Original Article

Investigating Antiuroliathiatic Potential of *Phyllanthus niruri* L. A Member of the Family Euphorbiaceae

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ABSTRACT

The inhibition of *in-vitro* calcium-oxalate crystal formation by *Phyllanthus niruri* L. extract was investigated by different methods i.e. nucleation assay and synthetic urine assay. In nucleation assay, the aim was to evaluate the effectiveness of different concentrations of the extract on calcium oxalate crystallization *in vitro* while in synthetic urine method the percentage inhibition and growth of the calcium oxalate monohydrate crystals from synthetic urine at different % concentrations of extract was investigated. Results revealed that in both the assay % inhibition for calcium oxalate crystal formation was found to be directly proportional to the increase in concentration of the plant extract with maximum inhibition of 61.97% ± 0.78 at 1000mg/ml in nucleation assay, while in synthetic urine assay maximum inhibition was 58.62 ± 0.02 %at 100% concentration of extract. Thus, the extract showed potent antilithic ability in both the assays studied.

Keywords: *Phyllanthus niruri* L., Urinary stone, Calcium oxalate monohydrate, *In-vitro*, Synthetic urine.

INTRODUCTION

With the increasing modernization the tremendous changes in the lifestyle of a common man along with the abnormal changes in the environment has resulted in an increase in frequency and variety of diseases prevailing today throughout the world. One such disease is lithiasis which is reported to be the third most common problem of the urinary tract prevailing in Asia, Europe, America and Middle East. Urolithiasis is considered to be one of the oldest known diseases[1].

The deposition or formation of stones in any part of the urinary system, i.e. the kidney, the ureters or the urinary bladder is called urolithiasis. A stone is an aggregation of solute materials from urine such as calcium, oxalate, phosphate and uric acid which forms stones. In India, calcium oxalate is found to be the most predominant

constituent of urolithiasis. Stone formation is the culmination of a series of physiochemical events i.e. supersaturation, nucleation, growth of the crystal and aggregation that occurs as the glomerular filtrate traverses through the tubules of nephron[2].

Calcium oxalate stones represent up to 80% of analyzing stones[3].They are of primary two types, calcium oxalate monohydrate (whewellite) and calcium oxalate dihydrate (weddellite). The occurrence frequency of whewellite is 78%, while that of weddellite is 43%[4].

Several modern techniques have been introduced for the treatment of urolithiasis like extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), and percutaneous nephrolithotomy (PNL). Studies show that ESWL is less effective in calcium oxalate monohydrate (COM) and cystine stones than calcium oxalate dihydrate (COD) and uric acid stones[1].Still some of the drawbacks of these methods exists which includes their being too costly for a common man and recurrence of stone formation along with a number of other side effects[5].

The Mother Nature has provided cures for every disease, the only need is to search for the proper natural cure. Hence the mankind has started searching for new herbal cures that could be more cost effective and dependable with respect to their side effects. India is blessed with rich traditional knowledge which could be harnessed in order to search for herbal remedies. Since ancient times Ayurveda has played a major role in providing successful cures for a variety of diseases. It has also provided cures for stone disease. Many plants having the property of disintegrating and dissolving the stone are referred to as "pashanbheda" in ayurveda. Although the herbal cures confer least side effects still their use is limited to only those who have faith in it. But things proven by scientific research can generate its worldwide acceptance. So an urgent need exists for proving the potential of herbal medicines by analyzing them through scientific research. This will not only help in providing safer remedies for various diseases as well as it will help in preserving the floral diversity and ancient medicinal knowledge from permanent eradication.

Phyllanthus niruri L., commonly known as 'bhuiamla' is an annual herb of the Euphorbiaceae family with a worldwide distribution. It is used as a medicine in the ayurvedic systems since ancient times. In Chraka Samhita it is reported to be useful in treating various ailments. The extract of this plant is reported to be useful in hepatitis, AIDS, as well as it has anti-inflammatory, anti-fungal, anti-viral, anti-bacterial, anti-oxidant, hepatoprotective, hypoglycemic, hypotensive, and analgesic effect. It is used as an ingredient of almost 175 ayurvedic formulations[6].

Its fresh root is believed to be an excellent remedy for jaundice, dropsy and genitor urinary infections. The fruits are used in the treatment of tubercular ulcers, wounds, sores, scabies, ring worms, cancer and oxidative stress[6].

The active component of *Phyllanthus niruri* is niruriside, considered responsible for antiviral activity against HIV virus. It also has antiplasmodial activity. It has several bioactive molecules such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides, tannins, alkaloids, ellagitannins, triterpenes, phenylpropanoids, steroids, ricinolic acid and phyltetralin. The alkaloids have the antispasmodic activity leading to smooth muscle relaxation. A protein isolated from its aqueous extract possesses protective activity against number of drugs & toxins induced organ pathophysiology. The protein possesses antioxidant activity and it even enhances the intracellular antioxidant

property. Aflavon glycoside was isolated from the aerial parts of this plant[6].

The aqueous infusion of the whole plant is employed as a stomachic, appetizer, anti-spasmodic, laxative, diuretic and carminative. It is also used in cases of malaria, hepatitis B, dysentery, gonorrhea, syphilis, tuberculosis, cough, diarrhea, vaginitis and as hepatoprotective agent[6].

It is commonly known as 'stone breaker' due to its popular use in lithiasis. Many biochemicals identified from this plant are proven to have inhibitory action on stones. The triterpenes have been found to inhibit the cytotoxicity induced by calcium oxalate as well as it also reduces excretion of stone forming constituents and the markers of crystal deposition in the kidneys. Moreover, methanolic extract of its leaves contain substances such as lignans and phyllanthin which showed uricosuric activity in hyperuricemic rats. Alkaloids extracted from plants of the genus *Phyllanthus* were found to show antispasmodic activity leading to smooth muscle relaxation, mostly evidenced in the urinary tract, which would facilitate the elimination of urinary calculi[7]. Root of this plant is also reported to promote stone elimination in patients with kidney stones, as well as normalization of calcium levels in hypercalciuric patients[6].

Several workers have reported antilithic activity in aqueous extract of this plant.

Phyllanthus niruri (aqueous extract) was found to strongly inhibit the growth of matrix calculus and reduced the number of stones in rats, Freitas *et al.,*(2002)[8].Barros *et al.,* (2003) reported an inhibitory effect of its aqueous extract on CaOx crystal growth and aggregation in human urine, which suggests that it may interfere with the early stages of stone formation and may represent an alternative form of treatment or prevention for urolithiasis[9]. Ramsout *et al.,* (2011) have reported the effect of *Phyllanthus niruri* on CaOx crystallization in synthetic urine (prepared according another method) as well as on human urine[10].Campos *et al.,* (1999) have demonstrated the potent inhibitory effect of *Phyllanthus niruri* on CaOx crystal adhesion in rats[11]. Nishiura *et al.,*(2004), reported that its aqueous extract was found to normalize the elevated urinary calcium levels in calcium stone forming patients[12]. Micali *et al.,*(2006) showed that patients submitted to extracorporeal shock wave lithotripsy and treated with *Phyllanthus niruri* presented lower incidence of residual stone fragments after lithotripsy[13]. Murugaiyah *et al.,* (2009), reported that *Phyllanthus niruri* induced an increase in calcium oxalate dihydrate forms and reduced the amount of calcium oxalate monohydrate crystals, responsible for higher potential risk for stone formation in an *in vitro* model for precipitation of calcium oxalate using human urine from healthy individuals. These data strongly suggest that *Phyllanthus niruri* may be a potential source of many substances with antilithiatic properties[14]. Acute toxicity of its aqueous leaf extract was investigated in which no toxicity was observed at the levels administered[15].

Thus the aim of the present study is to evaluate the effectiveness of aqueous extract of its whole plant for its anti-urolithiatic activity using two *in-vitro* methods; nucleation assay and synthetic urine assay. In nucleation assay the aim was to evaluate the effectiveness of different concentrations of the extract on calcium oxalate crystallization *in-vitro* while in synthetic urine method the percentage inhibition and growth of the calcium oxalate monohydrate crystals in synthetic urine at different % concentration of extract was evaluated.

MATERIALS & METHODS

Plant collection, identification and preparation of extract

The whole plant of *Phyllanthus niruri* was collected from Kolar road, Bhopal, Madhya Pradesh, during the month of September 2012 and the plant was identified with the help of regional Floras[16]and taxonomists and finally confirmed with the herbarium of Botanical Survey of India (BSI), Allahabad, with voucher specimen No.1370-151.01-699B.

Fresh plant, after collection was shade dried at room temperature and then grinded. The plant material was extracted with distilled water. Then the extract was concentrated to dryness at 30-40°c temperature, obtaining dried extract which was stored in refrigerator until used for further analysis.

Estimation of antilithic potential

1. Nucleation assay

The classical model for the study of oxalate crystallization was chosen because of its simplicity and satisfactory reproducibility[17]. This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of the plant extract used. Solution of calcium chloride and sodium oxalate were prepared at the final concentrations of 5mmol/L and 7.5mmol/L respectively in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. 950mL of calcium chloride solution mixed with 100mL of extracts at different concentrations. Crystallization was started by adding 950 mL of sodium oxalate solution. The temperature was maintained at 37 ºC. The OD of the solution was monitored at 620nm using spectrophotometer (Systronics digital spectrophotometer 166) after 30 minutes. The rate of nucleation was estimated by comparing the induction time in the presence of the extract with that of control. Data was represented in percentage inhibition.

The growth of crystals was expected due to the following reaction:

$$CaCl2 + Na2C2O4 \rightarrow CaC2O4 + 2NaCl$$

2. Synthetic urine assay

Preparation of synthetic urine

The classical model for the study of oxalate crystallization was chosen because of its simplicity and satisfactory reproducibility[18].This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of the plant extract. Two solutions of following composition were mixed: solution A: $Na_2C_2O_4$ (2mmol/1) and solution B: $CaCl_2 2H_2O$ (10mmol/1). The two solutions were prepared along with adding NaCl 9g to obtain the ionic force like the indoor environments. Synthetic urine is prepared by mixing and stirring two equal volumes of 50ml of solutions A and B at constant temperature (37°C) in capped vessels to give final artificial urine. Mixture agitation was maintained to prevent sedimentation.

Simulation of the sedimentary crystal formation

The crystal size development was monitored in sample drops by polarized microscope. A drop of the sample was put on hemacytometer counting chamber and it was observed under microscope after 30 minutes. The number of crystals was calculated and subsequently its photograph was taken. A series of experiments corresponding to different% concentrations of plant extract was conducted. The crystal size development by microscope was carried out after 30 minutes of formation of crystals and their photographs were taken. The percentage of Inhibition (I %) was calculated with the help of following formula

I% = [(TSI- TAI) / TSI] * 100

TSI- represents the number of calcium oxalate monohydrate crystals without inhibitor.

TAI- represents the number of calcium oxalate monohydrate crystals after addition of inhibitor.

RESULT AND DISCUSSION

1. Effect on Nucleation assay

Incubation of the salt forming solutions of calcium chloride and sodium oxalate resulted in the formation of calcium oxalate crystals. The rate of nucleation was estimated by comparing the induction time in the presence of the extract with that of control. The O.D. was monitored at 620nm after 30 minutes. The turbidity of solution in the presence of herb extract was lower in comparison to the control, showing that oxalate crystallization was less in the presence of the extract. Data represents that % inhibition for calcium oxalate crystal formation was directly proportional to the increase in concentration of the plant extract, with minimum inhibition of 39.65± 0.48% at 100mg/ml to a maximum inhibition of 61.97± 0.78 % at 1000mg/ml extract concentration.(Figure1.)

2. Effect on synthetic urine assay

The formation and growth of the calcium oxalate monohydrate crystals from synthetic urine at different extract concentrations was studied. The crystallization of oxalate in the absence of inhibitor, led to the formation of calcium oxalate monohydrate crystals which was monitored by polarized light microscopy. The number of calcium oxalate monohydrate crystals in control, was found to be maximum (362.5/mm^3) with no inhibition. In order to assess the inhibiting potential of plant extract for oxalate crystallization different percentages of plant extract was tested. It was found that the plant used in this study inhibited potently the crystal development with maximum number of crystals 262.5/mm^3 at 25% extract concentration (Figure2.) while minimum numbers of crystals 150/mm^3 were formed at 100% concentration of extract (Figure3.). Results show that the decrease in the number of crystal as well as % inhibition of the formation of calcium oxalate monohydrate crystals was directly proportional to the increase in percentage of plant extract, with minimum inhibition of 27.59± 0.45% at 25% extract while maximum inhibition of 58.62± 0.02% was obtained at 100 % extract concentration.(Figure4.)

Stone formation occurs as a result of culmination of various steps. The initial stage being with the supersaturation of urine with calcium oxalate while later factors being nucleation, growth and aggregation. Thus, if supersaturation or initial stages in crystallization can be prevented, then lithiasis could be avoided. The *in-vitro* results revealed that the plant extract has potent antiurolithiatic ability in both nucleation assay and synthetic urine assay. Along with this in synthetic urine assay minimum number of crystals were formed at maximum concentration studied.

However, these *in-vitro* results should be confirmed *in-vivo*. As reported earlier the presence of alkaloids and triterpenes[7] may be considered responsible for this inhibitory action on the growth of calcium oxalate monohydrate crystals, thus phytochemicals in this particular extract will be analyzed in future studies.

Similar work on antilithic potential of *Phyllanthus niruri* has been reported by several workers like Barros *et al.,* (2003)[9], Ramsout *et al.,* (2011)[10], Campos *et al.,* (1999)[11], Nishiura *et al.,*(2004)[12], Micali*et al.,*(2006)[13] and Murugaiyah *et al.,* (2009)[14].

Although Freitas *et al.,* (2002)[8] have reported the inhibitory effect of aqueous extract of whole plant of *Phyllanthus niruri* on calcium oxalate crystallization in rats, but

to the best of our knowledge and in accordance with the literature survey, no previous report on antiurolithiatic potential in aqueous extract of whole plant of *Phyllanthus niruri* by Atmani *et al.,* (2000) or Beghalia *et al.,* (2008) method was found.

CONCLUSION

In the present work the inhibition of calcium oxalate crystal formation by aqueous extract of whole plant of *Phyllanthus niruri* was studied. The extract showed potent antilithic ability and the percentage inhibition and amount of crystal formation was found to be directly proportional to the increase in concentration or percentage of the plant extract respectively. These *in vitro* results should be confirmed *in vivo*.

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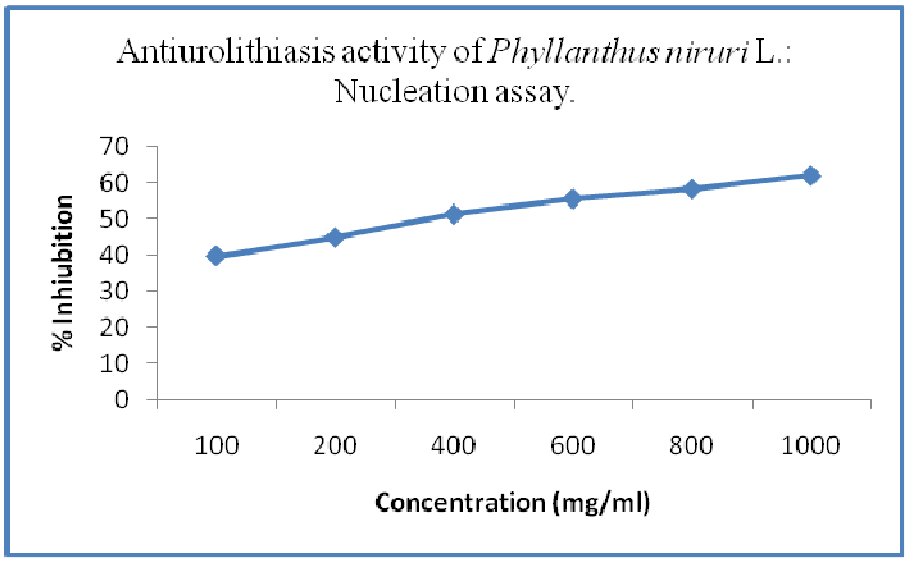

Figure 1. The effect of different concentrations of *Phyllanthusniruri*L. on Calcium oxalate crystal inhibition by nucleation assay.

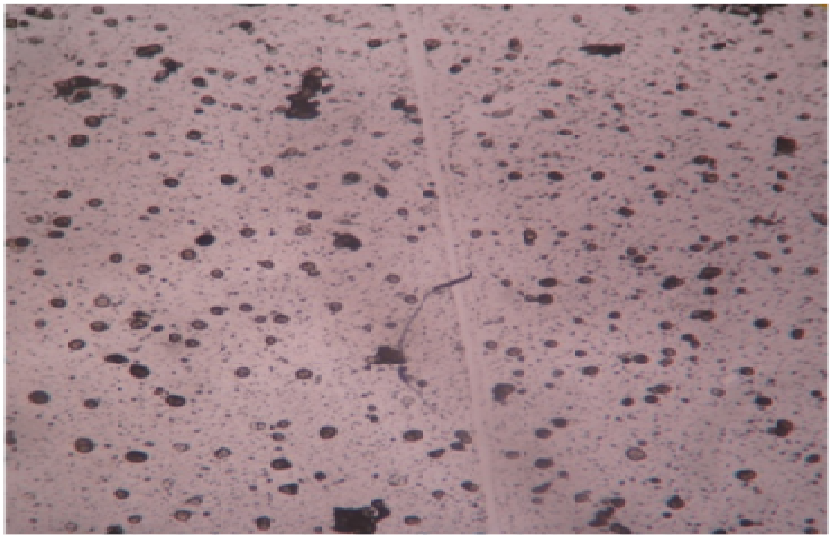

Figure 2. Calcium oxalate monohydrate crystal development in 25% extract concentration of *Phyllanthusniruri* L. in synthetic urine assay.

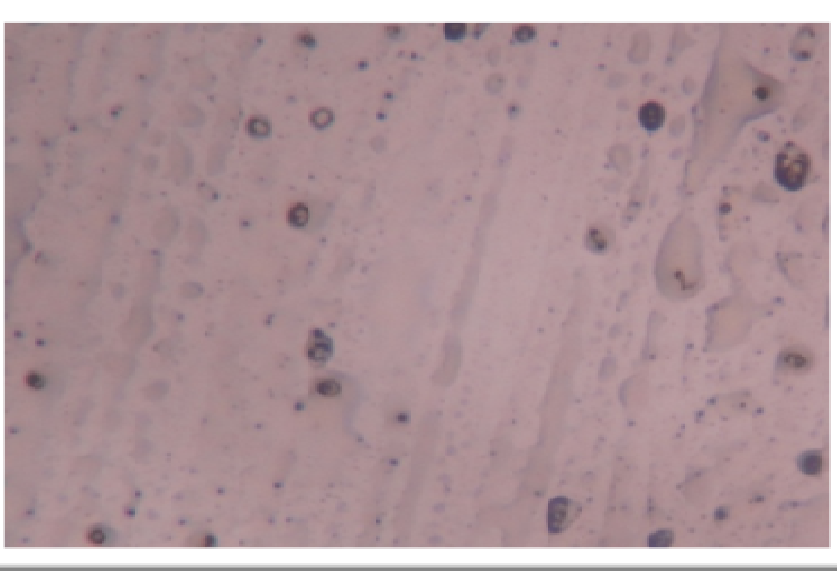

Figure 3. Calcium oxalate monohydrate crystal development in 100% extract concentration of *Phyllanthusniruri* L. in synthetic urine assay.

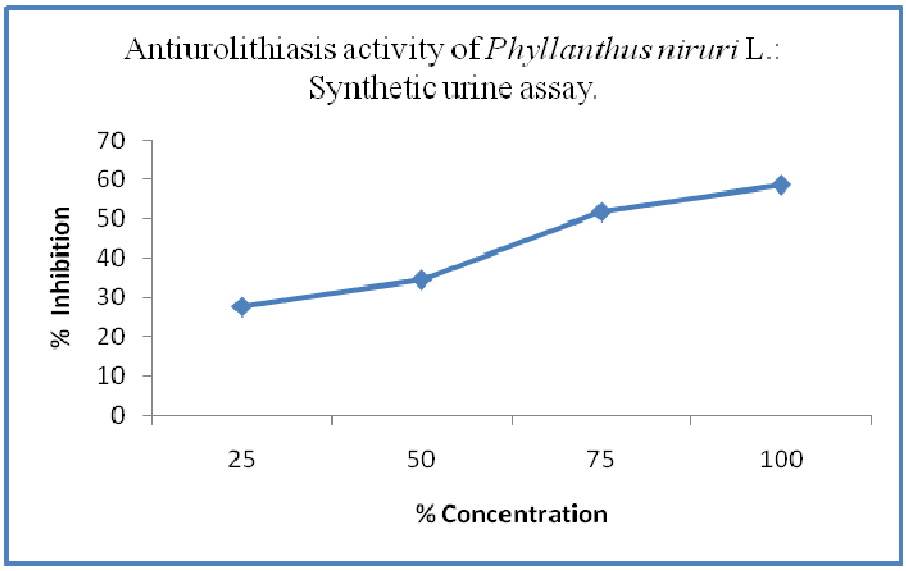

Figure 4. The effect of different percentages of *Phyllanthusniruri* L. extract on calcium oxalate monohydrate crystal inhibition in