

Lithotriptic Effect of *Cinnamomum verum* and *Nigella sativa* on Ureteric Calculi

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ABSTRACT

Introduction: Urolithiasis is one of the most prevailing diseases in the society. Conventional treatments are symptomatic and are not targeted to direct the dissolution of ureteric calculi. Thus, in this study plants like *Cinnamomum verum* (Lauraceae) (*C. verum*) and *Nigella sativa* (Ranunculaceae) (*N. sativa*) are selected to assess for their lithotriptic activity. **Materials and Methods:** The combinations of hydroalcoholic extract of these plants were prepared (HaNsCv). Ureteric calculi of human origin were sampled from patients post lithotripsy. The sampled calculi were weighed and incubated *ex vivo* with various concentrations of HaNsCv extract like 50, 100, 200 and 400 mg in tris buffer (pH 7) at 37°C. The change in the size of ureteric calculi was monitored at interval of 24 hr post for a period of 9 days. **Results and Discussion:** Amongst various concentration tested, the dissolution of ureteric calculi was initiated within 24 hr at dose 400 mg whereas remaining concentrations exhibited lithotriptic activity after 24 hr of incubation. Dissolution of ureteric calculi observed was found to be both dose dependent and time dependent. At the end on 9th day 100% dissolution of ureteric calculi was observed at dose 400 mg of HaNsCv extract. LCMS study depicted presence of flavonoids like quercetin, rutin, etc., which would have been responsible for the observed lithotriptic effect. Results obtained indicate that HaNsCv possess lithotriptic potential useful for management of urolithiasis.

Keywords: Antirolithiatic activity, Ureteric calculi, *Cinnamomum verum*, *Nigella sativa*, *ex vivo*.

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Received: 24-05-2022;

Revised: 07-09-2023;

Accepted: 16-05-2024.

INTRODUCTION

Nephrolithiasis is one of the most prevalent urologic diseases in Asia. The worldwide prevalence, incidence, and composition of calculi vary and have changed in the last several decades, with prevalence ranging from 7% to 13% in North America, 5%-9% in Europe, and 1%-5% in Asia.¹ It is characterized by high morbidity and low mortality but with significant socio-economic impact and serious consequences like severe pain in the back or belly, pain and burning during urination, blood in urine, fever, or chills. Furthermore, individuals who experienced renal stones once, often have a 50% recurrence rate.² Currently, open renal surgery for urolithiasis is unusual and is rarely performed due to the introduction of Extracorporeal Shockwave Lithotripsy (ESWL), which has become a standard treatment to eliminate kidney stones. Shockwaves are used in ESWL to break stones but the traumatic effects of these shockwaves lead to acute renal injury, a decline in the renal function, and an increase in stone

recurrence. At the same time, the cost involved in treatment is also high.³

The allopathic treatments with calcium channel blockers, α -adrenergic blockers, and corticosteroids are also associated with side effects like allergic reaction, severe hypotension, and congestive heart failure. Also, these drugs just ease out the passage of small to medium-sized stones by relaxing the renal tubules and do not act by dissolving renal calculi.

Herbs have been documented in Ayurveda for their kidney stone dissolving activity. A few of them to name are *Bryophyllum pinnatum*,⁴ *Pedaliium murex*,⁵ *Biophytum sensitivum*,⁶ etc. Also, various phytoconstituents have been reported for their anti-urolithiasis activity such are catechin,⁷ epigallocatechin-3-gallate,⁸ diosmin,⁹ rutin,¹⁰ and quercetin.¹¹

Hence leaves of *Cinnamomum verum* and seeds of *Nigella sativa* were selected to probe its lithotriptic effect. *Cinnamomum verum* is known to possess antibacterial,¹² antioxidant,¹³ and anti-inflammatory properties.¹⁴ *Nigella sativa* is known to possess antibacterial,¹⁵ antioxidant,¹⁶ and anti-inflammatory,¹⁷ properties. Thus, efforts have been made in this study to prove the efficacy of leaves of *C. verum* and seeds of *N. sativa* to dissolve ureteric calculi by *ex vivo* method.



DOI: 10.5530/ijper.58.3.93

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Renal calculi formed usually comprise calcium oxalate as a major constituent. Uroliths are generally composed of calcium as calcium oxalate monohydrate and calcium hydrogen phosphate dihydrate (75-90%), magnesium as ammonium magnesium phosphate hexahydrate (10-15%), uric acid and urates (3-10%); and 0.5-1% is composed of cystine, hippuric acid, L- tyrosine and xanthine.¹⁸

In vitro urolithiasis models can evaluate the ability of the test extract or plant compound to dissolve preformed crystals. The crystals are allowed to grow in a suitable medium for a period of time then inhibition or promotion of urinary crystal aggregation and growth usually being observed. Titrimetric estimation measures undissolved calcium oxalate by using KMnO_4 ,¹⁹ Turbidimetric method measures the turbidity in terms of calcium oxalate formation in synthetic urine using a spectrophotometer at 620 nm and crystallization inhibition measured by turbidity reduction.²⁰

But there are some limitations as *in vitro* tests just early-phase testing strategy and cannot provide a mechanistic approach to further explain *in vivo* findings. These models are also unable to provide and represent exact physiological conditions such as the body temperature of animals, the extracellular matrix, or the extent of cell contents.²¹ Generally, the extensive interactions among cells and tissues and other physiological reactions cannot be completely duplicated in a previous model which uses laboratory-made calcium oxalate crystals. The conventional method for *in vitro* analysis of lithotriptic activity makes use of synthetic calcium oxalate crystals. These crystals are devoid of stone matrix and do not mimic real physiology. Understanding this constraint, in this study ureteric calculi procured from human volunteers post lithotripsy were used. Hence *ex vivo* studies using natural ureteric calculi have been carried out. This study will provide a more realistic approach towards testing lithotriptic effect and will account for the matrix composition of the stone in totality.

MATERIALS AND METHODS

Approval of Experimental Protocol

The experimental protocol was approved by the Institutional Ethics Committee of Sir J.J. Group of hospitals and Grant Government Medical College, Mumbai, [Protocol no. IEC/RP/331/Feb/2021] and was carried out in accordance with Good Clinical Practices.

Chemical and Reagents

Ureteric calculi of human volunteers were procured immediately after lithotripsy from the Urology department, Sir J.J. Groups of Hospitals and were stored in airtight containers at room temperature.

0.1 M Tris buffer hydrochloride was procured from Loba chemicals, Mumbai. All the solutions were prepared in distilled

water. Dimensions of ureteric calculi were measured using Vernier callipers [SSU O-125 mm] and weights were recorded using digital balance [Electro lab].

Preparation of the Extracts

The dried leaves of *C. verum* and seeds of *N. sativa* were procured from the local market in Mumbai and were authenticated at Blatter Herbarium under specimen voucher no.916 of M. R Almeida and no. PVK-63 of P.V Kale respectively. Leaves and seeds were crushed and ground to obtain a coarse powder which was subjected to maceration using 70% hydroalcoholic solvent in a 1:5 ratio (solid: solvent) for 48 hr followed by 2 hr of ultrasonication.²² The mass was filtered and excess solvent from each extract was evaporated in a steam bath to obtain a semi-solid mass. All extracts were stored in an airtight container in a refrigerator at 2-8°C. The percent yield of HaCv was found to be 23.2% w/w and that of HaNS was found to be 21.2% w/w.

Collection and Analysis of Ureteric Calculi

Protocol for procurement of ureteric calculi was approved by Institutional Ethics Committee of Sir J.J. Group of hospitals, [Protocol no. IEC/RP/331/Feb/2021]. The calculi were collected following good clinical practice. The longest diameter of collected ureteric calculi was measured (mean diameter=9.8±0.18 mm) and stored at 2-8°C. The collected calculi were analysed qualitatively for confirming presence of calcium and oxalate. 0.5 g of ureteric calculi were powdered and treated with ammonium chloride (NH_4Cl), boiled and cooled to room temperature. To this ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$) was added and observed for white precipitate of calcium carbonate indicating presence of calcium.

For estimation of oxalate prepared sample was treated with dilute acetic acid and calcium chloride (CaCl_2) solution. The development of white precipitate of calcium oxalate was observed. To this dilute nitric acid (dil. HNO_3) was added and observed for appearance clear solution formation indicating dissolution of calcium oxalate formed.

Ex vivo Analysis

HaCV and HaNS were combined at the ratio of 1:1 (HaNsCv) according to the results of *in vitro* experimental results which indicated equipotency of HaCv and HaNs. Hence both the extracts were combined at following four dose levels as follows:

Sample No.	Doses of extract combined
1	25 mg HaNS+25 mg of HaCV (HaNsCv 50).
2	50 mg HaNS+50 mg of HaCV (HaNsCv 100).
3	100 mg HaNS+100 mg of HaCV (HaNsCv 200).
4	200 mg HaNS+200 mg of HaCV (HaNsCv 400).

A combination of extracts at four dose levels was added separately to four separate beakers each containing 200 mL of 0.1 M Tris buffer hydrochloride [pH 7] to simulate renal tissue physiological pH.²³ Ureteric calculi were collected, and their initial weights were accurately recorded using digital balance [Electrolab] and initial diameter was measured using vernier callipers [SSU O-125 mm]. Readings were taken in triplicate and their average mean values were reported for each parameter. Each calculus was dipped individually in four different HaNsCv extract solutions at four doses (Figure 1). For each concentration of HaNsCv the beaker was covered with foil and the whole assembly was kept in an incubator at 37°C to simulate the physiological conditions. Further their diameter was measured with vernier callipers at a time interval of 24 hr. A similar procedure was subjected to control.

RESULTS

Analysis of ureteric calculi

For each dose ureteric calculi of weight $10 \text{ mg} \pm 1.23$ were collected and analysed for presence of calcium and oxalate. Tests confirmed presence of calcium and oxalate for all samples.

Effect on the Dissolution of Ureteric Calculi

Time dependent graded lithotriptic effect was observed at doses 50,100,200, 400 mg of HaNsCv (Table 1). Among four doses used 400 mg initiated dissolution of $15 \pm 1.56\%$ within 24 hr whereas remaining doses exhibited dissolution of ureteric calculi after 24 hr (Figure 2) The dose of 50,100,200, 400 mg exhibited $42 \pm 1.22\%$, $52 \pm 1.12\%$, $82 \pm 1.23\%$ and $100 \pm 1.07\%$ dissolution of ureteric calculi respectively at 216th hr (9th day) (Figure 2) of incubation. Thus, complete dissolution of ureteric calculi was observed at 400 mg on 9th day of incubation (Figure 3).

LCMS Profiling of HaNsCv for the Presence of Phytoconstituents

The LCMS studies indicated the presence of various phytoconstituents like flavonoids such as quercetin, rutin, afzelin,

etc., in abundance along with pentacyclic triterpenes like lupeol and ursolic acid (Figure S1).

DISCUSSION

Current treatments for urolithiasis are majorly symptomatic and or involve surgical interventions, hence there is a need to probe an effective alternative treatment for urolithiasis. As urolithiasis is also associated with inflammation, bacterial infections, and severe pain, supportive multiple drug therapy is also required which includes the use of spasmolytic, antibiotics, opioids, and Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) thereby increasing the risk of side effects. Herbs are composed of multiple phytoconstituents with an array of therapeutic potentials and hence can offer an effective treatment option for urolithiasis along with minimization of side effects. *Cinnamomum verum* and *Nigella sativa* are commonly used herbal condiments in India. Ayurveda, Unani and other traditional medicinal literature report the antiurolithiatic effect of *Cinnamomum verum* and *Nigella sativa*.²⁴ In addition, both the condiments are reported in the literature to possess anti-bacterial, anti-inflammatory,²⁵ and diuretic properties,^{26,27} which can play a vital role in the management of complications associated with urolithiasis thereby minimizing the use of multiple drug therapy and associated side effects.

The conventional allopathic drugs act either by vasodilation and or diuresis thereby flushing out occluded ureteric calculi. Shock wave therapy disintegrates preformed larger stones into smaller fragments which are surgically removed. However, remnants pose a risk of recurrence. Presently no therapeutic treatment is available which can dissolve the ureteric calculi and thus can prevent the recurrence of kidney stones. The present study attempts to evaluate the therapeutic potential of *Cinnamomum verum* and *Nigella sativa* to dissolve ureteric calculi. To perform this study ureteric calculus of human origin were isolated from patients suffering from urolithiasis post lithotripsy at JJ Hospital, Mumbai. Previously performed *in vitro* studies on synthetic calcium oxalate crystals have revealed an equipotent



Figure 1: Ex vivo lithotriptic evaluation..

Table 1: Mean percent dissolution of ureteric calculi at various doses and time intervals.

Dose of HaNsCv (mg)	Mean Percent Dissolution (%)									
	Time Intervals in Hr									
	0 hr	24 hr	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr	192 hr	216 hr
0	0	0	0	0	0	0	0	0	0	0
50	0	0	9±1.26	14±1.65	17±1.55	22±1.26	24±1.45	31±1.55	40±1.43	42±1.22
100	0	0	12±1.54	16±1.35	20±1.15	27±1.55	33±1.26	49±1.07	52±1.15	52±1.12
200	0	0	24±1.27	33±1.56	40±1.45	43±1.48	47±1.78	64±1.43	71±1.55	82±1.23
400	0	15±1.56	29±1.43	42±1.54	49±1.65	58±1.85	72±1.71	85±1.65	93±1.26	100±1.07

N=3 (dissolution was observed in three ureteric calculi for each dose). mean percent dissolution (%) = $\frac{(\text{size of the ureteric calculi at 0 hrs} - \text{size of the calculi at } t \text{ hrs})}{\text{size of the calculi at 0 hrs}} \times 100$.

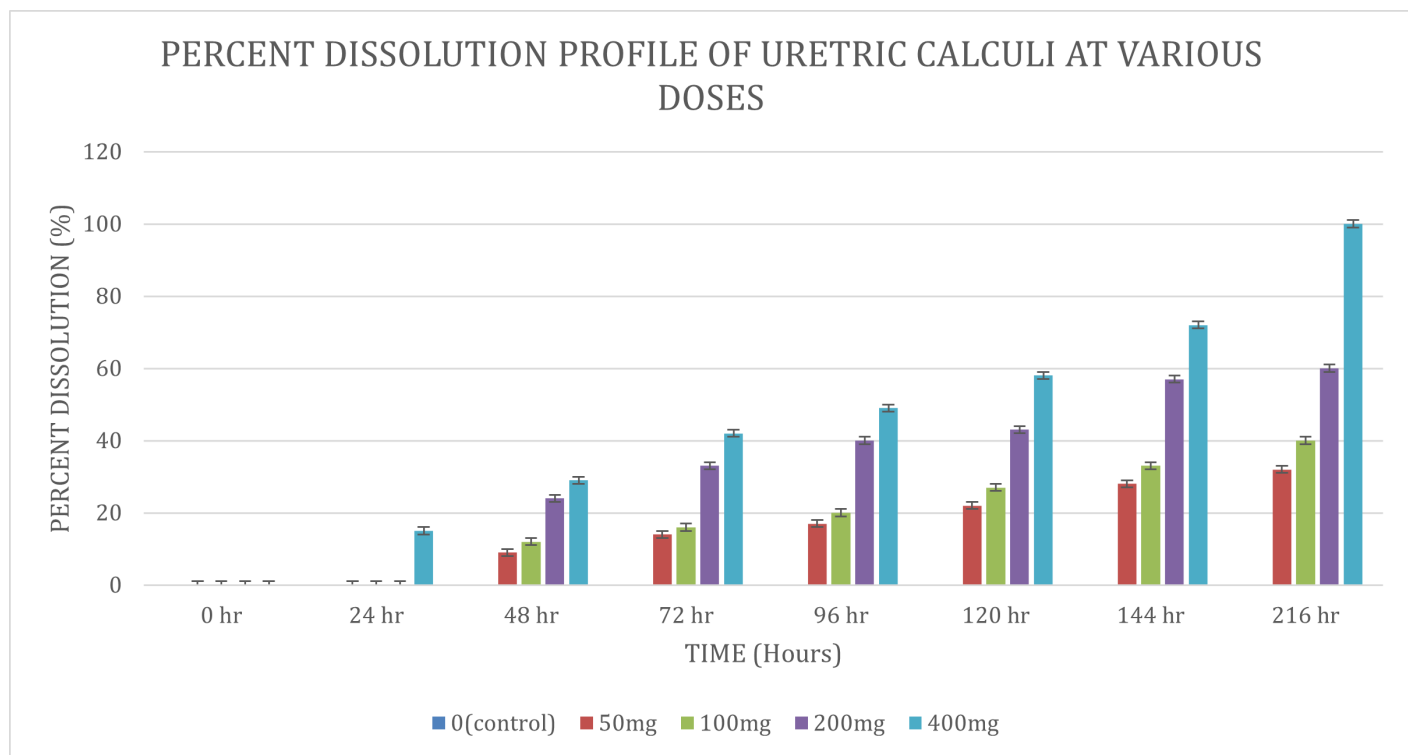


Figure 2: Percent dissolution profile of ureteric calculi at various doses and time intervals. The X-axis represents time points in hr. The Y-axis represents the mean percent dissolution (%) N=3 (dissolution was observed in three ureteric calculi for each dose). The vertical bar indicates the standard deviation of 3 readings.

lithotriptic effect of hydroalcoholic extracts of *C. verum* and *N. sativa* within 8 hr. Thus, in the present study on basis of this *in vitro* data combination of hydroalcoholic extract *C. verum* and *N. sativa* (HaNsCv) was prepared in the ratio of 1:1 respectively. The present study revealed the initiation of dissolution of ureteric calculi within 24 hr after incubation. Among various doses (50, 100, 200, and 400 mg) tested for *ex vivo* dissolution of ureteric calculi, dissolution was found to be initiated within 24 hr at a dose of 400 mg whereas, for all other concentrations, dissolution of ureteric calculi was observed after a period of 48 hr. Graded and dose depended dissolution of ureteric calculi was observed. Complete dissolution of ureteric calculi was obtained at 400 mg

over an incubation period of 216 hr (9 days). These results thus reveal the lithotriptic effect of HaNsCv.

LCMS studies of a combination of extracts HaNsCv revealed the presence of ursolic acid which is reported in the literature to possess antiurolithiatic activity.²⁸ Flavonoids like rutin, afzelin, and quercetin found in the extracts are reported in the literature for their lithotriptic activity via multiple modes of mechanisms viz. maintaining the balance of oxalate metabolism by decreasing the enzyme synthesizing oxalate, diuresis and facilitating the dissolution of ureteric calculi by shifting the crystal structure of Calcium Oxalate Monohydrate (COM) which is more stable and less soluble to a lesser stable and more soluble Calcium

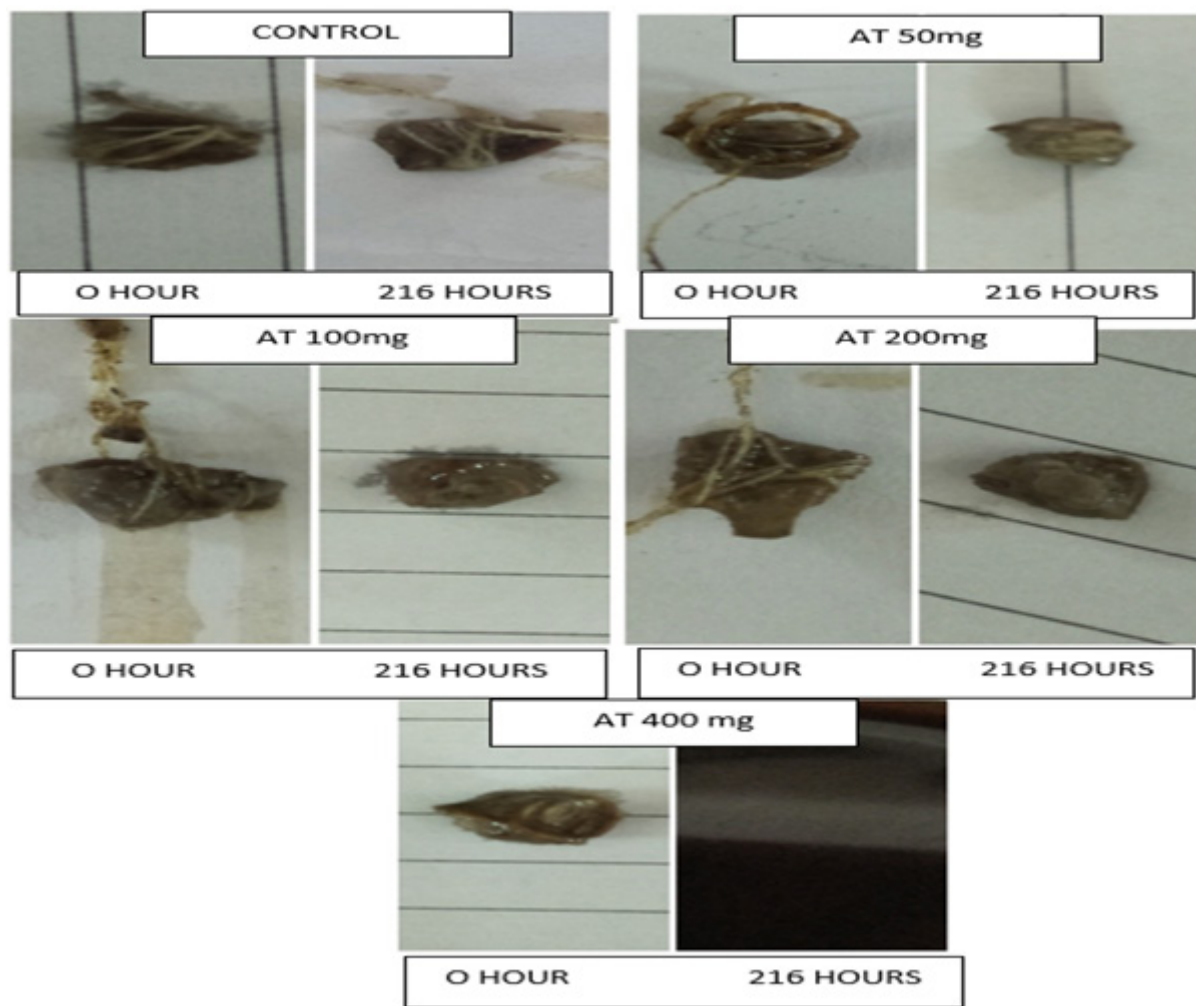


Figure 3: Sample calculi before and after the study at various doses.

Oxalate Dihydrate (COD) form.²⁹ Flavonoids are also proven to modulate the synthesis and expression of stone-promoting and inhibiting factors.³⁰ Flavonoids found in the extract HaNsCv possess antibacterial activity which can additionally play a vital role in the management of urolithiasis-associated infection. Scopoletin, a coumarin found in *C. verum* is reported to possess anti-inflammatory, antioxidant, and antibacterial properties.³¹ Rutin, kaempferol, and quercetin found in extracts are reported to possess Angiotensin-Converting Enzyme (ACE) inhibition activity *in vitro*.³² Renin-Angiotensin-Aldosterone System (RAAS) causes activation of NADPH oxidase in renal cells resulting in the production of ROS. Inhibition of Angiotensin-Converting Enzyme (ACE-I) thus decreases ROS production thereby significantly reducing calcium oxalate crystal deposition and consequently renal inflammation.^{33,34} The observed lithotriptic effect in present study might be an integration of these multiple modes of action exerted by various active phytoconstituents present in extract HaNsCv. Thus, HaNsCv possesses the ability to dissolve ureteric calculi and has a promising therapeutic potential in the treatment of urolithiasis.

CONCLUSION

The results obtained thus, indicate the significant lithotriptic therapeutic potential of HaNsCv. This effect thus plays a vital role dissolving ureteric calculi. To evaluate the antiurolithiatic effect of HaNsCv *in vivo* studies will be required to be conducted in the future to probe its exact mode of action and other pharmacological effects.

ACKNOWLEDGEMENT

Urology Department of Sir J. J groups of Hospitals.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

C. verum: *Cinnamomum verum*; **N. sativa:** *Nigella sativa*; **HaNsCv:** Hydroalcoholic extract of *Cinnamomum verum* and *Nigella sativa*; **hr:** Hour; **LCMS:** Liquid Chromatography and mass spectroscopy; **ESWL:** Extracorporeal Shockwave Lithotripsy;

KMnO₄: Potassium permanganate; **NSAIDs**: Nonsteroidal anti-inflammatory drugs; **COM**: Calcium oxalate monohydrate; **COD**: Calcium Oxalate Dehydrate; **ACE**: Angiotensin-converting enzyme; **RAAS**: Renin-angiotensin-aldosterone system; **ROS**: Reactive oxygen species.

SUMMARY

Nephrolithiasis is one of the most prevalent urologic diseases in Asia. Renal calculi formed in urolithiasis usually comprise calcium oxalate as a major constituent. Conventional *in vitro* urolithiasis models using calcium oxalate crystals to evaluate lithotriptic effect of plant extracts are devoid of stone matrix and do not mimic real physiology. Understanding this constraint, present study involves use of ureteric calculi procured from human volunteers post lithotripsy. Hydroalcoholic extracts of *Cinnamomum verum* and *Nigella sativa* were selected to probe their lithotriptic effect. *Cinnamomum verum* and *Nigella sativa* are reported in literature for antibacterial, antioxidant, and anti-inflammatory properties. HaCV and HaNS were combined at the ratio of 1:1 (HaNsCv) at four dose levels and incubated with ureteric calculi. Dose dependent graded lithotriptic effect was observed at doses 50,100,200, 400 mg of HaNsCv. Complete dissolution of ureteric calculi was obtained at 400 mg over an incubation period of 216 hr (9 days). These results thus reveal the lithotriptic effect of HaNsCv. LCMS studies of a combination of extracts HaNsCv revealed the presence of ursolic acid, flavonoids like rutin, afzelin, and quercetin which are reported in the literature for their lithotriptic activity via multiple modes of mechanisms. Thus, the present phytoconstituents impart lithotriptic efficacy to HaNsCv and thus has a promising therapeutic potential in the treatment of urolithiasis.

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Cite this article: Sanaye M, Khan N, Dhodi D, Parveen S. Lithotriptic Effect of *Cinnamomum verum* and *Nigella sativa* on Ureteric Calculi. *Indian J of Pharmaceutical Education and Research*. 2024;58(3):854-60.

SUPPLEMENTARY DATA

LCMS Analysis Data

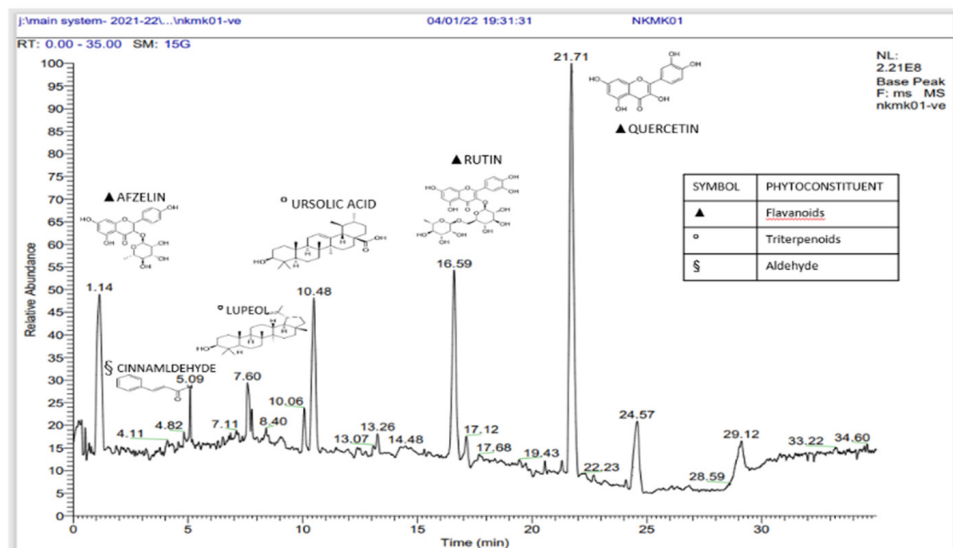


Figure S1: LCMS chromatogram of HaNsCv showing the presence of various phytoconstituents.

In vitro testing for Antiurolithiatic activity of HaNs and HaCv and HaNsCv

Antiurolithiatic activity of HaNs as per in vitro analysis

Doses (mg)	8 hr (% dissolution)	18 hr (%dissolution)	24 hr (% dissolution)
12.5	62.45±0.11	71.26±0.23	69±0.11
25	85.5±0.26	86.1±1.20	87±0.48
50	98.2±0.48	98.68±0.23	98.9±1.26
100	98.34±0.11	98.68±0.15	98±0.78
200	98.5±0.56	98.87±0.18	98.1±0.26

Antiurolithiatic activity of HaCv as per in vitro analysis

DOSE (mg) HaNs+HaCv (mg)	8 hr (% dissolution)	18 hr (% dissolution)	24 hr (% dissolution)
3.15±3.15	65.59±0.35	74.15±0.28	81.48±0.58
6.25+6.25	73±0.18	78.5±1.26	87.2±0.84
12.5+12.5	87±0.23	90±0.26	92.06±0.48
25+25	97.22±0.45	98.26±0.65	98±0.28
50+50	98.62±0.41	98.3±0.58	98.64±1.03

Antiurolithiatic activity of HaCv as per in vitro analysis

DOSE (mg)	8 hr (% dissolution)	18 hr (% dissolution)	24 hr (% dissolution)
12.5	59.59±0.45	69.15±0.28	72.48±0.58
25	74±0.18	78.5±1.26	80.2±0.84
50	87±0.48	88±0.26	89.06±0.48
100	97.22±0.45	98.2±0.65	98±0.28
200	98.56±0.48	98.3±0.58	98.64±1.03