealed flavonoids, phenolic compounds, saponins, and tannins. These components are often linked with the prevention of the development of urinary stones. Saponins are also recorded to have antilithic effects due to the degradation of mucoproteins, which are important binding substrates in the stone matrix [17]. Tannins and other polyphenols have the ability to inhibit the development of calcium oxalate (CaOx) crystals and could also help to dissolve existing crystals by stabilizing the calcium complexation [27]. Flavonoid was also associated with the dissolving action of CaOx crystals, and phenolics and flavonoid have a strong antioxidant effect, thus alleviating oxidative stress, which causes renal tubular damage and following crystal retention [28].

CONCLUSION

The anti-crystallization, anti-aggregatory, and crystal growth-inhibitory activities exhibited by GLE seem to be a synergistic result of these phytoconstituents. Although *in vitro* findings cannot be directly extrapolated to the complexity of *in vivo* systems, such models provide valuable preliminary insight into the intrinsic efficacy of test extracts and compounds. The current study revealed strong inhibitory effects of GLE on CaOx crystallization, reinforcing the concept that its phytochemical composition plays a crucial role in its antiurolithic properties.

CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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