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Evaluation of *in-vitro* anti-urolithiasis activity of *Nerunjil kudineer*

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Abstract

Purpose: *Nerunjil kudineer* is a Siddha Herbal formulation was carried out to evaluate the anti-urolithiatic activity.
Materials and methods: Homogenous precipitation method was used to prepare artificial stones such as calcium oxalate and calcium phosphate and semi-permeable membrane of eggs was used as dissolution bags. Dissolution models were incubated in 72 hrs and after that, the entire content in dissolution bags was estimated spectrophotometrically. The inhibitory activity of *Nerunjil Kudineer* on the nucleation of calcium-oxalate crystals (a major component of many renal stones) and calcium phosphate crystals and its rate of aggregation was determined by spectrophotometric assay.
Results: The effect of *Nerunjil kudineer* exhibited the inhibition of calcium oxalate and calcium phosphate crystal formations.
Conclusion: The present studies suggest that *Nerunjil kudineer* has a potential inhibition of calcium oxalate and calcium phosphate crystal formations in *In-vitro* studies. Further procedure of *In-vivo* study about *Nerunjil kudineer* may be required. Correlation between *in-vitro* and *in-vivo* studies may be helpful to understand the molecular mechanism of litholysis process and to reveal phytochemicals of the *kudineer* responsible for dissolving or disintegrating renal calculi.

Keywords: *Nerunjil kudineer*, calcium oxalate, calcium phosphate, Anti-urolithiatic activity, Nucleation assay, Aggregation assay.

Introduction

Urolithiasis is also called as Uroliths or Calculi. Urolithiasis is stone formed or located in any part of urinary tract. Calculi formation in urinary bladder, ureter or any part of urinary tract rather than kidney is known as urolithiasis while nephrolithiasis is characterized calculi formation in kidney. Nowadays stone formation is very common and it is a serious painful urologic disease due to change in lifestyle and dietary factors.

Urinary Calculi can be treated by traditional system of medicines especially the Siddha medicine. In Siddha system, urolithiasis was treated well effectively without surgery.

Nerunjil kudineer is one of the Siddha herbal formulations which is used to treat Renal calculi, Burning micturation, Stricture of urethra and Anuresis. *Nerunjil kudineer* contains fruits of *Nerunjil* (*Tribulus terrestris Linn*) and seeds of *Koththumalli* (*Coriandrun sativum).*

Tribulus terrestris (zygophyllaceae) – seeds are used as diuretic in afflictions of urinary discharges and impotency. It is used in kidney stones, gout, uterus disorders, calculous affection, etc. seeds are also stomachic. *Coriandrum sativum* (Umbelliferae) – seeds are diuretic, tonic, carminative, stomachic, antibilious, refrigerant and aphrodisiac.

It cures a host of digestive disorders ranging from indigestion, nausea, dysentery, colitis to diarrhea and chronic dysentery. Coriander leaves stimulate the appetite and act as a tonic.

The present study was undertaken to investigate the effect of *Nerunjil kudineer* on in-vitro urolithiasis using semi-permeable membrane of eggs in dissolution model and its anti-crystallization capacity through analyzing nucleation and aggregation assays.

Materials and Methods

Chemicals:

Sodium oxalate, p-phenylene diamine, calcium chloride dehydrate were purchased from Alpha Chemicals Ltd. Potassium permanganate, tris-buffer and sodium meta-bisulfite were purchased from Anju Chemicals Ltd. Cystone® was purchased from Himalaya Drug Company.

The dry raw materials *(Tribulus terrestris)* fruit and (*coriandrum sativum)* seeds were obtained from the raw country drug shop at Nagercoil, Kanniyakumari district and local market in Palayamkottai, Tirunelveli district. The raw drugs were authenticated by the experts from the Dept. of Gunapadam, Govt.Siddha medical college, Palayamkottai, Tirunelveli.

Selection of the drug:

The formulation "Nerunjil kudineer" was selected from the Siddha literature "Gunapadam Mooligai Vagupu" authored by Vaidhiya Rathinam K.S.Murugesa mudaliyar.

Preparation of *Nerunjil Kudineer*:

Take 68 grams of *Nerunjil* (*Tribulus terrestris Linn*) and 8 grams of *Koththumalli* (*Coriandrum sativum*). Both are mixed thoroughly and made it into a coarse powder and then it possessed through the sieve number 60. Then add 680 grams (660 ml) of water into the mixture and boiling well until the amount of the water reduced to half measure. Then cool it. Now the *Nerunjil kudineer* is prepared.

Preparation of Semi-permeable membrane from the Eggs:

Apex of eggs was punctured by a glass rod in order to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting 4ml concentrated HCl in 200ml distilled water.

It was kept for overnight which led to the complete decalcification of semi permeable membrane.

On the next day, semi permeable membranes were removed carefully from egg shells; washed thoroughly with distilled water and placed it in ammonia solution for neutralization of acid traces, and then rinsed it with distilled water.

It was stored in the refrigerator at a pH of 7-7.4 in the moistened condition.

10mg of the calcium oxalate was suspended in 10ml of distilled water as negative control.

5ml of *Nerunjil Kudineer* was taken.

500mg tablet of Cystone® was placed in absolute ethanol for removing colour coating and 400mg was obtained. Cystone® tablet was crushed into powder form and dispersed into 100ml of distilled water and filtered. Filtrate of Cystone® was used as positive control for *in vitro* anti-urolithiatic activity.

Spectrophotometric estimation of calcium oxalate by using dissolution model:

Synthesis of calcium oxalate by homogenous precipitation

1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water and 1.34gm of sodium oxalate was dissolved in 100 ml of 2N H_2SO_4. Both were mixed equally in a beaker to precipitate out calcium oxalate with constant stirring. The resultant calcium oxalate was freed from traces of sulphuric acid by ammonia solution and washed with distilled water. Then dried it at a temperature of 60 °C for two hours.

Preparation of 0.02M KMnO4 solution:

0.32gm of $KMnO_4$ was dissolved in 100ml of distilled water. It was boiled for 30min. After cooling, excess of MnO_4 was removed by filtration.

Methods:

Group I	1 ml of Calcium oxalate (1 mg/ml)+ 1 ml of Distilled Water
Group II	1 ml of Calcium oxalate (1 mg/ml)+ 1 ml of Cystone solution (400 mg/ml)
Group III	1 ml of Calcium oxalate (1 mg/ml)+ 1 ml of *Nerunjil Kudineer* (20mg/ml)

All groups were packed it together in egg semi permeable membrane tied with thread at one end and were suspended in a conical flask containing 150 ml 0.1 M Tris buffer each. At another end of thread tied by a stick placed on the mouth of conical flask and covered with aluminum foil.

All groups were kept in an incubator, pre heated to 37^0C for 4 hours, kept for three days. The entire content of each group was removed from sutured semi permeable membrane and was transferred into test tube individually.

4 ml of 1N H_2SO_4 and 60-80µl of 0.02M $KMnO_4$ were added and kept aside for 2 hours. Colour change from dark pink to colourless was observed after 2 hours. Change of colour intensity was measured against 620nm spectrophotometrically.

Concentration of undissolved calcium was determined from standard calibration curve of calcium oxalate by using the measured absorbance readings as shown in Graph 1.

Synthesis of calcium phosphate by homogenous precipitation:

1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water and 1.42gm of disodium hydrogen phosphate was dissolved in 100 ml of 2N H_2SO_4. Both the solutions were mixed equally in a beaker. A precipitate of calcium phosphate can be obtained by constant stirring the solutions.

The resultant calcium phosphate was freed from traces of sulphuric acid by ammonia solution then washed with distilled water and dried up at a temperature of 60 °C for two hours.

Preparation of Molybdate-sulphuric acid reagent:

Molybdate-sulphuric acid reagent was prepared by adding 5% w/v of sodium molybdate solution and 13ml of conc. H_2SO_4 in 80ml of distilled water. Finally the volume was adjusted to 100ml with distilled water.

Preparation of reducing solution:

1gm of p-phenylene diamine was dissolved in 100 ml of 3 % w/v of sodium meta-bisulfite solution.

Methods:

Group I	1 ml of Calcium phosphate (1 mg/ml)+ 1 ml of Distilled Water
Group II	1 ml of Calcium phosphate (1 mg/ml)+ 1 ml of Cystone solution (400 mg/ml)
Group III	1 ml of Calcium phosphate (1 mg/ml)+ 1 ml of *Nerunjil Kudineer* (20mg/ml)

All the three groups were packed it together in an egg semi permeable membrane and tied it with thread at one end and were suspended in a conical flask containing 150 ml 0.1 M Tris buffer each.

The other end of the thread was tied by a stick placed on the mouth of conical flask and covered with aluminum foil. All groups were kept in an incubator; pre heated to 37^0C for 4 hours then kept it for three days.

The entire content of each group was removed from the sutured semi permeable membrane and was transferred into the test tube individually. 4ml of 1N H_2SO_4 and 3ml of molybdate-sulphuric acid reagent, 1 ml of reducing solution were added and kept aside for 2 hours. Colour change from dark pink to colourless was observed after 2 hours. Change of colour intensity was measured against 620nm spectrophotometrically.

Concentration of undissolved calcium was determined from standard calibration curve of calcium phosphate by using the measured absorbance readings as shown in Graph 2.

In a pilot study, we found that the inhibition of nucleation and aggregation by the Nerunjil kudineer was not effective at below 5mg/ml. Nerunjil kudineer exhibited good inhibition activity above at >10mg/ml, and thus concentrations 10-60mg/ml have prepared in the present study. Cystone® was dissolved in distilled water to give concentrations of 10-60mg/ml for both the nucleation and aggregation assays.

Nucleation assay (Turbidity method):

The inhibitory activity of the *Nerunjil kudineer* on the nucleation of calcium oxalate crystals was determined by a spectrophotometric assay.

Crystallization was initiated by adding 100µl of 4 mM calcium chloride and 100µl of 50 mM sodium oxalate solutions to 0.5ml of human normal urine, both prepared in a buffer containing 0.5ml of 0.05 mM Tris buffer and 0.5ml of 0.15mM NaCl solution at pH 6.5 and 37^0C and adjusted to volume by adding 1.5ml of distilled water.

The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the kudineer and that of the control with no kudineer.

The optical density (OD) was recorded at 620nm, and the percentage inhibition calculated as (1-OD (experimental)/OD (control))/100.

Aggregation assay:

The rate of aggregation of the calcium oxalate crystals was determined by a spectrophotometric assay with slight modifications.

The calcium oxalate monohydrate (COM) crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate of 50 mM each.

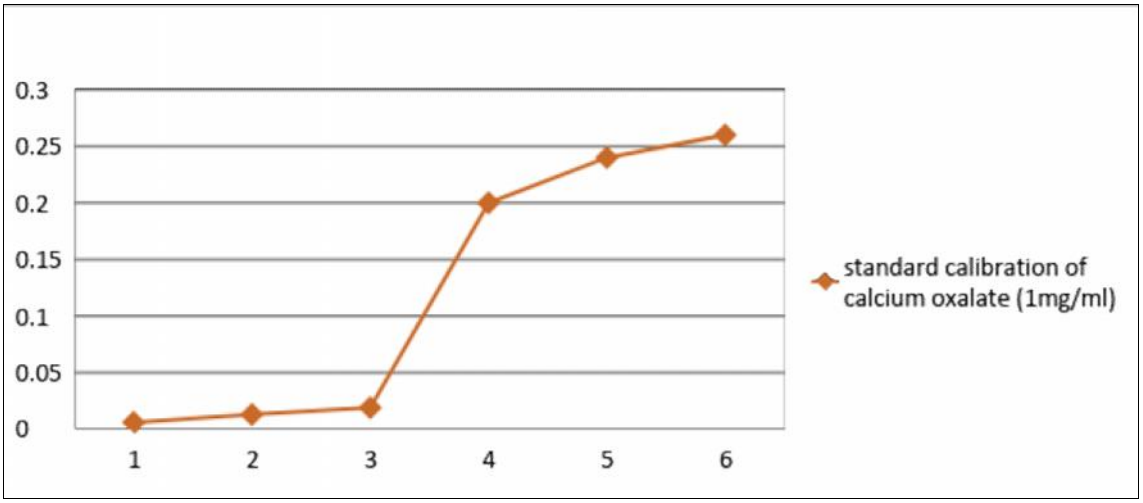
Both solutions were then equilibrated.

The solutions were then cooled to 37^0C and then evaporated. The COM crystals were then dissolved with 0.5ml of 0.05mM Tris buffer and 0.5ml of 0.15mM NaCl solution at pH 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded.

The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the kudineer against control.

Results and Discussion

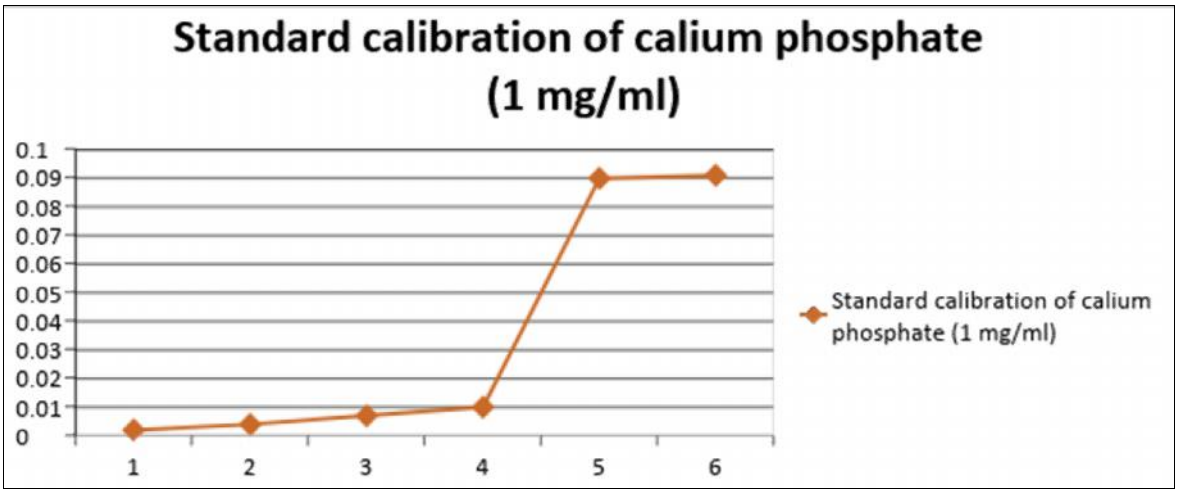
Spectrophotometric Assay:



Graph: 1

Spectrophotometric estimation of calcium oxalate:

The *Nerunjil kudineer* has greater capability to dissolve calcium oxalate as foremost element for stone forming in urinary tract as shown in the Graph: 1.



Graph: 2

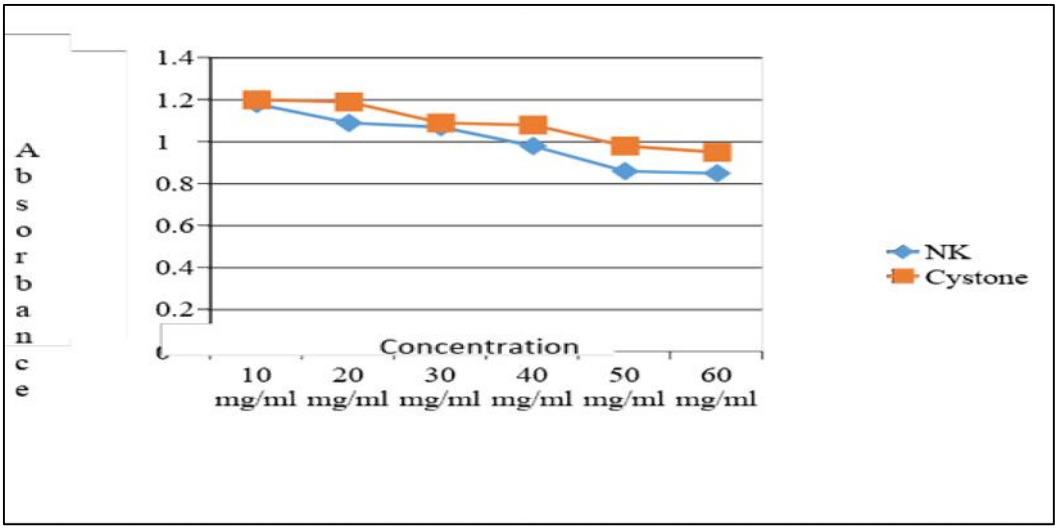
Spectrophotometric estimation of calcium phosphate:

The *Nerunjil kudineer* has greater capability to dissolve calcium phosphate as secondary element for stone forming in urinary tract as shown in the Graph: 2.

Nucleation assay:

Table 1: Nucleation Assay

S. No:	Concentration	Absorbance (620nm)	
		Nerunjil kudineer	Cystone solution
1.	10 mg/ml	1.18	1.20
2.	20 mg/ml	1.09	1.19
3.	30 mg/ml	1.07	1.09
4.	40 mg/ml	0.98	1.08
5.	50 mg/ml	0.86	0.98
6.	60 mg/ml	0.25	0.95



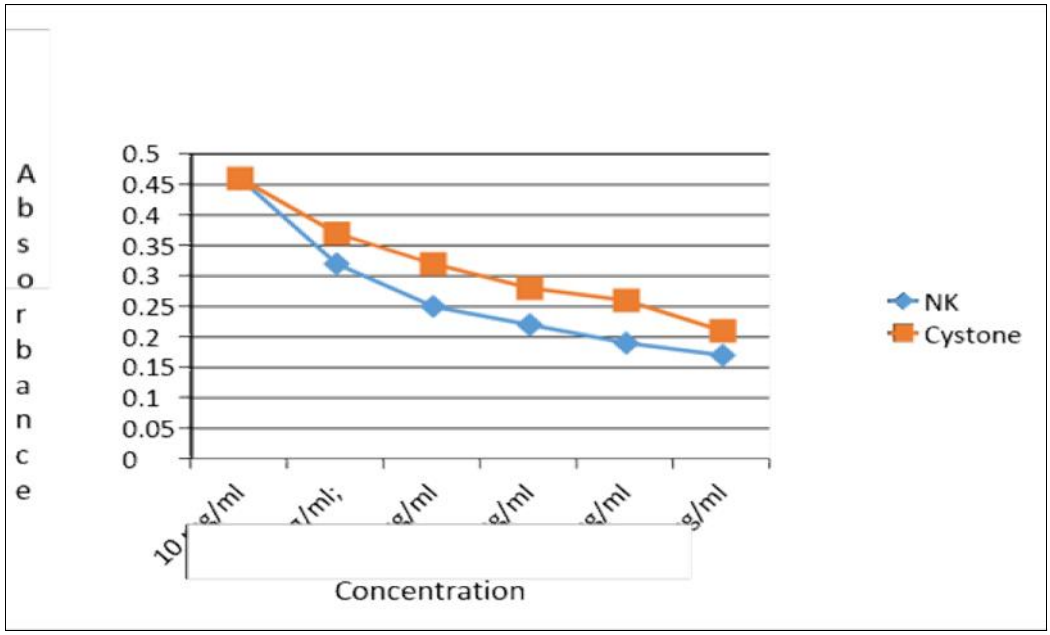
Graph 3: Nucleation Assay

The above table and graph shows that the anti-urolithiatic activity of *Nerunjil kudineer* and the cystone solution through the Nucleation assay method. The result shows that the *Nerunjil kudineer* has the significant anti-urolithiatic action. But the anti-urolithiatic activity of *Nerunjil kudineer* has slightly reduced when compared to the standard cystone solution

Aggregation Assay:

Table 2: Aggregation Assay

S. No:	Concentration	Absorbance (620nm)	
		Nerunjil kudineer	Cystone solution
1.	10 mg/ml	0.46	0.46
2.	20 mg/ml	0.32	0.37
3.	30 mg/ml	0.25	0.32
4.	40 mg/ml	0.22	0.28
5.	50 mg/ml	0.19	0.26
6.	60 mg/ml	0.17	0.21



Graph 4: Aggregation Assay

The above table and graph shows that the anti-urolithiatic activity of *Nerunjil kudineer* and the cystone solution through the Aggregation assay method. The result shows that the *Nerunjil kudineer* has the significant anti-urolithiatic action. But the anti-urolithiatic activity of *Nerunjil kudineer* has slightly reduced when compared to the standard cystone solution.

No pharmacologic intervention has definitively been shown to be effective for lithiasis. The present investigation will be supportive as additional information to the scientific evidences regarding *in-vitro* studies. Since mechanism of anti-urolitholytic activity in the *Kudineer* is exact unknown till date, correlation between *in-vitro* and *in-vivo* studies should be further investigated to reveal the phytochemicals of the *Nerunjil kudineer* are responsible for dissolving or disintegrating renal calculi and to know better understanding in the molecular mechanism of litholysis.

Conclusion

Nerunjil kudineer exhibited the significant anti-urolithiatic activity in *In-vitro* study.

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