ORIGINAL ARTICLE

Anti-urolithiatic potential of *Hydrangea arborescens* and *Lycopodium clavatum*: *In silico* and *in vitro* approach

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Abstract

Background: Accumulation of inorganic substances in the kidney leads to crystallization of deposited matter after a prolonged period of time and elevates inflammation and dysfunction of the urinary tract. Urolithiasis is a painful urologic condition characterized by the formation of hard crystals in any part of the urinary system such as kidney, ureter, or bladder. **Objectives:** The present study aimed to evaluate the anti-urolithiatic potential of *Hydrangea arborescens* and *Lycopodium clavatum* using *in silico* and *in vitro* methods. **Materials and Methods:** The potential of *H. arborescens* and *L. clavatum* was studied against the dissolution capacity of calcium oxalate (CaOx) crystals. The percent dissolution of calcium oxalate crystal was estimated by titrimetric and ultraviolet-spectrophotometric methods. Further, the phytochemicals from both plants were retrieved from the PubChem database to identify the molecular docking score and explore anti-urolithiatic activity. **Result:** The *H. arborescens* and *L. clavatum* have shown significant anti-urolithiasis activity by dissolving calcium oxalate crystals at 63.15 ± 3.53% and 27.80 ± 2.71%, respectively. Diepiserratenediol (−4.73) and apigenin (−4.71) from *L. clavatum* and quercetin (−6.12) and kaempferol (−4.97) from *H. arborescens* showed high docking scores compared to cystone (−3.56). **Conclusion:** The tincture of *H. arborescens* was found to be more effective than *L. clavatum* in dissolving CaOx crystals by *in vitro* methods.

Key words: Lycopodium clavatum, Hydrangea arborescens, Titrimetric estimation, Nephrolithotomy, anti-urolithiatic potential

INTRODUCTION

Kidneys are the primary excretory organs in both animals and humans, and kidney stone is the major urological problem with the highest prevalence, 19.7% in male.[1] Urolithiasis is a painful urologic condition characterized by the formation of hard crystals in any part of the urinary system such as kidney, ureter, or bladder. Renal calculus is formed when the urine is concentrated with insoluble ions of calcium oxalate, uric acid, and phosphate, leading to the formation of a kidney stone.[2] Kidney stones are the most common conditions affecting the urinary tract significantly; around 12% people worldwide suffer from urolithiasis with a recurrence rate is 70–81% in men and 47–60% in women. Renal calculus causes in men are 3 times more than in women, due to stimulation of testosterone and inhibition of estrogen.[3]

Kidney stones basically consist of a high concentration of calcium oxalate along with calcium carbonate and calcium phosphate. The multistep process of nucleation, crystal development, crystal aggregation, and crystal retention is involved in the pathophysiology of calcium oxalate stone formation.[4] In higher plants, at the time of metabolism, there is formation of oxalic acid from glycolate, glyoxylate and ascorbic acid. The calcium ions bind with free oxalic acid or oxalate, and crystals of calcium oxalate are formed which may lead to urolithiasis.

Surgical option for the urolithiasis treatment is nephrolithotomy, which is costly and recurrence of kidney

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stones is high.[5] Kidney stones can be prevented by lithotripsy; sound waves or laser techniques are employed to break kidney stones into small fragments and are removed through the urinary tract. This may cause renal injury and infections in the urinary tract.[6] Increased fluid intake, balanced diet, urinary alkalizing, and calcium-chelating drugs such as sodium bicarbonate, potassium citrate, and sodium citrate are current preventative agents of renal stones.[7] In addition, allopurinol and thiazide diuretics are given to treat calcium oxalate (CaOx) stone illness. These medicines have serious side effects and may not be successful in preventing stone recurrences, despite their potential for prophylactic use.[8] Hence, finding efficient medications to treat kidney stones with minimal or no side effects is essential.

The homeopathic medicines work based on the principle of "*Similia similar similibus*" which means "*like cureslike*."[9] In the present study, we have selected two homeopathic medicines which are been used in folklore practice as herbal drugs for the treatment of urolithiasis, namely *Hydrangea arborescens* and *Lycopodium clavatum*.[10] *H. arborescens* is a homeopathic mother tincture traditionally used to treat urinary tract infections, kidney stones, and prostate enlargement.[11] *L. clavatum* is used to treat gallstones, renal colic, gout disorders, rheumatism, menstrual problems, and urinary issues. It helps to reduce stone recurrence.[12] The claims of the marketed preparations of both drugs for treating urinary tract diseases, especially for urolithiasis, have not been scientifically proven. Hence, a study was undertaken to find the potential of homoeopathic formulations of *H. arborescens* and *L. clavatum* as an anti-urolithiatic agent using an *in silico* and *in vitro* approach.

MATERIALS AND METHODS

Molecular Docking Study

Protein preparation

The phytochemicals from both plants were analyzed for molecular docking score to explore their anti-urolithiatic activity. The crystal structure of oxalate oxidase (PDBID:2ETE) was retrieved from the Protein Data Bank.[13] For protein preparation, the standard protocol of protein preparation wizard (Schrodinger, LLC) was followed and minimized the protein structure until RMS gradient for heavy atom reached 0.3 A°.

Ligand preparation

The phytochemicals were downloaded from PubChem, and ligand was prepared using LigPrep version 4.8 (Schrodinger LCC). LigPrep generates an energy-minimized structure with multiple tautomers and stereoisomers.

Receptor Grid Generation and Molecular Docking

Molecular docking simulations were carried out using the GLIDE docking module of Schrodinger Suite software. The glide docking produced different poses for each input ligand, and each pose was scored and ranked by the glide docking scores in KcaL/mol.[14]

Collection of *H. arborescens* and *L. clavatum*

Homeopathic mother tincture of *H. arborescens* and *L. clavatum* was procured from Bakson Drugs and Pharmaceuticals Pvt. Ltd., India. The 100 mL tinctures of *H. arborescens* and *L. clavatum* were taken separately in a China dish and evaporated on an electric water bath at 100°C to obtain the concentrated residue.

In Vitro Anti-urolithiatic Activity

Preparation of semi-permeable membrane from egg

The apex of the eggs was pierced with a glass rod, and egg contents were removed. The empty eggshell was washed thoroughly with distilled water and immersed in a solution containing 50 mL concentrated HCl and 100 mL distilled water for 20 min. which results in complete decalcification of the semi-permeable membrane. Further, the semi-permeable membrane was separated completely from the egg shell and neutralized to remove the traces of HCl using ammonia solution. Once again, the membrane was washed with distilled water and then stored in an isotonic solution of pH 7–7.4 in a refrigerator [Figure 1].[15]

Preparation of synthetic kidney stones

- Solution 1: 1.47 g of calcium chloride dehydrate dissolved in 100 mL of distilled water
- Solution 2: 100 mL of 2N sulfuric acid (H_2SO_4) is dissolved in 1.34 g of sodium oxalate.

Both solutions in equal volume were mixed in a beaker and continuously stirred until calcium oxalate precipitated. After

Figure 1: Egg membranes stored in isotonic solution

Figure 2: Calcium oxalate crystals

complete precipitation, ammonia solution was used to remove excess traces of H_2SO_4; crystals were washed with distilled water and dried for 2 h in an oven at 60°C [Figure 2].[5]

Preparation of phosphate buffer

Potassium dihydrogen phosphate (5 mL) of 136 g/L solution was mixed with 29.5 mL of 1M sodium hydroxide, and the volume was adjusted to 100 mL with distilled water with pH of 7.4 ± 0.1.

Titrimetric Estimation

The content of each group [Table 1] was added into isolated semi-permeable egg membranes separately; the open end of the membrane was tied with thread and suspended in a conical flask containing 100 mL of phosphate buffer and covered with aluminum foil. All conical flasks were set for incubation for 24 h at 37°C. After an incubation period, the whole content of the individual egg membrane was removed and the content was used for the determination of undissolved CaOx crystals by titrimetric and ultraviolet (UV)-spectrophotometry methods.[5]

Standardization of potassium permanganate ($KMnO_4$) - 20 mL of oxalic acid was taken in a beaker and 5 mL of concentrated H_2SO_4 was added and boil it up to 70°C. The mixture was titrated against $KMnO_4$. The endpoint was observed as a pale pink color.

Titration

The contents of semi-permeable membrane were collected into a clean conical flask, and 2 mL of 1N H_2SO_4 was added and titrated against 0.09132N $KMnO_4$ until the pale pink color was obtained as endpoint. The % dissolution of CaOx was calculated using the following formula.[16]

% Dissolution = Weight of undissolved CaOx from control - Weight of undissolved CaOx from test/Weight of undissolved CaOx from control*100.

Table 1: Titrimetric estimation procedure

Groups	Contents
Group 1 (Control)	1 mg CaOx+1 mL Distilled water
Group 2 (*H. arborescens*)	1 mg CaOx+10 mg *H. arborescens*+1 mL distilled water
Group 3 (*L. clavatum*)	1 mg CaOx+10 mg *L. clavatum*+1 mL distilled water
Group 4 (standard drug) Cystone	1 mg CaOx+10 mg cystone+1 mL distilled water

H. arborescens: *Hydrangea arborescens*, *L. clavatum*: *Lycopodium clavatum*, CaOx: Calcium oxalate

Table 2: UV-visible spectrophotometric estimation

Sets	Groups	Contents
Set-A	Group 1	1 mg CaOx+10 mg *H. arborescens*+1 mL DW
	Group 2	1 mg CaOx+10 mg *L. clavatum*+1 mL DW
Set-B	Group 1	1 mg CaOx+20 mg *H. arborescens*+ 1 mL DW
	Group 2	1 mg CaOx+20 mg *L. clavatum*+1 mL DW
Set-C	Group 1	1 mg CaOx+30 mg *H. arborescens*+ 1 mL DW
	Group 2	1 mg CaOx+30 mg *L. clavatum*+1 mL DW
Standard drug		1 mg CaOx+10 mg cystone+1 mL distilled water (cystone tablet 500 mg purchased from Himalaya)

UV: Ultraviolet, DW: Distilled water, *H. arborescens*: *Hydrangea arborescens*, *L. clavatum*: *Lycopodium clavatum*

UV-visible (UV-vis) Spectrophotometric Estimation

The content of semi-permeable membrane as given in [Table 2] was collected in a clean test tube separately. 4 mL of 1N H_2SO_4 and 0.6 mL of 0.02M $KMnO_4$ were added and kept it for 15 min. The change in color intensity of the contents of each test tube was measured using UV-visible spectrophotometer at 620 nm; absorbance was plotted against the concentration of CaOx.[17]

RESULTS

Identification of Molecular Docking Score

The molecular docking score of compounds showing anti-urolithiatic activity from *H. arborescens* and *L. clavatum* phytochemicals was retrieved [Table 3] and compared against cystone, which is a marketed drug used for the treatment of kidney stones.

Titrimetric Estimation

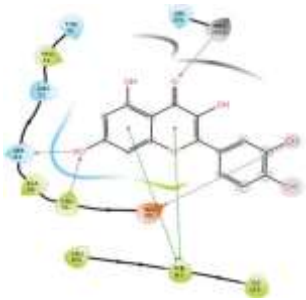
The amount of CaOx dissolved was calculated from the total quantity of CaOx used in the experiment. The percent CaOx

Table 3: List of phytochemicals and molecular docking score

S No.	Phytochemical name	Molecular formula	Docking score	3D structure
	***Lycopodium clavatum* phytochemicals and molecular docking score**			
1.	Diepiserratenediol	$C_{30}H_{50}O_2$	−4.73	
2.	Benzoic acid, 2-hydroxy-4-((2-hydroxy-4-methoxy-3,6-dimethylbenzoyl) oxy)-3,6-dimethyl-	$C_{19}H_{20}O_7$	−4.71	
3.	Apigenin 7-O-(2'',6''-di-O-E-p-coumaroyl) glucoside	$C_{39}H_{32}O_{14}$	−4.63	
4.	Diffractaic acid	$C_{20}H_{22}O_7$	−4.57	
5.	(14-Hydroxy-15-methyl-6-azatetracyclo[8.6.0.01,6.02,13] hexadecan-11-yl) acetate	$C_{18}H_{29}NO_3$	−4.24	
6.	Dihydrocaffeic acid	$C_9H_{10}O_4$	−4.084	
7.	d-Usnic acid	$C_{18}H_{16}O_7$	−3.56	

(Contd...)

Table 3: *(Continued)*

S No.	Phytochemical name	Molecular formula	Docking score	3D structure
8.	Cystone	$C_{27}H_{38}O_5$	−3.56	
	***Hydrangea arborescens* phytochemicals and molecular docking score**			
1.	Quercetin	$C_{15}H_{10}O_7$	−6.12	
2.	Kaempferol	$C_{15}H_{10}O_6$	−4.97	
3.	Jamaican	$C_{22}H_{18}O_6$	−2.31	
4.	Durmillone	$C_{22}H_{18}O_6$	−2.17	
5.	Cystone	$C_{27}H_{38}O_5$	−3.56	

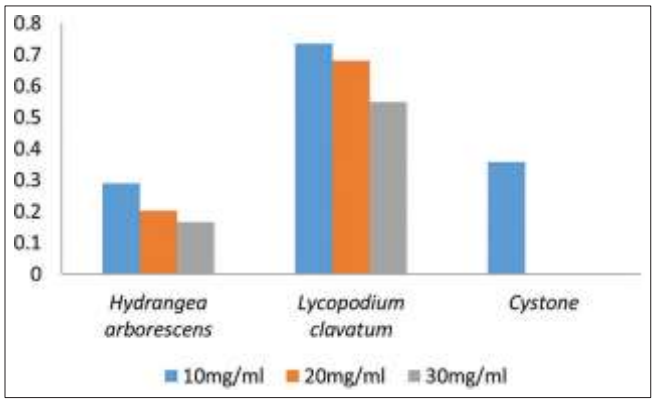


Figure 3: Absorbance of samples and standard

dissolved by *H. arborescens* and *L. clavatum* was 63.15 ± 3.53 and 27.80 ± 2.71, respectively [Table 4].

UV-vis Spectrophotometric Estimation

The color intensity of $KMnO_4$ in the sample was observed under a UV-vis spectrophotometer at the wavelength of 620 nm [Figure 3].

DISCUSSION

Urolithiasis is a condition of urinary tract dysfunction due to the deposition of crystals of CaOx in the kidney or in the ureter. Hypertension, chronic kidney diseases, and metabolic disorders such as diabetes, obesity, and gout elevate urolithiasis.[18] Lifestyle habits like alcohol consumption, smoking, and the addition of narcotic drugs like opium increase the chances of developing kidney stones.[19]

In the present study, homeopathic drugs *H. arborescens* and *L. clavatum* were used in view of the traditional practice of these medicines for the treatment of urinary tract infections such as kidney stones. *In silico* molecular docking score was retrieved for both the plant phytochemicals: diepiserratenediol (−4.73) and apigenin (−4.71) from *L. clavatum* and quercetin (−6.12) and kaempferol (−4.97) from *H. arborescens* which showed high docking score compared to cystone (−3.56).

A researcher Dinnimath *et al*., reported that quercetin isolated from *Aerva lanata* had shown significant anti-urolithiasis activity.[20] Moreover, Azimi *et al*. reported the anti-urolithiatic potential of Apigenin.[21] Hence, due to the presence of these phytochemicals, the plant might be showing significant anti-urolithiasis activity. The docking score and 3D structure of compounds from both plants are listed in [Table 3].

The mother tincture of both plants showed significant anti-urolithiasis activity by dissolving CaOx crystals of 63.15 ± 3.53% and 27.80 ± 2.71% from *H. arborescens* and *L. clavatum*, respectively, where *H. arborescens* had comparatively high dissolution capacity of CaOx crystals than cystone which was found as 37.17 ± 0.69%. The significant dissolution of CaOx crystals by *H. arborescens* might be because of the presence of quercetin and other secondary phytochemicals. Therefore, *H. arborescens* was found to be more effective than *L. clavatum* in dissolving CaOx crystals.

Table 4: Percent dissolution of CaOx

Groups	Undissolved CaOx (mg)	Dissolved CaOx (mg)	Percentage dissolution of CaOx
Control	0.99±0.02	0.05±0.02	0.00
Hydrangea arborescens	0.35±0.04	0.64±0.04	63.15±3.53
Lycopodium clavatum	0.68±0.02	0.31±0.02	27.80±2.71
Cystone	0.59±0.01	0.40±0.01	37.17±0.69

CaOx: Calcium oxalate

The basic principle of the estimation of dissolved CaOx in the sample is that when $KMnO_4$ reacts with oxalic acid, it shows a fading of the pink color of $KMnO_4$; hence, color intensity decreases. If the drug resembles anti-urolithiasis potential and dissolves CaOx crystals, titrating this content of semi-permeable egg membrane with $KMnO_4$ results in decreased endpoint and utilizes less amount of KMnO4 to reach endpoint; hence, the absorbance decreases.[22]

The *in silico* and *in vitro* evaluation for anti-urolithiatic activity of *H. arborescens* and *L. clavatum* evidences the traditional claims of the plants mentioned in homeopathic medicines. To collect more scientific data and to understand the mechanism behind the anti-urolithiatic potential of the drug, we will further investigate in the future.

CONCLUSION

In the present study, both the drugs *H. arborescens* and *L. clavatum* are effective against urolithiasis. In the titrimetric method, it reveals that CaOx dissolution percentage has increased using *H. arborescens* compared to *L. clavatum*. Whereas, in the UV-vis spectrophotometric method, the *H. arborescens* has shown a reduction in absorbance compared to *L. clavatum* which indicates the significant anti-urolithiasis activity which is proportional to concentration. The comparative evaluation of anti-urolithiatic activity between *H. arborescens* and *L. clavatum* demonstrated that *H. arborescens* exhibited significantly anti-urolithiatic activity than *L. clavatum*. The results obtained from the present studies are found encouraging and may be helpful to design the therapies for the cure of renal calculi; however,

further research needs to be performed using *in vivo* models to confirm the anti-urolithiatic potential of *H. arborescens*.

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