# *In Vitro* Effects of *PHYLLANTHUS NIRURI* Extract on Urolithiasis

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## Abstract

Urolithiasis is a condition that involves the formation of stones in the kidneys, bladder and / or urethra. Kidney stone is one of the oldest and most widespread diseases known to man. Many stones are asymptomatic until they start to move down from the urethra, causing pain due to blockage. The pain starts abruptly when a stone moves in the urinary tract, causing irritation or obstruction.

Usually, a person experiences sharp, tingling pain in the area of the kidneys or in the back and side of the abdomen. Sometimes nausea and vomiting occur. Additional corporeal shockwave lithotripsy, ultrasonic waves or shock waves are used to break up the stones so that they can be excreted in the urine or removed through an endoscope. Lithotripsy may be an alternative to surgery.

Medicinal plants play a significant role in various ancient traditional methods of medicine. Medicinal plants are the main source of new medicines that are considered to be very safe, with few or no side effects. Inhibition of the formation of calcium oxalate crystals .

The in vitro anterolytic function was performed by single diffusion gel growth technology, dual diffusion gel growth techniques and turbidometric method. The growth of hydroxyapatite crystals in the presence of 3% leaf methanol extract has a relatively short diffusion length and is more resistant to growth than 1%, 2% leaf methanol extract and cystone. At the same time in the study of growth inhibition of calcium hydrogen phosphate dihydrate crystal in the presence of 3% leaf methanol extract had a much higher growth inhibition capacity than different concentrations of leaf extracts and cystone. Similarly 3% leaf methanol extract produced

maximum inhibition of calcium oxalate crystal growth over different concentrations of leaf juice and cystone. In the turbidometric method, the maximum growth block was removed in 15mg / 5ml of leaf methanol extract rather than in different concentrations of leaf extract and cystone. Overall b. To clarify the chemical components of the extract and the mechanism responsible for its anterolytic activity.

# Introduction

### Urolithiasis

Urolithiasis (from Greek *oûron*, "urine", + *lithos*, "stone", + *-iasis*) is the formation of urinary calculi or urinary stones, which are formed or located anywhere in the urinary system (Pearle *et al*., 2007). It comprises of nephrolithiasis (the formation of kidney stones), ureterolithiasis (the formation of stones in the ureters) and cystolithiasis (the formation of bladder stones). Stone formation in kidney is one of the oldest and the most widespread medical problem known to humans. Urinary calculi have been found in Egyptian mummies dating back to 4000 BC and in the remains of 1500-1000 BC old North American Indians. Reference to stone formation is also made in early Sanskrit documents from India between 3000 and 2000 BC (Patankar *et al*., 2008). Uolithiasis is a recurrent renal disease which affects 4 % - 8 % of the population in UK, 15 % in US, 20 % in the Gulf countries and 11 % of the population in India. The disease affects all age groups from less than 1 year old to more than 70, with a male to female ratio of 2:1(Chauhan *et al*., 2009).

Approximately 80% of these calculi are composed of calcium oxalate and calciumphosphate (Daudon et al., 1993). Calcium oxalate crystals, primary constituent of humanrenal stones, exist in the form of calcium oxalate monohaydrate (COM) and calciumoxalate dihydrate (COD). The stone formation requiressupersaturated urine which depends on urinary pH, ionic strength, concentration ofcalcium, phosphate, oxalate, sodium and uric acid ions and complexations. Varioussubstances in the body have an effect on one or more of the above processes, therebyinfluencing a person’s ability to promote or prevent stone formation. Hydroxyapatite, Struvite, Brushite, Uric acid stone are also found in urinarysystem (Pathak et al., 2010).

# Materials and Methods

### Plant collection and extraction

The leaves of *Phyllanthus niruri* were collected fromPerambalur in December, 2016. Ten grams of dried powder was first defatted with petroleum ether and thenindividually extracted with ethyl acetate, methanol and water by using Soxhlet apparatus(Lin et al., 1999). The solvent was evaporated to dryness and the dried crude extract wasstored in air tight bottle at 4°C. The extraction was done at least three times and the mean values are presented. Then the extract was reconstituted with distilled water to produce the desired concentrations and used for further analysis.

### Phytochemical analysis

Phytochemical analysis (qualitative) was performed using standard procedures (**Trease and Evans., 1989**; **Sofowora, 1993**).

### *In vitro* antiurolithiatic studies

### Determination of hydroxyapatite crystal growth (HP)

The single diffusion gel growth technique was used to grow hydroxyapatite crystals (Parekh et al., 2014). Analytical grade sodium meta-silicate powder was used for preparation of the gel medium. To remove the impurities, 250 g sodium meta-silicate was dissolved in one liter of water in a beaker. On stirring it thoroughly, a dense milky solution of sodium meta-silicate was formed. It was left for a couple of days, so that heavy insoluble impurities could accumulate at the bottom of beaker. This was decanted into another beaker and filtered twice with Whatman filter paper no. 1 of 12.5 cm diameter. Then the solution was centrifuged on MSE high speed centrifuge unit for half an hour at 10,000 rpm. Practically the solution got rid off all suspended impurities and as a result, transparent, slightly golden colored solution of sodium meta-silicate was obtained. Sodium metasilicate solution of specific gravity 1.06 was acidified by 1N orthophosphoric acid so that the pH of the mixture could be set within 6.0- 6.5. The pH values

selected were between 6.0 to 6.5. This solution was transferred to different test tubes for setting the gel.

Glass test-tubes of 25 mm diameter and 150 mm length were used as crystal growth apparatus. The above mentioned mixture was poured in equal volumes in different test tubes and allowed to set into the gel form. Within 48 h good quality gel was set. For growth inhibition studies, 1%, 2% and 3% bark methanol extract was added along with 1 M calcium chloride solution as the supernatant solution to be poured on the set gels. Good quality, very small crystals were found in the form of liesegang rings. The distance between two liesegangs rings and thickness of liesegang ring in test tube were measured at different time intervals by using traveling microscope having a least capacity of measurement 0.001 cm.

**Determination of *in vitro* antiurolithiatic activity by turbidometric method**

The effect *P. niruri*leaf methanol extract on calcium oxalate crystallization was determined by the time course measurement of turbidity changes due to the crystal formation and aggregation in the metastable solutions of calcium and oxalate. Stock solutions of calcium chloride (8 mM/L) and sodium oxalate (1 mM/L), containing 200mM/L sodium chloride and 10mM/L sodium acetate were adjusted to pH 5.7. The second centrations were chosen because they are close to physiological urinary concentrations. pH value was adjusted to 5.7. The calcium chloride solution (5 ml) was stirred constantly both in the absence and presence of different concentrations (5 mg, 10mg, 15 mg) of leaf, stem and bark extract of *P. niruri*and standard drug (cystone) atroom temperature. After obtaining a stable base line, crystallization was induced by the addition of sodium oxalate solution (5 ml) to obtain the final concentration of calcium as4.25 mM and oxalate as 0.75 mM. The percentage inhibition was calculated by comparing the turbidity in the presence of different concentrations of leaf extract using the formula:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{Turbidity of sample}}{\text{Turbidity of control}}\right) \times 100$$

To determine the effect of incubation with the test material on calcium oxalate crystalformation, stock solutions of calcium chloride and sodium oxalate having compositionsimilar to those in the kinetic study were used. The optical density was measured at over30 min at 620 nm using a digital spectrophotometer (Shimadzu UV-1601, Japan).

**Statistical analysis**

The results are expressed as mean ± SEM. The statistical significance was assessed using One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison tests and value of $p < 0.05$ was considered significant.

# Results and Discussion

The standardization of a crude drug is an integral part for establishing its correct identity. Before any crude drug can be included in an herbal pharmacopeia, pharmacognostic parameters and standards must be established.

**Table 1. Qualitative analysis of *Phyllanthus niruri* flower extract with various solvents**

| S. No | Test | Aqueous extract | Methanolic extract |
|---|---|---|---|
| 1. | Carbohydrate | + | + |
| 2. | Saponins | + | + |
| 3. | Flavonoid | ++ | ++ |
| 4. | Anthocyanin & β Cyanin | ++ | + |
| 5. | Phenol | + | ++ |
| 6. | Acid | - | + |
| 7. | Protein & Amino acids | + | + |
| 8. | Alkaloids | + + | + + |

+ Presence, ++ Rich, - Not present

**Hydroxyapatite crystal growth (HA)**

In the present study, three different concentrations of leaf extract (1%, 2% and 3%) were used for growth inhibition study of HA crystal. Photographic image of HA crystals in the form of liesegang rings in different concentrations of leaf methanol extract is given in Fig 1.

The comparison was done by comparison of liesegang ring thickness, distance between two rings, number of rings and diffusion length between control and different concentrations of leaf extract. The pure $CaCl_2$ gave thick and sharp rings with deep diffusion in the gel. It served as control test. In 1 % leaf extract, thick sharp rings were formed in the gel; but thickness of ring was less than that of the control test (Fig. 2) whereas distance between two rings increased as compared to control (Fig. 3). The spiral rings formed were separate in control test while they were interconnected with each other in 1% leaf extract. However the diffusion length (4.8 cm) was same as those of pure $CaCl_2$ (control) (5cm) while number of rings was less than that of control (Table 2).

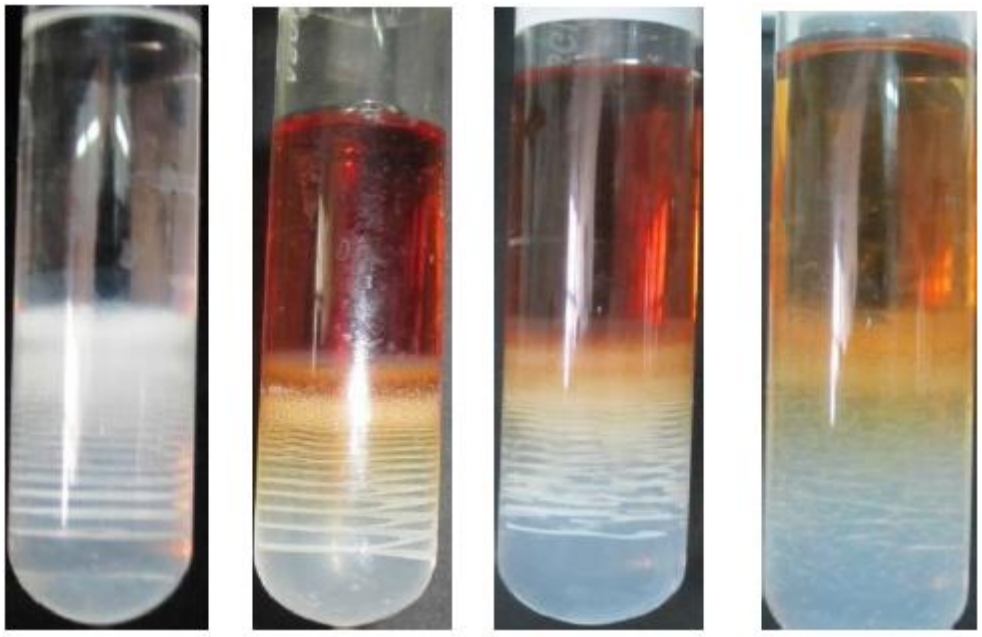


**Fig. 1 Photographic image of HA crystals in the form of liesegang ring in different concentrations of leaf methanol extract of *P. niruri*.**

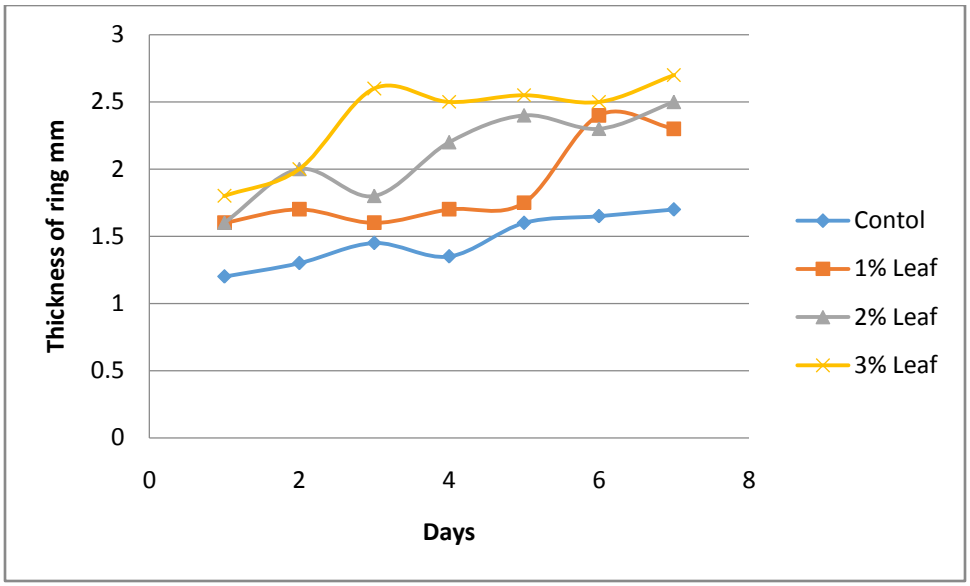


**Fig 2. Comparison of liesegang ring thickness between control and different concentrations of methanol leaf extract of *P. niruri***

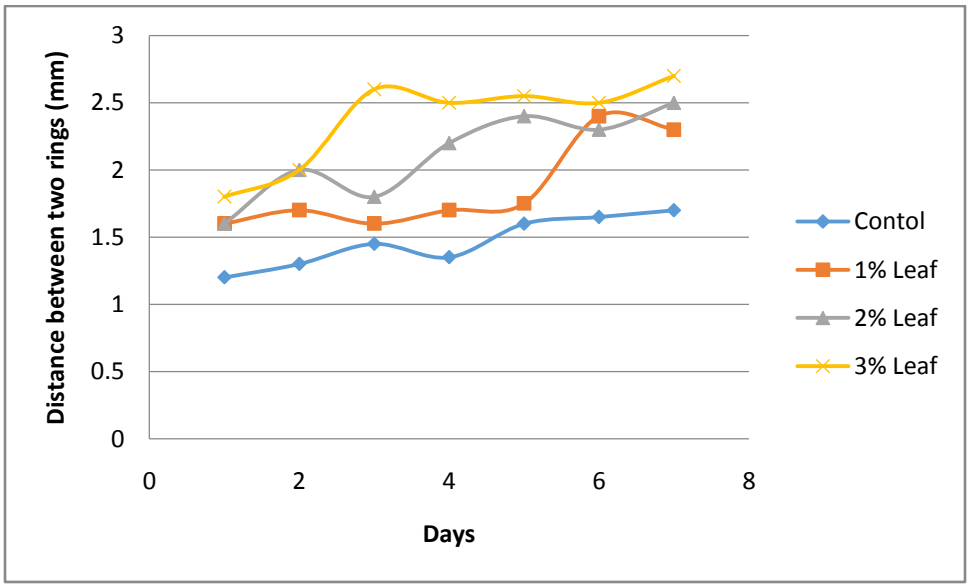


**Fig 3. Comparison of distance between two liesegang ring of control and different concentratons of methanolic leaf extract of *P. niruri***

In 2 % leaf extract, after 24 h of addition of 2 % leaf extract in gel, the ringsformed were slightly dissolved (Fig. 1) and thin as compared to those formed in control test and 1 % leaf extract contained gel (Fig. 2) whereas distance between two ringsincreased as compared to control (Fig.

3).The number of rings formed and diffusionlength also was comparatively less in 2% leaf extract as compared to control and 1% leaf extract (Table 2).In 3 % leaf extract, thickness of rings was less than that of 1% leaf, 2% leaf andcontrol (Fig. 2) while distance between two rings increased as compared to control and1%, 2% leaf extract (Fig. 3). However diffusion length (3.5 cm) and number of rings(8) decreased than control and 1%, 2% leaf extract (Table 2).

**Table 2. Comparison of number of liesegang rings and diffusion lenth between two liesegang rings in control and different concentrations of methanolic leaf extract**

| S.No | Leaf extracts | No. of liesegang ring | Diffusion length (cm) |
|---|---|---|---|
| 1 | Control | 14 | 5 |
| 2 | 1% | 13 | 4.8 |
| 3 | 2% | 10 | 3.5 |
| 4 | 3% | 8 | 3.5 |

In the gel growth technique, growth occurs due to the reaction between two solutions in a gel medium or achieving super-saturation by diffusion in gel medium. Thegel-based *in vitro* growth inhibition study of hydroxyapatite was carried out atphysiological temperature. Slow and controlled diffusion of reactants in gels could mimicthe growth of hydroxyapatite crystals at physiological conditions (Joshi et al., 2005a;Joseph et al., 2005). The inhibitive power of various medicinal plant extracts on the growth of hydroxyl apatite crystals is reported (Parekh et al., 2008; Parekh et al., 2014).

Hydroxyapatite crystals were grown in the silica gel media in the form ofliesegang rings. The effect of three different concentration of leaf methanol extract of *P.niruri*on hydroxyapatite crystals was noticed in terms of changes in the liesegang ringpattern, thickness of ring, diffusion length and number of rings formed. The number ofliesegang rings and diffusion length decreased in 3% methanol leaf extract containingsolution as compared to 1%, 2% methanol leaf extract and control while distancebetween two rings increased in 3% leaf methanol extract than the 1%, 2% methanol leaf extract and control. The methanol extract of all the three different concentration of

leaf was very effective in inhibiting the growth of hydroxyapatite crystals under in vitro gelconditions but the best effect was shown by 3% leaf extract. The phytochemical studiesof showed the presence of alkaloids, cardiac glycosides, flavonoids, tannins,carbohydrates, terpenoids and saponins (Desai and Chanda, 2014) i.e. leaf methanolextract contains substances that may inhibit the growth of hydroxyapatite crystals.

**Calcium hydrogen phosphate dihydrate crystal growth (CHPD)**

The effect of different concentration (1%, 2% and 3%) of standard drug cystone,methanol extract of leaf is given in Fig. 4. The average size of crystals indifferent concentration of cystone and leaf is given in Table 3.

The size of CHPD crystal gradually increased in control and differentconcentration of cystone and leaf extracts. Irrespective of the extractconcentration, maximum size of the crystal was found on 10th day and almost remainedconstant till day 14. From day 1 to day 14, crystal size was maximum in control ascompared to 1% leaf, (Fig. 4). Effect of 3% leaf extract on CHPD crystalgrowth is given in Fig. 4. Again a leaf effect of the concentration of the test solution onCHPD crystal growth was observed. The average crystal size was maximum in control (5.34 mm).In cystone, theaverage size of the crystals decreased as compared to control; the effect of concentrationwas also found.

**Table 3. Comparison of average size of crystals (mm) in different concentrations of cystone and leaf extract of *P. niruri***

| Concentration | Control | Cystone | Leaf |
|---|---|---|---|
| 1% | 5.37 ±0.570 | 4.88 ± 0.227 | 4.14 ± 0.349 |
| 2% | 5.34 ± 0.531 | 3.79 ± 0.305 | 3.33 ± 0.193 |
| 3% | 5.34 ± 0.570 | 3.46 ± 0.348 | 3.30 ± 0.270* |

Value of average crystal size was expressed in (mm) as mean ± SEM. One-way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett test. Comparisons are made against control and cystone *$p<0.01$

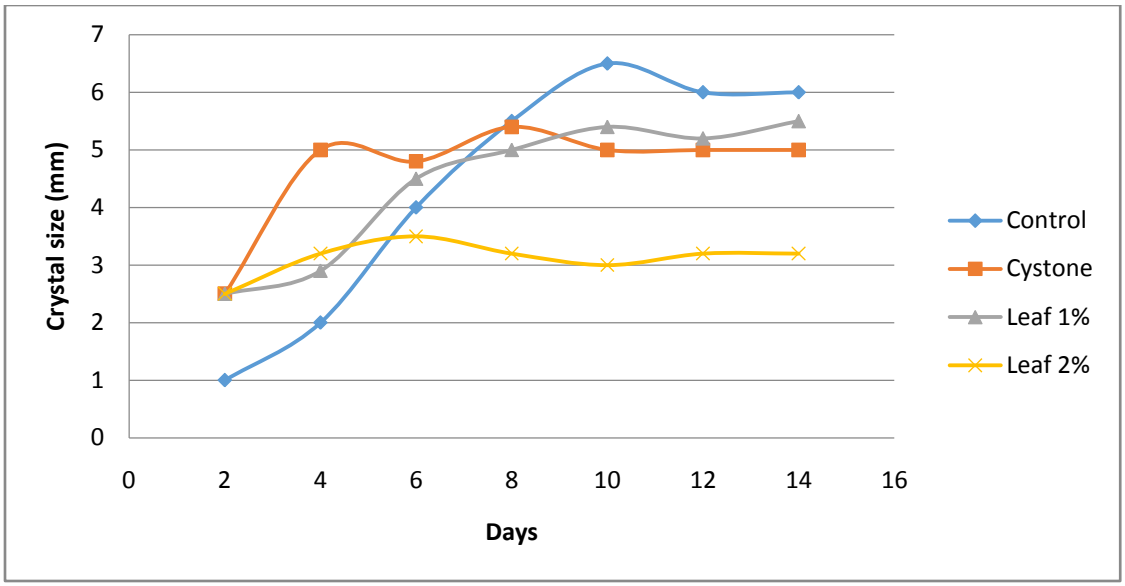


**Fig 4 : Growth of CHPD crystals size in different concentrations of cystone and leaf extract of P. nirurui**

The growth of crystals by gel is well known. This is a simple and convenientmodel to grow various types of urinary crystals and study the inhibitory effect ofmedicinal plant extracts. In the present study, Calcium hydrogen phosphate dehydrate (CHPD) crystals were grown by single diffusion gel growth technique. The effect of three different concentrations of leaf, of *P.niruri*on CHPD crystal was noticed in terms of changes in the growth and size of crystals was investigated. The size of crystals decreased with increase in the concentration of leaf, methanolextract of *P.niruri*. The methanol extract of all the three parts was very effective ininhibiting the growth of CHPD crystals under in vitro gel conditions but the best effectwas shown by 3% leaf extract. The size was minimum in it. This may be due to thepresence of phytoconstituents present in them, which inhibited the growth of crystals byforming soluble complexes. Inhibitory influence of citric acid and lemon juice on thegrowth of CHPD urinary crystals was reported by Joshi and Joshi (2003); while Dianaand George (2014) and Verghese et al. (2014) reported CHPD crystal growth inhibitionby *E. superbum* seed extract and *A. lanata* extract respectively.

### *In vitro* antiurolithiatic activity by turbidometric method

The effect of different concentrations of methanolic extract of leaf, of *P. niruri* on calcium oxalate crystallization was determined by time coursemeasurement of turbidity in the metastable

solutions containing $Ca^{2+}$ and oxalate at theconcentrations of 4 and 0.5 mM, respectively.The formation of crystals was estimated in terms of the turbidity of the solution.

There was a gentle decrease in the absorbance in 5 mg/5ml concentrations of leafextract and cystone as compared to control. The slopes of calcium oxalate crystalgrowth decreased in 1% leaf as compared to leaf while %inhibition increased significantly ($p< 0.01$) as compared to cystone (Table 4). Thepercent inhibition in 5mg/5ml concentration of cystone and leaf was 4.30 % and7.24 %, respectively (Table 4).

Kidney stone function is a complex process that results from a succession ofseveral physico-chemical events including supersaturation, nucleation, growth,aggregation and retention within renal tubules (Khan, 2010). Thus if supersaturation orlater steps in crystallization can be prevented, then stone formation can be avoided.Indeed, several measures are usually taken to reduce supersaturation like increasing fluidintake and medical therapy.

It is thought that supersaturation of urine is primarily responsible for calciumoxalate stone formation. When urinary supersaturation with calcium oxalate exceeds thelimit of metastability, nucleation of microcrystals occurs. Two separate processes governurinary stones, namely nucleation and aggregation of calcium oxalate crystals. Theformation of crystals was estimated in terms of turbidity of the solution with and withoutdifferent concentrations of leaf, stem and bark methanol extract of *P.niruri*.Significant inhibiting effect of extracts of leaf, stem and bark of *P.niruri*wasobserved as compared with control conditions and cystone. T*max* i.e. the maximum timecorresponding to the time between the addition of oxalate and the moment at whichmaximum absorbance (equilibrium) is measurable increased and the slopes of calciumoxalate crystal growth decreased in leaf, stem and bark methanol extract. Turbiditydecreased because of number of crystals decreased on addition of plant extract. Tmax and% inhibition increased with increased concentration of leaf, stem and bark extracts, butbest effect was shown by bark extract. Bark extract contains substances that inhibit thegrowth of calcium oxalate crystals. This property of plants may be important inpreventing the growth of kidney stone (Patel et al., 2012; Mittal et al., 2015; Ram et al.,2015).

Table 4. % inhibition and T*max* of calcium oxalate in different concentrations of cystone and leaf extract of *P. niruri*

| % Inhibition | | | | |
|---|---|---|---|---|
| Control | Cystone (5 mg/5ml) | Cystone (5 mg/ 10ml) | Leaf (5 mg/5ml) | Leaf (5 mg/ 10ml) |
| 0 | 4.30 ± 0.76 | 17.60 ± 0.91 | 7.24 ± 2.82 | 31.52 ± 4.73** |
| $T_{max}$ (min) | | | | |
| 12 | 14 | 14 | 14 | 16 |

Values are expressed in % and min as mean ± SEM. ANOVA followed by Dunnett test.

Comparison between 5mg/5ml of cystone and 5mg/5ml leaf extract . **$p<0.01$

The mechanism underlying this effect is possibly mediated through nephro protective properties.Also, these results indicate administration of bark extract of *P.niruri* reduced and

prevented the growth of urinary crystals. Therefore, the bark extract of *P.niruri* is helpful to prevent and cure stone development. Finally, these results stimulate further investigations to extract and identify the active chemical compounds responsible for the effects observed here.

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